EFFECTS OF IMMUNOSUPPRESSIVE DRUGS UPON THE MURINE IMMUNE SYSTEM

PENGARUH OBAT IMUNOSUPRESIF PADA SISTIM IMUNOLOGIK MENCIT

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to my wife : Ramlahwati.

and my son : Munsir Fadjri.

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   (Surat Mursalaat, ayat 7).-

ABSTRACT

The influence of immunosuppressive drugs (dexametha - sone sodium phosphat (Decadron) and cyclophosphamide (Endox- an)upon the murine immune system was studied in Department - of Cell Biology,Immunology and Genetics, Erasmus University Rotterdam The Netherlands.

We studied the effects of dexamethasone and cyclophos phamide upon the Ig production at four different level :

1. the serum Ig concentration.
2. the 'background' Ig secreting cells that occur in virtual ly all lymphoid tissue without deliberated immunization.
3. the surface Ig^+ B cells.
4. the B cells that give rise to clone of Ig secreting cells upon in vitro stimulation with bacterial lipopolysacchari des.

We investigated the effect of single intraperitoneal injection as well as short-term daily intraperitoneal admini stration of dexamethasone and cyclophosphamide upon the abo- ve four parameters.

The dose of dexamethasone employed ranged from 16 up to 144 mg/kg body weight(BW) for a single injection and from 1 up to 50 mg/kg BW for daily injections. For treatment with cyclophosphamide the mice received either a single injection of 300 mg/kg BW or daily injections of 1 or 100 mg/kg BW. A single injection of dexamethasone (16 to 144 mg/kg BW appe ared to cause a substantial decrease of the number of IgM, - IgG and IgA secreting cells in spleen and mesenteric lymph- nodes within one day, but hardly affected their number in - bone marrow. A single injection of dexamethasone, even a mas sive dose as 144 mg/kg BW, affected the serum IgM, IgG and IgA-level less severely than 7 daily injections of e.q. 1 mg/ kg BW. A single injection of 144 mg/kg BW dexamethasone, the persentage of B cells among the remaining spleen and bone - marrow cells was not affected. Also the height of the poly - clonal response after stimulation of the remaining B cells with LPS in vitro and the capacity to switch from IgM to IgG and IgA secretion were not affected.

Injection of cyclophosphamide on the other hand, not only greatly decrease the number of spleen and bone marrow - cells, but also the proportion of B cells among the remai - ning spleen and bone marrow cells. Furthermore, these B cell could no longer be polyclonally activated by LPS in vitro,in dicating that they were inhibited in their functional capaci ty.

It was found that 10^{-8}M dexamethasone in vitro redu - ced the response of splenic B cells to LPS by more 80 per - cent,while a similar reduction of the response by bone mar - row B cells required a 1000-fold higher concentration.

After multiple injections of 100 mg cyclophosphamide/ kg BW, the minimum values of background Ig-secreting cells in the various lymphoid organs were lower than after a sing- le injection of 300 mg cyclophosphamide/kg BW.-

SARI BACAAN

Penulis telah melakukan penelitian di Laboratorium Cell Biologi, Imunologi dan Genetika Universitas Erasmus, Rotterdam Nederland mengenai pengaruh obat imunosupresif (fosfat Natrium deksametason (Decadron) dan siklofosfamid (Endoxan) terhadap sistim pembentukan imunoglobulin tikus. Hal-hal yang telah diteliti ialah pengaruh kedua jenis obat ini terhadap pembentukan imunoglobulin pada empat level yang berbeda yaitu :

1. konsentrasi imunoglobulin dalam serum.
2. sel-sel yang menghasilkan imunoglobulin yang sesungguhnya terjadi dalam semua jaringan limfoid tanpa pemberian imunisasi.
3. limfosit B yang memiliki imunoglobulin permukaan.
4. limfosit B yang dapat menimbulkan sel-sel yang menghasilkan imunoglobulin dalam bentuk "clone in vitro" yang dirangsang dengan lipopolisakharida bakteri.

Penyuntikan deksametason, siklofosfamid kedalam rongga peritoneum tikus dilakukan dengan dua cara yaitu penyuntikan dosis tunggal dan dosis multipel selama 7 hari. Dosis deksametason untuk kedua cara tersebut berkisar antara 16 sampai 144 mg/kg berat badan (BB) dan 1 sampai 50 mg/kg BB. Dosis siklofosfamid 300 mg/kg BB pada dosis tunggal dan 1 atau 100 mg/kg BB untuk dosis multipel.

Satu hari sesudah penyuntikan tunggal deksametason (16 sampai 144 mg/kg BB) terdapat penurunan yang nyata jumlah sel yang membentuk IgM, IgG dan IgA dalam limpa dan kelenjar limfa mesenterium, tetapi hal ini hampir tidak dijumpai dalam sumsum tulang.

Pengaruh deksametason pada nilai IgM, IgG dan IgA dalam serum sangat kurang dengan penyuntikan tunggal dosis tinggi (144 mg/kg BB dari pada pemberian tiap hari selama satu minggu dosis rendah (1 mg/kg BB).

Pada penyuntikan tunggal deksametason (144 mg/kg BB) prosentase limfosit B diantara kelompok sel yang tertinggal dalam limpa dan sumsum tulang ternyata tidak dipengaruhi. Tidak terpengaruh pula peninggian poliklonal yang terbentuk dari limfosit B yang tertinggal setelah perangsangan dengan lipopolisakharida in vitro dan kemampuan perubahan IgM menjadi IgG dan IgA.

Penyuntikan seklofosfamid mengakibatkan bukan saja jumlah sel dalam limpa dan sumsum tulang yang sangat menurun melainkan pula limfosit B diantara sel-sel yang tertinggal dalam limpa dan sumsum tulang. Selanjutnya limfosit B itu tidak dapat lebih lama menghasilkan poliklonal yang diaktifkan oleh lipopolisakharida in vitro, hal ini menunjukkan bahwa limfosit tersebut dihambat fungsinya.

Pemberian deksametason langsung kedalam in vitro 10^{-8} M menurunkan lebih dari 80 persen limfosit B limpa yang dirangsang lipopolisakharida, sedangkan penurunan yang sama limfosit B sumsum tulang memerlukan konsentrasi 1000 kali lipat lebih tinggi. Sesudah penyuntikan siklofosfamid 100 mg/kg BB berulang-ulang maka nilai minimal sel-sel yang menghasilkan imunoglobulin bermacam-macam organ limfoid ternyata lebih rendah dari pada penyuntikan tunggal siklofosfamid 300 mg/kg BB.

CONTENTS

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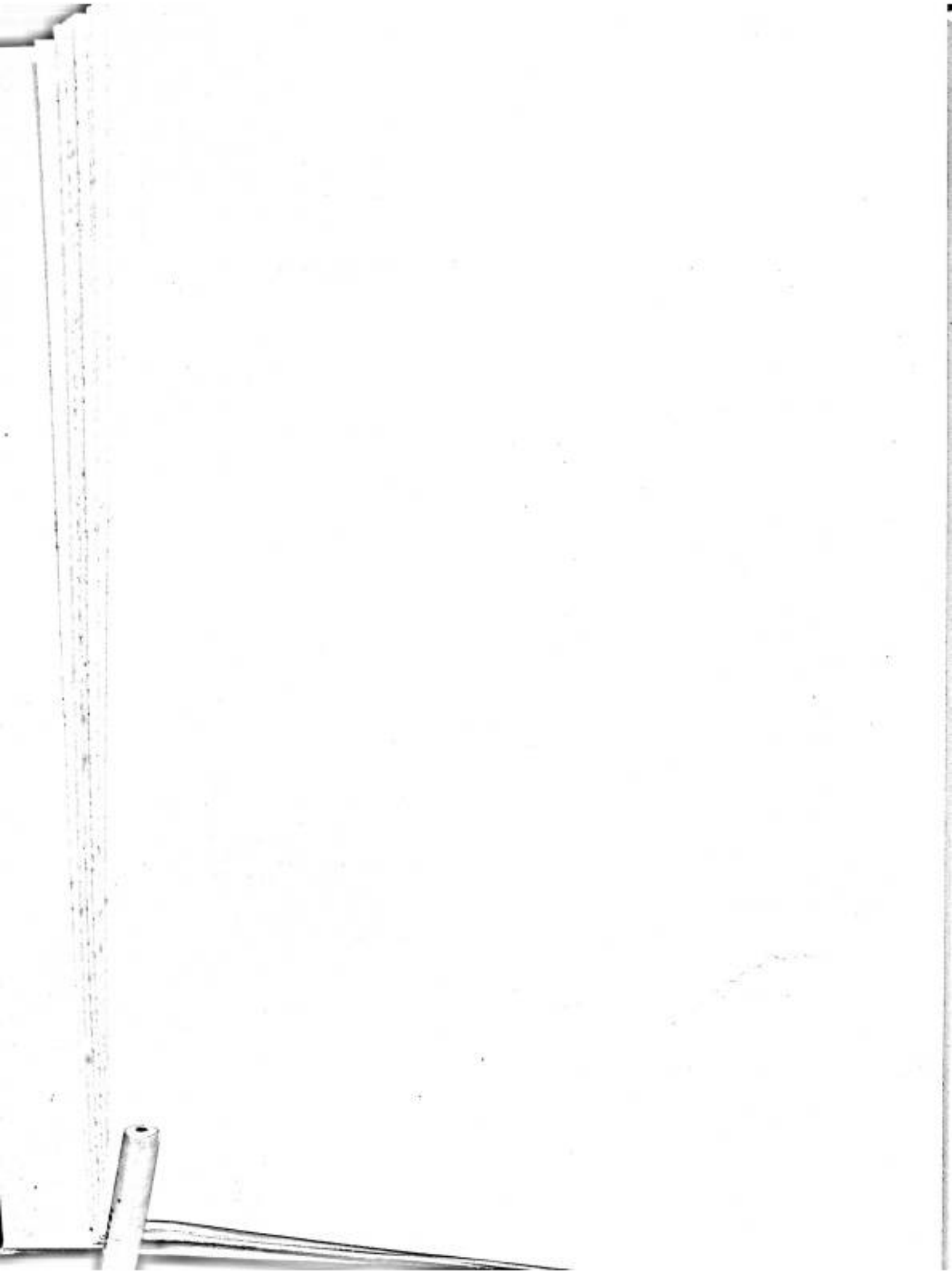
CHAPTER 1

Introduction



1. INTRODUCTION

1.1. General aspects of immunity

Man and higher animals are protected against infection by three lines of defence. The first is formed by the skin and the various other types of epithelium lining the portions of the body in close contact with the environment. The second line consists of a variety of host mechanisms compromising the growth or survival of microorganisms (e.g., sneezing, coughing, the ciliary sweeping of epithelium lining in for instance the trachea, the pH of secretions, etc.).

When microorganisms pass the epithelium as may happen normally in gut and in lungs and after an injury, the body is able to react with a third line of defence comprising a nonspecific and a specific system. The nonspecific system consists of a number of humoral factors such as C-reactive protein, complement and interferon, and of mononuclear phagocytic cells, granulocytes and natural killer cells. Phagocytic cells are able to move to the site of infection and to engulf and destroy the infecting organisms by lysosomal enzymes. The nonspecific response is operative within a few hours after the intrusion. In addition to the nonspecific response, man and higher animals can produce a slower emerging, but highly specific response: an immune response. Two kinds of specific immune responses can be distinguished: one is mediated by humoral factors, called antibodies ('humoral immunity'), the other by lymphoid cells ('cellular immunity'). Chemically, the antibodies are proteins. Such proteins with known or unknown antibody activity are called immunoglobulins (Ig). On the basis of their structure, revealed by their physicochemical and antigenic properties, the Ig can be subdivided into five different classes: IgM, IgG, IgA, IgD and IgE. The humoral immune response is particularly effective in the defence against bacteria and viruses occurring extracellularly. Also, a number of well-known immune phenomena which are harmful to the individual, such as immediate hypersensitivity (e.g., hay fever, asthma), are based on this type of defence mechanism. In contrast, the cell-mediated immune response is the major mechanism for destroying cells infected with viruses (e.g., influenza virus, measles virus) and for enhancing the capacity of phagocytic cells to destroy bacteria and parasites growing intracellularly (e.g., *Listeria monocytogenes, Mycobacterium tuberculosis, Mycobacterium leprae, Leishmania tropica*). The rejection of tissue and organ transplants from genetically non-identical individuals, and delayed type hypersensitivity reactions (e.g., Mantoux reaction in humans) are other examples of cell-mediated immune responses.

Two subpopulations of morphologically identical lymphocytes are responsible for both types of specific immune responses: B and T lymphocytes. Both types of lymphocytes are generated from the pluripotent hemopoietic stem cells which are present in the bone marrow. B cells proliferate and differentiate mainly within the bone marrow itself, whereas T cells do so in the thymus. Both, B and T cells have the capacity of recognizing entities which are not normal constituents of the organism. Such entities are called antigens. The antigen recognition by B cells is achieved by Ig receptors on their cell surface. After antigenic stimulation, B cells can differentiate into antibody-secreting plasma cells. T cells can enhance the proliferation and differentiation of B cells into antibody-secreting plasma cells. These T cells are called helper T cells. Helper T cells can also mediate cellular immune responses (e.g., delayed type hypersensitivity reactions and killing of bacteria and parasites growing intracellularly). In addition to these helper T cells, there are other T cells which are responsible for cellular immune responses: the killer T cells. Killer T cells lyse foreign antigenic cells (e.g.,

virus infected cells, organ and tissue grafts). Finally, suppressor T cells are able to suppress both humoral and cellular immune responses.

After primary antigenic stimulation, effector cells are produced, which mediate the above mentioned immune functions. These effector cells are mostly short-lived end-stage cells. Among the progeny of activated lymphocytes, however, are also cells which are long-lived and retain the capacity to become restimulated by the original antigen. They are responsible for the immunological 'memory' and cause the much faster and more vigorous immune response which is induced after second contact with the same antigen. The memory lymphocytes largely determine the effectiveness of vaccination procedures against infectious agents.

B and T cells differentiate into effector cells mainly within the spleen, lymph nodes, bone marrow, gut-associated lymphoid tissue and bronchus-associated lymphoid tissue. The major immunological function of these organs is to remove antigens from the bloodstream and the intercellular spaces, to concentrate them and to present them to the lymphocytes, so that the latter can proliferate and differentiate into antigen-specific effector T cells and antibody-forming cells. The peripheral lymphoid organs are provided with both accessory cells and the appropriate tissue architecture needed to fulfill these tasks.

Lymphocytes not only perform a variety of effector functions towards invading microorganisms and other antigens, they also regulate their own activity by the release of 'helper' and 'suppressor' factors, lymphocyte-specific growth hormones and antibodies. Other mononuclear blood cells also produce factors that regulate the activity of the lymphoid system. Together these factors account for the well-controlled homeostasis of the immune system. However, in a variety of diseases and after tissue and organ transplantation, the enhanced activity of the immune system has unfavorable effects which require medication. In such cases immunosuppressants may be used. Over the years many different immunosuppressants have been developed, biological (e.g., specific antisera against subpopulations of lymphocytes, hormones) as well as chemical (e.g., cytostatic drugs). In spite of their proven beneficial effect in a variety of diseases and their wide-spread use in medical treatment, there is in many cases still a lack of insight into their precise effects on the immune system. In view of this, we decided to study the influence of two frequently used immunosuppressive drugs, dexamethasone sodium phosphate and cyclophosphamide, upon the immune system. In these studies we employed a preclinical model, using mice as experimental animals.

The experimental work described in this thesis is aimed to analyse the effects of corticosteroids and cyclophosphamide upon the B cell lineage. This was done at two different levels: (a) unactivated B cells, and (b) actively Ig-secreting cells. As a background for these studies this thesis firstly describes the present state of knowledge of the immune system and the effects of corticosteroids and cyclophosphamide on the immune system. Subsequently we review the literature on the induction and measurement of antibody formation, followed by the data emerging from the experimental work performed.

1.2. *Structure of the immune system*

1.2.1. *The various lymphoid organs*

According to their contribution to antibody formation the lymphoid organs can be subdivided in two groups (Miller and Davies, 1964): the 'central' or 'primary' lymphoid organs, which are the breeding sites of lymphoid cells, and the 'peripheral' or 'secondary' lymphoid organs, which are mainly engaged in the induction of immune responses.

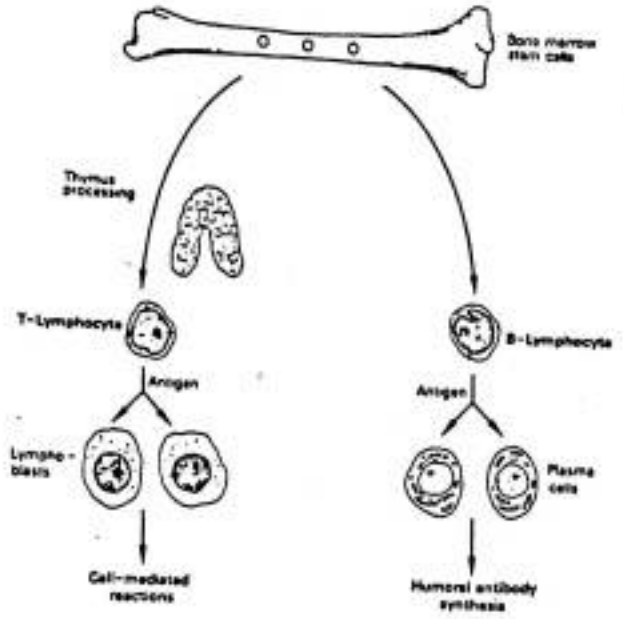


Fig. 1. Differentiation of stem cells into immunocompetent B lymphocytes in the bone marrow, and into T lymphocytes in the thymus. Proliferation and transformation to cells of the lymphoblast and plasma cell series occurs on antigenic stimulation. Modified from Roitt, 1980.

The primary lymphoid organs include the bone marrow and the thymus gland in mammals and the Bursa of Fabricius in birds. The bone marrow and the thymus are involved in the generation of B and T lymphocytes, respectively (Fig. 1).

Spleen, lymph nodes and unencapsulated lymphoid tissue constitute the secondary lymphoid organs. Unencapsulated lymphoid tissue is not constrained by a connective tissue capsule and is dispersed in the subepithelial parts of respiratory, alimentary and genitourinary tracts.

Lymphoid tissue guarding the gastrointestinal tract (tonsils, Peyer's patches, appendix) is unencapsulated and somewhat structured or present as diffuse cellular collections in the lamina propria. Together with the epithelial accumulation of the cells lining the respiratory and genitourinary tracts, they form the so-called mucosal-associated lymphoid tissue (Roitt, 1980).

1.2.2. *The function of the primary and the secondary lymphoid organs*

1.2.2.1. *Bone marrow*

The bone marrow is the central organ in hemopoiesis as well as lymphopoiesis. It contains the pluripotent hemopoietic stem cells which have the capacity of self renewal and are able to generate blood cells of the erythroid, myeloid, megakaryoid (Curry and Trentin, 1967; Trentin et al., 1967) and lymphoid series (Ford et al., 1966). With regard to the latter, the bone marrow is the major site of generation of immunocompetent virgin B lymphocytes (Phillips et al., 1977). Furthermore, it contains a substantial number of long-lived, potentially recirculating small lymphocytes (Miller and Osmond, 1975), partly of the B cell lineage and partly of the T cell lineage (Press et al., 1978; Röpke et al., 1975). These long-lived lymphocytes are thought to be B and T memory cells which are produced as a concomittant of immune responses leading to humoral and cellular immunity (Benner and Van Oudenaren, 1975; Osmond, 1980).

Lymphopoiesis in the bone marrow is dually regulated by local (microenvironmental) factors and by exogenous stimuli which can enhance the production rate (Osmond et al., 1981).

Several types of immunological effector cells occur in the bone marrow: effector T cells, suppressor T cells, natural killer cells and Ig-producing cells (Asherson and Zembala, 1973; Youdim et al., 1973; Zembala and Asherson, 1973; Kiessling et al., 1975; Herberman et al., 1975; Benner et al., 1981a; Bril and Benner, 1982). From the small lymphocytes in the bone marrow of young adult mice about 80% belong to the B cell lineage while about 12% of the marrow small lymphocytes are 'null' cells (i.e., can neither be classified as B lineage cells nor as T lineage cells) (Rosse, 1976). Thus, the bone marrow is not only a quantitatively important breeding site of virgin lymphocytes, but also a reservoir of long-lived potentially recirculating B and T lymphocytes.

McMillan et al. (1972, 1976), who measured the *in vitro* IgG production by lymphoid cells from human bone marrow, spleen, blood, lymph nodes and thymus, calculated that the bone marrow produced more than 95% of all IgG synthesized by the organs which were evaluated. By employing the protein A plaque assay, Benner et al. (1981[b]) have shown that, in mice, IgG and IgA secreting cells are relatively frequent in the bone marrow, as compared to IgM secreting cells. The absolute and relative contribution of the bone marrow to the total Ig synthesis of mice increases enormously with increasing age. At 4, 8, 14, 40 and 100 weeks of age the relative contribution of the bone marrow was found to be 17, 29, 46, 72 and 75%, respectively (Benner et al., 1981[a]).

The gradually increasing importance of the bone marrow as a site of Ig synthesis throughout the lifespan probably reflects the gradual adaptations of the individual to its antigenic environment. As an invididual becomes older, more antigenic stimuli from the environment will have been experienced and secondary type responses will prevail. Such secondary type responses to thymus dependent antigens involve a quantitatively important antibody production in the bone marrow.

1.2.2.2. Thymus gland

Embryologically, the thymus is derived from a tubular epithelial structure and some branchial pouches (Le Douarin, 1977). In a later phase, bone marrow derived prethymic stem cells migrate to the thymus to differentiate into T cells (Gorczynsky and MacRae, 1979[a], 1979[b]). Within the thymic microenvironment of epitheloid cells they proliferate and acquire characteristic T cell markers. The thymocytes differentiate into functionally distinct subpopulations with the competence to respond in the mixed lymphocyte reaction, to mediate allograft cytotoxicity, to generate antigen-specific help for B cells and to produce lymphokines. The lymphocytes in the thymus are held together by a sessile matrix of specialized connective tissue and epithelium (Clark, 1973).

The thymus contains subpopulations of lymphocytes with a different sensitivity to corticosteroids. Most cortical thymocytes are immature, medium and large-size cells actively synthesizing DNA and sensitive to corticosteroids and therefore are eliminated by enhanced levels of this hormone (Dougherty, 1952; Ishidate and Metcalf, 1963). Most medullary thymocytes, on the other hand, are immunocompetent and resistant to corticosteroid administration (Ishidate and Metcalf, 1963).

The extensive proliferation of thymocytes is localized in the more superficial layer of the thymus (Metcalf, 1966; Weissman, 1973). Some thymocytes migrate from the cortex into the periphery, while others migrate into the

medulla (Stutman, 1978). Several investigators claim that a high proportion of the lymphocytes produced by the thymus die *in situ* (Joel et al., 1977; Shortman, 1977). The cortex of the thymus contains a much higher concentration of lymphocytes than the medulla. Neonatal thymectomy grossly impairs cell mediated immunity and, in addition, has some effect on antibody production (Miller, 1960). Adult thymectomy, on the other hand, has a much smaller effect, although disturbance of certain functions has been noted (Taylor, 1963; Miller, 1965).

The thymus hardly contains antibody producing plasma cells. Most investigators who have studied this aspect reported a frequency of about 1% (Benner et al., 1974[b], 1974[c]; Vitetta et al., 1973). Thymic antibody-forming cell responses can only be evoked by high doses of antigen (Benner et al., 1974[c]), probably because antigen scarcely enters the thymus tissue.

1.2.2.3. Bursa of Fabricius

In birds, yet another primary lymphoid organ occurs, termed the Bursa of Fabricius. Embryologically, this organ is also derived from gut epithelium (Le Douarin, 1977). The Bursa of Fabricius is responsible for the development of immunocompetence in cells destined to make humoral antibody. Chickens bursectomized in embryonic life, have reduced levels of serum Ig, a smaller number of plasma cells (Cooper et al., 1969) and a profound depletion of small lymphocytes bearing surface Ig (Kincade et al., 1973). Injection of radioactive cell markers into the Bursa rapidly leads to the appearance of labeled cells in other lymphoid organs, such as the spleen (Hemmingsson, 1972; Back and Linna, 1973). Bursectomy usually does not influence cell mediated immunity (Cooper et al., 1965, 1966).

1.2.2.4. Secondary lymphoid organs

The secondary lymphoid organs have four main distinguishing features (Miller and Davies, 1964; Miller and Osoba, 1967). Firstly, they contain a mixture of T and B lymphocytes. Secondly, they usually show prominent cytological evidence of ongoing immune responses, e.g., the occurrence of germinal centers and plasma cells. Thirdly, they are reduced in size in animals raised in a germfree environment, i.e., where contact with exogenous antigen is reduced. Fourthly, lymphopoiesis in these organs occurs at a relatively slow rate.

The majority of cells in the secondary lymphoid organs are not sessile but migrate continuously from one organ to another via the bloodstream and lymph (Cottier et al., 1964; Ford and British, 1968). This can explain why treatment with anti-lymphocyte serum induces not only a marked lymphocytopenia, but also a profound cellular depletion of the secondary lymphoid tissues (Parrott, 1967). The proportion of T and B lymphocytes in these tissues varies considerably. In rodents, most cells in the spleen and Peyer's patches are B cells (50-60%), while T cells are the most predominant cells in lymph nodes (60-70%) (Raff, 1971; Sprent, 1977). The virgin B cells settle predominantly in the lymphoid follicles, while T cells mainly occur in the periarteriolar lymphatic sheath (PALS) of the splenic white pulp, the paracortex of the lymph nodes and the interfollicular areas of the gut-associated lymphoid tissue (McGregor and Gowans, 1963; Goldschneider and McGregor, 1968; Dineen and Adams, 1970; Sprent, 1973). The B cells can proliferate and differentiate into antibody producing plasma cells. This process starts at the intermediate of the B and T cell areas. The produced plasmablasts migrate into the medullary cords of the lymph nodes and the red pulp of the spleen. While migrating into the medullary cords and the red pulp, the plasmablasts mature into plasma cells (Van Ewijk et al., 1977).

B lymphocytes are a prominent feature of the cortex of lymph nodes and the white pulp of the spleen. In unstimulated secondary lymphoid organs they are present as spherical collections of cells termed primary nodules. After antigenic challenge they form secondary follicles, which consist of a corona or mantel of concentrically packed small B lymphocytes surrounding a pale staining germinal centre (Van Ewijk et al., 1977).

The site of generation of immune effector cells after immunization is determined by the route of administration of the antigen. Generally, after intravenous (iv) immunization, the immune reactivity, humoral or cellular, takes place in the spleen. After subcutaneous (sc) and intradermal immunization, on the other hand, the immune reaction is confined to the lymph nodes draining the site of antigen administration. Finally, after intraperitoneal (ip) immunization, the immune reaction takes place predominantly in the spleen, para-aortic and parathymic lymph nodes. After multiple iv immunization, antibody-producing cells appear not only in the spleen, but also in the bone marrow (Benner et al., 1974[d]). The capacity of bone marrow antibody formation coincides with, and is most likely dependent on, the occurrence of B memory cells (Benner et al., 1981[a]; Koch et al., 1981).

A major distinguishing feature of the effector cells involved in humoral and cellular immunity is that plasma cells keep localized in the lymphoid organs where they are generated, whereas effector T cells involved in cell-mediated immunity migrate out of the lymphoid organs to perform their function wherever they are needed in the body.

1.2.3. *B and T lymphocytes and their function*

It has already been mentioned that the small lymphocytes comprise of T lymphocytes responsible for cell-mediated immunity and the helper effect in the humoral immune response, and B lymphocytes whose progeny produces the circulating antibodies. The characteristic property of B lymphocytes in all vertebrate species is their easily detectable surface Ig. Each lymphocyte presents about 10^5 Ig molecules on its surface (Warner, 1974). The Ig on the surface of B lymphocytes acts as the receptor for antigen (Warner, 1974).

T cells are characterized by one or more surface antigens that are nowadays defined by a set of monoclonal antibodies. For the recognition of the human T cell antigens the monoclonal antibodies of the OKT and Leu series can be used (Reinherz and Schlossman, 1980; Engleman et al., 1981). In mice, Thy-1, Lyt-1 and Lyt-2 are the most characteristic surface antigens of T cells (Cantor and Boyse, 1975). Evidence that T cells are required for optimal B cell function was presented by Claman et al. (1966), who showed that antibody production to sheep erythrocytes (SRBC) in irradiated mice was greater following transfer of a mixture of T and B cells than in mice receiving either B or T cells. T cells can also suppress antibody production and thereby regulate the humoral immune response (Gershon, 1974). Helper T cells and suppressor T cells can regulate the humoral response at the level of antibody class, subclass, allotype and idiotype. Next to these two subpopulations, amplifier T cells and cytotoxic T cells ('killer T cells') occur (Cerottini et al., 1970; Feldman et al., 1977; Wolters and Benner, 1980). The amplifier T cells have been described to enhance the generation of active helper ('inducer') T cells (Feldman et al., 1977). The interaction of inducer cells with suppressor T cells is proposed to be involved in the feedback control of the humoral immune response (McDougal et al., 1979; Green et al., 1981).

The mechanism of feedback control by T cells is particularly relevant for the development and intensity of IgE hypersensitivity. This is apparent from the observation that selective elimination of helper and amplifier T cells abolished the suppression of the IgE antibody response (Watanabe et al.,

1977). It is generally accepted that prevention of B cell hyperreactivity is at least in part the consequence of inhibition of the T helper activity (Eardly et al., 1978). The generation of cytotoxic T cells (e.g., those which can lyse virus-infected cells and cells of non-identical organ and tissue transplants) is largely dependent on helper activity from helper T cells (Bach et al., 1976; Bach and Barbara, 1978). It is proposed that all different T cell subsets are already committed to their effector function before antigen contact. Each subset is characterized by a particular combination of cell surface antigens (Cantor and Boyse, 1975).

1.2.4. *Recirculation of lymphocytes*

As stated above, most lymphocytes are not confined to a particular lymphoid organ, but exchange between the various lymphoid tissues from one time to the other. Part of them are 'sessile cells' which merely circulate with the blood for some time or migrate via the bloodstream to another organ. The other lymphocytes recirculate, i.e., migrate from the blood to the lymph and back again to the blood (Gowans and Knight, 1964). When such cells again migrate to the lymph, they are called, by definition, 'recirculating lymphocytes'.

Migration from the blood to the lymph can occur via either of two pathways: via specialized venules with a high endothelium, the so-called post-capillary high endothelial venules (HEV) in the paracortex of the lymph nodes and via the capillary beds and the interstitial spaces of all other organs (Gowans, 1971). The great majority of all recirculating lymphocytes recirculate via the HEV (Gowans, 1971).

Within the lymphoid organs, different subsets of lymphocytes follow a characteristic migration pathway. This can be studied by iv injection of radioactively labeled lymphocytes and tracing the labeled cells at various intervals after injection. Such studies have revealed that, in the spleen, labeled T cells appear within a few minutes after iv injection in the marginal zone and the red pulp venous sinusoids (Sprent, 1977). From there the cells migrate to the PALS. The precise route by which T cells leave the spleen from the white pulp is not clear, although it may be via 'bridging channels' (pseudo lymphatics) connecting the PALS with the red pulp sinusoids (Mitchell, 1972). The lymphatic supply to the spleen is very limited in most species and the majority of cells probably leave via the splenic vein. In the lymph nodes, lymphocytes appear in the walls of the HEV soon after injection and from there they pass between (Schoefl, 1972) or through (Marchesi and Gowans, 1964) the endothelial cells to enter the paracortex. By some as yet undefined route, particular cells can migrate into the medullary sinuses, thus gaining access to the efferent lymphatics (Schoefl, 1972; Marchesi and Gowans, 1964).

Certain regions of the secondary lymphoid tissues are of normal cellularity in adult thymectomized, lethally irradiated, bone marrow reconstituted mice ('B mice') (Parrott et al., 1966; Van Ewijk et al., 1975). These 'thymus independent' areas include (a) the peripheral white pulp and primary follicles of the spleen; (b) the superficial cortex, follicles and medulla of lymph nodes; and (c) the follicles of Peyer's patches. Furthermore, the germinal centres in these regions are thymus-independent with regard to their cellular composition. In lymph nodes, iv administered B cells, like T cells, are first seen in the walls of the HEV (Sprent, 1977; Van Ewijk and Van der Kwast, 1980). The cells then migrate selectively to the primary follicles and the superficial cortex. Whether B cells reach these areas by penetrating the walls of the HEV where these vessels approach the follicles or, conversely, leave the HEV as the latter traverse the paracortex, is not clear yet. Later on, labeled B cells reach the medullary cords and by 24 hours they are also seen in the medullary sinuses (Sprent, 1977; Van Ewijk and Van der Kwast, 1980).

In the spleen, labeled B cells, like T cells, are first detected in the marginal zone and the reticulin sheaths surrounding terminal arterioles. From there the B cells migrate through the peripheral and central PALS to the peripheral region of the white pulp to accumulate in the follicles (Van Ewijk and Van der Kwast, 1980). Presumably, they leave the spleen via the splenic vein (Veerman and Van Ewijk, 1975; Anderson and Weiss, 1976).

In Peyer's patches, B cells probably gain access to the follicles via the HEV (Parrott et al., 1971). Like T cells, they appear to leave these structures through fine lymphatics. Both B and T lymphocytes can leave the lymph nodes via the efferent lymphatic vessels. Via these vessels and the thoracic duct they return to the blood stream. In this way lymphocytes are able to recirculate continuously between the lymphatics and the blood (Gowans, 1959; Ford, 1975). Although both B and T lymphocytes are able to recirculate, B lymphocytes traverse the peripheral lymphoid organs at the distinctly slower rate (Howard and Scott, 1972; Sprent, 1973; Ford, 1975). Most of the recirculating lymphocytes are long-lived cells, probably memory cells specific for antigens encountered in an earlier phase of life (Howard and Scott, 1972; Sprent, 1973; Ford, 1975).

1.3. *Antibody formation*

1.3.1. *Thymus-dependent and thymus-independent antigens*

Commonly, a distinction is made between two classes of antigens with respect to their requirement for T cell help to elicit an immune response: the thymus-dependent and the thymus-independent antigens. Thymus-dependent antigens, as are most conventional antigens, are the antigens which require collaboration between T lymphocytes and B lymphocytes to induce antibody production. Thymus-dependent antigens usually induce antibody formation of different classes and subclasses. The Ig-isotype distribution of the antibodies depends upon the type of antigen, the antigen dose, the route of immunization, whether or not an adjuvant is used and the availability of T cells (Wortis et al., 1969; Pritchard et al., 1973; Benner et al., 1973[c]; Benner and Van Oudenaren, 1976; Koch et al., 1981). Thymus-dependent antigens include heterologous erythrocytes, proteins and their haptened derivatives.

Thymus-independent antigens are the antigens which can initiate antibody production in the absence of T cell help. Some highly polymerized immunogens with repeated antigenic determinants, such as polymerized flagellin prepared from *Salmonella adelaide* (Feldman and Basten, 1971[a]), bacterial lipopolysaccharides (Andersson and Blomgren, 1971; Möller and Michael, 1971), polyvinyl pyrollidone (Andersson and Blomgren, 1971) and pneumococcal polysaccharides (Howard et al., 1971) are thymus-independent antigens. These antigens can provide both signals necessary to initiate the clonal growth and differentiation of B cells. One signal is provided by the antigenic determinants of the molecule, while the other is represented by a portion of the molecule directly capable of activating B cells irrespective of their antigenic specificity (Coutinho and Möller, 1974). This portion of thymus-independent antigens therefore acts as a mitogen.

The mechanism by which thymus-independent antigens induce antibody formation is still not completely clear, but the immune response to such antigens differs from the response to more typical thymus-dependent antigens in that the antibodies produced are largely confined to the IgM class (Koch et al., 1982[a], 1982[b]). Furthermore, little or no immunologic memory is engendered (Koch et al., 1982[a]). Substantial production of IgG and IgA antibodies is usually not induced by thymus-independent antigens, probably due to their simple structure and/or their failure to stimulate T helper cells. This fin-

ding indicates that T cells are required for the switch of IgM secreting cells to IgG and IgA secreting cells.

1.3.2. *Primary and secondary type responses*

Two types of adaptive immunological reaction may occur when antigen enters the body:

- The synthesis and release of free antibody molecules into the blood circulation and other body fluids. This type of immunity is called humoral immunity. The antibody molecules act, for example, by coating bacteria to enhance their phagocytosis and by combination with and neutralization of bacterial toxins.
- The production of sensitized T lymphocytes. These activated T lymphocytes might instruct macrophages to enhanced phagocytic and killing activity or by themselves act as the effector cells (T killer cells). This type of immunity is called cell-mediated immunity. It confers protection against microorganisms such as tubercle bacilli and viruses, which are characterized by their ability to live and replicate within the cells of the host.

After antigen has been injected for the first time, several days pass before detectable amounts of antibody appear in the serum. These few days comprise the latent or induction period. The length of the latent period is affected very strongly by the kind, dosage, and route of antigen administration, as well as by the age, species and general physical condition of the host (Cooper, 1982[b]). During the latent phase there is no identifiable antibody in the serum, but antibody secreted by single cells can be shown in the so-called plaque assay (Jerne and Nordin, 1963). As the latent period ends, the serum concentration of antibody gradually increases during the next few days, but it does not become very high. This is called the 'primary response'. The serum antibody concentration is often maintained for a few weeks and then starts to drop (Anderson and Dresser, 1972).

If rats are depleted from their small lymphocytes by chronic drainage of their thoracic duct lymph, they have a grossly impaired ability to mount a primary antibody response to antigens such as tetanus toxoid and SRBC, and to reject skin grafts (McGregor and Gowans, 1963). The immunological reactivity can be restored by injecting thoracic duct lymphocytes obtained from a syngeneic rat. The same effect can be obtained if, before injection, the thoracic duct cells are first incubated at 37°C for 24 hours under conditions which kill the large and medium size lymphocytes and leave only the small lymphocytes intact (Gowans, 1962; Gowans and McGregor, 1965). Thus small lymphocytes are necessary for the primary response to antigen.

When the same antigen is given for the second time, the remaining antibody from the primary response usually combines with the injected antigen. Consequently, the serum antibody concentration decreases. However, within 1 to 3 days after antigen injection usually a spectacular rise in the serum level of antibody can be detected. This accellerated response is called the 'secondary response'. Secondary antibody responses can often be 10 to 50 times greater than primary ones. All the conditions that govern the primary response also pertain to the secondary response. However, after reimmunization with the same dose of antigen the latent period is greatly abbreviated, the titer is significantly hightened, and the amount of antibody that is detectable is greatly increased (Cooper, 1982[b]).

Immunological memory, which is the underlying cause of secondary type responses, is carried by both T and B lymphocytes (Miller and Sprent, 1971; Benner and Van Oudenaren, 1976). Thus, in responses to thymus-dependent hapten-carrier conjugates both hapten-primed B cells and carrier primed T cells

are required to give maximal secondary responses (Mitchison et al., 1970). While T memory cells arise within one week after priming and can be elicited by very low doses of antigen, maximal development of B cell memory requires several weeks and higher antigen doses (Cunningham and Sercarz, 1971; Niederhuber and Möller, 1973). It has been shown that primed cells respond to a lower concentration of antigen than unprimed cells (Tao, 1968; Pierce, 1969) and that this probably reflects the greater avidity of memory cells. The high avidity of memory cells for antigen is due to selective clonal expansion of high-affinity receptor bearing cells during the primary response.

1.3.3. *Cooperation between T and B cells*

Interaction of antigen with immunologically competent T and B lymphocytes is the key event in the initiation of immune responses to thymus-dependent antigens. In the immune response to thymus-dependent hapten-protein conjugates T cells recognize determinants on the carrier molecule while B cells respond to the hapten determinants (Fig. 2).

Although the T lymphocytes do not by themselves secrete antibody they actively divide after contact with antigen (Oppenheim et al., 1966). This involvement of the T lymphocyte in antibody synthesis without itself producing antibody is to provide 'helper' factors to the B cells which helps the antigenic stimulation of these B cells to be more effective (Taussig, 1974[a]). When mice are thymectomized at birth or as adults and then given a heavy dose of total body irradiation followed by bone marrow transplantation, they show a diminished antibody response to heterologous antigens such as erythrocytes and serum proteins (Pritchard et al., 1973; van Muiswinkel et al., 1975). This deficient response, however, can be restored again by injecting either thymus cells or thoracic duct lymphocytes (Mitchell and Miller, 1968).

Murine T cells *or* B cells injected together with the antigen SRBC into irradiated recipient mice give only poor or modest antibody production. But when T and B cells are injected together, many times more antibody forming cells are induced by the antigen (Claman et al., 1966; Mitchell and Miller, 1968).

The mechanism of cooperation between T and B cells is not yet fully understood. They involve complex interactions between macrophages, T cells and B cells in which association of the carrier determinants with Ia determinants of the macrophages is crucial. Furthermore, the involvement of various soluble growth factors has been demonstrated. It has been proposed that the

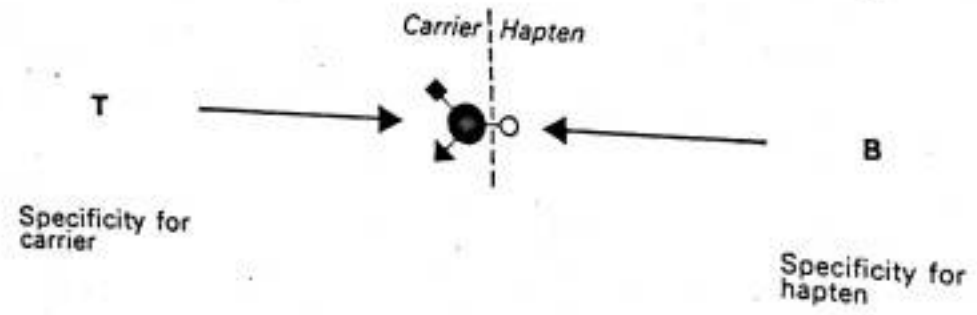


Fig. 2. Model showing the specificity of cooperating B and T lymphocytes for different determinants of a hapten-carrier conjugate.

sequence of events in the cooperation of T and B lymphocytes is as follows:
- binding of the antigen to the surface of macrophages or other adherent cells;
- recognition of this antigen in combination with macrophage Ia determinants by the receptors of helper T cells;
- induction of blastic transformation of these T cells and the release of 'helper factors' (B cell growth factor and B cell maturation factor);
- stimulation of B cells by antigen and the helper T cell-derived growth factor;
- clonal growth and differentiation of the stimulated B cells into antibody-secreting plasma cells under influence of the continued stimulation by antigen and helper T cell-derived growth and maturation factors (Farrar et al., 1982; Swain et al., 1982).

1.3.4. *Antigen presentation by macrophages*

Macrophages develop from blood monocytes (Van Furth and Cohn, 1968). One of the most important properties of macrophages is their ability to take up and degrade large amounts of foreign material. Most of the engulfed antigen is catabolized within 2-6 hours (Kölsch and Mitchison, 1968; Unanue and Askonas, 1968); 10% is retained for at least 2 days in macromolecular form. The macrophages or other adherent cells play an important role in both the induction and the expression of immune responses:
- macrophages can bind antigens either directly if sticky or indirectly if in the form of immune complex by way of their Fc and C3 receptors (Mandel et al., 1980);
- macrophages display Ia antigens on their surface and are therefore capable of participating, at least indirectly, in the regulation of immune responsiveness (David et al., 1973; Hauptfeld et al., 1973);
- macrophages carry receptors not only for B cell products (i.e., Ig) but also for T cell products (e.g., macrophage migration inhibition factor (MIF)) and lymphocytes themselves (Mandel et al., 1980; Schwartz et al., 1978);
- macrophages can ingest and degrade antigens, thereby altering their immunogenicity in a positive or negative sense (Feldman and Palmer, 1971[b]; Gallily and Feldman, 1967; Shortman and Palmer, 1971);
- macrophages can release products which can enhance and suppress immune responses (Liew and Parish, 1972; Allison, 1978; Smith and Ruscetti, 1981).

The central role of macrophages in antigen presentation to T lymphocytes is apparent from the observation that lymphocytes do not undergo blast transformation when exposed to mitogens or to antigens such as streptolysin-O, purified protein derivative (PPD) or vaccinia vaccine after passage over a glass bead column which removes the adherent cells (Hersch and Harris, 1968; Oppenheim and Rosenstreich, 1976; Smith and Ruscetti, 1981).

1.3.5. *Regulation by T suppressor cells*

T cells not only can enhance, but also can suppress antibody production and thereby regulate the humoral immune response (Gershon, 1974). Suppressor T cells regulating the antigen-specific IgM antibody response have been reported for thymus-dependent (Eardly and Gershon, 1975) and thymus-independent antigens (Baker et al., 1970, 1973). After immunization of mice with the thymus-independent antigen pneumococcal polysaccharide, the IgM response could be enhanced by pretreatment of the mice with anti-thymocyte serum (ATS) (Baker et al., 1970). For thymus-dependent antigens like keyhole limpet hemocyanine conjugated with trinitrophenyl (TNP-KLH) it was observed that *in vitro*

induction of antigen-specific suppressor T cells can be achieved by adding KLH to the culture. These cells were able to abrogate selectively the primary IgM response of normal spleen cells to DNP-KLH (Kontianen and Feldman, 1976).

Suppressor T cells can also regulate the IgG antibody response. Antigen-specific suppression of IgG antibody production by suppressor T cells has been observed in several mouse strains (Basten et al., 1974, 1975[b]). It has been shown that antigen-specific suppressor T cells regulate the avidity of IgG antibodies during a secondary humoral response against thymus-dependent DNP-conjugated proteins, both *in vivo* and *in vitro* (De Kruyff and Siskind, 1980; De Kruyff et al., 1980).

So far, no IgA-specific suppressor T cells have been found in mice. Human and mouse T lymphocytes, when stimulated by the polyclonal activator Concanavalin A (Con A) in culture, on the other hand, are able to inhibit the antibody response of various Ig classes (Dutton, 1975; Miyawaki et al., 1981; Rich and Pierce, 1973). In clinical studies it has been shown that certain patients with a selective IgA deficiency have T cells which can selectively prevent the production of IgA: coculturing of Con A activated T cells from such patients with normal lymphocytes caused a suppression of the IgA synthesis, whereas the IgM and IgG production remained unaffected (Waldmann et al., 1977).

The murine IgE response can be suppressed by two subsets of suppressor T cells, one is normally present in untreated mice and another arises after hyperimmunization. After adoptive transfer these two types of suppressor T cells act synergistically in suppressing IgE antibody formation (Itaya and Ovary, 1979). The suppression is mediated by non-specific suppressor T cells (Watanabe et al., 1976; Watanabe and Ovary, 1978). Mice of the SJL/J strain possess suppressor T cells that are not specific for antigen, carrier or hapten, and that selectively affect the production of the IgE class of antibody (Watanabe et al., 1976). These cells were found to belong to the Lyt-1+ subset of T cells (Watanabe et al., 1977).

1.3.6. *Helper and suppressor factors*

1.3.6.1. *Helper factors*

T cell helper factors are soluble products which can occur in supernatants and lysates of antigen-primed T cells. There are two types of T helper factors, one antigen-specific and the other non-specific (Klein, 1976). The non-specific helper factors possess helper T cell replacing activities for a variety of humoral immune responses. These T cell replacing factors (TRF) can be obtained from allogeneic mixed lymphocyte cultures (MLC) (Schimpl and Wecker, 1972), from antigen-stimulated cultures of primed cells (Waldmann, 1977) and from mitogen-activated normal lymphoid cells (Weicker et al., 1975). According to their physicochemical characteristics, these factors are not likely Ig (Hübner et al., 1978).

TRF obtained from allogeneic MLC cannot replace all helper T cell functions. For instance, after administration to DNP-primed B cells in culture, allogeneic helper factor does not help a secondary IgG response to DNP when the hapten is presented in a soluble complex with an irrelevant carrier-protein. Only if the antigen is present in particulate form (e.g., on the surface of macrophages or in a complex with antibodies) non-antigen-specific triggering of the IgG antibody response could be induced by this factor (North et al., 1977; Kemshead and Askonas, 1979). Another observation relevant in this context is that helper factors from allogeneically or Con A stimulated spleen cells can only enhance the IgM and not the IgG antibody production in nude mice to thymus-dependent antigens like heterologous erythrocytes and various hapten-carrier complexes (Kindred et al., 1979).

Helper T cells can also release an antigen-specific factor capable of triggering primed B cells *in vitro* (Feldman and Basten, 1972). This factor was restricted in the sense that only syngeneic B cells could be activated, and this factor could only exert its activity if it was administered to the culture together with the relevant antigen.

An *in vivo* active antigen-specific TRF has been described by Taussig (1974[a]). This factor is produced during *in vitro* incubation of primed T cells with synthetic antigens like (TG)AL (a heteropolymer of L-tyrosine, L-glutamine, L-alanine and L-lysine) and can be found in the supernatant within the first day of culturing. Via adoptive transfer experiments it was shown that the factor can help only those B cell clones which are specific for (TG)AL. It has been shown *in vivo* as well as *in vitro* that the latter factor is equally effective in inducing thymus-dependent antibody responses in syngeneic and allogeneic spleen cells (Taussig, 1974[a]; Taussig et al., 1975; Taussig and Munro, 1976; Howie and Feldman, 1977). Benacerraf and Germain (1978) have described that this factor bears determinants encoded for by the major histocompatibility complex (MHC).

Antigen-specific helper factors are characterized by their antigen-binding capacity and Ig-like determinants. This was concluded from the observation that a heterologous antiserum against mouse IgM can bind the factor (Howie and Feldman, 1977).

In vivo acting antigen-specific helper factor can also occur in the supernatant of allogeneically activated T cells. By injecting nude mice with such supernatants it was shown that the factor has the capacity to replace helper T cells during primary and secondary antibody responses of B cells to histocompatibility antigens, and not to antigens like SRBC (Kindred and Corley, 1977). Such a TRF from supernatants of allogeneically activated T cells was not active *in vitro* (Waterfield et al., 1979).

1.3.6.2. Suppressor factors

Not only helper T cells, but also suppressor T cells can secrete soluble factors. Many investigators have described that suppressor T cells inhibit the humoral immune response by releasing factors which suppress the functioning of helper T cells. Some of these suppressor molecules are genetically restricted in their action and have the capacity to bind the priming antigen. Others are non-specific and can suppress responses to several antigens and B cells from various mouse strains.

