THE MECHANISM OF ANTIBODY FORMATION IN MOUSE BONE MARROW

PROEFSCHRIFT

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GUUS KOCH

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Promotoren:	Prof. Dr. R. Benner Prof. Dr. O. Vos
Co-referenten:	Prof. Dr. R. E. P. A. Ballieux Prof. Dr. W. Hijmans

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Aan Adrianne

Aan mijn ouders

CONTENTS

BA	*Brucella abortus* bacteria
BALT	Bronchus-associated lymphoid tissue
B cells	Bone marrow-derived lymphocytes
BM	Bone marrow
C-ig cells	Mature cytoplasmic immunoglobulin-containing cells
Con A	Concanavalin A
CR	Receptor for the third component of the complement system
CTL	Cytotoxic T lymphocytes
CTL-P	Cytotoxic T lymphocyte precursors
C-μ cells	Lymphoid cells which contain μ heavy chains in the cytoplasma, but do not express Ig on the cell surface
dLPS	Detoxified lipopolysaccharide
DNP	Dinitrophenyl
Fc	Crystallizable fragment of immunoglobulin molecules
FcR	Receptor for the Fc part of immunoglobulin molecules
GALT	Gut-associated lymphoid tissue
GvH	Graft-versus-Host
HSC	Pluripotent haemopoietic stem cell
Ig	Immunoglobulin
Igh	Gene loci coding the heavy chain of immunoglobulin molecules
KLH	Keyhole limpet haemocyanin
LPS	Lipopolysaccharide
MLR	Mixed lymphocyte reaction
NK cell	Natural killer cell
PFC	Antibody-producing plaque-forming cell
PHA	Phytohaemglutinin
Pn	*Pneumococcus pneumoniae* bacteria
Pre-T cell	Pre-thymic T lymphocyte
PTL-P	Proliferating T lymphocyte precursor
s-IgD cells	Lymphocytes expressing IgD molecules on the cell surface
s-IgM cells	Lymphocytes expressing IgM molecules on the cell surface
SRBC	Sheep red blood cells
T cell	Thymus-derived lymphocyte
TD	Thymus-dependent
TI	Thymus-independent
TNP	Trinitrophenyl

ability to discriminate between self and non-self antigens. The antigen recognition on B cells is achieved by Ig molecules, receptors, present on the cell surface of mature B cells. After antigenic stimulation, B cells can differentiate into plasma cells. Plasma cells secrete Ig with specificity identical to the specificity of the antigen receptor of their B cell progenitor. 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 hypersensivity 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.

Another important feature of the immune system, besides the specificity of its responses, is the capacity of carrying memory. After primary antigenic stimulation effector cells are induced, which mediate the immune functions. These effector cells are mostly short-lived, end-stage cells. Among the progeny of activated lymphocytes are also cells which are long-lived, and which retain the capacity of being (re)stimulated by the original antigen. They cause the much faster and more vigorous immune response which is induced after the second contact with the same antigen. The memory lymphocytes largely determine the effectiveness of vaccination procedures against infectious agents, i.e., the protection against infectious diseases obtained by vaccination is mainly dependent upon the quality and quantity of the memory lymphocytes.

B and T cells exercise their effector function mainly within the spleen, lymph nodes, 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, and to present them to the lymphocytes. The peripheral lymphoid organs are provided with both accessory cells and the appropriate tissue architecture needed to fulfill these tasks. After contact with the antigen, thus processed, T and B lymphocytes differentiate and proliferate into antigen-specific effector T cells and antibody-forming cells, respectively.

After primary immunization the antibody-forming cells are mainly localized in the peripheral lymphoid organs. However, after the second contact of lymphocytes with the antigen the localization of antibody-forming cells is not confined to the peripheral lymphoid organs, but they also occur in the BM (Benner et al., 1974a). In fact, after the first week of secondary type responses the BM is the major site of localization of antibody-forming cells. The purpose of the studies described in this thesis has been to elucidate the mechanism underlying antibody formation in the BM.

In the next chapter some characteristics of the immune system which are important for a better understanding of the subsequent chapters will be summarized. In the outline the mouse is in the limelight because the experiments described in Chapter 4 and the Appendix papers are almost exclusively performed with mice. Chapter 3 reviews the literature about the occurrence of immunological effector cells within the BM.

2. *STRUCTURE OF THE IMMUNE SYSTEM*

2.1. Introduction

The immune system consists of different lymphoid tissues dispersed throughout the body. These tissues do not stand isolated, but are functionally interrelated and physically united by the blood and lymphatic vessels into one functional system. Lymphocytes populating the lymphoid system are able to move from one organ to the other through the blood and lymphatic vessels. Two sorts of lymphoid organs can be distinguished according to their main function (Miller and Davies, 1964): the primary or central lymphoid organs which are the breeding sites of lymphoid cells, and the secondary or peripheral lymphoid organs, which are mainly engaged in the induction of the immune response. This subdivision of the lymphoid system will be used to review briefly some features which are important for a better understanding of the experiments described in Chapter 4 and the Appendix papers.

2.2. Central or primary lymphoid organs

The central lymphoid organs include the BM, which is present in amphibia and all higher vertebrates, the thymus, which is present in almost all vertebrates with the exception of cyclostomi, and the bursa of Fabricius, which is found in birds only. Besides their difference in function, central and peripheral lymphoid organs also have a different microarchitectural structure. The thymus and bursa of Fabricius are lympho-epithelial organs. In thymus and bursa the epithelial cells are predominantly derived from endoderm. The mesenchymal component of both organs originates from different embryonic tissues: the mesenchyme of the thymus arises from ectomesenchyme, i.e. ectoderm, while the mesenchyme of the bursa is derived from mesoderm (Douarin, 1977). The lymphoid cells present in both organs are derived from blood-borne stem cells. Neither thymic nor bursal epithelium and mesenchyme can give rise to lymphoid cells. The microenvironment formed by epithelial cells is supposed to support the extensive proliferation and differentiation of lymphocytes which takes place within these organs.

2.2.1. Bone marrow

In adults the BM is the major localization of the pluripotent haemopoietic stem cells (HSC). The HSC have the capacity of self-renewal. Moreover, they are able to generate blood cells of the erythroid, myeloid, megakaryoid, and lymphoid series. The BM is the major site of generation of immunocompetent virgin B lymphocytes (Phillips et al., 1977). Large lymphoid cells, which contain cytoplasmic μ chains ($c\text{-}\mu^+$), putatively form the first detectable stage of B cell differentiation in mice (Raff et al., 1976; Owen, 1979; Levitt and Cooper, 1980; Osmond et al., 1981). These $c\text{-}\mu^+$ cells do not show expression of IgM on the cell surface. Many of the $c\text{-}\mu^+$ cells are cycling cells (Owen et al., 1977a, 1977b; Cooper and Lawton, 1979; Rusthoven and Phillips, 1980). They give rise to small $c\text{-}\mu^+$ lymphocytes. These small $c\text{-}\mu^+$ lymphocytes subsequently start to express IgM molecules on the cell surface (s-IgM)

without apparent further cell proliferation (Melchers and Andersson, 1973). Along with an increasing density of s-IgM, Ia antigen and a receptor for the Fc portion of the IgG molecule (FcR) appear on the surface of the immature B lymphocytes (Osmond and Rahal, 1978; Chan and Osmond, 1979). Only after a lag period, a receptor for the C3 component of the complement system (CR) and IgD molecules appear on the cell surface (s-IgD) (Yang et al., 1978; Chan and Osmond, 1979). Within the BM mature B cells with low density s-IgD (Lala et al., 1979) occur, whereas lymphocytes with a high density of s-IgD are present in the spleen and the lymph nodes. There is evidence that during receptor maturation, B cells migrate from the BM via the bloodstream to the red pulp and subsequently to the white pulp of the spleen (Osmond, 1975; Yoshida and Osmond, 1978). Especially, the follicles in the spleen form the sites of B cell accumulation (Martin and Leslie, 1977). The maturation of B lymphocytes is thought to be completed during the migration of B cells into the splenic white pulp (Yoshida and Osmond, 1978; Osmond, 1980). It should be noted that the ontogeny of B lymphocytes as described above, is mainly based upon evidence obtained from experiments on heterogeneous cell population. Therefore, the existence of other differentiation pathways of B lymphocytes cannot be definitely excluded. However, a similar differentiation pathway for B lymphocytes is obtained by analysis of surface markers on leukaemic cells of patients with different subtypes of acute lymphoblastic leukaemia (ALL). For this purpose one has to assume that, in a given patient, ALL results from proliferation of a single cell arrested at a certain stage of differentiation, and that the surface markers of the leukaemic cells are identical to the ones present on that single cell (Greaves and Janossy, 1978; reviewed by Foon et al., 1980).

The regulation of the lymphocyte production in the BM is essentially unknown. Thymic humoral factors or T lymphocytes do not influence the lymphocyte production, since the same rapid turnover of lymphocytes is found in the BM of neonatally thymectomized mice, congenitally athymic nude mice, and thymus-bearing control mice (Osmond and Nossal, 1974; Osmond et al., 1981). In mice which are repeatedly injected with anti-IgM serum from birth, s-IgM$^+$ lymphocytes fail to develop. Moreover, in mice thus treated, circulating IgM is absent and the serum levels of other Ig except of IgG1 depressed (Lawton and Cooper, 1974), which also indicates that the B lymphocyte population is highly deficient in these mice (Cooper and Lawton, 1979; Osmond and Gordon, 1979). However, a normal production rate of small lymphocytes is found in the marrow of the anti-IgM suppressed mice (Osmond et al., 1981). Thus, the marrow small lymphocyte production rate seems to be independent of feedback control by the mature B lymphocytes and their products. In mice reared under sterile conditions, the incidence of small lymphocytes and s-IgM$^+$ lymphocytes in the BM is normal (Osmond and Nossal, 1974). In contrast, the production rate is markedly reduced as compared to that in conventionally reared controls. Exogenous antigenic stimuli can temporally enhance the marrow lymphocyte production in normal mice as well as in T cell-deprived mice and anti-IgM suppressed mice. Moreover, a similar increase in marrow lymphocyte genesis is induced by nonimmunogenic irritants (e.g., mineral oil) (Osmond et al., 1981). All these findings suggest that the actual marrow lymphocytopoiesis is the consequence of two regulating factors: (a) the regulation by local (micro-

environmental?) factors, which cause a 'basal level' lymphocytopoiesis, and (b) the regulation by exogenous stimuli which can amplify the production rate.

By criteria of c-μ^+ and s-IgM, 80% of the small lymphocytes in the BM of young adult mice are of the B cell lineage. Most of these cells are recently generated from large lymphoid cells termed transitional cells (reviewed by Rosse, 1976). Approximately 12% of the marrow small lymphocytes have neither B nor T cell markers and are therefore frequently indicated as 'null' cells. Probably these 'null' cells form a heterogeneous population consisting of functionally unrelated cells. Some of the 'null' cells may represent precursor cells committed to the T cell lineage.

The BM of adult mammals contains a population of cells capable of maturing to T cells in the thymus (Ford et al., 1966). These pre-thymic (pre-T) cells are characterized by their capability of repopulating the thymus of an irradiated host (Kadish and Basch, 1976; Basch and Kadish, 1977), of expressing T cell markers (Bach, 1971; Komura and Boyse, 1973), and of expressing the enzyme terminal deoxynucleotidyl transferase (Pazimo et al., 1978). Furthermore, pre-T cells are able to differentiate into functional T cells (Gorzynsky and MacRae, 1979a, 1979b) after *in vitro* exposure to thymus derived humoral factors, thymic extracts, or mitogens, i.e., phytohaemagglutinin (PHA) and Concanavalin A (Con A) (Cohen et al., 1975; Press et al., 1977). The pre-T cells can be separated from both the HSC and mature T cells in the BM on the basis of the average cell density (El-Arini and Osaba, 1973; Basch and Kadish, 1977). These latter results, however, have been challenged by Boersma et al. (1981), who were unable to segregate pre-T cells, HSC, and mature T cells according to buoyant density, velocity sedimentation, and cell surface charge. The pre-T cells, which are the responsive cells in the above mentioned assays, are generated independent of the presence of the thymus, because they occur in BM and spleen of neonatally thymectomized (Röpke, 1977a, 1977b) and congenitally athymic mice (Scheid, 1975; Roelants et al., 1976; Röpke, 1977b). The PHA responsive cells, also belonging to the pre-T cell pool, are mainly rapidly renewing cells (Press and Rosse, 1978).

The fate of most of the rapidly renewing pre-T cells produced in the BM is not known. Only a few of these pre-T cells have been shown to migrate to the thymus according to local marrow labelling experiments (Brahim and Osmond, 1970; Parrott and de Sousa, 1971; Basch and Kadish, 1977; Yoshida and Osmond, 1978). Migration of BM cells to the thymus has been demonstrated using chromosome markers (Ford et al., 1966) and recently by an *in vivo* homing assay of fluorescence labelled BM cells (Lepault and Weissman, 1981). The BM cells express the Thy-1 antigen in the thymus within 3 hours after reconstitution of irradiated mice. This finding correlates well with the Thy-1 antigen expression induced by mitogen *in vitro* (Cohen and Patterson, 1975).

2.2.2. Thymus

The thymus is crucial to the development of functional T lymphocytes (Miller and Osoba, 1967). Within the thymus, immature immuno-incompetent pre-T cells, which seed in from the BM, proliferate and differentiate into mature immunocompetent T lymphocytes. These T lymphocytes eventually emigrate to the peripheral lymphoid organs (reviewed

by Cantor and Weissman, 1976; Stutman, 1978).

From a histological point of view the thymus can roughly be divided into the cortex and the medulla. In most staining procedures the cortex is stained more intensively than the medulla, due to the higher concentration of lymphocytes in the cortex. The lymphocytes in the thymus are held together by a sessile matrix of specialized epithelium and connective tissue (Clark, 1973). In the cortex extensive proliferation takes place particularly in the more superficial layers. Labelling experiments show that DNA synthesis in the thymus is mainly confined to the medium and large lymphocytes. These cells are predominantly localized in the outer cortex (Metcalf, 1966; Weissman, 1973). Small thymocytes are not labelled. Nevertheless, labelled small thymocytes are found in the medulla a few hours after selective labelling of the outer cortex (Weissman, 1967). Intrathymic migration of lymphocytes from the cortex to the medulla can explain the occurrence of labelled small thymocytes in the medulla. Existence of such a migrational pathway has been shown more directly by *in situ* local labelling of the superficial cortex with fluorescein (Scollay et al., 1980).

Thymocytes mature during the intrathymic migration, as is apparent from the expression of cell surface markers (e.g., Thy-1, TL, and Lyt antigens) some of which are only transient (e.g., the TL antigen). Also the densities of the surface antigens change (e.g., the density of Thy-1 decreases, while the density of Lyt-1 and H-2 antigens increases).

The anatomical localization of thymocytes closely correlates with their sensitivity to the cytolytic activity of corticosteroids. The cortical thymocytes lyse after *in vivo* administration of high doses of cortisone, while medullar thymocytes are much less affected (Dougherty, 1952; Ishidate and Metcalf, 1963). Functional studies have shown that the medullary, cortisone-resistant thymocytes are mainly immunocompetent cells, endowed with, among other things, mitogen reactivity, mixed lymphocyte reactivity, Graft-versus-Host (GvH) reactivity, and T helper activity (reviewed by Cantor and Weissman, 1976).

The thymus produces more lymphocytes than eventually leave this organ to repopulate the T-cell compartments within the peripheral lymphoid organs (Metcalf, 1966; Shortman, 1977; Cantor and Weissman, 1976; Scollay et al., 1980). Large numbers of recently formed thymocytes die within the thymus cortex (Joel et al., 1977; Shortman, 1977). It is an attractive hypothesis to suppose that these dying cells belong to 'forbidden' self-reactive T cell clones (von Boehmer and Byrd, 1972; Gorczynsky and MacRae, 1979a) or to T cell clones restricted for inappropriate histocompatibility antigens (Zinkernagel and Doherty, 1978; Jenkins et al., 1981). Probably, the thymus epithelium plays a major role not only in the selection of the appropriate T cells but also in the proliferation and differentiation of pre-T cells, in the sense that intimate contact is needed between pre-T cells and the epithelium. Humoral factors, produced by the epithelial cells, are involved in this interaction (reviewed by Bach and Carnaud, 1976; Goldstein et al., 1975; Trainin et al., 1977). Most of these factors act within a short range only. The existence of thymic humoral factors which are also active at longer distances and, thus, have hormonal properties, has been hypothesized. These latter factors might play a role in the maturation of the immature post-thymic T cells in the periphery (Cantor and

Weissman, 1976; Kruisbeek, 1978; Stutman, 1978).

2.2.3. Bursa of Fabricius

The bursa of Fabricius, which is present in birds only, arises as an evagination of the dorsal wall of the cloaca. This organ is populated with haemopoietic stem cells early in embyronic life. Chickens bursectomized in embryonic life, e.g., at 17 days of incubation, have reduced levels of surface Ig-bearing small lymphocytes, of plasma cells, and of serum Ig (Cooper et al., 1972). Thus, the bursa is involved in the generation of B lymphocytes in birds (reviewed by Schaffner et al., 1974, and by Glick, 1978).

2.3. Peripheral or secondary lymphoid organs

As stated above, virgin B and T lymphocytes generated within the bone marrow and thymus, respectively, migrate to the peripheral lymphoid organs. Within these organs the antigen-driven differentiation of lymphocytes takes place. The most important peripheral lymphoid organs are the spleen, the lymph nodes, the gut-associated lymphoid tissue (GALT) and the bronchus-associated lymphoid tissue (BALT). These tissues provide the architecture and accessory cells appropriate for the antigen processing and for the presentation of the antigen to the lymphocytes.

Within the peripheral lymphoid organs a B and a T cell compartment can be recognized (reviewed by Parrot and de Sousa, 1971). Virgin B cells settle predominantly in the lymphoid follicles (Rozing et al., 1978), 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 (Waksman, 1962; Gutman and Weissman, 1972; van Ewijk et al., 1977). These compartments have no rigid boundaries, and cells from either compartment are able to migrate to the other.

Upon primary antigen injection small B lymphocytes can be stimulated to transform into B cell blasts in the peripheral part of the PALS (van Ewijk et al., 1977). The B cell blasts proliferate and differentiate into antibody producing plasma cells (Nossal, 1962; Keuning et al., 1963). These plasma cells are the effector cells of the B cell lineage (Fagreus, 1948). During the differentiation of B cells into plasma cells the cells migrate to the red pulp of the spleen and to the medullary cords of the lymph nodes (Thorbecke and Keuning, 1956). Antigen specific T lymphocytes can greatly enhance the response of B cells (Claman et al., 1966; Miller and Mitchell, 1968; Mitchison, 1969).

The activation of T lymphocytes by antigen most likely occurs within the T cell areas. Blast transformation and proliferation of T cells precede the B cell blastogenesis (van Ewijk et al., 1977).

As a consequence of antigenic stimulation, a focus of blast cells, dividing cells, as well as active macrophages form a follicle centre or germinal centre. The germinal centre formation compresses the follicular dendritic cells to a crescent around this centre (Miller, 1964).

It is difficult to reconcile the segregation of B and T cells within the lymphoid organs with the need of T-B cell interaction, which is necessary for a T cell-dependent antibody response. The question where the T-B cell interaction is precisely localized has not completely been

frequency analyses in an *in vitro* limiting dilution assay. A major increase in the CTL-P frequency can also explain why *in vitro* cytotoxic responsiveness to ectromelia virus-infected cells can only be induced by primed spleen cells, but not by spleen cells of uninfected mice (Gardner and Blanden, 1976). An increase of antigen-specific CTL-P has also been observed in mice, that had rejected tumours induced by murine sarcoma-leukaemia virus (MSV-MLV). These tumours regress spontaneously *in vivo* (reviewed by Levy and Leclerc, 1977). Following rejection of the tumour, the frequency of CTL-P to syngeneic MLV-induced lymphoma cells is increased 10- to 20-fold as compared to normal mice (MacDonald et al., 1980).

In contrast to priming in a MLR, the increase of the frequency of alloantigen-reactive CTL-P observed in the spleen of allo-immunized mice is relatively small, i.e., 3- to 4-fold. Moreover, the frequency of proliferating T lymphocyte precursors (PTL-P) is not increased after *in vivo* alloimmunization, in contrast to priming in MLR. One possible explanation for these findings is that primary immunization with alloantigens *in vivo* results in a selective increase of CTL-P among the alloreactive PTL-P. However, qualitative differences between normal and alloimmune CTL-P can also explain these results. For example, alloimmune spleen CTL-P may have a lower proliferating capacity than CTL-P in normal spleen, or alternatively, alloimmune CTL-P may give rise to CTL with higher cytolytic efficiency. In accordance with such a hypothesis, some results obtained in MLR mass cultures, established with normal and alloimmune responding spleen cells, reveal differences between 'virgin' and 'memory' CTL in their antigen requirements and the temperature dependency of their cytolytic activities (Cerottini and Brunner, 1977).

It should be emphasized here that there is, at present, no methodology to delineate qualitative differences between virgin and memory T lymphocytes. Possibly, such selective pressure can be reflected by, for instance, changes in the T cell products or alternatively, an increase of the affinity of the antigen receptors on T lymphocytes.

2.5. Lymphocyte recirculation

Whereas the spleen is mainly drained by blood vessels, lymph nodes are intimately connected with the lymphatic system. This system consists of thin walled vessels, which originate in the interstitial spaces of the tissues, and thereby are able to drain these places. The lymphatic vessels of different parts of the body fuse and form larger vessels which eventually end up in the thoracic duct. The thoracic duct empties into the bloodstream.

Lymph nodes are also traversed by some small blood vessels. Within the lymph nodes lymphocytes are able to traverse the walls of the blood vessels (Gowans and Knight, 1964; Sedgley and Ford, 1976). This only occurs in the post-capillary part of these vessels at sites which are lined by high-endothelial cells, i.e., the so-called 'high-endothelial venules'. The high-endothelial venules possess recognition structures for lymphocytes (Woodruff et al., 1977; Butcher et al., 1979; Stevens et al., 1982). Both B and T cells are able to cross the walls of the high-endothelial venules after binding to these structures. Upon entry, B cells migrate to the outer cortex and to a lesser extent to the cortico-medullary junctions. T cells settle in the paracortical region,

where the high-endothelial venules are situated. Both B and T lymphocytes can leave the lymph nodes again via the efferent lymphatic vessels. Via these vessels and the thoracic duct they return to the bloodstream. 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 a distinctly slower rate (Howard and Scott, 1972; Sprent, 1973a; Ford, 1975). Most of the recirculating lymphocytes are long-lived cells, probably memory cells with specificity for antigens encountered in an earlier phase of life. This can be concluded from the percentage of labelled cells found in the thoracic duct lymph of animals subjected to a period of continuous administration of ^{3}H-thymidine (Howard and Scott, 1972; Sprent and Basten, 1973b). Only a minority of the virgin B lymphocytes recirculate (Strober, 1972; Strober et al., 1973a, 1973b). Antigenic stimulation extends the pool of specific recirculating B lymphocytes. Most likely, these memory B cells are produced in the germinal centres of the follicles of the various peripheral lymphoid organs (Wakefield and Thorbecke, 1968a, 1968b; Nieuwenhuis, 1971; Nieuwenhuis et al., 1974).

3. *THE BONE MARROW AS SITE OF IMMUNOLOGICAL EFFECTOR CELLS*

3.1. *Introduction*

In the preceding chapter a distinction was made between central and peripheral lymphoid organs. In the central lymphoid organs an antigen-independent proliferation and differentiation of lymphocytes takes place, whereas in the peripheral lymphoid organs the lymphocyte differentiation is an antigen-dependent process. This separation into compartments is an oversimplification since it has been shown that the production of B lymphocytes and precursors of T lymphocytes can also take place outside the BM, and in the absence of a functional BM microenvironment (Kincade et al., 1975; Rozing et al., 1976). On the other hand, effector cells can be found not only in peripheral lymphoid organs, but also within the central lymphoid organs. The purpose of this chapter is to give an overview of the lymphoid effector cells which can reside in the BM.

3.2. *Antibody- and immunoglobulin-producing cells within the bone marrow*

Only for the sake of clarity we will distinguish between antibody- and Ig-producing cells. It should, however, be noticed that this distinction is only arbitrary; it only consist in that the antigen specificity of the Ig molecules secreted by the Ig-producing cells is not known, whereas in the case of the antibody-forming cells it is.

Already at the turn of the century, at the time that the role of lymphocytes and plasma cells in antibody synthesis was not yet recognized, Pfeiffer and Marx (1898) reported the presence of antibodies within BM extracts of rabbits immunized with *Vibrio cholerae*. Almost simultaneously Deutsch (1899) showed the occurrence of anti-typhoid antibodies in the BM of rabbits and guinea pigs immunized with typhoid vaccine. In fact, these results do not prove that the antibodies were actually produced by cells within the BM. More suggestive evidence was obtained by Lüdke (1912), Reiter (1913), and, later, by deGara and Angevine (1943) and Thorbecke and Keuning (1953), who showed that BM cells of immunized animals released specific antibodies during *in vitro* cultivation. By adding radioactive amino acids to the culture medium, Askonas et al. (1956a) proved that the BM really produced specific antibodies upon *in vivo* immunization by *de novo* protein synthesis. Thereafter, a large number of papers appeared, which reported antibody formation to take place not only in the peripheral lymphoid organs but also in the BM. At present the evidence that the BM is a site of antibody formation can be summarized as follows:

(a) extracts of BM of rabbits and guinea pigs contain specific antibodies (Pfeiffer and Marx, 1898; Deutsch, 1899; deGara and Angevine, 1943; Thorbecke and Keuning, 1953; Thorbecke et al., 1962).
(b) BM cells cultured *in vitro* can produce antibodies (Carrel et al., 1912; Lüdke, 1912; Reiter, 1913; Thorbecke et al., 1953, 1956, 1961, 1962; Askonas et al., 1956a, 1958, 1965; Langevoort et al., 1963; McMillan et al., 1976) and Ig (Askonas et al., 1956a, 1956b, 1958, 1965; Langevoort et al., 1963; Thorbecke, 1954, 1960; Asofsky

and Thorbecke, 1961; Thorbecke et al., 1964; van Furth et al., 1966; McMillan et al., 1972, 1976; Vaughan et al., 1976; Kutteh et al., 1982).

(c) in rabbits (Kolouch et al., 1938, 1947; Bjørneboe and Gormsen, 1943; Fagreus, 1948) and in man (Gormsen and Heintzelmann, 1941; Good and Campbell, 1950; Good, 1955) a positive correlation exists between the number of plasma cells in the BM and the serum antibody level after intensive antigenic stimulation.

(d) the BM of immunized mole rats (Rice et al., 1972; Jankovic and Paunovic, 1973), rabbits (Landy et al., 1965; Donnelly and Sussdorf, 1975; Blijham, 1975), mice (Benner et al., 1974a), rats (Knothe et al., 1979), chickens (Keily and Abramoff, 1969; Weber, 1972; Janković et al., 1973) and adult frogs (Eipert et al., 1979) can contain large numbers of antibody-forming cells as determined with the plaque-assay. Furthermore, BM cells of rats immunized by an intradermal injection of xenogeneic cells produce antibodies directed to these latter cells. This is apparent from the activity of the immune BM cells in the antibody-dependent cell-mediated cytotoxicity assay (Harding and MacLennan, 1972).

Some reports are going as far as to suggest the BM as the major site of Ig-synthesis. This is especially apparent if the Ig-synthesis is calculated per whole organ (Askonas and White, 1956a; Askonas and Humphrey, 1958; McMillan et al., 1972, 1976; Haaijman et al., 1979; Benner et al., 1981b). Evidence for this suggestion has also been obtained in humans from the comparison of the ratio of the numbers of cytoplasmic Ig-containing cells (C-Ig cells) in the BM containing the various heavy and light chain isotypes, and the ratio of the serum levels of the same Ig isotypes. Both ratios are equal in the following combinations: IgM-IgG-IgA (Hijmans et al., 1971; Turesson, 1976), IgG1-IgG2-IgG3-IgG4 (Morell et al., 1975), IgA1-IgA2 (Skvaril and Morell, 1974), monomeric-polymeric IgA (Radl et al., 1974), κ-λ ratio (Hijmans et al., 1971; Vossen, 1975; Turesson, 1976), and IgD versus all other heavy chain isotypes together (van Camp et al., 1978). In contrast, the distribution profile of C-Ig cells in the peripheral lymphoid organs is different from the serum levels of the Ig classes and subclasses. The BM has also found to be the major location of autoantibody-forming cells in humans (McMillan et al., 1976), mice (Cohen, 1980), and rats (Weetman, 1980) suffering from diseases attendant with autoimmune phenomena.

The numbers of C-Ig cells (Haaijman et al., 1977, 1978) found within the BM of mice are substantial as compared with the numbers found in other lymphoid organs. The same results were obtained, when the number of Ig-secreting cells were assayed more directly with the protein A plaque-assay (Benner et al., 1981b). The absolute and relative contribution of the BM to the total number of Ig-secreting cells in mice increases enormously with age. For instance, in normal BALB/c mice the total number of Ig-secreting cells per animal increases 20- to 25-fold between 4 and 100 weeks of age. In the same period the number of Ig-secreting cells present in the BM increases 100-fold. As a consequence the percentage of all Ig-secreting cells per animal localized in the marrow increases from 17% at 4 weeks to 75% at 100 weeks of age (Benner et al., 1981b). Other mouse strains show similar distribution patterns of the Ig-secreting cells over the life-span. The accumulation

of Ig-secreting cells in the BM of mice during aging is independent of the thymus, since this phenomenon has also been found in congenitally athymic nude mice. However, the accumulation of Ig-secreting cells in the BM of these mice is retarded as compared with their heterozygous littermates (Haaijman et al., 1979; Benner et al., 1981b).