With respect to the antigen-specific factors, it has been shown that KLH specific suppressor factors can selectively suppress the secondary IgG antibody response to DNP-KLH by primed spleen cells *in vitro* (Taniguchi et al., 1976). The suppressor factor was not released from primed T cells by a short-term culture with antigen, but could be extracted from sonicated thymocytes and spleen cells from mice previously primed with KLH. The target of this T cell substance was the helper T cell since no effect was seen in the absence of helper T cells with identical specificity to that of the factor (Taniguchi et al., 1976). The characterization of this antigen-specific suppressor T cell factor revealed a heat labile nature, MHC determinants, no Ig determinants and genetic restriction of its activity (Tada et al., 1976; Taniguchi et al., 1976). Similar antigen-specific T cell derived suppressor factors for IgG antibody formation have been extracted from thymus and spleen of non-responder mouse strains which had been immunized with GAT (a heteropolymer of L-glutamine, L-alanine and L-tyrosine) or GT (a heteropolymer of L-glutamine and L-tyrosine) (Kapp et al., 1977; Waltenbaugh et al., 1977[a]). However, in contrast to the Tada factor, this T cell product mediates its suppressor activity by stimulating GAT or GT specific suppressor T cells (Waltenbaugh et al.,

1977[b]). The GAT/GT specific suppressor factor was found to be not strain specific (Waltenbaugh et al., 1977[a]) and to exert its activity only in association with the specific antigen (Kapp et al., 1977; Germain et al., 1978).

Antigen-specific suppressor T cell factors can also be obtained from hybridoma T cell lines. Such cell lines can be obtained by hybridization of thymoma cells and antigen-activated suppressor T cells. Such somatic cell hybrids have been found to release a suppressor factor in the supernatant with a specific combining site for the original stimulator antigen, and has MHC determinants and Ig-like determinants (Kontianen et al., 1978). This factor can selectively suppress the IgM and IgG antibody formation to DNP-KLH *in vitro*. Taussig et al. (1979) have described another type of antigen-specific suppressor T cell factor produced by a hybridoma T cell line. This factor inhibits the primary IgM antibody response against the specific antigen *in vitro*, has no Ig determinants, binds to the specific antigen and carries determinants of the MHC complex.

In the supernatants of cultures of T cells that had been primed previously with DNP-conjugated mycobacteria, IgE specific suppressor factors have been found. When injected into mice, such supernatants cause a profound inhibition of only the IgE production in the secondary anti-DNP antibody response. The active principle bears MHC determinants and is genetically restricted in its activity. It was shown that it can bind specifically to DNP-primed B cells, among them B cells bearing IgE on their cell membrane (Kishimoto et al., 1978).

Passively infused allogeneic cells and MLC supernatants have a similar capacity to cause a selective IgE suppression. Also in this case the suppressor substances were only effective in combination with syngeneic cells (Katz, 1979).

Nabel et al. (1981) have produced clones of a variety of T cell subsets which grow indefinitely *in vitro*. They described that all Lyt-2+ clones mediate suppressive activity and secrete a characteristic pattern of polypeptides that differ from Lyt-2- T cell clones. Cells of SRBC-specific suppressor T cell clones specifically bound glycophorin from SRBC. After incubation with ^{35}S-methionin-containing medium, the supernatant from this clone appeared to contain a biosynthetically labeled 70,000 mol.wt. protein that specifically bound to SRBC. This binding could be inhibited by glycophorin from SRBC but not from other erythrocytes. This antigen binding 70,000 mol.wt. peptide specifically and completely suppressed the *in vitro* primary anti-SRBC response by a mixture of primed Lyt-1+2- T cells and B cells. Suppression by this antigen binding peptide reflects the direct inhibition of helper T cell activity (Nabel et al., 1980; Fresno et al., 1981[b]).

Although the antigen binding peptide completely inhibited the *in vitro* primary response to a complex antigen, the suppression might reflect the combined biologic activities of many different 70,000 mol.wt. polypeptides or polypeptides associated with the 70,000 mol.wt. material by non-covalent interactions. The inactivation of helper T cells by the 70,000 mol.wt. molecule is rapid, specific and requires the presence of antigen. The mechanism of specific suppression of helper T cell function may depend upon two functionally distinct regions of the 70,000 mol.wt. molecule, one that binds antigen and a second that mediates suppression (Fresno et al., 1981[a]).

Papain splits the 70,000 mol.wt. molecule into two peptides: one with a 45,000 mol.wt. and another with a 24,000 mol.wt. The 45,000 mol.wt. peptide non-specifically suppresses antibody responses to several antigens and lacks antigen binding activity. The 24,000 mol.wt. peptide does not suppress but retains antigen binding activity. Thus papain splits the suppressor molecule into a 'variable' region responsible for antigen binding and a 'constant' region responsible for function. Since binding of the 70,000 mol.wt. molecule

to antigen results in the release of the 45,000 mol.wt. subunit, this cleavage may allow suppressor T cell molecules specific for one determinant to simultaneously suppress immunity to several antigenic determinants (Fresno et al., 1982).

1.4. *Cell-mediated immunity*

1.4.1. *Transplant rejection*

Replacement of defective or severely injured tissues and organs has been a medical objective as long as medicine has been practiced. The terms used for transplants between individuals and species are (Roitt, 1980):

- Autografts: tissue grafted back onto the original donor. Such transplantations almost invariably succeed and are especially important in treatment of burn patients (skin transplants).
- Isografts (syngeneic grafts): grafts between syngeneic individuals (i.e., of identical genetic constitution) such as identical twins or mice of the same pure inbred strain. Such transplantations also almost invariably succeed.
- Allografts (homografts): grafts between allogeneic individuals (i.e., members of the same species but of different genetic constitution), e.g., from man to man and from one mouse strain to another. Allografts usually do not succeed without additional immunosuppressive treatment of the recipient.
- Xenografts (heterografts): grafts between individuals of different species, e.g., rat to mouse. Just like allografts, xenografts usually do not succeed without additional immunosuppressive treatment of the recipient.

The major reason for graft rejection is a T cell mediated immune response to the cell surface antigens of the graft that are different from the host's cell surface antigens. The tissue antigens that induce an immune response in other individuals are called histocompatibility antigens and the genes that specify their structure are called histocompatibility genes. Two types of histocompatibility antigens can be discriminated, depending on the tempo of graft rejection. They are coded for by different gene families. The first category, genes of the MHC, specify histocompatibility antigens that induce rapid graft rejection. In man, the MHC is called the human leukocyte antigen (HLA) complex, in mouse the histocompatibility-2 (H-2) complex. The second category, minor histocompatibility genes, specify histocompatibility antigens that cause a slower graft rejection when acting alone.

Antigens encoded for by the MHC were originally discovered as the primary barrier to allograft transplantation (Snell and Stimpfling, 1966). Graft rejection was subsequently defined as a T cell response (Miller and Mitchell, 1967; Wortis, 1971). Two basic forms of cellular immunity probably play a central role in allograft rejection: development of delayed type hypersensitivity (DTH) and the generation of cytotoxic lymphocytes (Huber et al., 1976). Allogeneic skin grafts can induce a state of DTH to histocompatibility antigens in many species, including mice (Hoy and Nelson, 1969) and guinea pigs (Brent et al., 1962). After skin allografting in mice (Canty and Wunderlich, 1971) and rats (Peter and Feldman, 1972) graft specific cytotoxic lymphocytes are generated as well. Cytotoxic activity is usually assessed by measuring the release of ^{51}Cr from labeled target cells *in vitro* (Brunner et al., 1970; Canty and Wunderlich, 1971). In a comparative study of the cytotoxic and MIF activities of spleen cells during the primary response to alloantigens, Brunner and Cerottini (1971) found that both activities reach peak values at the same time. Furthermore, both activities could be abrogated by treatment of the spleen cells with anti-Thy-1 serum and complement. Huber et al. (1976)

found that DTH and cytotoxic responses are mediated by distinct T cell subclasses. The ability to generate graft specific DTH is confined to T cells with Lyt-1 determinants on their membrane. The cytotoxic activity to the allogeneic target cells, on the other hand, is build up and mediated by Lyt-23+ T cells (Cantor and Boyse, 1975).

Rejection of tissue and organ transplants may be caused not only by DTH effector T cells and cytotoxic T cells, but also by other mechanisms. Consideration of the different ways in which kidney allografts can be rejected may illustrate this point (Roitt, 1980):

Hyperacute rejection, within minutes of transplantation, characterized by sludging of red cells and microthrombi in the glomeruli, occurs in individuals with preexisting humoral antibody, either due to blood group incompatibility or presensitization through blood transfusion. About 80% of the patients who have preformed cytotoxic antibody to donor T lymphocytes in their sera reject their allografts immediately after transplantation. Some episodes of hyperacute rejection are due to the presence of antibodies directed against non-HLA endothelial antigens. Hyperacute antibody-mediated rejections are refractory to standard immunosuppressive therapy.

Acute early rejection, occurring up to about 10 days after transplantation, is characterized by dense cellular infiltration and rupture of peritubular capillaries and appears to be a cell-mediated hypersensitivity reaction involving T lymphocytes.

Acute late rejection, which occurs from 11 days onwards in patients suppressed with prednisone and azathioprine, is probably caused by the binding of Ig (presumably antibody) and complement to the arterioles and glomerular capillaries, where they can be visualized by immunofluorescent techniques. These Ig deposits on the vessel walls induce platelet aggregation in the glomerular capillaries leading to acute renal shut down.

In classic experiments, specific sensitivity to grafts can be transferred by lymphoid cells to syngeneic recipients but not by serum containing anti-donor antibody. Although a variety of mononuclear cell types are present in the rejection lesion of non-presensitized hosts (Strom et al., 1977), there is evidence that rejection of solid tissue allografts is in fact initiated by T lymphocytes. T lymphocytes have been shown to accumulate in graft infiltrates, and since allosensitized T cells produce target cell injury when confronted with cells bearing the antigens of the immunizing donor, it has been assumed that this is the primary mode of graft injury (Carpenter and Strom, 1980). Cellular rejection episodes are usually reversed by high dose corticosteroid therapy (Billingham et al., 1951; Bach and Strom, 1985[a]).

Insidious and late rejection of kidney transplants associated with subendothelial deposits of Ig and C3 on the glomerular basement membrane may sometimes be an expression of an underlying immune complex disorder that originally necessitated the transplant (Glassock et al., 1968) or, possibly, of complex formation with soluble antigens derived from the graft kidney.

1.4.2. *Delayed type hypersensitivity*

In appropriately immunized animals, cutaneous injection of antigen may elicit a slowly evolving inflammatory reaction known as DTH (Turk, 1980). At the site of a DTH reaction, the majority of infiltrating cells are nonspecific cells recruited by a small population of specifically sensitized T lymphocytes. Histological and adoptive transfer studies have demonstrated these points (McCluskey et al., 1963; Feldman and Najarian, 1963). Histologically, skin reactions of delayed onset are characterized by a local infiltration of mononuclear and polymorphonuclear cells (Lubaroff and Waksman, 1967; Schreier et al., 1982).

DTH is encountered in many allergic reactions to bacteria, viruses and fungi, in contact dermatitis resulting from sensitization to certain simple chemicals and in the rejection of transplanted tissues. The reaction is characterized by erythema and induration which appear only after several hours (hence the term 'delayed') and reaches a maximum at 24-48 hours, thereafter subsiding. The earliest phase of the reaction is seen as a perivascular cuffing of mononuclear cells followed by a more extensive exudation of mono- and polymorphonuclear cells. The latter soon migrate out of the lesion leaving behind a predominantly mononuclear cell infiltrate, consisting of lymphocytes and cells of the monocyte-macrophage series. This contrasts with the essentially 'polymorph' character of the Arthus reaction (Turk, 1980).

In mice, DTH reactions are generally elicited in a hind foot, by local challenge with the antigen that had induced the state of DTH. The DTH reaction is subsequently measured as the percentage increase of foot thickness, and is corrected for the non-specific swelling that can be elicited in non-immunized mice as well. A radioisotopic method has been introduced to measure murine DTH reactions more objectively. Yet the cell-bound radioactivity detectable at the site of challenge only gives a measure of the influx of nonspecific cells (Vadas et al., 1975). Also, the data obtained with this assay are not as reproducible as those obtained in the foot swelling assay.

In mice receiving, iv, a dose of antigen optimal for induction of antibody formation, no DTH reaction is detectable. In contrast, in mice receiving a dose of antigen too small to induce B cell activation, a DTH reaction is elicitable shortly and transiently after immunization (Lagrange et al., 1974[a]; Mackaness et al., 1974). A reasonable hypothesis is that shortly after their activation in T areas, specifically activated T cells recirculate through the blood until they encounter activated B cells and help them in the production of antibodies. On the basis of this hypothesis, if the dose of antigen is too small to activate B lymphocytes, activated T cells continue to circulate and are available transiently in the periphery, where they can be detected as effectors of DTH, if antigen is injected in the skin. Indeed, Milon et al. (1983) recently presented evidence that T cells activated by antigen can function as helper cells for B cells as well as DTH effector cells, depending on the microenvironment they reach during their migration. The absence of peripheral DTH reactivity in mice primed iv with a high dose of antigen (e.g., 10^9 heterologous red blood cells) would result neither from the absence of activation and clonal expansion of DTH mediating cells nor from induction of suppressive mechanisms, but would result from a decreased circulation of DTH mediating cells. Thus, DTH mediating T cells would disappear from the blood to enter the spleen only when specific B cells are present and activated by a high dose of antigen (Milon et al., 1983).

Cloned murine helper T cells (Bianchi et al., 1981) as well as cloned murine cytotoxic T cells (CTL) (Dennert et al., 1981) can mediate Jones-Mote type DTH reactions upon local transfer together with the antigen. Marchal et al. (1982) have claimed for cloned helper T cells that under highly sensitive conditions one cell is sufficient to create the specific DTH reaction and that every cell of a clone has the ability to induce such a DTH reaction.

Tuberculin type DTH in guinea pigs may be associated with a basophil-rich infiltration of the skin test site (Askenase et al., 1976). It has been suggested that antibodies as well as T cell factors play a role in the accumulation of this basophil-rich infiltration at the reaction site (Askenase et al., 1976). Convincing evidence exists that functionally short-lived T cells can perform an effector function in both Jones-Mote type DTH and the initial stage of tuberculin type DTH (Kettman and Mathews, 1975; Kettman and Turner Lubet, 1976; Askenase et al., 1977; Van der Kwast and Benner, 1977). Consequently,

it was proposed that after immunization for tuberculin type DTH short-lived blast cells would mediate initial DTH reactivity, while after longer periods long-lived effector cells would account for the DTH responsiveness (North, 1975; Lefford and McGregor, 1978). Persisting antigen or a high antigen dose would be required to drive the cells of a particular T cell subclass to become such long-lived cells (Askenase et al., 1977).

1.4.3. *Immunity to viruses*

A virus infection is the consequence of an imbalance between the infecting agent's virulence and the host's ability to limit viral spread. Clearing of infection may result from specific (immune response) and/or nonspecific mechanisms of defence. Defence mechanisms in either category can be classified further as:

- humoral, such as antibodies, complement and interferon;
- cellular, such as specific CTL and natural killer cells;
- combined humoral and cellular, such as antibody-dependent cell-mediated cytotoxicity (ADCC) (Casali and Michael, 1982).

Infection with *Ectromelia* virus results in rapid lysis of the infected cells. The virus disseminates via lymph and blood to the spleen and the liver. Recovery depends on control of virus growth in the liver parenchyma (Blanden, 1970). The recovery process starts between 4 and 6 days after infection and seems to depend mainly upon the presence of activated T cells, which recognize the viral antigen in the liver lesions (Blanden, 1970, 1971[a], 1971[b]). Macrophages present in the early infiltrate ingest and destroy infectious virus and necrotic tissue. Antibody does not appear in the circulation before the 7th or 8th day after infection and does not seem to play a role in recovery from primary infection.

Immune CTL can kill infected target cells by a single hit (Gardner et al., 1974[a]). By using ^{51}Cr-release assays, Gardner et al. (1974[b]) found significant cytotoxic activity for virus-infected target cells in the spleen of *Ectromelia*-infected mice as early as 2 days after infection. Maximal cytotoxic activity was attained by day 6.

CTL induced by virus have a dual specificity, i.e., they are specific for the virus which induced their generation as well as for the MHC antigens of the infected host. The latter is apparent from the observation that virus specific CTL lyse syngeneic virus infected target cells 30-300 times more efficient than allogeneic target cells infected with the same virus. Analysis of this restricted virus specific killing of infected target cells has shown that the restriction mapped to the H-2 complex and, more precisely, to the H-2K and H-2D region, but not to the H-2I region (Blanden et al., 1975; Koszinowski and Ertl, 1976).

The Blue-tongue virus (BTV) is an orbivirus affecting sheep, goats, cattle and wild ruminants. Stott et al. (1979) have suggested that a cell-mediated immune response is involved in protection against BTV re-infection and that sheep produce such a response on administration of an inactivated vaccine. BTV is known to be also highly effective in producing interferon (Rinaldo et al., 1975), a powerfull augmenter of natural killer cell activity (Trinchieri and Santoli, 1978; Gidlund et al., 1978; Welsh, 1978). The protective effect of both the humoral and the cell-mediated components of the immune system in BTV infections still awaits evaluation.

CTL actively killing *Influenza* virus infected cells are also able to induce a DTH skin reaction to homologous and heterologous type A *Influenza* viruses (Lin and Askonas, 1981). The variety of viruses that can be recognized suggests that virus specific CTL predominantly recognize certain viral proteins (Zweerink et al., 1977[a], 1977[b]; Ennis et al., 1977; Effross et al.,

1977; Zinkernagel et al., 1978). The major CTL recognition site on infected target cells is the viral hemagglutinin (HA) (Finberg et al., 1979, 1981). Finberg et al. (1982) have recently shown that reovirus specific CTL primarily recognize one distinct antigenic domain on the HA. The CTL response is directed against the region of the HA that is also responsible for the neutropism and the binding to the neutralizing antibodies.

1.4.4. Killing of intracellularly growing bacteria and parasites

Activated macrophages are an important component in host resistance to intracellularly growing bacteria and parasites (Jones et al., 1975; Mauel et al., 1975; Kress et al., 1977). The elimination of such microorganisms by macrophages is dependent on activated T lymphocytes (Mackaness, 1969). Induction of this microbicidal activity *in vitro* does not require contact with lymphocytes, but is mediated by soluble products released by the activated T lymphocytes (Fowles et al., 1973; Godal et al., 1971; Borges and Johnson, 1975; Nogueira and Cohn, 1978). The degree of microbicidal activity was found to be dependent upon both the lymphokine concentration and the time of incubation with the lymphokine.

Macrophage activating factor (MAF), a lymphokine that is produced by e.g. Con A stimulated spleen cells, can enhance the capacity of mouse peritoneal macrophages to kill *Schistosoma Mansoni schistosomulum*, a multicellular helminth parasite (Bout et al., 1981). The conditions required for activation of the macrophages to enable them to kill the *Schistosomula* were comparable with those described for cytotoxicity against microorganisms (Ruco and Meltzer, 1978; Nacy and Meltzer, 1979). Resident macrophages were not able to kill *Schistosomula*, but inflammatory macrophages were shown to be highly cytotoxic. Macrophages from mice treated with *Bacillus Calmette-Guérin* (BCG) or *Corynebacterium parvum* were able to kill *Schistosomula* as well (Mahmoud et al., 1979). Apparently the microbicidal activity of macrophages is by itself not antigen specific.

The immune response to the intracellularly growing bacterium *Listeria monocytogenes* has been studied in detail. Unanue et al. (1980) has described some of the consequences of the interaction between this bacterium and macrophages:

- secretion of biologically active molecules by macrophages;
- proliferation of T lymphocytes;
- acquisition of microbicidal properties by macrophages.

Hahn and Kaufmann (1982), who have also studied immunity to *Listeria monocytogenes*, propose the following sequence of events: *Listeria* specific Lyt-1+ T lymphocytes interact with Ia associated *Listeria* antigen on macrophages which leads to the production of Interleukin 1 (IL-1 = lymphocyte activating factor) by these macrophages. The IL-1 and *Listeria* antigens together activate the *Listeria* specific Lyt-1+ T lymphocytes that then produce Interleukin 2 (IL-2). The antigen and IL-2 bring about the recruitment and/or activation of Lyt-1+ T lymphocytes from the Lyt-1+2+ T lymphocyte pool. Proliferation of Lyt-1+ T lymphocytes takes place in parallel and the complex of reactions result in the secretion of lymphokines. These in turn attract macrophages and activate them for greater bactericidal activity (Weir and Blackwell, 1983).

Zinkernagel et al. (1977) have shown that effector T cells involved in the protection against *Listeria* are specific for the *Listeria* antigens in association with H-2I subregion coded histocompatibility antigens. These effector T cells are non-lytic, but produce several lymphokines that attract phagocytic cells. The similarity of these T cells with those involved in DTH reactions (North, 1970) has already been mentioned. H-2I region encoded determinants are thought to be expressed on a few cell types only, including lympho-

cytes, macrophages, sperm and epidermal, endothelial and mammary gland cells (Klein, 1982).

Injection of bacterial lipopolysaccharide (LPS) can increase as well as suppress resistance to several unrelated microorganisms, depending on the timing of the infection. LPS given 24 hr earlier reduces the extent of infection as measured by bacterial multiplication in the liver and spleen, thus enhancing non-specific resistance (Galelli et al., 1981). At the same time the protected mice show a depressed DTH reaction to *Listeria* antigen, depressed DNA synthesis in the spleen and depressed immune lymphocyte function. Thus, despite interference with lymphocyte function the anti-*Listeria* microbicidal activity of the macrophages induced by the LPS appeared to predominate (Galelli et al., 1981).

In conclusion, in defence against intracellularly growing bacteria and parasites, antigen-specific effector T cells release lymphokines that activate macrophages to increase their microbicidal activity. This microbicidal activity by itself is not antigen specific.

1.5. *Effects of corticosteroids on the immune system*

1.5.1. *General aspects*

The major natural glucocorticoid in rodents is corticosterone, whereas the major natural glucocorticoid in man is cortisol (Peterson, 1959). Among the numerous synthetic steroids which have been made available, mainly as anti-inflammatory agents, a few of them also possess immunosuppressive activity. The main common characteristics of all immunosuppressive steroids include an unsaturated cyclopentanophenanthrene-ring, a ketone at the 3rd C-position, O or OH in 11, a ketone and an alcohol function in 20 and 21 (Newson and Darrach, 1956) (Fig. 3). Not all compounds show the same relative anti-inflammatory, immunosuppressive and sodium retention potency. However, whereas plenty of data are available concerning the relative degree of anti-inflammatory and mineralocorticoid actions (c.f. Thorn and Lauler, 1972), there are few data concerning the relative immunosuppressive effects of corticosteroids.

The main products which have been used in immunological studies are the natural steroids hydrocortisone (cortisol) and cortisone and the synthetic compounds prednisone, prednisolone, methylprednisolone (Medrol®) and dexamethasone (Decadron®). The various corticosteroid preparations have been found to have different half lives *in vivo* (Table 1). Since also the anti-inflammatory activity greatly differs (Table 2), it is difficult to compare data from studies that employed different corticosteroids.

Table 1. Half lives of some corticosteroids in vivo

Cortisol	90 min
Cortisone	30 min
Prednisone	60 min
Prednisolone	200 min
Dexamethasone	200 min
Corticosterone	60-80 min

From Baxter and Forsham, 1972.

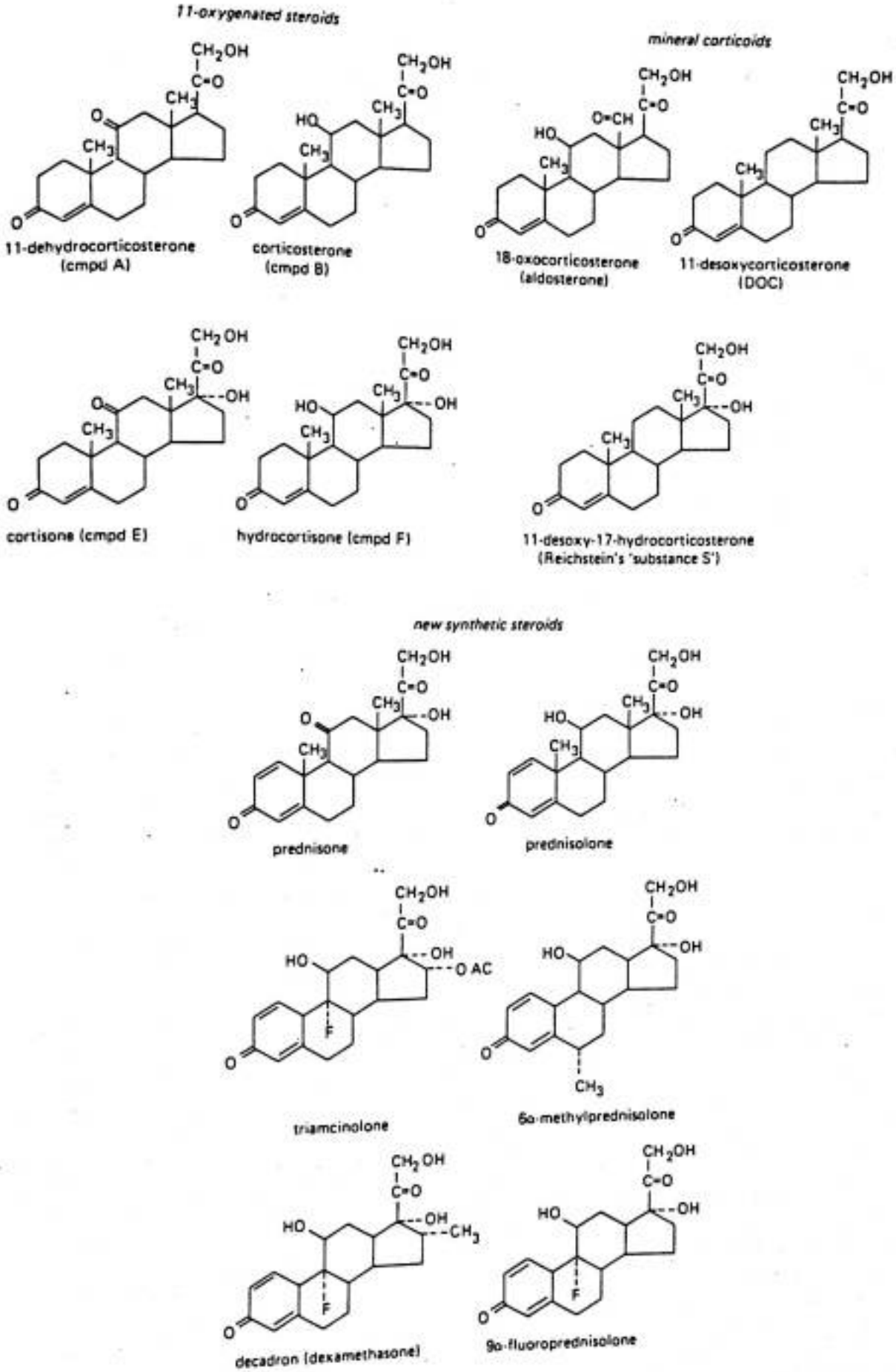


Fig. 3. Formulae of natural and synthetic steroids.

Table 2. Dose equivalences of some corticosteroids

	Anti-inflammatory activity	Dose equivalence
Hydrocortisone	1	20 mg
Cortisone	0.8	25
Prednisone	3.5	5
Prednisolone	4	5
Methylprednisolone	5	4
Triamcinolone	5	4
Paramethasone	10	2
Betamethasone	25	0.6
Dexamethasone	30	0.75

From Thorn and Lauler, 1972.

Cortisone and hydrocortisone acetate have been mainly used for *in vivo* studies, whereas prednisolone and hydrocortisone hemisuccinate have been generally used in *in vitro* studies because they are much more soluble than acetates. Conversely, because of their lower solubility, acetates have a depot effect and thus are more suitable for *in vivo* studies (Bach and Stron, 1985[a]). Dexamethasone sodium phosphate (DEXA), which is a well-soluble synthetic corticosteroid, is frequently used in man as well as in experimental animals and *in vivo* as well as *in vitro*.

Dependent on the extent of lymphoid cell depletion by corticosteroid injection, Claman (1972) has divided animal species into two groups: corticosteroid sensitive species (hamster, mouse, rat, rabbit) and corticosteroid resistant species (ferret, monkey, guinea pig, man). In corticosteroid sensitive species, the most dramatic result of the systemic administration of corticosteroids is the rapid decrease of thymic weight (Shewell and Long, 1956). Furthermore, the corticosteroid treatment causes lymphocytopenia (Quittner et al., 1951; Branceni and Arnason, 1966) and shrinkage of the spleen and lymph nodes.

Lymphoid cells of corticosteroid resistant species are quite resistant to the lytic effects of pharmacologic concentrations of corticosteroids (Claman et al., 1971; Claman, 1972). In guinea pigs, cortisone acetate given intramuscularly twice daily for seven days at a dose of 40 mg/kg BW produced only 37 per cent reduction in thymic weight (Blau et al., 1968). *In vitro*, however, several immature human lymphoid subsets are readily lysed by upper physiological and pharmacological concentrations of cortisol (Galily, 1983). Chronic lymphocytic leukemia cells and malignant cells from part of the acute lymphoblastic leukemia patients were also found to be highly sensitive to the *in vitro* cortisol induced lysis. The leukemic cells from all acute and chronic myeloid leukemias and from some acute lymphoblastic leukemia patients, however, are completely resistant to cortisol-induced lysis, even at supra-pharmacological levels of the hormone. The lysis of the sensitive cell populations is specifically induced by glucocorticoids but not by other steroid hormones (Galily, 1983). The cytolytic action of DEXA on mouse thymocytes *in vitro* could be blocked by cycloheximide, an inhibitor of protein synthesis (Durant and Homo-Delarche, 1983). This suggests that corticosteroids control the synthesis of some protein(s) involved in the triggering of cell lysis.

A frequently observed effect of corticosteroid administration is the induction of lymphocytopenia, due to 'redistribution' of circulating lymphocytes to other lymphoid compartments (Fauci, 1973[a], [b]; ...ise

mechanism of corticosteroid induced redistribution of cells has not been clearly defined so far. The traffic of (re)circulating lymphocytes is dependent on the endogenous corticosteroid level (Thompson et al., 1980; Abo et al., 1981; Kawate et al., 1981).

The effects of corticosteroids upon cells are mediated via glucocorticoid receptors. The following sequence of events has been proposed (Fig. 4):

- the corticosteroid passes the cell membrane and binds to a tissue-specific cytoplasmic glucocorticoid receptor;
- the corticosteroid-receptor complex enters the nucleus in an activated form;
- binding to the target cell DNA with subsequent increased synthesis of specific messenger RNAs.
- increased synthesis of specific proteins with subsequent expression of the corticosteroid effect on the target cells (Chan and O'Malley, 1978).

Specific cytoplasmic glucocorticoid receptors have been identified in normal human lymphocytes (Lippman and Barr, 1977), monocytes (Werb et al., 1978), neutrophils and eosinophils (Peterson et al., 1981). On the basis of the amino acid sequence of the human glucocorticoid receptor deduced from the cloned cellular DNA (Hollenberg et al., 1985), the locations of functionally and immunologically important regions of the protein have been proposed. These include an immunological domain located in the amino-terminal half of the protein, a DNA-binding domain that shows structural similarities with other DNA binding proteins, and the glucocorticoid-binding site localized near the carboxyl terminus of the molecule (Weinberger et al., 1985; Giguère et al., 1986).

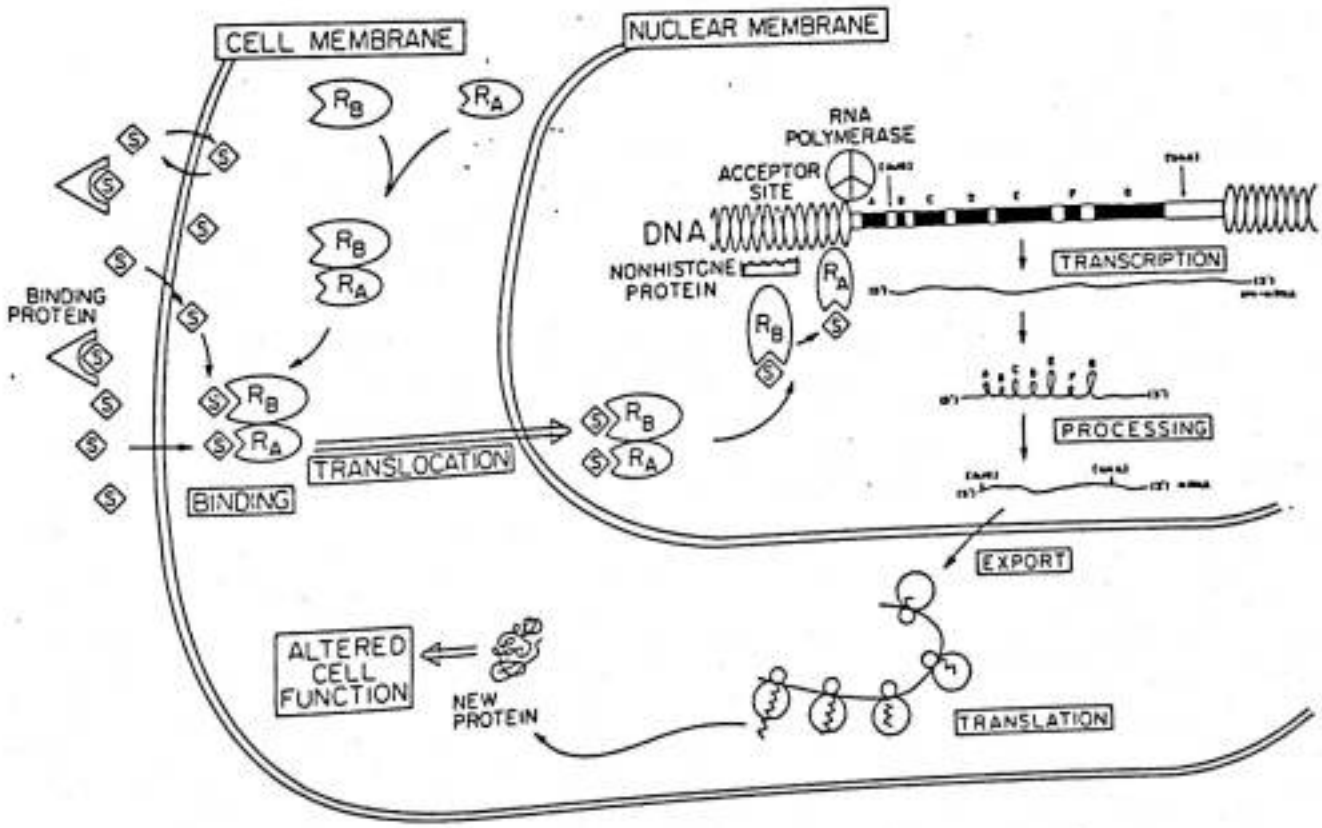


Fig. 4. Molecular mechanism of steroid hormone action. From Chan and O'Malley (1978).

Mitogen stimulated lymphocytes in the S-phase and post S-phase of the cell cycle have a 2 to 3-fold increased number of glucocorticoid receptors (Crabtree et al., 1980).

Corticosteroid resistance in man has been shown to be associated with a decreased density of the glucocorticoid receptor in peripheral tissues (Chrousos et al., 1983). There is evidence that corticosteroids can modulate their own receptor concentration in tissue culture (Cidlowski and Cidlowski, 1981; Lacrois et al., 1984; Svec and Rudis, 1981) as well as *in vivo* (Loeb and Rosner, 1979; Dahlberg et al., 1981; Mayer and Rosen, 1978). Excess corticosteroids have been shown to decrease glucocorticoid receptors in both the hippocampus (Tornello et al., 1982) and the pituitary (Svec and Rudis, 1981), areas thought to be involved in glucocorticoid feedback regulation of the hypothalamic-pituitary-adrenal axis (Sapolski et al., 1984). Several studies have reported that corticosteroid administration also decreases glucocorticoid receptor content in human lymphocytes (Bloomfield et al., 1981; Schlechte et al., 1982), but others have found no effect of Cushing's syndrome or exogenous corticosteroid administration on glucocorticoid receptors in lymphocytes (Junker, 1983; Kontula et al., 1980; Brentani et al., 1986).

Several authors have postulated that corticosteroid resistance might be associated with a decrease of the number of cellular glucocorticoid receptors (Hollander and Chiu, 1966; Rosenau et al., 1972; Lippman, 1973; Kaiser et al., 1974). This concept, however, is questionable since it has been demonstrated that corticosteroid resistant cells can have as many receptors as sensitive ones (Sibley and Tomkins, 1974; Duval et al., 1976, 1977; Lippman et al., 1974; Lippman and Barr, 1977; Smith et al., 1977; Waddell et al., 1977).

Corticosteroids have been extensively employed as therapeutic agents in a variety of inflammatory and immunologically mediated diseases (Fauci et al., 1976; Schreiber, 1977; Rees and Lockwood, 1982). These agents have been clearly demonstrated to cause several anti-inflammatory and/or immunosuppressive effects including decreased migration of cells into inflammatory sites (Rebuck and Mellinger, 1953; Boggs et al., 1964), circulating lymphocytopenia (Fauci, 1974, 1975[a]; Yu et al., 1974, Webel et al., 1974) and monocytopenia (Fauci and Dale, 1974; Fauci, 1975[a]; Yu et al., 1974), decreased phagocyte function (Fauci, 1979; Becker and Grasso, 1985; Shezen, 1985) as well as decreased Ig levels (Butler and Rossen, 1973). The principle mechanism whereby corticosteroids suppress inflammation is impeding the access of neutrophils and monocytes to an inflammatory site. This has been quantitated in many species (Allison et al., 1955; Perper et al., 1974).

Corticosteroid administration produces neutrophylic leukocytosis that reaches a peak 4 to 6 hours after the drug is given (Bishop et al., 1968; Dale et al., 1975). The neutrophylic count rise is attributable to at least two processes: their accelerated release from the pool of mature neutrophils in the bone marrow and their reduced egress from the blood to inflammatory sites (Bishop et al., 1968).

The capacity of corticosteroids to stabilize lysosomal membranes and thereby to inhibit lysosomal enzyme release may be an important aspect of the anti-inflammatory property of corticosteroids (Weissmann and Thomas, 1968). Since lysosomal enzymes are involved in the destruction of material ingested by macrophages, corticosteroids may also influence the capacity of macrophages to digest and hydrolyse phagocytosed material (Schreiber, 1977).

Corticosteroids have a direct effect in antagonizing bronchoconstriction by histamin and slow-reacting substance-A (Goldyne, 1982). Furthermore, corticosteroids can influence the binding of IgE and IgG immune complexes to the mast cell or platelet membrane receptor site and thereby inhibit the dis-

charge of pharmacologically active mediators (Schreiber, 1977). Corticosteroids also inhibit the histamin release by basophils (Schleimer et al., 1981).

Corticosteroids are also effective in the therapy of both IgG induced autoimmune hemolytic anemia (Atkinson and Rosse, 1980) and immune thrombocytopenic purpura (Branehög and Weinfeld, 1974; McMillan et al., 1976; McMillan, 1981). Their mechanism of action may be related to:

- decreasing the synthesis of IgG antibodies (McMillan et al., 1976);
- elution of the IgG antibody from the red blood cell or platelet membrane;
- suppressing the clearance of IgG coated erythrocytes and platelets, probably by interfering with macrophage IgG and C3 receptor function (Schreiber, 1977; Atkinson and Rosse, 1980) and phagocytic activity (Fauci, 1979; Becker and Grasso, 1985; Shezen et al., 1985).

Corticosteroids are known to be also effective in immunoproliferative disorders characterized by the accumulation of immature lymphoid cells, e.g., common acute lymphocytic leukemia and null cell acute lymphocytic leukemia (Henderson, 1969; Mauer, 1980).

Corticosteroids have been demonstrated to decrease fibroblast proliferation and to inhibit collagen fibril production, the ingrowth of blood vessels and the production of mucopolysaccharides, all necessary in the wound-healing process and in the formation of granulation tissue at the inflammatory site (Germuth, 1956; Lorenzen, 1969).

1.5.2. *Effects on mononuclear cells*

There are two distinct intravascular populations of lymphocytes. They are distinguished on the basis of circulatory and migratory capabilities and have been termed 'recirculating' and 'non-recirculating' lymphocyte populations (Gowans, 1959; Everett et al., 1964; Ford and Gowans, 1969). About two-third of the lymphocytes within the intravascular space belong to the recirculating lymphocyte pool. All cells in this pool can freely migrate or recirculate into and out of the intravascular space in constant equilibrium with the vast total body recirculating lymphocyte pool. The extravascular portion of the recirculating lymphocyte pool is distributed over the intercellular tissue fluid and the lymph in the thoracic duct and other lymph vessels and certain areas of the spleen and the lymph nodes (Gowans, 1959; Everett et al., 1964; Ford and Gowans, 1969). A small fraction of lymphocytes within the bone marrow parenchyma also belongs to the recirculating lymphocyte pool (Röpke and Everett, 1974; Fauci, 1975[c]). Many of these recirculating lymphocytes are long-lived T cells (Sprent, 1973).

A smaller portion of the intravascular lymphocytes belong to the 'non-recirculating' pool, so called because these cells are not readily capable of free migration and most likely live their whole life span within the intravascular space (Everett et al., 1964).

The precise mechanisms whereby the circulation of lymphocytes is altered and lymphocytopenia results from corticosteroid administration remain unclear. Corticosteroids may affect the endothelium of small vessels resulting in a change in the distribution pattern of cells passing through these vessels. However, it has been clearly shown that alteration of the molecular configuration of the surface of lymphocytes results in a striking change of their circulation pattern (Gesner and Ginsburg, 1964; Woodruff and Gesner, 1968). It is reasonable to assume that corticosteroid-induced effects on the lymphocyte surface may account for the redistribution of cells from the circulation to other body compartments (Fauci, 1975[a]).

Lymphocytes which normally recirculate between intravascular and extravascular compartments are readily redistributed out of the peripheral circu-

lation by corticosteroid administration, while the pattern of the non-recirculating, intravascular lymphocytes is relatively unaltered by corticosteroid therapy (Fauci, 1975[6]). Those lymphocytes that redistribute out of the circulation due to corticosteroid treatment accumulate in other body compartments such as the bone marrow (Cohen et al., 1970; Levine and Claman, 1970; Cohen, 1972).

In man, administration of a single dose of 400 mg hydrocortisone iv (Fauci and Dale, 1974), 60 mg prednisone orally (Yu et al., 1974) or 1 g methylprednisolone iv (Webel et al., 1974) causes a marked, but transient, lymphocytopenia that is maximal at 4 to 6 hours after administration. Also circulating monocytes, eosinophils and basophils decrease in number, reaching minimum values 6 hours after corticosteroid administration. Peripheral leukocyte counts usually return to normal by 24 hours, but occasionally, e.g., after massive doses such as 1 g of methylprednisolone (Webel et al., 1974), suppression of the leukocyte count may last up to 48 hours.

Corticosteroid treatment decreases the absolute number of both T and B cells in the peripheral blood. The circulating T lymphocytes are proportionally more decreased than the circulating B lymphocytes (Fauci and Dale, 1974; Fauci, 1975[6]; Yu et al., 1974). In humans, circulating helper T cells drop to a greater extent than circulating suppressor/cytotoxic T cells, thereby causing a decreased helper/suppressor ratio (Slade and Hepburn, 1983; Cupps et al., 1984). This is not due to a selective lympholytic effect since *in vitro* exposure of human lymphocytes to methylprednisolone does not result in significant lympholysis or in alteration of the relative numbers of helper and suppressor/cytotoxic T cells (Slade and Hepburn, 1983).

Monocytes are more sensitive than lymphocytes to the redistribution effect of low doses of corticosteroids (Hahn et al., 1980). The function of the monocyte in mixed lymphocyte reaction is particularly sensitive to inhibition by corticosteroids (MacDermott and Stacey, 1981).

Corticosteroids have long been known to exert a particularly drastic cytolytic effect on the thymus of corticosteroid sensitive species (Claman, 1972). Acute exposure *in vivo* and *in vitro* causes intrathymic cell death and rapid nuclear pyknosis as shown by vital staining and electron microscopy (Dougherty et al., 1964; Lundin and Schelin, 1969; La Pushin and De Harven, 1971; Alvares and Truitt, 1977; Wyllie, 1980) and progressive abrogation of protein synthesis, with eventual lysis (Burton et al., 1967; Trainin et al., 1974; Rothenberg, 1980). More than 90% of the lymphoid cells in the thymus are killed within 48 hours of treatment with pharmacological doses of cortisone, hydrocortisone, cortisol or DEXA (Dougherty et al., 1964; La Pushin and De Harven, 1971; Boersma et al., 1979). In morphological terms, the cortex of the thymus is especially sensitive to corticosteroids, whereas the medulla, which comprises less than 10% of the adult mouse thymus, is resistant (Ishidate and Metcalf, 1963; Van Ewijk et al., 1981). In terms of cell turnover rates, the short-lived cells in the thymus are more sensitive than the long-lived cells (Esteban, 1968).

Several studies have reported that T lymphocytes (Andersson and Blomgren, 1970; Cohen et al., 1970; Cohen and Claman, 1971; Moorhead and Claman, 1975; Lee, 1977) and accessory cells (Thompson and Van Furth, 1970, 1973; Balow and Rosenthal, 1973; Lee et al., 1975; Lee, 1977) are major targets of corticosteroid action and that their functional loss accounts for the major part of the immunosuppressive effects of these hormones. However, Dracott and Smith (1979) have shown that a single injection of mice with hydrocortisone affected B lymphocytes in the spleen, mesenteric lymph nodes and popliteal lymph nodes more severely than T lymphocytes.

Corticosteroids depress the bactericidal and fungicidal activity of macrophages *in vitro* (Rinehart et al., 1974, 1975; Schaffner, 1985) and suppress phagocytosis and intracellular digestion of endocytosed material (Becker and Grasso, 1985; Shezen et al., 1985). Since corticosteroids stabilize lysosomal membranes, it has been argued that corticosteroid-induced immunosuppression may be due to interfering with the antigen-processing function of macrophages (Zurier and Weissmann, 1973). Also the corticosteroid-induced suppression of antibody formation of mice to thymus-dependent antigens has been claimed to be mainly due to a dysfunction of the accessory cells (Mantzouranis and Borel, 1979).

The observation described in chapter 3 of this thesis that after a week of daily DEXA injections of mice the proportional decrease of cytoplasmic Ig-containing (C-Ig) cells is much greater than the proportional decrease of Ig-secreting plaque forming cells (PFC), suggests that the immunosuppressive effect of DEXA in mice is not only due to an effect upon peripheral B cells, but also to an effect upon helper T cells, either directly or via the antigen presenting accessory cells (Sabbele et al., 1983). Indeed, there is evidence that, in mice, the generation as well as the expression of helper T cell activity is affected by corticosteroids (Markham et al., 1978). More recently, the immunosuppressive effect of corticosteroids has been attributed to interference with IL-2 production (Ezine and Papiernik, 1984; Goodwin et al., 1986) and IL-2 receptor expression by T cells (Piccolella et al., 1986). See also section 1.5.3.

1.5.3. *Effects of in vitro corticosteroids on different stages of the immune response*

Pharmacologic levels of corticosteroids *in vitro* cause a striking lymphocytosis and reduction of cell viability in certain rodent species (Claman et al., 1971). However, mature human lymphocytes are relatively resistant to these direct cytolytic effects of corticosteroids (Caron, 1969; Claman et al., 1971). Corticosteroids added to cultures of human lymphocytes in concentrations that are not directly lethal to cells, do inhibit DNA, RNA and protein synthesis (White and Makman, 1967), proliferation induced by phytohemagglutinin (PHA; a T cell mitogen) and other lectins (Ono et al., 1968) and the mixed lymphocyte reaction (Roath and Cuppari, 1965; Heilman and Leichner, 1972). Activated lymphocytes are less sensitive to the immunosuppressive effect of corticosteroids than unactivated lymphocytes (Baxter and Harris, 1975). Other investigators (Larsson, 1980) have described that DEXA does not inhibit the triggering of antigen-sensitive cells, but suppresses the proliferation of T lymphocytes via the inhibition of IL-2 production. Recently, evidence has been presented that the inhibition of IL-2 production by corticosteroids is due to inhibition of the endogenous leukotriene B4 production (Goodwin et al., 1986). However, dependent on the dose employed, corticosteroids may also directly affect B and T cells. For example, *in vivo* generated CTL are sensitive to inhibition by high doses of corticosteroids, not only indirectly by their IL-2 dependence, but also directly (Schleimer et al., 1984).

It has been shown that pharmacological concentrations of corticosteroids displace the dose-response curves of PHA and pokeweed mitogen (PWM; a mitogen for B cells as well as for T cells) to the right, but under optimal conditions the same maximum response is achieved (Fauci et al., 1976; Gordon and Nouri, 1981), indicating selective inhibition at suboptimal levels of stimulation. Corticosteroids also inhibit the proliferative response of human lymphocytes stimulated by phorbol myristic acetate. This inhibition, however, was found to be not mitogen-dose dependent (Gordon and Nouri, 1981). These results sug-

gest that a corticosteroid-sensitive mechanism or lymphocyte subpopulation may be selectively activated by phorbol myristic acetate and low doses of PHA and PWM.

Blomgren (1974) has shown that human monocytes and media conditioned by them partially reversed the inhibitory effects of corticosteroids on the proliferative responses of human peripheral blood cells to PHA and PWM.

So far it is unclear whether the inhibition of the mixed lymphocyte reaction by corticosteroids (Roath and Cuppari, 1965; Heilman and Leichner, 1972) has a similar underlying cause as the inhibitory effect on mitogen stimulated cultures. It has been shown that corticosteroids decrease the expression of β2-microglobulin and HLA-A, -B and -C histocompatibility antigens on human peripheral blood lymphocytes *in vitro* (Hokland et al., 1981) which might lead to suboptimal stimulation of the responder cells. Recently, Dennis and Mond (1986) showed that iv injection of mice with 40 µg DEXA causes a 35 to 40% reduction of surface-Ia antigens on B cells within 3 hours. The maximum effect was found after 6 hours, which on average resulted in 75% suppression of control values of surface-Ia. By 12 hours after injection the values began returning towards baseline levels. The same effect was observed in athymic nude mice, indicating that the suppressive effect of DEXA on B cell expression of surface-Ia is not a T cell dependent phenomenon. Taken together, these data suggest that suppression of the surface-Ia expression by corticosteroids may be a means whereby endogenous or exogenous corticosteroids are able to influence the normal as well as abnormal immunologic state, and thus also may account for the inhibitory effect of exogenous corticosteroids on mitogen stimulated cultures and mixed lymphocyte reactions.