The Ig-production in the BM is facilitated by exposure of the animal to exogenous antigens. Exposure to exogenous antigen is the major factor determining the total number of Ig-secreting cells as well as the distribution of these cells over the various lymphoid organs and the heavy chain isotype distribution of the Ig-secreting cells. In normal, conventionally reared mice the majority of the IgG- and IgA-secreting cells are localized in the BM. In contrast, in antigen-deprived mice the number of IgG- and IgA-secreting cells present in the BM is highly deficient (Haaijman et al., 1979; Benner et al., 1981b). Moreover, when germ-free mice are completely deprived of exogenous antigens, by feeding them with a chemically defined low molecular weight diet from weaning and by keeping them on stainless steel grids instead of the usual bedding, Ig-producing cells hardly appeared in the BM (Benner et al., 1981d). The deficiency of IgG- and IgA-secreting cells in the BM of antigen-deprived mice suggests that the produced IgG and IgA are predominantly directed against environmental antigens, that penetrate by passing the epithelium lining the gastro-intestinal and respiratory tracts.

In spite of the extensive evidence, reviewed above, in favour of the BM as a major site of Ig- and antibody synthesis in comparison with the spleen, lymph nodes, and gut-associated lymphoid tissue, this conclusion has not yet been generally accepted. Probably, this is a consequence of the fact that antibody formation can hardly be found after immunization of rabbits (Thorbecke, 1961; Langevoort et al., 1963) and mice (Friedman, 1964; Eidinger and Pross, 1967; Chaperon et al., 1968; Mellbye, 1971; Nedelkova and Dobreva, 1971; Anderson and Dresser, 1972; Cohen, 1972; Cunningham, 1973, 1974; Lozzio and Wargon, 1974) with thymus-dependent (TD) antigens. However, in most of these investigations antibody formation has been assayed in the different lymphoid organs only during the primary response. This, however, is crucial, because antibody formation in the BM is only induced after two or more immunizations with the same antigen (Thorbecke et al., 1961; Langevoort et al., 1963; Benner et al., 1974a; 1977b; Hill, 1976).

Studies on antibody formation in BM have mostly been done with mice using sheep erythrocytes (SRBC) as an antigen. The secondary response to SRBC is characterized by high numbers of antibody-forming cells present in the spleen and/or lymph nodes during the first week after the booster immunization. After the first week, however, the number of these cells in the total BM is substantially higher than in all other lymphoid organs together (Benner et al., 1974a). This characteristic kinetics of the secondary response in mice is independent of the booster dose, route of immunization and type of TD antigen, and is found for IgM, IgG, and IgA antibody production (Benner et al., 1974a; Hill, 1976). Furthermore, IgE antibody production by BM cells has been observed in mice immunized with alum-precipitated ovalbumine (OV) and with mixtures of Ov and Con A (Kind and Malloy, 1974; Gollapudi and Kind, 1975). This IgE antibody production has been determined by the adoptive passive cutaneous anaphylaxis (PCA) reaction. It should be noted here, that

anti-host DTH response during the delayed GvH reaction (Bril and Benner, in press), which usually develops in lethally irradiated mice reconstituted with allogeneic BM cells. This suggests that antigen-activated suppressor T cells, which are mainly derived from the peripheral lymphoid organs (Bianchi et al., unpublished results), localize also in the BM.

Other suppressive activities have also been found in the BM. It has been reported that murine BM cells can suppress the *in vitro* humoral immune response (Singhal et al., 1972; Drury and Singhal, 1974; Petrov et al., 1975; Adler et al., 1976; Gorzcynsky and MacRae, 1977a; Duwe and Singhal, 1979a, 1979b; Dauphinée and Talal, 1979), and the proliferative and cytotoxic response of lymph node and spleen cells to alloantigens in the MLR (Gorczynsky and MacRae, 1977a; Dorshkind et al., 1980; Maraoka and Miller, 1980). In certain studies it has been shown that the suppressor cells are present in the lymphocyte-enriched fractions of the BM, but are insensitive to treatment with anti-Thy-1 serum and complement, and to treatment with anti-Ig serum and complement, and, therefore, probably are 'null' cells. Dauphinée and Talal (1979) have described that a radiosensitive cell population in the BM is responsible for the observed suppression of the *in vitro* antibody response. These suppressor cells are also insensitive to treatment with anti-Thy-1 serum and complement. However, when the BM cells are incubated with thymosin or thymus-derived humoral factors and then treated with anti-Thy-1 serum and complement, the suppressor activity is nearly abolished. These results suggest that the suppression is mediated by a precursor cell that can be induced by thymosin to express Thy-1 antigen on the cell surface. However, the suppression mediated by these BM cells is not antigen-specific, which is an additional argument in favour of the inference that the suppression observed in the latter experiments is not mediated by mature suppressor T cells.

3.4. Natural killer cells in the bone marrow

Natural killer (NK) cells are believed to be a subpopulation of lymphoid cells that are present in normal individuals of mammalian and avian species. NK cells display a 'spontaneous' - that is without prior intentional sensitization - cytolytic activity *in vitro* against a variety of tumour cells, some virus-infected cells, and certain normal cells (Kiessling et al., 1975a; Herberman et al., 1975; Nunn, 1977).

The NK cells are medium-sized mononuclear cells which neither express Ig's nor Ia antigen (reviewed by Kiessling, 1979). Although NK cells are thymus-independent in the sense that they are present in congenitally athymic nude mice and neonatally thymectomized mice and rats (Herbermann et al., 1978a; Reynolds, in press), a substantial proportion expresses cell surface markers which are characteristic for T cells. About half of the NK cells in humans express low-affinity receptors for SRBC, and the majority react with monoclonal antibodies directed against T cell-associated antigens (West et al., 1977; Eisenbart, 1980; Ortaldo et al., 1981). Similarly, 50% of the NK cells in the mouse express a low density of the Thy-1 antigen, and 20% are weak Lyt-1 positive (Herberman et al., 1978b; Mattes et al., 1979; Koo and Hatzfeld, 1980). No other typical T cell markers have been detected on NK cells so far (Pollack et al., 1979; Koo et al., 1980). Another cell surface marker of NK cells is the receptor for the Fc portion of IgG

(FcR). This marker is readily detected on most human NK cells. When appropriate depletion procedures are used, this receptor can also be detected on most of the NK cells of mice and rats (Herberman et al., 1977; Oehler et al., 1978). In contrast to these phenotypical characteristics, which suggest that NK cells belong to the T cell lineage, NK cells share some surface markers with macrophages and polymorphonuclear leukocytes in both man (Zarling and Kung, 1980; Kay and Horwitz, 1980; Breard et al., 1981; Ault and Springer, 1981; Ortaldo et al., 1981), mice (Lohman-Matthes and Domzig, 1980), and rats (Reynolds et al., 1981). At present, insufficient evidence exist to decide whether NK cells are cells belonging to the T cell lineage, to the monocyte lineage or to a separate cell lineage that is derived from a common precursor for lymphocytes, monocytes, and polymorphonuclear leucocytes.

It has been shown in mice that NK cells are BM derived cells (Haller et al., 1977a, 1977b) and, moreover, are dependent on the functional BM microenvironment for full expression of their cytolytic activity (Kumar et al., 1979). Thus, NK precursor cells are most probably generated within the BM and emigrate to the periphery, mainly the spleen. Also the peripheral blood contains substantial NK activity. Low NK activity, can be found within the BM itself (Kiessling et al., 1975b; Herberman et al., 1975b). It has not been investigated yet whether this low activity is a consequence of a low frequency of NK cells in this organ or of a relative immaturity. Both the low density Thy-1-bearing small lymphocytes and the rapidly renewed, Fc receptor bearing 'null' small lymphocytes in the BM (Chan and Osmond, 1979) are possible candidates for the BM NK precursor cells and the mature NK cells.

4. *INTRODUCTION TO THE EXPERIMENTAL WORK*

As stated in Chapter 2 the subdivision of lymphoid organs into 'central' and 'peripheral' was based upon the view that the antigen-independent formation of virgin lymphocytes and the induction of immune responses by antigen takes place in different organs. According to this view hardly any antibody formation would take place outside the peripheral lymphoid organs. In accordance, when mice, the most frequently used experimental animal in immunological research, are immunized with a thymus-dependent (TD) antigen, antibody formation would be confined mainly to the peripheral lymphoid organs. However, as mentioned before (Chapter 3), during the last 90 years papers have appeared in the literature in which evidence has been presented, indicating that the BM is also an important site of antibody formation. This incongruity was removed by the finding of high numbers of PFC in the BM of mice after secondary immunization (Benner et al., 1974a, 1974c; Hill, 1976). Adoptive transfer experiments, and experiments using parabiotic and splenectomized mice (Benner et al., 1974b, 1975, 1977b) revealed a coincidence between the presence of memory cells and the capacity of antibody formation in the BM. The peripheral lymphoid organs not only produce these memory cells, but are also needed for the initiation of the BM PFC response (Benner, 1977b). Bacterial lipopolysaccharides (LPS), on the other hand, induce antibody formation in the BM not only during the secondary, but also during the primary response (Benner and van Oudenaren, 1976). These data formed the background at the start of the investigations described in this thesis. *The purpose of the investigations presented* in this thesis was to get insight into the mechanism underlying the antibody formation within the BM after immunization with TD and thymus-independent (TI) antigens. These studies are described in the Appendix papers of this thesis.

In *Appendix paper I* the methods have been described, which were used to induce and to measure antibody formation in the mouse BM. Furthermore, a number of critical factors influencing the BM PFC response have been discussed.

In *Appendix paper II* experiments have been described in which the antibody production by spleen and by BM cells during the secondary response to SRBC has been quantified. Insight into the quantitative aspects of antibody formation in the BM is important since all previous studies in mice have been done with the plaque assay which detects the number of antibody-forming cells, but which gives no data about the quantity of antibodies produced.

The role of the peripheral lymphoid organs in the initiation of the BM PFC response has been investigated using parabiotic mice consisting of members congenic for the Igh-1 locus. Furthermore, the requirement for memory B and memory T lymphocytes in the BM antibody formation has been investigated *(Appendix paper III)*.

In order to exclude the possible facilitating influence of hapten-specific memory T cells and circulating anti-DNP antibodies we have studied the cellular requirements of the BM response in more detail by the technique of adoptive antibody formation in lethally irradiated syngeneic mice *(Appendix paper IV)*.

In *Appendix paper V* we have investigated the ability of several TI antigens (i.e., TNP-LPS, TNP-conjugated detoxified LPS, DNP-Ficoll, *Pneumococcus pneumoniae* bacteria, TNP-conjugated *Brucella abortus* organisms and α-(1,6)dextran) to induce antibody formation in bone marrow after primary immunization. It was found that only TNP-LPS and DNP-Ficoll induce a significant BM PFC response. In this paper also the mechanism underlying BM antibody formation to these two antigens has been investigated.

In *Appendix paper VI* the ability of TI antigens to induce antibody formation in the BM has been investigated after secondary immunization.

Appendix paper VII deals with the proliferative activity of antibody forming cells in the BM of mice immunized with either the TD antigen SRBC, or the TI antigens TNP-LPS and DNP-Ficoll.

5. *DISCUSSION OF THE EXPERIMENTAL WORK*

The BM is a major site of localization of Ig-secreting cells in mice and a variety of other mammals (reviewed by Benner, 1981a; Chapter 3.1). However, when mice are immunized with a TD antigens, PFC were found almost exclusively in spleen and lymph nodes (Friedman, 1961; Eidinger and Pross, 1967; Chaperon et al., 1968; Mellbye, 1971; Nedelkova and Dobreva, 1971; Anderson and Dresser, 1972; Cohen, 1972; Cunningham, 1973, 1974; Lozzio and Wargon, 1974). Secondary immunization of mice with TD antigens induces a PFC response not only in the spleen and the lymph nodes but also in the BM. During the early phase of the secondary response, much higher numbers of PFC are localized in the spleen and/or the lymph nodes than in the BM. After this first phase of about one week duration, the PFC response in the BM is several times higher than in all other lymphoid organs together (Benner et al., 1974a; Appendix paper I). Quantitative analysis of the mean antibody production per PFC in spleen and BM shows that there is no major difference in production per cell per unit of time in both compartments during the second phase of the response (Appendix paper II). Therefore, the BM is the major source of serum antibodies during this phase of the secondary response to a TD antigen.

Studies on the mechanism underlying antibody formation in the BM have been greatly facilitated by the above mentioned finding that in mice BM antibody formation can be induced by two separate injections of a TD antigen (Benner et al., 1974a). This discovery and the observation that only weak PFC activity is found in the BM after primary immunization with TD antigens, suggested that memory cells are required for BM antibody formation. In subsequent experiments, Benner et al. (1974b, 1975, 1977b) noticed a coincidence between the occurrence of B and T memory cells and the ability to respond to challenge with the relevant antigen with antibody formation in the BM.

By priming and boosting of mice with heterologous hapten-carrier conjugates, we have shown that BM antibody formation is strictly dependent upon the presence of memory B cells, but not on memory T cells, before the booster immunization (Appendix paper III). Likewise the adoptive antibody formation in the BM to hapten-carrier conjugates is dependent upon the presence of hapten-specific memory B cells, and not upon carrier-specific memory T cells (Appendix paper IV). However, memory T cells do enhance the adoptive BM PFC response mediated by infused hapten-specific memory B cells. We have also investigated whether hapten-specific memory T cells, which might replace carrier-specific memory T cells, are involved. This was done by transferring hapten-specific memory B cells, which were depleted of T cells by treatment with anti-Thy-1 serum plus complement, together with normal spleen cells as a source of unprimed helper T cells. The data show that also hapten-specific memory T cells, if they would occur under these particular experimental conditions, are not required for BM antibody formation. Furthermore, these adoptive transfer experiments showed that specific antibodies are not necessary for antibody formation in the BM (Appendix paper IV).

The origin of the antibody-forming cells that appear in the BM during secondary type responses to TD antigens was investigated in

parabiotic for the Igh-1 locus. From analysis of the allotype of the IgG2a-antibodies produced by PFC in the BM of such parabiotic mice it appeared that BM antibody formation is dependent on the immigration of memory B cells activated by the booster antigen in the peripheral lymphoid organs (Appendix paper III).

The immigration of activated memory B cells from the spleen takes place during the first few days after the booster immunization. This has to be concluded from the experiments of Benner et al. (1977c) in which mice were splenectomized at different intervals after the booster injection of a TD antigen. These experiments revealed that splenectomy four or more days after the booster injection no longer influences the BM PFC response, whereas splenectomy at day two could completely prevent the BM PFC response (Benner et al., 1977c). Consistent with such a migration of activated cells toward the BM, radioautographic studies in guinea pigs demonstrated an influx of newly formed mononuclear cells via the bloodstream into the BM after intravascular antigen administration (Appendix paper III). Furthermore, enhanced migration of lymphocytes from the spleen toward the BM has recently been observed in pigs during the first week after secondary immunization with SRBC (Pabst et al., in press).

The antibody formation to TD antigens in the BM during secondary type responses is characterized by its sustained pattern (Appendix paper I). This BM PFC response is not maintained by a continuous influx of antibody forming cells or their precursors from the periphery into the BM. This appears from the above-described splenectomy experiments (Benner et al., 1977c) and from experiments with parabiotic mice. In the latter experiments it was shown that in case of parabiosis of mice with an ongoing BM PFC response and non-immunized mice or recently primed mice, only minor numbers of PFC can be found in the BM of the latter (Benner et al., 1977a).

Immunization of mice with LPS (Benner and van Oudenaren, 1976), TNP-LPS, and DNP-Ficoll leads to antibody formation in the BM already during the primary response. TNP-conjugated *Brucella abortus* bacteria

TABLE I

Mitogenic Properties of TI Antigens *In Vitro*

Antigen	Polyclonal B cell stimulation	Polyclonal DNA synthesis
(TPN-)LPS	+	+
(TNP-)dLPS	-	-
TNP-BA	-	-
DNP-Ficoll	-	-
Dextran	±	±
Pn	-	-

(TNP-BA), *Pneumococcus pneumoniae* bacteria (Pn), and α-(1,6)dextran, on the other hand, do not induce a BM PFC response (Appendix paper V). The mitogenic properties of the TI antigens employed are presented in Table I.

The kinetics of the BM response to TNP-LPS is basically different from the response to DNP-Ficoll. While primary immunization of mice with LPS or TNP-LPS leads to a substantial PFC response in the BM already by day 4 after immunization, injection of DNP-Ficoll does not induce a PFC response in the BM before day 5. Furthermore, the BM PFC response to TNP-LPS equals or surpasses the response in the spleen already on day 6, whereas the BM PFC response to DNP-Ficoll does not exceed the level of the splenic response and attains this level later, namely around day 10.

The mechanisms underlying antibody formation in the BM are also different for TNP-LPS and DNP-Ficoll. The BM-localizing PFC induced by TNP-LPS are formed within the BM itself from small B lymphocytes recently produced by this organ. This appears from the observation that the BM anti-TNP-LPS response is sensitive to treatment of the mice with hydroxyurea (HU; a drug that kills cells which are in the S-phase of the cell cycle) before immunization, and is resistant to splenectomy. This local activation of newly formed B cells requires, in addition to the antigenic signal of TNP-LPS, the mitogenic signal of the lipid A component of LPS. This is apparent from the observation that detoxification of LPS, which inactivates the lipid A component that is responsible for the mitogenic and adjuvant properties of LPS, abolishes the ability of LPS to induce antibody formation in the BM. The *in situ* induction of antibody formation to (TNP-)LPS in the BM is compatible with the recent observation that B cells in the mouse BM are fully immunocompetent *in vitro* (Benner et al., 1981c).

In contrast to the BM PFC response to TNP-LPS, the response to DNP-Ficoll in the BM is resistant to HU treatment before immunization, and is reduced in splenectomized mice. Thus, antibody formation in the BM to DNP-Ficoll is mainly dependent on long-lived B cells that migrate, after antigenic stimulation, from the peripheral lymphoid organs into the BM (Appendix paper V).

As stated above, TD antigens give rise to antibody formation in the BM after multiple immunization only, because antigen-specific memory B cells, induced during the primary immune response, are indispensible. Therefore, the question arises as to whether TI antigens, which do not induce antibody formation in the BM after primary immunization, do so after secondary injection of the same antigen. This has been investigated for TNP-conjugated detoxified LPS (TNP-dLPS), TNP-BA, and Pn. Of these TI antigens only TNP-BA induces a PFC response in the BM after secondary immunization. Furthermore, this BM PFC response has the same kinetics as secondary type BM responses to TD antigens (Appendix paper VI).

The inability of the certain TI antigens (e.g., Pn and TNP-dLPS) to induce substantial secondary BM antibody formation is not due to a failure to induce memory B cells. This is apparent from the quantitatively and/or qualitatively different secondary PFC response to these antigens in the spleen. This indicates that they do induce and activate memory B cells.

Since TD antigens hardly induce antibody formation in congenitally athymic nude mice, one would expect that the antibody-producing cells found in these mice are mainly directed against TI antigens. Therefore,

the weak BM PFC response to most TI antigens, which is also found after repeated immunization, is difficult to reconcile with the occurrence of high numbers of background Ig-secreting cells in the BM of congenitally athymic mice (Haaijman et al., 1979; Benner et al., 1981d). This incongruity can be explained either by the fact that the panel of TI antigens that we have used, is not representative for the normal situation, or that most exogenous antigens that normally stimulate the immune system of nude mice are associated with a mitogenic principle.

After it had been established that antibody formation to TD antigens and DNP-Ficoll in the BM is dependent upon immigrant cells from the peripheral lymphoid organs, the question arose whether these cells represent end-stage cells that no longer proliferate, or cells that can still proliferate within the BM *in situ*. Likewise the question arose whether BM antibody formation to TNP-LPS is associated with a local clonal expansion of the activated B cells. As stated above, the immigration of SRBC-activated memory B cells and their progeny is restricted to the first four days after the booster immunization with the TD antigen SRBC. Thus, splenectomy on day 4 after the booster immunization with SRBC has no influence on the BM PFC response (Benner et al., 1977c). To investigate the proliferative activity of the immigrant B cells in the BM we have treated such boosted and splenectomized mice with HU on different days after the booster injection, and subsequently assayed the anti-SRBC PFC response. It was found that treatment with HU on day 4 and 5 reduces the number of PFC in the BM dramatically. Beyond day 6 HU treatment did not have a significant effect upon the BM PFC response. This reducing effect of HU upon the BM PFC response of splenectomized mice is a clue for the proliferative activity of the relevant cell compartment. Apparently the proliferative activity within the antibody-forming cell lineage within the BM is substantial, and maximal 4 to 6 days after immunization. Similar data were found for the BM PFC response to DNP-Ficoll. These results indicate that the magnitude of the BM PFC response to TD antigens and the TI antigen DNP-Ficoll is highly dependent upon two events: the migration of antigen-activated (memory) B cells from the peripheral lymphoid organs into the BM, and the proliferative activity of the immigrant cells within the BM. Both events are mainly restricted to the first few days after immunization in spite of the sustained character of the BM PFC response (Benner et al., 1974c; Appendix paper I).

Examination of histological sections of the BM of rabbits hyperimmunized with pneumococcal vaccines have shown that the number of plasma cells in the BM increases after immunization and that they occur as small islands of 5 to 6 cells (Bjørneboe and Gormsen, 1943). This observation also suggests that plasma cells, or more likely, their progenitor cells proliferate within the BM.

In contrast to DNP-Ficoll, antibody formation in the bone marrow to TNP-LPS is caused by a local activation of newly formed B cells by the antigen, and a subsequent differentiation into antibody-forming cells. The differentiation of the activated B cells into PFC is clearly associated with proliferation, because the BM PFC response to TNP-LPS is sensitive to treatment with HU during the first few days after immunization (Appendix paper VII).

In view of the above described data, the sustained character of the BM PFC response can only be explained by a relatively long lifetime of

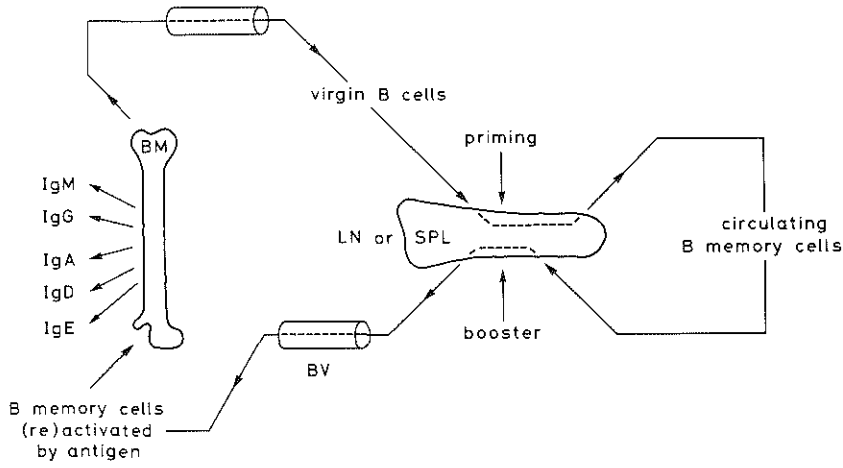

Figure 1. Schematic representation of the mechanism underlying antibody formation in bone marrow. See text for explanation. BM = Bone marrow; BV = Blood vessel; LN = Lymph node; SPL = Spleen.

the antibody-producing cells in the BM. Evidence for a long lifetime of at least a part of the plasma cells in the BM as compared to plasma cells in the spleen has been presented by Taylor and Everett (1972). A long lifetime of plasma cells has also been observed in cultures of human BM fragments (Mendelow et al., 1980). The life-span of plasma cells in the BM of experimental animals can be investigated by occluding one hind limb for a short period, during which ^{3}H-TdR is infused systemically. By the enumeration of the number of labelled plasmablasts and plasma cells in the BM at different intervals after the labelling the life-span of these cells can then be estimated.

The above outlined mechanism underlying antibody formation in the BM is schematically represented in Figure 1. It should be noted here once again, that the BM is unable to mount an antibody response to TD antigens and to most TI antigens by local activation of the B cells by antigen. Instead the BM antibody response is dependent upon the immigration of antigen-activated cells of the B cell lineage from elsewhere. After arrival in the BM these blast cells further proliferate and mature into PFC, and produce large amounts of antibodies of the IgM, IgG, IgA (Benner et al., 1974b; Appendix paper I) and IgE (Kind and Malloy, 1974; Gollapudi and Kind, 1975) classes. By means of immunofluorescence techniques such cells are revealed as C-Ig cells. In normal not intentionally immunized mice large numbers of background C-Ig cells, that is, C-IgM, C-IgG and C-IgA (Haaijman et al., 1977, 1978) as well as C-IgD and C-IgE positive cells (Benner, van Oudenaren and Radl, personal communication) occur in the BM.

The occurrence of antibody formation within the BM does not agree with the classical definition of central and peripheral lymphoid organs (c.f. Chapter 2). Therefore, it is preferable to distinguish between central and peripheral lymphoid organs by the exclusive ability of the

latter to initiate immune responses. So far, the PFC response to LPS and its mitogenic derivatives is the only exception. Presumably, the central lymphoid organs lack the appropriate microenvironment and/or quantity or quality of cells required for the early steps in the induction of immune responses.

So far no compelling evidence exists to suppose that antibody formation in human BM is dependent on another mechanism than antibody formation in mouse BM. Human BM can display histological features that are characteristic of peripheral lymphoid tissues, e.g., the occurrence of lymphoid follicles. In some rare cases even germinal centres have been observed in these follicles (Duhamel, 1968; Rywlin et al., 1974; Maeda et al., 1977). These follicles are more frequently observed in BM samples of individuals suffering from various types of diseases. Furthermore, the occurrence of lymphoid follicles in the BM increases with the age of the individuals. Although, so far, no lymphoid follicles have been reported to be present in mouse BM this is not surprising in view of the fact that only relatively young and healthy mice are used for such studies. Therefore, it is interesting to examine histological sections of BM of mice suffering from well-known lymphoproliferative diseases such as the NZB and MLR/lpr strains to see whether lymphoid follicles are present in such mice. In rabbits, lymphoid follicles have not been found in normal marrows but some are present in abnormal marrow (Hashimoto et al., 1955).

It is unclear whether the accumulation of antigen-activated memory B cells outside the peripheral lymphoid organs is a specific or merely a random process. In the latter case, the activated memory B cells would disseminate from the peripheral lymphoid organs to any place in the body where the specific antigen is retained. According to this view, antibody formation in the BM would be the expression of a general property of secondary type responses. Thus, secondary PFC responses, in contrast to primary responses, would be less restricted to the peripheral lymphoid organs where the PFC originate, but would disseminate to all sites of the body where the relevant antigen is present. Therefore, the mechanism underlying ectopic antibody formation, for instance in granulomatas (reviewed by MacMaster et al., 1953), may be the same as the mechanism underlying BM antibody formation.

In case the migration of antigen-activated B cells is a specific process, specific recognition structures, comparable to those found in the high-endothelial venules in lymph nodes (Woodruff, 1977), may be involved. Bjørneboe and Gormsen (1943) have reported that in rabbits small clusters of plasma cells are diffusely scattered throughout the BM parenchyma. These clusters have been observed adjacent to the blood sinusoids. A similar distribution of plasmablasts and plasma cells has been observed in mice by Osmond (personal communication). Therefore, if such a mechanism would play a role, one would expect the recognition structures to be present on the walls of the blood sinusoids.

The characteristic kinetics of the PFC response in spleen and BM shows that the antibody-forming cell response is regulated in such a manner that the peripheral lymphoid organs respond rapidly, but only for a short period, whereas the BM response starts slowly, but takes care of a long-lasting massive production of antibodies to antigens which repeatedly challenge the organism. Thereby the peripheral lymphoid organs provide a fast defence to the challenging antigen, while the BM

provides a long-lasting protection against recurrent infections. Possibly the long-lasting antibody formation in the BM is caused by a lack of feedback suppression in this organ. Therefore, it would be interesting to investigate in future studies the factor(s) determining the different kinetics of the PFC response in the spleen and the BM.

Antibody formation in the BM may also form a local feedback regulation pathway for the production of B cells with the relevant antigenic specificity (Jerne, 1979). Recently, it has been proposed that anti-idiotypic antibodies can stimulate and take part in the selection of the antibody repertoires (reviewed by Coutinho et al., 1980). Likewise, it is conceivable that anti-idiotypic antibodies produced by the BM can affect the size of specific clones at the pre-B and B cell level.

6. SUMMARY

The bone marrow (BM) is the major production site of B lymphocytes. The newly formed small B lymphocytes migrate, after a maturation period of one or more days, to the peripheral lymphoid organs (spleen, lymph nodes, tonsils, etc.), where they can be activated by antigen (after which they can differentiate into antibody-forming cells) or die. According to this view, most of the immunoglobulin(Ig)- and antibody-producing cells are considered to be confined to the peripheral lymphoid organs. However, publications have appeared during the last ninety years presenting evidence that antibody formation can also take place in the BM (Chapter 3). This thesis further investigates this phenomenon with mice as experimental animals.

After primary immunization of mice with thymus-dependent (TD) antigens, antibody formation almost exclusively takes place in the peripheral lymphoid organs. However, after secondary immunization with the same antigen, substantial numbers of antibody-producing plaque-forming cells (PFC) are found not only in the peripheral lymphoid organs, but also in the BM. The first phase of the secondary response, about 1 week of duration, is characterized by much higher numbers of PFC in the spleen and/or lymph nodes than in the BM. But after this first phase, the PFC response in the BM is much higher than in all other lymphoid organs together (Appendix paper I).

Quantitative analysis of the mean antibody production per PFC in spleen and bone marrow shows that the production per cell per unit time in both organs is about the same during the second phase of the secondary response to sheep erythrocytes (SRBC) (Appendix paper II). Therefore, the BM is the major source of serum antibodies during this phase of secondary type responses to such TD antigens.

The subsequent experiments described in this thesis were aimed to elucidate the *mechanism* underlying antibody formation in the mouse BM.

By priming and boosting of mice with TD heterologous hapten-carrier conjugates, we have shown that BM antibody formation is strictly dependent upon the presence of memory B cells at the moment of the booster immunization, but not on memory T cells (Appendix paper III). Likewise, the adoptive antibody formation in the BM to TD hapten-carrier conjugates is dependent upon the presence of hapten-specific memory B cells. Neither hapten-specific memory T cells nor specific antibodies appeared to be involved in the induction of the adoptive antibody formation in the BM (Appendix paper IV).

The origin of the antibody-forming cells that appear in the BM during secondary type responses to TD antigens has been investigated in parabiotic mice consisting of members congenic for the Igh-1 locus. From analysis of the allotype of the IgG2a antibodies produced by PFC in the BM of such parabiotic mice it appeared that BM antibody formation is dependent on the immigration of memory B cells activated by the booster antigen in the peripheral lymphoid organs (Appendix paper III). This migration of (re)activated memory B cells from the peripheral lymphoid organs into the BM is restricted to the first four days after secondary immunization with the TD antigen. The immigrant cells continue to proliferate in the BM. but predominantly during the first few days after

arrival. This can be concluded from the profound and long-lasting suppression of the BM PFC response after elimination of the proliferating cells by treatment with hydroxyurea (HU; a drug that kills cells which are in the S-phase of the cell cycle (Appendix paper VII).

In contrast to TD antigens, certain thymus-independent (TI) antigens (e.g., LPS, TNP-LPS, and DNP-Ficoll) give rise to antibody formation in the BM already during the primary response. The mechanisms underlying the BM antibody formation to (TNP-)LPS and to DNP-Ficoll, however, are different. The PFC response to (TNP-)LPS in the BM is dependent on the local activation and subsequent proliferation of newly formed B cells. This appears from the observation that this response is sensitive to treatment of the mice with HU *before* (Appendix paper V) as well as after (Appendix paper VII) immunization, and is resistant to splenectomy. This local activation of newly formed B cells requires besides the antigenic signal of (TNP-)LPS the mitogenic signal of the lipid A component of LPS. This is apparent from the observation that detoxification of LPS, which inactivates the lipid A component that is responsible for the mitogenic and adjuvant properties of LPS, abolishes its capacity of inducing antibody formation in the BM.

In contrast to the BM PFC response to TNP-LPS, the BM response to DNP-Ficoll is resistant to treatment of the mice with HU before immunization, and is reduced in splenectomized mice. Thus, antibody formation to DNP-Ficoll in the BM is mainly dependent upon long-lived cells which migrate from the peripheral lymphoid organs into the BM (Appendix paper V). The maturation of these immigrant cells into PFC is, just as during secondary responses to TD antigens, associated with proliferation (Appendix paper VII).

In contrast to LPS, TNP-LPS, and DNP-Ficoll, other TI antigens (e.g., TNP-conjugated *Brucella abortus* bacteria (TNP-BA), *Pneumococcus pneumoniae* bacteria, and α-(1,6)dextran) do not induce a BM PFC response after primary immunization (Appendix paper V). After secondary immunization with the latter TI antigens, only TNP-BA gives rise to a weak PFC response in the BM. The failure of certain TI antigens to induce antibody formation in the BM is not due to an inability to induce memory B cells during the primary response or to an inability to reactivate these memory B cells (Appendix paper VI).

The different kinetics of the PFC response in spleen and BM shows that the antibody-forming cell response is regulated in such a manner that the peripheral lymphoid organs respond rapidly, but only for a short period, whereas the BM response starts slowly, but takes care of a long-lasting massive production of antibodies to antigens which repeatedly challenge the immune system. Thereby the peripheral lymphoid organs provide a fast defence to challenging antigens, whereas the bone marrow provides a long-lasting protection against recurrent infections. Possibly the long-lasting antibody formation in the BM is caused by a lack of feedback suppression. Therefore, it would be interesting to investigate the factor(s) determining the different kinetics of the antibody response in spleen and BM.

7. SAMENVATTING

Het beenmerg (BM) is de belangrijkste produktieplaats van B-lymfocyten. De nieuw gevormde B-lymfocyten migreren na een rijpingsproces van één of enkele dagen naar de perifere lymfoiede organen (milt, lymfklieren, tonsillen, enz.). In deze laatste organen kunnen de B-lymfocyten geaktiveerd worden door antigenen (waarna ze kunnen differentiëren tot antilichaamvormende cellen), of doodgaan. Gewoonlijk veronderstelt men dat de meeste immuunglobuline(Ig)- en antilichaamproducerende cellen in de perifere lymfoiede organen gelokaliseerd zijn. Sinds het einde van de vorige eeuw zijn er echter ook publikaties verschenen, waaruit blijkt dat antilichaamvorming tevens in het BM kan plaatsvinden (Hoofdstuk 3). In dit proefschrift is dit verschijnsel nader onderzocht, waarbij gebruik gemaakt is van muizen als proefdieren.

Na primaire immunisatie van muizen met thymus-afhankelijke antigenen vindt antilichaamvorming vrijwel uitsluitend plaats in de perifere lymfoiede organen. Na een tweede immunisatie met hetzelfde antigeen worden antilichaam-producerende plaque-vormende cellen (PFC) echter niet alleen in de milt en de lymfklieren, maar ook in het BM aangetroffen. Aanvankelijk, d.w.z., gedurende ongeveer de eerste week na secundaire immunisatie, bevinden de meeste PFC zich in de milt en/of lymfklieren. Na de eerste week is de PFC-respons in het BM veel hoger dan in alle andere lymfoiede organen samen (Appendix publicatie I).

Kwantitatieve bepalingen van de gemiddelde antilichaamproduktie per PFC in milt en BM toonden aan dat de produktie per cel en per tijdseenheid in beide organen ongeveer gelijk is, tijdens de tweede fase van de secundaire respons tegen schape rode bloedcellen (SRBC) (Appendix publikatie II). Daarom is het BM tijdens deze fase van secundaire responsen tegen zulke thymus-afhankelijke antigenen de belangrijkste bron van antilichamen.

De verdere experimenten die in dit proefschrift beschreven worden zijn uitgevoerd met het doel inzicht te verkrijgen in het mechanisme dat ten grondslag ligt aan antilichaamvorming in het BM.

Door de primaire en secundaire immunisatie uit te voeren met heterologe thymus-afhankelijke hapteen-carrier-conjugaten kon worden aangetoond dat de verschijning van PFC in het BM strikt afhankelijk is van de aanwezigheid van memory-B-cellen op het ogenblik van de secundaire immunisatie, en dat memory-T-cellen niet noodzakelijk zijn (Appendix publikatie III). Ook voor de antilichaamvorming tegen thymus-afhankelijke hapteen-carrier conjugaten in het beenmerg van letaal-bestraalde muizen zijn, naast normale niet eerder geaktiveerde T-cellen, alléén hapteen-specifieke memory-B-cellen noodzakelijk. Hapteen-specifieke memory-T-cellen, noch specifieke antilichamen spelen een rol bij de PFC-respons in het BM van bestraalde muizen (Appendix publikatie IV).

De oorsprong van de PFC, die in het BM verschijnen tijdens secundaire responsen tegen thymus-afhankelijke antigenen, is onderzocht door middel van parabiose van muizen die congeen zijn voor het Igh-1 (IgG2a allotype) locus. Tijdens de parabiose ontstaan verbindingen tussen de bloedbaan van beide muizen, waardoor o.a. uitwisseling van bloed(cellen) tussen de beide dieren mogelijk wordt. Analyse van het allotype van de IgG2a antilichamen, die in het beenmerg werden geproduceerd, toonde aan dat de antilichaamvorming in dit orgaan tot stand komt door

immigratie van memory-B-cellen die in de perifere lymfoiede organen gereaktiveerd zijn door (het betreffende) antigeen. De migratie van antigeen-gestimuleerde memory-B-cellen van de perifere lymfoiede organen naar het BM vindt plaats gedurende de eerste vier dagen na de secundaire immunisatie. De geimmigreerde cellen delen na aankomst in het BM nog gedurende enkele dagen. Dit kan geconcludeerd worden uit de langdurige en sterke verlaging van de PFC-respons in het BM na eliminatie van de delende cellen door behandeling van de muizen met hydroxyureum (HU; een stof die cellen doodt die in de S-fase van de celcyclus zijn) (Appendix publikatie VII).

In tegenstelling tot thymus-afhankelijke antigenen induceren sommige thymus-onafhankelijke antigenen (b.v. LPS, TNP-LPS en DNP-Ficoll) antilichaamvorming in het BM al na primaire immunisatie. De mechanismen die ten grondslag liggen aan de antilichaamvorming tegen (TNP-)LPS en DNP-Ficoll in het BM zijn echter verschillend. De PFC-respons tegen (TNP-)LPS in het BM ontstaat door lokale stimulatie en daarop volgende proliferatie van nieuwgevormde B-cellen. Dit blijkt uit experimenten met muizen die vóór (Appendix publikatie V) of na (Appendix publikatie VII) immunisatie met (TNP-)LPS behandeld werden met HU, en het feit dat het verwijderen van de milt vóór immunisatie geen invloed heeft op de hoogte van de respons in het BM. De lokale stimulatie door (TNP-)LPS is afhankelijk van de mitogene aktiviteit van het LPS. Dit blijkt uit het feit dat geen PFC-respons in het BM wordt gevonden na primaire immunisatie met gedetoxificeerd LPS of met TNP-geconjugeerd gedetoxificeerd LPS (TNP-dLPS). Detoxificatie van LPS inactiveert de lipid A component van LPS. Dit lipid A is er verantwoordelijk voor dat LPS niet alleen als antigeen, maar ook als mitogeen en als adjuvant kan optreden bij de aktivatie van lymfocyten.

In tegenstelling tot de respons tegen TNP-LPS, is de PFC-respons tegen DNP-Ficoll in het BM niet gevoelig voor behandeling van de muizen met HU vóór primaire immunisatie. Bovendien is de PFC-respons tegen DNP-Ficoll in het BM verlaagd wanneer de milt is verwijderd vóór de immunisatie met DNP-Ficoll. De PFC-respons in het BM tegen DNP-Ficoll is daarom, net zoals de respons tegen thymus-afhankelijke antigenen, afhankelijk van de migratie van lang-levende B cellen van de perifere lymfoiede organen naar het BM (Appendix publikatie V). De rijping van de geimmigreerde cellen tot PFC gaat, net als bij secundaire responsen tegen thymus-afhankelijke antigenen, gepaard met celdeling (Appendix publikatie VII).

In tegenstelling tot LPS, TNP-LPS en DNP-Ficoll induceren andere thymus-onafhankelijke antigenen (b.v. TNP-geconjugeerde *Brucella abortus* (TNP-BA) bakteriën, *Pneumococcus pneumoniae* bakteriën en α-(1,6)-dextraan) geen PFC-respons in het beenmerg na primaire immunisatie. Van deze laatste groep thymus-onafhankelijke antigenen induceert alleen TNP-BA een zwakke PFC-respons in het BM na secundaire immunisatie. Het feit, dat ook na secundaire immunisatie met de meeste van deze antigenen niet of nauwelijks een respons in het BM wordt gevonden, wordt niet veroorzaakt doordat na immunisatie met thymus-onafhankelijke antigenen geen memory-B-cellen zouden worden gevormd, en ook niet doordat deze antigenen niet in staat zouden zijn de geinduceerde memory-B-cellen te reaktiveren (Appendix publikatie VI).

De verschillende kinetiek van de antilichaamvorming in milt en BM toont aan dat de antilichaamrespons zodanig wordt gereguleerd dat in

de perifere lymfoiede organen de respons snel wordt opgebouwd, maar slechts kort duurt. De antilichaamrespons in het BM komt daarentegen langzaam op gang en zorgt voor een langdurige en massale produktie van antilichamen, gericht tegen antigenen die het immuunsysteem herhaaldelijk stimuleren. Door dit verschil in regulatie zorgen de perifere lymfoiede organen voor een snelle afweer tegen binnendringende antigenen, terwijl het BM het lichaam langdurig beschermt tegen terugkerende infekties. Mogelijk wordt de lange duur van de PFC-respons in het BM veroorzaakt, doordat een duidelijke 'feedback' suppressie ontbreekt. Het zou daarom interessant zijn om te onderzoeken welke factor(en) het verschil in kinetiek van de antilichaamvorming in milt en BM bepalen.

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en kundig alle manuscripten van de publikaties heeft getypt, maar ook met veel geduld ervoor heeft gezorgd dat dit boekwerkje werd wat het is.

Tenslotte wil ik van deze gelegenheid gebruik maken mijn ouders te bedanken voor de bijzondere wijze waarop zij mij in mijn jeugd hebben begeleid en gestimuleerd en voor de gelegenheid die zij mij geboden hebben mij verder te ontwikkelen.

Adrianne, de plaats waarop je hier in het dankwoord vermeld wordt, is misschien wel symptomatisch voor de plaats die je in de afgelopen jaren 'moest' innemen. Voor de vrijheid die je me daarvoor gegeven hebt ben ik je dankbaarder dan ik hier in een enkel woord kan zeggen. We hebben het er dus nog wel over onder de kersenbomen.

CURRICULUM VITAE

Na het behalen van het diploma HBS-B aan het Lorentz Lyceum te Eindhoven in 1965, begon de auteur van dit proefschrift in hetzelfde jaar een studie aan de subfaculteit Scheikunde van de Rijksuniversiteit te Groningen. In januari 1973 behaalde hij het doctoraal examen met biochemie als hoofdvak bij Prof.Dr. M. Gruber (doctoraal onderzoek naar conformatie-eenheden van immunoglobuline G van de mens onder leiding van Dr. H.G. Seyen) en vegetatieve fysiologie als bijvak bij Prof.Dr. W.G. Zijlstra.

Tijdens de militaire dienst was hij gestationeerd op het Medisch Biologisch Laboratorium RVO-TNO, alwaar hij betrokken was bij onderzoek naar de etiologie van de experimentele shock bij ratten (onderzoek o.l.v. Dr. M. Wijnans en Prof.Dr. C. van der Meer).

Na de vervulling van de militaire dienstplicht trad hij in 1975 in tijdelijke dienst van het Radiobiologisch Instituut GO-TNO (onderzoek o.l.v. Dr. P. Bentvelzen naar de betrokkenheid van RNA-tumor virussen in de etiologie van leukaemie bij de mens).

Sedert 1978 is hij verbonden aan de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Binnen de afdeling Celbiologie II werd het in dit proefschrift beschreven onderzoek verricht onder leiding van Prof.Dr. R. Benner

APPENDIX PAPER I

INDUCTION OF ANTIBODY FORMATION IN MOUSE BONE MARROW

Robbert Benner, Adrianus van Oudenaren and Guus Koch

Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands.

I. INTRODUCTION

Since the last decade of the nineteenth century, papers have appeared in the literature presenting evidence that antibody formation in frogs and a variety of higher vertebrates, including humans, may take place not only in peripheral lymphoid organs but also in the bone marrow (summarized in Benner and Haaijman, 1980). This evidence has been obtained in studies showing that after (often intensive) antigenic stimulation (1) extracts of bone marrow contain high concentrations of specific antibodies, (2) bone marrow cells produce and release antibodies and immunoglobulins (Ig's), (3) the incidence of plasma cells in the bone marrow increases, and (4) antibody-producing plaque-forming cells (PFCs) occur in the marrow.

Immunoglobulin synthesis occurs in the bone marrow of unintentionally immunized animals and in humans as well. This can be demonstrated (1) in short-term *in vitro* cultures (Askonas and White, 1956; McMillan et al., 1972); (2) by means of the protein A plaque assay, which is an appropriate assay for enumerating Ig-secreting cells irrespective of V region specificity (Benner et al., 1981); and (3) by methods involving cytoplasmic fluorescence of plasmablasts and plasma cells (Hijmans et al., 1971; Haaijman et al., 1979). In fact, in adult mice (Section VI, F) and in humans the marrow is the major site of Ig synthesis. For humans this conclusion is based upon parallel determination of serum Ig levels and the number of cytoplasmic Ig-containing plasmablasts and plasma cells (C-Ig cells) in various lymphoid organs of the same individual. The IgM-IgG-IgA class distribution profile and the κ-λ ratio of the C-Ig cells in the bone marrow correlated with the levels of the various Ig classes and light chains in the serum after correction for pool size and metabolic rate. This correlation was not found when the C-Ig cell patterns of other lymphoid organs were compared with the serum Ig spectrum. The same correlation between C-Ig cells in the bone marrow and Ig levels in the serum was found in separate studies involving IgD, the subclasses of IgG and IgA, and monomeric and polymeric IgA (Hijmans, 1975; Turesson, 1976).

Nevertheless, the bone marrow has never generally been accepted as a site of antibody formation in addition to the spleen, the lymph nodes and the gut-associated lymphoid tissues. This may be due to the fact that in virtually all studies involving the most popular experimental animal in immunological research, the mouse, little or no antibody formation was found in the marrow after immunization. This chapter will discuss the critical factors accounting for these negative results (Section VI).

II. COLLECTION OF MOUSE BONE MARROW CELLS

A. Collection of Marrow from Femurs

Mouse bone marrow is most easily collected from the femur. A mouse is killed with CO2 or cervical dislocation and pinned with its back upon a tray of cork; the fore- and hindlegs are then spread apart. Four pins are used per mouse, one for each leg. The skin can most easily be cut after wetting with 70% ethanol in water. The procedure for removing the femur is illustrated in Fig. 1. A large piece of skin should be cut from the hindleg and the lower abdomen in order to provide a good view of the working area (Fig. 1a and 1b). 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

Figure 1. Procedure for removing the femur of a mouse. For description see text.

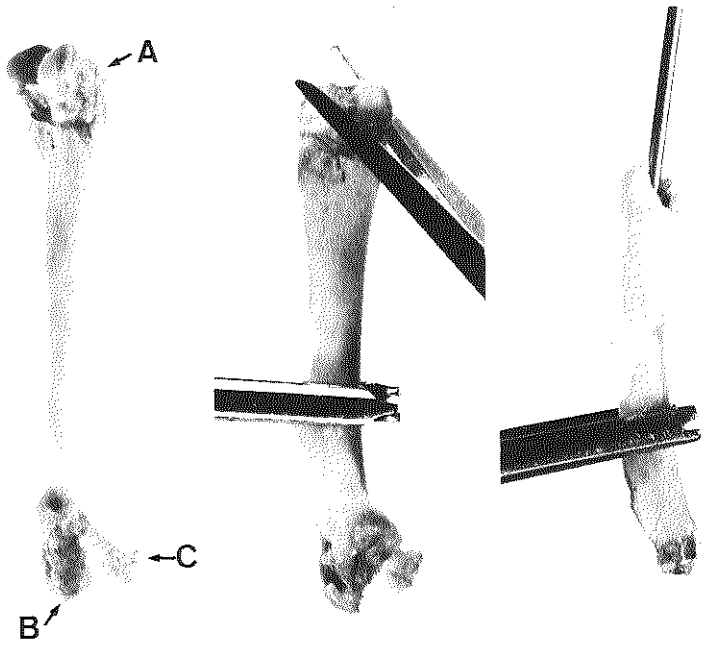

Figure 2. Procedure for the preparation of the bone marrow from a mouse femur. For description see text.

upper side of the femur (Fig. 1c and 1d). Subsequently the femur is slightly lifted with surgical forcepts, and the knee joint is cut (Fig. 1e). 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. 1f).

Before isolating the bone marrow from the femur, the residual muscle tissue is removed with a 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 a balanced salt solution (BSS) from the syringe (Fig. 2). Whether or not the marrow has been completely extracted from the femur can be judged from the color of the shaft. Bone marrow from long bones other than femurs can be similarly isolated by flushing the marrow cavity with BSS.

A single-cell suspension from the bone marrow can most easily be obtained by gently squeezing the latter through a nylon gauze filter with 100 µm openings (Stokvis and Smits Textielmij. Haarlem, The Netherlands). The nylon is stretched between two aluminium rings with a diameter of 3 cm. This setup is used in combination with a 50 ml glass beaker (Fig. 3).

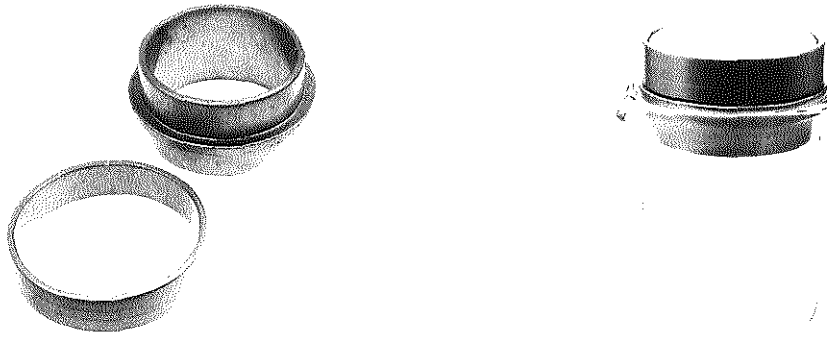

Figure 3. Nylon gauze filter for preparation of single-cell suspensions.

B. Collection of Marrow from Flat Bones

Bone marrow from a flat bone is not easily isolated by flushing the marrow cavity. Therefore we prefer to break the bone in BSS with a mortar and pestle. When this is done carefully, the viability of the bone marrow cells is equal to that obtained after flushing long bones with BSS, i.e., over 90%. Large bone fragments can be removed by centrifuging for 30 sec at 125 g. Tiny bone fragments can be separated from the marrow by gently squeezing the supernatant through a nylon gauze filter with 100 μm or, if necessary, 30 μm openings.

III. DISTRIBUTION OF ANTIBODY-FORMING CELLS OVER VARIOUS BONE MARROW COMPARTMENTS

After mice are immunized the plaque-forming cell (PFC) activity is approximately the same in different bone marrow compartments. This is apparent from studies in which the PFC activity was determined in bone marrow cell suspensions obtained from femur, tibia, humerus, rib, and sternum. Such a comparative study was made during the primary response to *Escherichia coli* lipopolysaccharide (LPS) and the secondary response to sheep erythrocytes (SRBCs) (Table I). In view of the absence of significant differences between the PFC responses of marrow cells from these different compartments, the femoral marrow should be considered as reliable representative of the total bone marrow.

In similar studies on the distribution of surface Ig-positive (B) cells over various bone marrow compartments, equally high frequencies were found (Rozing et al., 1978).

IV. CALCULATION OF THE ANTIBODY-FORMING CELL ACTIVITY OF THE TOTAL BONE MARROW

Comparison of the PFC response of the bone marrow with that of the other lymphoid organs after immunization requires quantitation of

the PFC response per whole organ. For spleen and lymph nodes this is easy to do, since the plaque assay can be performed with a sample from a cell suspension made from the whole organ. For bone marrow this is not feasible. In view of the comparable PFC activity in various bone marrow compartments (Table I), and in view of the fact that bone marrow cells can be easily isolated from femurs, we advocate determination of marrow PFC response in the femoral marrow, with subsequent calculation of the PFC response of the total marrow by using a conversion factor. This conversion factor can be deduced from the distribution of intravenously (iv) or intraperitoneally (ip) injected ^{59}Fe over the various parts of the skeleton. Three independent groups of investigators have performed such 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 value of the mean of these three percentages is 15.87. Thus the PFC response of the total bone marrow can be calculated to be 15.87 times the response of 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. But sometimes it is difficult to remove all the visible (red) bone marrow from a femoral shaft. Consequently, the calculated bone marrow PFC response is too low. In order to reduce this factor we prefer to collect the marrow from both femurs of a mouse. Then we can use a conversion factor of 7.9.

TABLE I

Comparison of Plaque-Forming Cell Activity in Various Compartments of Mouse Bone Marrow during the Primary Response to Lipopolysaccharide and the Secondary Response to Sheep Red Blood Cells[a,b].

Source of bone marrow cells	Anti-LPS PFCs	Anti-SRBC PFCs		
		IgM	IgG	IgA
Femur	2492	820	4914	1568
Tibia	3415	1037	4648	1935
Humerus	2937	1640	4971	1882
Rib	2986	1667	5795	2137
Sternum	2960	n.d.[c]	n.d.	n.d.

a. From Benner and van Oudenaren, 1975, 1976.

b. Female (C57BL x CBA)F1 mice were either immunized by a single iv injection of 10 µg *E.coli* LPS or primed with 10^7 SRBCs iv and boosted with 4 x 10^8 SRBCs iv 4 months later. The PFC assay was performed 5 days after immunization with LPS and 7 days after the booster injection of SRBCs. The figures represent the number of PFCs per 10^7 viable nucleated cells.

c. n.d., not determined.

V. KINETICS OF THE RESPONSE

Whether or not immunization of mice leads to antibody formation in the bone marrow is dependent on a number of factors (discussed in Section VI). Generally, secondary iv immunization with T-dependent antigens induces bone marrow antibody formation. The bone marrow PFC response has characteristic kinetics, independent of the type of T-dependent antigen and independent of the booster dose (Benner et al., 1974). The response can be divided into two phases. The first phase, of about 1 week duration, is characterized by a much higher number of PFCs in the spleen than in the marrow. During the second phase, on the other hand, the PFC response in the bone marrow is much higher than in all the other lymphoid organs combined (Benner et al., 1974). This relatively high PFC activity in the bone marrow is found for IgM as well as IgG and IgA antibody production (Fig. 4).

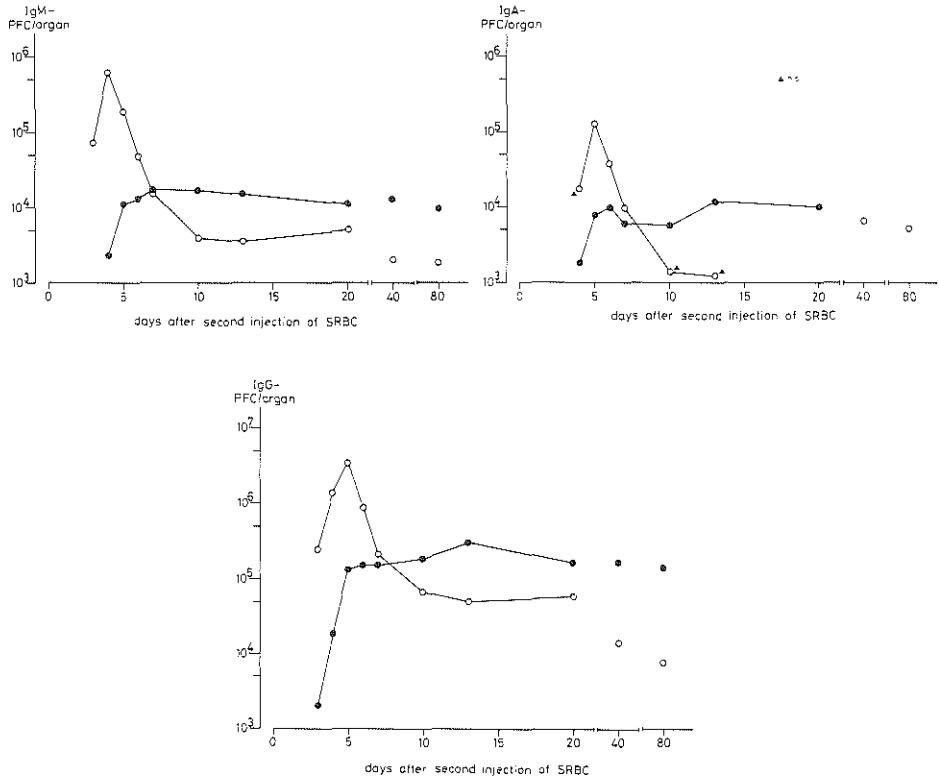

Figure 4. Number of IgM, IgG and IgA PFCs in (C57BL x CBA)F1 mouse spleen (open circles) and bone marrow (solid circles) after two iv injections of SRBCs. The mice were primed with 10^7 SRBCs and boosted with 4 x 10^8 SRBCs 3 months later. A triangle indicates that the number of IgA PFCs above the number of IgM PFCs in the indirect plaque assay was not significant.

VI. CRITICAL FACTORS

A. Primary versus Secondary-Type Responses

As stated in Section I, all initial studies in which the occurrence of antibody formation in mouse bone marrow was investigated revealed little or no PFC activity in this organ after immunization (reviewed in Benner and Haaijman, 1980). Almost all of these studies were limited in one aspect which proved to be crucial: Antibody formation in the bone marrow was assayed only during the primary response. Secondary immunization with a T-dependent antigen, however, induces antibody formation in the bone marrow. This result was found for heterologous erythrocytes (Fig. 4), protein antigen (Hill, 1976), and a variety of T-dependent hapten-carrier complexes (Koch et al., 1981) provided normal, thymus-bearing mice were used. Secondary immunization of nude mice with SRBCs does not lead to significant antibody formation in the bone marrow.

Sometimes antibody formation occurs in the bone marrow after a single injection of a high dose of a T-dependent antigen. However, in such cases PFCs only occur in the marrow during the late phase of the primary response. We have shown that antibody formation in mouse bone marrow is dependent on the availability of memory B cells at the time of the booster injection (Koch et al., 1981). Therefore, the most likely explanation for bone marrow antibody formation during the late phase of the primary response is that in fact a secondary-type response is occurring. The generation of memory B cells during the first phase of the primary response and the persistence of the injected antigen probably account for this phenomenon (see also Section VI, D).