The PWM induced generation of Ig-secreting cells by human peripheral blood B lymphocytes could be markedly enhanced by physiologic and pharmacologic concentrations of hydrocortisone *in vitro* (Fauci et al., 1977; Paavonen, 1985; Goodwin and Atluru, 1986). This effect was seen only when hydrocortisone was added within the first 24 hours of culture and only when hydrocortisone and PWM were present together in the cultures (Fauci et al., 1977). Evidence has been presented that the corticosteroid induced stimulation of *in vitro* Ig synthesis by human mononuclear cells is due to inhibition of suppressor T cells (Knapp et al., 1982; Paavonen, 1985; Piccolella et al., 1985). The inhibition of suppressor T cells by corticosteroids might be attributed to preventing endogenous arachiconic acid metabolism, perhaps the endogenous production of leukotriene B4 (Goodwin and Atluru, 1986). Several investigators have reported that leukotriene B4 causes induction of suppressor T cells (Rola-Plezczynski et al., 1982; Atluru and Goodwin, 1984). Exogenous leukotriene B4 at 10^{-10} M inhibits PWM stimulated Ig production in cultures of human peripheral blood mononuclear cells, and this effect is mediated via activation of radiosensitive $OKT8^+$ T cells (Atluru and Goodwin, 1984). It may be that the leukotriene B4 normally produced by resting peripheral blood mononuclear cells induces similar suppressor cells and that inhibition of the generation of these suppressor cells by hydrocortisone is responsible for the stimulation of Ig production by this agent.

Only supraphysiologic concentrations of hydrocortisone (10^{-3} M) were capable of suppressing human B cell proliferation (Fauci et al., 1977; Cupps et al., 1985). The response may be mediated by a direct effect on B cells or antigen-presenting cells or as the proliferative response of the activity of or *in vitro* activation are while such *vitro* corticosteroids (Cupps within the *in vitro* cell cycle, namely, the dif- effects of the cells in the human B

tant to suppression by even these extraordinarily high concentrations of corticosteroids (Fauci et al., 1977; Cupps et al., 1985).

In vitro specific antibody production induced by antigens is highly sensitive to physiologic concentrations of hydrocortisone, in mice (Mishell et al., 1977) as well as in humans (Galanaud et al., 1981). The nonspecific response produced under the same conditions was found to be much less sensitive (Galanaud et al., 1981). It is unclear whether this selective depression of specific antibody formation is due to an effect of the corticosteroid on the specific B cells or the helper T cells involved. For murine T cells it has been shown that *in vitro* exposition to doses of DEXA equivalent to elevated physiologic concentrations abolish the suppressor T cell activity (Bradley and Mishell, 1981). In contrast, under the same conditions the helper T cell function was resistant to even pharmacologic concentrations of DEXA. The apparent corticosteroid resistance of the helper T cells was found to be mediated by the products of activated macrophages. While macrophage factors protected helper T cells from corticosteroid inhibition, they did not prevent the effects of DEXA on suppressor T cells (Bradley and Mishell, 1981). In man, naturally occurring suppressor cells as well as the induction of Con A-activated suppressor cells is sensitive to pharmacologic levels of corticosteroids only (Haynes and Fauci, 1979; Paavonen, 1985).

After activation by either specific antigens or nonspecific mitogens, T lymphocytes begin to secrete soluble products that are involved in cell-mediated immune responses. The effects of corticosteroids on mediator production by T lymphocytes must be separated from their effect on the interaction of the mediators with their target cells. Studies of the direct effect of corticosteroids *in vitro* have shown that the production of MIF (Balow and Rosenthal, 1973), MAF (Weston et al., 1973) and skin reactive factor (Pick et al., 1970) is normal. However, corticosteroids directly antagonized the effects of MIF and MAF on the target macrophage.

So far, little data is available about the effect of corticosteroids on interferon synthesis. Adolf and Swetly (1979) showed that the Sendai virus induced interferon production by human tumor lines is augmented by corticosteroids. Piccolella et al. (1986), on the other hand, showed that corticosteroids inhibit the synthesis of interferon by human peripheral blood mononuclear cells cultured with soluble microbial antigens.

A final property of T lymphocytes that seems to be affected by corticosteroids is direct cell-mediated cytotoxicity. The relatively little evidence available from *in vitro* studies suggests that low concentrations of corticosteroids protect target cells from T lymphocyte killer activity (Rosenau and Moon, 1962; Stavy et al., 1973), but do not alter the functional capacity of corticosteroid treated killer cells later tested without the drug (Stavy et al., 1973, 1974). Higher concentrations of corticosteroids, on the other hand, directly inhibit *in vivo* activated CTL (Schleimer et al., 1984).

1.5.4. *Effects of in vivo corticosteroids on different stages of the immune response*

While a variety of data are available about the effects of corticosteroids on human lymphoid cells *in vitro*, data about their effects on the *in vivo* immune response in man are relatively scarce. Therefore, most data presented below about the *in vivo* effects of corticosteroids on the immune response were obtained in studies of experimental animals, particularly mice.

Many studies have shown that corticosteroids can suppress both humoral and cellular immune responses in experimental animals such as the mouse, rat and rabbit (Claman, 1972). Until the early sixties, it was thought that corticosteroids act by means of nonselective lymphoid cell destruction. Later

studies, however, indicated that some of the cells involved in the induction and expression of immune responses are corticosteroid resistant. This was first shown in graft-versus-host (GvH) reactions by Warner (1964) who demonstrated that the few cells (about 5 per cent) which remained in the chicken thymus after cortisone treatment had virtually all the thymic GvH potential. Similar data have been reported for the mouse thymus, not only with regard to GvH reactive T cells, but also to helper T cells (Blomgren and Andersson, 1969; Andersson and Blomgren, 1970). Memory B cells in the mouse thymus were found to be rather resistant to corticosteroid treatment *in vivo* (Benner et al., 1974[b]). The resistant thymocytes have been shown to reside almost exclusively in the medulla (Ishidate and Metcalf, 1963; Van Ewijk et al., 1981). In the spleen about 75 per cent of the cells are corticosteroid sensitive. The resistant population contains all the spleen's GvH inducing potential (Cohen et al., 1970). Also helper T cells in the spleen are corticosteroid resistant (Cohen and Claman, 1971). Splenic B cells, on the other hand, are sensitive to hydrocortisone, since in adoptive transfer experiments the immunocompetence of spleen cells from hydrocortisone treated mice can be restored by bone marrow cells and not by thymocytes (Cohen and Claman, 1971). Bone marrow cells (at least while in the marrow microenvironment) are quite resistant to corticosteroid treatment (Levine and Claman, 1970; Cohen and Claman, 1971).

Corticosteroids have been demonstrated to depress DTH in guinea pigs (Harris and Harris, 1950) and mice (Dietrich and Hess, 1970; Fachet and Parrott, 1972) as well as in man (Salomon and Angel, 1961). This effect is probably due, in part, to the capacity of corticosteroids to suppress inflammation by decreasing mononuclear cell chemotaxis (Thompson and Van Furth, 1970) and capillary permeability and by improving vascular tone.

Corticosteroids can also suppress graft rejection (Hamburger et al., 1972). It therefore is widely used in clinical organ transplantation. Despite the rapid accumulation of new data concerning their mechanism of action, clinical use of corticosteroids remains largely empirical. A major concern for physicians has been the avoidance of their serious side-effects, which is mainly achieved by reducing the amount of corticosteroids that is routinely given (Fauci et al., 1976; Salaman, 1983). The effects of corticosteroids on organ graft rejection have recently been reviewed (Dupont et al., 1984).

It is unlikely that the beneficial effect of corticosteroid treatment in clinical organ transplantation is due to true immunosuppression, i.e. suppression of lymphocyte sensitization, since the clinical effect is generally obtained in a few hours, which is probably too short a time to believe it to be a true immunosuppressive action at the sensitization level. It might well be that the rapid beneficial effect of corticosteroids on rejection crisis is due to their effect on lymphoid effector cells and/or an anti-inflammatory effect on the mononuclear cell infiltrate, e.g. by decreasing the production of lymphokines (Dupont et al., 1985).

The extent of corticosteroid mediated suppression of thymus dependent antibody formation is dependent on the lymphoid organ studied as well as on the interval between the start of the corticosteroid injections and the moment of antigenic stimulation. Injection of mice with corticosteroids shortly *before* primary immunization with a thymus-dependent antigen markedly suppresses the anticipated antibody response (Björneboe et al., 1951; Germuth, 1956; Berglund, 1956, 1962; Gabrielson and Good, 1967). Injection of a depot preparation such as cortisone acetate shortly *after* antigen administration causes a gradual, progressive reduction of the serum antibody level (Gabrielson and Good, 1967).

Corticosteroids depress both IgM and IgG production, as studied at the

serum level or at the level of antibody-secreting PFC (Craddock et al., 1967; Petranyi et al., 1971). However, depression of IgM production is more difficult to achieve than depression of IgG production (Petranyi et al., 1971). The extent of immunosuppression afforded by corticosteroids decreases when the antigen dose increases (Berglund, 1956; Dietrich, 1966), which is in contrast to the findings reported for cyclophosphamide (Bach and Strom, 1985[b]).

Generally, secondary type antibody responses are much more resistant to the suppressive effects of corticosteroids than primary ones (Ward and Johnson, 1958; Blumer et al., 1962; Durkin and Thorbecke, 1971; Benner et al., 1978). Corticosteroids may affect secondary type responses during the priming phase, thus by inhibiting the generation of memory cells, as well as during the booster phase, thus by inhibiting the generation of antibody-forming cells from memory B cells. As far as thymus-dependent antigens are concerned, the generation of antibody-forming cells from memory B cells is fully dependent on helper T cells (Benner et al., 1974[a]). Therefore, corticosteroids may affect secondary type antibody responses via an effect on B cells as well as T cells.

A single injection of mice with a relatively large dose of hydrocortisone, when given at the moment of priming with a thymus-dependent carrier, prevented carrier specific helper T cell activity. Hydrocortisone was also found to decrease the helper T cell activity when given after such activity had been generated. However, under the same experimental conditions, suppressor and amplifier T cell activities were unaffected, even in this corticosteroid sensitive species (Markham et al., 1978).

Daily injection of DEXA starting one day before immunization with the thymus-independent antigen LPS suppressed the anti-LPS PFC response in the spleen, but markedly enhanced the bone marrow PFC response. Daily DEXA injection starting several days after immunization with LPS hardly affected the PFC response, neither in the spleen nor in the bone marrow (Benner and Van Oudenaren, 1979).

Daily injection of a high dose of DEXA starting either one day before or five days after the booster injection of the thymus-dependent antigen SRBC suppressed the anti-SRBC IgM, IgG and IgA PFC responses in spleen. The bone marrow anti-SRBC PFC responses, on the other hand, were found to be rather resistant (Benner et al., 1978), and did not increase as did the bone marrow PFC response to thymus-independent antigens (Benner et al., 1979). Daily injection of a dose as high as 16 mg DEXA per kg BW, starting one day before the booster injection, did decrease the bone marrow PFC response (Benner et al., 1978).

The ongoing Ig-synthesis is also decreased by corticosteroid treatment, although to a smaller extent than primary antibody responses. This has been shown for several species and has been established by measuring the serum Ig levels (see ahead). We have studied this aspect by quantitating the numbers of background IgM, IgG and IgA secreting cells in the lymphoid organs of corticosteroid treated mice (see Chapter 3). In man, however, single-dose and several days of *in vivo* corticosteroid treatment increases the numbers of 'background' Ig-secreting cells in the peripheral blood (Cupps et al., 1984).

1.5.5. *Effects on serum immunoglobulin levels*

As stated above, it has been shown that corticosteroid treatment can decrease the serum Ig level in humans (Wollheim, 1967; Butler and Rossen, 1973; Butler, 1975; Settipane et al., 1978). To our knowledge, similar data about experimental animals are hardly available.

It has been shown that a 5 day course of a high dose of methylpredniso-

lone decreased the level of serum Ig in normal volunteers. Particularly the IgG level was affected, with a maximum decrease of 22 per cent 2 weeks after treatment. Decrease of IgM and, sometimes, IgA levels was less clearcut. Recovery was seen 3 to 8 weeks after the end of the treatment (Butler and Rossen, 1973; Posey et al., 1978).

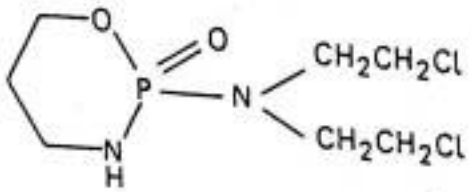
The decreased serum Ig levels after treatment with hydrocortisone is initially due to an increased catabolism of IgM, IgG and IgA (Levy and Waldmann, 1970; Griggs et al., 1972; Butler and Rossen, 1973). The decreased serum Ig level seen several weeks later, on the other hand, reflects a decreased rate of production (Butler and Rossen, 1973).

In man it has been shown that corticosteroid treatment not only can decrease the serum IgG and IgA levels, but also can increase the serum IgE level. After completion of corticosteroid therapy for an exacerbation of asthma, both total IgE and specific IgE increased dramatically, while the serum levels of the other Ig classes were depressed (Posey, 1978; Settipane et al., 1978). This different behaviour of IgE might be due to the fact that IgE production seems to be more sensitive to the regulatory suppressive effects of T lymphocytes than the other Ig classes (Saxon et al., 1980). The decrease in suppressor T cell activity after *in vivo* glucocorticosteroid treatment (Saxon et al., 1978) may account for this increase in serum IgE level. However, *in vitro* experiments employing purified B cells from atopic patients showed that corticosteroids also augment the 'background' IgE synthesis in the absence of suppressor T cells (Hemady et al., 1985).

1.6. *Effects of cyclophosphamide on the immune system*

1.6.1. *Pharmacology*

Shortly after its synthesis in 1958, cyclophosphamide (CY) was recognized as a potent cytotoxic agent with a high therapeutic index (Arnold et al., 1958). It is a phosphoric acid ester diamide (Fig. 5). Various analogues of CY have been used in experimental conditions such as iphosphamide and trophosphamide which for some tumors have shown a better therapeutic index than CY. It quickly became part of many multi-drug regimens for the treatment of solid tumors and leukemias. Its clinical use has widened to non-neoplastic diseases as well (Steinberg et al., 1972[a]; Gershwin et al., 1974; Bach and Strom, 1985[b]; Pirofsky and Bardana, 1977). Therapeutic benefits have been observed following prolonged administration of the drug in pathological situations where immune or autoimmune mechanisms are thought to be at play (Steinberg et al., 1971, 1972[b]; Fries et al., 1973; Steinberg and Decker, 1974; Decker et al., 1975; Townes et al., 1976; Donadio et al., 1978).



cyclophosphamide

Fig. 5. Formula of cyclophosphamide

CY is not active by itself but must be metabolized before becoming active (Foley et al., 1961). More precisely, CY must be split by a phosphoramidase which is present at high concentrations in certain malignant tissues (Gomori, 1948). This phosphoramidase, which splits P-N bonds, cleaves phosphorylated mustard, releases non-nitrogen mustard, and induces the appearance of biological activity. Such activation normally takes place mainly in the liver microsomes (Padgett and Colvin, 1972; Shand and Liew, 1980), through a process involving oxidation and O- and N-dealkylation (Shand and Howard, 1979; Shand and Liew, 1980).

Since CY needs to be metabolically transformed by liver microsomes to become lymphocytotoxic, it is essentially inactive *in vitro*. Therefore, for *in vitro* studies usually the biologically active CY metabolite 4-hydroperoxycyclophosphamide (4-HPCY) is used. This drug is also active *in vivo*, e.g. following local intradermal injection (Boerrigter and Scheper, 1984; Boerrigter et al., 1986).

It is thought that CY acts on cells by cross-linking the nuclear DNA and it is known that CY preferentially affects rapidly proliferating cells in the S-phase of the cell cycle (Turk and Poulter, 1972[b]). In fact, evidence for *in vivo* DNA cross-linking is scanty perhaps because the methods available to detect the cross-links are not sensitive enough. Cross-links can also be established between DNA and RNA or proteins. Cross-links will essentially mainly lead to cell death (Bach and Strom, 1985[b]).

1.6.2. *Effects on subpopulations of leukocytes in vitro*

Few studies deal with the *in vitro* activities of CY. As stated above, CY is essentially inactive, even at high concentration, unless it is activated by hepatic microsomes. This holds for both the cytotoxic and the functional effects. This can be accomplished *in vitro* (Connors et al., 1970). Sharma (1983), however, has noted some suppressive effect of CY on the mitogenic response of human lymphocytes *in vitro* at concentrations of 20 µg/ml and higher.

In vitro experiments have shown that the immunosuppressive effect of CY is not merely due to its cytotoxicity for certain mononuclear cell populations. Stevenson and Fauci (1980) have studied the differential effects of *in vivo* activated CY on subpopulations of normal human peripheral blood lymphocytes involved in the *in vitro* PWM induced PFC response against SRBC. It was found that the generation of PFC in this system is sensitive to the activated CY over a wide concentration range, including concentrations which have a minimal effect on overall cell viability. Similar data were obtained by Ozer et al. (1982) using 4-HPCY. Kinetic experiments revealed that activated CY exerts its inhibitory effect only if added very early in culture (Stevenson and Fauci, 1980). This might be due to its interference with Ig receptor expression since murine B cells incubated with activated CY *in vitro* lose their ability to re-express surface-bound Ig after capping with anti-Ig sera (Shand and Howard, 1978, 1979). Such cells when reinfused, are unable to mount antibody responses *in vivo*.

Stevenson and Fauci (1980) have also shown that when T cell enriched populations of peripheral blood lymphocytes are incubated overnight with a high concentration of activated CY and then added back in co-culture to fresh autologous B cells, the subsequent PFC response was significantly enhanced. This suggests that regulatory suppressor T cells are much more sensitive to the inhibitory effects of CY than helper T cells. Similar data have been presented by others, not only for human lymphocyte subpopulations, but also for murine lymphoid cells (Diamantstein et al., 1979, 1981; Kaufmann et al., 1980; Ozer et al., 1982). Precursors of suppressor T cells were found to be more

sensitive than already activated suppressor T cells (Kaufmann et al., 1979; Ozer et al., 1982; Klajman et al., 1984).

Hemopoietic progenitor cells (granulocyte/macrophage colony-forming units and erythroid burst-forming units) and pluripotent hemopoietic stem cells are sensitive to CY-derivatives *in vitro*. In general, a time- and dose-dependent effect on colony-forming unit survival was found, with a relative sparing of the pluripotent hemopoietic stem cells compared with the committed progenitors (De Jong et al., 1985).

1.6.3. *Effects on subpopulations of leukocytes in vivo*

CY is highly cytotoxic for lymphoid cells *in vivo*. A single injection of mice and guinea pigs with CY at doses of over 100 mg/kg BW drastically decreased cell numbers in spleen, thymus (Turk and Poulter, 1972[a]) and Peyer's patches (Chin and Hudson, 1970), while the cellularity of lymph nodes was moderately decreased (Turk and Poulter, 1972[a]). The minimum values for the spleen were about 10% of the original cell numbers and were reached on day 3 (Snippe et al., 1976). In the thymus, depletion of the cortex started earlier than depletion of the medulla, suggesting that CY selectively affects short-lived lymphocytes (Turk and Poulter, 1972[a]; Turk, 1980). The thymus weight was not normalized until day 21 (Schwartz, 1971; Turk and Poulter, 1972[a]). There was also a drastic decrease of the lymphocyte number in the peripheral blood, with slow recovery (Göing et al., 1970).

Turk and Poulter (1972[b]) have also studied the action of CY on rapidly dividing cells labeled with 5-iodo-2-deoxyuridine-^{125}J (IUdR). When CY was injected one or several days before IUdR it induced a rapid decrease in the number of labeled cells in all lymphoid tissues, but the greatest effect was on thymus and bone marrow. These data indicate that CY preferentially affects short-lived cells. The DNA synthesis as assessed by IUdR uptake by these tissues remained suppressed for 3 days in lymph nodes, spleen and bone marrow. Recovery was followed by a period of increased synthesis, returning to normal by 1¼ days, with an overshoot especially in the spleen (Turk and Poulter, 1972[b]; Kolb et al., 1977). DNA synthesis in the thymus remained suppressed for as long as 10 days (Turk and Poulter, 1972[b]).

Histopathologically, CY especially affects B cell areas in the lymphoid organs. Thus Turk et al. (1972; Turk and Poulter, 1972[a]) have shown that after injection of 300 mg/kg BW of CY into guinea pigs or C3H mice, there was in lymph nodes a specific depletion of follicles, medullary cords, germinal centers and corticomedullary junctions without any significant depletion of paracortical areas. Similar results have been reported for the spleen, the white pulp of which showed a more severe and longer-lasting effect on the B cell areas than on the T cell areas (Stockman et al., 1973). However, chronic treatment with daily doses of 10 or 20 mg/kg BW may induce depletion of thymus-dependent areas of lymph nodes and spleen in guinea pigs (Winkelstein, 1977).

When the number of Thy-1+ cells was determined in spleen, mesenteric lymph nodes and pooled peripheral lymph nodes of mice after 1, 2 and 3 sublethal injections of CY (300 mg/kg BW), a proportional increase of Thy-1+ cells was found in all lymphoid organs tested, reaching values of over 90% after 3 injections (Poulter and Turk, 1972). The proportional increase of Thy-1+ cells was greatest in the spleen. Marker studies have also shown a depletion of B cells in pigs (Mackie, 1981) and guinea pigs (Brouilhet et al., 1978). Also pre-B cells are destroyed by CY (Burrows et al., 1978).

The preferential effect of CY on the B cell compartment is compatible with its selective effects on short-lived lymphocytes (Turk and Poulter, 1972[b]; Turk, 1980) and the observation that B lymphocytes, on the mean, have

a shorter life span than T lymphocytes (Sprent and Basten, 1973; Sprent, 1977).

Later studies showed that CY also significantly affects T cells, not only after chronic treatment, but also after a single injection of a sub-lethal dose of CY (300 mg/kg BW) (Hunninghake and Fauci, 1976; Kolb et al., 1977). Long-term studies of patients with multiple sclerosis (Brinkman et al., 1983) and nephrotic syndrome (Feehally et al., 1984) have shown that CY selectively depresses OKT4+ helper T cells rather than OKT8+ cytotoxic/suppressor T cells. The effect was found to last 2 to 3 months after cessation of treatment.

Cupps et al. (1982) have shown that chronic low-dose (2 mg/kg BW/day) CY treatment of patients with non-neoplastic immune mediated diseases caused a total lymphocytopenia in the peripheral blood without affecting the relative proportions of B cells and T cells, indicating that both subpopulations were numerically affected to the same extent. The functional suppression of B cells as measured by PWM induced Ig secretion, however, was much greater than the quantitative depletion of cell numbers. T cell function, on the other hand, measured by *in vitro* blastogenic responses to the mitogens PHA, Con A and PWM, was not significantly suppressed (Cupps et al., 1982). In another study, however, a depressed *in vitro* blastogenic response to PHA was noted (Winkelstein et al., 1972). Also studies with guinea pigs have shown that CY treatment *in vivo* has a differential effect on the functional capabilities of different peripheral blood leukocyte subpopulations (Hunninghake and Fauci, 1976).

Treatment of mice with CY leads to decreased MLR (Milton et al., 1976; Smith et al., 1981) and CTL responses (Merluzzi et al., 1979) by their spleen cells *in vitro*. Detailed studies have shown that a single injection of a high dose of CY causes a diminution of the frequency of CTL precursors in the spleen to 10% of the original value as evaluated by a limiting dilution culture system (Taswell et al., 1979; Collavo et al., 1984). It is interesting to note that the inhibitory effect of CY on CTL generation is restored by adding helper T cells (Merluzzi et al., 1980; Varkila and Hurme, 1983), supernatant from mixed lymphocyte cultures (Merluzzi et al., 1981) and IL-2 (Merluzzi et al., 1983; Varkila and Hurme, 1983). All these data suggest that CTL precursors are more resistant to CY than helper T cells. Varkila and Hurme (1983), however, have presented evidence that treatment of mice with doses of CY as high as 150 mg/kg BW does not destroy helper T cells, but rather affect their *in vitro* restimulation, probably by destroying short-lived amplifier cells that synergize with helper T cells (Feldman et al., 1977; Benner et al., 1985).

Milton et al. (1976) have studied the kinetics of recovery of the *in vitro* proliferative activity of T cells from CY treated mice and the relationship between this and some *in vivo* immune responses. The proliferative response to PHA and allogeneic cells was depressed for up to 3 weeks after drug treatment in spleen and lymph node cells, responses recovering more rapidly in lymph node cells. It appeared that the *in vitro* proliferative activity is not a good indicator of the *in vivo* T cell functional capacity as indicated by the very rapid recovery of the ability to reject skin grafts and the fairly rapid recovery of the ability to produce cytotoxic cells compared to the slower recovery of *in vitro* T cell activities.

CY also depresses natural killer cell function in the mouse, an effect that can be corrected for by the administration of normal spleen cells (Riccardi et al., 1981). Limiting dilution analysis showed a substantial decrease of the number and frequency of natural killer cell precursors in mice treated with 10 mg/kg BW CY. The maximum effect was found 2 days after CY injection,

and a gradual recovery was achieved by day 12.

Remarkably, CY at a dose of 200 mg/kg BW can also induce suppressor cells which can suppress primary (Braciale and Parish, 1980) and secondary (McIntosh et al., 1979, 1982) *in vitro* antibody responses. These suppressor cells are adherent to nylon wool and resistant to treatment with anti-Ig serum and anti-Thy-1 antibodies and complement. They are generated in normal mice and in nude mice, and their suppressor activity is not H-2 restricted. Thus these suppressor cells are distinct from B cells, T cells, NK cells and macrophages (Segre et al., 1985).

Few data are available on the effects of CY on macrophage function. CY does induce a monocytopenia and abrogates the cellular reaction to thioglycollate (Hunninghake and Fauci, 1976; Buhles and Shifrine, 1977). CY does not affect macrophage glass adherence, phagocytosis and intracellular digestion of live *Candida* (Buhles and Shifrine, 1977). However, it does depress antigen trapping in the spleen of normal mice given aggregated human gammaglobulins, while it does not affect liver trapping or serum clearance in the same mice (Nettesheim and Hammons, 1970). High doses of CY may also inhibit antigen retention by lymphoid organs (Phipps et al., 1981).

CY can cause a profound neutropenia, both in experimental animals (Hunninghake and Fauci, 1976) and humans (Rodriguez et al., 1973; Atkinson et al., 1974). In guinea pigs treated with a single ip injection of 100 mg/kg BW CY, minimum values of neutrophils in the peripheral blood were found at day 5 (Hunninghake and Fauci, 1976). Circulating eosinophils are not depleted by high-dose CY treatment. After some days however, CY does cause a substantial eosinophilia, which is maximal at day 14 (Thomson et al., 1986).

1.6.4. *Effects on antibody formation*

CY is highly effective in suppressing antibody formation both in experimental animals and in immunologically mediated diseases. In experimental models, usually a large dose of the antigen is administered in combination with CY. Studies with mice have shown that there are substantial interstrain differences in the degree of suppression of antibody formation to SRBC (Pevnitsky and Smirnova, 1976). High or low reactivity to the antigen characteristic of different mouse strains, however, was not responsible for these differences (Pevnitsky et al., 1977). Apparently, the interstrain differences are due to different pharmacodynamics of CY in different mouse strains. Recent studies have shown that various mouse strains differ with regard to the activity of the liver microsomal enzyme system involved in the conversion of CY into its biologically active form. From a number of mouse strains studied, DBA/2 was found to be the most susceptible to the immunosuppressive activity of CY. The level of biologically active CY in these mice was higher than in any of the other mouse strains studied. Furthermore, their immunocompetent cells are more susceptible to the immunosuppressive effect of the activated CY (Pevnitsky et al., 1985). These data should be kept in mind when comparing data from studies on the immunosuppressive effect of CY employing different mouse strains or different animal species.

When comparing different immunosuppressants, CY appears to be one of the most potent suppressors of antibody formation in the mouse as well as in rats, guinea pigs, pigs and perhaps less regularly in humans (reviewed by Bach and Strom, 1985[a]). Antibody formation against all types of antigen including SRBC, protein antigens, bacterial antigens such as typhoid vaccine or *Brucella* antigens and tumor antigens have been suppressed, independent of whether it concerned agglutinating, hemolysing, precipitating or anaphylactic antibodies (Bach and Strom, 1985[b]). Furthermore, CY suppresses not only antibody formation to TD antigens, but also to TI antigens such as TNP coupled to lipopoly-

saccharide (TNP-LPS) (Smith et al., 1979) and pneumococcal polysaccharide (Howard and Courtenay, 1975; Shand and Howard, 1978; Howard and Hale, 1978; Havas and Schiffman, 1981).

The extent of suppression of antibody formation is dependent on the dose and immunogenicity of the antigen used. The lower the dose of antigen and the weaker the immunogenicity, the greater the immunosuppressive effect (Dukor and Dietrich, 1970). Similarly, when complete Freund adjuvant is used to potentiate the response towards the antigen, the immunosuppressive effect by CY is decreased or even absent (Maguire and Steers, 1963).

The extent of suppression of antibody formation is also highly dependent on the timing of drug administration. When given as a single dose, CY must be administered between day 0 and day 4 after immunization to induce the maximum suppression (Berenbaum, 1967; Dukor and Dietrich, 1970). When CY is given before the antigen or 4 days after, there is nearly no immunosuppressive effect, indicating a very rapid recovery of the cells affected by CY.

CY may have a differential effect on the formation of antibodies of different Ig (sub)classes (Chiorazzi et al., 1976; Kerckhaert et al., 1977; Drössler et al., 1981, 1983; De Macedo and Mota, 1982; Turk et al., 1984). The most consistent observation is the increase of IgE antibody formation that has been observed in mice (Chiorazzi et al., 1976; De Macedo and Mota, 1982) and guinea pigs (Turk et al., 1984) treated with CY around the time of immunization. This increased IgE antibody production has also been observed after low-dose total body irradiation, and is in both cases attributed to the elimination of suppressive T cells. Since CY also affects B cells, the enhancing effect of CY on IgE antibody formation is a summation between its effect on B cells and on the various T cell subsets, helper, suppressor and amplifier T cells.

The effect of CY on suppressor cells is most apparent in situations where the function of suppressor cells is normally predominent. Thus, CY may transform non-responding animals into responders, a phenomenon particularly well demonstrated by Debrè et al. (1976). These authors treated BALB/c mice, which are nonresponders for the antigen GT, with CY (200 mg/kg BW) 12 days before immunization with GT. Following this treatment the mice responded to GT with a substantial primary IgG antibody response. Similar results have been reported by Noble et al. (1977). Also IgE nonresponder Hartley strain guinea pigs can be converted into IgE high-responders by CY treatment (Graziano et al., 1981).

Primary antibody responses are more susceptible to CY induced suppression than secondary responses (Smith et al., 1979; Havas and Schiffman, 1981). Moreover, secondary responses are normal when the antigen is given without CY to an animal whose primary response has been suppressed by CY (Finger et al., 1969; Kool, 1973). A single injection of CY in the course of a secondary response can increase the IgG antibody production (Gagnon and MacLennan, 1979), but prolonged administration of CY does cause a small fall in the level of IgG antibody production (Gagnon and MacLennan, 1981). This fall, however, is rapidly reversed when the drug is withdrawn. These data suggest that the clinical benefit of CY treatment in diseases such as systemic lupus erythematosus (Steinberg and Decker, 1974), rheumatoid arthritis (Townes et al., 1976) and other inflammatory connective tissue disorders (Fauci et al., 1978, 1979; Steinberg et al., 1972[b]) in which autoimmune or immunologic phenomena are thought to play a predominant role, may not be due to the action of the drug on antibody producing cells or their precursors.

CY is one of the few immunosuppressive agents allowing the induction of tolerance to particulate antigens. Induction of tolerance by combined immunization and treatment with CY has been achieved for a variety of antigens in

mice, rats and guinea pigs (reviewed by Bach and Strom, 1985[b]). The tolerance is easier to induce and lasts longer with weak immunogens than with stronger ones (Dietrich and Dukor, 1968). Interestingly, tolerance for antibody production against horse erythrocytes in mice is associated with normal DTH against this antigen (Ramshaw et al., 1977).

CY induced tolerance may be relatively long-lasting (over 20-60 days) (Aisenberg, 1967; Miller and Mitchell, 1970) and can be transferred by spleen or lymph node cells to syngeneic recipients (Many and Schwartz, 1970; Aisenberg, 1973). Thymectomy increases the duration of tolerance significantly (Aisenberg and Davis, 1968), which is in contrast to the absence of the effect of thymectomy on the recovery from immunosuppression mentioned before. Transfer of normal spleen cells to tolerant mice does not break the tolerance where as ATS treatment does. The transfer of tolerance by spleen and lymph node cells to syngeneic mice is dependent on T cells, indicating that the tolerant state is mediated and maintained by suppressor T cells (Ramshaw et al., 1977), although in certain protocols both T and B cells may become tolerized (Jacobs et al., 1971; Talal et al., 1971).

1.6.5. *Effects on cell mediated immunity*

A variety of studies have shown that CY can suppress as well as enhance cell-mediated immune responses. The most extensive studies have been done on the effects of CY on DTH and contact sensitivity. The original studies showed that CY suppresses these T cell mediated reactions in a variety of species (reviewed by Bach and Strom, 1985[b]). In most of these studies CY was administered for several days starting shortly before or shortly after sensitization. Also in man CY decreases DTH, as has been shown for mumps, PPD and Candida (Alepa et al., 1970; Santos et al., 1971; Mackay et al., 1973).

Under appropriate conditions CY can suppress both the induction and expression of DTH (Campa et al., 1980). The influence on the induction phase is apparent from the observation that CY treatment of sensitized donors can prevent the transfer of DTH to syngeneic recipients. The effect on the effector phase has been shown in a comparable set-up, employing CY treated recipients that were infused with sensitized cells. In these recipients a clear anti-inflammatory effect was found. This anti-inflammatory effect has also been noted by several other investigators (Stevens and Willoughby, 1969; Currey, 1971; Perper et al., 1971; Winkelstein, 1973). Currey (1971) showed in rats that CY prevented adjuvant arthritis expression when administered at the time of onset of clinical symptoms, whereas it had no effect when injected at the time of sensitization. Similarly, high-dose CY treatment of rats immunized with tubular basement membranes in adjuvant may successfully inhibit the cellular immune response producing primary interstitial nephritis, provided the drug is given before the kidney damage is extensive (Agus et al., 1986). However, in the usual therapeutic protocols the anti-inflammatory effect is relatively modest, inferior to the immunosuppressive effect (Perper et al., 1971).

Due to the suppressive effects of CY on the induction of cell-mediated immune responses as well as on the inflammatory reaction mediated by sensitized T cells, the underlying cause of the beneficial effect of CY treatment on skin and organ transplant survival (reviewed by Bach and Strom, 1985[b]) is largely unclear. The same holds for the proven beneficial effect of CY treatment on diseases like reumatoid arthritis (Townes et al., 1976), Wegener's granulomatosis (Fauci et al., 1971, 1974; Steinberg et al., 1972[b]), systemic lupus erythematosus, psoriasis and psoriatic arthritis (Steinberg et al., 1972[b]; Steinberg and Decker, 1974), all characterized by immune system disturbances.

A single injection of CY one or several days *before* immunization can substantially increase DTH and contact sensitivity responses (Bach and Strom, 1985[b]; Enander et al., 1986). In mice intracutaneously immunized with SRBC emulsified in CFA, maximal enhancement occurred when the CY was applied 8 hours before the antigen (Kerckhaert et al., 1977). The enhancement can occur even when the concurrent antibody response is suppressed (Maguire and Ettore, 1967; Lagrange et al., 1974[b]; Turk and Parker, 1982). At first it was thought that the enhanced DTH response was due to the decreased level of the putative depressive antibodies (Kerckhaert, 1974; Lagrange et al., 1974[b]). Later studies, however, showed that enhancement of DTH can be obtained at CY doses which do not inhibit antibody formation (Askenase et al., 1975) or even enhance it (Gill and Liew, 1978). Furthermore, DTH is generally not suppressed by passive injection of hyperimmune antibodies (Mitsuoka et al., 1976) as would be expected if the antibody interpretation of CY mediated enhancement was correct.

Genetically determined low-responder mice for the antigen lactic dehydrogenase isoenzyme can be converted to high DTH responders when treated with CY before immunization (Miller et al., 1976). The enhancement occurred with low, medium and high antigen doses. The CY dose was not critical either, since both low doses (e.g. 100 mg/kg BW) and fiarly high doses enhanced DTH (Askenase et al., 1975). For an equal total dose, fractionated doses are more efficient (Rondinone et al., 1983). The only critical factor was found to be the respective timing of drug and antigen administration.

The CY induced enhancement has been documented for all forms of DTH, including contact sensitivity (Zembala and Asherson, 1976; Maguire et al., 1979), PPD induced DTH (Dwyer et al., 1981) and Jones-Mote type DTH reactions characterized by basophil-rich infiltrates (Nomoto et al., 1980; Taube et al., 1981).

The CY induced enhancement of DTH and related phenomena is generally attributed to inhibition of suppressor T cells. It should be kept in mind, however, that several subpopulations of suppressor T cells exist (Germain and Benacerraf, 1981) and that these subpopulations are not equally sensitive to CY (Germain and Benacerraf, 1981; Shukla and Chaturvedi, 1984). Most likely, CY acts on the precursors of suppressor T cells (Zembala and Asherson, 1976; Sy et al., 1977; Turk and Parker, 1982). Similar CY sensitive suppressor T cells can suppress the *in vivo* generation of CTL against hapten-conjugated syngeneic lymphocytes. Thus, pretreatment of mice with a single dose of CY converts their state of low responsiveness for this antigen into a state of high responsiveness (Röllinghoff et al., 1977). This, however, is not a general phenomenon in *in vivo* generation of murine CTL since CY does decrease the *in vivo* generation of CTL specific for murine lymphocytic choriomeningitis virus (Allan and Dougherty, 1985; Dougherty and Allan, 1985).

Interestingly, CY induced enhancement of DTH is not obtained in old or adult thymectomized mice (Mitsuoka et al., 1979), suggesting that the CY sensitive subpopulation of suppressor T cells is short-lived. Sy et al. (1979) have identified a subpopulation of auxiliary suppressor T cells that could represent a selective CY target. These auxiliary suppressor T cells occur in immunized lymph nodes only and are sensitive to adult thymectomy.

1.7. *Introduction to the experimental work*

As described above, corticosteroids and CY have a variety of effects on the immune system. They affect both the viability and the functional activity of lymphocytes and other leukocytes. These drugs are mostly known about their immunosuppressive effects, but, dependent on the dose and regimen of administration, they can also enhance the immune response to particular antigens.

For example, daily administration of the synthetic corticosteroid dexamethasone sodium phosphate (DEXA) can substantially enhance the antibody production in the bone marrow of mice immunized with the TI antigen LPS (Benner and Van Oudenaren, 1979). The immunostimulating effect of CY is even more prominent. Many studies have shown that a single injection of CY, especially when given shortly before immunization, can substantially enhance IgE antibody formation (Chiorazzi et al., 1976; De Macedo and Mota, 1982; Turk et al., 1984) and DTH and contact sensitivity responses (Bach and Strom, 1985[b]; Dwyer et al., 1981; Enander et al., 1986; Nomoto et al., 1980; Taube et al., 1981). Chronic treatment with CY, however, almost invariably leads to suppression of humoral and cell mediated immune responses (Bach and Strom, 1985[b]).

The enhancing effect of DEXA upon anti-LPS antibody formation in the bone marrow is most likely due to a decreased egress of newly formed B cells from the bone marrow or a redistribution of B lineage cells from the peripheral lymphoid organs towards the marrow. The enhancing effect of a single injection of CY upon subsequent immune responses is generally attributed to the relative susceptibility of suppressor T cell activity for CY (Bach and Strom, 1985[b]). It should be realized, however, that corticosteroids as well as CY can affect virtually all components of the immune system (c.f. sections 1.5 and 1.6 of this chapter), and that both humoral and cell mediated immune reactions depend on interactions between several subpopulations of lymphoid and non-lymphoid cells which can affect the final outcome positively as well as negatively. Therefore, the effect of administration of corticosteroids or CY upon a particular activity of the immune system is the summation of its effect on the various subpopulations of B and T (helper, suppressor, amplifier, cytotoxic) lymphocytes, antigen presenting cells and other subpopulations of non-lymphoid cells which can modulate the response.

The purpose of the investigations reported in this thesis was to obtain more insight into the effects of DEXA and CY upon the Ig production of normal mice that were not intentionally immunized and kept under clear-conventional housing conditions. Although during the experiments the mice were not barrier maintained according to the draft guidelines 'Accomodation and Care of Experimental Animals' of the Council of Europe, they had been bred and raised under such conditions and were maintained free of pathogens during the experiments.

The effects of DEXA and CY upon the Ig production were studied at four different levels: (a) the serum Ig concentration; (b) the number of 'background' Ig secreting cells; (c) the number of surface Ig positive B cells; and (d) the number of LPS reactive B cells. The latter was done in a culture system that allows the clonal growth of every LPS reactive B cell up to a cell input of 1200 murine spleen cells or 6000 bone marrow cells per culture. Under these conditions a large proportion of B cells develop into a clone of Ig secreting cells. The actual frequency of LPS reactive B cells in this culture system is somewhat different for different mouse strains. In the mouse strain C57BL/6J employed here, the frequency of LPS reactive B cells is 1 in about 3 surface Ig positive B cells.

We investigated the effect of a single injection as well as short-term daily administration of DEXA and CY upon the above four parameters. The doses DEXA employed ranged from 16 up to 144 mg/kg BW for a single ip injection and from 1 up to 50 mg/kg BW for daily ip injection which, in view of the high immunosuppressive and anti-inflammatory activities of the drug, are extremely large doses when compared with the doses employed in clinical practice. For treatment with CY the mice received either a single ip injection of 300 mg/kg BW or daily injections of 1 or 100 mg/kg BW.

Chapters 3 and 4 of this thesis describe the effect of a single and seven daily ip DEXA injections upon the serum IgM, IgG and IgA concentrations

at various intervals after the last injection. It turned out that the IgG level was more affected than the other Ig classes. The effect on the serum IgM, IgG and IgA levels did not correlate very well with the effect on the numbers of IgM, IgG and IgA secreting cells in spleen, bone marrow and mesenteric lymph nodes (Chapter 3). Therefore, we also determined the number of cytoplasmic Ig positive cells (plasmablasts and plasma cells) to see whether their frequency correlated better.

In Chapter 4 the effect of a single ip injection of a high dose (144 mg/kg BW) of DEXA upon the B cell compartment of spleen and bone marrow is described. The effect is compared with that of a single ip injection of 300 mg/kg BW CY. Within one day both drugs greatly reduced the number of nucleated cells in spleen and bone marrow, but their effect on the B cell system was markedly different. This was especially true for the *in vitro* LPS reactive B cells, which were almost completely inhibited in their functional capacity by CY.

The effect of DEXA upon the LPS reactive B cells in spleen and bone marrow was studied in detail in Chapter 5. This was done by ip injection of DEXA as well as by *in vitro* culture of spleen and bone marrow cells in the presence of increasing concentrations of DEXA. A single ip injection of a high dose of DEXA (144 mg/kg BW) caused a profound decrease of the number of nucleated cells, especially in the spleen, but the proportions of surface Ig positive B cells and LPS reactive B cells among the remaining cells were unaffected. Seven daily ip injections of a moderate dose of DEXA (50 mg/kg BW), however, did affect the proportion of total B cells in spleen and bone marrow as well as the proportion of LPS reactive B cells. The LPS induced Ig secreting cell responses by splenic B cells and bone marrow B cells appeared to be differentially susceptible to the suppressive activity of DEXA *in vitro*.

In Chapter 6 the effect of CY upon the B cell system is described. Single and daily ip injection of CY appeared to have substantially different effects upon the serum Ig concentrations. After a single CY injection (300 mg/kg BW), but not after 7 daily administrations (100 mg/kg BW/day), a temporary increase of the serum Ig level was found. Under both conditions, after some days CY decreased the serum Ig level, but not more than down to 50% of the original level. This was in clear contrast to the effect on the Ig secreting cells in the various lymphoid organs. Their number was much more severely depressed. The same holds for the functional capacity of the residual B cells as measured by polyclonal activation by LPS *in vitro* and by adoptive transfer *in vivo*.

1.8. *Materials and Methods*

1.8.1. *Experimental animals*

Female C57BL/6J (8-12 weeks old), BALB/c (8-12 weeks old) and (C57BL/Rij x CBA/Rij)F1 hybrid mice (18-20 weeks old), and female Lewis rats (4 weeks old) were used. The C57BL/6J mice were purchased from OLAC 1976 Ltd., Blackthorn, England. The BALB/c and (C57BL/Rij x CBA/Rij) mice were obtained from the Laboratory Animals Centre of the Erasmus University, Rotterdam, and the Medical Biological Laboratory, Rijswijk, The Netherlands. The Lewis rats were purchased from the Central Laboratory for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. All mice and rats received pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3.0) *ad libitum*.

The mice were housed maximally 3 per small-size cage (18 x 11.5 cm) (Fig. 6), 4-8 per middle-size cage (26 x 20 cm) and 9-18 per large-size cage (42 x 26 cm).

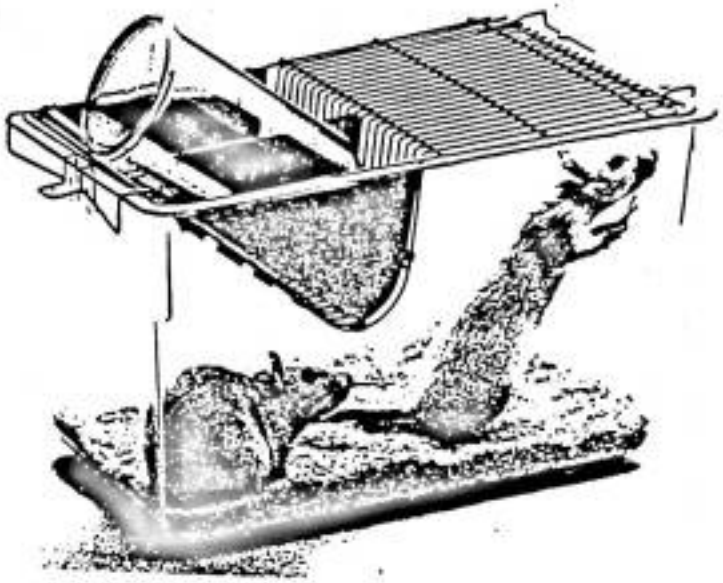


Fig. 6. Small size cage used for housing the mice.

The BALB/c and C57BL/6J mice were used to investigate the effect of DEXA upon B cells and the 'background' Ig synthesis, while the (C57BL/Rij x CBA/Rij)F1 hybrid mice were used to study the effect of CY.

1.8.2. Treatment with corticosteroids

For treatment of mice with corticosteroids, the synthetic corticosteroid dexamethasone sodium phosphate (DEXA; Decadron©, Merck & Co., Rahway, New Jersey, USA) was used. The mice received one or more ip injections of 0.5 ml balanced salt solution (BSS) containing 0, 1, 16, 48 or 144 mg DEXA/kg BW. The BSS was prepared according to Mishell and Dutton (1967). The injections were always done between 4 and 5 PM. The effect of DEXA on the immune system was determined at various intervals after the last injection. The applied corticosteroid treatment did not cause any sign of illness or loss of body weight. Also dissection showed no abnormalities.

1.8.3. Treatment with cyclophosphamide

For treatment of mice with cyclophosphamide (CY; Endoxan©, Mead Johnson & Co., Evansville, Indiana, USA), the mice received one or more ip injections of 0.5 ml BSS containing 0, 1, 3.3, 10, 33, 100 or 300 mg CY/kg BW. The injections were always done between 4 and 5 PM. The effect of CY on the immune system was determined at various intervals after the last injection. The applied CY treatment did not cause any sign of illness or loss of body weight. Also dissection showed no abnormalities.

1.8.4. Cell suspensions

Mice and rats were killed by exposing them to carbon dioxyde vapour. Immediately after killing, the organs to be used (spleen, lymph nodes and/or thymus), were removed and placed into a 15 ml tube containing BSS.

Spleens, lymph nodes and thymuses were separately minced with scissors and squeezed through a nylon gauze filter (Stokvis and Smits Textiel Mij., Haarlem, The Netherlands) with 100 µm openings to give a single cell suspension. The nylon was stretched between two aluminium rings with a diameter of 3 cm. This set-up was used in combination with a 50 ml glass beaker or glass container (Fig. 7). The cells were gently forced through the nylon filter with a plunger, and another 4 ml of BSS was used to collect the remaining

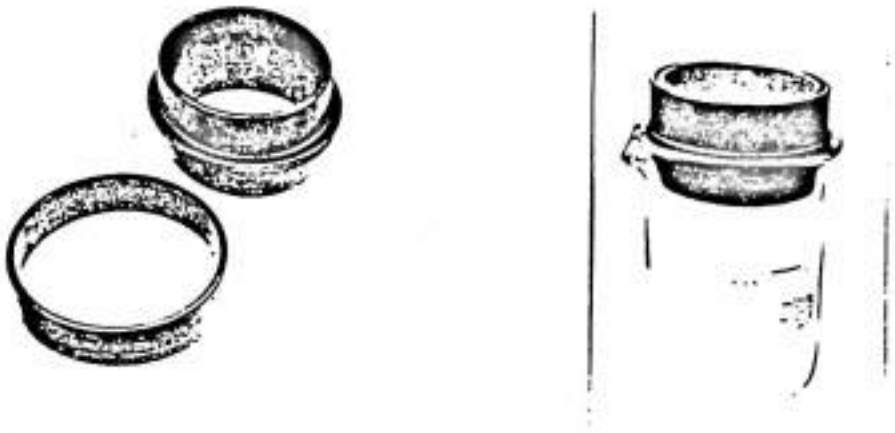


Fig. 7. Nylon gauze filter for preparation of single cell suspensions.

cells. The pooled cells were washed by spinning down in a Heraeus Christ centrifuge (type Minifuge; Osterode AM, Harz, Western-Germany) for 10 min at 1500 rpm and at 4°C. Immediately thereafter, the supernatant was discarded and the packed cells resuspended in 5 ml BSS by a Pasteur pipette. Viable and total nucleated cells were counted after resuspending.