B. Type of Antigen

Thus far, we did not observe exceptions to the rule that secondary iv or ip immunization of thymus-bearing mice with a T-dependent antigen dissolved in saline evokes the appearance of PFCs in the bone marrow. The kinetics of such bone marrow PFC responses has been described in Section V.

Although we have made only minimal investigations into whether antibody formation in the bone marrow also occurs after secondary immunization with T-independent antigens, we have studied the primary response to T-independent antigens. Intravenous immunization with a T-independent antigen such as pneumococcus, *E.coli* bacterium, α-1,6-dextran, or 2, 4-dinitrophenyl (DNP)-Ficoll leads to a weak antigen-specific PFC response in the bone marrow and induces large numbers of PFCs in the spleen (Koch et al., 1982). However, immunization of mice with 10 μg *E.coli* LPS or 100 μg 1,3,5-trinitrophenyl(TNP)-LPS induces high PFC activity in the marrow. After abolishment of the mitogenic moiety of LPS, the lipid A component, by detoxification according to von Eschen and Rudbach (1976), the residual polysaccharide was no longer capable of inducing bone marrow antibody formation (Benner and Haaijman, 1980). The same result was found for the TNP-detoxified LPS complex. Thus antibody formation in the bone marrow after primary immunization with a T-independent antigen seems to require a second signal from a lipid A-like component. T cells are not required for the formation of bone marrow antibody to LPS and TNP-LPS. This can be

deduced from the observation that BALB/c nude mice respond to an iv injection of LPS with bone marrow antibody formation. Although the kinetics of the response of nude mice is essentially the same as that of their euthymic littermates, the number of PFCs in the marrow of nude mice is generally 50-70% lower (Benner and van Oudenaren, 1979).

C. Route of Antigen Administration

Antibody formation is generally most prominent in the lymphoid organ draining the site of immunization. This rule also holds for antibody formation in bone marrow during the secondary response to T-dependent antigens. The highest bone marrow PFC responses are observed with iv or ip booster injections. At low booster doses a splenectomy just before the booster injection can almost completely prevent the bone marrow PFC response (Benner et al., 1977). Thereby the migration of antigen-activated memory B cells from the spleen into the bone marrow is prevented. These antigen-activated memory B cells, which normally migrate into the bone marrow during the first few days after the booster injection, account for the bone marrow PFC response (Koch et al., 1981).

TABLE II

Influence of the Route of Booster Immunization on the Extent of the Bone Marrow Plaque-Forming Cell Response[a].

Surgery	Booster dose of SRBCs	PFCs per bone marrow	
		IgM	IgG
Sham Sx	10^6, iv	8,830 (6,150-12,700)	45,760 (37,090-56,450)
Sx	10^6, iv	2,340 (1,570-3,990)	8,530 (7,380-9,860)
Sx	10^6, sc	860 (610-1,220)	8,340 (6,360-10,940)
Sham Sx	4×10^8, iv	29,510 (26,950-32,310)	255,400 (239,410-272,470)
Sham Sx	4×10^8, sc	8,480 (5,490-13,090)	91,030 (82,970-99,870)
Sx	4×10^8, iv	44,970 (33,110-61,060)	298,900 (232,920-383,590)
Sx	4×10^8, sc	21,820 (20,260-23,500)	96,760 (74,440-125,790)

a. Female (C57BL x CBA)F1 mice were primed with 10^7 SRBCs iv and boosted with either 10^6 or 4×10^8 SRBCs 3 months later. The mice were either splenectomized (Sx) or shamsplenectomized (sham Sx) 3 weeks before the booster immunization. The PFC assay was performed 7 days after the booster injection. Values shown are the geometric mean and 95% confidence limits.

The decrease in the bone marrow PFC response resulting from a splenectomy can be overcome by using a high booster dose. This is illustrated in Table II for SRBCs as antigen. High iv booster doses of SRBCs probably stimulate the lymph nodes so that these organs can replace the spleen. Subcutaneous (sc) booster injections also lead to bone marrow antibody formation, both in nonsplenectomized and in splenectomized mice, but only with high antigen doses (Table II). Thus the antigen must be present in the peripheral lymphoid organs as well as in the bone marrow for PFCs to appear in the latter organ.

Whether or not secondary immunization with a T-dependent antigen will lead to bone marrow antibody formation is independent of the route of primary immunization; iv, ip and sc priming are all effective. However, in the case of SRBCs as antigen, a 100-fold higher antigen dose is required for sc priming than for iv or ip priming in order to prepare for an equally high secondary bone marrow PFC response (Benner et al., 1974). The preparation for bone marrow antibody formation by iv priming with low or moderately high doses of SRBCs can be completely prevented by a prior splenectomy. A splenectomy before iv priming with a high dose of SRBCs is ineffective (Benner and van Oudenaren, 1975; Benner et al., 1977), probably as a result of the stimulation of lymph node tissue by the large amount of iv injected antigen.

D. Use of Adjuvants

The use of an adjuvant can interfere with bone marrow antibody formation. Adjuvants which induce excessive granulopoiesis within the bone marrow (e.g., complete Freund's adjuvant (CFA) and high doses of LPS) abolish the ongoing Ig synthesis in this organ. This is apparent from the observation that several days after such a treatment the presence of Ig-synthesizing cells in the bone marrow can no longer be revealed with the protein A plaque assay. This excessive granulopoiesis can last for 1-4 weeks, depending on the dose and type of adjuvant used.

Secondary immunization of mice with heterologous erythrocytes or hapten-carrier complexes emulsified in CFA does not lead to antibody formation in the bone marrow during the first few weeks after the booster injection. This is merely due to the adjuvant, since injection of an equal amount of the same antigen in saline induces bone marrow antibody formation. Adjuvants which do not induce excessive granulopoiesis, such as alum, do not interfere with antibody formation in the marrow.

In our experience, the occurrence of excessive granulopoiesis, and thus the possibility of detecting antibody formation in the bone marrow, can usually be predicted from the color of the femoral shaft. Excessive granulopoiesis in the marrow is associated with a severe depression of erythropoiesis in this organ. Thus the femur is observed to be white instead of reddish.

Primary immunization with an antigen emulsified in adjuvant leads to prolonged antibody synthesis as compared to that observed after immunization with the antigen dissolved in saline. This can be concluded from comparison of the serum antibody titers of animals treated differently. However, the adjuvant also affects the organ distribution of the antibody-producing PFCs. Several months after a single injection of antigen together with adjuvant, large numbers of PFCs occur in the bone

marrow (Table III). The primary response becomes a secondary-type response, probably because of the slow release of antigen from the adjuvant-induced granuloma.

E. Corticosteroid Level

The extent of the immune response is dependent upon the corticosteroid level (Garbielsen and Good, 1967). Generally, an increase in the corticosteroid level decreases the response, whereas a decrease in the corticosteroid level can increase the response (van Dijk et al., 1976). In mice, corticosteroids have a differential effect upon antibody formation in spleen and bone marrow (Benner et al., 1978; Benner and van Oudenaren, 1979). Daily injections of corticosteroid suppress the primary and secondary anti-SRBC IgM, IgG, and IgA PFC response in the spleen. Bone marrow IgM, IgG, and IgA PFCs were found to be rather resistant to corticosteroid-mediated suppression. These responses were not affected by daily doses of dexamethasone up to 4 mg/kg body weight. A daily dose of 16 mg/kg body weight decreased the bone marrow PFC response when the injections were started before the booster injection. When the corticosteroid injections were started 5 days after the booster injection, the dose of 16 mg/kg body weight did not suppress the

TABLE III

Influence of the Use of Adjuvant on the Number and Organ Distribution of Antibody-Forming Cells 3 Months after Primary Immunization with a Dinitrophenyl-Carrier Complex[a].

		PFCs per spleen		PFCs per bone marrow	
Antigen	Adjuvant	IgM	IgG[b]	IgM	IgG[b]
DNP-KLH	-	1,287 (1,005-1,647)	n.s.	500	3,790 (2,707-5,306)
DNP-KLH	Alum	3,815 (2,512-5,794)	n.s.	1,569 (581-4,238)	18,301 (8,200-40,845)
DNP-CGG	-	500	n.s.	500	n.s.
DNP-CGG	Alum	2,683 (1,010-7,125)	n.s.	865 (424-1,764)	23,924 (13,851-41,321)
DNP-CGG	CFA	2,639 (2,044-3,406)	n.s.	5,872 (2,499-13,799)	84,692 (71,912-99,744)

a. Female (C57BL x CBA)F1 mice were immunized with 100 µg DNP10-chicken gamma globulin (DNP-CGG) or DNP200-keyhole limpet hemocyanin (DNP-KLH) ip 3 months previously. The DNP/carrier ratio is expressed as the number of DNP groups per protein molecule. The antigen was administered either dissolved in saline, precipitated on alum, or emulsified in CFA. Values shown are the geometric mean and 95% confidence limits.

b. n.s., the number of IgG PFCs above the number of IgM PFCs in the indirect plaque assay was not significant.

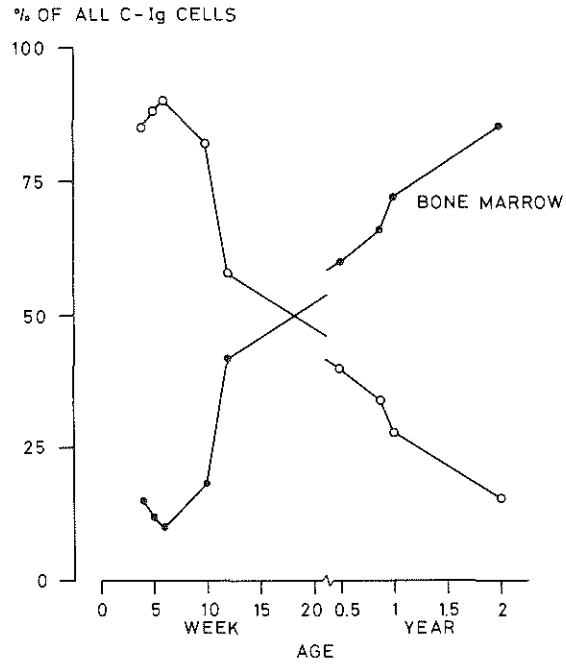

Figure 5. Relative contribution of the bone marrow (solid circles) and the other (open circles) lymphoid organs (spleen, mesenteric lymph nodes, and Peyer's patches combined) to the total number of C-Ig cells in male CBA mice of various ages. From Haaijman et al., 1977.

bone marrow PFC response, in contrast to the splenic PFC response (Benner et al., 1978).

The primary response to the thymus-independent antigen LPS is also affected by corticosteroid injection. In this case also, suppression of the splenic PFC response was proportional to the dose of corticosteroids administered. However, the anti-LPS bone marrow PFC response showed dose-dependent enhancement after daily corticosteroid injections. Thus antibody formation in mouse bone marrow, in contrast to antibody formation in mouse spleen, seems to be very resistant to corticosteroid-mediated suppression.

F. Age

Primary immunization of adult mice with LPS or TNP-LPS and secondary immunization with a T-dependent antigen lead, regardless of the age of the mice used, to antibody formation within the bone marrow. With aging, the day of peak PFC activity in the bone marrow occurs later, just as in the spleen. The extent of the bone marrow PFC response decreases somewhat with increasing age. This is especially true of the IgG PFC response (Blankwater, 1978).

Insight into the actual Ig-synthesizing activity of a mouse can be obtained by measuring the number of Ig-secreting cells or the number of C-Ig cells in various lymphoid organs. When this is done for mice of

various ages, a clear age-related increase is found in the total number of C-Ig cells in all lymphoid organs. At 8 weeks of age a plateau is attained, which is maintained until death (Haaijman et al., 1977). However, organ distribution of C-Ig cells changes considerably during aging. With time, C-Ig cells accumulate in the bone marrow (Fig. 5), and this holds for C-IgM cells as well as C-IgG and C-IgA cells (Haaijman et al., 1977). The gradual increase in the importance of the bone marrow as a site of Ig synthesis throughout the life span probably reflects the gradual adaptation of individual mice to their antigenic environment. As an individual ages, the more often antigenic stimuli from the environment will have been experienced before, and thus more secondary-type responses will prevail. As outlined in Section VI, A, such secondary-type responses involve the bone marrow.

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APPENDIX PAPER II

QUANTITATION OF ANTIBODY PRODUCTION IN MOUSE BONE MARROW DURING THE SECONDARY RESPONSE TO SHEEP ERYTHROCYTES

G. Koch, Christine Weerheijm-De Wit and R. Benner

Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands.

SUMMARY

The antibody production per organ was quantified during the secondary response to sheep erythrocytes (SRBC) by measuring the haemolysin production in short-term cultures of spleen and bone marrow cells. Using this assay it was shown that during the first 5 days of the response the spleen is the major site of antibody production, whereas thereafter the bone marrow is the major source. By comparing the haemolysin production per organ and the total number of haemolysin-producing plaque-forming cells (PFC) per organ it was shown that the mean antibody production per PFC varied both in spleen and bone marrow during the secondary response. The mean antibody production per PFC was minimal when the PFC response was maximal. In the bone marrow the mean production per PFC decreased from day 3 to day 5, probably due to immigration of low producing PFC from the spleen. Beyond day 6 the production per cell increased in both spleen and bone marrow, indicating a further maturation of the PFC.

INTRODUCTION

After primary immunization of mice with thymus-dependent (TD) antigens such as sheep erythrocytes, the spleen is the major site of localization of antibody-producing plaque-forming cells (PFC). The PFC response in the spleen is maximal on day 4 for IgM and day 6 for IgG, while IgA-PFC appear only after relatively high antigen doses and late in the response (7,24). In the early phase of the primary response there is a rather good correlation between the number of anti-SRBC PFC in the spleen and the titer of specific antibodies in the serum. After reaching the peak response, numbers of PFC in the spleen fall off much faster than serum antibody titers. This is by no means compensated for by an increase of PFC numbers in other lymphoid organs (1). Thus, during the second phase of the primary response there is a clear discrepancy between the total number of PFC that can be found in the animal and the serum antibody titer. This might be due to a larger antibody production per PFC during the second phase of the response. Indeed, recently it has been shown that the mean antibody production per PFC can vary during a primary response (23).

During the secondary response to intravenously (iv) injected TD antigens, PFC not only appear in the spleen, but also in large numbers in the bone marrow (7). Initially, the spleen contains the majori-

ty of all PFC of the animal, but after day 7 to 10 the response of the bone marrow becomes 3 to 10 times as high as in all other lymphoid organs together. This implies that, if the mean antibody production by PFC in the bone marrow would be the same as by PFC in the spleen, the bone marrow would be the major source of serum antibodies during the second phase of secondary type responses. However, such quantitative data on the antibody production per PFC in spleen and bone marrow are not available, yet. Therefore we considered it worthwhile to study the antibody production *in vitro* by spleen and bone marrow cells during the secondary response to SRBC. The results show that the antibody production *per PFC*, (a) varies in both spleen and bone marrow in different phases of the response, (b) is different for spleen and bone marrow during the early phase of the response, and (c) is both in spleen and bone marrow minimal during the peak response, and maximal during the late phase of the response.

MATERIALS AND METHODS

Mice. (C57BL/Rij x CBA/Rij)F1 female mice, 16-20 weeks old, were used. They were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Antigen and immunization. SRBC stored in Dulbecco's solution, were washed three times in phosphate buffered saline (PBS, pH 7.2) before use. Mice were primed by an iv injection of 10^7 SRBC. For secondary immunization mice received an iv booster injection of 4×10^8 SRBC. The interval between primary and secondary antigen injection was 2-3 months.

Assay for PFC. Cell suspensions were prepared and assayed for IgM- and IgG-PFC as described previously (7,8).

Quantitation of antibody production. The quantitation of antibody production by lymphoid cells *in vitro* was done as described extensively by van Dijk and Bloksma (23) with some minor modifications. Briefly, 0.5 ml of appropriately diluted lymphoid cells suspensions were mixed with an equal volume of washed SRBC (10^9/ml) in 15 ml tubes. After incubation for 45 min at 37°C, 0.1 ml 1:16 diluted rabbit antiserum directed against mouse IgG was added. This antiserum was raised as described previously by Zaalberg et al. (25). The same batch was used in the *in vitro* haemolysis assay and the plaque assay. The mixture was incubated for 15 min at 37°C. Subsequently, 0.5 ml of 1:4 diluted guinea pig serum (Flow batch 44022008) was added as a source of complement and incubated for another 20 min at 37°C. Finally, 2 ml of PBS was added whereafter the tubes were centrifuged for 5 min at 1500 g. The percentage of haemolysis was calculated from the extinction of the supernatant at 540 nm (E_{540}). As a positive control was used the E_{540} of supernatants from a similar number of SRBC lysed by freshly prepared 0.04% NH_4Cl. The data were corrected for the E_{540} of mixtures containing SRBC, complement and rabbit anti mouse IgG only.

The amount of antibody needed for 50% haemolysis was constant. Therefore the number of cells secreting this amount of antibody can be

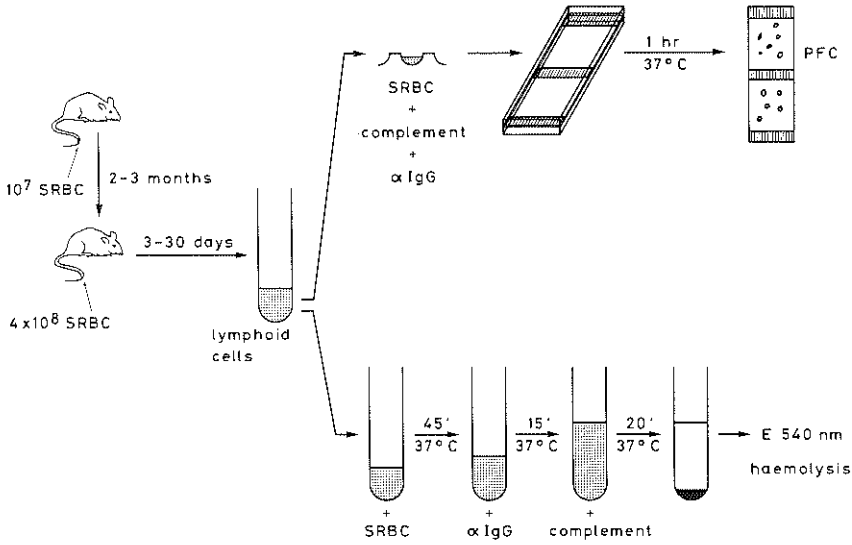

Figure 1. Scheme of the experimental set-up used to determine the mean amount of antibodies produced per individual PFC in spleen and bone marrow during the secondary response of mice to SRBC.

used as a measure for the antibody production. This number is called the lytic concentration (LC50).

Calculation of the antibody production per PFC. For quantitation of the antibody production per PFC, cell suspensions of spleen and bone marrow from individual mice were simultaneously assayed for the number of PFC per 10^7 nucleated cells and for the LC50 value. A scheme of the experimental set up is shown in Fig. 1. Since the antibody production is defined as the number of cells needed for 50% haemolysis (LC50), the amount of antibody secreted by 10^7 cells will be 10^7/LC50. The mean antibody production per PFC can be calculated as 10^7/LC50xPFC.

Calculation of PFC number and antibody production of the total bone marrow. Femoral bone marrow was used for preparing bone marrow cell suspensions. For the calculation of the PFC response and antibody production in the bone marrow of the whole animal we multiplied the response by the bone marrow cells of the two femurs with a factor of 7.9, since ^{59}Fe distribution studies have shown that 12.6% of the total bone marrow is located in both femurs together (11). Previously no differences could be detected between the PFC responses evoked by fixed numbers of nucleated bone marrow cells from femur, tibia, humerus, rib and sternum, which indicates that the PFC activity of the femoral bone marrow indeed is a reliable measure for the marrow of the whole animal (8,9).

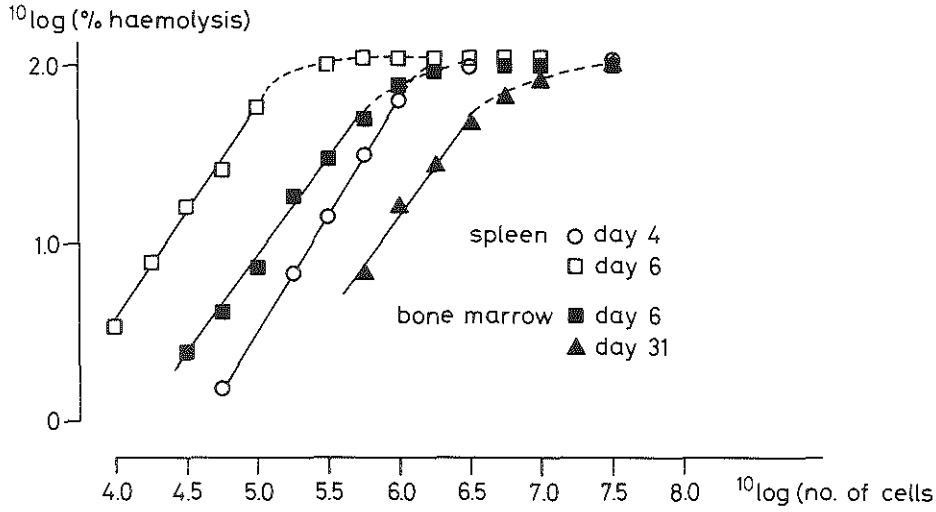

Figure 2. Haemolysin production by graded numbers of spleen and bone marrow cells. Data are presented of the responses by spleen cells on day 4 (○) and 6 (□) and by bone marrow cells on day 6 (●) and 30 (▲) after secondary immunization with 4 x 10^8 SRBC iv.

RESULTS

Dose effect curve during the secondary response in spleen and bone marrow

After primary immunization of mice with SRBC, the IgM-haemolysin production *in vitro* as a function of the number of spleen cells follows a sigmoid curve. After logarithmic transformation of both parameters a linear function is obtained for values between 10 and 80% haemolysis (23). We determined whether this also holds for spleen and bone marrow cells at different intervals after secondary immunization with SRBC, and for mixtures of IgM and IgG antibodies. This was indeed the case (Fig. 2). Furthermore, the slopes of curves did not vary either with the source of the haemolysin-producing cells (spleen *versus* bone marrow), the interval between booster injection and testing, or the class of antibodies tested. In figure 2 the data are shown obtained with spleen cells on day 4 and 6, and with bone marrow cells on day 6 and 30 after booster injection. For the curves found on other days, and for curves reflecting the IgM-haemolysin production only, similar slopes were found. From the similarity of the slopes of these curves we conclude that if the mean avidity of the antibodies would change during the secondary response against SRBC, or would be different for antibodies produced by spleen and bone marrow, it does not influence the method used to measure the antibody production per PFC.

Antibody production per PFC in spleen and bone marrow

The PFC response and antibody production in spleen and bone marrow of mice boosted with 4 x 10^8 SRBC were assayed at various intervals after booster injection according to the scheme shown in figure 1. The

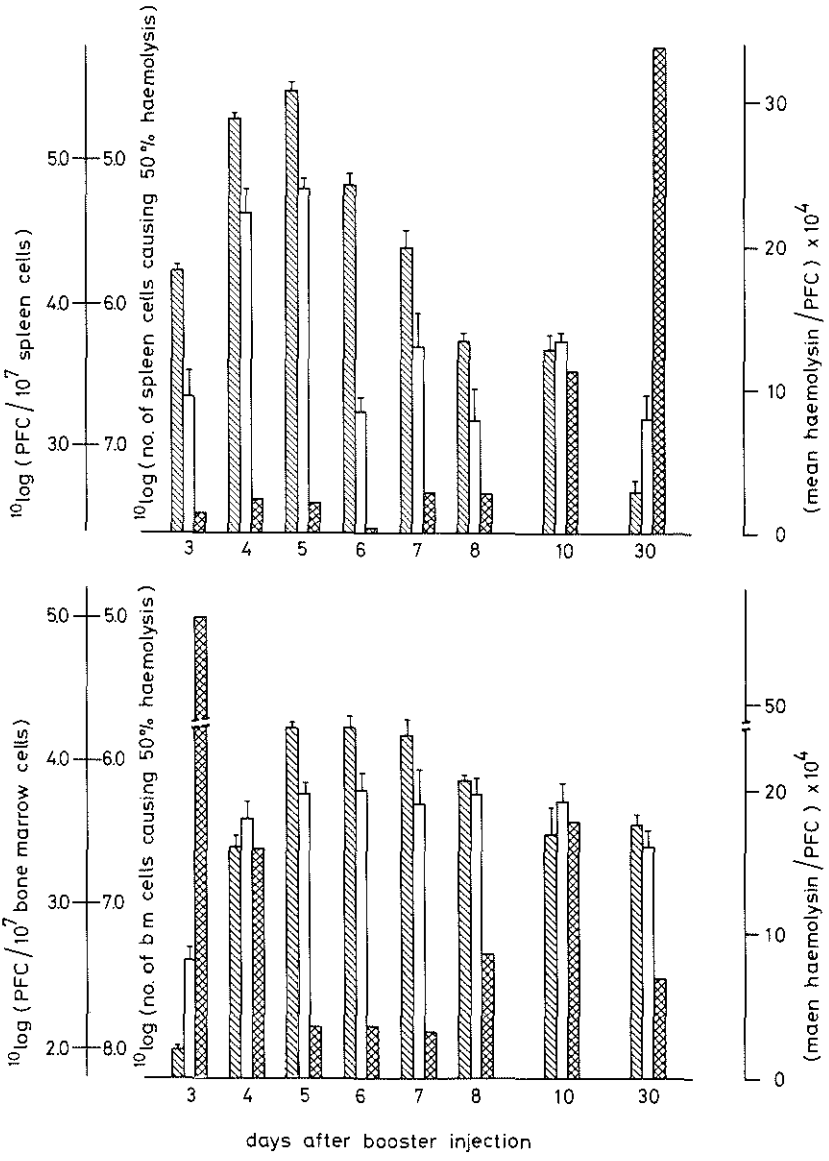

Figure 3. Quantitation of antibody production in spleen (upper part) and bone marrow (lower part). The number of PFC (hatched columns) and the number of cells needed for 50% haemolysis (open columns) was determined at different days after the booster injection of 4 x 10^8 SRBC iv. The same batch and the same concentration of anti-IgG was used in the PFC assay and the haemolysin production assay. From the mean number of PFC and the mean number of cells needed for 50% haemolysis the mean haemolysin production per PFC (cross-hatched columns) was calculated. The columns represent the geometric mean of 5-8 individually tested mice, and the vertical bars the standard error of the mean.

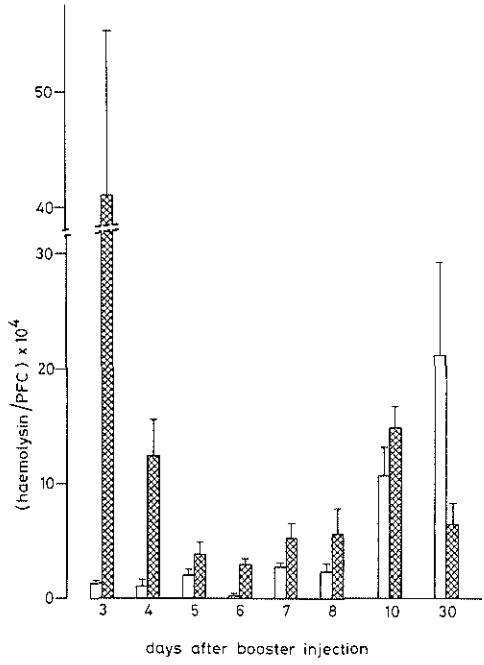

Figure 4. Quantitation of the mean antibody production per PFC in spleen (open columns) and bone marrow (cross-hatched columns). The figure summarizes the data of mean haemolysin production per PFC for spleen and bone marrow as shown in Fig. 3. The vertical bars represent the standard error of the mean.

data presented here (Fig. 3 and 4; Table 1) represent one out of three similar experiments, all yielding essentially the same results. In the spleen the PFC response and the antibody production were maximal on day 4 after booster injection (Fig. 3, upper part). Thereafter they gradually decreased. From the mean number of PFC and the mean number of cells required for 50% haemolysis, the mean antibody production per PFC was calculated. In the spleen, the mean production per PFC was nearly constant from day 3 until day 8 after booster injection, but gradually increased after day 8. One month after the booster injection the production per PFC was 5 to 25 times as high as was found during the first phase of the secondary response.

In the bone marrow the PFC response and the antibody production were maximal around day 6 (Fig. 3, lower part). After day 6 the response decreased only slightly. After day 10 the PFC response per 10^7 nucleated cells was higher in the bone marrow than in the spleen. This is in agreement with our previous results (7). The mean antibody production per PFC in the bone marrow was high at the beginning of the secondary response (Fig. 3), but decreased to 5-30% of that value from day 3 to day 5. From day 5 to day 7 the antibody production per PFC in the marrow was minimal. After day 7 the production increased again.

In figure 4 we have summarized the data of the mean antibody pro-

duction per PFC for spleen and for bone marrow. At all intervals after booster injection tested, except on day 30, the mean production per PFC was as high or higher in the bone marrow as in the spleen. This difference was most prominent during the early phase of the response.

Total antibody production in spleen and bone marrow

From the mean antibody production per PFC and the number of PFC per whole spleen and bone marrow the total antibody production by spleen and bone marrow was calculated. The data of Table 1 show that the spleen is the major source of specific serum antibodies during the first 5 days of the secondary response. However, after day 5 the total bone marrow is the major production site of specific antibodies.