For collection of the femoral bone marrow, the killed mice were pinned with their back upon a tray of cork, the fore- and hind legs spread apart. Four pins were used per mouse, one for each leg. The skin was cut after wetting with 70% ethanol in water. The procedure for removing the femur has been described by Benner et al. (1981[c]) and is illustrated in Fig. 8. A large piece of skin should be cut from the hind leg and the lower abdomen in order to provide a good view of the working area (Figs. 8a and 8b). The tendon of the knee is then cut, so that the rectus femoris can be lifted. It is crucial to do this carefully, so that no muscle tissue remains on the upper side of the femur (Figs. 8c and 8d). Subsequently, the femur is slightly lifted with surgical forceps and the knee joint is cut (Fig. 8e). The femur is lifted again and the muscles on the lower side are cut. Finally, the femur is elevated so that the joint capsule, which keeps the head of the femur toward the ilium, becomes stretched and can be cut (Fig. 8f).

Before isolating the bone marrow from the femur, the residual muscle tissue is removed with a paper tissue. The marrow can most easily be isolated after cutting the head of the femur and a small piece of the greater trochanter on the upper side and a small piece of the condyle on the lower side. By means of a 2 or 5 ml syringe equipped with a 25G x 0.6 needle, a hole is pricked in both spongious ends of the femur. Then the marrow is collected by flushing the marrow cavity with 1 or 2 ml of BSS from the syringe (Fig. 9). Whether or not the marrow has been completely extracted from the femur can be judged from the color of the shaft.

Single cell suspensions from the bone marrow were obtained by gently squeezing the latter through a nylon gauze filter with 100 µm openings (see above).

1.8.5. Counting of total nucleated cells

Nucleated cells were counted with a Coulter counter model BZI (Coulter Electronics Ltd., Harpenden, Herts, England). A 20 µl sample of the cells to be counted was suspended in 20 ml of an isotonic solution (Isoton; Coulter Electronics Ltd.). Subsequently, 2 drops of Zaponin (Coulter Electronics Ltd.) were added to lyse the cells, the mixture was homogenized and the nuclei counted.

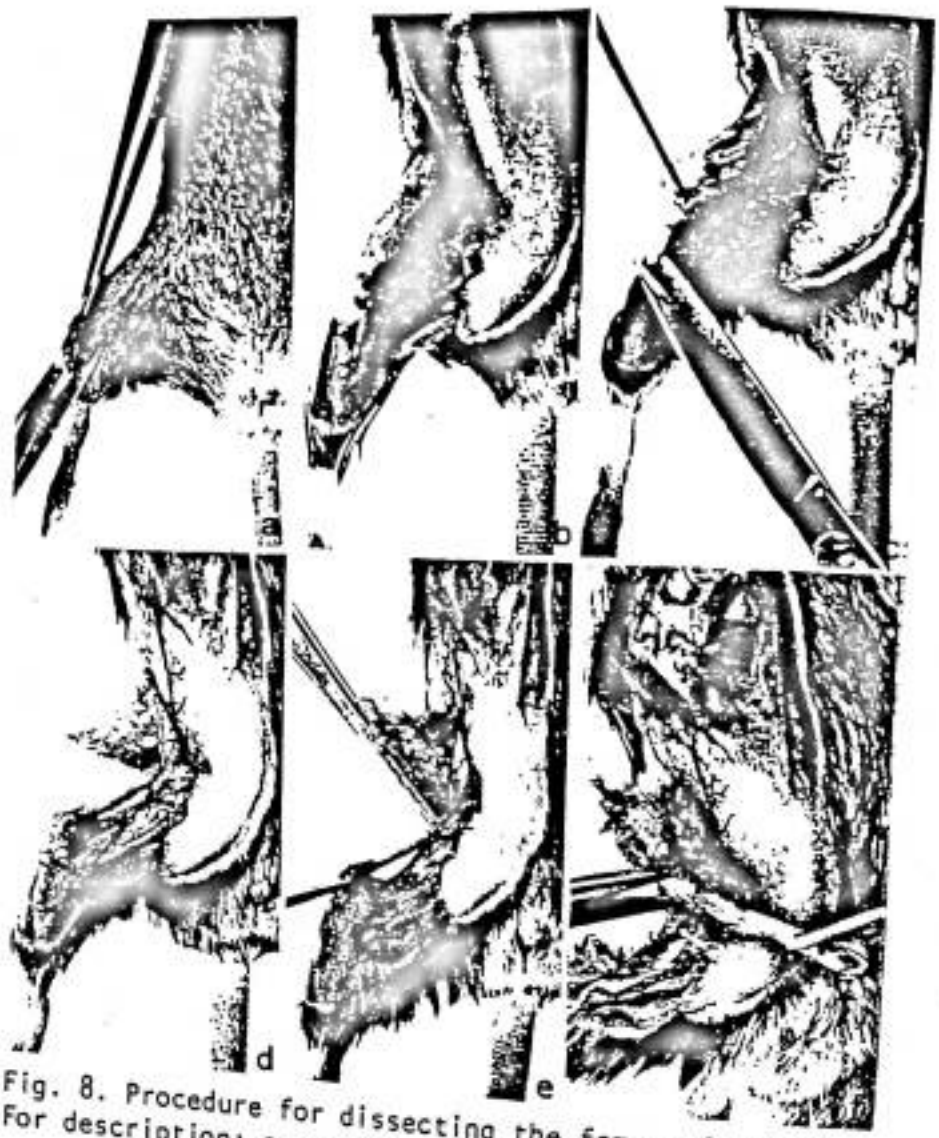


Fig. 8. Procedure for dissecting the femur of a mouse. For description: see text.

Fig. 9. Procedure for the isolation of the bone marrow from a mouse femur.

1.8.6. *Counting of viable nucleated cells*

Viable nucleated cells were counted by the trypan blue exclusion assay. The trypan blue was centrifuged for 5 min at 3000 rpm shortly before use. A 20 µl sample of the cells to be counted was mixed with 180 µl of 0.2% trypan blue in BSS solution in a well of a 96-well microtiter plate. Thereafter the mixture was pipetted into a Bürker bright-line hemocytometer (Arthur H. Thomas Company, Philadelphia, USA). The viable cells, characterized by their exclusion of the trypan blue, were counted under a light microscope. The cells in 25 large squares were counted. Their number was multiplied by a factor of 10^5 to find the total number of viable nucleated cells/mm^3.

1.8.7. *Protein A plaque assay*

Ig secreting cells were assayed by the hemolytic plaque assay as described by Gronowicz et al. (1976) and modified by Van Oudenaren et al. (1981). This assay is based upon binding of the Fc-portion of rabbit anti-mouse-Ig antibodies to *Staphylococcus aureus* protein A that has been coupled to target SRBC. Secretion of Ig by the Ig producing cells leads to the formation of immune complexes consisting of mouse Ig and the added rabbit antibodies. These complexes, bound to the protein A-coated SRBC, activate complement which leads to lysis of the target SRBC and thus to 'plaque' formation. This assay detects all cells secreting Ig that are recognized by the rabbit antiserum used.

In most strains of *S.aureus*, protein A is covalently linked to the cell wall (Sjöquist et al., 1972). This protein A binds Ig molecules with high affinity. The principal Ig class bound is IgG, although in many cases binding is restricted to certain IgG subclasses. In some species, IgM and IgA can also bind to protein A (Goding, 1978).

Protein A has proven to be useful for the study of antigens and receptors on the surface of intact cells, for the differential binding of different Ig (sub)classes and for the detection of Ig-secreting cells. Thus protein A is now the reagent of choice for many preparative and analytical purposes in immunology.

For the plaque assay, *S.aureus* protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) was coupled to SRBC with $CrCl_3.6H_2O$. For coupling, 1 ml of a protein A solution (1 mg dissolved in 1 ml 0.9% NaCl), 1 ml of washed, packed SRBC, 9 ml 0.9% NaCl and 50 µl of an aged (over 2 years old) solution of $CrCl_3.6H_2O$ (0.05 M in saline) were mixed and incubated for 1.5 hr at 37°C, with shaking every 15 min. Thereafter the cells were washed 3 times with 0.9% NaCl and finally resuspended in BSS. Protein A-coated SRBC can be used in the plaque assay for at least three days, provided they are stored at 4°C.

The protein A plaque assay was performed in BSS using Cunningham-type chambers as described by Lefkovits and Cosenza (1979). These incubation chambers are prepared by placing three strips of narrow double faced adhesive tape on a microscope slide (75 x 25 mm), dividing it into two areas of roughly equal surface. A second slide is pressed onto the first so that the two slides are face to face and separated only by the thin strips of tape. The total volume of the two chambers is 180-200 µl.

Each sample to be tested consisted of 100 µl of an appropriately diluted cell suspension, mixed with 15 µl of an optimal amount of diluted guinea pig complement (Behringwerke, Marburg Lahn, Western-Germany) purified over a Sepharose-protein A column (Van Oudenaren et al., 1981), 15 µl of appropriately diluted specific rabbit-anti-mouse-IgM, -IgG or -IgA antiserum and 15 µl of a 17% suspension of protein A-coated SRBC. The rabbit antiserum used was purified over a Sepharose-protein A column as described by Goding (1978) in order to isolate the protein A binding fraction of Ig.

Each sample was mixed and distributed over both chambers of a set of slides by a Pipetman Gilson pipette (Villiers Le Bel, France). The chambers were sealed by carefully dipping the slide edges into a melted mixture of equal portions of paraffin (Paramat®; Gurr Ltd., High Wycombe, Bucks, England) and wax (ACF-Chemifarma NV, Maarssen, The Netherlands). Subsequently, the chambers were placed on a tray and incubated at 37°C for 4-5 hr. The Ig secreting PFC were counted under a dissecting microscope with dark field illumination (Fig. 10). Careful handling of the chambers avoided distortion of the plaque morphology (Fig. 11).

1.8.8. *Calculation of the number of Ig secreting cells per organ*

1.8.8.1. *Spleen and mesenteric lymph nodes*

The number of Ig secreting cells in spleen and mesenteric lymph nodes was determined by applying the protein A plaque assay on a sample from a cell suspension made from the whole organ. Since all cells from an organ, after making the cell suspension, were suspended in a known volume of BSS (usually 5 ml), the number of Ig secreting cells per whole organ could be calculated from the number per sample assayed, the volume of BSS used for suspending the cells, the applied dilution of this cell suspension and the volume of the sample assayed (100 µl).

1.8.8.2. *Bone marrow*

Comparison of the number of Ig secreting cells in the bone marrow with that of the other lymphoid organs requires quantitation of the number of Ig secreting cells per whole organ. As described above, for spleen and lymph nodes this is easy to do, since the protein A plaque assay can be performed on a sample from a cell suspension made from the whole organ. For the bone marrow this is not feasible.

Benner and Van Oudenaren (1975, 1976) have previously shown that the frequency of antibody-producing cells in various bone marrow compartments (femur, tibia, humerus, rib, sternum) of immunized mice is approximately the same. In view of this comparable PFC activity in various bone marrow compartments and the fact that bone marrow cells can be easily isolated from femurs, we determined the number of Ig secreting cells in the femoral marrow (similarly as described above for spleen and mesenteric lymph nodes) with subsequent calculation of the number of Ig secreting cells per total bone marrow by using a conversion factor. This conversion factor was deduced from the distribution of iv or ip injected ^{59}Fe over the various parts of the skeleton. Three independent groups of investigators have performed such

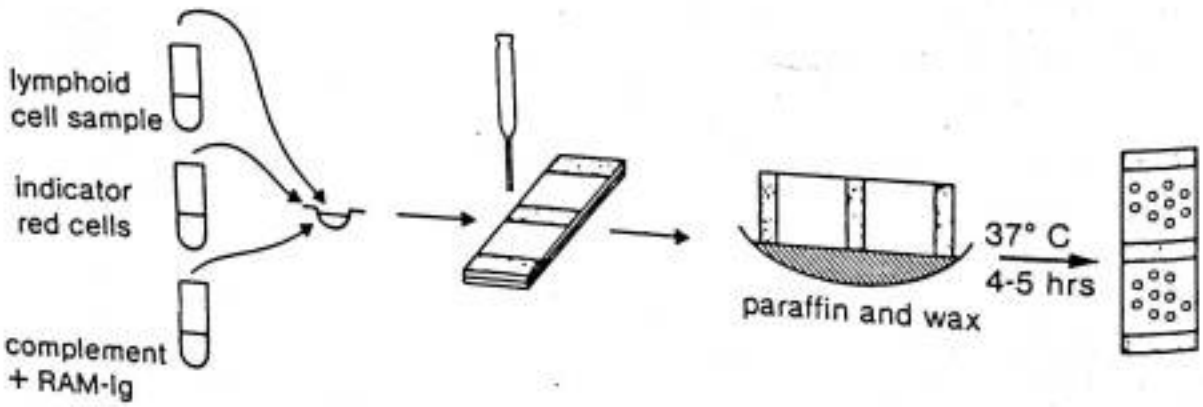


Fig. 10. Scheme of the procedure of the protein A plaque assay.

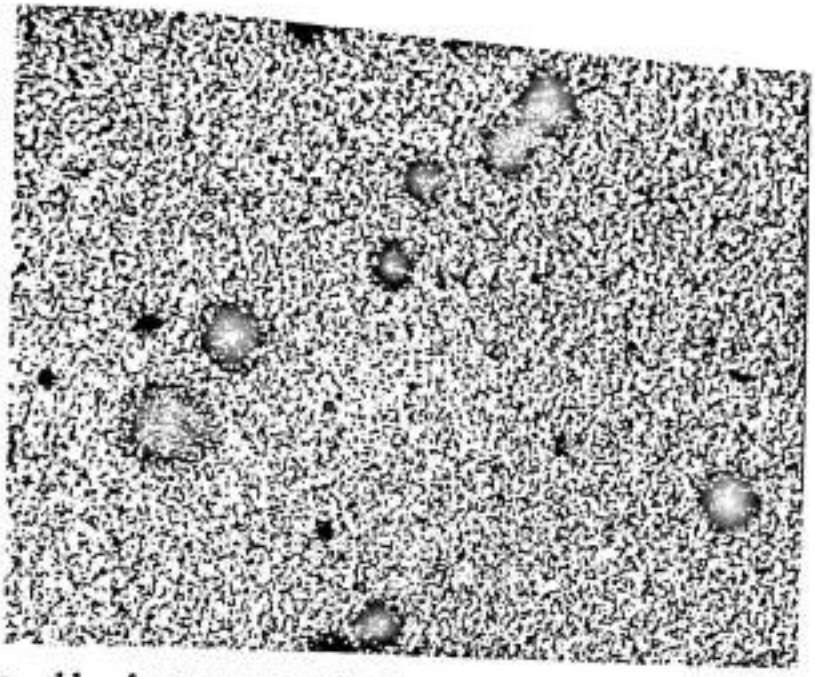


Fig. 11. Appearance of plaques caused by Ig secreting cells in the protein A plaque assay.

studies (Chervenick et al., 1968; Schofield and Cole, 1968; Smith and Clayton, 1970). They found that of the total amount of ^{59}Fe which localized in the bone marrow, 5.9%, 7% and 6%, respectively, was bound by the femoral marrow. The reciprocal of the mean of these three percentages is 15.87. Thus the number of Ig secreting cells per total bone marrow can be calculated to be 15.87 times the number found in the bone marrow of a single femur. This calculation is based on the assumption that all bone marrow cells can be extracted from a femur. Sometimes, however, it is difficult to remove all the visible (red) bone marrow from a femoral shaft. Consequently, the calculated number of Ig secreting cells per total bone marrow is too low. In order to reduce this variability we collected the marrow from both femurs of a mouse. Thus we used a conversion factor of 7.9 (Benner et al., 1981[c]).

1.8.9. *Determination of cytoplasmic Ig-containing cells*

1.8.9.1. *General aspects*

Cells secreting Ig are morphologically characterized by an increased amount of basophilic cytoplasm. Their morphology can range from lymphoblast to plasma cell. Many of these cells can be enumerated by means of immunofluorescence with heterologous antibodies specific for the Ig produced (Hijmans et al., 1969; Hijmans and Schuit, 1972). The availability of fluorescent reagents of high activity and specificity is a crucial requirement for reliable results. In practice, the choice is mainly between fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC). Although many conjugates are commercially available, they can be prepared easily as described by Bergquist et al. (1974) and Goding (1976). It has been found in cell suspensions of murine lymphoid organs that enumerations of the Ig producing cells by immunofluorescence and by the protein A plaque assay do not always give similar results (Van Oudenaren et al., 1984). The protein A plaque assay was found to detect as many or several times more Ig producing cells than the immunofluorescence assay, depending on the age and antigenic load of the mice, and upon the Ig class and organ studied. To detect intracytoplasmic Ig-containing (C-Ig) cells, the cells have to be fixed with reagents that allow the fluorescent antibody to enter them.

1.8.9.2. *Preparation of cytocentrifuge preparations*

Spleen cell suspensions were prepared as described in section 1.8.4. Cells were resuspended in phosphate-buffered saline (PBS; pH 7.8) with 5% bovine serum albumin (BSA; Povlat, Amsterdam, The Netherlands) in a concentration of 2 x 10^6 cells per ml. Cytocentrifuge preparations of 100.000 spleen cells were prepared according to Vossen et al. (1976). Briefly, the preparations were made by spinning down (5 min, 1000 rpm) in a cytocentrifuge (Nordic Immunological Laboratories, Tilburg, The Netherlands) 50 µl of the cell suspensions to be tested in slides precoated with 100 µl of PBS (pH 7.8) supplemented with 1% BSA (PBS-BSA). After air drying for 15 min, the location of the cells was marked by encircling with a glass pencil. The slides were then fixed in ethanol containing 5% acetic acid (5 parts of acetic acid to 95 parts of ethanol) for 15 min at -20°C, washed for 30 min in PBS while stirring, and dried by wiping with a fine paper tissue, except for the cell spot.

1.8.9.3. Visualization of cytoplasmic Ig containing cells

When the cytocentrifuge preparations are prepared as described in section 1.8.9.2, the cells occur in a monolayer of about 5 to 7 mm diameter. Therefore, they can be easily stained with a small volume (15-20 µl) of antiserum conjugated with e.g. FITC or TRITC. This was done for 30 min at room temperature in a humidified chamber. The conjugates were diluted in PBS-BSA containing 10-20 mM sodium azide to prevent bacterial growth. Excess conjugate was removed by flicking the slides and washing for 15 min in PBS. The slides were then dried and mounted in buffered glycerol (1 part of PBS to 9 parts of glycerol, p.a.; Merck AG, Darmstadt, Western-Germany) containing 1 mg phenylenediamine per ml (stored at -20°C) to prevent fading of the fluorochrome. A coverslip was sealed to the slide with paraffin wax (Paramat®; Gurr Ltd., High Wycombe, Bucks, England). For evaluation of immunofluorescence staining Zeiss fluorescence microscopes were used equipped with phase contrast facilities and an epiillumination system (Haaijman et al., 1979). The Ig class distribution of the C-Ig cells was determined by means of combinations of TRITC and FITC labeled antisera specific for the heavy chains of IgM, IgG, IgA and total Ig.

1.8.9.4. Conjugated antisera

A fluorescein-conjugated goat antiserum directed against mouse Ig (GAM-Ig-FITC, Lot no. 2-873; Nordic Immunological Laboratories, Tilburg, The Netherlands) was used to determine the total number of C-Ig cells per slide. The rhodamine and fluorescein-labeled antisera specific for the heavy chains of murine IgM, IgG and IgA were prepared, purified and generously supplied by Dr. J. Radl and Miss P. van den Berg from the Institute for Experimental Gerontology, TNO, Rijswijk, The Netherlands. The antisera fulfilled all specificity criteria described previously (Haaijman et al., 1979). The specificity of the reagents was corroborated by the fact that preparations of quite a number of normal BALB/c mice did not show any C-Ig cell containing Ig of two different classes, notably in mesenteric lymph nodes and Peyer's patches.

1.8.9.5. The number of cytoplasmic Ig containing cells per organ

From the number of C-Ig cells per slide, the number of nucleated cells spun down per slide and the number of nucleated cells per organ, the total number of C-Ig cells per organ was calculated.

1.8.10. Determinat[illegible] by membrane immunofluorescence

Spleen and bon[illegible] were prepared as described in section 1.8.4. Cell[illegible] min at 1000 rpm at 4°C) three

times with PBS-BSA (containing 10-20 mM sodium azide) to remove intercellular proteins and proteins secreted by the cells themselves. Finally the cells were resuspended in similar PBS-BSA and adjusted to a concentration of 10^7 cells per ml. For the detection of membrane bound Ig or other membrane glycoproteins the cell suspension need not to be fixed. For staining a volume of 100 µl cell suspension was mixed with 50 µl of an optimal concentration of FITC labeled goat antiserum directed against mouse Ig (GAM-Ig-FITC; lot no. 2-873; Nordic Immunological Laboratories, Tilburg, The Netherlands) and incubated for 30 min at 0°C. After two washes with the above mentioned BSA containing PBS the preparations were mounted in buffered glycerol and sealed with paraffin (see section 1.8.9.3). The percentage of nucleated cells with distinct membrane fluorescence was determined with the Zeiss fluorescence microscopes mentioned in section 1.8.9.3.

1.8.11. Polyclonal activation of B cells by lipopolysaccharide in vitro

1.8.11.1. Mass cultures

In so-called 'mass cultures', lymphocytes are cultured at relatively high density. Spleen cells were cultured at densities varying from 1200 to 30,000 cells per culture well of 0.2 ml. Bone marrow cells were cultured at densities varying from 6000 to 150,000 cells per 0.2 ml culture well. To improve B cell activation and clonal growth, in some experiments 7.2×10^5 rat thymus filler cells were added per well. These rat thymocytes were irradiated *in vitro* with 0.1 Gy X-irradiation shortly before culture to reduce the background number of Ig secreting cells in the thymocyte inoculum (Hooijkaas et al., 1982). This irradiation was performed in a Philips Müller MG300 X-ray machine. The physical constants of this machine have been described previously (Benner et al., 1974[8]).

The cells were cultured in 96-well tissue culture plates (Costar 3596; Costar, Cambridge, Massachusetts, USA) in 0.2 ml RPMI 1640 culture medium supplemented with glutamine (4 mM), sodium pyruvat (0.1 M), penicillin (100 U/ml), streptomycin (50 µg/ml), 2-mercaptoethanol (5×10^{-5} M) and fetal bovine serum (20%; lot B 663903 02 from Boehringer Mannheim GmbH, Mannheim, Western-Germany) that was specifically selected for growth-supporting properties and low endogenous mitogenic activity. For polyclonal activation of the B cells cultured an optimal concentration of 50 µg/ml LPS from *Escherichia coli* (strain 026:B6; Difco Laboratories, Detroit, Michigan, USA) was added. Cultures were assayed for IgM secreting cells on day 4, and for IgG and IgA secreting cells on day 5. For each determination at least 3 replicate cultures were assayed.

1.8.11.2. Limiting dilution cultures

The frequencies of LPS-reactive B cells that can give rise to clones of IgM secreting cells were determined in a limiting dilution culture system originally devised by Andersson et al. (1977). In this culture system varying numbers of spleen cells (maximally 60) or bone marrow cells (maximally 600) were cultured in 96-well tissue culture plates (Costar) together with 7.2×10^5 irradiated (0.1 Gy) rat thymus cells (Hooijkaas et al., 1982) to support growth and 50 µg/ml *E.coli* LPS (026:B6; Difco) in 0.2 ml RPMI 1640 medium that was supplemented as described in section 1.8.11.1. Routinely, 32 replicate cultures were set up for each cell concentration. Control cultures did not contain mouse cells but did contain rat thymus cells. The cultures were assayed individually for clones of IgM secreting cells on day 5. Cultures were scored as positive when they yielded more than 10 PFC above the maximum number of PFC found in the control cultures containing rat thymocytes only.

1.8.12. *Collection of serum samples*

Mice were killed by exposing them to carbon dioxide vapour. The skin was wetted with 70% ethanol in water and cut at the upper chest. Blood was taken by cardiac puncture with a 1 ml tuberculin syringe with a 35G x 5/8 needle at intercostal rate 2 or 3 at the left side of the sternum. Blood was collected in plastic tubes (Brand microtest tubes from Dijkstra, Amsterdam, The Netherlands). After clotting, the serum was isolated by spinning down in a Heraeus Christ centrifuge (type Minifuge) for 10 min at 3000 rpm and at 4°C. The isolated serum was placed in a plastic vial and stored at -30°C prior to use.

1.8.13. *Quantitative immunoglobulin measurement by immunodiffusion*

1.8.13.1. *General aspects*

Quantitative Ig measurements in sera from mice treated with DEXA were performed by single radial immunodiffusion (SRID). SRID is a technique in which a single partner of the antigen-antibody reaction, usually the antigen (Ag), diffuses radially from a small well punched into a gel layer of constant thickness, while the other partner, usually the antibody (Ab), has been uniformly incorporated into this gel layer and is, therefore, not considered as diffusing. In other words, the precipitating Ab is homogeneously incorporated into a layer of agar or agarose gel of uniform thickness, small identical reservoirs or wells are punched into this gel, and in each well is delivered an identical volume of Ag solution of known (standards) or unknown (test-samples) concentration. As the Ag radially diffuses into the Ab containing gel, it will give rise to a disk- or ring-shaped Ag-Ab precipitate, which will slowly increase in size until, after a variable period of time, it reaches a final end-point size which does not increase despite further time is allowed for diffusion. At this point and thereafter, there is a simple linear relationship between the amount or concentration of Ag introduced into a given well and the surface area of the precipitate ring surrounding it, and a linear inverse relationship between the surface area of the precipitate ring and the Ab concentration in the gel. Thus, at constant Ab concentration in the gel and at the end point of diffusion, there is a linear relationship between the surface area of the precipitate ring and the Ag concentration. On the other hand, the same amount of Ag, introduced into identical gels varying only in Ab concentration, will yield, at diffusion end point, precipitate rings whose areas will increase linearly with decreasing Ab concentration.

1.8.13.2. *Preparation of the agar*

For preparation of the agar gel, one standard tube of high resolution buffer (No. 1-979, pH 8.6; Nordic Immunological Laboratories, Tilburg, The Netherlands) is dissolved in 1 l distilled water. Subsequently, sodium azide and polyethylene glycol is added to a final concentration of 0.015% sodium azide and 3% polyethylene glycol, respectively. Dry agarose is added to this solution to a concentration of 1%. This suspension is placed in a boiling waterbath and stirred until all the agar has dissolved. Afterwards, distilled water is added to replace losses due to evaporation. The stock solution of agar is distributed over a number of bottles of 60 ml and stored at 4°C, never be frozen.

1.8.13.3. *Preparation of the agar–antiserum mixture*

The stock solution of agar is dissolved again in a boiling waterbath for a short time and allowed to cool down to 50°C. An appropriate amount of antiserum is added to the agar solution, so that the final concentration of

antiserum in different samples is 1%, 2%, 5% and 10%, respectively. The agar solution and the added antiserum are mixed as thoroughly as possible, while avoiding bubbling. A volume of 10 ml of the mixture is put into a petri dish of 9 cm diameter and placed at horizontal surface. The horizontal position is to be checked and adjusted with a spirit level (Schut, Groningen, The Netherlands).

1.8.13.4. Materials required to prepare and fill the Ag reservoirs

To prepare Ag reservoirs in the gel layer, circular wells are punched out into the gel, using a needle of 3 mm diameter. The small cylinders of gel cut out by the needle are removed by suction without rupturing or deforming the gel. The important part of the puncher is that which penetrates into the gel: that part should be very thin-walled in order to minimize deformation and displacement of the gel when the puncher is lowered into it. Punchers should be regularly checked for accuracy and cleanliness.

Each of the wells is filled with 7.5 µl of antigen solution which is delivered by means of a Pipetman Gilson pipette (20 µl size). The petri dish is kept in a strictly horizontal position, until the Ag solution has been soaked up by the gel, which takes 10-30 min. The plates are kept in a humid chamber at room temperature.

1.8.13.5. Measurement of the size of the precipitates

The ringshaped precipitates which form around the antigen wells grow in size during a few days, after which no further increase in diameter is observed. The gels are washed with PBS (pH 7.2) in large petri dishes for two or three days, with several daily changes of the PBS. After drying at room temperature or at 37°C, the gels are stained for 10-15 min in an aqueous solution containing 0.5 g of Amido Black, 0.25 g thiazine red, 50 ml concentrated acetic acid and 450 ml methanol. The background color is removed by an aqueous solution containing 200 ml of concentrated acetic acid, 1000 ml methanol and 800 ml distilled water. This is done for 10-15 min.

For the measurement of the precipitin areas, the dishes are placed on an optical enlarging system and the diameters of the successive precipitin rings are measured. One usually measures two perpendicular diameters of the same ring and multiplies them to compensate for slight deviations from circularity. If the rings are quite circulair, suffice it to measure one diameter and to square it (Mancini et al., 1965). The data of different antigen concentrations are plotted (Fig. 12) and a regression line is calculated with its statistical parameter. This is done with the formula $S=So+K.Cag$ (Mancini et al., 1965), in which S represents the terminal size of the precipitate, Cag the amount of antigen, So the intercept (obtained by extrapolating the curve back to zero concentration) and K the slope of the line.

In our experiments the day 0 values (100%) were calculated as the arithmetic mean of the absolute Ig levels of all BSS-treated control mice. These values were for IgM 18.1 $\pm$ 1.5 mg/dl, for IgG 603 $\pm$ 86 mg/dl and for IgA 25 $\pm$ 3.6 mg/dl.

1.8.14. Quantitative immunoglobulin measurement with an enzyme-linked immunosorbent assay

Quantitative Ig measurements in sera from mice treated with CY were performed with an enzyme-linked immunosorbent assay (ELISA). This assay was adopted to measure mouse serum Ig levels of the various classes quantitatively. Details of this method will be described in Chapter 2, section 2.4.3. Briefly, diluted serum samples were coated onto the bottom of Terasaki trays (Greiner Nürtingen, Western-Germany). After washing with Dulbecco's PBS (DPBS) the

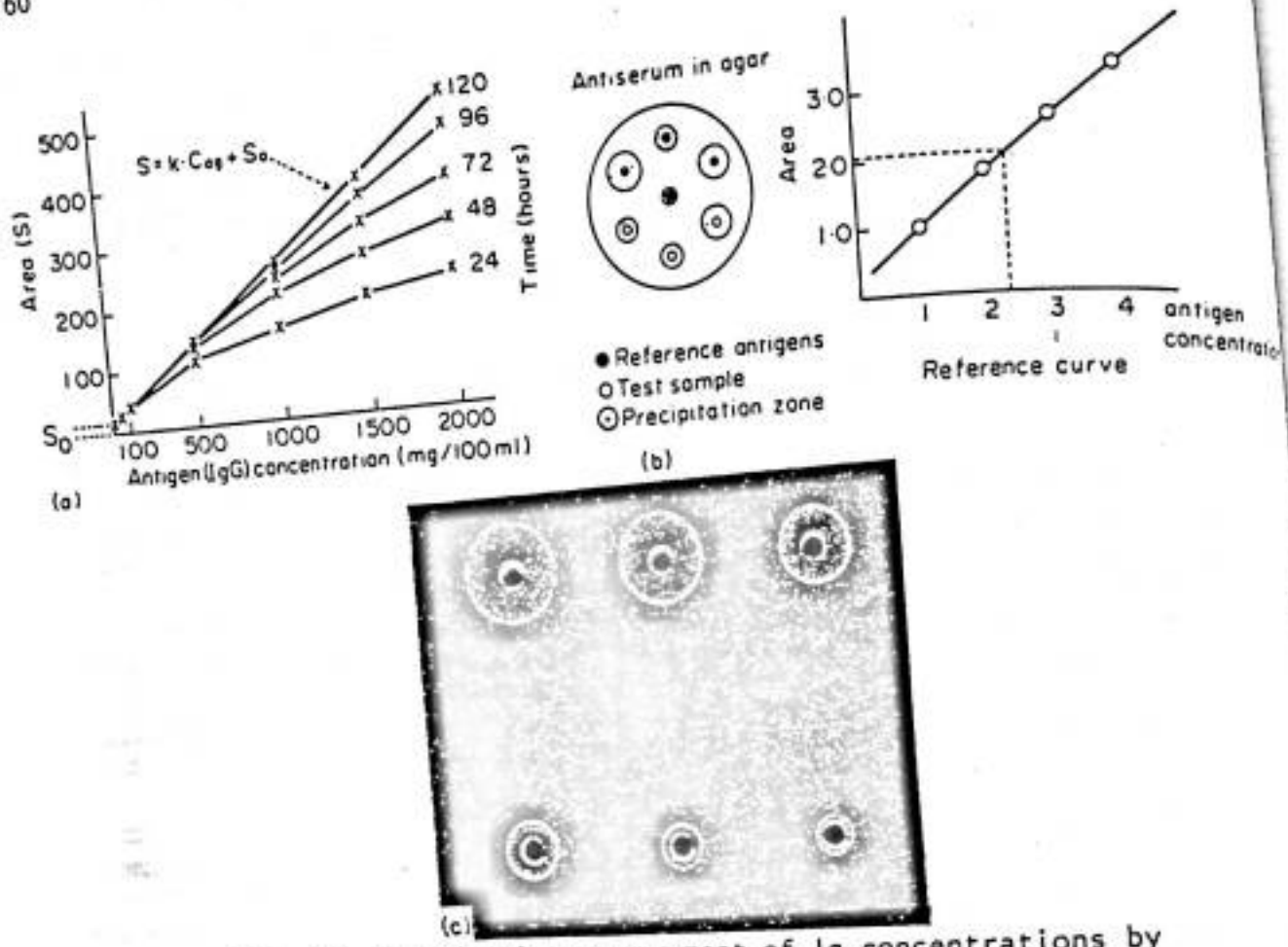


Fig. 12. Quantitative measurement of Ig concentrations by immunodiffusion. (a) Time-dependent increase of the precipitin surfaces. (b) Constructed reference curve. (c) Precipitin surfaces at different antigen concentrations. For explanation: see text.

trays were incubated for 1 hr at 37°C with DPBS-gelatin to coat non-specific binding sites. Subsequently the plates were repeatedly washed with DPBS-Tween-gelatin and incubated for 1 hr at room temperature with rabbit-anti-mouse immunoglobulin (RAM-Ig) or isotype-specific antisera of rabbit origin (RAM-IgM, RAM-IgG and RAM-IgA). These antisera were affinity-purified and found to be specific for the respective mouse Ig classes according to immunoelectrophoresis. The antigen-antibody complex in the wells was incubated for 1 hr at 20°C with a protein-A-β-galactosidase conjugate. Binding of the detecting antibodies to the serum Ig was visualized by incubation with the substrate 4-methylumbelliferyl galactoside. Fluorescence in the individual wells of the Terasaki trays was then quantitatively analysed within 2 min using a scanning inverted microfluorometer, connected to a digital voltmeter and a desk-top calculator.

To quantitate the Ig levels, a reference standard of a large pool of normal mouse serum obtained from approximately one-year-old CBA/Rij mice wa used on each plate (Mink and Benner, 1979). This reference serum contained 38.5 mg IgM, 230.2 mg IgG1, 432.8 mg IgG2 and 178.8 mg IgA per 100 ml.

1.8.15. Statistics

1.8.15.1. *General aspects*

Numerous quantities in the natural sciences seem to be so-called 'norm ly distributed' and many statistical procedures are based on the assumptio underlying normal distribution. This normal distribution is derived by int gration of the probability density function of (a) continuous random varia

(b) the estimates of discrete random variables, and (c) the relative frequency distributions of symmetrically distributed variables. Many distributions are bell-shaped and fairly symmetrical with respect to the mean. Such a distribution is called the 'normal' or 'Gaussian' distribution with a mean and a standard deviation. The density function reaches only one maximum and is therefore called 'unimodal'.

For application of statistics to biological problems, especially to immunological data, it is important to realise that many data show an extremely asymmetric distribution ('skew'), the so-called 'asymmetric continuous frequency distribution'.

One of the problems of statistical interference is to know whether this lack of fitting is due to simple sampling errors or to a non-normality of the population of measures from which the samples are taken. In any case, it is important to give in a single figure some indication of the general 'trend' of a series of measurements. Such a figure is called a 'measure of location' and the most familiar among the measures of location is the 'arithmetic mean'. If there are N observations of the variable x, they will be denoted by x_i ($i = 1, 2, \ldots N$). The arithmetic mean of x_i is defined by m_x:

$$m_x = \frac{\sum_{i=1}^{N} x_i}{N} \qquad (1)$$

which is simply the average value of the sample data.

The 'geometric mean' is another measure of central tendency. It is especially useful in averaging ratios, percentages and rates of chance. The geometric mean is found by computing the product of the n sample results and then taking the n^{th} root of this product.

The geometric mean will never exceed the arithmetic mean value. Thus for very positively skew data, i.e. data which contain a fairly high proportion of very large values, the geometric mean value is nearer the median than is the arithmetic mean.

One advantage of the geometric mean is that if the readings are such that their logarithm is normally distributed, then log(geometric mean) = arithmetic mean of the logarithm of the individual readings.

The use of the geometric mean is usually advocated where the recorded readings are proportional to some applied effect. The geometric mean is only applicable if the quantities involved are measured on a ratio scale. But for such readings the logarithm of the reading has, as often as not, more desirable statistical properties than the initial readings themselves. When dealing with the logarithms of the readings, one should use the arithmetic mean. In the final process of converting back to the actual readings, the anti-log of the arithmetic mean of the log(readings) will, of course, be the geometric mean.

When the mean value of a series of measurements has been obtained, it is usually of interest to express the degree of variation around this mean. The most common measure of dispersion is the mean value of the squared deviations from the mean:

$$s^2 = \frac{\sum_{i=1}^{N} (x_i - m_x)^2}{N} \qquad (2)$$

which is called 'variance'. The total number of observations is usually subtracted by 1, when the mean (one parameter) has already been calculated from the sample. This conversion is essentially based upon the assumption of estimating the mean and the standard deviation in a population by determining the mean and the standard deviation of a sample from this population. The square root of the variance is called 'standard deviation' (SD).

$$SD = \frac{\sum_{i=1}^{N}(x_i - m_x)}{\sqrt{N-1}} \qquad (3)$$

In a large population of measurements, a large number of random samples of certain size are taken from that population and these will show arithmetic means that differ from each other. This variation can be expressed as standard deviation of the mean. Generally, however, only one sample representing a particular population is available. The standard deviation decreases when N is greatly increased. The standard deviation of an indefinitely large population of measurements divided by the square root of the number of observations minus one, gives the 'standard error of the mean' (SEM).

$$SEM = \frac{SD}{\sqrt{N-1}} \qquad (4)$$

The mean of a number of measurements may only be taken when the given data express a normal distribution. One may consider the distribution to be symmetric when $-0.5 < \text{Skewness} < +0.5$. The distribution is highly skewed when this value exceeds ± 1. As many statistical tests are based on the assumption of normality, it is often convenient to look for ways to transform skewed data to an approximately normal distribution. Three of the mostly used normalizing transformations are the harmonic $(100/x_i)$, the square root $(\sqrt{x_i})$, and the logarithmic $(\log x_i)$ transformations.

The success of normalization is assessed by the statistic:

$$\text{Skewness} = \frac{\Sigma(x_i - m)^3}{(SD)^3} \qquad (5)$$

When a logarithmic transformation is used to obtain a normal distribution ($y_i = \log x_i$), the arithmetic mean can be transformed back to the original scale of observations by taking

$$m_x = \text{antilog } m_y \qquad (6)$$

in which m_x is called the 'geometric mean' of x. This geometric mean is often used to express the mean of a number of plaque-forming cell measurements or serum titer determinations, because these data generally are asymmetrically distributed.

1.8.15.2. Statistical analysis of data from limiting dilution cultures of B cells

If B cells are randomly and independently distributed throughout a large number (e.g., 32) of replicate microculture wells, the number of responding cells per well follows a Poisson distribution. This is true since the total number of microcultures is usually very large ($n > 20$) while, on the other hand, the probability p that a particular well out of this large number contains responding cells is very small ($p < 0.1$). For these reasons, the Poisson distribution is the limiting distribution of a binomial distribution when n tends to infinity and p to zero. The Poisson distribution is an asymmetrical right (or the third moment is positive) skewed distribution around the mean when a histogram of all integers ($r = 0, 1, 2, 3, \ldots$) is constructed. It is clear that the mean and the variance of a Poisson distribution have the same numerical value when n tends to infinity. The mean number of responding cells can be calculated from the observed proportion of negative cultures using the Poisson formula

$$F_r = \lim_{n \to \infty} \binom{n}{r} p^r . q^{n-r} = \frac{u^r . e^{-u}}{r!} \qquad (7)$$

where Fr is the fraction of cultures containing r responding cells, $q = 1-p$, u is the mean number of responding cells per well and r is the actual number of responding cells in a microculture well (0, 1, 2, 3, ...).
The zero term of the Poisson equation is

$$F_o = e^{-u} \qquad (8)$$

The logarithms of the equation are

$$\ln F_o = -u \qquad (9)$$

$$-\ln F_o = u$$

which means that the negative logarithm of the fraction of nonresponding cultures is linearly proportional to the mean number of responding cells per well.

If the cell input is plotted on the x-axis on linear scale and the negative logarithm of the fraction of nonresponding cultures ($-\ln Fo$) on the y-axis, the experimental points are expected to fit a straight line.

The above equation simplifies for $u = 1$:

$$F_o = e^{-1} = 0.37 \qquad (10)$$

Thus, when the mean number of responding cells per well is one, 37% of the wells will remain negative.

Figure 13 shows the plot of a limiting dilution analysis of LPS-stimulated B cells. This is an example of the semilogarithmic plot. Interpolating at the level of 0.37 one reads 3. Thus 3 B cells contain, on average, one responding B cell.

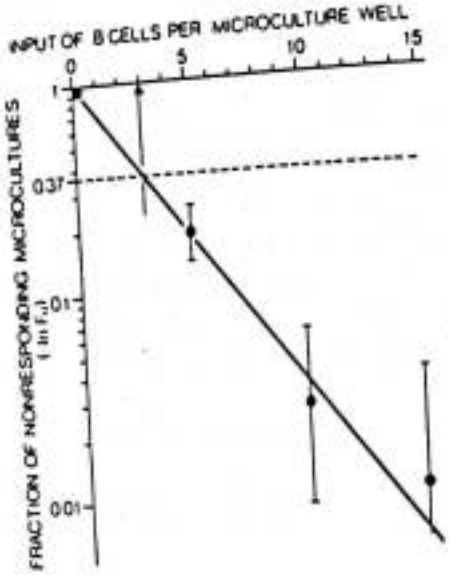


Fig. 13. Limiting dilution culture of B cells. All cultures contained a constant number of irradiated thymocytes (7.2×10^5 per culture. The abscissa indicates the input of B cells per culture (0, 5, 10 or 15 B cells/well). The ordinate indicates the fraction of nonresponding cultures (-ln Fo). Each point is based on PFC tests from 32 replicate cultures. An input of about 3 B cells per well yielded 37% negative cultures in this particular experiment (Lefkovits, 1979).

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CHAPTER 2

Generation and measurement of antibodies

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2. GENERATION AND MEASUREMENT OF ANTIBODIES *)

2.1. Introduction

The antibody molecule has evolved to perform two distinct functions: epitope recognition and elimination of foreign antigens. Antibody activity presides in immunoglobulin (Ig) molecules, each of which can interact with some out of a virtually unlimited number of fysico-chemically different antigens (ag). These antigens include exogenous ('foreign or nonself') antigens as well as endogenous ('self') antigens, including Ig-idiotopes (Eichmann, 1978; Jerne, 1984; Köhler et al., 1984)). In spite of the large variety of different antibodies (ab) specificities, ab destroy or eliminate foreign ag by a small number of effector mechanisms. To carry out its dual function, the antibody molecule has evolved discrete globular domains: one of these domains binds ag, whereas the others mediate effector mechanisms (Edelman, 1973). Thus the functional duality of the ab molecule is reflected in its three-dimensional structure. The organization of ab-gene clusters also reflects this duality (Honjo, 1983; Calvert et al., 1984).

A state of humoral immunity to a certain ag is characterized by the presence of sufficient ab in the blood to counteract the ag and/or the ability to mediate an accelerated and increased ab production as compared to a nonimmune state. Since the majority of the ab produced are released into the blood stream, it is generally assumed that the Ig levels in the serum reflect the overall activity of the humoral immune system. The total activity of all B cell clones together will yield, under normal conditions, a heterogeneous spectrum of serum Ig molecules.

In disease, however, some disorders of the immune system can lead to imbalanced B cell activity, which is reflected in excessively high or low serum levels of one or more Ig (sub)classes (Radl et al., 1972; Radl et al., 1978). Moreover, this imbalanced activity can lead to a restriction in serum Ig heterogeneity and the appearance of temporary or permanent homogeneous Ig components or paraproteins (Radl, 1979; Radl et al., 1980). Excessive production of such components is often the result of malignant B cell transformation (McIntyre, 1979).

Insight into the regulation of the overall activity of the humoral immune system requires reliable measurement of the normal and the diseased concentration and heterogeneity of the various Ig classes and subclasses in the blood. Together with the determination of the numbers of ab and Ig secreting cells after immunization, measurement of circulating ab gives insight into the regulation of Ig synthesis and ag-specific ab-induction under normal conditions. Moreover, determination of these parameters might help in the rapid diagnosis and the understanding of the underlying cause of diseases in which the B cell system is involved.

In this chapter we focus on the determination of ab and Ig secreting cells and the measurement of circulating ab in the mouse. Applications for studying human ab formation are mentioned in the various sections. The reader is referred to the references quoted for further information.

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2.1.1. B cell development leading to antibody production

All individuals, whether mice or men, that are not deliberately immunized, have naturally occurring Ig in their serum and at secretory sites. The Ig levels are built up and maintained by Ig secreting cells in the various lymphoid organs. In mice, the number of these spontaneously occurring ('background') Ig secreting cells vary, together with the serum level and serum Ig heterogeneity, with the genetic background, T cell function and exogenous antigenic load (Benner et al., 1982b; Hooijkaas et al., 1985). The breeding of germfree mice, fed an ultrafiltered solution of chemically-defined low molecular weight nutrients, made it possible that mice became practically free of exogenous ag like microorganisms or food constituents (Pleasants et al., 1981; Wostmann et al., 1982). Nevertheless, these mice have Ig-secreting cells with completely normal functional characteristics, though there are isotype-dependent differences in cell numbers (Hooijkaas et al., 1984; Hooijkaas et al., 1985)). Moreover, in these studies, along with others, it became apparent that the specificity repertoire of B cells is 'complete' (Coutinho 1980; Coutinho et al., 1983; Countinho et al., 1984a; Coutinho, 1984b), probably due to idiotype-anti- idiotype interactions as propounded in the network theory of Jerne (1974, 1984).

When mice are deliberately immunized with thymus-dependent (TD) ag, ab-secreting cells were found, dependent on the route of immunization, in the spleen, lymph nodes, gut-associated and bronchus-associated lymphoid tissues, but not in the bone marrow. Upon secondary immunization of mice with TD ag, the bone marrow becomes the major source of ab-formation, dependent on migration of reactivated memory B cells from peripheral lymphoid organs into the bone marrow (Benner et al., 1977). Thymus-independent (TI) ag can also induce ab-secreting cells in the various peripheral lymphoid organs. Some TI antigens (e.g. lipopolysaccharides (LPS), hapten- Ficoll conjugates), however, induce bone marrow ab formation after primary immunization, while others are not capable of inducing an ab-secreting cell response in the bone marrow at all (Koch et al., 1982a).

B lymphocytes, the precursors of ab-secreting cells, are destined to produce Ig-molecules of a unique ag-binding specificity. Maturation of B cells involves ag-independent differentiation from virgin B cells to immunocompetent B cells. In this largely ununderstood process B cells acquire specific homing and cell-recognition properties, ag-specific cell surface Ig receptors and eventually B cell effector functions.

In adult mammals, the bone marrow is the major site of generation of these immunocompetent B lymphocytes that are thought to be derived from pluripotent hemopoietic stem cells (Osmond, 1980a; Osmond, 1980b). Large pre-B cells, containing cytoplasmic u-chains, give rise to small cu^+ cells that start to express IgM together with other cell markers like Ia antigens and IgG Fc receptors and after many hours IgD and receptors for C3b, on their cell membrane (Osmond, 1984). Many of the cu^+ cells are cycling cells. Surface IgM positive B cells acquire the capacity, by the process of gene-rearrangement, to switch to the synthesis of other Ig isotypes (Cooper et al., 1980; Jeske and Capra, 1984). The short-lived immunocompetent B cells migrate, after this ag-independent differentiation, via the blood stream into the spleen, lymph nodes and othe peripheral lymphoid tissues where they can be activated by ag or mitogen to clonal proliferation and terminal differentiation to ab-secreting plasma cells (Osmond et al., 1981; Osmond and Owen, 1984).

Alternatively, they can give rise to memory B cells by becoming involved in a so-called 'germinal center reaction' (Opstelten and Osmond, 1983). A large proportion of the B lymphocytes that emigrate from the bone marrow migrate to the red pulp and subsequently to the white pulp of the spleen (Osmond and Batten, 1984). While doing so, the maturation is completed. This process involves macrophages and T cells and their soluble products such as B cell growth and maturation factors (Paige et al., 1982). The memory B cells are long-lived recirculating B cells which can respond to subsequent stimulation by the same antigen with an enhanced ('secondary type') ab response.

The in vivo Ig production is usually estimated by quantitating the serum Ig

concentration or the numbers of Ig containing or Ig secreting cells in the various lymphoid organs.