DISCUSSION

The quantitative data of antibody production per organ presented in this paper show that the mouse bone marrow is the major source of antibodies during the second phase of the secondary response to SRBC. This extends our previous data showing that during secondary immune response of mice the majority of all PFC are localized in the marrow beyond the first week after booster injection (7). Furthermore, the results show that the mean haemolysin production per PFC can be different in different lymphoid organs of the same animal and at different

TABLE I

Total Antibody Production in Spleen and Bone Marrow

Days after booster injection[a]	(IgM+IgG)-PFC/organ $\times 10^{-3}$		Antibody production/organ[b]	
	spleen	bone marrow	spleen	bone marrow
3	508(1.1)[c]	3(1.3)	66(1.1)	16(1.4)
4	2,908(1.7)	88(1.2)	1,095(1.2)	122(1.2)
5	6,886(1.2)	638(1.1)	1,451(1.2)	222(1.1)
6	1,768(1.3)	744(1.2)	53(1.3)	268(1.2)
7	671(1.4)	764(1.2)	174(1.4)	244(1.2)
8	72(1.1)	333(1.1)	19(1.1)	283(1.1)
10	82(1.2)	44(1.3)	93(1.2)	153(1.2)
30	10(1.2)	148(1.2)	31(1.3)	102(1.2)

a. Mice were primed with 10^7 SRBC iv and boosted with 4×10^8 SRBC iv after 2 to 3 months. On different days after the booster injection the numbers of direct and indirect PFC were measured.

b. From the number of PFC per organ and the mean antibody production per PFC the total antibody production per organ was calculated.

c. Geometric mean $\pm$ SEM of five to eight mice.

intervals after secondary immunization.

Calculation of the total antibody production per organ shows that around day 6 the bone marrow becomes the major site of antibody production. This is in agreement with earlier findings on the late phase of the primary response of guinea pigs to bacteriophage ØX174 (15) and ovalbumin (4,6). Also in tissue culture experiments with cells from various lymphoid organs of rabbits after primary and secondary immunization with ovalbumin and type 3 pneumococci it was shown that the bone marrow made the major contribution to the overall antibody and immunoglobulin synthesis (5). Similar *in vitro* experiments with human lymphoid tissues indicated that also in man the bone marrow is the major site of antibody (19) and immunoglobulin production (18).

In the present study we have used an *in vitro* haemolysis assay, and did not study the plaque diameter, because the plaque diameter is not only dependent on the amount of antibodies secreted by the PFC, but also on the affinity of the secreted antibodies (12-14). Our method, however, is not influenced by changes in the avidities of the antibody molecules, as can be concluded from the fact that throughout the experiments the same slope was found for all titration curves of antibody production (e.g., Fig. 2). This allows us to interpret the data in terms of mean amount of antibodies produced per PFC.

Recently van Dijk and Bloksma (23) reported data on the haemolysin production per PFC in the spleen during the primary response to SRBC. They found that the mean production varied during the primary response and decreased during the exponential increase of the PFC number. The production per PFC was minimal during the peak PFC response. We found a similar variation of antibody production per PFC in spleen and bone marrow during the secondary response to SRBC. As in the primary response, the production in bone marrow and spleen was minimal when the PFC response was maximal. The high antibody production per PFC found on day 3 in the bone marrow is dependent on antigenic stimulation since on the day of the booster injection no antibody production could be demonstrated in this organ with our haemolysin assay.

The sharp decrease of the production in the marrow from day 3 to day 5 does not necessarily means that the production by these "high producer" PFC decreased. It might also be due to PFC with a low production immigrating into the marrow, thereby decreasing the mean production per PFC. Indeed, experiments with parabiotic mice and experiments involving splenectomy after secondary stimulation have shown that the bone marrow PFC are derived from memory B cells activated by antigen in the spleen (10,17).

Maturation of the antibody-producing cells probably causes the increase of antibody production per PFC during the second phase of the secondary response in the spleen and the marrow. Electron microscopic studies of PFC have shown that early in the response lymphoblasts and immature plasma blasts predominate, whereas later on nearly all PFC have the morphology of mature plasma cells (16,21,22). During this maturation the cells develop more rough and smooth endoplasmic reticulum, i.e., are better equipped for protein synthesis. Moreover, Andersson et al. (3) and Melchers and Andersson (20) have shown that during maturation of immunoglobulin-synthesizing cells after *in vitro* stimulation of B cells with lipopolysaccharide the ratio between the immunoglobulin and protein synthesis increases. Also different IgM-

producing myelomas can have a different Ig production per cell. These myelomas may represent different maturation stages of the plasma cell lineage (2).

In conclusion we have shown that the mean antibody production per PFC during the secondary response to SRBC (a) can be different in spleen and bone marrow of the same animal, (b) is different for spleen and bone marrow early in the response and (c) is minimal both in spleen and bone marrow when the PFC response is maximal. Furthermore, we have shown that in mice the bone marrow is the major site of antibody production during the second phase of the secondary response to SRBC.

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APPENDIX PAPER III

THE MECHANISM OF THYMUS-DEPENDENT ANTIBODY FORMATION IN BONE MARROW

Guus Koch[1], Dennis G. Osmond[2], Michael H. Julius[3] and Robbert Benner[1]

[1] *Department of Cell Biology and Genetics, Erasmus University Rotterdam,*
[2] *Department of Anatomy, McGill University, Montreal, Canada,*
[3] *Basel Institute for Immunology, Basel, Switzerland.*

SUMMARY

During the primary immune response of mice to intravenously administered thymus-dependent antigens the spleen is the major site of localization of antibody-producing plaque-forming cells (PFC). During the secondary response, on the other hand, large numbers of PFC not only appear in the spleen, but also in the bone marrow. By inducing B memory cells with a DNP-carrier complex and activating the DNP-specific B memory cells with the same hapten conjugated to a heterologous carrier, we show in this paper that B memory cells, but not necessarily T memory cells, must be present before booster immunization for PFC to appear in the bone marrow. The origin of the PFC that appear in the bone marrow during secondary type immune response was studied in parabiotic mice consisting of members congenic for the Igh-1 locus. From analysis of the allotype of antibodies produced by PFC in the marrow of such pairs of parabionts it appeared that antibody formation in bone marrow is dependent on the immigration into the marrow of B memory cells activated in peripheral lymphoid organs. Consistent with such a migration of activated cells, radioautographic studies in guinea pigs demonstrated an influx of newly formed mononuclear cells into the bone marrow via the blood stream during the first three days after intravascular antigen administration.

INTRODUCTION

After primary immunization of mice with thymus-dependent antigens spleen and lymph nodes are the major sites of localization of antibody-producing plaque-forming cells (PFC), dependent on the route of antigen administration. No substantial antibody formation occurs in the bone marrow during the primary response to thymus-dependent antigens. This has been reported by several independent investigators (reviewed by Benner and Haaijman (1)). However, after secondary immunization with the same thymus-dependent antigen PFC appear not only in the spleen, but also in the bone marrow (2,3). During the first week of the secondary response most PFC are localized in the peripheral lymphoid organs, but beyond that period the majority of all PFC of the animal are localized in the bone marrow. After the first week also the majority of the antibodies are produced by the bone marrow (Appendix paper II).

Adoptive transfer experiments and experiments with parabiotic mice have shown that the appearance of PFC in the bone marrow of mice during a secondary type immune response is dependent on potentially circulat-

ing memory cells (4,5). The peripheral lymphoid organs such as spleen and lymph nodes not only produce these memory cells (6), but are also needed for the initiation of the PFC response in the bone marrow (7). Splenectomy until two days after an iv booster injection completely prevents the appearance of PFC in the marrow. At day 4, however, splenectomy no longer prevents antibody formation in the bone marrow.

In the present study we investigated the cellular requirements for antibody-formation in the bone marrow and the origin of the antibody forming cells. The data presented show that hapten-primed B memory cells, but not carrier-primed T memory cells, must be present at the time of the booster injection for hapten-specific PFC to appear in the marrow. In addition, we show that antigen-dependent activation of B memory cells in the spleen is followed by their migration into the marrow where they account for antibody formation.

MATERIALS AND METHODS

Animals. Female (C57BL/Rij x CBA/Rij)F1, BALB/c ($H\text{-}2^d$), BAB/14 ($H\text{-}2^d$), CWB/13($H\text{-}2^b$) and CSW($H\text{-}2^b$) mice were used. The (C57BL x CBA)F1 mice, 14-20 weeks old, were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. The BALB/c CWB/13 and CSW mice, 8-12 weeks old, were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland. The BAB/14 mice were bred at the Basel Institute for Immunology. Male Hartley guinea pigs were used at 350-450 g body weight, approximately 6 weeks of age.

Antigen and immunization. Keyhole limpet hemocyanin (KLH; lot no. 630054) was obtained from Calbiochem-Behring Corp. (La Jolla, CA) as a slurry in 65% ammonium sulfate. Before use it was centrifuged at 25,000 g for 30 min. The precipitate was dissolved in phosphate-buffered saline (PBS; pH 7.2) and desalted on Sephadex G-25. Chicken gamma globulin (CGG) was prepared by ammonium sulfate precipitation of normal chicken serum. DNP-KLH and DNP-CGG were prepared according to the method of Eisen (8). KLH was dinitrophenylated to a level of 6-8 DNP molecules per 10^5 Dalton units, and CGG to a level of 8-10 DNP molecules per protein molecule. In case of immunization with hapten-carrier complexes, the mice were primed by an intraperitoneal (ip) injection of 100 µg of DNP-KLH or DNP-CGG adsorbed on alum, unless stated otherwise. Three months later the mice received a booster injection of 100 µg DNP-KLH intravenously (iv) or 100 µg DNP-CGG adsorbed on alum ip. Some groups of mice received a supplemental immunization with 10 or 100 µg of carrier protein homologous to the carrier of the booster antigen. Such a supplemental immunization was given 5 weeks before the booster injection. Sheep erythrocytes (SRBC) from a single donor were used. They were stored in Alsever's solution and washed three times in a balanced salt solution (BSS) before use. Secondary responses to SRBC were induced by primary immunization with 10^7 SRBC iv and secondary immunization with 2×10^6 SRBC iv. The interval between primary and secondary antigen injection was 3 months.

Parabiotic mice. Parabionts were joined laterally under Avertin (Merck-Schuchardt) anesthesia (9). The mice were united by suturing the peritoneal and abdominal wall muscles without leaving a communication between the two abdominal cavities. The connective tissue along the thorax was also sutured. In addition, the scapulae were joined. The skin was closed with linen thread from behind the ears to the femora.

Splenectomy. Splenectomy (Sx) of mice that had to be parabiosed was done during the surgery for parabiosis. Splenectomy and sham-splenectomy (ShSx) of non-parabiotic mice was done under Avertin anesthesia as described previously (2).

Assay for PFC. Cell suspensions were prepared and assayed for IgM- and IgG-PFC as described previously (2,6). TNP-conjugated SRBC were used for assaying DNP-specific PFC. SRBC were conjugated with trinitrobenzenesulfonic acid as described by Rittenberg and Pratt (10). Bone marrow PFC activity was determined in the marrow of both femurs. For the calculation of the PFC response in the bone marrow of the whole animal we multiplied the response by the marrow cells of the two femurs with a factor of 7.9, since ^{59}Fe-distribution studies have shown that 12.6% of the total bone marrow is located in both femurs together (11). Previously, no differences could be detected between the PFC activity of bone marrow cells from femur, tibia, humerus, rib and sternum, which indicates that the PFC activity in various compartments of mouse bone marrow is about the same (7,12). IgG-PFC were developed by using a rabbit antiserum directed to mouse IgG. This antiserum was prepared as described previously by Zaalberg et al. (13). The mouse antiallotype antisera specific for the Igh-1^a and Igh-1^b allotype were prepared and kindly provided by Dr. Michel Seman at the Laboratory of Immunodifferentiation, University of Paris, France.

Radioautography of immigrant cells in bone marrow. To provide direct evidence of a blood-borne migration of cells into the bone marrow the method of partial body labelling with ^{3}H-thymidine was used in guinea pigs, as previously described by Osmond and Everett (14,15). Guinea pigs were given an intracardiac injection of TAB vaccine (0.5 ml; 750 x 10^6 typhoid organisms; 500 x 10^6 paratyphoid A and B organisms) under light ether anesthesia. At daily intervals from 1 to 5 days thereafter, ^{3}H-thymidine (1 µCi/g body weight; specific activity 6.7 Ci/mM) was administered by intracardiac injection under ether anesthesia while the circulation to the left hind limb was occluded by an elastic compression bandage. After allowing 27 min. for the clearance and cellular incorporation of most circulating ^{3}H-thymidine, any residual free ^{3}H-thymidine was diluted by intracardiac injection of 150 mg nonradioactive thymidine, approximately 10^4-fold greater than the original dose of ^{3}H-thymidine. Three min. later the occlusive bandages were released, restoring the circulation to the hind limb, and the anesthesia was terminated. This procedures produces an initial labelling of DNA-synthesizing cells in all tissues except those of the occluded hind limb (14). Guinea pigs were killed by ether overdose at a constant interval of 24 hr after the injection of ^{3}H-thymidine. Tibial marrow cells were suspended, counted, smeared, dipped in melted NTB-2 emulsion (Eastman Kodak, Rochester, N.Y.), exposed for 7 days, developed and stained

through the fixed emulsion with a modified MacNeal's stain. Control experiments, performed at each post-antigen interval, were identical to the above except for the injection of vaccine suspending fluid alone, instead of TAB organisms. Approximately 10,000 nucleated cells were examined along the entire length of each bone marrow smear. The number of radioautographic grains overlying individual cells was recorded, grouping the labelled cells (<10 grains) into morphological categories, as described in Table IV.

RESULTS

Cellular requirements for antibody formation in the bone marrow

From previous studies we know that antibody formation to T-dependent antigens in the bone marrow only occurs after multiple injections of the same antigen (4,5). The phenomenon is dependent upon the presence of long-lived lymphocytes induced by priming which are operationally defined as memory cells. To investigate whether only B memory cells, only T memory cells, or B as well as T memory cells are required for the bone marrow PFC response, we primed B and T cells separately by means of heterologous hapten-carrier conjugates. Thus, we compared the response to iv administered DNP-KLH of five different groups of mice (Table 1). Group I was a control group that was primed to DNP-CGG at the same time as other DNP-CGG-primed mice, and was used to measure the background (DNP-CGG-induced) anti-TNP PFC activity at the moment that the other groups of mice received a booster injection of DNP-KLH. B and T cells of the mice of group II were neither primed to DNP nor to KLH before immunizing with DNP-KLH. In the mice of group III KLH-specific T memory cells were induced by separate priming with KLH only. In this group DNP-specific B cells were unprimed. In the mice of group IV DNP-specific B memory cells were induced by priming with DNP-CGG, while in mice of group V DNP-specific B memory cells as well as KLH-specific T memory cells were induced by separate priming with DNP-CGG and KLH, respectively. The priming with DNP-CGG was done 3 months, and the priming with KLH was done 5 weeks before immunization with DNP-KLH. In the groups I-V, the number of TNP-specific PFC was enumerated in spleen and bone marrow on day 4 and 6 after the iv DNP-KLH injection. These intervals were chosen because the PFC response in the spleen is maximal on day 4, and in bone marrow between day 6 and 12 (2). T cell priming appeared to have no enhancing influence on the PFC response in spleen and bone marrow (Table 1). In contrast, DNP-specific B cell priming greatly facilitated the TNP-specific PFC response in the bone marrow and enhanced the response in the spleen. However, this result was only found for the IgG response.

In Table II data are presented from a similar experiment, but here B cell priming was done with DNP-KLH, and T cell priming with CGG. The TNP-specific PFC response was elicited by booster injection with DNP-CGG. In this experiment all injected antigens had been adsorbed on alum and were administered ip. The number of TNP-specific PFC were enumerated on day 8 and 10 after the booster injection and not on day 4 and 6, because the maximal PFC response is delayed when the antigen is given together with adjuvant, and when it is injected ip instead of iv (data not shown). High numbers of TNP-specific PFC only appeared in the bone

TABLE II

The Influence of Hapten and Carrier Priming upon the Anti-TNP PFC Response to DNP-CGG in the Bone Marrow

Group	Primary immunization	Supplemental immunization[a]		Anti-TNP PFC/Organ			
				Spleen		Bone Marrow	
				IgM	IgG	IgM	IgG
I	DNP-KLH	-	day 0	1,763 (1,484-2,092)	n.s.[c]	1,721 (1,498-1,977)	20,323 (16,430-25,138)
II	PBS	PBS	day 8	57,738 (45,091-79,144)	1,365,052 (1,067,570-1,745,429)	1,867 (1,345-2,591)	21,118 (15,516-28,744)
II	PBS	PBS	day 10	5,150 (4,228-6,274)	74,198 (42,718-128,877)	<500	23,823 (15,230-37,267)
III	PBS	CGG	day 8	4,228 (3,738-4,920)	n.s.	<500	2,244 (1,549-3,251)
III	PBS	CGG	day 10	4,728 (3,878-5,763)	n.s.	<500	3,014 (1,638-5,547)
IV	DNP-KLH	PBS	day 8	6,647 (4,938-8,946)	822,722 (611,115-1,107,785)	3,560 (2,477-5,117)	622,355 (462,040-838,294)
IV	DNP-KLH	PBS	day 10	3,312 (2,725-4,027)	310,974 (230,428-419,677)	1,537 (630-3,472)	289,490 (226,838-369,447)
V	DNP-KLH	CGG	day 8	2,518 (1,531-4,140)	7,586 (5,571-10,330)	1,399 (629-3,109)	67,273 (51,301-83,217)
V	DNP-KLH	CGG	day 10	2,973 (2,375-3,722)	8,180 (4,477-14,944)	4,495 (3,429-5,894)	43,222 (31,819-58,171)

a. Mice were primed by ip injection of 100 µg DNP-KLH. Two months later the mice of the groups III and V received a supplemental ip injection of 100 µg CGG. Five weeks later all mice, except those of group I, were boosted with an ip injection of 100 µg DNP-CGG. All antigens were adsorbed on alum. The numbers of anti-TNP PFC were measured 8 days and 10 days after the booster injection of DNP-CGG. The mice of group V were only primed with DNP-KLH. Each group consisted of 5 mice.

b. Geometric mean ± 1 SEM.

c. n.s. means that the number of IgG-PFC above the number of IgM-PFC in the indirect plaque assay was not significant.

TABLE I

The Influence of Hapten and Carrier Priming upon the Anti-TNP PFC Response to DNP-KLH in the Bone Marrow

Group	Primary immunization	Supplemental immunization		Anti-TNP PFC/Organ			
				Spleen		Bone Marrow	
				IgM	IgG	IgM	IgG
I	DNP-CGG	-	day 0	2,639 (2,407-2,893)	n.s.[c]	5,847 (4,137-7,489)	84,692 (79,846-89,833)
II	PBS	PBS	day 4	391,281[b] (345,900-442,616)	n.s.	<500	<500
II	PBS	PBS	day 6	29,500 (24,102-36,229)	n.s.	2,672 (2,067-3,452)	5,242 (3,711-7,405)
III	PBS	KLH	day 4	139,280 (115,522-167,924)	n.s.	1,145 (815-1,597)	n.s.
III	PBS	KLH	day 6	12,770 (10,487-15,549)	n.s.	4,176 (2,820-6,182)	n.s.
IV	DNP-CGG	PBS	day 4	25,209 (23,746-26,762)	132,352 (92,394-189,591)	2,818 (1,702-4,665)	53,430 (51,468-55,468)
IV	DNP-CGG	PBS	day 6	93,246 (73,083-118,971)	1,274,041 (1,037,798-1,564,061)	9,851 (4,294-22,548)	303,566 (246,748-373,468)
V	DNP-CGG	KLH	day 4	94,828 (83,324-107,920)	1,589,475 (1,372,128-1,841,251)	2,068 (1,358-3,149)	94,375 (85,179-104,563)
V	DNP-CGG	KLH	day 6	28,234 (22,364-35,694)	957,583 (759,499-1,207,328)	8,558 (7,389-9,413)	286,805 (228,883-359,385)

a. Mice were primed by ip injection of 100 µg DNP-CGG emulsified in CFA. Two months later the mice of the groups III and V received a supplemental iv injection of 10 µg KLH. Five weeks later all mice, except the mice of group I, were boosted by iv injection of 100 µg DNP-KLH. The numbers of anti-TNP PFC were measured 4 and 6 days after the booster injection of DNP-KLH. The mice of group I were only primed with DNP-CGG and assayed for anti-TNP PFC three months later. Each group consisted of 5 mice.

b. Geometric mean ± 1 SEM.

c. n.s. means that the number of IgG-PFC above the number of IgM-PFC in the indirect plaque assay was not significant.

marrow after B cell priming by DNP-KLH. CGG-specific T cell priming had no improving influence on the anti-TNP PFC response in the bone marrow. Instead, the anti-TNP PFC response upon immunization with DNP-CGG after priming with both DNP-KLH and CGG was even 10-fold lower than after priming with DNP-CGG only (Table II).

The above results show that for antibody formation in bone marrow the presence of B memory cells is obligatory. In contrast, T memory cells need not to be present at the time of the booster immunization.

The origin of antibody-forming cells in the bone marrow

The spleen is needed until 4 days after the booster injection of SRBC for PFC to appear in the bone marrow (8). At day 4 Sx can no longer prevent bone marrow antibody formation. To investigate the role of the spleen in the initiation of the PFC response in the bone marrow we parabiosed mice congenic for the Igh-1 locus. This was done 3 months after priming with 10^7 SRBC iv. One member of each pair of parabionts was Sx. The surgery for joining the mice by means of parabiosis and the Sx were performed at the same time. Five days after the surgery all mice were boosted by iv injection of 2×10^6 SRBC. This low booster dose of SRBC was chosen, because higher doses of SRBC can overcome the effect of Sx upon the antibody formation in the bone marrow, probably because of antigenic stimulation of lymph node tissue (8). Eight days after the booster immunization the numbers of IgM, Igh^{a+} and Igh^{b+}-PFC were determined in the bone marrow of both members. Parabiosis of a non-splenectomized mouse and a Sx mouse could account for a clear anti-SRBC PFC response in the bone marrow of the Sx member of the parabiotic pair (Table III), confirming our previous results (8). In parabionts consisting of one splenectomized CWB mouse (Igh^b) and one CWS mouse (Igh^a) 80-90% of the indirect PFC in the bone marrow of the CWB member were of the Igh^a allotype (Table III). The number of Igh^{b+}-PFC found in the bone marrow of the parabiosed Sx CWB mouse was not higher than in the marrow of non-parabiotic Sx CWB control mice. Comparable results were found in similar experiments with Sx CSW mice parabiosed with non-Sx CWB mice, and with Sx BAB/14 mice (Igh^b) parabiosed with normal BALB/c mice (Igh^a) (Table III). Thus, the PFC that appear in the bone marrow of both members of the parabiotic mice secrete antibody molecules of the allotype of the spleen-bearing member. Thus, antibody formation in bone marrow appears to be mediated by the immigration of B memory cells into the bone marrow, which are activated by antigen in the periphery.

Migration of cells into the bone marrow after antigen administration

Radioautography revealed an immigration of cells into guinea pig bone marrow that was substantially increased after antigenic stimulation (Table IV). In control animals not given antigens, small numbers of well-labelled cells appeared in tibial marrow 24 hr after administering a single pulse of ^{3}H-thymidine, the incorporation of the isotope having been initially excluded from the hind limb itself by local circulatory occlusion. Such labelled cells were thus derived from DNA-synthesizing cells that were located originally in extramyeloid tissues and subsequently migrated into the bone marrow during the next 24 hr. The number of labelled cells entering the bone marrow at various intervals after control injections showed no change with time, and are grouped together in Table IV. In contrast, for the first 3 days after intra-

TABLE III

Secondary PFC Response in the Bone Marrow of Parabiotic Ig-Congenic Mice Consisting of 1 Sx and 1 ShSx member

Mice[a]	IgM-PFC		Igh-1^{a+}-PFC		Igh-1^{b+}-PFC	
	Sx	ShSx	Sx	ShSx	Sx	ShSx
CWB (Sx) - CSW parabionts[b]	2,315[c] (1,714-3,128)	7,190 (5,574-9,266)	15,200 (10,629-21,690)	35,450 (29,790-42,310)	2,560 (2,151-3,051)	n.s.
CWB (Sx) and CSW controls	3,044 (2,611-3,550)	9,240 (6,372-13,412)	n.s.	43,280 (37,635-49,765)	3,832 (3,361-4,378)	n.s.
CSW (Sx) - CWB parabionts	5,100 (3,410-7,628)	7,395 (5,320-10,310)	6,503 (4,116-10,274)	n.s.	13,989 (9,998-19,258)	34,807 (27,625-43,826)
CSW (Sx) and CWB controls	4,463 (3,188-6,290)	10,820 (9,409-12,465)	5,168 (2,936-9,110)	n.s.	n.s.	52,875 (42,988-65,300)
BAB/14(sx) - BALB/c parabionts	1,927 (1,567-2,365)	5,220 (3,810-7,172)	18,241 (13,029-25,553)	31,590 (25,476-39,181)	2,116 (1,571-2,852)	n.s.
BAB/14(Sx)and BALB/c	2,680 (2,330-3,094)	8,290 (6,579-10,451)	n.s.	71,740 (65,817-78,225)	4,358 (3,857-4,939)	n.s.

a. The allotype of CSW and BALB/c mice is Igh^{a} and of CWB and BAB/14 mice is Igh^{b}.
b. Mice congenic for the Igh-1 locus were primed with 10^7 SRBC. Three months later these mice were primed for parabiosis and at the same time one of the members was splenectomized (Sx). Control mice primed with 10^7 SRBC were Sx or ShSx 3 months later. Five days after surgery all mice were boosted by iv injection of 2×10^6 SRBC. Eight days after the booster injection the number of Igh-$^{a+}$- and Igh^{b+}-PFC were determined in the bone marrow of all mice.
c. Geometric mean ± 1 SEM from five or six mice.
d. n.s. means that the number of Igh^{a+} resp. Igh^{b+}-PFC above the number of IgM-PFC in the indirect plaque assay was not significant.

TABLE IV

The Influence of Systemic Antigen Administration upon the Migration of Blood-borne Cells into the Bone Marrow

Cell type	Influx of labeled blood-borne cells in the bone marrow[a] (per 1,000 nucleated cells) at various days after injection of TAB vaccine: 0[b]	2 days	3 days	4 days	5 days	6 days
Total cells	1.7 ± 0.3	7.1	5.8	6.5	2.4	2.6
Small lymphocytes[c]	0.1 ± 0.06	0	0.1	0.3	0	0.2
Large mononuclear cells[d]	0.7 ± 0.2	1.5	2.4	3.3	0.7	0.8
Immature plasma cells[e]	0.1 ± 0.02	0.6	0.4	0.7	0.6	0.5
Damaged cells[f]	0.8 ± 0.2	5.0	2.9	2.2	1.1	1.1

a. Labelled cells (10 grains) in radioautographs of guinea pig tibial marrow, 24 hr after ^{3}H-thymidine had been administered systemically but excluded from hind limb tissues by temporary occlusion of the hind limb circulation.

b. Mean ± S.E. of control experiments at 5 intervals after injecting fluid devoid of TAB organisms.

c. Lymphocytes measuring 8.0 μ nuclear diameter.

d. Including large lymphoid (transitional) cells, 8.0 μ nuclear diameter, having a leptochromatic nucleus and high ratio of nucleus to cytoplasm; large basophilic blast-like cells, 15-17 μ diameter; and monocytoid cells, 13-17 μ diameter, with irregular-shaped nucleus and moderate to plentiful amounts of pale-staining cytoplasm.

e. Large cells, 12-15 μ diameter, with an indented or flattened nucleus, plentiful moderately basophilic cytoplasm and a clear paranuclear area.

f. Well-defined, but structureless "smudge" cells.

vascular injection of TAB vaccine the number of labelled immigrants increased 3- to 4-fold. At long post-antigen intervals the influx of labelled cells returned towards normal values (Table IV).

Morphologically, the labelled marrow immigrants in control animals comprised a few small lymphocytes and putative plasmablasts together with larger proportions of various large mononuclear cells and fragile cells that appeared as damaged forms in smears, respectively. All

categories except small lymphocytes were increased after antigen stimulation, as detailed in Table IV.

The radioautographic criteria used ensured that all cells scored as labelled (10 grains) represented only the actual immigration of labelled cells and excluded any weak labelling that might result from local reutilization of radiolabelled materials circulating from the extramyeloid tissues. Although some cells in the initially occluded marrow showed minimal labelling of a few grains per cell, the labelling incidence dropped rapidly with increasing grain count to zero at 10 grains per cell. Separate counts of granulocyte progenitors (myeloblasts, myelocytes, metamyelocytes), known to be locally produced in the bone marrow, showed that whereas some cells (2 to 3%) had small numbers (3 to 5) of overlying grains, none exceeded 5 to 7 grains. In contrast, the labelled immigrants often exceeded 30 grains per cell. There was no difference in the mean labelling intensity of labelled immigrants either between the bone marrow of control (21.3 $\pm$ 1.5 grains per cell) and experimental (22.7 $\pm$ 1.4 grains per cell) series of experiments, respectively, or at various post-antigen intervals.

Similar labelled cells were detected in the blood. During the elevated post-antigen influx of cells into the marrow (4 days post-TAB; 24 hr post-^{3}H-thymidine) a count of 1000 mononuclear cells in blood smears revealed labelling (<10 grains) of 5.3% of the small lymphocytes and 20.0% of the larger mononuclear cells, together with some highly labelled damaged "smudge" cells.

In absolute numbers the labelled immigrants detected in the bone marrow after antigenic stimulation represented approximately 10,000 to 15,000 cells per cu. mm of marrow. Being the basis of labelling with only a single pulse of ^{3}H-thymidine, this figure represents a minimal estimate of the total influx of cells. The substantial size of this population indicates that the labelled cells had localized within the marrow parenchyma, being greatly in excess (1,000-fold) of the number that could be attributed simply to blood cells in the marrow circulation, which itself accounts for only approximately 5% of the total marrow volume in the guinea pig tibia (15). Moreover, the proportions of labelled cells of various types differed markedly in blood and marrow; small lymphocytes comprised approximately 30% of the labelled cells in the blood, but only about 3% of those in the marrow.

DISCUSSION

The origin of the antibody-producing cells found in the bone marrow has to date remained unresolved. Evidence that antibody formation can take place in the bone marrow has been presented in the literature during the last decade of the 19th century. In most species, thymus-dependent antigens induce the appearance of antibody-forming cells in the bone marrow only after repeated stimulation with the same antigen. However, thymus-independent antigens often induce bone marrow antibody formation already in the primary response (see for extensive review Reference 1). Studies reported herein show that PFC appearing in the bone marrow after secondary immunization with thymus-dependent antigens are the progeny of immigrant cells that originate from the spleen (Table III).

The migration of cells from the spleen to bone marrow takes place during the first few days of the secondary response, as can be concluded from experiments in mice Sx at different intervals after the booster injection. These experiments revealed that Sx 4 or more days after the second booster injection no longer influences the bone marrow PFC response, whereas Sx on day 2 can completely prevent bone marrow antibody formation (7). Thus, the appearance of bone marrow PFC (4,5) requires activation of memory cells in the periphery rather than *in situ* (Table III).

Bone marrow antibody formation is dependent on the availability of specific memory cells (4) at the time of reimmunization (5). To date it has been unclear whether only B memory cells, only T memory cells, or both B and T memory cells are required. The present study demonstrates that the presence of B memory cells is the only obligate requirement (Table I and II). The T-dependent activation of B memory cells and the first part of their differentiation into PFC likely occur in peripheral lymphoid organs. In these organs B-T collaboration can occur more efficient than in the marrow (6).

Previously, the entry of individual cells into the bone marrow after antigen stimulation has not been demonstrated directly or characterized morphologically. The present radioautographic studies in guinea pigs reveal that certain newly formed cells normally enter the bone marrow from the blood stream and that this migration is substantially increased for the first 3 days following administration of TAB vaccine, thus corresponding closely in timing with the migration of PFC precursors from the spleen to the marrow during the secondary immune response to thymus-dependent antigens in mice. Although the effects in guinea pigs are observed after a single dose of TAB vaccine, the response to these complex antigens probably includes a substantial secondary component.

Morphologically, the immigrant cells are diverse, consistent with the inclusion of immunoglobulin-secreting plasmablasts, activated lymphoblasts, and other cells. In contrast, although labelled newly formed small lymphocytes are present in the blood, very few of these cells enter the marrow. Marrow immigrants in control and antigen-stimulated animals, although differing in numbers, show a similar range of morphology as well as comparable cell kinetics, judged by their ^{3}H-thymidine labelling intensity. The immigration in control animals may reflect a flow of cells activated in peripheral lymphoid tissues by continuous exposure to environmental antigens. It is also not excluded that some marrow immigrants may have non-immunologic haemopoietic potentials in the marrow.

The cells demonstrated radioautographically to enter the bone marrow after antigenic stimulation are derived from cells that previously had been actively proliferating in extramyeloid tissues. As mentioned above, the bone marrow PFC response in mice is not affected by splenectomy four or more days after the booster injection (7). The long-lasting maintenance of the PFC response in the bone marrow cannot be explained by a continuous influx of antibody-forming cells from the periphery. This can be concluded from our previous experiments showing that in the case of parabiosis of mice with an ongoing antibody-forming cell response and non-immunized mice or recently primed mice, only minor numbers of PFC could be found in the bone marrow of the latter

(16). This raises the intriguing question whether the immigrant cells can still proliferate in the bone marrow. If the immigrant cells cannot continue to proliferate in the bone marrow, then the normal, long-lasting maintenance of the PFC response can only be explained by a long lifetime of the PFC in the bone marrow. Evidence for a long life span of at least a part of the plasma cells in the bone marrow as compared to plasma cells in the spleen has been presented by Tyler and Everett (17).

B cell priming with DNP-carrier conjugate greatly enhanced the TNP-specific IgG-PFC response in the bone marrow after a booster injection of DNP conjugated to a heterologous carrier (Tables I and II). In contrast, pre-immunization with the homologous carrier protein only did not facilitate the antibody formation in the bone marrow. This latter result shows that the occurrence of T memory cells only is not sufficient. We did not always find a clear helper effect of pre-immunization with carrier protein on the anti-hapten PFC response in the spleen. Probably this is caused by the immunization schedule used. Especially the high booster dose used to get optimal antibody formation in the bone marrow could have masked such a helper effect. This aspect is subject for further studies.

When the mice were primed with DNP-KLH and boosted with DNP-CGG, additional immunization with CGG caused a clear suppression of the anti-TNP PFC response in spleen and bone marrow. Similar data concerning antibody formation in the spleen have been reported by others (18,19). Whether this suppression is carrier specific or hapten specific remains to be established. Evidence for both possibilities have been presented (18,19). Important in the context of the present study is that the suppression leads to a lower PFC response in both the spleen and the bone marrow.

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APPENDIX PAPER IV

DIFFERENTIAL REQUIREMENT FOR B MEMORY AND T MEMORY CELLS IN ADOPTIVE ANTIBODY FORMATION IN MOUSE BONE MARROW

G. Koch and R. Benner

Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands

SUMMARY

During the secondary response of mice to T-dependent antigens, antibody-producing plaque-forming cells (PFC) appear not only in peripheral lymphoid organs, but also in the bone marrow. This bone marrow antibody formation is feeble after primary immunization. The capacity of bone marrow antibody formation is dependent on the presence of antigen-specific memory cells at the moment of secondary immunization. We investigated whether hapten-primed B memory, carrier-primed T memory or both B memory and T memory cells are required for the adoptive PFC response in the bone marrow to T-dependent hapten-carrier conjugates. Adoptive antibody formation in the bone marrow was found after transfer of hapten-primed spleen cells, but not after transfer of carrier-primed spleen cells or virgin spleen cells. Thus, B memory cells are obligatory for adoptive antibody formation in the bone marrow, in contrast to T memory cells. However, T memory cells did facilitate the bone marrow PFC response mediated by the infused B memory cells.

INTRODUCTION

Secondary, but not primary, immunization of mice with T-dependent antigens, induces large numbers of antibody-producing plaque-forming cells (PFC) in the bone marrow (Benner, Meima, van der Meulen & van Muiswinkel, 1974b). During the first week of the response most PFC are localized in spleen and lymph nodes. After the first week, however, the number of PFC localized in the bone marrow is 3- to 10-fold greater than in all other lymphoid organs together.

During the life span the number of immunoglobulin (Ig)-secreting cells in the bone marrow increases enormously (Benner, 1981). This raises questions about the mechanism underlying antibody formation in the bone marrow, and about the regulation of the distribution of antibody-forming cells over the various lymphoid organs.

Several experiments with mice have revealed a coincidence between the occurrence of B and T memory cells and the capacity of bone marrow antibody formation in response to the antigen used for priming (Benner, Meima & van der Meulen, 1974a). However, there is only little information as to whether the presence of both B and T memory cells, only B memory cells or only T memory cells is required. To study the cellular requirements of the marrow response we employed the technique of adoptive antibody formation in lethally irradiated syngeneic mice.

MATERIALS AND METHODS

Mice. Female (C57BL/Rij x CBA/Rij)F1 mice, 14-20 weeks of age, were used. They were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Antigen and immunization. Keyhole limpet hemocyanin (KLH) was dinitrophenylated to a level of 6-8 DNP molecules per 10^5 Dalton units, and chicken gamma globulin (CGG) to a level of 8-10 DNP molecules per protein molecule as described previously (Koch, Osmond, Julius & Benner, 1981). SRBC from a single donor were used. They were stored in Alsever's solution and washed three times in a balanced salt solution (BSS) before use.

Donor mice were primed by an intraperitoneal (ip) injection of 100 µg alum-adsorbed carrier or hapten-carrier conjugate, or with an intravenous (iv) injection of 10^7 SRBC. Mice immunized with hapten-carrier conjugate or SRBC were used 2-3 months after priming, and mice immunized with carrier only after 2-6 weeks.

Preparation of cell suspensions. Cell suspensions were prepared in a balanced salt solution (BSS) as described previously (Benner et al., 1974b).

Cell counts. Nucleated cells were counted with a Coulter Counter Model B. Viable nucleated cells were counted in a haemocytometer using 0.2% trypan blue in BSS as a diluent. The viability of the transferred cell suspensions was more than 80%.

Selective depletion of T lymphocytes. Monoclonal mouse anti-Thy-1.2 (from clone F7D5) was purchased from Olac 1976 Ltd. For the cytotoxic depletion of T cells 5 x 10^7 spleen cells/ml were incubated for 30 min at 4°C with 1:120 diluted anti-Thy-1.2 antibodies. Spleen cells incubated with BSS instead of anti-Thy-1.2 were used as a control. After washing the cells were resuspended in 10 µl undiluted guinea-pig complement per 10^6 cells and incubated for 20 min at 37°C. Finally the cells were washed twice and gently resuspended in BSS.

Selective depletion of B lymphocytes. The method described by Julius, Simpson & Herzenberg (1973) was used for the depletion of B lymphocytes, with one minor modification. The nylon wool columns (Fenwool Laboratories, Merton, Gevel, III) were prewashed with BSS supplemented with 5% bovine serum albumin (BSA) instead of fetal calf serum. Approximately 90-95% of the recovered spleen cells were T lymphocytes as shown by indirect membrane fluorescence staining.

Irradiation. The recipient mice received 7.5 Gy whole body irradiation generated in a Philips Müller MG 300 X-ray machine (Benner et al., 1974a). During irradiation the dose was measured with a NE Ionex 2500/3 dosimeter.

Cell transfer. Recipient mice were iv injected with the appropriate cell suspensions together with antigen within 4 hr after irradiation. They were immunized with 100 µg of the relevant hapten-carrier conjugate without adjuvant.

Assay for plaque forming cells (PFC). Cell suspensions were prepared and assayed for IgM and IgG anti-TNP PFC as described previously (Koch et al., 1981). CGG was coupled to SRBC by the $CrCl_3$ method (Gold & Fudenberg, 1967). For the calculation of the PFC response in the bone marrow of the whole animal we multiplied the response by the marrow cells of the two femurs with a factor of 7.9 (Benner, van Oudenaren & Koch, 1981).

RESULTS

Antibody formation in spleen and bone marrow after transfer of graded numbers of primed B and primed T cells.

The contribution of B memory and T memory cells to antibody formation in the bone marrow was investigated by means of adoptive transfer of spleen cells to irradiated syngeneic mice, and heterologous hapten-carrier conjugates as antigen. To determine the optimal dose of carrier-primed spleen cells for the adoptive PFC response in spleen and bone marrow, we transferred graded numbers of KLH-primed spleen cells together with unprimed spleen cells, 4×10^7 DNP-CGG-primed spleen cells and 100 µg DNP-KLH. All recipient mice received in total 1.4×10^8 spleen cells. The number of KLH-reactive T cells had no influence on the number of IgM- and IgG-producing PFC in the spleen (Fig. 1, left). In contrast, the bone marrow PFC response increased with increasing number of KLH-primed cells transferred. When the logarithm of the number of anti-TNP PFC in the bone marrow was plotted against the logarithm of the number of KLH-primed cells transferred, a linear relationship was observed. The same result was observed, when nylon wool-enriched KLH-primed T cells were transferred (data not shown).

In a comparable approach we investigated the relationship between the number of DNP-primed B cells and the adoptive anti-TNP PFC response in spleen and bone marrow.

The requirement for B memory and T memory cells in adoptive antibody formation in bone marrow.

The requirement for B memory and T memory cells in the adoptive bone marrow PFC response to DNP-KLH was studied by comparing the responses in four different groups of mice (Fig. 2). Group 1 was a control group which received spleen cells from normal mice only and was used to measure the primary adoptive PFC response to DNP-KLH. Mice of group 2 received KLH-primed spleen cells as a source of KLH-reactive T memory cells along with spleen cells of unprimed mice. Mice of group 3 were given spleen cells from donor mice immunized with DNP-CGG as a source of DNP-reactive B memory cells together with unprimed spleen cells. Both, DNP-primed spleen cells and KLH-primed spleen cells were transferred to the recipient mice of group 4. All recipient mice received DNP-KLH together with the transferred cells. Transfer of DNP-reactive B memory cells greatly facilitated the anti-TNP PFC response in the bone marrow. In the spleen an enhancement was found as well, but especially of the IgG-PFC response (data not shown). Carrier-reactive T memory cells could enhance the bone marrow PFC response, provided they were transferred together with DNP-primed spleen cells. No PFC response was found in the bone marrow after transfer of carrier-reactive T

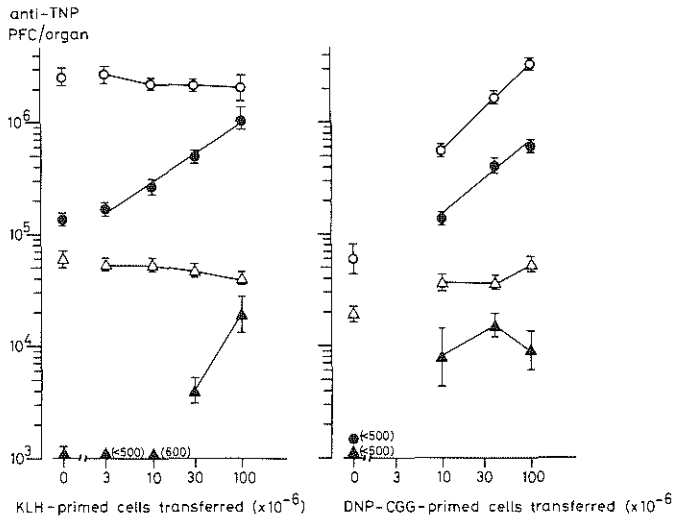

Figure 1. Adoptive PFC response in spleen (open symbols) and bone marrow (closed symbols) to 100 µg DNP-KLH. Irradiated syngeneic recipient mice were reconstituted with graded numbers of KLH-primed spleen cells and 4 x 10^7 DNP-CGG-primed spleen cells (left) or graded numbers of DNP-CGG-primed spleen cells together with 4 x 10^7 KLH-primed spleen cells (right). Spleen cells of non-immune mice were added to the transferred primed cells so that every recipient received 1.4 x 10^8 cells. The number of IgM- (△,▲) and IgG-producing (○,●) anti-TNP PFC were enumerated 7 days after cell transfer. Each point represents the geometric mean of 5 mice ± 1 SEM. Wherever the geometric mean is below the value of the abscissa the actual number of PFC is indicated by the number between brackets.

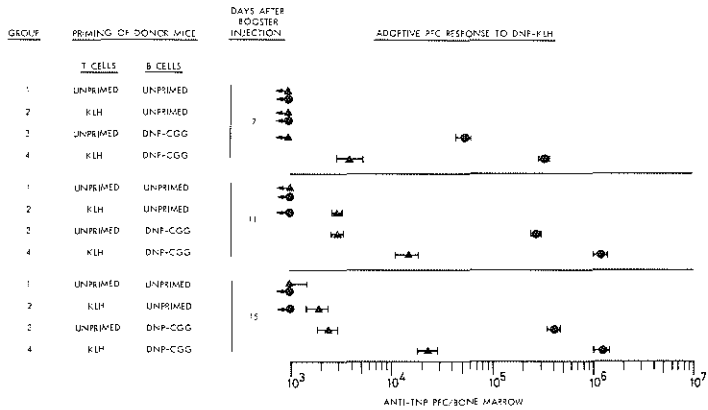

Figure 2. Influence of B memory and T memory cells upon the adoptive PFC response to 100 µg DNP-KLH. Irradiated mice were reconstituted with 4 x 10^7 spleen cells from non-immune mice and/or from mice immunized as indicated, so that each recipient received 8 x 10^7 spleen cells. The number of IgM- (▲) and IgG-producing (●) anti-TNP PFC were enumerated in the bone marrow at 7, 11, and 15 days after cell transfer. Each point represents the geometric mean of 5 mice ± 1 SEM.

TABLE 1

Requirement for B and T Memory Cells in Adoptive Antibody Formation in the Bone Marrow to DNP-KLH[a]

Group	Priming of donor mice		PFC assay on day	anti-TNP PFC/organ			
				Spleen		Bone marrow	
	T cells	B cells		IgM	IgG	IgM	IgG
1	unprimed	DNP-CGG α Thy-1.2 + C	7	35,301[b] (27,322-45,609)	1,851,232 (1,588,179-2,157,856)	4,287 (3,857-4,765)	13,987 (11,408-17,150)
2	KLH	DNP-CGG α Thy-1.2 + C	7	43,417 (36,309-47,249)	1,382,250 (1,238,628-1,542,525)	1,856 (1,310-2,633)	22,613 (20,802-24,581)
3	unprimed	DNP-CGG BSS + C	7	39,977 (31,858-50,165)	3,335,886 (2,882,932-3,860,007)	828 (623-1100)	44,747 (36,995-54,152)
4	none	DNP-CGG α Thy-1.2 + C	7	11,175 (8,837-14,131)	131,522 (112,173-154,207)	839 (524-1,342)	n.s.[c]
1	unprimed	DNP-CGG α Thy-1.2 + C	15	6,668 (6,273-7,087)	282,306 (275,705-289,064)	540 (374-781)	84,701 (73,166-98,055)
2	KLH	DNP-CGG α Thy-1.2 + C	15	5,737 (5,646-5,830)	137,371 (127,967-147,466)	1,029 (786-1,344)	77,604 (71,245-84,559)
3	unprimed	DNP-CGG BSS + C	15	4,998 (4,485-5,569)	196,605 (174,617-221,361)	2,120 (1,821-2,467)	105,390 (97,516-113,901)
4	none	DNP-CGG α Thy-1.2 + C	15	2,519 (2,381-2,665)	22,265 (20,411-24,287)	< 530	6,057 (4,503-8,148)

a. Irradiated mice were reconstituted with 2×10^7 anti-Thy-1.2 or 4×10^7 BSS plus complement-treated DNP-CGG-primed spleen cells together with 4×10^7 unprimed or KLH-primed spleen cells. All reconstituted mice were boosted with 100 µg DNP-KLH iv.

b. Geometric means of 5 mice ± 1 SEM.

c. n.s. means that the number of IgG-PFC above the number of IgM-PFC in the indirect assay was not significant.

memory cells along with unprimed spleen cells. Essentially the same results were obtained when the DNP-CGG primed spleen cells were depleted of T cells by treatment with anti-Thy-1.2 serum plus complement (Table 1).

In Figure 3 data are presented of similar experiments using DNP-KLH-primed spleen cells together with CGG-primed spleen cells. High numbers of anti-TNP PFC appeared in the bone marrow provided DNP-reactive B memory cells were transferred. Transfer of CGG-reactive T memory cells together with unprimed spleen cells was not sufficient for the appearance of an anti-TNP PFC response in the bone marrow, although they could enhance the marrow anti-TNP response mediated by DNP-KLH-primed spleen cells (Fig. 3, upper part). Anti-CGG PFC appeared only in the bone marrow of recipients which received spleen cells of mice immunized with CGG. Both anti-TNP and anti-CGG PFC were found in the marrow of irradiated recipients inoculated with DNP-primed B cells as well as CGG-primed B cells.

T cell depletion of SRBC-primed spleen cells by treatment with anti-Thy-1.2 plus complement abrogated the anti-SRBC PFC response in the bone marrow, while only a marginal PFC response in the spleen was found. Addition of nylon wool purified primed T cells to the T cell-depleted spleen cells restored the PFC response in the bone marrow and in the spleen. Partial restoration of the marrow PFC response was obtained with nylon wool purified spleen cells of unprimed mice (Table 2).

These experiments show that PFC can only appear in the marrow when antigen-specific memory cells are present on the time of the booster injections and that B memory cells, but not T memory cells, are crucial for antibody formation in the bone marrow. Although the T memory cells are not obligatory, they do enhance the bone marrow response by the B memory cells.

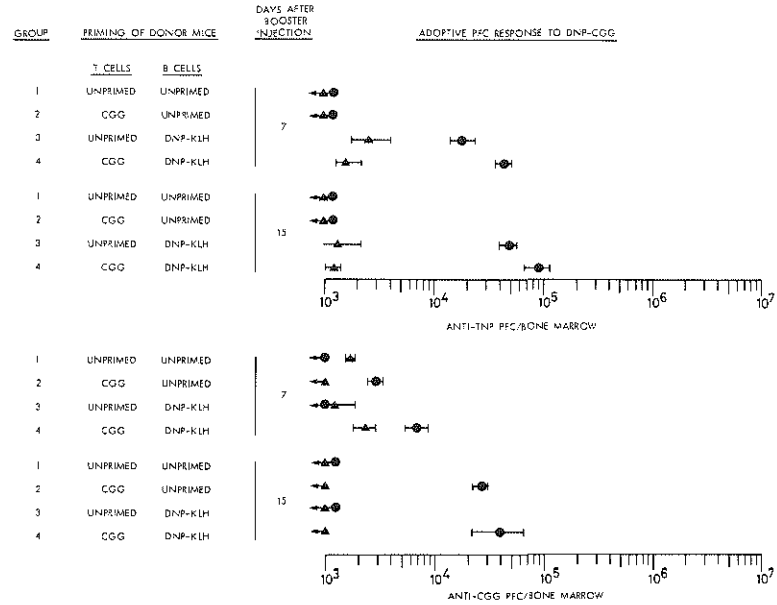

Figure 3. Influence of B memory and T memory cells upon the adoptive PFC response to 100 µg DNP-CGG iv. Irradiated mice were reconstituted with 4×10^7 spleen cells from non-immune mice and/or from mice immunized as indicated, so that each recipient received 8×10^7 spleen cells. The number of IgM- (▲) and IgG-producing (●) anti-TNP PFC (upper part) and anti-CGG PFC (lower part) were enumerated in the bone marrow at 7 and 15 days after the cell transfer. Each point represents the geometric mean of 5 mice ± 1 SEM.

TABLE 2

Requirement for B and T Memory Cells in Adoptive Antibody Formation in the Bone Marrow to SRBC[a]

Group	T cells nylon wool purified	B cells	PFC assay on day	anti-SRBC PFC/organ spleen IgM	spleen IgG	bone marrow IgM	bone marrow IgG
1	SRBC	SRBC α Thy-1.2 + C	7	64,434[b] (40,467-102,596)	1,181,460 (1,099,334-1,269,697)	22,195 (20,276-24,295)	63,660 (57,573-70,391)
2	unprimed	SRBC α Thy-1.2 + C	7	37,100 (33,664-40,888)	275,320 (249,889-303,318)	9,007 (8,167-9,936)	32,476 (29,818-35,372)
3	none	SRBC BSS + C	7	97,971 (66,820-143,644)	1,563,355 (1,318,616-1,853,518)	3,937 (3,279-4,726)	29,208 (33,430-36,411)
4	none	SRBC α Thy-1.2 + C	7	17,893 (14,043-22,798)	142,816 (119,758-170,313)	500	500
1	SRBC	SRBC α Thy-1.2 + C	15	33,362 (32,110-34,662)	447,476 (415,949-481,393)	2,059 (1,931-2,196)	47,657 (44,594-50,930)
2	unprimed	SRBC α Thy-1.2 + C	15	18,503 (16,678-20,528)	152,565 (131,155-177,469)	560 (423-672)	27,217 (25,113-29,497)
3	none	SRBC BSS + C	15	19,244 (18,412-20,114)	206,602 (199,532-213,923)	983 (726-1,332)	46,265 (42,019-50,940)
4	none	SRBC α Thy-1.2 + C	15	2,582 (2,341-2,848)	26,201 (24,380-28,158)	500	1,044 (793-1,467)

a. Irradiated mice were reconstituted with 2×10^7 anti-Thy-1.2 or 4×10^7 BSS plus complement treated SRBC-primed spleen cells together with 1.5×10^7 nylon wool-purified SRBC-primed or unprimed spleen cells. All reconstituted mice were boosted with 4×10^8 SRBC iv.

b. geometric mean of 5 mice ± 1 SEM.

Effect of booster dose on the adoptive antibody formation in bone marrow

Irradiated mice reconstituted with 4 x 10^7 DNP-CGG-primed and 4 x 10^7 KLH-primed spleen cells were given an iv booster injection of 0, 0.01, 1 or 100 µg DNP-KLH. At each booster dose tested a PFC response was found in the spleen as well as in the bone marrow. For the spleen the optimum dose DNP-KLH was found to be 1 µg when tested 7 days after cell transfer. In contrast, in the bone marrow the PFC response increased with increasing booster dose of DNP-KLH (Table 3). This result shows that adoptive bone marrow antibody formation to hapten-carrier conjugates is a regular phenomenon, and not the consequence of a non-physiological high booster dose of antigen.

DISCUSSION

This paper shows that adoptive antibody formation in the bone marrow to T-dependent hapten-carrier conjugates is completely dependent upon the transfer of hapten-primed B memory cells. Unprimed B cells do not give rise to bone marrow-localizing PFC, independent of whether they are transferred together with carrier-primed T memory cells or in combination with virgin T cells (Figs. 2 and 3). Apparently, virgin and memory B cells are basically different with regard to the capacity to mount a bone marrow PFC response. When B memory cells in the peripheral lymphoid organs are reactivated by the antigen used for priming, they can migrate toward the bone marrow via the blood stream and there further mature into antibody-secreting PFC (Benner et al., 1974b; Koch et al., 1981). The experiments reported in this paper and elsewhere (Koch et al., 1981) show that virgin B cells and their direct offspring do not have this capacity.

TABLE 3

Effect of the Booster Dose upon Adoptive Antibody Formation in the Bone Marrow[a]

Booster dose	PFC assay on day	anti-TNP PFC/organ			
		Spleen		Bone Marrow	
		IgM	IgG	IgM	IgG
0	7	2,115[b] (1,749-2,558)	<500	<500	<500
0.01	7	12,702 (11,322-14,250)	303,433 (287,502-320,427)	<500	4,280 (3,670-4,956)
1	7	48,475 (44,945-52,282)	762,043 (695,264-879,653)	2,679 (2,320-3,093)	19,516 (18,367-20,737)
100	7	38,292 (32,445-45,193)	765,023 (674,568-867,608)	2,398 (2,090-2,752)	22,454 (20,444-24,662)
0	15	1,799 (1,395-2,320)	<500	<500	<500
0.01	15	3,490 (3,550-4,372)	116,272 (106,872-126,360)	<500	26,519 (25,168-27,941)
1	15	9,380 (8,540-10,303)	182,917 (177,328-188,681)	681 (368-1,259)	43,442 (37,561-50,242)
100	15	9,776 (8,179-11,864)	290,738 (260,039-825,060)	1,264 (780-2,048)	119,582 (93,131-154,575)

a. Irradiated syngeneic recipient mice were reconstituted with 4 x 10^7 DNP-CGG-primed and 4 x 10^7 KLH-primed spleen cells. The reconstituted mice were given an iv booster injection of the indicated dose of DNP-KLH.

b. Geometric means of 5 mice ± 1 SEM.

T memory cells were found to enhance the bone marrow PFC response by transferred B memory cells in a dose-dependent way (Fig. 1, left). A similar effect was not observed for the PFC response in the spleen. This suggests that adoptive antibody formation in the bone marrow requires more T cell help than adoptive antibody formation in the spleen. So far it is unclear whether antibody formation to T-dependent antigens in the bone marrow requires T cell help within the marrow itself. The present data would be compatible with such a supposition, since infused T cells localize more readily in spleen and lymph nodes than in the bone marrow (Taub & Lance, 1968).

It is unlikely that T memory cells and virgin T cells differ qualitatively with respect to their capacity to enhance the adoptive bone marrow PFC response by restimulated B memory cells. This view is based upon adoptive transfer experiments with DNP-CGG-primed lymph node cells and DNP-KLH as booster antigen. In these experiments KLH-primed cells were unable to improve the bone marrow response (data not shown). This is probably due to the fact that lymph nodes contain a larger proportion of T cells than spleen (Raff, 1971).

In previous studies with intact mice primed and boosted with heterologous T-dependent DNP-carrier conjugates we have also observed that B memory cells are required and sufficient for antibody formation in the bone marrow (Koch et al., 1981). However, in these studies a possible facilitating influence of circulating anti-DNP antibodies and hapten-specific T memory cells induced by the primary immunization could not be ruled out. The adoptive transfer experiments reported here show that specific antibodies are not required for antibody formation in the bone marrow. Furthermore, passive transfer of IgG1 and IgG2 anti-DNP antibodies did not enhance the adoptive PFC response in the bone marrow of the recipients (data not shown).

The present experiments are also relevant with regard to the possibility that in intact mice the requirement for carrier-specific T memory cells for bone marrow antibody formation was by-passed by using hapten-specific T memory cells. We tested this possibility by transferring anti-Thy-1.2 treated DNP-CGG-primed spleen cells together with normal spleen cells using DNP-KLH as booster antigen. In these experiments we found that DNP-primed B cells together with unprimed T cells mounted a normal anti-TNP PFC response in the bone marrow of the recipient mice (Table 1).