It is also known that upon immunization with TD as well as TI ag and independent of the ag dose, Ig with no binding capacity for the eliciting ag are synthesized (Urbain-Vansanten, 1970; Moticka, 1974; Pachmann et al., 1974). The ratio of this so called ag non-specific response to the ag- specific response is isotype-dependent and is greater during a primary immune response than during a secondary one (Benner et al., 1982a). Whether this is caused by ag non-specific B cell growth and maturation factors or induction of autoantibodies or anti idiotypic antibodies is still controversial. This non-specific Ig production together with the ag-specific ab- production upon immunization, is also reflected in the serum Ig fraction (Cazenave et al., 1974). Therefore, measurement of serum Ig levels is not a reliable measure for the capacity to produce ag-specific ab upon immunization. This is all the more true since a substantial proportion of the circulating Ig is withdrawn from the circulatory system at secretory sites (Van Muiswinkel et al., 1979).

The improvement of in vitro culture techniques, both for murine and human B cells, allows the analysis of normal B cell development at the cellular level. Under these conditions Ig production or secretion by B cells can also be measured by many different and reliable techniques (sections 2.3. and 2.4. of this chapter).

Studies on B cell function in man are mainly restricted to the functional analysis of peripheral blood mononuclear cells. In man, study of the regulation of the ag-induced immune response became feasible only after the development of appropriate in vitro culture techniques and ag-specific plaque-forming cell assays.

The ontogenic maturation time of the lymphoid system relative to birth differs from one species to another. As measured by the onset of immune responsiveness as well as by the appearance of peripheral T and B cells, mice develop their immune system just before and after birth, whereas humans develop a functional immune system early in gestation. In man, neonatal B cells express surface IgM, IgG and IgA molecules in adult proportions (Van Tol et al., 1984) but they have only a limited capacity to produce Ig which, furthermore, is mainly restricted to IgM (Hayward and Lawton, 1977; Van Tol et al., 1983). It is not clear as yet, whether neonates lack the appropriate helper T cell signals for isotype-switch or have a diminished susceptibility for extracellular differentiation and maturation signals. However, at the DNA level the mechanism for gene rearrangement or heavy chain isotype switching is not defective.

2.1.2. Antibodies as diagnostic tools

It is well established that the formation of an ag-ab complex is the basic event for a humoral immunological reaction. This complex formation is driven by thermo-dynamical principles. Together with the nature of the non- covalent bonds involved, this ag-ab complex formation suggests the chemical basis of the immunological reaction. Antibodies can be studied from these immunochemical points of view as a screening system for B cell activity in fundamental immunology.

It is also obvious that ab, because of their molecular recognition properties, can be used as detecting reagents for a variety of proteins by virtue of their high specificity and the sensitivity of ag-ab complex formation. A variety of soluble antigens like glyco- and lipoproteins, polysaccharides, hormones, vitamins and haptens, whether linked to cells, bacteria, latex particles carrier molecules or otherwise can thus be detected. Therefore many techniques have been designed, all based upon detection of this ag-ab complex formation. They are applied among others in clinical chemistry, food and dairy technology, microbiology as well as in immunology, pathology, molecular biology and endocrinology. The various techniques, some of which will be discussed in greater detail below, have their own detection limits, sensitivity ranges, advantages and disadvantages. It is important to note that we will discuss in this chapter only those methods that we have experience with and by no means review all the other methods that are available and/or applicable.

2.2. Induction of antibody formation

The clonal selection theory proposed by Burnett in 1959 remains one of the most important integrating concepts in immunology. When the immune system encounters an antigen, only those lymphocytes carrying the appropriate receptors will be able to bind that particular antigen. As a consequence intracellular processes are initiated (i.e. activation) which can lead to cell division and differentiation.

An essential aspect in the discussion of activation is the cell cycle. Most of the mature mIgM$^+$/IgD$^+$ B lymphocytes are in a resting state (G_o- phase), exhibiting a low rate of metabolic activity and no net DNA synthesis (Chan and Osmond, 1979). Activation is a general term referring to a complex process by which the cell, after contact with the stimulting agent, progresses from the G_o-phase into the cell cycle (Black et al., 1980; Herzenberg et al., 1980).

2.2.1. Induction of antibody formation in vitro

In studying the processes related to activation and subsequent proliferation and differentiation of B lymphocytes in vitro, specific ag or polyclonal B cell activators (mitogens) are used. The latter stimulate a large proportion of the B cell population and thus evoke responses of sufficient magnitude to permit analysis of membrane related and intracellular changes. Polyclonal B cell activators (PBA) can roughly be divided into two groups. One group of substances can bind to the antigen receptor, i.e. membrane bound Ig, and mitogens of the second group bind to other (mostly unknown) membrane associated structures. Examples of the latter category are pokeweed mitogen (PWM), lipopolysaccharide (LPS), purified protein derative (PPD), dextran and Epstein-Barr virus (EBV). PBA binding to the ag receptor are anti-immunoglobulin antibodies (anti-Ig; anti-IgM; anti-IgD; anti-IgG; anti-idiotypic antibodies) and Staphylococcus aureus Cowan I strain (Sta) bacteria (Sieckmann et al., 1978; Romagnani et al., 1981; Purè and Vitetta, 1980; Yoshizaki et al., 1982).

PBA and ag can be subdivided according to their requirement for T cells to facilitate the B cell response. T cell-dependent (TD)- and T cell- independent (TI)-PBA and ag can be discerned. TD-PBA and ag can only induce a B cell response in the presence of T cell help (e.g., PWM, PPD and most soluble proteins). In contrast TI-PBA and ag are able to stimulate B cells in the absence of T cells (e.g., LPS, EBV and certain polysaccharides). T cell help in the B cell response against TD-PBA and ag requires physical contact of the helper T cell and the responding B cell. The T cell recognizes the ag, probably bound to the ag receptor of the B cell, in context of class II molecules encoded for by the major histocompatibility complex (MHC) (Schwartz, 1984).

The second way in which a B cell can be helped to proliferate and differentiate, is through soluble ag-specific helper factors and/or non- specific factors - often referred to as lymphokines - produced by activated T cells. Among these are growth factors, regulating the proliferation of activated B cells and differentiation inducing factors.

The initial activation of helper T cells participating in TD B cell responses is based on the dual recognition of the ag (or polyclonal T cell activator) and class II MHC-molecules, usually present on the surface of ag presenting cells (APC) (Vitetta et al., 1984). The factor dependent differentiation of B cells is thought to be a linear sequence of steps leading from an initial activation, subsequent DNA synthesis and cell division to Ig production. In studying these processes two membrane Ig-binding PBA are most frequently used: anti-IgM (mice, humans) and Sta (humans) (Kishimoto et al., 1984; Howard et al., 1984; Kehrl et al., 1984). Furthermore, in murine studies LPS is frequently used as a PBA.

Binding of an antigen or PBA to surface Ig or another receptor can trigger a cascade of events taking place in cytoplasm and nucleus, eventually resulting in the generation of a clone of Ig secreting B cells. One of these early changes is the enhanced transcription of the c_μ-gene. This results, by unknown posttranslational events, h...

transcription, to a decrease in the membrane IgD density. Lamson and Koshland (198
shown that the increase in the transcription of the c_μ-gene is accompanied by a shift
μ_m-RNA to μ_s-RNA. This shift begins shortly after PBA exposure and within 48 hou
μ_s-RNA is the predominant RNA in the activated B cells. This event takes place bef
activated cells enter the S-phase. Upon activation with a low dose of anti-IgM resting
cells enter the cell cycle (G_o --> G_1), which is accompanied by cell enlargement and
expression of receptors for B cell growth factor (BCGF like BSF-1; Rabin et al., 198
B cell differentiation factor (BCDF). Upon binding this BCDF and growth promoting
the B cell can enter the S-phase (Sidman et al., 1984).

Resting human B cells (G_o phase) can be activated by anti-IgM or Sta to expres
number of activation markers like the transferrin receptor in the G_1 phase before ent
the S phase. The activated cells are then responsive to BSF-1 (Ambrus and Fauci, 198

One of the characteristics of an activated B cell is its ability to secrete pentamer
IgM. Necessary for the assembly and secretion of pentamer IgM is an other protein, t
J-chain. At 72 hours after initiation of the activation and just before the secretion of
pentamer IgM, newly transcribed J-chain sequences appear. The secretion of large am
of IgM is preceded by a rapid amplification of μ_s- and J-chain RNA. One can hypot
that these events are initiated at the cellular level after B cells have bound differentia
factors (e.g., BCDF), which are known to promote B cell differentiation towards an Ig
secreting B blast together with a monokine, IL-1 (Lipsky et al., 1983).

Isotype-specific BCDF have been described for IgE in the rat system (Delespess
al., 1975), IgG in the mouse system (Isakson et al., 1982) and IgA in both the mouse
(Kiyono et al., 1982) and human (Mayer et al., 1982) systems.

It is generally accepted that $sIgM^+$ B cells give rise to cells that can synthesize o
Ig isotypes still expressing the same specificity and the same idiotype as their ancestor
By this mechanism, called Ig class
switching or isotype switch, the full range of antibody isotypes can be expressed in
molecules having the same specificity and thus the same idiotype (Gearhart et al., 197
Gearhart, 1982; Hooijkaas et al., 1981; Baumhäckel et al., 1982; Alt et al., 1982). The
ching process in known to occur through a complex set of DNA and RNA splicing an
recombination events, involving either deletion of the DNA intervening the rearrangec
V-gene sequence and the newly expressed C-region gene, or sister chromatid exchang
(Obata et al., 1981; Radbruch and Sablitzky, 1983). The deletion model (Honjo and K
1978) suggests a sequential isotype or subclass expression after switching, correspondir
the localization of the heavy chain C-genes in the genome (in humans chromosome 1
mice chromosome 12).

A prerequisite for <u>in vitro</u> growth of normal B lymphocytes is the use of suita
culture conditions, which allow every single lymphocyte, having the capacity to be
stimulated by the PBA employed, to grow and develop into a clone of cells. Growth
requires, in addition to RMPI 1640 medium (for mouse lymphocyte cultures) and the
presence of for example the mitogen LPS, growth-supporting fetal bovine serum and
use of 2-mercaptoethanol, while for maturation mouse or rat thymus filler cells are
necessary (Hooijkaas et al., 1982; Lefkovits, 1972, 1979). Serum-free media have also
used (Iscove and Melchers, 1978). Recently, it was reported that macrophages are a
prerequisite for the <u>in vitro</u> activation of B cells by LPS (Melchers and Corbel, 1983).
general, every third murine splenic B cell is responsive to LPS and will grow up to 7
dividing every 18 hours under these <u>in vitro</u> culture conditions. Cultures are normally
lyzed on day 5 for IgM secretion and on day 7 for the secretion of IgG or IgA in the
appropriate plaque assays. For the analysis of supernatants in radio-immuno assay (R
enzyme-linked immunosorbent assay (ELISA) the cells are generally cultured up to 1

We use the following culture conditions: varying numbers of mouse spleen or ly
node cells (maximally 1200 per 0.2 ml) or bone marrow cells (maximally 6000 per 0.2
are cultured in 96-well tissue culture plates (Costar 3596; Costar, Cambridge, MA, U
together with 7.2 x 10^5 irradiated (0.1 Gy) rat thymus cells to support growth and 50
LPS B from E. coli (026:B6; Difco Laboratories, Detroit, MI, USA) in 0.2 ml RPMI

medium supplemented with glutamin (4 mM), penicillin (100 IU/ml), streptomycin (50 µg/ml), 2-mercaptoethanol (5 x 10^{-5}M) and fetal bovine serum (20%) (lot B 66390302; Boehringer Mannheim GmbH, Mannheim, Western-Germany), specifically selected for growth-supporting properties and low endogenous mitogenic activity. Cultures are incubated in a humidified atmosphere containing 5% CO_2 at 37°C (Heraeus, Hanau, FRG).

2.2.2. Induction of antibody formation in vivo

Several lymphoid organs are involved in antibody formation in vivo. The bone marrow and thymus are called the 'central' or 'primary' lymphoid organs because they are the breeding sites of lymphocytes. The bone marrow generates the immunocompetent virgin B lymphocytes and pre-T cells. These pre-T cells are generated independent of the presence of the thymus, the lymphoid organ to which these immature, immuno-incompetent cells normally home, proliferate and differentiate into mature immunocompetent T lymphocytes. The 'peripheral' or 'secondary' lymphoid organs like spleen, lymph nodes, the gut-associated (GALT) and the bronchus-associated lymphoid tissue (BALT) provide the architecture and accessory cells (macrophages and dendritic cells) appropriate for the antigen processing and for the presentation of the antigen to the lymphocytes. In these organs, therefore, the antigen-driven differentiation of lymphocytes takes place. Within these peripheral lymphoid organs a B and T cell compartment can be recognized. Virgin B cells settle predominantly in the lymphoid follicles, whereas T cells are found within the peri-arteriolar lymphoid sheath (PALS) of the splenic white pulp, the paracortex of lymph nodes and the interfollicular areas of the GALT (Van Ewijk et al., 1977; Rozing et al., 1978).

2.2.2.1. Primary versus secondary-type responses

Upon primary antigen injection small B lymphocytes can be stimulated to transform into B cell blasts in the peripheral part of the PALS. These B cell blasts proliferate and differentiate into antibody producing plasma cells. During this differentiation the cells migrate to the red pulp of the spleen and to the medullary cords of the lymph nodes.

Although thymic humoral factors or T cells do not influence the lymphocyte production in the bone marrow, after ag stimulation of B cells ag- specific T cells can greatly enhance their response. The activated B cells give rise to clones of plasma cells, which, remain mainly localized in the lymphoid organs. Their secretory products, however, the antibodies, are carried by the blood and lymph to places where they have to perform their function.

The humoral immune response to an antigen is characterized not only by the production of plasma cells, but also by the induction of antigen- specific immunological memory. By comparison with the primary response, the secondary response is characterized by : a shorter lag period between the encounter with ag and the ab production, ab production that has a higher rate and is more persistent, higher ab titers at the peak of the response, a predominance of IgG molecules and ab with a higher affinity for ag than those produced in the primary response.

Both the T and B lymphocyte population carry immunological memory and such cells are, in contrast to virgin cells, long-lived small cells with a life-span of several weeks to several months (Sprent, 1977). Furthermore these memory cells are recirculating cells, while virgin lymphocytes are mainly non-recirculating sessile cells (Strober and Dilley, 1973). The amounts and the types (affinity, isotypes) of ab formed vary widely with the conditions of immunization, some of which are discussed in the following sections.

2.2.2.2. Type of antigen

The induction of maximal antibody production to most ag requires the participation of ag-specific T helper cells (hence such ag are called "thymus-dependent" [TD]). Upon primary immunization of mice with TD ag, PFC are almost exclusively found in spleen and lymph nodes. Secondary immunization, however, induces a substantial PFC response in the bone marrow as well. During the secondary response the bone marrow becomes the major source of serum ab (Benner et al., 1974). This bone marrow ab form

the immigration and the subsequent proliferation of memory B cells that are reactivated by the booster ag in the peripheral lymphoid organs (Benner et al., 1974; Koch et al., 1982b).

Presumably the bone marrow lacks the appropriate microenvironment and/or the quantity or quality of cells required for the early steps in the induction of immune responses. In contrast to mouse bone marrow, human bone marrow has features which are characteristic of peripheral lymphoid tissues, e.g. the occurence of follicles with germinal centers (Nieuwenhuis and Opstelten, 1984). Therefore, antibody formation in the human bone marrow may not be similarly dependent upon an influx of antigen-activated B cells from the periphery.

Germinal centres arise as discrete sites of B cell differentiation in B cell areas of lymphoid tissues after antigenic stimulation. An important role has been suggested for germinal centres in heavy chain class switching (Kraal et al., 1982, 1985).

The characteristic kinetics of the PFC response in spleen and bone marrow shows that the ab-forming cell response is regulated in such a manner that the peripheral lymphoid organs respond rapidly, but only for a short period, whereas the bone marrow response starts slowly, but takes care of a long-lasting massive production of ab to ag which repeatedly challenge the individual. Thereby the peripheral lymphoid organs provide a fast defence to the challenging ag, while the bone marrow provides a long- lasting protection against recurrent infections (Tayler and Everett, 1972; Mendelow et al., 1980).

In contrast to TD ag, certain thymus-independent (TI) antigens give rise to ab formation in the bone marrow already during the primary response (Koch et al., 1982a, 1982c). The difference in capacity of the various TI ag that we have tested to induce a PFC response in the bone marrow does not correlate with the subdivision of TI ag into two classes (TI-1 and TI-2) according to the response they induce in CBA/N mice. These mice are unable to raise an immune response to TI-2 ag such as DNP-Ficoll, levan, dextran etc. (Sher et al., 1975). It is thought that TI-2 ag stimulate the more mature B cells that express IgD, complement receptors, Lyb-3 and Ly-5 surface ag, and predomiantly occur in the spleen (Huber et al., 1977; Ahmed et al., 1977). TI-1 ag such as TNP-LPS and TNP-Brucella abortus stimulate, besides the more mature B cells, a population of less mature B cells that are also present in the bone marrow (Mond et al., 1977).

2.2.2.3. Route of antigen administration

Normally, lymphatic tissues are almost constantly encountered with ag from transiently invasive or indigenous microbes (normal flora of skin, intestines, etc.) and those that enter the body by inhalation (e.g., plant pollens), by ingestion (e.g., food, drugs) and by penetration of the skin.

For debliberate immunization, immunogens are usually injected into skin (intradermally or subcutaneously (s.c.)) or muscle (intramuscularly (i.m.)), depending on the volume injected and the irritancy of the immunogen. Intraperitoneal (i.p.) and intravenous (i.v.) injections are also used in experimental work, especially with particulate ag. Regardless of the route, most ag eventually become distributed throughout the body via lymphatic and vascular channels. Because most ag's are degraded in the intestines, feeding is effective only under special circumstances. Allergic responses to food are probably due to ag that resist degradation by intestinal enzymes. Immunization can also be performed by aerosol administration of the ag.

Ab formation is generally most prominent in the lymphoid organ draining the site of immunization. The highest bone marrow PFC responses are observed with i.v. or i.p. booster injections (Benner et al., 1977).

Secondary immunization with a TD ag will lead to ab formation in the bone marrow independent of the route of primary immunization: i.v. and i.p. are equally effective; s.c. priming is effective with relatively high doses of ag.

Every ag has an optimal immunogenic dose range. Much larger amounts elicit high-zone tolerance. With TD ag, but not with TI ag, lower amounts can also cause tolerance (low-dose tolerance). Also the physical state of an ag influences the immunogenicity: aggregated molecules of bovine gamma globulin, for instance, are immunogenic, while monomers are tolerogenic. It is thus difficult to induce tolerance to

particulate ag, which are usually highly immunogenic. The route of administration is another determinant: soluble ag tend to be immunogenic when injected into tissues, but to be tolerogenic when given i.v.

The surface of a protein ag consists of a complex array of overlapping potentially antigenic determinants; in aggregates they approach a continuum. Most determinants depend upon the conformational integrity of the native protein molecule. Those to which an individual responds is dictated by the structural differences between an ag and the host's self-proteins and by host regulatory mechanisms, and is not necessarily an inherent property of the protein molecule reflecting restricted antigenicity or limited number of antigenic sites. As stated above, protein ag therefore, are more immunogenic when administered in aggregated than in soluble form. Thus chemically cross-linked protein molecules (e.g., by glutaraldehyde) and ag-ab complexes, prepared in slight ag excess are usually highly immunogenic. When, however, the complexes are prepared in ab excess their immunogenicity is greatly reduced, probably because the antigenic determinants are blocked.

The level of ab in the serum reflects the balance between rates of synthesis and degradation. When the rates are equal, the serum ab concentration is constant (steady state). The rate of synthesis depends upon the total number of ab producing cells, which varies enormously with conditions of immunization. By contrast, the rate of degradation (expressed as half- time) is determined by the H chain class; e.g., IgM and IgA usually have a shorter half life than IgG molecules.

2.2.2.4. Use of adjuvants

Adjuvants are agents that by non-specific mechanisms can modify the humoral or cellular immune response by simultaneous injection of ag and adjuvant. Adjuvants not only can stimulate the response to the antigen, but, dependent on the conditions, they can also suppress; furthermore, their effect can be ag-specific as well as non-specific. A large variety of substances can act as adjuvants and their modes of action are very heterogeneous (Borek, 1977; Whitehouse, 1977; Hilgers et al., 1985).

Originally, most adjuvants used were of bacterial origin that were themselves good immunogens. Adjuvants like complete Freund adjuvant (CFA) have a strong stimulating effect on most immune responses. However, it also induces excessive granulopoiesis within the bone marrow, which abolishes the ongoing Ig synthesis in this organ. Other adjuvants which do not induce excessieve granulopoiesis, such as alum, do not interfere with ab formation in the marrow. Many adjuvants probably mediate their effect by protecting the ag for prolonged periods of time against breakdown, while they simultaneously stimulate the mononuclear phagocyte system. Adjuvant induced immunomodulation, on the other hand, can also be mediated by macrophages.

2.2.2.5. Age

A variety of membrane changes can occur during aging, including the appearance of new differentiation ag, modification of the cell membrane by continued exposure to environmental chemical haptens, quantitative differences in the expression of normal cell surface ag (e.g., Ig idiotype by monoclonal expansion of a B cell line), changes in cholesterol/phospholipid ratio, expression of viral ag or by insertion into the cell membrane of altered proteins arising from somatic mutations or errors in protein synthesis (Orgel, 1973; Heidrick, 1973; Burnet, 1974; Rivnay et al., 1979; Callard, 1981).

The proliferation and differentiation of B cells is only moderately impaired with age leading to a decrease in the number of effector cells generated (Price and Makinodan, 1972). However, the pattern of differentiation of B cell precursors found in the bone marrow of aged mice may be altered, since in the in vivo transfer systems it was suggested that bone marrow from aged mice contained a significantly greater number of differentiated immunocompetent cells than the marrow of young mice (Farrar et al., 1974). The age related changes in the central lymphoid organs are to a large extent based upon deficiencies in T cell function: old bone marrow has a decreased capacity to provide the thymus with T cell progenitors because of a reduced stem cell population. Also the number

of cortisone- resistant mature thymocytes declines with age together with a progressive involution of the thymus after sexual maturation (Tyan, 1981).

In mice high-avidity IgG antibody-forming cells preferentially decline during aging (Goidl et al., 1976). This finding is in contrast to the notion that the secondary immune potential is less severely affected by age than the primary potential (Finger et al., 1972). Moreover, the response to TD ag is more susceptible to age-related decline than the response to the TI ag LPS (Blankwater et al., 1975). Also the responsiveness towards low doses of ag declines faster than towards high doses (Price and Makinodan, 1972).

It was shown in non intentionally immunized mice that the total number of cells containing cytoplasmice immunoglobulins (C-Ig) summed over spleen, bone marrow, mesenteric lymph nodes and Peyer's patches did not change appreciably during aging, in contrast to the relative contribution of the different lymphoid organs to the total (Haaijman et al., 1977; Haaijman and Hijmans, 1978). The majority of C-Ig cells in young mice was localized in the spleen, whereas in mice older than six months of age the predominant site was the bone marrow. Comparative studies with athymic (nude) mice revealed that the differential role of spleen and bone marrow during aging does not dependent on the presence of a functional thymus while the class distribution of C-Ig cells does (Haaijman et al., 1979). Ideally, the total number of Ig-producing cells in the various lymphoid organs should correlate with the Ig concentration in the serum. In man such correlations have been reported for the various Ig classes and subclasses (Hijmans, 1975; Turesson, 1976). In these studies Ig-producing cells were enumerated by immunofluorescence. In mice, similar studies, also with immunofluorescence did not reveal a simple linear correlation. During aging in normal mice a slow increase of background C-Ig positive cells was found (Van Oudenaren et al., 1981). However, the number of Ig-secreting cells increases more rapidly (Benner et al., 1982a), leading to a change in the ratio between the number of Ig-producing cells found in the protein A plaque assay and by immunofluorescence. The underlying cause of these discrepancies is probably that in immunofluorescence only plasma blasts and plasma cells can be identified with certainty. However, earlier stages of the plasmacytic series are also able to produce and secrete Ig, and thus are able to cause plaques (Melchers and Andersson, 1973, 1974).

The number of Ig-producing cells per organ depends on age. During the first week of life of mice almost only IgM secreting cells are found. At 14 weeks of age the number of Ig-secreting cells reaches a maximum in lymph nodes and Peyer's patches and then decreases gradually. However, the number of Ig-secreting cells in the bone marrow increases constantly throughout life (Benner et al., 1982a).

Qualitative aspects concerning serum Ig levels in the aging human and laboratory animal population show an increase of the average concentrations of IgG and IgA, while those of IgM and IgD show a (generally nonsignificant) decrease (Buckley et al., 1974). Increased levels of IgG1 and IgG3 subclasses in volunteers over 95 years of age were found to be responsible for the elevated values of the IgG class as compared to young adult controls (Radl et al., 1975). The IgG2 and IgG4 subclasses did not seem to contribute substantially to the increased total concentration of IgG in the aged. Increased variations with age among the Ig levels of different individuals were generally found in both human and mouse species. The age-related changes were, therefore, selective (Radl, 1981). In old age the occurrence of autoantibodies, paraproteins and immunodeficiencies at the B cell as well as T cell level can occur. This is most likely due to a selective effect influenced by genetic factors.

2.3. Measurement of immunoglobulin secretion at the cellular level

2.3.1. Preparation of cell suspensions

The analysis of antibody production in different organs can be performed at the cellular level under normal *in vivo* or controlled *in vitro* conditions.

Before describing the most frequently used detection techniques for Ig secretion we shall discuss the preparation of cell suspensions from lymhoid organs of mice and man. For quantitating the Ig-secreting cell activity it is essential to use procedures for the preparation of cell suspensions that allow the recovery of as many viable Ig-secreting cells as possible. Below we shall discuss the procedure that, in our hands, gives the best results.

2.3.1.1. Cell suspensions from lymphoid organs of mice

The preparation of cell suspensions from mice has been described in detail in section 1.8.4. of Chapter 1 of this thesis.

2.3.1.2. Cell suspensions of human origin

Most frequently heparinized peripheral blood samples are obtained while occasionally samples of lymphoid organs like bone marrow or lymph nodes are available. After mincing the organs (if necessary) and aspiration of the cells through a needle or flushing through a nylon gauze filter with 100 µm openings, mononuclear cells are collected by density centrifugation
on Ficoll-Paque (density : 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). Before use in one of the various immuno-assays, the cells have to be washed three times in a balanced salt solution (BSS).

2.3.2. Antibody-secreting cell assays

The objective of hemolytic plaque assays is to enumerate and study individual ab-secreting cells, especially in situations where only a few of such cells are present among many cells that do not release antibodies. For example, using the plaque assay as few as one hundred ab-secreting plaque- forming cells (PFC) can be detected within a population of 10^8 spleen cells. This great sensitivity is very useful during the early phase of the immune response after a primary antigenic stimulus and in attempts to induce antibody formation in vitro.

The original Jerne-type direct plaque assay detects single cells which secrete IgM antibodies that bind to determinants on the target red blood cells, usually sheep erythrocytes (SRBC) (Jerne and Nordin, 1963a; Jerne et al., 1963b). These determinants may be naturally occurring red blood cell surface ag, or determinants artificially coupled to the red cell surface (Jerne et al. 1974). Cells secreting antibodies of a (sub)class other than IgM may also be detected in this assay. Therefore rabbit ab specific for that Ig (sub)class has to be added as developing antiserum (Dresser and Wortis, 1965).

Cells secreting Ig without known antibody specificity ('background' Ig secreting cells) can also be assayed in a hemolytic plaque assay. Two systems have mainly been developed to determine isotype-specific Ig secreting cells: the reverse plaque assay (Molinaro and Dray, 1974; Molinaro et al., 1978) and the protein A plaque assay (Gronowicz et al., 1976). Both are completely dependent on the presence of anti-Ig antibodies on the surface of the target SRBC. In the reverse plaque assay (section 2.3.2.2.) the SRBC are directly coated with the antibody molecules. In the protein A plaque assay (section 2.3.2.1.) this is done indirectly by coating the target SRBC with protein A by $CrCl_3$ followed by the addition of antibodies of the IgG class with high binding affinity to protein A.

As the detection of PFC depends solely upon the specificity of the developing antiserum, isotype-specific assays can be performed and it is thought plausible that these assays are applicable to any molecule secreted in sufficient amounts and for which a specific developing antiserum is available. For example, the secretion of albumin by hepatocytes (Primi et al., 1981).

In the original hemolytic plaque assay of Jerne and Nordin, lymphoid cells together with a suspension of target red blood cells are immobilized in a agar gel. Cunningham (1965) and Cunningham and Szenberg (1968) proposed a modification of the original assay by incubating the lymphoid cells and the target red blood cells in a medium without supporting gels as monolayers in sealed chambers. This method is simple, sensitive and rapid for the

enumeration of PFC and therefore used routinely throughout the different plaque assays described in this thesis.

2.3.2.1. Protein A plaque assay

Ig secreting cells can be assayed by the hemolytic plaque assay as described by Gronowicz et al. (1976) and modified by Van Oudenaren et al. (1981). This assay has been extensively described in section 1.8.7. of Chapter 1 of this thesis.

Since its introduction, the protein A plaque assay has been succesfully employed for the detection of Ig-secreting cells of a variety of species including man (Hammerström et al., 1979; Smith et al., 1979; Hammerström et al., 1980).

2.3.2.2. Reverse plaque assay

Contaminating Ig molecules in the guinea pig complement used for protein A as well as reverse plaque assays, reduces the number of plaques and their size by competitive inhibition of the binding of the specific rabbit anti-Ig molecules via the protein A to the target SRBC. For the protein A plaque assay this problem can be overcome by affinity chromatography of the guinea pig complement on a protein A-Sepharose column (Van Oudenaren et al., 1981).

In the reverse plaque assay, developed by Molinaro and Dray (1974), SRBC are directly coated with antibodies using the chromium chloride coupling method (see section 1.8.7. of Chapter 1 of this thesis). This assay is based upon diffusion of the Ig (e.g. mouse Ig) from the Ig secreting cells into an agar gel containing SRBC coated with rabbit anti-mouse- Ig. Localized hemolysis occurs around the cells upon addition of a rabbit anti-mouse Ig developing antiserum and complement. The method, essentially being a modification of Mancini's single radial immunodiffusion assay, has the same incubation of 48 hrs but a greater sensitivity (0.1 µg/ml compared to 100 µg/ml). Ig-secreting cells form hemolytic plaques independent of the antibody specificity of the secreted Ig. This assay can therefore be applied to enumerate subpopulations of Ig-secreting cells on the basis of the isotype, allotype or idiotype they secrete (Molinaro et al., 1978).

The protein A plaque assay, however, is more economical since it requires a smaller amount of rabbit-anti-Ig antibodies. Moreover, affinity purification of the developing anti-Ig antibodies, required in the reverse plaque assay, is not necessary in the protein A assay. Furthermore, the same batch of protein A-coated SRBC can be used to enumerate Ig-secreting cells in cell suspensions obtained from different species.

2.3.2.3. Antigen specific plaque assays

The plaque-forming cell assay can be adapted to the detection of cells forming antibodies to ag like proteins, polypeptides, polysaccharides and a variety of haptens. The only requirement is that the red cells be coated to exhibit an adequate density of ag determinants without causing excessive fragility of the red cells or altering their susceptibility to lysis by complement.

Two general approaches to coupling determinants to red cells are used: the chemical approach where the ag or haptens are directly coupled to the red cells, or the immunological approach where proteins or haptens are conjugated to anti-erythrocyte antibodies which then attach to red cells by virtue of their ab activity. This is most effectively accomplished by using monovalent Fab fragments of anti-red cell antibodies. Such conjugates, that should not by themselves lyse in the presence of complement, have been described (Strausbauch et al., 1970; Miller and Warner, 1971).

In the above described direct plaque assay, the only antibodies detected are of the IgM class, since IgG, IgA and IgE antibodies are not efficient enough in complement binding to mediate cell lysis under these experimental conditions. To detect these antibodies of non-IgM isotype, the so- called indirect plaque assay has to be applied. In the latter assay, an IgG-, IgA- or IgE-specific rabbit antiserum is added, which will bind to antibody

of the relevant Ig class produced by the cell suspension and bound to the indicator erythrocytes. The class specific antibodies crosslink the coating antibodies and generate conditions for efficient complement binding and hence for cell lysis. Since also direct plaques will develop, it is essential to note that the number of such 'facilitated' or 'indirect' plaques can in principle be determined by subtracting from the number of plaques developed with the antiserum, the number of plaques obtained in its absence.

In this section an example of a protein-antigen specific as well as a number of hapten-specific plaque assays will be given.

2.3.2.3.1. Coupling of haptens to target cells for use in plaque assays

Haptenic groups may be linked directly to the erythrocyte surface or be attached as a hapten-protein conjugate which is especially useful to space out the haptenic group or when the coupling conditions are damaging to the red cell membrane.

The widely used nitro-phenyl derivatives 2,4-dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP) are completely cross-reactive. Preferably, TNP is coupled to SRBC by using water-soluble trinitrophenyl benzene sulphonic acid (TNBS) as the active form, essentially as described by Rittenberg and Pratt (1969). Briefly, 30 mg of TNBS (Eastman Kodak Co., Rochester, NY, USA) is dissolved in 4 ml Na-cacodylate buffer (0.28 M, pH 6.9)(BDH Chemicals Ltd., Poole, UK). Together with 1 ml washed, packed SRBC, the mixture is wrapped in foil and incubated for 10 min at room temperature on a rotor. Subsequently, the cells are washed two times in glycyl-glycin (0.05 M in BSS) and then two times in BSS. Cells are resuspended to 17% (v/v) in BSS. When 3 mg of TNBS is used for coupling to 1 ml SRBC, cells are referred to as TNP3-SRBC instead of TNP30-SRBC in the above described protocol.

Other nitrophenyl derivatives like 4-hydroxy-5-iodo-3-nitrophenyl (NIP) or 4-hydroxy-3,5-dinitrophenyl (NNP) are coupled to SRBC by adding e.g. 0.4 or 4 mg and 0.2 or 2 mg of the hapten succinimide active esters (Biosearch, San Rafael, CA, USA) per milliliter of washed and packed SRBC. The coated cells are referred to as $NIP_{0.4}$-SRBC, NIP_4-SRBC, $NNP_{0.2}$-SRBC and NNP_2-SRBC, respectively. This mild coupling procedure is essentially as described by Pohlit et al., (1979): SRBC (1 ml) are resuspended in 10 ml carbonate-bicarbonate buffer (0.12 M, pH 9.2). Addition of the esters is as follows: for NNP_2-SRBC 50 µl of a 40 mg/ml solution is used, for $NNP_{0.2}$- SRBC 50 µl of a 4 mg/ml solution, while for NIP_4 100µl of a 40 mg/ml and for $NIP_{0.4}$ 100 µl of a 4 mg/ml solution is used. These ester solutions are added dropwise to the mixture.

The coupling mixture is wrapped in foil and incubated for 40 min at room temperature wrapped in foil on a rotor. Cells are then washed in saline and BSS (2 times) and finally resuspended in BSS and adjusted to a 17% solution (Hooijkaas et al., 1983, 1985).

2.3.2.3.2. Coupling of proteins to target cells for use in plaque assays

For the coupling of large protein antigens to target cells for use in complement dependent lysis it is essential that indicator cells of optimal sensitivity are produced. The simplest procedure for coating red cells with proteins like ovalbumine and chicken gamma globulin (Koch and Benner, 1982), is the one that makes use of $CrCl_3$ as coupling agent according to the method described by Gold and Fudenberg (1967). We recently described a rapid procedure in which different proteins like protein A or ovalbumin can be coupled to SRBC within 4 minutes (Savelkoul et al., submitted). This procedure is also based upon $CrCl_3$ as the coupling agent but it can essentially be prepared freshly in order to give more reproducible coupling ratios compared to the aged solutions that are routinely used (Perucca et al., 1969; Sweet and Welborn, 1971; Kofler and Wick, 1977; Ling et al., 1977; Truffa-Bachi and Bordenave, 1980). SRBC are washed four times (5 min, 1500 x g) in 10 vol freshly prepared $CrCl_3$ in saline (12.5 ug/ml). For coupling 1 ml washed, packed SRBC, 1 ml of protein A or ovalbumin in saline (1 mg/ml) and 1 ml $CrCl_3$ in saline (1 mg/ml) are mixed and incubated for 4 min at room temperature. Subsequently, 20 vol 1% fetal calf serum (FCS) in saline are added and the cells are washed in this solution 3 times for 5 min at 600 x g. Finally, the cells are resuspended in BSS and used within 3 days of

2.3.2.4. Practical aspects of plaque forming cell assays

- The simplest procedure for coating red cells with proteins like protein A (section 2.3.2.1. and 2.3.2.3.2.) and ovalbumin (OVA) (section 2.3.2.3.2.) and the one requiring the least protein, is the use of $CrCl_3$ according to the method described by Gold and Fudenberg (1967). The coupling conditions that produce indicator cells of optimal sensitivity must be found empirically. Relevant points to consider are: an optimal protein concentration should be between 0.25 and 2.5 mg/ml and phosphate ions which 'stop' the reaction, should be excluded from the mixture until coupling has been completed. For a given number of SRBC, the volume of $CrCl_3$ solution may differ but the final concentration of $CrCl_3$ per cell must be kept constant. The same holds true for the desired protein to be coupled.

For mysterious reasons, usually aged solutions of $CrCl_3$ have to be used for the coupling reaction. In our alternative method we use freshly prepared $CrCl_3$ solution for reproducible coupling. Moreover, by prewashing the cells in a low concentration of $CrCl_3$ the coupling time can be decreased to 4 minutes. Stopping the reaction by washing with a 1% FCS solution prevents clumping of the cells, which is a well known problem with the $CrCl_3$ coupling method.

Most important, optimal conditions for sensitization of red cells that is optimal for hemagglutination and reverse plaque assays is inadequate for plaque assays. Such cells have a low density of determinants so that the rate of attachment of the secreted ab to red cells is too low to cause a plaque. Dependency on a high degree of coupling varies with the stage of the immune response (the affinity of the ab) and the Ig class of the PFC.

Other protein coupling procedures, e.g. with carbodimide, benzidine and benzoquinone (Ternynck and Avrameas, 1976; Dresser, 1978) will not be discussed here.

- Inhibition of plaque formation by free ag or hapten confirms the specificity of the plaques. The avidity of the ab produced by the PFC can be analyzed by measuring this inhibition: high avidity ab plaque formation is inhibited by lower concentrations of free ag or hapten than plaque formation by low avidity ab (Jerne et al. 1974; Fazekas de St.Groth, 1979).

- Optimal hemolytic plaque formation requires carefully controlled reagents and conditions. A batch of SRBC should be selected on the basis of a low autohemolytic activity. Most batches of guinea pig complement are most effective at a 1:20 final dilution of fresh serum or a 1:10 dilution after passage of the guinea pig complement through a protein A-Sepharose column.

SRBC in Alsever's solution should be aged for one to three weeks, since fresh cells are less susceptible to lysis while older cells are excessively fragile. The optimal final red cell concentration is 2%, thus 17 µl of a 17% (v/v) suspension of ag or protein A coated SRBC per 147 µl incubation mixture.

Prolonged incubation times till 4-5 hrs do not affect plaque morphology and numbers, provided the guinea pig complement and developing rabbit antiserum have been carefully absorbed with the target-type red blood cells in order to remove antibodies that can lyse the whole monolayer of target red blood cells. After incubation at 37° and 40°C approximately the same numbers of plaques are found, while lower temperatures cause a reduction in plaque numbers.

- True plaques must fulfill the following criteria:

a) Microscopic examination must reveal the antibody-releasing cell in the centre of a plaque and not tissue fragments that cause false plaques.

b) Toward the periphery of a plaque a diluting-out effect of lysed SRBC should be visible, since the concentration of ab becomes too low to cause lysis of all target red cells in that area.

c) Plaques should be round and not sharp-edged like air bubbles.

d) Plaques are never totally clear, that is, lysis of the red cell mono layer is never complete. Fully transparant, clear plaques in the monolayer are almost always the result of bacterial activity, since the described plaque assays are essentially performed under non-sterile conditions.

- Counting of the plaques in the Cunningham liquid layer plaque assay is preferably done immediately after the incubation. The convenient range of plaques to be counted per

slide is 30-150. Outside this range the counts are less reliable. Plaques can be counted under low magnification (20-40 x) and dark field illumination using a dissecting microscope.

The frequency distribution of the number of plaques in each of a large number of slides containing PFC from a single cell population is not normal but follows a Poisson distribution. This means that the estimate of variance is equal to the total number of plaques counted. The variance of the square root of the plaque count or its 2 x SD range is ± 1. Thus the 95% Poisson confidence range associated with a plaque count of 100 is from 9^2 (=81) to 11^2 (=121). In these circumstances it is necessary to calculate the geometric means of the data. However, in the event of a group of animals involving some non-responders, the arithmetic mean calculated from the whole group will give a closer approximation to the mean response of responders than the geometric mean (Jerne et al., 1974).

The plaque counts are expressed either as PFC per whole organ or as PFC per 10^6 viable nucleated cells. This can easily be done for spleen and lymph nodes, with a sample from a cell suspension made from the whole organ. But for the total bone marrow of a mouse this is not feasible. Since the bone marrow from femurs can easily be isolated, a conversion factor has been determined: the PFC response of the total bone marrow of a mouse can be calculated to be 7.94 times the response by the bone marrow from two femurs (Benner et al., 1981).

If the samples are taken from cultures, then the number of PFC may be expressed per culture, per 10^6 input cells, or per 10^6 recovered viable cells.

The size of the plaques depends both on the quality and quantity of antibodies released: cells secreting high-affinity ab will produce smaller plaques than low affinity ab, given the same number of ab molecules secreted per unit of time. The smallest recognizable plaque is about 0.1 mm, corresponding to the angular resolution of the human eye.

Another important factor is the epitope density of the ag determinants on the indicator red cells: higher epitope density leads to smaller plaques by a given number of ab molecules. Also the production rate of the ab- secreting cell (the number of ab molecules secreted per cell per unit of time) determines the size of the plaque. This might be related to the isotype of the secreted ab, since usually large plaques are found when tested for IgM-secreting cells, medium sized plaques for IgG-secreting cells and small plaques for IgE-secreting cells.

2.3.2.5. Solid-phase enzyme-linked immunospot assay

Hemolytic plaque assays have extensively been used for evaluating the kinetics of the humoral immune response and the regulatory mechanisms underlying proliferation and maturation of B-lymphocytes to antibody-secreting cells. These assays have, as outlined in section 2.3.2.4., several inherent limitations when the assay is applied to soluble protein antigens.

Inconsistent results have generally been associated with difficulty in coupling enough ag onto the target red cells, the instability of the antigen-coated target red cells, variable lysability of different batches of target red cells, uncertainty about the isotype involved in the formation of direct plaques and the inability to quantitate the ab molecules secreted. Recently methods have been described in which the ag was adsorbed onto polystyrene or nitrocellulose surfaces (Sedgwick and Holt, 1983; Czerkinsky et al., 1983; Moore and Calkins, 1983; Holt et al., 1984; Miller and Borrebaeck, 1985; Holt and Plozza, 1986). Application of ab-secreting cells results in an immobilized antigen-specific focus of the Ig produced. These foci are subsequently developed in an ELISA by the addition of the appropriate enzyme-anti-Ig conjugate and an enzyme substrate in agarose which yields an insoluble product after incubation resulting in clearly visible spots of different size and intensity. Apart from ag-specific Ig secreting cells also total Ig-secreting cells of a particular isotype can be determined in this ELISPOT assay using protein A coated surfaces.

The procedure we use is essentially according to Sedgwick and Holt (1983) with modifications. Proteins (e.g., ovalbumin) are coated at a co

phosphate buffered saline (pH 7.2; PBS) in 100 µl per well c
microtiterplates (Titertek, Flow, Irvine, Scotland) for 1 hr a
three times with PBS and subsequently the remaining active
PBS containing 1% BSA (Boseral, Organon Teknika, Oss, Th
washed one time with PBS-BSA containing 0.05% Tween-20
Western-Germany). After drying, the wells are slowly filled
washed two times with PBS and diluted in PBS-BSA or BSS-
densities must be determined critically by testing many diffe
10^3 cells per ml. These cell suspensions are incubated at a st
Incubation times can be prolonged till 16 hrs at 4°C by work
Cells are then resuspended in culture medium containing BS
5% CO_2-atmosphere. Cells are discarded by flicking the plat
0.05% Tween-20 in distilled water to lyse the remaining cells
washed thoroughly with PBS-BSA-Tween for 2 hrs at 37°C b
conjugate. Conjugates can be prepared according to Kearney
enzyme alkaline phosphatase to specific affinity purified anti

The plates are washed three times in PBS-BSA-Tween
is added. The substrate buffer is prepared as follows: 150 mg
X-405 (Sigma, St. Louis, USA) and 1.0 g sodium azide are di
distilled water and 95.8 ml 2- amino-2-methyl-1-propanol (A
stirring. Distilled water is added to about 90% of the final vo
10.25 with concentrated HCl. The solution is left overnight at
Distilled water is then added to bring the volume to 1.0 l and
A 2.3 mM solution of the substrate
5-bromo-4-chloro-3-indolyl phosphate (5-BCIP; Sigma, St. L
AMP buffer in a light-protected bottle and the substrate is fi
µm about an hr after preparation to sterilize and to remove a
solution should then be stored at 4°C in the dark.

For use in the assay the substrate is mixed with agarose.
agarose (Type I; Sigma, St. Louis, USA) is prepared in distille
aliquots and stored at 4°C. On the day of assay, 1 aliquot is he
is liquified, it is cooled to 40°C at which temperature the sub
forewarmed. Subsequently a mixture of 12 ml substrate and 3
µl of this is immediately added to each well of the plate, and
After 5 min, when the mixture is hardened, the plates are clo
at 37°C until the blue dots, each representing an Ig secreting c
min). Spots can be easily counted over a light microscope equ
(Belco Glas Inc., Vineland, U.S.A.). Critical points to note in

- The filtration of the substrate solution to avoid the occur
above the agarose/solid-phase interface.
- The addition of a protein (like BSA) in all washing and
high number of small 'false' spots.
- The avoidance of cell movement during the incubation o
movement of the agarose during spot development which cou
- The likelihood of lack of sufficient isotype specificity of
especially with many of the commercially available conjugate

2.3.2.6. Immunofluorescence assays

Cells producing Ig are morphologically characterized by
basophilic cytoplasm. Their morphology can range from lymp
these cells can be enumerated by means of immunofluorescen
specific for the Ig produced (Hijmans et al., 1969; Hijmans a
availability of fluorescent reagents of high activity and spec
for reliable results. In practice, the choice is mainly between
(FITC) and tetramethylrhodamine isothiocyanate (TRITC). A
commercially available they can be prepared easily as describ

and Goding (1976). It has been found in cell suspensions of murine lymphoid organs that enumerations of the Ig producing cells by immunofluorescence and by the protein A plaque assay do not always give similar results (Van Oudenaren et al., 1984). The protein A plaque assay was found to detect as many or several times more Ig producing cells than the immunofluorescence assay, depending on the age and antigenic load of the mice, and upon the Ig class and organ studied. To detect intracytoplasmic Ig-containing (C-Ig) cells, the cells have to be fixed with reagents that allow the fluorescent antibody to enter them. The preparation of cytocentrifuge preparations and the visualization of C-Ig cells have been described in the sections 1.8.9.2. and 1.8.9.3. of Chapter 1 of this thesis. The Ig class distribution of the C-Ig cells can be determined by means of combinations of TRITC and FITC labeled antisera specific for the heavy chains of IgM, IgG, IgA, IgD and IgE. It is also possible to perform indirect staining in that the cells are first exposed to the relevant antiserum at the appropriate dilution and then, after washing, to a fluorescent antiserum to detect the first antibody as an antigen.

The use of fluorescent labeled antibodies is more abundant in the detection of membrane bound glycoproteins. However, such a membrane labeling can well be combined with detection of intracytoplasmic Ig. For the detection of membrane bound Ig or other membrane glycoproteins, cell suspensions need not to be fixed. They are carefully washed (5 min at 100 g at 4 °C) 3 times with PBS-BSA to remove serum proteins or proteins secreted by the cells themselves. The pellet is resuspended and adjusted to a cell density of 1-2 x 10^7 cells per ml. From this suspension 50 µl is mixed with an equal volume of the appropriate conjugated antiserum and incubated for 30 min in melting ice. Then 2 ml of ice cold PBS-BSA is added and cells are washed two times with cold PBS-BSA. Then the cells are spun on a slide by a cytocentrifuge (direct staining) or stained once more with one or two other antibody conjugated to other fluorochromes before preparing slides (double or triple staining). To block remaining active sites of the heterologous antiserum, cells are incubated with normal serum of the species in which the antiserum was raised.

Using such techniques much has been learned about the differentiation and maturation pathway(s) of B cells both in mouse and man, depending on their membrane-bound as well as intracytoplasmic markers. Since many, if not all, differentiation stages of normal human B cell development seem to be parallelled by leukemic disorders, the above mentioned techniques are extremely valuable in the detection of malignant cells (Van Dongen et al., 1984, 1985).

2.4. Measurement of circulating immunoglobulins

As outlined in the section 2.1.2. of this chapter, ab can be used as detecting reagents for a variety of molecules (e.g., Ig) by virtue of their molecular recognition properties. A vast amount of literature exists on different techniques developed to detect Ig in a qualitative, semi-quantitative or quantitative way. Such assays are based on different molecular properties of ab, ag or ab-ag complexes. As will be discussed below, in this respect it is important to note that different Ig isotypes because of differences in protein (antigenic) structure can behave differently in the various assays resulting in false conclusions. In all of these assays detection and, if possible, quantitation is based upon detecting (anti- isotypic) ab. The specificity, affinity and titer of these ab determines the reliabilities of the assay. The formation of ag-ab complexes on which all described assays are based determine the conditions under which the assays should be performed. Most ab-ag complex formation occurs optimally at an pH of 6.5 - 7.0 although the reaction is not greatly affected between pH 5.5 - 5.8. Monoclonal ab, however, can show drastic changes in specificity with altering pH. The rate of association can be increased by lowering the ionic strength of the buffer especially when dealing with ab of relatively low affinity (Ka $<10^5$ M^{-1}). In many reactions the affinity of ab specific for a certain ag is increased by incubation at lower temperatures (4°C instead of room temperature or 37°C). Changes in reaction temperature do influence the reaction speed but only minor changes occur in the affinity

constant. The stability of the ag-ab complex both under in vivo and in vitro conditions depends largely upon non-covalent interactions, mainly hydrophobic forces and hydrogen bonds and, to a lesser extent, on electrostatic-charge interactions. These hydrophobic forces contribute to the overall shape of a protein molecule in an aqueous system when the hydrocarbon-like parts of the protein chain are together and the polar parts of the chain are in contact with the solvent.