The occurrence of antibody formation within the bone marrow in adoptive transfer experiments is highly dependent upon the number of B memory cells transferred (Fig. 1). In our previous studies with heterologous erythrocytes as antigen and transfer of 2×10^7 viable nucleated immune spleen cells, we did not find antibody-producing PFC in the marrow of the recipients (Benner et al., 1977). However, after transfer of 4×10^7 immune spleen cells or more, PFC can appear in the bone marrow, both with heterologous erythrocytes as antigen (Table 2), and with hapten-carrier complexes (Figs. 1-3). Apparently, a threshold number of B memory cells has to be present in the animal before secondary immunization can lead to antibody formation in the bone marrow. This can also explain why in the present adoptive transfer experiments the height of the bone marrow PFC response as compared to the splenic PFC response is smaller than in intact mice (c.f. Benner et al., 1974b). The dependence of bone marrow antibody formation on large numbers of B

memory cells stands in clear contrast to the dependence of the booster dose of antigen. Low booster doses of antigen, which lead to antibody formation in the spleen, concomittantly give rise to PFC in the bone marrow (Table 3).

In conclusion, antibody formation in the bone marrow is a regular phenomenon during secondary responses to T-dependent hapten-carrier complexes. The phenomenon is independent of the dose of antigen used for booster immunization, of the presence of circulating hapten-specific antibodies and of T memory cells, but absolutely dependent upon the availability of hapten-reactive B memory cells.

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APPENDIX PAPER V

THE CAPACITY AND MECHANISM OF BONE MARROW ANTIBODY FORMATION BY THYMUS-INDEPENDENT ANTIGENS

Guus Koch, Beatrix D. Lok, Adrianus van Oudenaren and Robbert Benner

Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands and the Basel Institute for Immunology, Basel, Switzerland

SUMMARY

Primary immunization of mice with certain thymus-independent (TI) antigens (i.e., TNP-LPS and DNP-Ficoll) leads to antibody formation in the bone marrow (BM). TNP-*Brucella abortus*, *Pneumococcus pneumoniae* organisms and α-(1,6)dextran, on the other hand, do not induce a BM antibody-producing plaque-forming cell (PFC) response. This paper deals with the mechanism underlying antibody formation in the BM to TNP-LPS and DNP-Ficoll. The majority of the BM-localizing PFC induced by TNP-LPS are formed within the BM from the proliferating lymphocyte pool, because this response was found to be resistant to splenectomy, and sensitive to treatment with hydroxyurea (HU) before immunization. This local activation of newly formed B cells requires in addition to the antigenic signal of TNP-LPS the mitogenic signal from the lipid A component of LPS. In contrast, the BM PFC response to DNP-Ficoll was reduced in splenectomized mice, and resistant to HU treatment before the primary immunization. Thus, antibody formation in the BM to DNP-Ficoll is mainly dependent on long-lived B cells that migrate from the peripheral lymphoid organs into the BM.

INTRODUCTION

After primary immunization of mice with the thymus-independent (TI) antigen lipopolysaccharide (LPS) antibody-producing plaque-forming cells (PFC) appear not only in the spleen, but also in the bone marrow (BM) (1). Data have been presented suggesting that these PFC are not immigrants from the peripheral lymphoid organs, but have developed from their precursors *in situ* (2). Such a mechanism underlying the BM PFC response to LPS would be in clear contrast to the mechanism of antibody formation in the BM to thymus-dependent (TD) antigens. The antibody formation to TD antigens in the BM is strictly dependent upon the immigration of antigen-activated B lineage cells from peripheral lymphoid organs (3).

These putatively different mechanisms underlying antibody formation in the marrow to TD antigens and LPS might be explained by the mitogenic properties of LPS (4-6). The hypothesis can be put forward that antibody formation in the BM after primary immunization with LPS requires in addition to the antigenic component of LPS a second, mitogenic, signal provided by the lipid A component of LPS. According to this hypothesis, abolishment of the lipid A component of LPS should prevent the anti-LPS PFC response in the BM. Also, primary immunization

with TI antigens that have no mitogenic properties should not lead to the appearance of PFC in the BM. Furthermore, the marrow PFC response to LPS should be dependent on the number of newly formed B lymphocytes *in situ*.

The present paper verifies these predictions by investigating (a) the effect of detoxification of LPS upon its ability to evoke BM antibody formation, (b) different TI antigens with regard to their ability to evoke antibody formation in the BM, (c) the effect of conjugation of lipid A to the TD antigen sheep erythrocytes (SRBC) upon its ability to cause a primary anti-SRBC PFC response in the BM, and (d) the effect of depletion of the population of newly formed B cells upon the PFC response to TNP-LPS in the BM.

MATERIALS AND METHODS

Mice. Female (C57BL/Rij x CBA/Rij)F1, C57BL/6J, CBA/J and BALB/c mice were used. The (C57BL/Rij x CBA/Rij)F1 hybrid mice, 14 to 20 weeks of age, were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands; female C57BL/Ka-nu/nu mice, 13 to 14 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands; and female CBA/J, C57BL/6J and BALB/c mice, 8 to 12 weeks of age, were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland.

Antigen and immunization. LPS from *E.coli* 055:B5 (Difco Laboratories, Detroit, MI), prepared according to the phenol-extraction method, was conjugated with 2,4,6-trinitrobenzenesulfonic acid (TNBS) essentially as described by Quintans and Lefkovits (7) with only one minor modification. An amount of 100 µg of LPS was dissolved in 10 ml of 2% (w/v) sodiumcarbonate and 100 µg TNBS was added. After stirring for 16 hours at 25°C, TNP-LPS was extensively dialyzed against balanced salt solution (BSS). LPS was detoxified by alkaline hydrolysis, carried out by heating at 56°C in 1NNaOH during 1 hr (8). Alkali-treated lipid A was a gift from Dr. C. Galanos from the Max-Planck Institut für Immunobiologie (Freiburg, FRG). Sensitization of SRBC with lipid A was performed by incubation of a 5% (v/v) suspension of SRBC with lipid A at a concentration of 50 µg/ml for 30 min at 37°C. Ficoll, m.w. 400,000 daltons, was purchased from Pharmacia (Uppsala, Sweden). It was derivatized to N-(2-aminoethyl)carbamylmethylated Ficoll (AECM-Ficoll) according to the method described by Inman (9). AECM-Ficoll was conjugated with dinitrobenzenesulfonic acid as described by Eisen (10). Whole heat-killed formalinized pneumococci (*Pneumococcus pneumoniae* strain R 36A) was obtained from Dr. C. Berek from the Basel Institute for Immunology. Dextran B512 (α-(1,6)dextran) was kindly provided by Dr. A. Coutinho from the same Institute. Whole heat-killed *Brucella abortus* bacteria were obtained from Dr. P. van der Heyden from the Central Veterinary Institute, Schiedam, The Netherlands. These bacteria were conjugated with TNBS as described by Mond et al. (11). All immunizations were done by i.v. injection in a tail vein, unless stated otherwise.

Preparation of cell suspensions. Cell suspensions of spleen and femoral bone marrow were prepared in BSS as described previously (12).

Assay for PFC. Cell suspensions were assayed for IgM- and IgG-PFC as described previously (12,13). Target SRBC were conjugated with TNBS as described by Rittenberg and Pratt (14). SRBC were coated with LPS as described previously (1). Para-diazonium-phenylphosphorylcholine, kindly provided by Dr. C. Heusser of the Basel Institute for Immunology, was synthesized according to Chesebro and Metzger (15), and was coupled to SRBC as described by Claflin et al. (16). To enumerate the number of anti-dextran PFC, donkey red blood cells (DRBC) were coated with stearyldextran conjugate (kindly provided by Dr. A. Coutinho). Shortly, 40 mg of α-(1,6)dextran conjugated with stearylchloride were incubated for 60 min at 37°C with 0.5 ml packed DRBC in 9.5 ml saline, according to the method described by Leon et al. (17). The complement (C) used was purified by affinity chromatography on Sepharose-protein A according to van Oudenaren et al. (18). PFC activity in the BM of individual mice was determined in the pooled marrow of both femurs. For the calculation of the PFC response in the BM of the whole animal we multiplied the response by the BM cells of two femurs with a factor of 7.9 (12). Calculation of the PFC response per 10^6 nucleated bone marrow cells from the data presented, is possible by taking into account that one mouse femur contains approximately 2×10^7 nucleated cells.

Hydroxyurea (HU) treatment. Mice were exposed to HU according to the schedule devised by Hodgson et al. (19). Mice were given two i.p. injections of HU at a dose of 1 g/kg body weight. The second injection was given 7 hr after the first. The rationale for using two doses instead of one was to allow the proliferating cells not in the S-phase at the time of the first dose to enter the S-phase and thereby to become susceptible to killing by the second dose of HU.

Splenectomy. Splenectomy and sham-splenectomy were performed 1 mo before immunization with TNP-LPS or DNP-Ficoll. Mice were anaesthetized by an i.p. injection of 0.1 ml/10 g body weight of a 1:40 diluted stock solution of Avertin (stock solution: 1 g 2,2,2-tribromoethanol (Merck-Schuchardt, München, FRG) in 0.5 g 2-methyl-2-butanol). The incision was made in the left upper abdomen. For splenectomy, the splenic blood vessels were tied in a single suture, then cut, and the spleen was removed. The incision was closed in two layers. There was no post-operative mortality.

RESULTS

Effect of detoxification of LPS upon the bone marrow PFC response to TNP-LPS

Primary immunization of mice with 100 µg TNP-LPS caused a substantial anti-TNP PFC response in both spleen and BM. On day 6 the response in the BM was as high as in the spleen (Fig. 1). Detoxification of LPS by alkaline hydrolysis abolished the ability of TNP-LPS to induce antibody formation in the BM, whereas it had only a marginal effect on the PFC response in the spleen (Fig. 1). The kinetics of the anti-LPS PFC response in mice immunized with TNP-LPS was essentially the same as that of the anti-TNP PFC response, but the number of anti-LPS PFC was consistently lower (data not shown). The detoxified LPS used in these experiments induced only low numbers of IgM-secreting PFC in bulk cul-

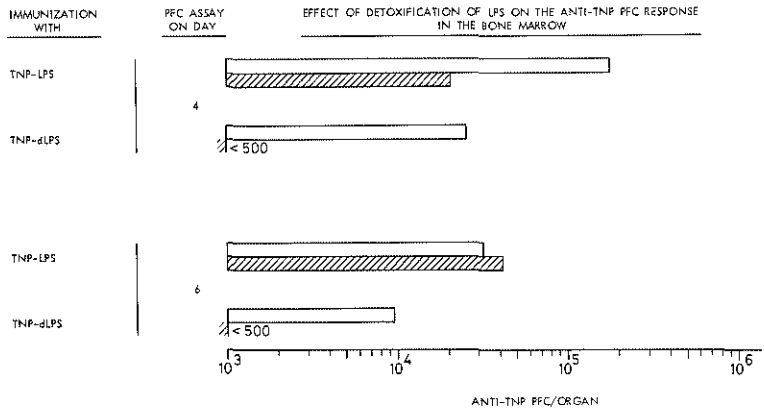

Figure 1. The anti-TNP PFC response in spleen (open columns) and BM (hatched columns) of (C57BL x CBA)F1 mice to 100 µg TNP-LPS or TNP-conjugated detoxified LPS (TNP-dLPS). The number of anti-TNP PFC were enumerated 4 and 6 days after immunization. The PFC-assay was done on pooled spleen cells and pooled BM cells. Each group consisted of five mice. Wherever the number of PFC did not differ significantly from the background level of about 500 PFC per organ this is indicated.

TABLE 1

Effect of Conjugation of Lipid A to SRBC upon the Anti-SRBC PFC Response in Spleen and Bone Marrow

Immunization[a]	PFC assay on day	anti-SRBC PFC/organ	
		spleen	bone marrow
SRBC-lipid A conjugate	4	139,386[b] (122,413-158,712)	6,837 (5,742-8.140)
SRBC + lipid A	4	76,413 (65,897-88,608)	761 (539-1,075)
SRBC	4	6,302 (4,432-8,979)	< 500
SRBC-lipid A conjugate	6	64,354 (45,113-91,803)	12,951 (9,601-17,461)
SRBC + lipid A	6	11,152 (9,840-13,483)	< 500
SRBC	6	26,520 (16,495-42,637)	< 500

a. C57BL/Ka-nu/nu mice were i.v. immunized with either 4×10^8 lipid A-conjugated SRBC, 4×10^8 SRBC plus 10 µg lipid A or 4×10^8 SRBC only. The number of IgM- and IgG-producing anti-SRBC PFC were enumerated 4 and 6 days later. The number of IgG-PFC above the number of IgM-PFC in the indirect plaque assay was not significant in any of the mice.

b. Geometric mean. In parenthesis ± 1 SEM. n = 5.

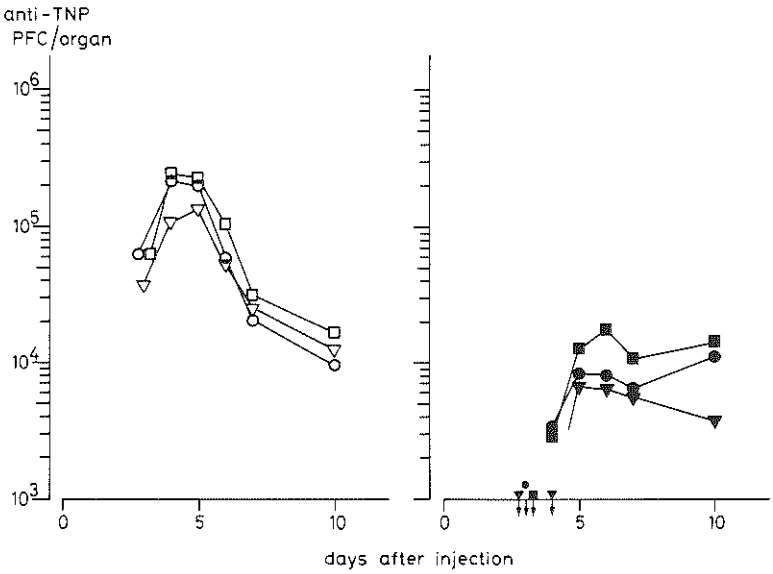

Figure 2. The number of anti-TNP PFC in spleen (left) and BM (right) after one injection of 1 (○,●), 10 (□,■) or 100 (▽,▼) µg DNP-Ficoll i.v. In neither group tested a significant number of IgG-PFC was found in the indirect plaque assay at any time after immunization. The PFC-assay was done on pooled spleen cells and pooled BM cells. Each group consisted of five mice. Wherever the number of PFC did not differ significantly from the background level of about 500 PFC per organ this is indicated with an arrow.

tures of spleen cells over the entire dose range of detoxified LPS tested (10^{-4} to 10^2 µg/ml). The native LPS, on the other hand, induced high numbers of IgM-secreting cells (data not shown). These data confirm the original data of Anderson et al. (4). Because detoxification of LPS by alkaline treatment inactivates the lipid A component (20), which is responsible for the mitogenic and adjuvant properties of LPS (4-6), lipid A seems to be required for antibody formation to TNP-LPS in the BM. The immune response to SRBC, normally a TD antigen, becomes partly thymus independent after complexation of the erythrocytes with purified lipid A. This is apparent from the occurrence of high numbers of PFC in the spleen of congenitally athymic nude mice after injection of 4×10^8 SRBC complexed with lipid A (Table 1). Moreover, a low but significant anti-SRBC PFC response was found in the BM of these nude mice, in contrast to nude mice injected with 4×10^8 SRBC together with unbound lipid A or with 4×10^8 SRBC only. Together, these results support the view that the mitogenic signal of the lipid A component is responsible for the antibody formation to TNP-LPS in the BM.

PFC response to DNP-Ficoll, α-(1,6)dextran, Pneumococcus pneumoniae and TNP-Brucella abortus

To investigate whether antibody formation in the BM after primary immunization with TI antigens is a regular phenomenon, we assayed the PFC response in spleen and BM after primary immunization with DNP-Ficoll, α-(1,6)dextran, formalinized *Pneumococcus pneumoniae* bacteria,

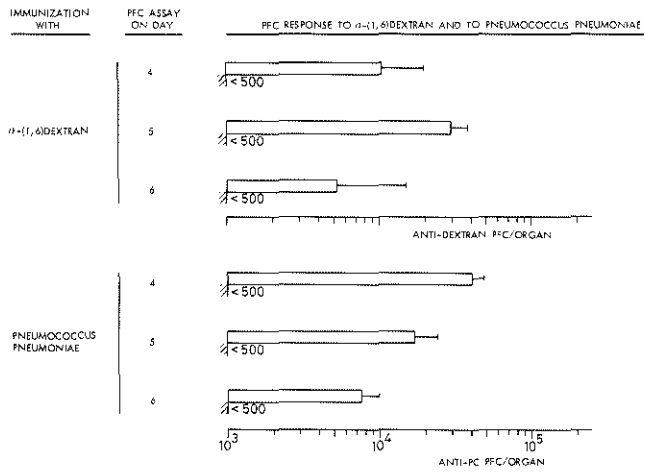

Figure 3. The PFC response to a single i.v. injection of 10 µg of α-(1,6)-dextran in CBA/J mice (upper part) and 10^9 pneumococci in BALB/c mice (lower part). The numbers of IgM-producing anti-dextran and anti-PC PFC were enumerated 4, 5 and 6 days after immunization in spleen (open columns) and BM (hatched columns). No significant number of PFC was found in the BM of any group of mice at any time after immunization. Each column represents the geometric mean ± 1 SEM of five mice.

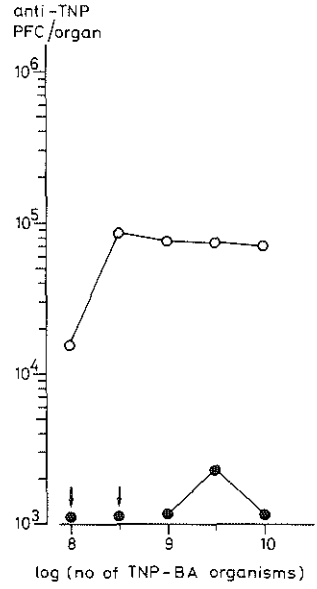

Figure 4. The anti-TNP PFC response in spleen (O) and BM (●) of mice immunized with 10^8, 3.3×10^8, 10^9, 3.3×10^9 and 10^{10} TNP-conjugated *Brucella abortus* organisms (TNP-BA) i.v. Each point represents the number of IgM-producing anti-TNP PFC of pooled spleen or BM cells from five mice at 4 days after immunization. Wherever the number of PFC is below the value of the abscissa this is indicated by an arrow.

and TNP-conjugated *Brucella abortus* organisms (TNP-BA).

Primary immunization with 1, 10, and 100 µg DNP-Ficoll i.v. evoked an anti-TNP PFC response in the spleen as well as in the BM (Fig. 2). Independent of the antigen dose, the PFC response peaked in the spleen between days 4 and 5 after immunization. The PFC response in the BM increased until a plateau was reached by day 5. At day 10 the number of PFC in the marrow equalled the PFC number in the spleen. The highest PFC response in the marrow was observed after immunization with 10 µg DNP-Ficoll. Immunization with doses of 0.1 and 100 µg led to a significant PFC response in the spleen only (data not shown). After immunization with optimal doses of α-(1,6)dextran (Fig. 3, upper part), formalinized pneumococci (Fig. 3, lower part) or TNP-BA (Fig. 4) PFC were found in the spleen only.

Influence of HU on the PFC response to TNP-LPS and DNP-Ficoll in the bone marrow

To investigate whether the PFC that appear in the BM during the primary response to TNP-LPS originate from a rapidly renewing B cell pool or from the pool of long-lived B cells, we treated mice with HU. Treatment of mice with HU inhibits the pre-B and B cell activity resulting in a minimal number of mitogen-reactive B cells in spleen and BM on day 6 after treatment (21). HU also diminishes the number of nucleated cells in spleen and bone marrow with minimal numbers at 2 days after the HU injection. By day 4, the total numbers of nucleated cells are back to normal (21). In Figure 5 (upper part) the effect is shown of the treatment of mice with HU 6 days before primary immunization with 10 µg TNP-LPS. No significant influence of HU treatment was found on the PFC response in the spleen when assayed on days 4, 6, and 21 after primary immunization. However, the number of PFC in the BM of HU-treated mice varied between 9 and 16% of the number in the BM of the control mice.

In contrast to the effect of HU treatment upon the response to TNP-LPS, no influence of HU treatment could be found on the response to DNP-Ficoll, neither in the spleen nor in the BM (Fig. 5, lower part). These results show that the anti-TNP PFC that appear in the BM after primary immunization with TNP-LPS mainly originate from the rapidly proliferating B cell pool, whereas the anti-TNP PFC that appear in the BM after primary immunization with DNP-Ficoll mainly originate from slowly renewing, long-lived B cells. Because HU treatment does not affect the anti-TNP PFC response induced in the spleen by TNP-LPS and DNP-Ficoll, these PFC probably originate from long-lived B cells.

Effect of splenectomy upon the PFC response to TNP-LPS and DNP-Ficoll in the bone marrow

The origin of PFC appearing in the BM after immunization with TNP-LPS and DNP-Ficoll was examined in splenectomized and sham-splenectomized mice immunized with either 10 µg TNP-LPS or 10 µg DNP-Ficoll i.v. The number of anti-TNP PFC was enumerated in spleen and BM 4, 6, 10, and 21 days after immunization. Splenectomy before immunization with 10 µg TNP-LPS had no influence on the number of anti-TNP PFC that appeared in the BM on all days tested (Fig. 6, upper part). In contrast, splenectomy before immunization with DNP-Ficoll significantly reduced the anti-TNP PFC response in the BM, especially during the early phase of the response (Fig. 6, lower part).

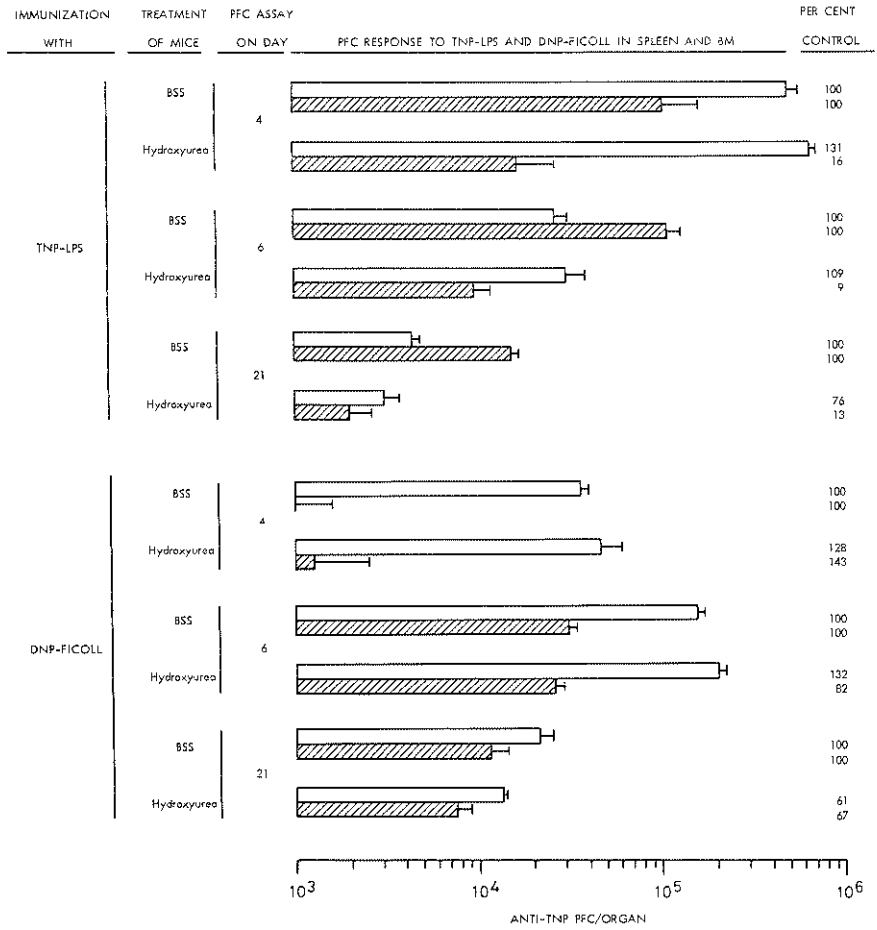

Figure 5. Influence of HU on the PFC response to TNP-LPS (upper part) and DNP-Ficoll (lower part) in mouse spleen and BM. Mice were treated with two i.p. injections of HU or BSS (control) 6 days before immunization. The number of IgM-producing anti-TNP PFC in spleen (open columns) and BM (hatched columns) was enumerated 4, 6 and 21 days after immunization. Each column represents the geometric mean ± 1 SEM of 5 mice. The data presented in this figure represent one out of three similar experiments, all yielding essentially the same results.

DISCUSSION

After primary immunization of mice with TNP-LPS and DNP-Ficoll, anti-TNP PFC appeared not only in the spleen, but also in the BM (Figs. 1 and 2). However, the kinetics of the PFC responses to both antigens are basically different. After one injection of LPS or TNP-LPS the BM PFC response is already substantial by day 4 (Figs. 1 and 5, lower part) after immunization, whereas after injection of DNP-Ficoll no PFC response is found in the BM before day 5. Furthermore, the BM PFC

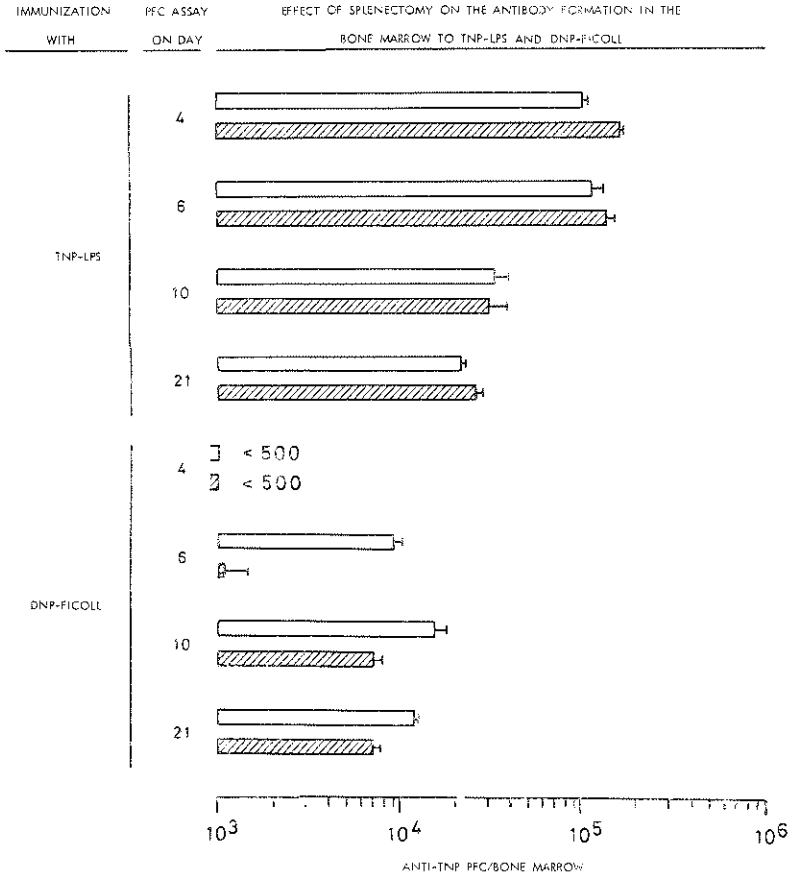

Figure 6. Effect of splenectomy upon the PFC response to TNP-LPS and DNP-Ficoll in mouse BM. Splenectomized (hatched columns) or sham-splenectomized (open columns) mice were immunized with either 10 µg TNP-LPS (upper part) or 10 µg DNP-Ficoll (lower part) i.v. The number of IgM-producing anti-TNP PFC was enumerated 4, 6, 10 and 21 days after priming. Each column represents the geometric mean ± 1 SEM of 5 mice. The data presented in this figure represent one out of three similar experiments all yielding essentially the same results.

response to TNP-LPS equalled or surpassed the response in the spleen already on day 6, whereas the BM PFC response to DNP-Ficoll does not surpass the level of the splenic response and attains this level later, namely around day 10. We did not find antibody formation in the BM after primary immunization with optimal doses of formalinized *Pneumococcus pneumoniae* bacteria, TNP-BA and α-(1,6)dextran (Figs. 3 and 4). Previously, Baker et al. (22) reported that no PFC response could be detected in the mouse BM after primary immunization with optimal doses of type III pneumococcal polysaccharide (SIII).

The appearance of PFC in the BM after primary immunization with TNP-LPS is dependent on a rapidly renewing B cell population, as is

apparent from the effect of HU treatment before immunization (Fig. 5, upper part). Furthermore, splenectomy before primary immunization with TNP-LPS did not influence the BM PFC response (Fig. 6, upper part). This indicates that antibody formation to TNP-LPS in the BM is dependent on local activation of newly formed B cells. These activated B cells subsequently differentiate and proliferate (23) into PFC *in situ*.

The local activation of the B cells by LPS within the BM is dependent on the mitogenic signal provided by the lipid A component of LPS (Fig. 1). This view is also suggested by experiments in which the TD antigen SRBC was artificially complexed with lipid A. This complex, in contrast to native SRBC, behaves as a TI antigen, and induces specific antibody formation within the BM during the primary response (Table 1). The *in situ* induction of antibody formation in the BM is compatible with our recent observation that B cells in the BM are fully immunocompetent *in vitro* (24).