The specificity of an ab is dependent on the degree of cross reactivity with closely related antigenic determinants. Such interactions mostly occur with a lower affinity. Below are described a number of assays that are routinely performed in our laboratory.

2.4.1. Precipitation - based assays

When an optimal concentration of ag with multiple antigenic determinants is present in a solution together with ab, a large three-dimensional network is formed that precipitates out of solution. Protein ag with molecular weights of 40-60 kD give only precipitates under narrow optimal concentrations (equivalence zone of this so-called Heidelberger-Kendall curve). Polysaccharides, virusses and denaturated or very large proteins result in very broad curves. This reaction is especially very sensitive to changes in pH, ionic strength or temperature. The affinity of the ab used determines the rate at which the precipitate is formed. It can be envisaged that ab of the IgM class have a greater ability of complex formation than ab of the IgG and IgA classes.

Detection of the developed precipitate is based upon the visibility of the sediment. The sensitivity of detection can be increased by allowing the ag and the ab to diffuse into an agar gel. At the point where ag and ab meet in an equivalent concentration ratio they will form an insoluble precipitate. Since the excess of ag or ab will remain soluble outside the equivalence zone and can be washed out of the gel, the remaining precipitate can be stained by a regular protein staining method (e.g. Coommassie Brilliant Blue, Silver staining, etc.). In the original double immunodiffusion technique (according to Ouchterlony) it is very difficult to assess the concentration of an ag because of the necessity to keep both the reagent concentrations within the equivalence zone. The same holds true for two quantitative modifications: single radial immunodiffusion (SRID) according to Mancini et al. (1965) and the rocket electroimmunoassay (Laurell, 1966). In the single radial immunodiffusion a known amount of ab is mixed in the gel restricting the amount of the unknown ag that can be determined after concentric diffusion has resulted in circular precipitate formation. The diameter of such a precipitation circle is proportional to the ag concentration (see section 1.8.1.3. of Chapter 1 of this thesis). In the rocket electrophoresis technique an ag is moved (by an unidirectional electric force) into an ab containing agarose gel. When precipitation occurs, a stationary precipitate is formed which has the appearance of a rocket. Its length is related to the ag concentration.

The accuracy of quantitation of Ig levels is highly dependent on the quality of the reference standard that is tested in parallel and from which the absolute values are derived. For quantitation of murine Ig we employed SRID as well as rocket electrophoresis with a secondary standard consisting of normal mouse serum, which generally is more reliable than a primary standard of a myeloma protein (Mink and Benner, 1979; Mink et al., 1980). To determine the absolute Ig concentration in human sera, SRID is commonly used (De Bruyn et al., 1982; Kornman-Van den Bosch et al., 1984).

Another branch of precipitation-based techniques is immunoelectrophoresis (IEP) and immunofixation. In IEP mixtures of ag are separated in an unidirectional electric field in an agar gel. Subsequently, slots are cut out of the agar next to the sample wells and filled with an anti- isotypic antiserum. Diffusion of the ab is allowed and at the optimal ag-ab ratio a precipitation bow is formed. Any serum proteins can be identified in this way provided the specific ab is available. The technique is primarily qualitative but is extremely valuable in the detection of monoclonal gammapathies both in mice and humans (Radl et al., 1980; Radl., 1981). Immunofixation is a more sensitive modification of IEP employing the same ag separation in an electric field but the separated ag are deteted by overlaying the gel with an ab impregnated strip of cellulose acetate or filter paper. This technique is used

for sera of experimental animals as well as for sera and cerebrospinal fluid samples from patients suffering from gammapathies or multiple sclerosis (Cejka and Kithier, 1976; Radl, 1981).

2.4.2 Agglutination-based assays

Agglutination occurs when ab react with ag determinants either on native antigens or artificially attached to molecules, cells or particles. Apart from these direct agglutination reactions by ab directed against ag determinants on the surface of red blood cells (so-called hemagglutination reactions), bacteria and molds, indirect agglutination reactions also occur by ab directed against soluble ag passively adsorbed or chemically coupled to the surface of red blood cells or inert particles like latex, carbon, bentonite, collodion or Sepharose-beads to which a second (detecting) ab is added as a developing reagent. It is obvious that in these assays also IgM ab are more effective than IgG and IgA ab. Sometimes the agglutinating capacity of ab can be improved by adding heterologous ab specific for the light chain or Fc-part of the heavy chain of the agglutinating ab.

Agglutination-like assay for detecting Ig-secretion is the rosette forming cell assay. In this assay, anti-isotypic ab are coupled to erythrocytes, so that they form rosettes when incubated with ab-secreting cells (Haegert, 1981).

All techniques described so far are semi-quantitative in that the detection of formation of an ag-ab precipitate in solution or gel or an ag- ab agglutinate are limited to the smallest visible complexes. Therefore much effort has been put in improving the detection of such complexes in solution based upon Rayleigh light scattering by them. For the absolute quantitative determination of ab in human sera techniques like nephelometry and turbidimetry have been used (De Bruyn et al., 1982).

2.4.3. Ligand-binding assays

After the introduction of the radioimmuno-assay (RIA) a variety of immunoassays have been developed in which the detection of Ig is based upon a (ligand) labeled anti-isotypic ab. Many different labels are available for this purpose: radioisotypes, enzymes, fluorescent dyes, stable free radicals, electron-dense components, etc. Here we shall deal with enzyme immunoassays (EIA). Moreover we shall focus on heterogeneous assays in which the bound and the free fraction of the ligand are physically separated by a washing procedure and in which the ag (Ig) to be determined is either directly or indirectly physically attached to a solid phase (e.g. the well of a microtiter plate or Terasaki tray). Therefore such assays are called enzyme-linked immunosorbent assays (ELISA).

For the detection of the antigen-specific fraction or the total amount of a particular Ig isotype in serum we apply a two site system. In both cases, in the assay the Ig to be determined is sandwiched between the known ag or an anti-isotypic catching ab adsorbed to a solid phase and an enzyme labeled second ab, respectively. The catching as well as the detecting ab are heterologous compared to the Ig to be determined. After adsorbing the ag or catching ab to the solid phase, all subsequent incubations are done until equilibrium has been reached and competition can no longer occur between the labeled and unlabeled ligand involved. Moreover all other reagents, except the sample, are in slight excess, so that only the Ig that is bound out of the sample is the limiting step. In this way the signal is directly related to the amount of Ig bound. Other EIA's, whether competitive, homologous, one-site, homogeneous or non-equilibrium reaching, will not be discussed. The reader is refered to the available reviews (Schuurs and Van Weemen, 1977; Wisdom, 1976) and books (Voller et al., 1981; Avrameas, 1983; Bizollon, 1984). For use in sandwich ELISA, the quality of the antisera is extremely important in determining the specificity and the sensitivity of the assay (Shields and Turner, 1986). Some relevant points to consider before performing

ELISA will be described after which essential parameters lil
and accuracy will be discussed.

2.4.3.1. Set-up of the assay

The majority of ELISA techniques are performed in n
equipment facilitating easy and automatic handling is comm
moderately sensitive ELISA system a serum sample of 100 µ
concentration of IgM or IgG ab (Stokes et al., 1982; Ohlson
et al., 1985; Jenum, 1985). This holds for mice as well as hu
a number of Ig classes, especially rare ones like IgD, IgE, a
antibodies, however, does require a fair amount of serum. I
longitudinal studies in sera of mice or children it is often di
to perform the various assays. Moreover, in the production c
would like to test for Ig production by the hybrid cells as so
fusion, at a moment that only very small amounts of mediur
amount of reagents required for such micro- ELISA is also f
to our decision to perform ELISA in 60-well Terasaki trays

2.4.3.2. Coating of solid phases

In microtiter systems different solid phases can be used
in sufficiently high amounts, like polystyrene, polyvinyl chlo
polycarbonate, nitrocellulose, silicons etc. For the Terasaki sy
plates are available that can be coated easily and reproducibl
plastic surfaces, probably due to hydrophobic interactions be
matrix and protein molecules having a net charge of zero. Th
coating depends on the diffusion coefficient of the adsorbing
surface area to be coated to the volume of the coating solutic
adsorbing substances as well as the temperature and the dura
With regard to the last two parameters, 3 hrs at 37°C or over
found to be suitable. Moreover, to ensure sufficient saturatio
sample for coating is dissolved preferably in a high ionic stre
carbonate buffer) at a high pH (9.6) (Engvall and Perlmann,
by preincubation of the desired protein at elevated temperatu
the protein (Parish and Higgins, 1982).

During coating microtitre plates show a distinct 'edge e
plate adsorb more protein than those in the interior (Kricka e

There is evidence that during the set up and performan
adsorbed protein is released from the solid phase depending c
(Metzger et al., 1981). This is a prozone phenomenon know a
1983, Klasen and Rigutti, 1983). Such an effect can greatly i
precision of one-site type ELISA while it does not affect san
and Van Weemen, 1977).

We also tried to coat plates by allowing them to dry cor
with a fan) so as to ensure a 100% coating of protein. We an
conditions a higher degree of protein denaturation will occur
of the assay is not improved (unpublished results). Since the
heavily depends on the quality of the coating reaction, we us
preferably a standerd coat of a heterologous monoclonal catc
isotype of the Ig molecule to be assayed.

To detect polyclonal ab (serum) or monoclonal ab (hyb
surface antigenic determinants, monolayers of cells are coate
very low concentration of glutaraldehyde (0.02% for 15 min
and Heusser, 1979; Lansdorp et al., 1980; Van Soest et al., 1

of glutaraldehyde does not alter the conformation of antigenic determinants at the cell surface (Van Ewijk et al., 1984).

When testing the occurrence of ab directed against small ag it is advisable to precoat the plates with a polylysine spacer, while in the case of haptens preferably hapten-carrier conjugates are used for coating. The conformation of ag determinants, however, can be altered more by such procedures than by the coating process itself.

Coated plates can be stored either wet in PBS with 1-2 mM sodium azide at 4°C or dried in an excicator for periods of up to 4 weeks. After the coating has completed, trays have to be washed and all the remaining binding sites saturated with an unrelated protein (1% (w/v) BSA or 0.02% (w/v) gelatin in a 1 M Na_2CO_3 buffer or PBS) and incubated for 0.5 - 1 hr at 37°C. Husby et al. (1983) suggested use of 0.1% (v/v) human serum albumin preincubated at 56°C for 30 min to reduce non-specific binding. In all the subsequent washing and incubation steps this protein is present along with a small amount (0.05% (v/v)) of Tween-20 - a nonionic detergent - to prevent non-specific adsorption (Schönheyder and Anderson, 1984).

2.4.3.3. Choice and preparation of ligand

The sensitivity of ELISA systems depend heavily upon the quality and the specificity of the detecting ab. For use in ELISA, generally heterologous ab are employed that are purified out of hyperimmune sera. After immunization of the animal with the purified ag or Ig there is an increase in ab quality and affinity. We shall discuss this in greater details in section 2.4.3.5.

In addition, the specific activity and the turnover number (or molecular activity) of the enzyme to be applied for conjugating the detecting ab, play a major role. We have chosen in our assays for the enzyme β- galactosidase from Escherichia coli (Enzyme Code 3.2.1.23) for a number of reasons. Firstly, the enzyme is available with high specific activity (400 U/mg), has a high turnover number (12,500 substrate molecules transformed per molecule of enzyme per minute) and is sufficiently large (MW of 540.000 Da) to be linked to ab by a number of chemical methods with a good degree of retention of enzymatic and immunological activity. Secondly, the conjugate is soluble and stable under assay conditions and can be stored for prolonged times. Finally, the enzyme itself is absent in the sample and so are the substrates, inhibitors and other disturbing factors.

Several methods have been successfully used to link enzymes to antibodies as extensively reviewed by Kennedy et al. (1976), Schuur and Van Weemen (1977), Avrameas et al. (1978) and O'Sullivan and Marks (1981). The most popular cross-linking methods employ succinimidyl-3-(-2-pyridyldithiopropionate) (SPDP) and the one-step and the two-step glutaraldehyde method in which the homobifunctional aldehyde reacts with the aminoresidues in the protein to form a Schiff's base, the periodate-oxidation method in which carbohydrate residues of the enzyme are oxidated to form aldehyde groups, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), a heterobifunctional reagent coupling to sulfhydryl residues on the enzyme molecule.

We have employed the one-step glutaraldehyde method of Avrameas and Ternynck (1969). The ab to be coupled is extensively dialysed against 0.1 M potassium phosphate buffer, pH 7.8. Routinely we couple 1 ml ab solution of 5-10 mg protein per ml. Of the enzyme β-galactosidase (specific activity 600 U/mg; Boehringer, Mannheim, Western Germany) 2 mg is dissolved in one ml of the same buffer. The reaction mixture is prepared by mixing 1 ml enzyme (2 mg/ml with 1 ml ab (4 mg/ml) and 10 ul of a 25% (v/v) glutaraldehyde solution (TAAB Labotories Eq. Ltd, Reading, Berks, UK), stored in the dark at 4°C. The reaction is allowed to occur for 30 min at room temperature on an end-over-end mixer. The reaction is blocked by the addition 1 ml of 1 M lysine (Sigma, St. Louis, USA) in the same buffer and followed by overnight at 4°C. Extensive dialysis against PBS is performed and after addition of either 1% BSA or 0.02% gelatin or glycerol to a final concentration of 50% (v/v) the conjugate is stored at 4°C. With these additions the conjugate is stable for a few months, while in the absence of these additives activity is lost within a few weeks.

Another way of performing biotinylated ag and an avidin lab avidin-biotin binding (Ka=10^{15} M the Ig in the sample (Yolken et a

In sandwich ELISA enzyme (Engvall, 1978; Surolia and Pain, assaying Ig with no binding affin mouse cell surface determinants ((Savelkoul et al., 1985). Another detecting ab having very high af fractions). In this way various un ab. Coupling of protein A to β-ga method described by Deelder and

2.4.3.4. Substrate reaction and pr

We use the substrate 4-meth Louis, USA) which generates a hi β-galactosidase. The set-up of our measurement of the reaction endp compared favorably with those of o-nitro-phenyl-β-galactopyranosi 10^{-5}M, respectively.

The microfluorometric analy performed as described by De Jos The readings are expressed as arb in 0.25 mg/ml in 0.05 M potassiu till 60°C. After dissolving the sub aliquots can be stored at -20°C fo 60°C to dissolve before use. It sho binding reactions, so they are ext time should be always kept exactl

2.4.3.5. Quantitation in immuno a

Quantitation based upon EL different ways. One procedure is standard sample (Van Weemen et al., 1981; Giallongo et al, 1982; T Savelkoul et al., 1985). This proce standard graph, the limited numb of the geometry of the graph at l prepare calibration graphs by ass procedure is based upon the Lan Scatchard analysis of myeloma m (Porstmann et al., 1984; Pesce et a clonal antiserum is involved with 1983), an index of heterogeneity Petty, 1972; Lew, 1984). Both ty of affinity constants and allow as doesn't hold for real polyclonal a such situations a third method of theories developed for RIA, a ba described equations are derived (were formulated to describe the s spline function, Y = A + B/x + C et al., 1985).

Especially noteworthy is the four-parameter log-logit plot that is very useful for microprocessor-based data analysis (Ukraincik and Piknosh, 1981; Duggleby, 1981; Ritchie et al., 1983; Caulfield and Schaffer, 1984). The general form of this logistic equation may be expressed as $Y = (a-d)/[1 + (X/c)^b] + d$ and Logit $Y = \ln [Y/(100-Y)] = b \ln (X) + a$, in which

a = the response when X = 0,
b = the slope of the curve,
c = the concentration resulting from a response 50% of (a-d), and
d = value of control (X dilution is infinite).

Commonly a calibration curve of AFU versus log dilution of the standard is plotted, and the concentration of the Ig in the samples thus determined. In our experience, however, probably because of differing affinities of the Ig for the coat, the standard and the sample curves often have different slopes complicating interpretation of the data obtained. Butler et al. (1978, 1985) considered the logarithmic transformation of the Mass Law and arrived at the conclusion that the system can become independent of affinity when log-log plots are used.

While it is assumed that the ratio of solid-phase antigen : added antibody in the so-called 'linear region' of the log-log ELISA titration curve is such that all antibody becomes bound to solid-phase antigen, it has not been empirically demonstrated. If ELISA are influenced by affinity (Butler et al., 1978; Butler, 1981), the amount of antibody bound in this linear region may be less than 100%. The nature of such binding curves may also be influenced by avidity and heterogeneity.

The binding of detecting reagents (antibody-enzyme conjugates) at high concentrations of primary antibody appears sterically inhibited in direct proportion to the size of the conjugate system used for detection, leading to marked deviation from linearity in this region of the binding curve. Discrepancies between the slope of the binding curve for the primary antibody and the binding of the detection system in the linear region of the titration plot result from changing ratios of bound enzyme to bound primary antibody rather than altered activity of the former. Alternatively, the standard curve can be transformed into a four-parameter log-logit fit from which the sample Ig concentration can be determined.

Controversy has arisen over whether the ELISA measures antibody concentration or antibody affinity (Butler et al., 1978). As a discontinuous solid-phase assay comprising usually 2 cycles of 3 washes and a second antibody step during which time the first antibody may be dissociating, it would not be surprising from first principles that low affinity antibodies may not be detected. Because low affinity antibodies may predominate early in an immune response (and hence are important in the early diagnosis of infections) and may be important immunopathologically (Steward and Steensgaard, 1983), it was considered worthwhile to investigate the influence of antibody affinity in ELISA.

The study of the effect of affinity on in vitro assays has until recently depended upon immune sera raised in vivo. These sera contain a heterogeneous population of antibodies of varying class and affinity, so that interpretation of results is based on overall affinity estimation. Although often called 'average' affinity, the affinity value estimated empirically for a particular antiserum may not represent the mean, mode or median. This is due to the fact that so far there is no satisfactory experimental or mathematical way of describing the distribution of affinities of the antibodies in an antiserum in terms of its shape (which may be a skewed Gaussian or bimodal distribution) or its range (Steward and Steensgaard, 1983). Monoclonal antibody techniques now provide the opportunity of studying individual homogeneous antibodies of known affinity, class and concentration.

2.4.3.6. Features of enzyme immunoassays

Some common characteristics of ligand-binding immuno assays determine the quality and the final applicability of such an assay. These are the specificity, sensitivity, precision and reproducibility, and practicability.

The specificity of the assay
ab employed. As mentioned earlier
to be of a high affinity to be of u
However, it was not until stu
DNP-hapten system, that thermod
made. These studies clearly illustra
following immunization with DNP
Freund's complete adjuvant (FCA)
effect of antigen dose on affinity
affinity maturation is greater than
maturation but low levels of antib
when the amount of antigen is not
affinities (including low affinity)
antigen becomes limiting, cells bea
antigen and produce antibody. Thu
production of progressively higher
Since the specificity of such
immunoelectrophoresis, retesting o
(Shields and Turner, 1986). Many
necessary when employing commer
have to be taken into consideration
retained and secondly the affinity
Thus the formulation (pH, molarity
plays a major role in the specificit
the conjugate to the solid phase, w
detergents, should be checked and
highest possible dilution. In a sand
much better sensitivity is reached
ag determinants, by the catching a
detecting ab. Moreover in such an
pure ag for coating.
The sensitivity of an assay is
giving a response which differs sig
(detection limit). Apart from the fa
assay conditions play a role in the
washing steps, optimal reagent dilu
molecular weight of the ag (for ag-
diluent ionic strength and pH, incu
enzyme label per detecting ab. Alsc
product formed determines the sen-
tative assays described reach detect
example for IgG having a molecula
0.3-3 ng per ml) (Kato et al., 1975
The precision and reproducib
dose-response curve and the standa
of ag. The steeper the log dose- re
this line, the higher will be the pre
The practicability of the assa
but also to the possibility of autom
steps, conjugates, substrate etc., an
necessary calculations.

2.4.3.7. Recommended procedure
antibodies

- Clear polystyrene 60 well Tera
monoclonal anti-isotype mouse ab

Na_2CO_3 buffer; pH 9.6) in all wells of a tray. After the application of distilled water the lid on each tray is closed and the trays incubated overnight at 4°C.

- Trays are washed 3 times with PBS, 3 times with PBS containing 0.05% (v/v) Tween-20 (PBS-Tw) and 3 times with PBS containing 0.02% gelatin with a low bloom number (PBS-Gel). Wells are filled with PBS-Gel, the lids are closed and then the trays are incubated for 30 min at 37°C.
- Serum samples are diluted four till eight times with a dilution factor of 2, 3.3 or 10 after which 5 µl/well is applied very carefully with a standardized and calibrated pipette (Hamilton, Bonaduz, Switzerland).

Two rows of each plate are filled with PBS-Gel: one as a blank and the other as a serum control. Trays are closed in a humidified atmosphere (for this as well as all subsequent steps) and incubated for 1 hr at room temperature.

- Samples are removed from the wells by sucking out the liquid with a replicator (Biotec, Basel, Switzerland). Plates are washed extensively with PBS-Tw-Gel three times with intermittant drying.
- The detecting antibody-β-galactosidase conjugate is applied to all 8 rows plus the serum control row. The conjugate is diluted in PBS with 10 mM $MgCl_2$, 0.05% Tween and 0.02% gelatin and plates are incubated 30 min at 37°C, or 60 minutes at room temperature.
- After extensive washing (5 times with PBS-Tw-Gel) 5 µl (3.7 M per well) of substrate solution (4-methyl-umbelliferyl-β-D-galactopyranoside) is applied and the plates are incubated for exactly 60 min at 37°C.
- Finally, to each well 5 µl stopping buffer (0.1 M Na_2CO_3 buffer; pH 10.4) is added and the resulting fluorescence is determined in an automatic inverted microfluorimeter.

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CHAPTER 3

The effect of corticosteroids upon the number and organ distribution of 'background' immunoglobulin-secreting cells in mice

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The Effect of Corticosteroids upon the Number and Organ Distribution of "Background" Immunoglobulin-Secreting Cells in Mice *)

SUMMARY

The influence of the synthetic corticosteroid dexamethasone sodium phosphate (DEXA) upon the immunoglobulin (Ig)-secreting cells was studied in not intentionally immunized BALB/c mice. This was done for IgM-, IgG-, and IgA-secreting cells in spleen, mesenteric lymph nodes (MLN), and bone marrow (BM). A single injection of DEXA (16 to 144 mg/kg body wt) markedly reduced the number of Ig-secreting cells in spleen and MLN within 1 day, but hardly affected their number in the BM. The decrease was immediately followed by a recovery and, at the highest doses and especially in MLN, by an overshoot. Two weeks after the initial decrease a second decrease was found. When mice were subjected to daily treatment with DEXA during 1 week, initially a recovery pattern was found in spleen and MLN similar to that found after a single injection of a high dose. In this case, however, the effects were less dose dependent, and the overshoot reaction was followed by a period of subnormal numbers of Ig-secreting cells which lasted at least 1 week. This late effect of DEXA not only occurred in spleen and MLN, but also in the BM. The most prominent effect of daily treatment with DEXA was the long-lasting decrease of the number of IgG-secreting cells starting 1 week after withdrawal of treatment. This decrease was associated with a severely decreased serum IgG level.

INTRODUCTION

Corticosteroids are widely used as therapeutic agents in a variety of immunologically mediated and inflammatory diseases. Regarding the influence upon the immune system, corticosteroids affect the distribution pattern of lymphoid cells and mononuclear phagocytes as well as their functional activities (1).

The effect of corticosteroid administration upon antibody formation is dependent on the timing of the treatment. Administration shortly before immunization can suppress the response (2–4), while administration after antigenic exposure often has a smaller effect upon serum antibody levels (4, 5). The effect upon serum immunoglobulin (Ig) levels, which are a measure for all ongoing humoral immune responses together, is different for the various classes, and never as severe as upon primary antibody formation (6, 7).

The effect of corticosteroid treatment upon the ongoing Ig synthesis in various

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lymphoid organs of the same individual has hardly been studied. For man, McMillan and coworkers (5) compared the extent of suppression in spleen and bone marrow. These authors found no effect of corticosteroids upon splenic IgG production rates, while marrow IgG production rates started to decrease 3 weeks after beginning therapy and reached levels approximately one-fourth of pretreatment rates after 6 weeks.

In experimental animals, the effect of corticosteroids upon the total Ig production has not been assayed at the organ level. Such studies are now feasible in view of the recent development of plaque assays which can detect all Ig-secreting cells of a particular isotype, irrespective of antigen-binding specificity (9, 10).

We have applied the protein A plaque assay to investigate the effects of single and multiple injections of dexamethasone sodium phosphate (DEXA) upon the number and organ distribution of the "spontaneously" occurring ("background") Ig-secreting cells in mice. The results show that the influence of DEXA upon the ongoing Ig synthesis differs for various lymphoid organs and is, in mice, the smallest in the bone marrow (BM).

MATERIALS AND METHODS

Mice. Female BALB/c mice, 8 to 12 weeks old, were used. They were obtained from the Laboratory Animals Center of the Erasmus University.

Corticosteroid treatment. The synthetic corticosteroid dexamethasone sodium phosphate (Decadron; Merck & Co., Rahway, N.J.) was used. The mice were intraperitoneally (ip) injected with this drug dissolved in 0.5 ml of a balanced salt solution (BSS). Injections were always done between 4 and 5 PM. The applied DEXA treatments did not cause any sign of illness or loss of body weight (BW). Also, dissection showed no abnormalities.

Cell suspensions. Cell suspensions of spleen, mesenteric lymph nodes (MLN), and femoral bone marrow of individual mice were prepared in BSS as described in detail in a previous paper (11).

Protein A plaque assay. The protein A plaque assay for Ig-secreting cells was originally described by Gronowicz *et al.* (9), and has been modified in our laboratory as described in detail elsewhere (10).

Assay for cells containing cytoplasmic immunoglobulin (C-Ig cells). C-Ig cell determinations were done as described in detail in a previous paper (12).

Calculation of the total number of Ig-secreting cells and C-Ig cells per organ. Total numbers of Ig-secreting cells and C-Ig cells per organ were calculated by using the number of cells per slide and the total cell yield per organ (11, 12).

Serum IgM, IgG, and IgA levels. Serum IgM, IgG, and IgA levels were determined in Mancini immunodiffusion plates according to the standard procedure. The absolute standard used has been described in a previous paper (13).

Origin and specificity of anti-Ig antisera. All antisera used in the protein A plaque assay and in Mancini immunodiffusion were of rabbit origin. They were either kindly provided by Dr. C. Martinez-Alonso (Basel Institute for Immunology, Basel, Switzerland) or prepared in our own laboratory. The specificity of all antisera used to detect IgM-, IgG-, and IgA-secreting cells was confirmed in the protein A plaque assay with appropriate myeloma cell suspensions. The specificity of all antisera used in Mancini immunodiffusion was confirmed in Ouchterlony with the appropriate myeloma proteins. To detect IgG, we used a mixture of antisera recognizing mouse IgG1, IgG2a, IgG2b, and IgG3, but no light chains.

Effect of a Single Injection of DEXA upc

The effect of a single injection of eitl studied upon the background Ig synthe caused a profound decrease of the numb MLN. The number in the BM, however, the following days the numbers of Ig-sec doses tested, an overshoot was found in s

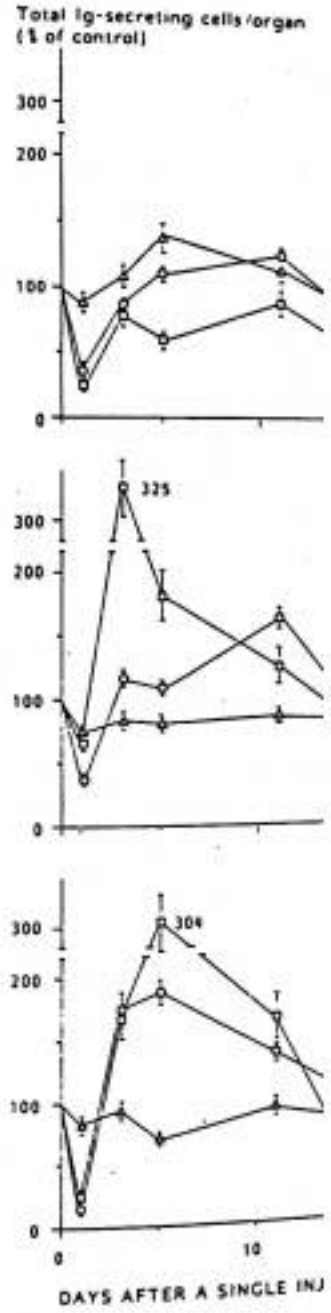

FIG. 1. Effect of a single injection of DEXA upon mouse spleen (○), MLN (□), and BM (△). Number determined at various intervals after injection of either a group of BSS-treated control mice was assayed as w as the arithmetic mean of all these controls. These va ± 1000; and for BM 115,000 ± 9000.

inent in the latter organ. Two weeks after the initial decrease a second decrease was found. Thereafter the figures gradually normalized.

In Fig. 2 the absolute numbers of Ig-secreting cells are plotted for spleen, lymph nodes, and BM together at 1 and 5 days after injection of 144 mg DEXA/kg BW. Figure 2 clearly shows that the highest dose of DEXA tested reduced the total number of Ig-secreting cells per mouse to about one-third within 1 day. Furthermore, it shows that the overshoot reaction in spleen and lymph nodes causes a doubling of the total number of Ig-secreting cells per mouse at 5 days after treatment.

In subsequent experiments the decrease of the number of Ig-secreting cells in the spleen was simultaneously determined in the protein A plaque assay and the cytoplasmic immunofluorescence assay. The first assay detects all cells secreting Ig, and the latter assay detects plasmablasts and plasma cells. In both assays we found the same proportional decrease in the number of Ig-secreting cells by DEXA (Fig. 3A).

A single injection of the highest of three doses of DEXA tested also decreased the serum IgM, IgG, and IgA levels. This decrease lasted more than 3 weeks and was found for all three isotypes. At all time points investigated, however, the decrease was not more than 25%. Remarkably, all three doses tested caused a substantial but short-lasting increase of the IgG concentration at 3 days after treatment. For all three doses this increase was found to be 25% (data not shown).

Effect of Multiple Injections of DEXA upon "Background" Ig

The effect of multiple DEXA injections was studied after applying a daily and an alternate-day treatment protocol, both lasting 1 week. In the protocol for daily in-

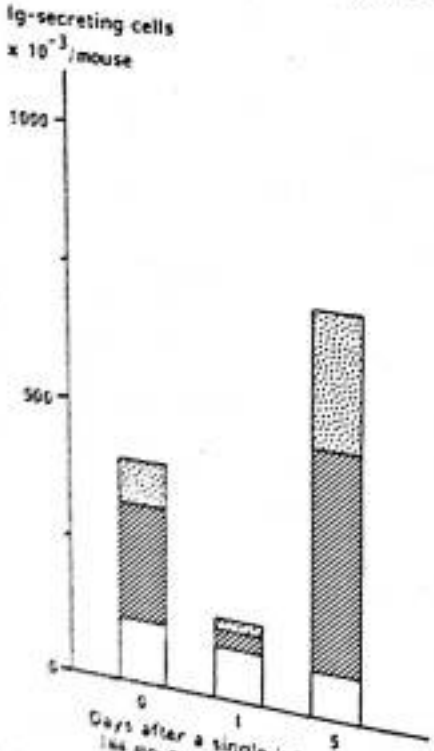

FIG. 2. Number of Ig-secreting cells in mouse spleen (hatched), total lymph node tissue (dotted), and total BM (open) of mice injected with 144 mg DEXA/kg BW. Ig-secreting cells were determined on the day of DEXA injection and 1 and 5 days after. The number of Ig-secreting cells per total lymph node tissue of the same animals at three times the number found in the MLN. Data were obtained from

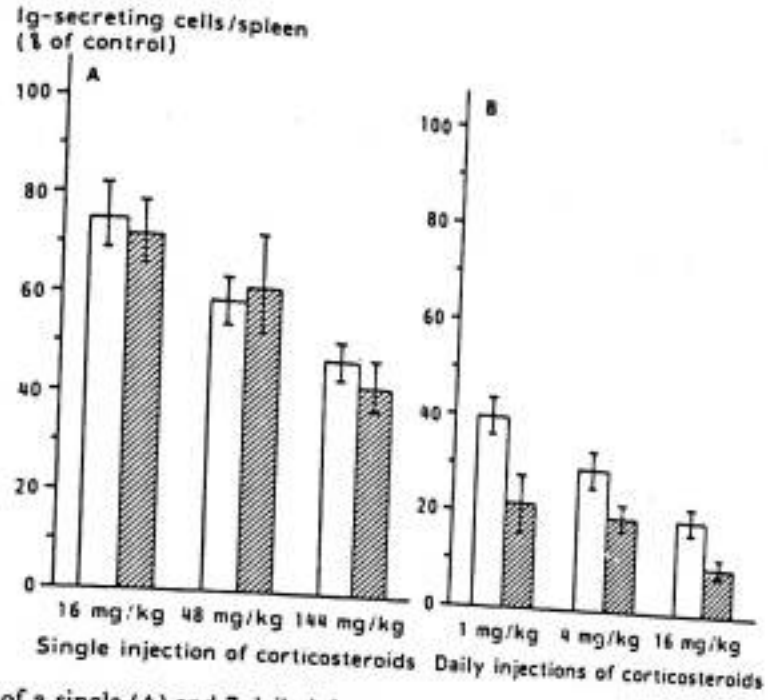

FIG. 3. Effect of a single (A) and 7 daily injections (B) of DEXA upon the total number of Ig-secreting cells per spleen as determined in the protein A plaque assay (open) and by cytoplasmic immunofluorescence (hatched). Numbers of Ig-secreting cells were determined 1 day after the only (A) or the last (B) injection of the indicated dose of DEXA. A group of BSS-treated control mice was assayed simultaneously. In the spleen of the control mice for a single injection of DEXA 187,000 ± 15,000 Ig-secreting cells were found in the protein A plaque assay and 76,000 ± 6000 by cytoplasmic immunofluorescence. In the control group for 7 daily injections of DEXA these values were 136,000 ± 11,000 and 71,000 ± 7000, respectively ($n = 6$).

jection, two doses of DEXA, 1 and 16 mg/kg BW, were tested. Both doses caused virtually identical effects. Initially a substantial decrease of the number of IgM-, IgG-, and IgA-secreting cells was found in spleen and MLN, followed by a rapid recovery of the number of Ig-secreting cells in the MLN, which, in the case of IgM and IgA, surpassed the normal value substantially (Fig. 4). In the spleen, on the other hand, the recovery was slower and did not overshoot. During the first few days after termination of the DEXA treatment, increased numbers of Ig-secreting cells were found in the BM, especially for IgM. Thereafter, another decrease was found, occurring in all three organs tested, but especially in the MLN. This decrease started about 1 week after termination of DEXA treatment and was found to be most prominent for IgG and IgA. The number of IgG-secreting cells in spleen and MLN was clearly depressed for more than 5 weeks. At the end of the observation period of 40 days, the figures for the numbers of IgM- and IgA-secreting cells in the various lymphoid organs were still not all normal (Fig. 4).

Determination of the number of Ig-secreting cells in the spleen of DEXA-treated mice by application of the protein A plaque assay and the cytoplasmic immunofluorescence assay on the same cell suspensions showed a different proportional decrease. At all three doses tested (1, 4, and 16 mg/kg BW) a much greater decrease was found by immunofluorescence than in the plaque assay (Fig. 3B). Since in the immunofluorescence assay, on the mean, more mature Ig-secreting cells are detected than in the protein A plaque assay (14), these data suggest that after 1 week of daily DEXA injections the Ig-secreting cells in the spleen are, on the mean, less mature than in the BSS-treated control mice.

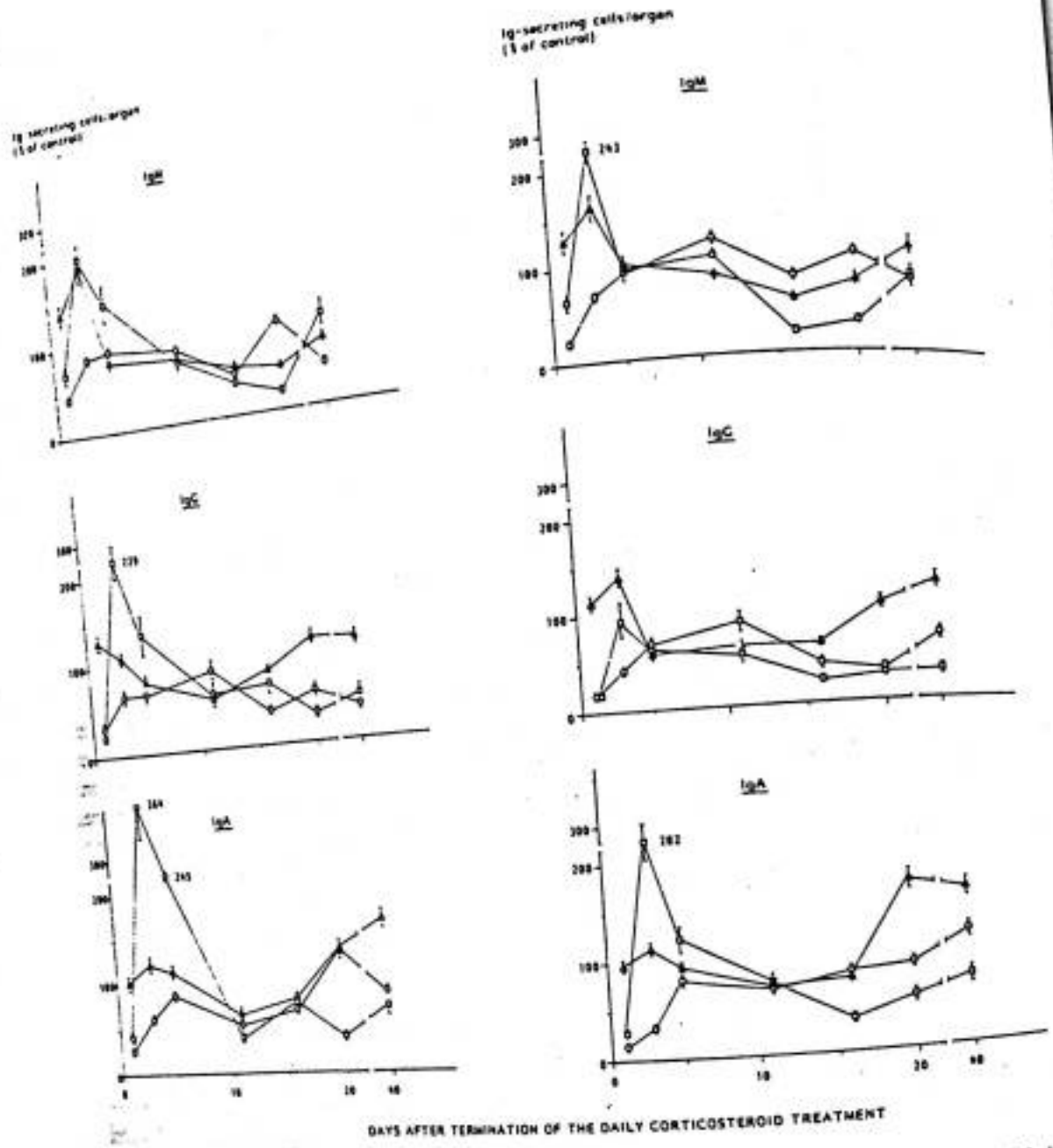

FIG. 4. Effect of 7 daily injections of DEXA upon the number of "background" IgM-, IgG-, and IgA-secreting cells in mouse spleen (○), MLN (□), and BM (△). Numbers of Ig-secreting cells were determined at various intervals after the last injection of either 1 (left) or 16 (right) mg DEXA/kg BW. At all time points a group of BSS-treated control mice was assayed as well (n = 6). The Day 0 values (100%) were calculated as the arithmetic mean of all these controls. The values for IgM, IgG, and IgA are for spleen 71.000 ± 4000, 58.000 ± 7000, and 57.000 ± 4000; for MLN 1400 ± 200, 7000 ± 1300, and 3300 ± 600; and for BM 18.000 ± 2000, 31.000 ± 2000, and 45.000 ± 3000, respectively.

Alternate-day treatment of mice with 16 mg DEXA/kg BW had a similar effect upon the number of Ig-secreting cells in the various lymphoid organs as daily treatment with this dose (data not shown).

A daily injection of 1 or 16 mg DEXA/kg BW substantially decreased the serum Ig level. This decrease lasted throughout the whole observation period of 40 days, was largest for the 16-mg/kg BW dose, and was found to be most prominent for IgG (Fig. 5). At 20 and 40 days after termination of the daily treatment with 16 mg DEXA/kg BW, the serum IgG levels were even less than 50% of normal. The lowest serum IgM and IgA concentrations were found directly after termination of the DEXA treatment. These isotypes recovered faster than IgG, but were still subnormal by the end of the observation period (Fig. 5).

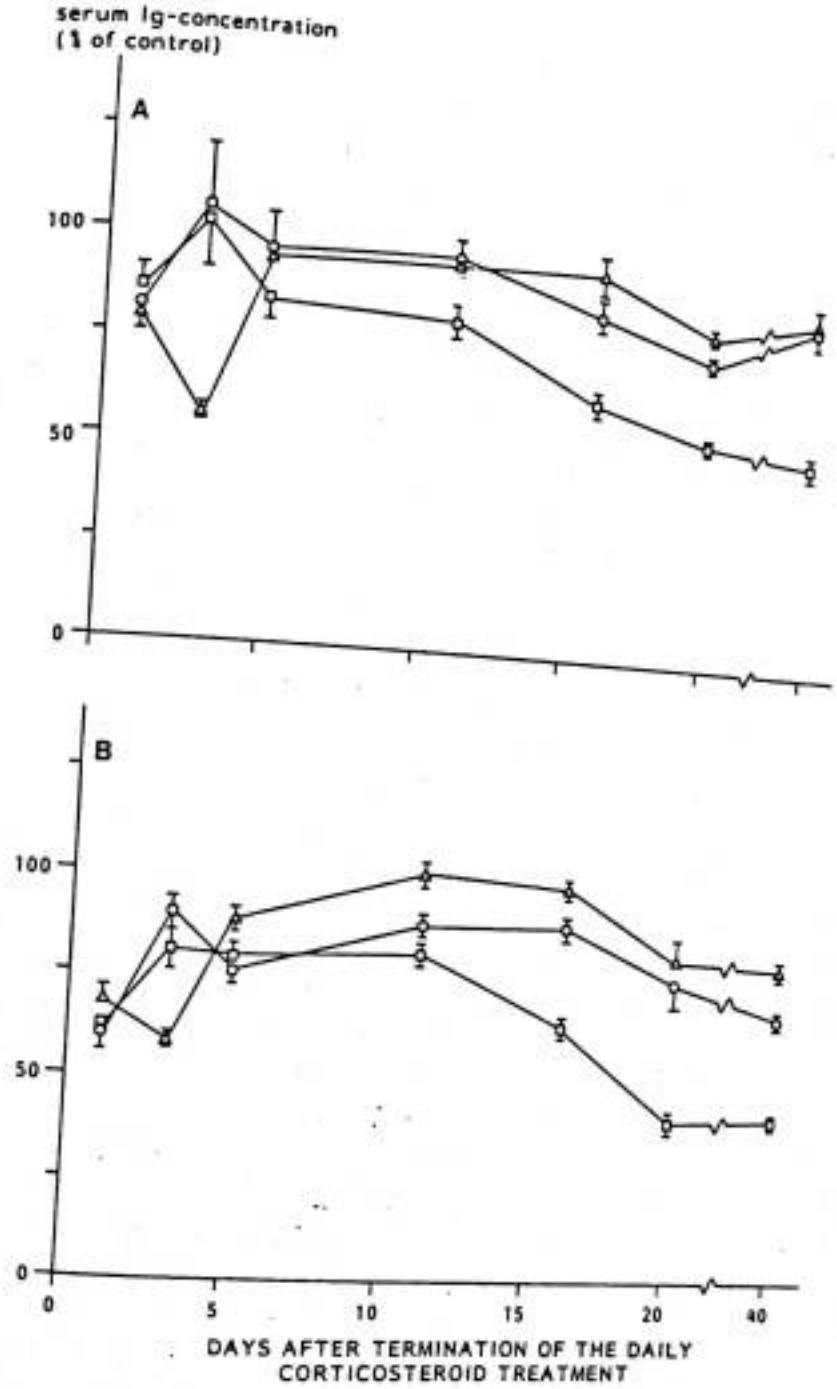

FIG. 5. Effect of 7 daily injections of DEXA upon the serum levels of IgM (O), IgG (□), and IgA (Δ). (A) 1 mg DEXA/kg BW; (B) 16 mg/kg BW. Sera were obtained from the same mice as used for determination of the number of IgM-, IgG-, and IgA-secreting cells in spleen, MLN, and BM (Fig. 3). In the Mancini, all Ig measurements were related to an absolute standard of normal mouse serum. The Day 0 values (100%) were calculated as the arithmetic mean of the absolute Ig levels of all BSS-treated control mice. These values are for IgM 18.1 ± 1.5 mg/dl, for IgG 603 ± 86 mg/dl, and for IgA 25 ± 3.6 mg/dl.

DISCUSSION

This paper shows that one or more injections of mice with a relatively high dose of corticosteroids cause a fast and severe decrease of the number of "background" IgM-, IgG-, and IgA-secreting cells in spleen and MLN, while their number in the BM is, initially, hardly affected. The effects were greater after multiple injections than after a single injection. Since at the age that the experimental animals were used (8–12 weeks) the majority of all Ig-secreting cells of the animals are localized in spleen

(14), this decrease was found to be associated with lowered serum IgM, IgG, and IgA levels. The effect upon IgG was most prominent. Little difference was found between mice treated according to a daily or an alternate-day protocol, both with regard to numbers of Ig-secreting cells and serum Ig levels. Also little difference was found between mice treated with 1 and 16 mg DEXA per kg BW per day. Remarkably, seven daily injections of 1 mg DEXA/kg BW were more effective in causing a long-lasting suppression than a single injection of 144 mg/kg BW.

Data available about the effect of corticosteroids upon serum Ig levels in man indicate that these drugs can cause a long-lasting moderate suppression of IgG and weak suppression of IgA. Serum IgM levels in man have been reported to be hardly susceptible to corticosteroid-mediated suppression (6, 7, 15, 16). It is remarkable that similar basic information for mice was not available in the literature so far. On the other hand, a variety of papers have appeared about the influence of corticosteroids upon serum antibody levels induced by deliberate immunization (17–19). In recent years there is, in addition, much interest in the cellular targets of corticosteroid-induced suppression of antibody formation. Both for man and mouse there is ample evidence that various subpopulations of lymphocytes have a different susceptibility (20–22).

Corticosteroid treatment did not cause an early decrease of the number of Ig-secreting cells in the BM. We have previously shown that secondary-type antibody formation to T-dependent antigens in the BM, but not in the spleen, is also highly resistant to DEXA (23). Also B lymphocytes in the BM and hemopoietic and proliferative responses by BM cells are resistant to corticosteroids (24, 25). It might well be that microenvironmental factors in the BM account for this resistance. It has been shown by Mishell *et al.* that activated accessory cells can prevent immunosuppression by corticosteroids *in vitro* (22, 26). Studies on lymphocyte homing using donor and recipient mice treated with cortisone acetate have indeed presented evidence for local factors determining the effect of corticosteroids (27).

The early decrease of the number of Ig-secreting cells in spleen and MLN after a single or daily treatment with DEXA was followed by a rapid recovery, which was the fastest in the MLN. Dracott and Smith (28), studying the cell numbers in various lymphoid organs of mice at different intervals after a single dose of hydrocortisone acetate, also found the fastest recovery in MLN. The reason might be the high antigenic pressure from the intestine. The overshoot of Ig-secreting cells in the MLN was confined to IgM and IgA and could not be detected as an increase of the serum IgM and IgA levels (Fig. 5). This might be due to an increased catabolic rate of serum Ig. In several species there is evidence for such an effect of corticosteroids (2, 6, 15, 29, 30).

Most studies indicate that, in mice, peripheral B cells are the prime cellular targets for corticosteroid-mediated immunosuppression *in vivo* (24, 25). We have previously argued that by cytoplasmic immunofluorescence, on the mean, more mature Ig-secreting cells are detected than in the protein A plaque assay (14). Furthermore, we have shown that the differentiation of B cells into fully mature C-Ig cells is dependent on the availability of enough T-cell help (14). The present observation that after a week of daily DEXA injections the proportional decrease of C-Ig cells is much greater than the proportional decrease of Ig-secreting plaque-forming cells suggests that immunosuppression of DEXA is not only due to an effect upon peripheral B cells, but

also to an effect upon helper T cells, either directly or via the antigen-presenting accessory cells. Indeed, there is evidence that, in mice, the generation as well as the expression of helper-T-cell activity is affected by corticosteroids (21). Since IgG and IgA syntheses are more dependent upon the helper-T-cell activity than IgM synthesis (31, 32), this can also explain why the latter is the less affected by DEXA (Fig. 5).

Mice, treated daily with relatively high doses of corticosteroids, display, after the early decrease of Ig-secreting cells and subsequent overshoot reaction, a long-lasting decrease in the number of Ig-secreting cells and in the serum Ig levels. Others have shown that ongoing antibody formation in mouse (3) and rat (17) and Ig synthesis in man (15), when measured in the serum, become suppressed only some weeks after termination of the treatment. The initial resistance of serum antibody and Ig levels is in most studies probably due mainly to the resistance of the Ig-secreting cells in the BM to immunosuppression by corticosteroids. Secondary-type responses depend on a quantitatively important contribution of the BM to the overall antibody synthesis (11, 33), so that with increasing age an increasing and, in fact, major proportion of all serum Ig is produced by the BM (33). These Ig-secreting cells in the BM originate in the peripheral lymphoid organs (34). The immediate destruction and redistribution of lymphocytes by corticosteroid treatment and the differential susceptibility of different T-lymphocyte subpopulations for these effects (20–22) cause a long-lasting unbalance of B- and T-lymphocyte subpopulations in the peripheral lymphoid organs (28) and, consequently, a long-lasting decreased Ig synthesis, not only there, but also in the marrow.

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CHAPTER 4

The effect of corticosteroids and cyclophosphamide upon immunoglobulin synthesis in mice

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The effect of corticosteroids and cyclophosphamide upon immunoglobulin synthesis in mice *)

SUMMARY

The influence of the synthetic corticosteroid dexamethasone sodium phosphate (DEXA) and cyclophosphamide (CY) was studied upon the B cell compartment of mice. A single injection of a high dose of DEXA (144 mg/kg BW) caused a profound decrease of the number of spleen cells and a moderate decrease of the number of bone marrow cells. The percentage of B cells among the remaining spleen and bone marrow cells was not affected. Also the polyclonal response after stimulation of the remaining B cells with E. coli lipopolysaccharide (LPS) in vitro was not affected. These data show that, on the mean, the population of LPS-reactive B cells are not more or less sensitive to DEXA than the other cells of the lymphohemopoietic system. Injection of CY, on the other hand, not only greatly decreased the number of spleen and bone marrow cells, but also the proportion of B cells among the remaining cells. Furthermore these B cells could no longer be polyclonally activated by LPS in vitro.

INTRODUCTION

Immunosuppressive agents have been categorized by Berenbaum in Class I and Class II agents depending on their ability to affect antibody formation when given prior to immunization (1).

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Class I agents (e.g., radiation, corticosteroids, cyclophosphamide, melphalan and nitrogen mustard) suppress antibody production when given either before or after antigen. Class II agents (e.g., 6-mercaptopurine, 6-thioguanine, methotrexate and vincristine), on the other hand, have little or no effect on antibody production when given prior to immunization, but cause suppression when administered 1 or 2 days after immunization (1). From the various immunosuppressive drugs, corticosteroids and cyclophosphamide (CY) have been most extensively studied, in experimental as well as in clinical situations.

Corticosteroids and CY have a different mechanism of action. Corticosteroids cause lymphoid cell destruction as well as redistribution of potentially circulating lymphoid cells (2,3). CY, on the other hand, is an alkylating agent which acts preferentially on actively dividing cells. It causes a direct latent damage of DNA; if affected cells undergo a cycle of division before DNA repair occurs, this effect leads to the death of the cells (4).

Corticosteroids and CY affect lymphoid as well as nonlymphoid cells, although the various subpopulations to a different extent (2,3,5). Several studies have shown that these drugs not only affect the lymphoid cell numbers, but also their functional capacity (reviewed in references 2 and 5). B cells have been shown to be highly susceptible to the immunosuppressive activity of corticosteroids and CY. This effect, however, has never been quantified at the level of newly-formed B cells. In view of our current interest in the effects of immunosuppressive drugs on the murine B cell system (6-8), we decided to study the effects of the synthetic corticosteroid DEXA and CY upon the compartment of newly-formed B cells. This was done in a culture system that allows the clonal growth of every mitogen reactive B cell up to a cell input of 1200 spleen cells or 6000 bone marrow cells per culture (9). We employed E. coli lipopolysaccharide as a mitogen which, in C57BL/6J mice, induces every third B cell to develop into a clone of Ig-secreting cells (10).

The effect of DEXA upon the compartment of newly-formed B cells was compared with the effect upon the serum concentration of IgM, IgG and IgA.

MATERIALS AND METHODS

Animals. Female BALB/c and C57BL/6J mice, 8 to 12-week-old, were used. They were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and OLAC Ltd, Bicester, United Kingdom, respectively. Female Lewis rats, 5-week-old, were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands.

Treatment with corticosteroids and cyclophosphamide. The synthetic corticosteroid dexamethasone sodium phosphate (Decadron; Merck & Co., Rathway, N.J., U.S.A.) and cyclophosphamide (Endoxan; Koch-Light Laboratories, Colnbrook, United Kingdom) were used as immunosuppressants. The mice received a single intraperitoneal (ip) injection of the drug dissolved in 0.5 ml of a balanced salt solution (BSS). The applied doses are indicated in the Resultssection. Injections were always done between 4 and 5 PM. The applied treatments did not cause any sign of illness or loss of body weight. Also, dissection did not show abnormalities.

Cell suspensions. Cell suspensions of spleen, mesenteric lymphnodes (MLN) and femoral bone marrow of individual mice and cell suspensions of rat thymuses were prepared in BSS as described in detail in a previous paper (11). Viable nucleated cells were counted by trypan blue exclusion.

Membrane fluorescence. Immunofluorescence staining of Ig's on the surface of B cells was done as described in detail elsewhere (12).

Culture conditions. C57BL/6J spleen cells (1200 viable nucleated cells per culture) or bone marrow cells (6000 viable nucleated cells per culture) were cultured together with 5×10^5 growth supporting rat thymus cells (irradiated with 110 mgray) and 50 µg/ml Escherichia coli LPS in 0.2 ml RPMI 1640 medium supplemented as described previously (13). The cultures were set up in Microtest II tissue culture plates (Costar 3596; Costar, Cambridge, MA, U.S.A.).

Protein A plaque assay. The protein A plaque assay for IgM-, IgG- and IgA-secreting cells was originally described by Gronowicz et al (14), and has been modified in our laboratory as described in detail elsewhere (15). The origin and specificity of the rabbit antisera employed in the protein A plaque assay

have been described previously (8).

Serum IgM, IgG and IgA levels. Serum IgM, IgG and IgA levels were determined in Mancini immunodiffusion plates according to the standard procedure. The absolute standard (16) and the antisera (8) used have been described in previous papers.

RESULTS

Effect of a single injection of DEXA upon the B cell compartment. The effect of a single injection of C57BL/6J mice with 144 mg/kg BW DEXA was studied in spleen and bone marrow with regard to the number of nucleated cells, the percentage of surface-Ig positive (B) cells and the polyclonal B cell response upon stimulation with LPS in vitro. Within 1 day this dose of DEXA caused a decrease of the number of nucleated cells in the spleen and the bone marrow of 78 and 30 per cent, resepectively. The percentage of B cells among the remaining nucleated cells was not significantly affected (Table 1). Also in vitro polyclonal response by the remaining B cells in the spleen and the bone marrow from DEXA treated mice was not significantly affected. This was found for the IgM-response as well as for the IgG- and IgA-response (Table 1).

A single injection of BALB/c mice with 144 mg/kg BW DEXA caused a long-lasting disturbance of the serum IgM, IgG and IgA levels. The concentration of all three Ig classes fell to about 75 per cent of the original value. The kinetics of the effect, however, was different for the different classes. At 1 day after DEXA administration, the IgM concentration slightly increased and subsequently decreased till about 80 per cent of normal. The IgG level initially decreased , subsequently rose till 125 per cent of the original value an then decreased again. IgA, finally, immediately decreased. At 40 days after DEXA injection, the serum concentrations of all three Ig classes were still below normal (Fig. 1).

A single injection of a smaller dose of DEXA (48 or 16 mg/kg BW caused similar effects as the dose of 144 mg/kg BW, although the decrease observed was smaller. The transient increase of the serum IgG concentration till 125 per cent of the original value, however, was also found at the doses of 48 and 16 mg/kg BW (Fig.1).

Table 1. Effect of a single injection of dexamethasone and cyclophosphamide upon the in vitro polyclonal response to E. coli lipopolysaccharide.

Organ	Treatment[a]	Nucleated cells ($\times 10^{-6}$)[b]	B cells[c]	IgM-PFC[d]	IgG-PFC	IgA-PFC
Spleen	-	115±8.8	45±2.0	18,354±1135	9588±602	713±157
Spleen	DEXA	25±7.0	40±0.9	16,332±961	7663±785	725±109
Bone marrow	-	35±1.8	5.3±0.7	11,334±692	7738±974	663±206
Bone marrow	DEXA	25±1.3	5.4±0.3	12,786±897	9050±438	550±124
Spleen	-	110±8.3	46±0.6	13,430±807	10,990±1640	n.d.
Spleen	CY	19±4.8	15±1.9	20±3.8	31±14	n.d.
Bone marrow	-	28±2.2	5.2±0.3	15,340±595	18,810±1830	n.d.
Bone marrow	CY	13±1.4	3.3±0.7	34±5.7	18±5.2	n.d.

a. C57BL/6J mice were ip injected with either 144 mg/kg BW dexamethasone (DEXA) or 300 mg/kg BW cyclophosphamide (CY) one day before harvesting the spleen and bone marrow cells.
b. Figures represent the number of nucleated cells ($\times 10^{-6}$) per whole spleen or per 2 femurs.
c. The percentage of surface-Ig positive B cells was determined by immunofluorescence.
d. The number of Ig-secreting cells (PFC) was determined in cultures of LPS-stimulated spleen cells (1200 viable nucleated cells per well) and bone marrow cells (6000 viable nucleated cells per well). At these and at lower cell inputs the number of IgM-, IgG- and IgA-PFC induced by the mitogenic moiety of LPS is lineary related to the number of B cells cultured (Hooijkaas et al., unpublished observations). IgM-PFC were determined on day 5 and IgG- and IgA-PFC were determined on day 6 of culture.

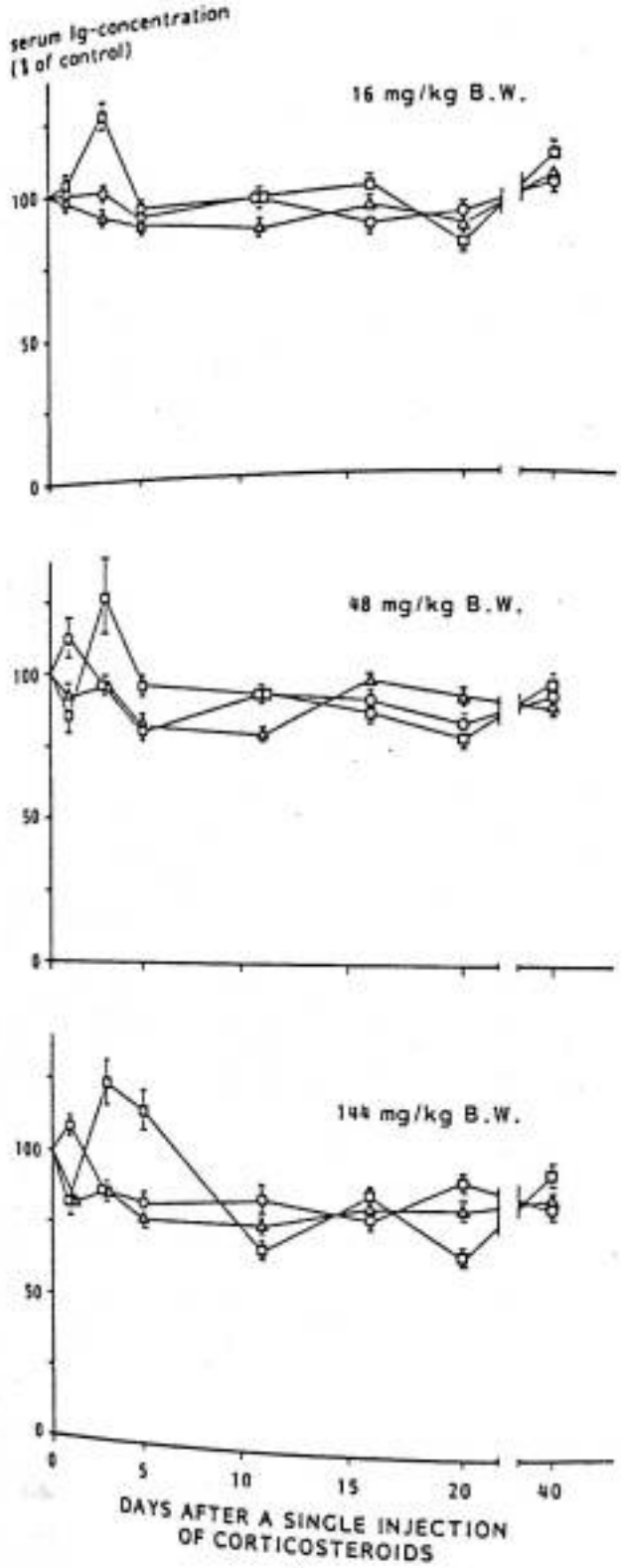

Fig. 1. Effect of a single injection of BALB/c mice with either 16, 48 or 144 mg DEXA per kg BW upon the serum levels of IgM (O), IgG (□) and IgA (Δ). All Ig measurements were related to an absolute standard of normal mouse serum. The Day 0 values (100%) were calculated as the arithmetic mean of the absolute Ig-levels of all BSS-treated control mice. These values were for IgM 18.1 ± 1.5 mg/dl, for IgG 603 ± 86 mg/dl, and for IgA 25 ± 3.6 mg/dl.

A single injection of a smaller dose of DEXA (48 or 16 mg/kg BW) caused similar effects as the dose of 144 mg/kg BW, although the decrease observed was smaller. The transient increase of the serum IgG concentration till 125 per cent of the original value, however, was also found at the doses of 48 and 16 mg/kg BW (Fig. 1).

Effect of a single injection of CY upon the B cell compartment. A single injection of C57BL/6J mice with 300 mg/kg BW CY decreased the number of nucleated cells in the spleen and the bone marrow within 1 day by 83 and 54 per cent, respectively. The number of B cells decreased even more, as the percentage of B cells in the spleen and the bone marrow of CY treated mice was considerably lower than in the BSS injected controls. The residual B cells were incapable of a polyclonal response upon stimulation with LPS in vitro (Table 1). The numbers of IgM- and IgG- secreting cells detected in these cultures were hardly above the values observed in non-stimulated cultures of spleen and bone marrow cells from CY treated mice (data not shown).

DISCUSSION

This study shows that a single injection of a high dose of DEXA or CY has a profound effect upon the B cell compartment in spleen and bone marrow. When comparing the effects of DEXA and CY in the doses tested, CY was found to have a greater effect upon the number of nucleated cells in the bone marrow and, in contrast to DEXA, to reduce the percentage of B cells among the remaining spleen and bone marrow cells. The most striking difference between the effects by DEXA and CY, however was that DEXA apparently did not influence the LPS-induced generation of IgM-, IgG- and IgA-secreting cells by the residual spleen and bone marrow B cells, while CY did. This is not only apperent from the studies of mass cultures described in this paper, but also from limiting dilution experiments employing the same culture conditions. The latter studies showed that the absolute frequency of LPS-reactive B cells among the viable nucleated cells as well as their capacity to switch from the synthesis of IgM to the synthesis of other isotypes was not affected by DEXA-treatment (Sabbele NR et al, to be published). Thus, DEXA-treatment does not affect the functional and

proliferative capacity of the newly-formed LPS-reactive B cells in spleen and bone marrow, which stands in contrast to its effects upon other lymphoid (17,18) and non-lymphoid cell types (17,19).

The effect of a single high dose of DEXA upon the serum concentrations of IgM, IgG and IgA was much less dramatic than upon the B cell compartment of the spleen. However, the decrease observed lasted several weeks, probably due to a long-lasting unbalance of B and T lymphocyte subpopulations in the various lymphoid organs (3).

A single injection of 300 mg/kg BW Cy was found to have a dramatic effect upon the population of LPS-reactive B cells. This treatment completely abolished the in vitro polyclonal response to this mitogen. This is most likely due to the fact that, in mice, LPS can only activate short-lived B cells (20,21). As Cy preferentially alkylates the DNA of actively dividing cells (4), the continuously replaced (22,23) pre-B cells and short-lived B cells also become affected (24). After stimulation of these cells in vitro with LPS, all reactive B cells are drawn into cycle within 30 hrs (25). As it takes much more time to repair the CY-induced DNA-damage (26), all B cells that start to divide upon stimulation with LPS, will die.

Other investigations have shown that 'memory-type' B cells and T cells are more resistant tot the immunosuppressive effects of CY than virgin B cells (5,27). This may explain why secondary-type antibody responses are less affected by CY than primary antibody formation (28).

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CHAPTER 5

The effect of corticosteroids upon B cells **in vivo** and **in vitro** as determined in the LPS-culture system

Submitted for publication.

THE EFFECT OF CORTICOSTEROIDS UPON MURINE B CELLS IN VIVO AND IN VITRO AS DETERMINED IN THE LPS-CULTURE SYSTEM *)

SUMMARY

The influence of the synthetic corticosteroid dexamethasone sodium phosphate (DEXA) upon mouse B cells was studied. This was done by in vivo treatment of mice with a single or multiple injection of DEXA and by culturing murine spleen cells and bone marrow cells in vitro in the presence of different concentrations of DEXA. The effect of DEXA on the B cell compartment was assayed by polyclonal stimulation of the B cells by Escherichia coli lipopolysaccharide (LPS) in vitro and subsequent measurement of the Ig-secreting cell response in the protein A plaque assay. DEXA treatment could greatly reduce the number of B cells in the spleen, but the bone marrow B cell compartment was quite resistant to DEXA. The in vitro LPS-induced IgM-response of the residual B cells from both spleen and bone marrow and their capacity to switch from IgM- to IgG- and IgA-secretion were not affected. These data indicate that DEXA can decrease the total number of B cells, but not the functional capacity of the residual LPS-reactive B cells. This was confirmed at the clonal level by limiting dilution culture experiments.

The contrasting effects of DEXA on splenic and bone marrow B cells was also found when the cells were exposed to the drug in vitro. It was found that 10^{-8} M DEXA in vitro reduced the response of splenic B cells to LPS by more than 80%, while a similar reduction of the response by bone marrow B cells required a 1000-fold higher concentration.

INTRODUCTION

Corticosteroids have at least three effects on cells of the immune system: destruction, inhibition of function, and redistribution (reviewed by Claman, 1972, and by Bach and Strom, 1985). The susceptibility to these effects depend on the animal species and cell type, and its location, physiological maturity and state of activation (Claman, 1972; Haynes and Fauci, 1978; Bach and Strom, 1985). Given the complex interactions between various subsets of lymphoid and non-lymphoid cells in immune reactions and all the above variables, in vivo experiments on the effects of corticosteroids on immune reactions are difficult to interpret. This urges for the choice of assay systems that allow the selective analysis of the various components of the immune system.

In previous studies we have shown that a single and multiple injection of mice with the synthetic corticosteroid dexamethasone sodium phosphate (DEXA) have contrasting effects upon the antibody production (Benner et al., 1978; Benner and Van Oudenaren, 1979) and 'background' immunoglobulin (Ig) production (Sabbele et al., 1983) in spleen and bone marrow. These studies revealed that antibody formation to the T-dependent antigen sheep red blood cells, and the background IgM-, IgG- and IgA-synthesis in the bone marrow were quite resistant to massive doses of DEXA, whereas the response of the spleen was greatly reduced (Benner et al., 1978; Sabbele et al., 1983). Bone marrow antibody formation to the T-independent antigen Escherichia coli lipopolysaccharide (LPS) was even substantially increased by high-dose DEXA treatment (Benner and Van Oudenaren, 1979). Also other studies have shown that B lineage cells in the murine bone marrow are relatively resistant

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to exogenous corticosteroid administration (Claman, 1972; Levine and Claman, 1970; Dracott and Smith, 1979), although this does not hold for all stages. Pre-B cells, for example, are decreased by at least 100-fold following in vivo corticosteroid treatment (Ku and Witte, 1986).

In the present study we have further investigated the effect of corticosteroid treatment upon the B cell compartment in spleen and bone marrow. This was done by using an in vitro culture system that allows the clonal growth of every B cell that can be activated by the mitogen LPS (Andersson et al., 1977a; Melchers and Andersson, 1984). The data obtained indicate that the differential effect of corticosteroid administration on splenic and bone marrow B cells not only holds in vivo, but also in vitro. Furthermore, DEXA did not affect the functional capacity of the B cells that resisted the destructive and redistribution-inducing effects of DEXA in vivo.

MATERIALS AND METHODS

Mice

Female C57BL/6J mice, 8 to 12 week old, and female Lewis rats, 4 weeks old, were used. The C57BL/6J mice were purchased from HARLAN OLAC 1976, Ltd., Blackthorn, England. The Lewis rats were purchased from the Central Laboratory for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. All mice and rats received pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3.0) ad libitum.

Corticosteroid treatment

The synthetic corticosteroid dexamethasone sodium phosphate (Decadron©; Merck and Co., Rahway, N.J., U.S.A.) was used. The mice were intraperitoneally (ip) injected with the drug dissolved in 0.5 ml of a balanced salt solution (BSS). This BSS was prepared according to Mishell and Dutton (1967). Injections were always done between 4 and 5 PM. The applied dexamethasone (DEXA) treatments did not cause any sign of illness or loss of body weight (BW). Also, dissection showed no abnormalities. In the experiments in which we added DEXA to the culture medium, the contribution of the drug solution was maximally 1% of the total culture volume.

Cell suspensions

Cell suspensions of spleen and femoral bone marrow of individual mice and cell suspensions of rat thymuses were prepared in BSS as described in detail in a previous paper (Benner et al., 1981b). Viable nucleated cells were counted by trypan blue exclusion.

Membrane fluorescence

For membrane immunofluorescence of B cells, the cells were resuspended to a concentration of 10^7 cells/ml in phosphate-buffered saline (PBS; pH 7.8) supplemented with 1% bovine serum albumin (BSA) and 0.02% NaN_3. A volume of 100 μl cell suspension was mixed with 50 μl fluorescein-conjugated goat antiserum directed against mouse immunoglobulins (GAM-Ig-FITC, lot no. 2-873; Nordic Immunological Laboratories, Tilburg, The Netherlands) and incubated for 30 min at 0°C. After two washes with PBS-BSA the preparations were mounted in buffered glycerol (p.a.; Merck A-G, Darmstadt, F.R.G.) and sealed with paraffin (Paramat©, Gurr, Ltd., High Wycombe, Bucks, England).

Culture conditions

The frequencies of LPS-reactive B cells that can give rise to clones of IgM-secreting cells were determined in a limiting dilution culture system originally devised by Andersson et al. (1977a). In this culture system varying numbers of spleen and bone marrow cells were cultured together with 7.2×10^5 irradiated (0.1 Gy) growth supporting rat thymus cells and 50 μg/ml *Escherichia coli* LPS (026:B6; Difco Laboratories, Detroit, MI, U.S.A.) in 0.2 ml RPMI 1640 medium supplemented as described previously (Hooijkaas et al., 1982). The cultures were set up in 96-well tissue culture plates (Costar 3596; Costar, Cambridge, MA, U.S.A.). Routinely, 32 replicate cultures were [illegible] tion. Control

et al., 1982). The cultures were set up in 96-well tissue culture plates (Costar 3596; Costar, Cambridge, MA, U.S.A.). Routinely, 32 replicate cultures were set up for each cell concentration. Control cultures did not contain mouse cells but did contain rat thymus cells. The cultures were assayed individually for clones of IgM-secreting plaque-forming cells (PFC) on Day 5. Cultures were scored as positive when they yielded more than 10 PFC above the maximum number of PFC found in the control cultures containing rat thymocytes only.

Polyclonal activation of B cells cultured at high density ('mass cultures') was performed under essentially the same culture conditions as used for the limiting dilution cultures. Triplicate cultures of 1200 viable nucleated spleen cells and 6000 viable nucleated bone marrow cells were set up for each experimental point. Separate cultures were assayed on day 5 for total IgM-secreting cells and on day 6 for IgG- and IgA-secreting cells.

Protein A plaque assay

The protein A plaque assay for IgM-, IgG- and IgA-secreting cells was originally described by Gronowicz et al. (1976) and has been modified in our laboratory as described in detail elsewhere (Van Oudenaren et al., 1981). The origin and specificity of the rabbit antisera employed in the protein A plaque assay have been described previously (Sabbele et al., 1983).

RESULTS

Effect of in vivo exposure of B cells to DEXA

The effects of a single and multiple injection of DEXA were studied on the B cell compartment in spleen and bone marrow of C57BL/6J mice. One day after a single injection of 144 mg DEXA/kg BW the number of nucleated cells in the spleen was decreased by 80%. The bone marrow, however, was hardly affected. The percentage of surface Ig^+ B cells among the remaining nucleated cells in spleen and bone marrow was not affected (Tables 1 and 2). When the residual spleen and bone marrow cells were cultured in vitro in the presence of LPS, similar numbers of IgM-, IgG- and IgA-secreting cells were observed as after culture of the same number of spleen and bone marrow cells from untreated mice (Table 1).

After 7 daily injections of 50 mg DEXA/kg BW, the number of spleen cells was reduced to 8% of the original number whereas the bone marrow, again, was hardly affected. This regimen, however, affected the B cell compartment more severely than the total nucleated cell compartment. In spleen as well as in bone marrow these multiple DEXA injections reduced the percentage of B cells among the residual nucleated cells to half the original value, namely to 27 instead of 50% of the spleen cells and to 2.9 instead of 5.4% of the bone marrow cells (Table 1). This means that the absolute number of B cells per spleen was reduced by about 95%.

The residual B cells in the spleen and the bone marrow of mice subjected to multiple DEXA injections were not affected in their capacity to become activated by LPS in vitro and to give rise to clones of IgM- and IgG-secreting cells. The Ig-secreting cell responses observed were half as high as those obtained after culture of the same number of spleen or bone marrow cells from untreated mice (Table 1).

The effect of a single and multiple DEXA injection upon spleen and bone marrow B cells were more in-depth analysed by employing a limiting dilution culture system. This system allows the activation and growth of single B cells, so that frequency estimates can be made. It was found that a single injection of 144 mg DEXA/kg BW did not affect the frequency of IgM-secreting clones upon culture of the spleen and bone marrow cells. Also the clone size and the frequency that IgM-secreting clones switch to IgG-secretion were not affected (data not shown). Multiple DEXA injections did affect the frequency of IgM-secreting clones. However, after correction for the decreased B cell incidence in the spleen and the bone marrow of the DEXA-treated mice, the same frequency of LPS-reactive B cells was calculated (Table 2). Also after multiple DEXA injections the

Table 1

Effects of single and multiple dexamethasone injection on the B cell compartment in spleen and bone marrow

Organ	Treatment[a]	Nucleated cells($\times 10^{-6}$)[b]	% B cells[c]	IgM-PFC[d]	IgG-PFC	IgA-PFC
Spleen	-	115 ± 8.8	42	18,354 ± 1135	9588 ± 602	713 ± 157
Spleen	single	25 ± 7.0	40	16,332 ± 961	7663 ± 785	725 ± 109
Bone marrow	-	35 ± 1.8	4.2	11,334 ± 692	7738 ± 974	663 ± 206
Bone marrow	single	25 ± 1.3	5.9	12,786 ± 897	9050 ± 438	550 ± 124
Spleen	-	103 ± 9.9	50	17,860 ± 899	7640 ± 703	N.D.[e]
Spleen	daily	8 ± 1.8	27	10,960 ± 766	5370 ± 344	N.D.
one marrow	-	29 ± 4.7	5.4	14,420 ± 1043	7040 ± 757	N.D.
one marrow	daily	24 ± 1.4	2.9	6,300 ± 603	6520 ± 266	N.D.

)C57BL/6J mice received either a single ip injection of 144 mg DEXA/kg BW or seven daily ip injections of 50 mg DEXA/kg BW. Cells were harvested one day after the last injection.
)Figures represent the number of nucleated cells ± SEM ($\times 10^{-6}$) per whole spleen or per 2 femurs.
)The percentage of surface-Ig^+ B cells was determined by membrane immunofluorescence.
)The number of Ig-secreting cells (PFC) was determined in cultures of LPS-stimulated spleen cells (1200 viable nucleated cells per well) and bone marrow cells (6000 viable nucleated cells per well). IgM-PFC were determined on day 5, and IgG- and IgA-PFC were determined on day 6 of culture.
e)N.D. means that the numbers of IgA-secreting cells were not determined in these experiments.

Table 2

Effect of single and multiple dexamethasone injection on the frequencies of LPS-reactive B cells in spleen and bone marrow as determined by limiting dilution analysis

Organ	Treatment[a)]	Frequency of IgM-secreting clones[b)]	% B cells[c)]	Frequency of LPS-reactive B cells[d)]
Spleen	-	1 in 5 (5.4, 5.3, 5.4)	45 ± 2.0	1 in 2 (2.6, 2.4, 2.3)
Spleen	single	1 in 6 (6.3, 6.6, 4.1)	40 ± 0.9	1 in 2 (2.5, 2.4, 1.6)
Bone marrow	-	1 in 33 (48, 26, 24)	5.3 ± 0.7	1 in 2 (3.2, 1.4, 1.0)
Bone marrow	single	1 in 38 (51, 30, 33)	5.4 ± 0.3	1 in 2 (2.8, 1.4, 2.0)
Spleen	-	1 in 6 (8.9, 5.1, 5.1)	47 ± 2.2	1 in 3 (4.4, 2.2, 2.6)
Spleen	daily	1 in 13	21 + 3.5	1 in 1

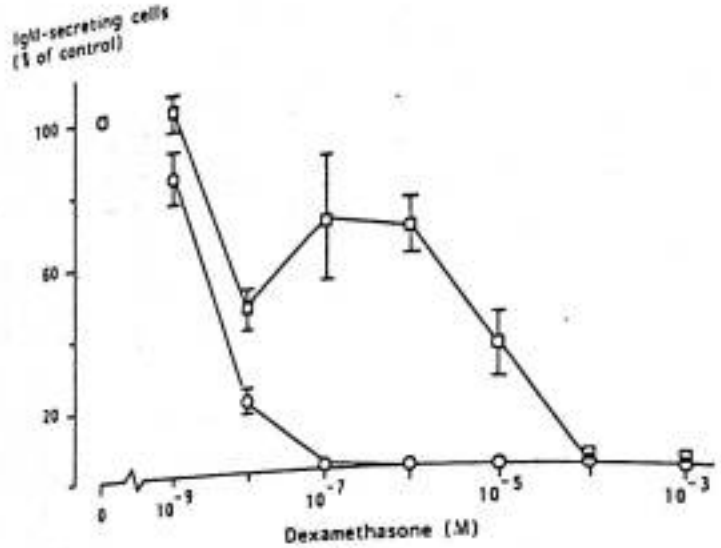

Fig.1. Effect of increasing concentrations of DEXA upon the in vitro LPS-induced IgM-secreting cell response by C57BL/6J spleen (○) and bone marrow (□) cells. Numbers of IgM-secreting cells were determined on Day 5 after culturing 1200 spleen cells or 6000 bone marrow cells per well. Spleen and bone marrow cells cultured in the absence of DEXA gave rise to 41,200 ± 2459 and 20,200 ± 1320 IgM-secreting cells, respectively. Figures represent the responses by B cells cultured in the presence of DEXA calculated as percentage (± SEM) of the response obtained from similar cultures without DEXA.

clone size and the switch frequency to IgG of the LPS-reactive B cells were the same as those observed after culture of spleen and bone marrow cells from untreated mice (data not shown).

Effect of in vitro exposure of B cells to DEXA

The sensitivity of splenic and bone marrow B cells to DEXA was compared by in vitro culture of spleen and bone marrow cells in the presence of LPS and varying concentrations (10^{-9} to 10^{-3} M) DEXA. It was found that increasing concentrations of DEXA decreased the LPS-induced IgM-secreting cell response by splenic B cells as well as by bone marrow B cells. The sensitivity of both populations of B cells for the immunosuppressive activity of DEXA, however, differed substantially. While 10^{-8} M DEXA reduced the response of the splenic B cells by more than 80%, a similar reduction of the response by bone marrow B cells required a 1000-fold higher concentration of DEXA (Fig.1).

DISCUSSION

A variety of studies have shown that corticosteroid administration of sensitive species like mouse, rat, hamster and rabbit can severely decrease the number of viable nucleated cells in thymus, spleen, and lymph nodes, the most severely affected being the thymus (Claman, 1972; Dracott and Smith, 1979). The bone marrow, however, is hardly affected as far as total cell numbers are concerned. Also when the various lymphohemopoietic cell lineages in the bone marrow are evaluated individually, major alterations are not found in the compartment size of most lineages (Levine and Claman, 1970; Claman, 1972; Dracott and Smith, 1979).

Although the bone marrow seems to be resistant to corticosteroid treatment, the production of granulocytes (Metcalf, 1969), monocytes (Thompson and Van Furth, 1973) and lymphocytes (Brahim, 1978) is inhibited by corticosteroid administration. The apparent

resistance is therefore attributed to a decreased egress of newly formed cells from the bone marrow and redistribution of mature cells, a.o. T cells (Moorhead and Claman, 1972; Fauci, 1975; Dracott and Smith, 1979), from the peripheral lymphoid organs towards the marrow. Consequently, the proportion of short-lived cells in the bone marrow decreases while the proportion of long-lived cells increases (Esteban, 1968).

Dracott and Smith (1979) have presented evidence that only a minor part of all peripheral leukocytes are redistributed to the bone marrow after corticosteroid treatment. Since all other tissues show severe depletion, the latter must be mainly due to destruction. This is also supported by in vitro experiments (Claman et al., 1971).

In mice, the decrease of the incidence of B cells in the peripheral lymphoid organs after corticosteroid treatment is larger than the decrease of the T cell number. After a single subcutaneous injection of 2.5 mg hydrocortisone acetate, minimal numbers of B cells are found in the spleen on Day 3 and in the mesenteric lymph nodes on Day 5 (Dracott and Smith, 1979). We did not find a decreased percentage of B cells after a single DEXA injection. However, we assayed on Day 1 only. Dracott and Smith (1979) also found normal percentages of B cells on the first day after the hydrocortisone acetate injection.

Just as in previous studies of other investigators, we observed a severe decrease of the number of nucleated spleen cells while the bone marrow cellularity was only moderately affected. This was found after a single ip injection of 144 mg DEXA/kg BW as well as after 7 daily injections of 50 mg DEXA/kg BW. Remarkably, multiple DEXA injections did not have a more severe effect on the bone marrow cellularity than a single injection, whereas it did on the spleen. Both in spleen and in bone marrow, multiple DEXA injections decreased the proportion of B cells among the residual nucleated cells (Tables 1 and 2). In both organs the B cell incidence decreased till about 50 per cent of the normal value. This probably is due to two factors. Firstly, to the severe decrease of the number of pre-B cells in the bone marrow by corticosteroid treatment, down till less than one per cent of normal (Ku and Witte, 1986), causing a decreased production of B cells. Secondly, to the rapid turnover of the majority of B cells in the mouse (Sprent and Basten, 1973; Sprent, 1977). Under these conditions the B cell compartment in the bone marrow as well as the peripheral lymphoid tissues will be rapidly exhausted.

The residual B cells that still occurred in spleen and bone marrow after single or multiple injection of DEXA were assayed functionally in vitro. This was done in a culture system employing LPS as a mitogen. This culture system allows the clonal growth of every LPS-reactive B cell, independent of the availability of T cells and macrophages in the cell suspension to be assayed. In the C57BL mouse strain employed in this study one out of every three surface Ig$^+$ B cells gives rise to a clone of IgM-secreting cells under these conditions (Andersson et al., 1977a, 1977b). During clonal expansion, individual daughter cells can express other heavy chain isotypes (Coutinho and Forni, 1982), giving rise to subclones of IgG- and IgA-secreting cells (Benner et al., 1981a; Coutinho and Forni, 1982). Using this culture system, both in mass cultures (Table 1) and under limiting dilution conditions (Table 2) no differences could be detected between the residual B cells from DEXA-treated mice and B cells from untreated mice. Similar frequencies of LPS-reactive B cells were found after correction for B cell incidence, similar IgG- and IgA-responses, and similar clone sizes. This indicates that a single and multiple injection of mice with a high dose of DEXA can decrease the number of surface Ig$^+$ B cells, but do not affect the functional capacity of the LPS-reactive subpopulation of those B cells that resist the cytolytic and redistribution-inducing activities of DEXA. This is in sharp contrast to the effects of cyclophosphamide upon murine B cells. A single injection 300 mg of this drug per kg BW completely abolishes the functional capacity of all B cells (Sabbele et al., submitted for publication).

Most interesting, the differential susceptibility of splenic and bone marrow B cells to DEXA was found not only in vivo, but also in vitro. While the LPS-induced Ig-secreting cell response by splenic B cells from naive mice was severely decreased by 10^{-8} M DEXA, a similar reduction of the response by bone marrow B cells required a

1000-fold higher concentration (Fig. 1). This might suggest that bone marrow B cells are intrinsically more resistant to corticosteroid-mediated immunosuppression than splenic B cells. Alternatively, accessory cells in the bone marrow might prevent the corticosteroid-mediated immunosuppression. Indeed, Mishell and coworkers have presented evidence that products of activated macrophages can prevent corticosteroid-mediated immunosuppression (Mishell et al., 1977; Bradley and Mishell, 1981). These authors showed that while macrophage factors protected helper T cells from corticosteroid-mediated suppression, they did not prevent the effects of corticosteroids on suppressor T cells.

It should be emphasized that to all our cultures of 1200 spleen cells and 6000 bone marrow cells 7.2×10^5 rat thymocytes were added to support B cell activation and clonal growth. This thymocyte feeder also contained a low proportion of macrophages (Van Ewijk, 1984). Still they could not protect the splenic B cells from DEXA-mediated immunosuppression. Therefore, if the resistance of bone marrow B cells to DEXA is due to the presence of protecting accessory cells in the bone marrow inoculum, these cells should be extremely potent. Cell separation studies will reveal whether the resistance of bone marrow B cells is an intrinsic property of these cells or is due to protecting accessory cells.

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CHAPTER

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THE EFFECT OF CYCLOPHOSPHAMIDE UPON B CELLS AND 'BACKGROUND' IMMUNOGLOBULIN-SECRETING CELLS IN MICE *)

SUMMARY

The influence of cyclophosphamide (CY) was studied upon the B cell compartment of mice. This was done at five different levels: (a) the serum immunoglobulin (Ig) levels; (b) the numbers of 'background' Ig-secreting cells; (c) the incidence of surface Ig^+ B cells; (d) the capacity of lipopolysaccharide (LPS) reactive B cells to give rise to a polyclonal IgM- and IgG-response in vitro; and (e) the capacity of long-lived memory B cells to give rise to an adoptive anti-sheep red blood cell plaque-forming cell response in vivo.

A single injection of 300 mg CY/kg body weight (BW) decreased the numbers of background IgM-, IgG- and IgA-secreting cells in spleen, bone marrow and lymph nodes to minimum values of about 25% of normal at day 7. The incidence of surface Ig^+ B cells also gradually decreased after CY treatment. The functional capacity of the B cells, however, was completely abolished one day after a single injection of 300 mg CY/kg BW. This was found for the LPS-reactive B cells, which largely represent newly-formed, short-lived B cells as well as for long-lived memory B cells.

The decrease of background Ig-secreting cells following a single injection of 300 mg CY/kg BW was followed by a gradual recovery with a substantial overshoot peaking about 40 days after CY injection. After multiple injections of 100 mg CY/kg BW, the minimum values of background Ig-secreting cells in the various lymphoid organs were lower than after a single injection of 300 mg CY/kg BW, but in this case the recovery was not associated with an overshoot reaction. Remarkably, the serum Ig levels were much less decreased than the numbers of Ig-secreting cells in the various lymphoid organs, suggesting that the half life of the circulating Ig in CY treated mice is lengthened.

INTRODUCTION

Cyclophosphamide (CY) has been shown to be able to affect a variety of lymphoid and non-lymphoid cell populations (reviewed by Bach and Strom, 1985). The available data suggest that a single injection of a high dose of CY and short-lasting courses of daily injection of a low dose of CY preferentially affect B cells and suppressor T cells. Most likely, the subpopulations of T cells involved in the suppressor cascade are not equally sensitive to CY, the subpopulation of short-lived amplifier suppressor T cells being the most sensitive (Germain and Benacerraf, 1981; Shukla and Chaturvedi, 1984).

Labeling studies employing 5-iodo-2-deoxyuridine-^{125}J (IUdR) have shown that rapidly dividing cells are more susceptible to the action of CY than non-cycling cells (Turk and Poulter, 1972). This probably is one of the major causes of the observation that the suppressive effect of CY on the peripheral B cell compartment is more severe than on the peripheral T cell compartment since the latter, on the mean, consists of a larger proportion of long-lived cells (Sprent, 1977). Another reason might be that CY interferes with the immunoglobulin (Ig) receptor expression and capping by B cells (Shand and Howard, 1978, 1979).

So far, hardly data are available about the susceptibility of different subpopulations of

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murine B lineage cells for the immunosuppressive activity of CY. Also no data are available about the influence of CY upon the ongoing ("background") immunoglobulin (Ig) synthesis. Therefore we decided to study the effect of CY upon the B cell compartment at five different levels: (a) the serum Ig concentration; (b) the numbers of background IgM-, IgG- and IgA-secreting cells in various lymphoid organs; (c) the incidence of surface Ig^+ B cells; (d) the functional capacity of lipopolysaccharide (LPS)-reactive B cells, which predominantly comprise newly-formed short-lived cells (Rusthoven and Phillips, 1980); and (e) the functional capacity of long-lived memory B cells induced by priming with sheep red blood cells (SRBC). The data presented show that CY reduces the functional capacity of B lineage cells to a much larger extent than their number and functional activity.

MATERIALS AND METHODS

Mice

Female (C57BL/Rij x CBA/Rij)F1 hybrid mice, 5 weeks old and 12 to 18 weeks old, and female C57BL/6J mice, 8 to 12 weeks old, and female Lewis rats, 4 weeks old, were used. The (C57BL/Rij x CBA/Rij)F1 mice were obtained from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. The C57BL/6J mice were purchased from HARLAN OLAC Ltd., Blackthorn, Bicester, United Kingdom. The Lewis rats were purchased from the Central Laboratory for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. All mice and rats received pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3.0) ad libitum.

Treatment with cyclophosphamide

For treatment with cyclophosphamide (Endoxan®; Koch-Light Laboratories, Colnbrook, United Kingdom) the mice received a single intraperitoneal (ip) injection of 300 mg CY/kg body weight (BW) in 0.5 ml balanced salt solution (BSS) or seven daily ip injections of 1 or 100 mg CY/kg BW in BSS. The BSS used was prepared according to Mishell and Dutton (1967). Injections were always done between 4 and 5 PM. The applied treatments did not cause any sign of illness or loss of body weight. Also dissection did not show abnormalities. Each experimental group consisted of five mice.

Preparation of cell suspensions

Cell suspensions of spleen, mesenteric lymph nodes (MLN) and femoral bone marrow of individual mice and cell suspensions of rat thymuses were prepared in BSS as described in detail in a previous paper (Benner et al., 1981). Viable nucleated cells were counted by trypan blue exclusion.

Antigen and immunization

SRBC were obtained from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. Before use, the cells were washed three times in phosphate buffered saline (PBS; pH 7.2). (C57BL x CBA)F1 mice, 12 to 18 weeks old, were primed by an intravenous (iv) injection of 10^7 SRBC in a volume of 0.05 ml BSS. The interval between the priming and the adoptive cell transfer was 3 months.

Adoptive transfer of spleen and bone marrow cells

For adoptive transfer experiments, the recipient mice received 750 rad whole body irradiation generated in a Philips Müler MG 300 X-ray machine. Animals were irradiated in well-aerated circular Perspex cages. Physical constants of the irradiation have been described previously (Benner et al., 1974). Radiation control mice died in 9 - 16 days. The recipients were iv injected with 2×10^7 viable nucleated cells of the appropriate lymphoid cell suspension and 5×10^8 SRBC within 4 hrs after irradiation. Within another 4 hrs all recipients were iv injected with 10^7 corticosteroid resistant thymocytes as a nonlimiting source of helper T cells. These thymocytes were obtained from 5-week-old syngeneic mice injected with 30 mg dexamethasone sodium phosphate (Decadron®) 2 days before. On the fourth day all recipient mice were boosted with 5×10^8 SRBC ip. SRBC-specific antibo-

dy-secreting plaque-forming cells (PFC) in the spleen of the recipient 7 days after cell transfer as described previously (Benner et al., 1974). donors and recipients consisted of five mice.

Membrane fluorescence

For immunofluorescence staining of B cells, the cells were resus tration of 10^7 cells per ml in PBS (pH 7.8) supplemented with 1% bov (BSA) and 0.02% NaN3. 100 µl cell suspension was mixed with 50 µl f goat antiserum directed against mouse immunoglobulins (GAM-Ig-FIT Immunological Laboratories, Tilburg, The Netherlands) and incubated After two washes with PBS-BSA the preparations were mounted in bu Merck A-G, Darmstadt, F.R.G.), sealed with paraffin (Paramat©, Gur Bucks, Great Britain) and evaluated under a Zeiss fluorescence micros

Culture conditions

C57BL/6J spleen cells (6000 viable nuceated cells per well) or bo (30,000 viable nucleated cells per well) were cultured together with 50 coli LPS and 5 x 10^5 growth-supporting rat thymus cells (irradiated wi in 0.2 ml RPMI 1640 medium supplemented as described previously (H The cultures were set up in microtest II tissue culture plates (Costar 35 bridge, MA, U.S.A.). The cultures were harvested and assayed on Day cells.

Protein A plaque assay

The protein A plaque assay for IgM-, IgG- and IgA-secreting cel described by Gronowicz et al. (1976) and has been modified in our labo in detail elsewhere (Van Oudenaren et al., 1981). The origin and specif antisera employed in the protein A plaque assay have been described p al., 1983).

Assay for cells containing cytoplasmic immunoglobulin (C-Ig cells)

C-Ig cell determinations were done as described in detail in a pre man et al., 1979).

Calculation of the total number of Ig-secreting cells and C-Ig cells per organ

Total numbers of Ig-secreting cells and C-Ig cells per organ were the number of positive cells per slide, the number of cells tested per sli cell yield per organ (Haaijman et al., 1979; Benner et al., 1981).

Serum IgM- IgG- and IgA-levels

A Terasaki ELISA system was adopted to measure quantitatively of the various classes. Details of this method have been described previ al., 1984). Briefly, diluted serum samples were coated onto the bottom After washing with Dulbecco's PBS (DPBS) the trays were incubated fo DPBS-gelatin to coat non-specific binding sites. Subsequently the plates washed with DPBS-Tween-gelatin and incubated for 1 hr at room temp specific antisera of rabbit origin (RAM-IgM, RAM-IgG and RAM-IgA purified and found to be specific for the respective mouse Ig classes by resis. The antigen-antibody complex was incubated for 1 hr at 20°C wit β-galactosidase conjugate. Binding of antibodies to the wells was visual with the substrate 4-methylumbelliferyl galactoside. Fluorescence in the the Terasaki trays was then quantitatively analysed within 120 sec using inverted microfluorometer, connected to a digital voltmeter and a desk- quantitate the Ig levels, a reference standard of a large pool of normal obtained from approximately one-year-old CBA/Rij mice was used on

Benner, 1979).

RESULTS

Effect of a single injection of CY upon the background Ig synthesis

The effect of a single injection of 300 mg CY/kg BW was studied upon the background Ig synthesis of (C57BL x CBA)F1 mice. This was done by determining the numbers of background IgM-, IgG- and IgA-secreting cells and the serum concentrations of IgM, IgG and IgA at various intervals after the CY injection.

Immediately after injection of the CY, the numbers of IgM-, IgG- and IgA-secreting cells in spleen, bone marrow and MLN substantially decreased. In all three organs minimal values were reached at 7 days after CY injection (Fig. 1). These minimum values ranged from 5 to about 40% of the original Ig-secreting cell numbers and, within a particular lymphoid organ, hardly differed for IgM, IgG and IgA. The minimum values in MLN tended to be lower than in spleen and bone marrow, especially with regard to the IgG-secreting cells.

At one day after the CY injection, the number of Ig-secreting cells in the spleen was determined not only with the protein A plaque assay (which is a functional assay), but also by cytoplasmic immunofluorescence (which detects plasmablasts and plasma cells). It appeared that the percentage decrease of positive cells as detected by these assays hardly

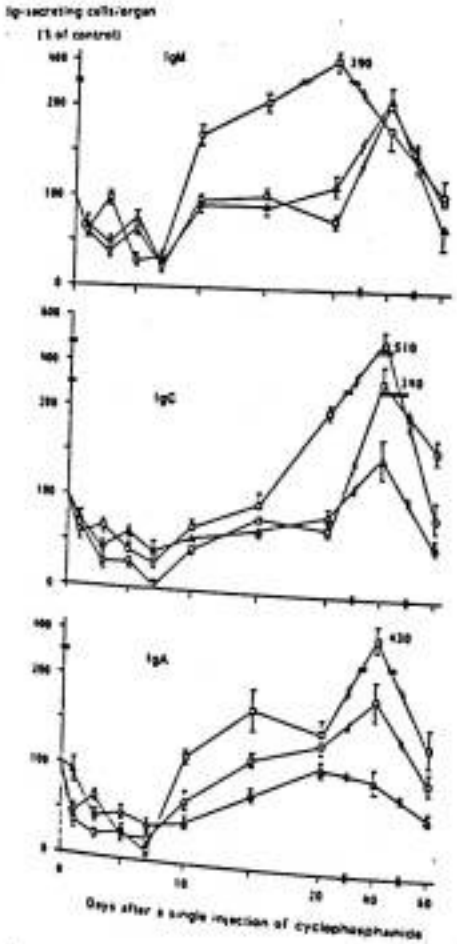

Fig. 1. Effect of a single injection of 300 mg CY/kg BW on the number of background IgM-, IgG- and IgA-secreting cells in mouse spleen (○), MLN (□) and bone marrow (△). Numbers of Ig-secreting cells were determined at various intervals after drug administration. At all time points, a group of BSS-treated control mice were assayed as well. The Day 0 values (100%) were calculated as the arithmetic mean of all these controls. These values (IgM, IgG, IgA) are for spleen 106,666, 65,000 and 90,416; for MLN 3,183, 16,600 and 6,583; and for bone marrow 202,470, 170,710 and 214,380, respectively.

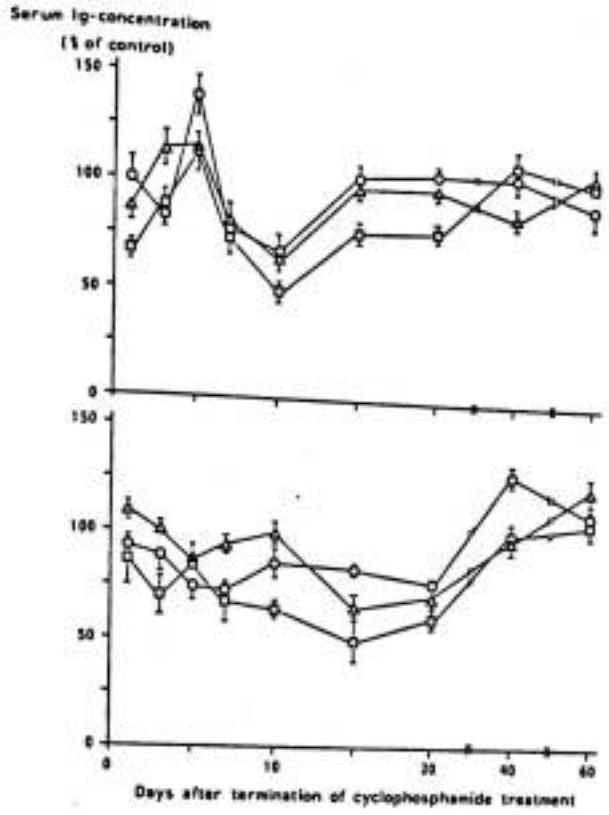

Fig. 2. Effect of a single injection of 300 mg CY/kg BW 7 daily injections of 100 mg CY/kg BW (lower part) on the IgM (○), IgG (□) and IgA (△). Sera were obtained from used for determination of the numbers of background Ig-se (Fig. 1 and Fig. 3, right part). The Day 0 values (100%) the arithmetic mean of the absolute Ig levels of all BSS- mice. These values are for IgM 18 ± 1.5 mg/dl; for IgG 60 for IgA 25 ± 3.6 mg/dl.

differed (data not shown), indicating that the Ig-secreting cells were n inhibited, but also to a considerable extent no longer detectable as plas cell.

Starting at Day 7, the numbers of IgM-, IgG- and IgA-secreting and for all three organs substantially surpassed the control level. Peak of normal, were found 40 days after the CY injection (Fig. 1).

The effect of CY injection on the serum concentration of IgM, I pronounced than on the numbers of Ig-secreting cells. Here, minimum of normal were found at 10 days after the CY injection. Within a few levels recovered till the control values without displaying an overshoot upper part).

Effect of multiple injections of CY upon the background Ig synthesis

The effect of multiple CY injections was studied after 7 daily in CBA)F1 mice with either 1 or 100 mg CY/kg BW. These doses had a g upon the numbers of IgM-, IgG- and IgA-secreting cells in the organs tions of 1 mg CY/kg BW did not cause an immediate substantial decre IgM-, IgG- and IgA-secreting cells, whereas the injections of 100 mg 3). Instead, immediately after discontinuing the injections of 1 mg CY/ reactions occurred, especially in MLN, the latter peaking 1 day after t (Fig. 3). These overshoot reactions were followed by a substantial decr of Ig-secreting cells, especially for IgG and IgA.

After discontinuing the daily injections of 100 mg CY/kg BW the initial overshoot reactions did not occur. This regimen almost completely abolished the IgG- and IgA-secreting activity in spleen, bone marrow and MLN, while the number of IgM-secreting cells in the MLN was the least affected (Fig. 3). The numbers of Ig-secreting cells in the various organs tested returned to normal without displaying substantial overshoot reactions.

At one day after the last CY injection, the decrease of the number of C-Ig cells in the spleen was much larger than the decrease of the number of Ig-secreting cells detected in the protein A plaque assay. The incidence of C-Ig cells had decreased till 3.4% of the number found in the BSS-treated control mice, whereas the incidence of Ig-secreting cells detected in the protein A plaque assay was 11% of the number found in the control mice (Table 1).

The serum IgM, IgG and IgA levels of the mice treated with 7 daily injections of 100 mg CY/kg BW were again much less affected than the Ig- secreting cell compartment. After the last CY injection the serum Ig levels, especially IgG and IgA, gradually decreased, reaching minimum values about 2 weeks after the last injection (Fig. 2, lower part).

Effect of a single injection of CY upon the B cell compartment

The effect of a single injection of 300 mg CY/kg BW upon the B cell compartment was determined in spleen and bone marrow of C57BL/6J mice at various intervals after

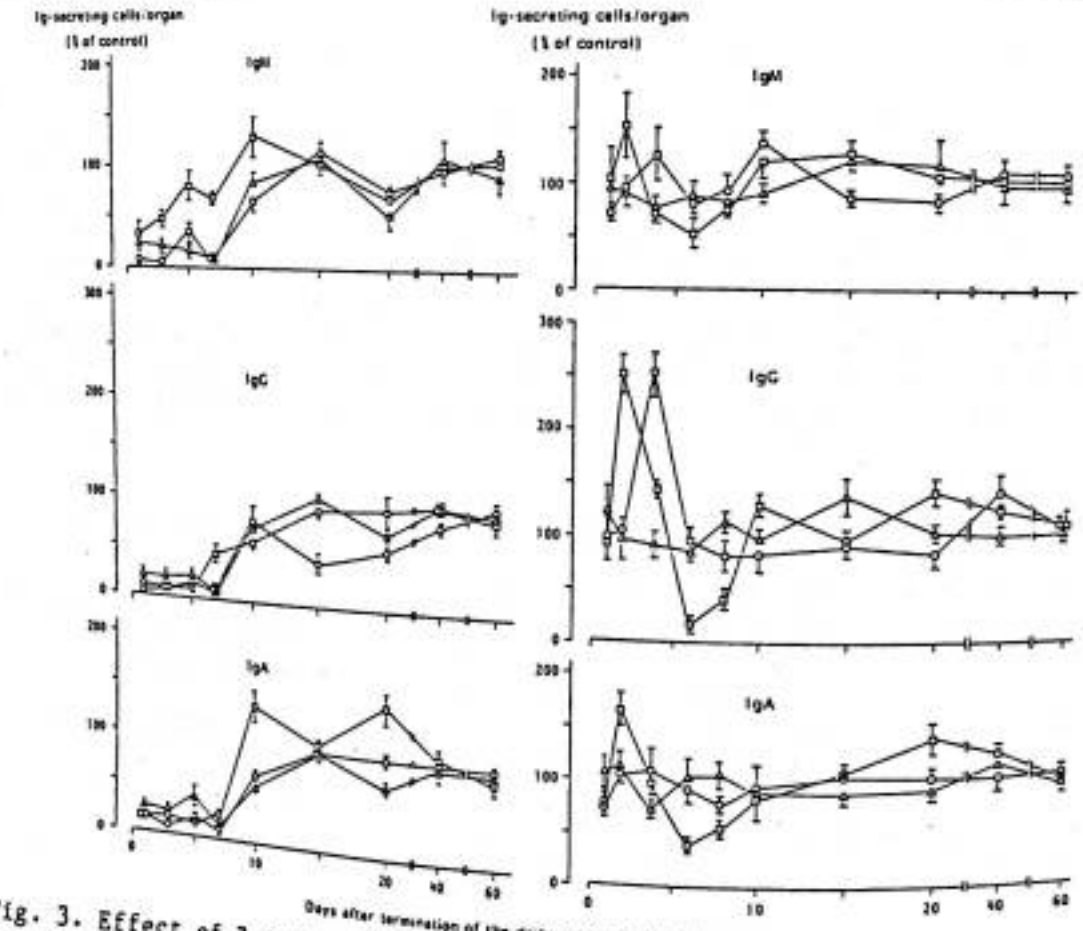

Fig. 3. Effect of 7 daily injections of either 1 mg CY/kg BW (left part) or 100 mg CY/kg BW (right part) on the number of background IgM-, IgG- and IgA-secreting cells in mouse spleen (○), MLN (□) and bone marrow (△). Numbers of Ig-secreting cells were determined at various intervals after the last drug administration. At all time points a group of BSS-treated control mice was assayed as well. The 100% values were calculated as the arithmetic mean of all these controls. These values (IgM, IgG, IgA) are for spleen 218,700 ± 16,950, 130,000 ± 32,100 and 130,600 ± 24,650; for MLN 4,400 ± 1,300; 10,150 ± 800 and 8,600 ± 1,750; and for bone marrow 211,998 ± 36,127, 210,807 ± 46,449 and 304,896 ± 86,546, respectively.

injection. This was done by enumerating the surface Ig^+ cells and by determining the functional capacity of the B cell compartment.

CY injection rapidly decreased the number of viable nucleated cells in the spleen and the bone marrow to a minimum value of about 8 per cent of normal at Day 3. The number of B cells also severely decreased in the spleen and in the bone marrow as appeared from the steadily decreasing percentages of surface Ig^+ cells with increasing interval after CY injection. Lowest incidences of B cells were found in spleen and bone marrow around Day 6 (Table 2).

The capacity to give rise to a polyclonal IgM-response in vitro was virtually completely abolished by a single injection of 300 mg CY/kg BW. From Day 6 on this capacity gradually recovered (Table 2). Normal or supranormal in vitro IgM-responses were found at 3 weeks after the CY injection, while the percentages of surface Ig^+ cells in spleen and bone marrow were still below their normal values.

The effect of CY treatment upon long-lived B cells was evaluated by means of adoptive transfer experiments. Therefore, (C57BL x CBA)F1 mice primed with 10^7 SRBC 3 months before, were treated with 300 mg CY/kg BW. One day later, 2×10^7 spleen cells and 2×10^7 bone marrow cells were transferred together with 5×10^8 SRBC to separate groups of irradiated syngeneic recipient mice. In order to circumvent a relative shortage of helper T cells in the recipients, all recipients received 10^7 corticosteroid resistant thymocytes. The adoptive anti-SRBC PFC responses in the recipient spleens were determined 7 days after cell transfer. It appeared that the CY treatment of the donors completely prevented the adoptive anti-SRBC PFC response (Table 3), indicating that the functional capacity of the long-lived memory B cells was abolished by the CY.

DISCUSSION

Cy is one of the most potent suppressors of antibody formation. Its activity has been shown in many studies employing mice, rats, guinea pigs, pigs and humans (reviewed by Bach and Strom, 1985; Mackie, 1981). Antibody formation to T-dependent as well as to T-independent antigens is suppressed by CY, and to soluble as well as to particulate antigens. CY can suppress antibody formation of all Ig classes and subclasses, although

Table 1

Effect of 7 daily CY injections upon the incidence of Ig-secreting cells in the spleen of (C57BL x CBA)F1 mice as determined in the protein A plaque assay and by cytoplasmic immunofluorescence

Treatment	protein A PFC		C-Ig cells	
	number ($\times 10^{-3}$)	% of control	number ($\times 10^{-3}$)	% of control
CY	34 ± 4	11 ± 1	7.5 ± 2.3	3.4 ± 0.8
control	319 ± 34	-	219 ± 38	-

Numbers of Ig-secreting cells (total of IgM-, IgG- and IgA-secreting cells) were determined one day after the last injection of 100 mg CY/kg BW. A group of BSS-treated control mice was assayed simultaneously. Figures represent the arithmetic mean ± 1 SEM (n = 5).

Table 2

Effect of a single injection of 300 mg CY/kg BW upon the incidence of surface Ig^+ B cells and the LPS-induced *in vitro* Ig-secreting cell response by spleen and bone marrow cells from C57BL/6J mice at various intervals after the CY administration

Interval (days)	spleen			bone marrow		
	nucleated cells	B cells	LPS response	nucleated cells	B cells	LPS response
1	18 ± 3*	35 ± 8	0.3 ± 0.1	43 ± 7	56 ± 11	0.3 ± 0.1
3	8 ± 3	30 ± 5	5.4 ± 3.5	9 ± 2	40 ± 5	0.3 ± 0.3
6	20 ± 4	12 ± 4	4.4 ± 1.7	80 ± 3	3.8 ± 1.0	0.1 ± 0.1
13	130 ± 52	13 ± 2	9.4 ± 3.3	96 ± 7	19 ± 3	9.4 ± 2.3
21	73 ± 13	69 ± 12	153 ± 31	91 ± 9	67 ± 10	152 ± 13

*All figures are calculated as percentage of the values found for spleen and bone marrow of untreated control mice. For the spleen, these values are $123 \pm 10 \times 10^6$ nucleated cells, 45 ± 0.7% B cells and a LPS-induced *in vitro* IgM-secreting cell response of 16,992 ± 4,094 protein A PFC. For the bone marrow of a single femur these values are $17 \pm 1 \times 10^6$, 5.2 ± 0.5% and 10,679 ± 1,886, respectively.

Table 3

Effect of a single injection of 300 mg CY/kg BW upon the adoptive anti-SRBC PFC response by spleen and bone marrow cells from (C57BL x CBA)F1 mice primed with 10^7 SRBC 3 months previously

Donor mice primed with[a]	Treated with CY	Cells transferred[b]	Anti-SRBC PFC/recipient spleen ($\times 10^{-3}$) IgM	IgG	IgA
-	-	spleen	262[c] (235-311)	1406 (1203-1635)	301 (264-351)
-	+	spleen	<0.5	<0.5	<0.5
10^7 SRBC	-	spleen	422 (333-534)	4,360 (3,684-5,160)	836 (729-959)
10^7 SRBC	+	spleen	0.5 (0.4-0.6)	<0.5	<0.5
-	-	bone marrow	26 (23-30)	71 (58-91)	N.S.[d]
-	+	bone marrow	<0.5	<0.5	<0.5
10^7 SRBC	-	bone marrow	63 (51-79)	381 (321-453)	50 (13-74)
10^7 SRBC	+	bone marrow	0.5 (0.3-0.8)	<0.5	<0.5

a) Mice were either primed with 10^7 SRBC in BSS iv or injected with BSS only 3 months before cell transfer.
b) For adoptive transfer, lethally irradiated recipient mice were iv infused with either 2×10^7 viable nucleated spleen cells or 2×10^7 viable nucleated bone marrow cells. All recipients received 5×10^8 SRBC and 10^7 corticosteroid resistant thymocytes as a non-limiting source of helper T cells. Anti-SRBC PFC were determined in the recipient spleens at 7 days after cell transfer.
c) Geometric mean with 95% confidence limits.
d) N.S. means that the number of IgM- plus IgA-PFC in the indirect plaque assay did not differ significantly from the number of IgM-PFC found in the direct plaque assay.

different Ig (sub)classes may be affected to a different extent (Drössler et al., 1981, 1983; Turk and Parker, 1982). A single injection of a high dose of CY shortly before immunization often enhances IgE antibody formation, while it usually suppresses antibody formation of other classes (Chiorazzi et al., 1976; Graziano et al., 1981; De Macedo and Mota, 1982).

In view of all these data on the effects of CY on antibody formation it is surprising that hardly data are available about the effect of CY on serum Ig-levels and on the Ig-secreting cells in the various lymphoid organs that build up and maintain these levels. Aisenberg did study the effect of CY injections on the numbers of background anti-SRBC IgM- and IgG-secreting cells in naive mice at various intervals after drug administration (Aisenberg, 1967; Aisenberg and Wilkes, 1967). He found a dramatic decrease of the numbers of antibody forming cells of both isotypes within 4 days after the first injection of 85 mg CY/kg BW.

The data presented in this paper show that a single as well as multiple CY injections of mice cause a decrease of both the number of background IgM-, IgG- and IgA-secreting cells and the serum IgM-, IgG- and IgA-levels. A single injection of 300 mg CY/kg BW decreases the IgM-, IgG- and IgA-levels down to 50 to 70 per cent of normal at Day 10 (Fig. 2, upper part). The IgG-level proved to be the most severely affected. This was also found to be the case after 7 daily injections of 100 mg CY/kg BW (Fig. 2, lower part). In previous studies on the effect of multiple injections of a high dose of corticosteroids, the most severe decrease was also found for the IgG class (Sabbele et al., 1983).

It is remarkable that the background IgM-, IgG- and IgA-secreting cells (Figs. 1 and 3) were much more severely depressed by CY treatment than the serum concentrations of the various Ig classes. It is tempting to speculate that the smaller effect on the circulating Ig is due to a decreased catabolic rate. For corticosteroids evidence has been presented that treatment with this drug does affect the catabolic rate of serum Ig (Butler and Rossen, 1973; Butler, 1975; Levy and Waldman, 1970; Griggs et al., 1972).

The severe decrease of background IgM-, IgG- and IgA-secreting cells was found not only in spleen and lymph nodes, but also in the bone marrow. This is in clear contrast to the effects observed after single or multiple injections of a high dose of corticosteroids. In the latter case the Ig-secreting cell compartment in the bone marrow was hardly affected (Sabbele et al., 1983). This might well be due to properties of the bone marrow microenvironment that protect marrow cells against damage by enhanced corticosteroid levels (Bradley and Mishell, 1981; Zatz, 1976).

In view of the above discussed differential sensitivity of the various Ig (sub)classes for the immunosuppressive and immunostimulating activities of CY it might be surprising that the IgM-, IgG- and IgA-secreting cell compartments are all decreased to virtually the same extent. This might well be due to the high doses of CY employed by us in most experiments (Figs. 1 and 3, right). Indeed, after 7 daily injections of a dose of CY as low as 1 mg/kg BW, much more variation was found, both between Ig-secreting cells of different classes within the same organ and between Ig-secreting cells of the same class in different organs (Fig. 3, left).

After a single dose of 300 mg CY/kg BW a substantial overshoot reaction of IgM-, IgG- and IgA-secreting cells was found in various lymphoid organs at 40 days after injection (Fig. 1). This overshoot reaction could not be detected at the serum level (Fig. 1, upper part), suggesting an increased catabolic rate of the serum Ig.

After multiple CY injections a late overshoot reaction did not occur (Fig. 3). Multiple CY injections affect both B and T lymphocytes (Winkelstein, 1977). A single injection of a high dose of CY, however, preferentially affects B cells (Turk and Poulter, 1972; Poulter and Turk, 1972). This leads to a B-T dysbalance, which is known to favor transient overshoot reactions of B cell clones (Van den Akker et al., 1983; Radl, 1981).

After multiple CY injections, the proportional decrease of C-Ig cells was much greater than the proportional decrease of Ig-secreting PFC (Table 1). We have previously argued that by cytoplasmic immunofluorescence, on the mean, more mature Ig-secreting cells are detected than in the protein A plaque assay (Benner et al., 1982). This suggests that during chronic CY treatment antigen-activated B cells do no longer differentiate into fully mature C-Ig cells, a process that is dependent on the availability of enough T cell help (Benner et

al., 1982).

Several studies suggest that primary a
mediated suppression than secondary respo
Havas and Schiffman, 1981). In view of th
and Turk, 1972; Mackie, 1981), this datum
susceptible to CY than long-lived memory
effect of CY on the functional activity of t
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data show that the capacity of B cells to gi
faster and more severely depressed by CY t
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for newly-formed (Rusthoven and Phillips,
memory B cells. In both cases the functiona
one day after a single injection of 300 mg (
decreased by only two-third (Table 2). This
induced by treatment with high doses of co
capacity of the residual spleen and bone ma
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In conclusion, the data presented in th
are less susceptible to the immunosuppressiv
resting B cells. The serum Ig-levels are ever
ting a decreased catabolic rate of Ig under c

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CHAPT

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S U M M A R Y

Immunosuppressive agents are wid
nt of immunologically mediated diseas
ssue transplantation. Many of these d
- lymphoid and non-lymphoid cell types

Antibody formation and cell-medi
pend on complex interactions between
hocytes and antigen-presenting cells.
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ions has enormously increased. Simulta
e been obtained about the influence of
rugs on immune reactions and about the

Advancement in this field of res
dent on the further development of met
ents that allow a more sensitive and o
f the effects of immunosuppressive dru
stituents of the immune system. We have
y developed methodologies to analyse i
f two frequently used immunosuppressiv
e sodium phosphat (Decadron) and cyclc
n the immunoglobulin (Ig) synthesizing
urine immune system.

We studied the effects of dexame
yclophosphamide (CY) upon the Ig produ
ent levels :

a. the serum Ig concentrations.
b. the 'background' Ig-secreting cells
ly all lymphoid tissues without del
c. the surface Ig^+ cells.
d. the B cells that give rise to clone
upon in vitro stimulation with bact-
des (LPS).

We investigated the effect of s

(ip) injection as well as short-term daily ip administration of DEXA and CY upon the above four parameters. The doses of DEXA employed ranged from 16 up to 144 mg/kg body weight(BW) for a single injection and from 1 up to 50 mg/kg BW for daily injection which, in view of the high immunosuppressive - and anti-inflammatory activities of DEXA are extremely large doses when compared with the doses employed in clinical practice. For treatment with CY the mice received either a single injection of 300 mg/kg BW or daily injections of 1 or 100 mg/kg BW.

As a background for these studies we review in Chapter 1 the present state of knowledge on the structure and - function of the immune system and the effects of corticosteroids and CY on the immune system.

In Chapter 2 we discuss in detail procedures for the generation and measurement of antibodies. Special attention is paid to technical aspects of the various assay systems - for the quantitation of antibodies and antibody-forming cells

Chapter 3 describes our studies on the influence of - DEXA on the Ig production and the serum Ig-levels. A single injection of DEXA (16 to 144 mg/kg BW) appeared to cause a - substantial decrease of the numbers of IgM, IgG, and IgA secreting cells in spleen and mesenteric lymph nodes (MLN) within one day, but hardly affected their number in the BM. The decrease was immediately followed by a recovery and, at the highest doses and especially in MLN, by an overshoot. Two weeks after the initial decrease a second decrease was found. When mice were subjected to daily treatment with DEXA (1 or 16 mg/kg.BW) during 1 week, initially a recovery pattern was found in spleen and MLN similar to that found after a single injection of a high dose. In this case, however, the effects were less dose-dependent and the overshoot reaction was - followed by a period of subnormal numbers of Ig-secreting - cells which lasted at least 1 week. This late effect of DEXA

of only marginal in spleen and BM, but also in PM. The most prominent effect of daily treatment with DEXA was the long - lasting decrease of the number of Ig A-secreting cells start - ing 1 week after withdrawal of treatment. This decrease was associated with a decrease of the serum IgA-level to about - 5 per cent of the normal value. The serum IgM and IgA- le - vels, on the other hand, were much less affected by multiple DEXA injections.

A single injection of DEXA, even a massive dose as - 144 mg/kg BW, affected the serum IgM, IgG and IgA-levels - less severely than 7 daily injections of e.g. 1 mg DEXA/kg. BW (Chapter 4). After a single injection of DEXA th IgG le- vel was decreased to the same extent as the IgM and IgA le- vel. A characteristic effect of a single injection of DEXA on the serum Ig pattern was the initial short-lasting increase of the IgG-level to about 125 per cent of the normal value.

In addition to the effects of a single DEXA injection on the serum Ig-levels, Chapter 4 describes the influence of a single injection of DEXA and CY upon the B cell compartment of mice. A single injection of 144 mg DEXA/kg BW caused a - profound decrease of the number of spleen cells and a mode - rate decrease of the number of bone marrow cells. The percentage of B cells among the remaining spleen and bone marrow - cells was not affected. Also the height of the polyclonal response after stimulation of the remaining B cells with LPS in vitro and the capacity to switch from IgM to IgG and Ig A secretion were not affected. These data show that, on the - mean, the population of LPS-reactive B cells are not more or less sensitive to DEXA than the other cells of the lymphohemopoietic system.

Injection of CY, on the other hand, not only greatly decreases the number of spleen and bone marrow cells, but also the proportion of B cells among the remaining spleen and

bone marrow cells. Furthermore, these B cells could no longer be polyclonally activated by LPS in vitro, indicating that they were inhibited in their funcional capacity.

The effects of DEXA and CY treatment on the B cell compartment are studied in more detail in the experiments reported in Chapter 5 and 6.

Chapter 5 describes the influence of DEXA upon B cells in vivo and during in vitro culture. One day after a series of 7 daily injections of 50 mg DEXA/kg BW, the number of spleen cells was reduced by more than 90 per cent, whereas the total number of nucleated cells in the bone marrow was much less affected. The frequency of B cells was reduced by about 50 per cent of the original percentage in both spleen and bone marrow. The in vitro LPS-induced Ig-secreting cell response was reduced accordingly. These data suggest that multiple injections of DEXA can reduce the number and percentage of B cells in the lymphoid system, but do not affect the functional capacity of the residual LPS-reactive B cells. This was confirmed at the clonal level by limiting dilution culture experiments.

The contrasting effects of DEXA on splenic and bone marrow B cells was also found when the cells were exposed to the drug in vitro. It was found that 10^{-8}M. DEXA in vitro reduced the response of splenic B cells to LPS by more than 80%, while a similar reduction of the response by bone marrow B cells required a 1000-fold higher concentration.

Chapter 6 deals with detailed experiments on the influence of CY on the circulating Ig-levels, background Ig-secreting cells and B cells.

A single injection of 300 mg CY/kg BW decreased the numbers of background IgM, IgG, and IgA-secreting cells in spleen, bone marrow and MLN to minimum values of about 25% of normal at day 7. The incidence of surface Ig^+ B cells also gradually decreased after CY treatment. The functional capacity of the B cells, however, was completely abolished

one day after a single injection of 300 mg CY/kg BW. This was found for the LPS-reactive B cells, with largely represent - newly-formed, short-lived B cells as well as for the long-lived memory B cells.

The decrease of the numbers of background Ig-secreting cells following a single injection of 300 mg CY/kg BW was - followed by a gradual recovery with a subtantial overshoot - peaking about 40 days after CY injection. After multiple in - jections of 100 mg CY/kg BW, the minimum values of background Ig-secreting cells in the various lymphoid organs were lower than after a single injection of 300 mg CY/kg BW, but in this case the recovery was not associated with an overshoot reacti on. Remarkably, the serum Ig-levels were much less decreased than the numbers of Ig-secreting cells in the various lymphoid organs, suggesting that the half-life of the circulating Ig in CY treated mice is lengthened.

In this study the following new findings were found :

1. The decrease of background Ig-secreting cells following a single injection of 300 mg/kg body weight of cyclophospha mide was followed by gradually recovery with a substantial 'overshoot peaking' about 40 days after cyclophosphami de injection.
2. The percentage of B cells among the remaining spleen and bone marrow cells were not affected by a single injection of dexamethasone (144 mg/kg body weight), but they were - completely abolished within a day after a single injecti- on of 300 mg/kg body weight of cyclophosphamide.
3. The study on the influence of the synthetic corticostero- id dexamethasone sodium phosphat upon the immunoglobulin- secreting cells were experimented on unintentionally immu nized Balb/C mice.-

R A N G K U M A N

Obat-obat imunosupresif telah dipakai secara luas dalam pengobatan penyakit-penyakit imunologik dan pada pencangkokan organ dan jaringan. Banyak obat tersebut mempengaruhi baik - bermacam-macam sel limfoid maupun bukan sel limfoid.

Pembentukan antibodi dan respons imunologik seluler ber gantung pada saling mempengaruh yang kompleks antara bermacam -macam subpopulasi limfosit dan sel-sel yang menyajikan anti gen. Lebih dari dua puluh tahun terakhir, mekanisme tentang reaksi imunologik telah meningkat dengan pesat. Serentak ba nyak data telah diperoleh mengenai pengaruh obat-obat imunosu presif terhadap reaksi-reaksi imunologik serta letak tempat bekerjanya. Kemajuan dalam lapangan penelitian ini sangat ber gantung kepada perkembangan lebih lanjut berbagai metode dan bahan yang lebih sensitif dan/atau analisa selektif pengaruh obat-obat imunosupresif atas bermacam-macam sistem imunologik yang dipilih.

Penulis menggunakan metode-metode yang telah dikembang kan dewasa ini untuk menyelidiki secara terperinci pengaruh 2 jenis obat imunosupresif yang sering digunakan, yaitu fosfat natrium deksametason (Decradon) dan siklofosfamid (Endoxan) - pada tempat pembentukan imunoglobulin sistem imunologik tikus

Penulis telah meneliti pengaruh deksametason, dan siklo fosfamid atas pembentukan imunoglobulin pada empat level yang berbeda, yaitu:

a. konsentrasi imunoglobulin dalam serum.
b. sel-sel yang menghasilkan imunoglobulin yang sesungguhnya terjadi dalam semua jaringan limfoid tanpa pemberian imuni sasi.
c. limfosit B yang memiliki imunoglobulin permukaan.
d. limfosit B yang dapat menimbulkan sel-sel yang menghasil kan imunoglobulin dalam bentuk "clone in vitro" yang di -

rangsang dengan lipopolisakharida bakteri.

Dengan 4 macam parameter tersebut, telah diteliti pengaruh penyuntikan deksametason, siklofosfamid ke dalam rongga peritoneum tikus dengan dua cara yaitu penyuntikan satu kali saja dan penyuntikan tiap hari selama satu minggu.

Dosis deksametason untuk ke dua cara tersebut, berturut-turut berkisar antara 16 sampai 144 mg/kg berat badan, dan 1 sampai 50 mg/kg berat badan; ternyata aktivitas imunosupresif dan anti inflamasi yang kuat deksametason memerlukan dosis yang sangat tinggi bila dibandingkan dengan dosis yang digunakan dalam praktek di rumah sakit.

Dosis siklofosfamid 300 mg/kg berat badan pada penyuntikan tunggal dan 1 atau 100 mg/kg berat badan untuk pemberian tiap hari selama satu minggu.

Dalam Bab.1 dijelaskan latar belakang penelitian ini, struktur dan fungsi sistem imunologik serta pengaruh kortikosteroid, siklofosfamid pada sistem imunologik.

Dalam Bab.2 diuraikan lebih terperinci cara kerja menentukan pembentukan dan pengukuran antibodi. Perhatian khusus ialah tentang teknik berbagai macam sistem pemeriksaan kuantitatif antibodi dan sel-sel yang menghasilkan antibodi.

Dalam Bab.3 diterangkan beberapa penelitian tentang pengaruh deksametason pada pembentukan imunoglobulin dan nilai imunoglobulin dalam serum. Satu hari sesudah penyuntikan tunggal deksametason dosis tinggi (16 sampai 144 mg/kg berat badan) terdapat penurunan yang nyata jumlah sel yang membentuk IgM, IgG dan IgA dalam limpa dan kelenjar limfa mesenterium, tetapi hal ini hampir tidak dijumpai dalam sumsum tulang. Penurunan ini segera diikuti oleh suatu perbaikan, terutama dalam kelenjar limfa mesenterium terjadi suatu penyimpangan ("overshoot") yang selanjutnya disusul penurunan kedua 2 minggu setelah penurunan awal.

Apabila tikus disuntik deksametason dosis rendah (1 atau

16 mg/kg berat badan) tiap hari selama satu minggu, maka per baikan dalam limpa dan kelenjar limfa mesenterium sama dengan hasil penyuntikan tunggal dosis tinggi. Dalam hal ini, penga ruh deksametason kurang bergantung kepada besarnya dosis dan reaksi menyimpang ("overshoot") diikuti oleh suatu periode subnormal jumlah sel yang mengeluarkan imunoglobulin selama - kurang lebih satu minggu. Pengaruh deksametason yang terakhir ini tidak hanya terjadi dalam limpa dan kelenjar limfa mesen terium tetapi juga dalam sumsum tulang. Pengaruh yang paling menonjol pemberian deksametason tiap hari ialah berlangsung nya lama penurunan jumlah sel yang menghasilkan imunoglobulin G (IgG) yang dimulai satu minggu setelah pemberian dihentikan Penurunan ini berhubungan dengan menurunnya nilai IgG dalam - serum kurang lebih 50 persen nilai normal. Sebaliknya nilai IgM dan IgA dalam serum kurang dipengaruhi oleh pemberian dek sametason yang berulang-ulang.

Dalam Bab.4 dibahas bahwa pengaruh deksametason pada ni lai IgM, IgG dan IgA dalam serum, sangat kurang dengan penyun tikan tunggal dosis tinggi (144 mg/kg berat badan) daripada - pemberian tiap hari selama satu minggu dosis rendah (1 mg/kg berat badan).

Sesudah penyuntikan tunggal deksametason ditemukan penu runan nilai IgG sama dengan nilai IgM dan IgA. Pengaruh khas penyuntikan tunggal deksametason ialah terjadinya mula-mula peninggian sementara nilai IgG dalam serum sampai 125 persen nilai normal. Selanjutnya diterangkan pengaruh pemberian tung gal deksametason, dan siklofosfamid pada populasi limfosit B tikus. Penyuntikan tunggal deksametason 144 mg/kg berat badan menyebabkan jumlah sel dalam limpa sangat menurun, sedangkan dalam sumsum tulang hanya terjadi penurunan jumlah sel yang sedang. Prosentase limfosit B di antara kelompok sel yang ter tinggal dalam limpa dan sumsum tulang ternyata tidak terpenga ruh. Tidak terpengaruh pula peninggian poliklonal yang terben tuk dari limfosit B yang tertinggal setelah perangsangan de

ngan lipopolisakharida "in vitro" dan kemampuan perubahan IgM menjadi IgG dan IgA. Data ini menunjukkan bahwa rata-rata populasi limfosit B yang bereaksi dengan lipopolisakharida tidak lebih sensitif terhadap deksametason daripada sel-sel lain dalam sistem limfohemopoitik.

Penyuntikan siklofosfamid mengakibatkan bukan saja jumlah sel dalam limpa dan sumsum tulang yang sangat menurun, melainkan pula limfosit di antara sel-sel yang tertinggal dalam limpa dan sumsum tulang. Selanjutnya limfosit B itu, tidak dapat lebih lama menghasilkan poliklonal yang diaktifkan oleh lipopolisakharida in vitro, hal ini menunjukkan bahwa limfosit tersebut dihambat fungsinya.

Pengaruh deksametason, dan siklofosfamid pada populasi limfosit B telah diteliti lebih terperinci dalam beberapa penelitian yang dilaporkan dalam Bab.5 dan Bab.6.

Dalam Bab.5 diuraikan pengaruh deksametason pada limfosit B in vivo dan in vitro. Pada penyuntikan deksametason 50 mg/kg berat badan, tiap hari selama 7 hari, dijumpai satu hari sesudah penyuntikan dihentikan, penurunan jumlah sel dalam limpa lebih dari 90 persen, sedangkan jumlah semua sel yang berinti dalam sumsum tulang kurang dipengaruhi. Jumlah limfosit B menurun kurang lebih 50 persen dari prosentase normal dalam limpa dan sumsum tulang. In vitro, sel-sel yang menghasilkan imunoglobulin dengan perangsang lipopolisakharida ternyata menurun. Data ini menyokong bahwa penyuntikan deksametason beberapa kali dapat menurunkan jumlah dan prosentase limfosit B dalam sistem limfoid, tetapi tidak mempengaruhi fungsi limfosit B yang tertinggal yang bereaksi dengan lipopolisakharida. Hal ini telah dikuatkan pada nilai clone pada penelitian in vitro dengan menggunakan cara pengenceran terbatas.

Pengaruh kontras deksametason pada limfosit B dalam limpa dan sumsum tulang juga telah ditemukan apabila sel-sel tersebut diberi obat langsung ke dalam in vitro, yaitu deksametason 10^{-8}M in vitro menurunkan lebih dari 80 persen limfosit B

limpa yang dirangsang lipopolisakharida, sedangkan penurunan yang sama limfosit B sumsum tulang memerlukan konsentrasi - 1000 kali lipat lebih tinggi.

Dalam Bab.6 dilaporkan penelitian yang terperinci menge nai pengaruh siklofosfamid pada imunoglobulin yang beredar de ngan latar belakang sel-sel yang menghasilkan imunoglobulin - dan limfosit B.

Penyuntikan tunggal siklofosfamid 300 mg/kg berat badan, menurunkan jumlah sel yang menghasilkan IgM, IgG dan IgA da lam limpa, sumsum tulang dan kelenjar limfa mesenterium sam pai 25 persen nilai normal pada hari ke 7 sesudah penyuntikan dihentikan. Pengaruh siklofosfamid pada limfosit B yang mempu nyai imunoglobulin permukaan ialah terjadinya penurunan perla han-lahan. Fungsi limfosit B dirusak jelas satu hari setelah penyuntikan tunggal 300 mg/kg berat badan. Hal ini dijumpai - pada limfosit yang dirangsang lipopolisakharida, yang banyak dalam bentuk baru, baik pada limfosit B dengan umur pendek - maupun pada sel memori limfosit B yang berumur panjang.

Penurunan jumlah sel yang mengeluarkan imunoglobulin se sudah penyuntikan tunggal siklofosfamid 300 mg/kg berat badan diikuti oleh suatu perbaikan yang perlahan-lahan dengan suatu puncak peninggian ("overshoot") yang nyata setelah 40 hari pe nyuntikan siklofosfamid dihentikan. Sesudah penyuntikan siklo fosfamid 100 mg/kg berat badan berulang-ulang, maka nilai mi nimal sel-sel yang menghasilkan imunoglobulin pada bermacam -macam organ limfoid ternyata lebih rendah daripada penyunti kan tunggal siklofosfamid 300 mg/kg berat badan, tetapi dalam hal ini perbaikan tidak berhubungan dengan keadaan yang sa - ngat meninggi tadi ("overshoot").

Yang menarik perhatian ialah nilai imunoglobulin dalam serum sedikit berkurang dibandingkan dengan jumlah sel yang - membentuk imunoglobulin pada berbagai macam organ limfoid.Hal ini menyokong bahwa lebih panjang "half-life" imunoglobulin yang beredar pada tikus yang disuntik siklofosfamid.

Pada penelitian ini telah di
sebagai berikut :

1. Penurunan sel-sel yang m
   pemberian dosis tunggal
   badan) diikuti oleh suat
   ngan suatu reaksi "overs
   berian siklofosfamid ini

2. Persentase sel limfosit
   dan sumsum tulang tidak
   dosis tunggal deksametas
   dangkan sel-sel tersebut
   lah pemberian dosis tung
   rat badan.

3. Pengaruh pemberian korti
   fosfat pada sel-sel yang
   diteliti pada tikus tan

CONCLUTIONS

1. A single injection of dexamethasone sodium phosphat (16 to 144 mg /kg body weight) as well as a multiple injections (1 to 16 mg /kg body weight) appeared to cause a substantial decrease of the numbers of IgM, IgG and IgA secreting cells in spleen, mesenteric lymph nodes within one day, but hardly affected their number in the bone marrow.

2. A single injection of dexamethasone, even a massive dose as 144 mg /kg body weight, affected the serum IgM, IgG and IgA-levels less severely than 7 daily injections of e.q. 1 mg dexamethasone /kg body weight.

3. It was found that 10^{-8}M. dexamethasone in vitro reduce the response of splenic B cells to lipopolysaccharides by more than 80 per cent, while the a similar reduction of the response by bone marrow B cells required a 1000-fold higher concentration.

4. The percentage of B cells among the remaining spleen and bone marrow cells was not affected by a single injection of dexamethasone (144 mg /kg body weight) while that cells was completely abolished one day after a single injection of 300 mg /kg body weight of cyclophosphamide.

5. A single injection of 300 mg cyclophosphamide /kg body weight, decreased the numbers of background IgM, IgG and IgA secreting cells in spleen, mesenteric lymph nodes and bone marrow to minimum values about 25 per cent of normal at day 7 after treatment.

6. The incidence of surface Ig^+B cells also gradually decrease after cyclophosphamide treatment.

7. Remarkably, the serum Ig-levels were much less decreased than the numbers of Ig secreting cells in the various lymphoid organs, suggesting that the half life of the circulating Ig. in cyclophosphamide treated mice is lengthened.

1. Dengan pemberian d
tunggal (16 sampai
multipel (1 sampai
runan sel-sel yang
limpa, kelenjar lir
tapi hal ini hampi

2. Pembentukan IgM, Ig
pada pemberian mult
kg berat badan) da
dosis tinggi (144 m

3. Pemberian deksamets
menurunkan lebih ds
rangsang LPS, sedar
sumsum tulang memer
lebih tinggi.

4. Prosentase limfosit
gal dalam limpa dan
ruhi dengan penyunt
berat badan, tetapi
berian siklofosfami
rat badan dalam sat
kan.

5. Penyuntikan dosis t
badan) menurunkan
IgG dan IgA dalam
sumsum tulang 25 pe
7 sesudah penyuntik

6. Pengaruh pemberian
mempunyai imunoglo
runan yang perlaha
hentikan.

7. Nilai imunoglobuli
banding dengan jum
lin pada macam-mac
bahwa "half life"
tikus yang disunti

ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
AFU	Arbitrary fluorescence units
Ag	Antigen
AMP	2-amino-2-methyl-1-propanol
APC	Antigen presenting cells
ATS	Anti-thymocyte serum
BALT	Bronchus-associated lymphoid tissue
BCDF	B cell differentiation factor
BCG	Bacillus Calmette-Guérin
BCGF	B cell growth factor
5-BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
BSS	Balanced salt solution
BTV	Blue-tongue virus
BW	Body weight
C3	Complement factor 3
CFA	Complete Freund adjuvant
C-gene	Gene coding for the constant part of the Ig molecule
C-Ig cells	Cytoplasmic immunoglobulin containing cells
Con A	Concanavalin A
CTL	Cytotoxic T lymphocytes
CY	Cyclophosphamide
DEXA	Dexamethasone sodium phosphate
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DPBS	Dulbecco's phosphate-buffered saline
DTH	Delayed type hypersensitivity
EBV	Epstein-Barr virus
EIA	Enzyme immuno assay
ELISA	Enzyme-linked immunosorbent assay
Fc	Crystallizable fragment of immunoglobulin molecules
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
GAT	Synthetic polypeptide of L-glutamine, L-alanine and L-tyrosine
GT	Synthetic polypeptide of L-glutamine and L-tyrosine
GvH	Graft-versus-Host
H chain	Heavy chain of Ig molecule
H-2	Mouse histocompatibility-2 complex
HA	Hemagglutinin
HEV	High endothelial venules
HLA complex	Human leukocyte antigen complex
4-HPCY	4-hydroperoxycyclophosphamide
hr	Hour
IEP	Immunoelectrophoresis
Ig	Immunoglobulin
IL-1	Interleukin 1
IL-2	Interleukin 2
ip	Intraperitoneal
IUdR	5-iodo-2-deoxyuridine
iv	Intravenous
KLH	Keyhole limpet hemocyanin

LPS	Lipopolysaccharide
M	Molar
MAF	Macrophage activating factor
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibition factor
min	Minute
MLC	Mixed lymphocyte culture
MLN	Mesenteric lymph nodes
mol.wt.	Molecular weight
NIP	4-hydroxy-5-iodo-3-nitrophenyl
NNP	4-hydroxy-3,5-dinitrophenyl
PALS	Periarteriolar lymphatic sheath
PBA	Polyclonal B cell activator
PBS	Phosphate-buffered saline
PBS-gel	PBS containing 0.02% gelatin
PBS-Tw	PBS containing 0.05% Tween-20
PFC	Plaque-forming cell
PHA	Phytohemagglutinin
PM	Post meridiem
PPD	Purified protein derivative of tubercle bacilli
PWM	Pokeweed mitogen
sc	Subcutaneous
sec	Second
SPDP	Succinimidyl-3-(2-pyridyldithiopropionate)
SRBC	Sheep red blood cells
SRID	Single radial immunodiffusion
Sta	*Staphylococcus aureus* Cowan I
RIA	Radio immuno assay
RNA	Ribonucleic acid
TD	Thymus-dependent
(TG)AL	Synthetic polypeptide of L-tyrosine, L-glutamine, L-analine and L-lysine
TI	Thymus-independent
TNBS	Trinitrophenyl benzene sulphonic acid
TNP	Trinitrophenyl
TRF	T cell replacing factor
TRITC	Tetramethylrhodamine isothiocyanate
V-gene	Gene coding for the variable part of the Ig molecule
Vol	Volume

CURRICULUM VITAE

Sabbele, N. Rewa was born on 20 July 1939 in Jeneponto, Indonesia. He graduated from high school in 1962 and later continued his study at the Medical Faculty, Hasanuddin University, Ujung Pandang from which he graduated as physician in 1976.

From September 1, 1959 he was employed by the school of Medicine, Hasanuddin University, Ujung Pandang and was engaged in the Department of Histology.

In Februari 1, 1966 he became assistant and worked at the Department of Histology, Faculty of Medicine, Hasanuddin University, Ujung Pandang, Indonesia under the guidance of dr. Tan Hian Tjo.

In 1971 he became lecturer in the Histology Department Medical Faculty of the same University.

From 1974 until 1975 as long as one year he became a trainee of the Consortium Medical Sciences about Medical Biology at the Department of Biology, School of Medicine, University of Indonesia, Jakarta under the guidance of Professor dr. M.K. Tajudin. Also he got a special course of immunology under the guidance of dr. Arjatmo Tjokronegoro Ph.D.

In 1978 he became acting head of the Department of Histology, School of Medicine, Hasanuddin University, Ujung Pandang. From January 1, 1980 until December 31, 1980 and from January 1, 1981 until October 31, 1981 he was appointed head of the Histology Department, Medical Faculty, Hasanuddin University, Ujung Pandang.

From November 1, 1981 until December 31, 1983 he studied immunology in the Department of Cell Biology, Immunology and Genetics, Medical Faculty, Erasmus University, Rotterdam The Netherlands under the leadership of Professor Dr. R. Benner.

During his study he did the investigation and wrote the immunology dissertation. From October 2, 1986 until February 15, 1987 he became once more a trainee in immunology at the Department of Cell Biology, Immunology and Genetics, Faculty of Medicine, Erasmus University, Rotterdam, The Netherlands, for the completion of his dissertation again under the Leadership of Professor Dr. R. Benner.

He was the best lecturer of the year in 1985 at Medical Faculty, Hasanuddin University, Ujung Pandang.

RIWAYAT HIDUP

Sabbele N.Rewa dilahirkan pada tanggal 20 Juli 1939 - di Jeneponto, Indonesia. Telah lulus Sekolah Menengah Atas bagian B. pada tahun 1962, dan kemudian melanjutkan sekolah nya ke Fakultas Kedokteran Universitas Hasanuddin Ujung Pan dang dan pada tahun 1976 tammat sebagai dokter umum.

Sejak tanggal 1 September 1959 menjadi pegawai negeri pada Fakultas Kedokteran Universitas Hasanuddin Ujung Pandang dan bertugas pada Bagian Histologi.

Mulai pada tanggal 1 Pebruari 1966 menjadi Asisten - Perguruan Tinggi dan bertugas pada bagian Histologi Fakultas Kedokteran Universitas Hasanuddin Ujung Pandang Indonesia di bawah pimpinan dr. Tan Hian Tjo.

Pada tahun 1971 menjadi dosen tetap pada bagian Histo logi Fakultas Kedokteran Universitas yang sama.

Dari tahun 1974 sampai 1975 selama 1(satu) tahun me - ngikuti pendidikan Consortium Medical Sciences pada bagian - Biologi Fakultas Kedokteran Universitas Indonesia Jakarta - dalam bidang Biologi Kedokteran dibawah pimpinan Professor - dr. M.K.Tajudin. Selama pendidikan ini memperoleh pula bimbi ngan khusus tentang imunologi dari dr. Arjatmo Tjokronegoro Ph.D.

Pada tahun 1978 menjadi Pejabat Ketua bagian Histologi Fakultas Kedokteran Universitas Hasanuddin Ujung Pandang.

Dari tanggal 1 Januari 1980 sampai dengan 31 Desember 1980 dan dari tanggal 1 Januari 1981 sampai 31 Oktober 1981 diangkat menjadi Ketua Bagian Histologi Fakultas Kedokteran Universitas Hasanuddin Ujung Pandang.

Mulai pada tanggal 1 November 1981 sampai dengan 31 - Desember 1983 mengikuti pendidikan imunologi pada Afdeling - Celbiologie, Immunologie en Genetica Faculteit der Geneeskun de Erasmus Universiteit Rotterdam Nederland dibawah pimpinan Professor Dr. R.Benner. Selama pendidikan ini berlangsung, penelitian dilakukan sambil menulis tesis dalam bidang imuno logi.

Dari tanggal 2 Oktober 1986 sampai dengan 15 Pebruari 1987 melanjutkan lagi pendidikan pada Afdeling Celbiologie, Immunologie en Genetica Faculteit der Geneeskunde Erasmus - Universiteit Rotterdam Nederland dalam bidang imunologi - dengan tugas perampungan tesis dibawah pimpinan Professor - Dr. R.Benner.

Terpilih sebagai dosen teladan Fakultas Kedokteran - Universitas Hasanuddin Ujung Pandang pada tahun 1985.

CONCLUTIONS

1. A single injection of dexamethasone sodium phosphat (16 to 144 mg /kg body weight) as well as a multiple injections (1 to 16 mg /kg body weight) appeared to cause a substantial decrease of the numbers of IgM, IgG and IgA secreting cells in spleen, mesenteric lymph nodes within one day, but hardly affected their number in the bone marrow.

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KESIMPULAN

1. Dengan pemberian deksametason sodium fosfat baik dosis tunggal (16 sampai 144 mg/kg berat badan) maupun dosis multipel (1 sampai 16 mg/kg berat badan) terjadi penurunan sel-sel yang membentuk IgM, IgG dan IgA dalam limpa, kelenjar limfa mesenterium dalam satu hari, tetapi hal ini hampir tidak dijumpai dalam sumsum tulang.

2. Pembentukan IgM, IgG dan IgA dalam serum lebih rendah pada pemberian multipel deksametason dosis rendah (1 mg/kg berat badan) dari pada pemberian tunggal deksametason dosis tinggi (144 mg/kg berat badan).

3. Pemberian deksametason langsung kedalam in vitro 10^{-8}M. menurunkan lebih dari 80 per cent limfosit B yang dirangsang LPS, sedangkan penurunan yang sama limfosit B sumsum tulang memerlukan konsentrasi 1000 kali lipat lebih tinggi.

4. Prosentase limfosit B diantara kelompok sel yang tertinggal dalam limpa dan sumsum tulang ternyata tidak dipengaruhi dengan penyuntikan tunggal deksametason 144 mg/kg berat badan, tetapi sel-sel tersebut dirusak dengan pemberian siklofosfamid tunggal dengan dosis 300 mg/kg berat badan dalam satu hari setelah pemberian obat dihentikan.

5. Penyuntikan dosis tunggal siklofosfamid (300 mg/kg berat badan) menurunkan jumlah sel-sel yang menghasilkan IgM, IgG dan IgA dalam limpa, kelenjar limfa mesenterium dan sumsum tulang 25 per cent dari nilai normal pada hari ke 7 sesudah penyuntikan dihentikan.

6. Pengaruh pemberian siklofosfamid pada limfosit B yang mempunyai imunoglobulin permukaan ialah terjadinya penurunan yang perlahan-lahan setelah pemberian obat ini dihentikan.

7. Nilai imunoglobulin dalam serum sedikit berkurangnya dibanding dengan jumlah sel-sel yang membentuk imunoglobulin pada macam-macam organ limfoid, hal ini menyokong bahwa "half life" dari imunoglobulin yang beredar pada tikus yang disuntik siklofosfamid adalah lebih panjang.