HU treatment of mice before immunization with DNP-Ficoll has no influence on the PFC response in the BM (Fig. 5, lower part). This indicates that the precursors of these anti-TNP PFC belong to the slowly renewing, long-lived B cell subpopulation. These long-lived B cells are most likely activated in the peripheral lymphoid organs and then emigrate toward the BM, as can be concluded from the reduced anti-TNP response in the BM of mice splenectomized before immunization. Thus, antibody formation to DNP-Ficoll in the BM is based upon a similar mechanism as BM antibody formation to TD antigens (3). In the latter case, the occurrence of antigen-specific B memory cells are a prerequisite for antibody formation in the BM, so that only booster immunization leads to substantial antibody formation in this organ. Possibly, BM antibody formation to DNP-Ficoll is similarly dependent on B memory cells. These B memory cells could well be generated during the early phase of the primary response, and be reactivated some days later, due to the persistence of DNP-Ficoll (25). A similar phenomenon has been observed after immunization with TD antigens emulsified in complete Freund's adjuvant, where a significant BM PFC response occurs after a single injection of the antigen (12).

The residual BM PFC response in the splenectomized mice immunized with DNP-Ficoll is probably due to emigration toward the BM of B cells activated by the antigen within the peripheral lymph nodes. However, it cannot be excluded that some long-lived or short-lived B cells are activated by DNP-Ficoll within the BM itself, so that a local PFC response emerges.

The difference in capacity of the various TI antigens tested to induce a PFC response in the BM does not correlate with the subdivision of TI antigens 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 antigens such as DNP-Ficoll, levan, dextran, SIII, and polyinosinic-polycytidilic acid (26-29). In contrast, CBA/N spleen cells do respond to TNP-LPS *in vitro* and TNP-BA *in vivo* (11,30). It is thought that TI-2 antigens stimulate the more mature B cells that express IgD, C receptor, Lyb-3 and Lyb-5 surface antigens (31-34), which predominantly occur in the spleen. TI-1 antigens such as TNP-LPS and TNP-BA should stimulate, besides the more mature B cells, a population of less mature B cells that are also present in the BM. Our data show

that TNP-LPS, which is mitogenic, does induce antibody formation in the BM, whereas the non-mitogenic (11) TI-1 antigen TNP-BA, does not. Thus, it is not likely that the differential capacity of TI antigens to induce antibody formation in BM is also based upon a different capacity to activate the less mature BM B cells. Furthermore, immunization with DNP-Ficoll, a nonmitogenic (35,36) TI-2 antigen, also leads to antibody formation in the BM. However, because different B cell subsets are involved in the marrow PFC response to TNP-LPS (TI-1) and DNP-Ficoll (TI-2) our results do not contradict the conventional classification of TI antigens into TI-1 and TI-2. Merely the fact that TNP-BA does not induce antibody formation in the bone marrow whereas TNP-LPS does, further subdivides the group of TI-1 antigens. Moreover the data presented argue for the further subdivision of the group of TI-2 antigens, because, of this group of TI antigens only DNP-Ficoll is able to induce a PFC response in the marrow.

In conclusion, different TI antigens differ in their capacity to induce antibody formation in the mouse BM. Two antigens were found to induce substantial antibody formation in the BM by primary immunization: LPS and its (mitogenic) derivatives, and DNP-Ficoll. The responses to these antigens follow different kinetics and are based upon different mechanisms.

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APPENDIX PAPER VI

ANTIBODY FORMATION IN MOUSE BONE MARROW DURING SECONDARY TYPE RESPONSES TO THYMUS-INDEPENDENT ANTIGENS

G. Koch, B.D. Lok and R. Benner

Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands

SUMMARY

The data presented in this paper shows that different thymus-independent (TI) antigens have a differential capacity of inducing antibody formation in mouse bone marrow, both after primary and secondary intravenous immunization. Primary immunization with certain TI antigens (e.g., lipopolysaccharide (LPS), TNP-LPS, DNP-Ficoll) induces the appearance of antibody-forming cells not only in the spleen, but also in the bone marrow. A single injection of certain other TI antigens (e.g., pneumococci (Pn), TNP-conjugated detoxified LPS (TNP-dLPS), TNP-conjugated *Brucella abortus* bacteria (TNP-BA)), on the other hand, induces antibody formation in the spleen only. After secondary immunization with these TI antigens only TNP-BA induces a PFC response in the bone marrow. Pn, TNP-dLPS and TNP-BA, but also DNP-Ficoll, are unable to induce bone marrow antibody formation after secondary injection of the antigen, in spite of the clear-cut secondary type character of the splenic response. Thus, the absence of a bone marrow PFC response after secondary immunization with these antigens is not due to a failure to induce memory B cells. This data implies that either two subpopulations of memory B cells exist, one giving rise to antibody formation in the spleen and the other accounting for the bone marrow response, or that antibody can selectively inhibit the secondary bone marrow antibody response to certain TI antigens.

INTRODUCTION

Thymus-dependent (TD) antigens evoke substantial bone marrow (BM) antibody formation only after multiple immunization (1). This is because antigen specific memory B cells, induced during the primary immune response, are indispensable (2). The memory B cells are activated by the antigen of the booster immunization in the peripheral lymphoid organs, and subsequently emigrate toward the BM (2,3). In contrast to TD antigens, some thymus-independent (TI) antigens induce antibody formation in the BM already during the primary response. Of a whole variety of TI antigens tested, TNP-LPS, LPS and DNP-Ficoll induce antibody formation not only in the peripheral lymphoid organs, but also in the BM (4), while *Pneumococcus pneumoniae* bacteria (Pn), TNP-conjugated *Brucella abortus* bacteria (TNP-BA), α-(1,6)dextran (4) and pneumococcal type III polysaccharide (S III) (5) do not.

The first week of the secondary response to TD antigens is characterized by much higher numbers of antibody-producing plaque-forming

cells (PFC) in the spleen and/or the lymph nodes than in the BM. In contrast, after the first week the PFC response in the BM is several times higher than in all other lymphoid organs together (6). After primary immunization with the TI antigen DNP-Ficoll, the marrow PFC response is feeble, but the kinetics and mechanism of this PFC response are essentially the same as in case of TD antigens (4). The kinetics of the primary response to TNP-LPS, however, is different in that substantial numbers of PFC appear in the BM already at day 3. Furthermore, the marrow PFC response to TNP-LPS equals or surpasses the response in the spleen already on day 6 (4,7).

Since memory B cells play a pivotal role in BM antibody formation to TD antigens, the question arises as to whether TI antigens, which do not induce antibody formation in the marrow after primary immunization, do so after multiple injection of the same antigen. The experiments reported in this paper were designed to answer this question as well as to get insight into the role of memory B cells in secondary antibody formation to TI antigens in the BM.

MATERIALS AND METHODS

Mice. Female (C57BL/Rij x CBA/Rij)F1 hybrid mice and BALB/c mice, 14-20 weeks of age, were used. They were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Antigen and immunization. Sheep erythrocytes (SRBC) from a single donor were used. They were obtained from the Central Veterinary Institute, Schiedam, The Netherlands. Heat killed *Brucella abortus* bacteria (BA) were kindly donated by Dr. P.D. van der Heyden from the same Institute. BA was conjugated with 2,4,6-trinitrophenylsulfonic acid (TNBS) as described by Mond et al. (8). Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Difco Laboratories, Detroit, Michigan, USA), prepared according to the phenol extraction method, was conjugated with TNBS as described previously (4). LPS was detoxified by alkaline hydrolysis according to Neter et al. (9). Ficoll with a molecular weight of 400,000 daltons was purchased from Pharmacia (Uppsala, Sweden). It was derivatized to N-(2-aminoethyl)carbamylmethylated Ficoll (AECM-Ficoll) and conjugated with dinitrobenzenesulfonic acid as described previously (4). The *Pneumoccoccus pneumoniae* bacterium (Pn) strain, R36A, was obtained from Dr. C. Berek of the Basel Institute for Immunology (Switzerland) and grown in Todd-Hewitt medium. Growth was terminated by heat-killing for 1 hr at 100°C. The bacteria were washed in saline and resuspended in saline containing 0.4% formalin. The formalinized Pn were washed three times immediately before immunization.

The mice were primed with either 10 µg TNP-LPS, 10 µg DNP-Ficoll, 10^9 TNP-BA, or 10^9 Pn bacteria. For secondary immunization the mice were boosted with the indicated antigen 2 months after priming. All immunizations were done by intravenous (iv) injection in a tail vein.

Preparation of cell suspensions. Cell suspensions of spleen and femoral BM were prepared in balanced salt solution (BSS) as described previously (10).

Adoptive cell transfer. Recipient mice received 7.5 Gy whole body X-irradiation (3), and were iv injected with the appropriated cell suspension within 4 hours after irradiation.

Assay for PFC. Cell suspensions were assayed for IgM- and IgG-PFC as described previously (10). Anti-TNP PFC responses were assayed with SRBC conjugated with TNBS (11) as targets. For the determination of anti-LPS PFC the target SRBC were coupled with LPS as described previously (7). To determine phosphorylcholine(PC)-specific PFC, para-diazonium-phenylphosphorylcholine, kindly provided by Dr. C. Heusser of the Basel Institute for Immunology, was coupled to SRBC as described by Claflin et al. (12).

RESULTS

Capacity of TNP-LPS, DNP-Ficoll, Pn, and TNP-BA to induce bone marrow antibody formation after secondary immunization

To investigate the capacity of several TI antigens to induce antibody formation in mouse BM, we studied the kinetics of the PFC response in spleen and BM of mice primed and boosted with optimal doses of TNP-

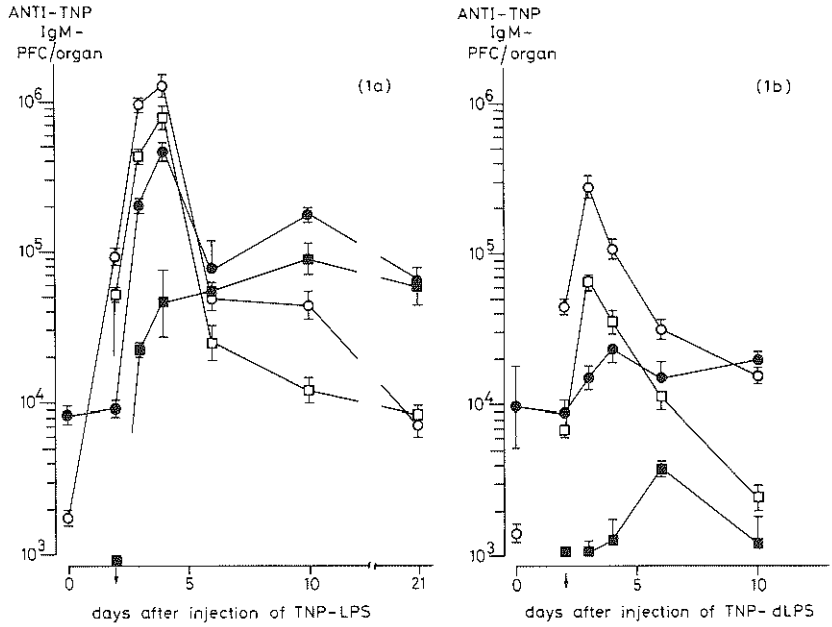

Figure 1. Primary and secondary anti-TNP PFC response in mouse spleen and bone marrow to TNP-LPS and TNP-dLPS. Mice injected with BSS (□,■) and mice primed by injection of 10 µg TNP-LPS (○,●) were immunized two months later with either 100 µg TNP-LPS iv (Fig. 1a) or 100 µg TNP-dLPS iv (Fig. 1b). The IgM- and IgG-PFC were enumerated in spleen (○,□) and bone marrow (●,■) at various intervals after secondary injection. A significant number of IgG-PFC was found in the spleen on day 6 (81,000 PFC) and day 10 (72,000 PFC) after secondary injection of TNP-LPS and at day 6 (33,000 PFC) and day 10 (12,000) PFC after secondary injection of TNP-dLPS. Wherever the number of PFC is below the level of the abscissa this is indicated by an arrow. Vertical bars represent 1 SEM (n = 5).

TABLE I

Primary and Secondary PFC Response to DNP-Ficoll in the Spleen and the Bone Marrow

PFC assay on day	Anti-TNP IgM-PFC ($\times 10^{-3}$)/organ[a] spleen primary	spleen secondary	bone marrow primary	bone marrow secondary
0	-	6.3[b] (5.6-7.2)	-	15.2 (13.1-17.8)
4	306.7 (264.9-355.2)	266.4 (225.7-319.1)	7.2 (6.0-8.6)	35.1 (29.8-41.3)
6	154.0 (135.2-175.5)	75.5 (62.3-91.6)	16.2 (13.0-18.2)	28.1 (23.7-33.3)
10	19.5 (17.4-22.0)	23.1 (19.5-27.4)	14.8 (12.9-17.0)	16.0 (13.2-19.4)
21	13.2 (12.2-14.4)	18.4 (14.9-22.8)	23.8 (20.7-27.4)	38.5 (31.4-47.2)

a. Mice injected with BSS and mice primed by an iv injection of 10 µg DNP-Ficoll were immunized with 10 µg DNP-Ficoll iv 2 months after the primary injection. The number of IgM- and IgG-PFC were enumerated 0, 4, 6, 10 and 21 days after the last injection of DNP-Ficoll. After secondary immunization with DNP-Ficoll significant numbers of IgG-PFC were found in the spleen on day 10 (10,400 PFC) and day 21 (16,900 PFC).

b. Geometric mean. In parenthesis $\pm$ 1 SEM (n = 5).

LPS, DNP-Ficoll, Pn and TNP-BA.

In mice primed with 10 µg TNP-LPS and boosted with 100 µg TNP-LPS 2 months later, high numbers of anti-TNP IgM-PFC appeared in the spleen as well as the BM. The secondary PFC response in both organs was higher than the primary response, but the kinetics was essentially the same (Fig. 1a). Significant numbers of IgG-PFC were found on days 6 and 10 after secondary immunization only. The kinetics of the anti-LPS PFC response in these mice was essentially the same as that of the anti-TNP PFC response, but the numbers of anti-LPS PFC were consistently lower (data not shown).

From previous studies we know that the primary BM PFC response to TNP-LPS is dependent on the mitogenic activity of the LPS carrier (4). This mitogenic activity resides in the lipid A component of LPS, and can be destroyed by alkaline hydrolysis (9,13). The question arises as to whether the secondary BM PFC response is similarly dependent on the mitogenicity of LPS. Therefore, we assayed the PFC response in spleen and bone marrow of mice primed with 10 µg TNP-LPS and boosted with 100 µg TNP-dLPS. Both after primary and secondary immunization with TNP-dLPS substantial numbers of anti-TNP PFC were found in the spleen only (Fig. 1b).

After secondary immunization with DNP-Ficoll, the anti-TNP IgM-PFC

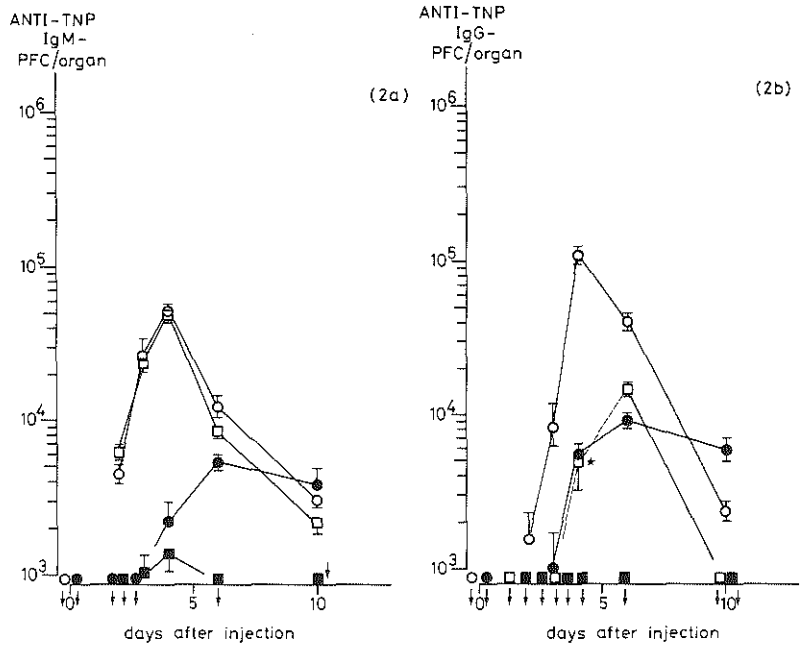

Figure 2. Primary and secondary anti-TNP PFC response to TNP-BA in mouse spleen and bone marrow. Mice injected with BSS (□,■) and mice primed with 10^9 TNP-BA iv (○,●) were boosted by iv injection of 10^9 TNP-BA 2 months later. The number of anti-TNP IgM-PFC (Fig. 2a) and IgG-PFC (Fig. 2b) were enumerated in spleen (○,□) and bone marrow (●,■) at various intervals after injection of TNP-BA. The persistent primary response was determined in mice which were primed with TNP-BA and assayed for anti-TNP PFC on the day of secondary immunization. Where an asterix is added to an experimental point, the number of IgG-PFC above the number of IgM-PFC in the indirect plaque assay was not significant. Wherever the number of PFC is below the level of the abscissa this is indicated by an arrow. Vertical bars represent 1 SEM (n = 5).

response in the spleen was considerably lower than after primary immunization (Table 1). However, during the secondary response low but significant numbers of IgG-PFC appeared in the spleen on day 10 and 21 after the booster injection (see footnote Table 1). The number of PFC occurring in the BM after secondary immunization with DNP-Ficoll was hardly higher than after primary immunization (Table 1). The increase that did occur may in fact be attributed to the PFC response induced by the primary immunization. This response persisted in the BM until the day of booster immunization (day 0 value in Table 1).

The secondary PFC response to TNP-BA was characterized by an increased number of anti-TNP IgG-PFC in the spleen and by the occurrence of IgM-and IgG-PFC in the BM (Fig. 2).

In mice primed and boosted with 10^9 Pn, the anti-PC IgM-PFC response was much higher than in mice immunized with Pn once. Low, but significant, numbers of anti-PC PFC were found in the bone marrow on day 4 after secondary immunization only (Fig. 3).

The capacity of TNP-LPS, DNP-Ficoll and TNP-BA to induce hapten-specific

TABLE II

The Influence of Priming with TNP-LPS, DNP-Ficoll and TNP-BA upon the Anti-TNP PFC Response to DNP-KLH

PFC assay on day	Anti-TNP IgM-PFC ($\times 10^{-3}$)/organ[a]							
	Spleen				Bone marrow			
Priming:	none	TNP-LPS	DNP-Ficoll	TNP-BA	none	TNP-LPS	DNP-Ficoll	TNP-BA
0	-	2.4[b] (2.2-2.8)	<5.0 (4.6-5.3)	<0.5	-	19.7 (18.7-20.7)	11.0 (10.1-11.9)	<0.5
4	62.8 (55.5-71.2)	46.4 (43.6-49.3)	217.1 (186.2-253.3)	16.5 (15.5-17.6)	<0.5	8.1 (6.4-10.2)	8.8 (7.9-9.7)	1.0 (0.5-1.9)
6	6.5 (6.2-6.8)	9.1 (8.1-10.4)	38.1 (35.2-41.2)	5.2 (4.7-5.7)	<0.5	10.7 (8.7-13.2)	8.2 (7.0-9.7)	1.4 (1.1-1.8)
10	1.6 (1.4-1.8)	3.3 (3.2-3.5)	5.2 (4.9-5.5)	1.5 (1.4-1.7)	<0.5	18.0 (12.2-26.4)	8.4 (6.9-10.2)	1.9 (1.7-2.2)
19	1.4 (1.2-1.6)	2.6 (2.3-3.0)	3.8 (3.5-4.0)	1.5 (1.4-1.6)	<0.5	11.2 (7.7-16.2)	8.1 (7.3-9.0)	5.6 (3.8-8.1)

a. Mice were primed by an iv injection of either 10 µg TNP-LPS, 10 µg DNP-Ficoll, or 10^9 TNP-BA. Two months later all mice, except the mice of the first line, were boosted by an iv injection of 100 µg DNP-KLH. The numbers of IgM- and IgG-PFC were determined at 0, 4, 6, 10 and 19 days after the booster injection. Significant numbers of IgG-PFC were only found in the spleen of unprimed mice at day 6 (21,000 PFC) and 10 (8,000 PFC), in the spleen of mice primed with TNP-LPS at day 6 (5,700 PFC), in the spleen of mice primed with DNP-Ficoll at day 10 (5,000 PFC), in the spleen of mice primed with TNP-BA at day 6 (9,100 PFC), and in the bone marrow of these latter mice at day 6 (11,000 PFC), 10 (6,600 PFC), and 19 (5,400 PFC) after the booster injection of DNP-KLH.

b. Geometric mean. In parentheses ± 1 SEM (n = 5).

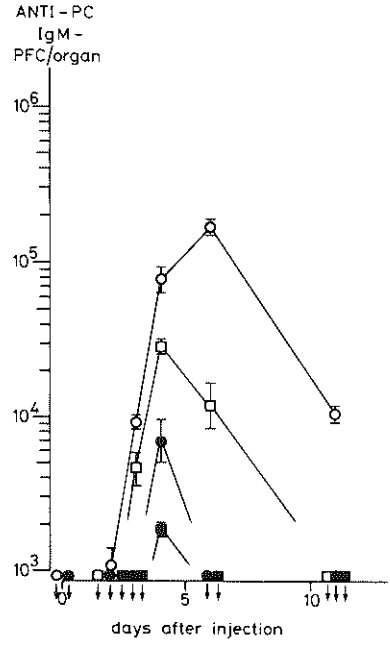

Figure 3. Primary and secondary anti-PC PFC response to Pn. Mice injected with BSS (□,■) and mice primed with 10^9 Pn (○,●) were boosted with 10^9 Pn iv 2 months later. The number of anti-TNP IgM- and IgG-PFC were enumerated in spleen (○,□) and bone marrow (●,■) at various intervals after injection of Pn. The number of IgG-PFC above the number of IgM-PFC in the indirect plaque assay was not significant at any day tested. The persistent primary PFC response (day 0 value) was determined on the day of secondary immunization. Wherever the number of PFC is below the level of the abscissa this is indicated by an arrow. Vertical bars represent 1 SEM (n = 5).

memory B cells

The BM PFC response to TD antigens is strictly dependent upon the presence of memory B cells at the moment of the booster immunization (2). The differential capacity of TI antigens (e.g., TNP-LPS, DNP-Ficoll and TNP-BA) to induce secondary BM antibody formation might be caused by their inability to induce memory B cells after primary immunization, or to (re)activate these cells after secondary immunization. To circumvent the latter possibility, mice primed with a TI antigen were boosted with DNP-KLH, an antigen that is known to be able to (re)activate DNP- and TNP-specific memory B cells. Thus, mice primed with either TNP-LPS, DNP-Ficoll, or TNP-BA, were boosted with DNP-KLH two months later, and the PFC were determined in spleen and bone marrow at various intervals after the secondary immunization. The data obtained (Table II) show that only priming with DNP-Ficoll can substantially increase the splenic anti-TNP IgM-PFC response. A significant anti-TNP IgG-PFC response in the bone marrow only occurred in mice primed with TNP-BA and boosted with DNP-KLH (see footnote Table II).

Since in intact mice several regulating factors operate, which might be absent in irradiated mice, we tested similarly primed mice for the presence of memory B cells in an adoptive transfer system. Thus, mice were primed with either 10 µg TNP-LPS, 10 µg DNP-Ficoll, or 10^9 TNP-BA. Two months later 10^8 nucleated spleen cells of primed or non-primed mice were transferred together with 100 µg DNP-KLH into irradiated syngeneic recipients. The number of IgM- and IgG-PFC was assayed in the spleen at day 7 after cell transfer. Mice which received non-immune spleen cells showed a substantial IgM- and IgG-PFC response.

TABLE III

Adoptive PFC Response to DNP-KLH by Spleen Cells from Non-Immune Mice and Mice Primed with TNP-LPS, DNP-Ficoll or TNP-BA

Priming	Anti-TNP PFC ($\times 10^{-3}$)/Spleen[a]	
	IgM-PFC	IgG-PFC
none	37[b] (33-41)	121 (110-133)
TNP-LPS	136 (115-161)	149 (126-178)
DNP-Ficoll	295 (258-338)	335 (262-428)
TNP-BA	64 (59-69)	207 (188-226)

a. Mice were primed with either 10 µg TNP-LPS, 10 µg DNP-Ficoll, or 10^9 TNP-BA iv. Spleen cells (10^8) of these mice were transferred together with 100 µg DNP-KLH into irradiated syngeneic mice two months later. The number of anti-TNP IgM- and IgG-PFC were enumerated 7 days after cell transfer.

b. Geometric mean. In parenthesis $\pm$ 1 SEM (n = 5).

Immunization of the donor mice with TNP-LPS, DNP-Ficoll, or TNP-BA significantly increased the adoptive IgM-PFC response in the recipients' spleen. Likewise, spleen cells from mice primed with DNP-Ficoll and TNP-BA gave rise to more IgG-PFC than unprimed spleen cells (Table III). Thus TNP-LPS, DNP-Ficoll, and TNP-BA do induce memory B cells.

The capacity of TNP-LPS and DNP-Ficoll to activate memory B cells

As stated in the previous section, a possible explanation for the failure of certain TI antigens to induce BM antibody formation after secondary immunization might be an inability of these TI antigens to activate memory B cells. Therefore, we induced DNP-specific memory B cells by priming with DNP-KLH, and investigated the capacity of TNP-LPS and DNP-Ficoll to (re)activate the memory B cells induced by this antigen. After primary immunization with 10 µg TNP-LPS high numbers of IgM-PFC, but no significant numbers of IgG-PFC were found in spleen and BM. Induction of DNP-specific memory B cells by pre-immunization with DNP-KLH reduced the anti-TNP-LPS IgM-PFC response in both spleen and BM, but facilitated, on the other hand, the appearance of anti-TNP IgG-PFC in both organs (Table IV, upper part). Essentially the same effect of preimmunization with DNP-KLH was found in the response to DNP-Ficoll (Table IV, lower part). These results show that TNP-LPS and DNP-Ficoll can activate DNP-specific B memory cells committed to IgG-synthesis.

DISCUSSION

The results of the experiments presented in this paper, show that TI antigens have a differential ability to induce antibody formation in the BM. This was found for the primary as well as the secondary response.

Both after primary and secondary immunization TNP-LPS induced a PFC response in the spleen *and* the bone marrow. These responses are completely dependent on the mitogenic signal of LPS. This is apparent from the

TABLE IV

The influence of Priming with DNP-KLH upon the Anti-TNP PFC Response to TNP-LPS and DNP-Ficoll[a]

Booster antigen	PFC assay on day	Anti-TNP PFC ($\times 10^{-3}$)/organ[b]							
		Spleen				Bone marrow			
		IgM		IgG		IgM		IgG	
	priming:	none	DNP-KLH	none	DNP-KLH	none	DNP-KLH	none	DNP-KLH
TNP-LPS	0	-	3.8 (0.6-7.2)	-	< 0.5	-	1.5 (0.7-2.3)	-	28.5 (18.0-46.0)
	4	282.1 (263.4-302.0)	87.5 (80.1-96.3)	< 0.5	224.0 (202.8-247.5)	47.9 (34.5-66.4)	6.8 (5.2-8.6)	< 0.5	75.3 (67.3-84.3)
	6	107.3 (90.3-127.5)	21.1 (17.4-25.7)	23.6(ns)[c] (8.1-68.9)	135.0 (100.2-182.1)	45.1 (19.8-102.9)	3.8 (3.6-4.4)	< 0.5	50.7 (47.1-54.6)
	10	13.0 (10.6-15.8)	23.8 (20.2-28.0)	2.2(ns) (1.6-3.1)	166.7 (134.3-207.0)	43.8 (34.1-56.2)	15.3 (11.6-19.7)	2.2(ns) (1.1-4.6)	92.6 (78.4-110.0)
DNP-Ficoll	0	-	3.8 (3.5-4.2)	-	< 0.5	-	1.6 (1.2-2.0)	-	18.5 (15.2-22.5)
	4	172.2 (159.3-186.1)	14.7 (12.8-16.8)	< 0.5	34.6 (27.2-43.9)	3.5 (2.9-4.3)	1.6 (1.3-2.0)	< 0.5	25.5 (22.8-28.6)
	6	145.8 (134.5-158.1)	18.4 (16.8-20.2)	< 0.5	116.6 (99.9-151.1)	26.9 (23.4-28.9)	1.8 (1.2-2.7)	< 0.5	31.8 (28.2-35.6)
	8	28.8 (25.5-32.5)	5.6 (3.8-8.1)	< 0.5	57.5 (39.0-84.9)	16.6 (11.1-25.6)	1.5 (1.1-2.0)	< 0.5	40.6 (35.0-47.7)

a. Unprimed mice and mice primed by ip injection of 100 µg of DNP-KLH adsorbed on alum, were boosted with either 10µg TNP-LPS or 10 µg DNP-Ficoll ip 2 months later. The number of IgM- and IgG-PFC were enumerated at various days after immunization with TNP-LPS and DNP-Ficoll, respectively. The day 0 values refer to mice primed with DNP-KLH and assayed for IgM- and IgG-PFC two months later.

b. Geometric mean. In parentheses ± 1 SEM (n = 5).

c. ns means that the number of IgG-PFC above the number of IgM-PFC in the indirect plaque assay was not significant.

observation that primary or secondary immunization with TNP-dLPS does not lead to substantial TNP-specific antibody formation in the bone marrow, whereas a lower secondary type splenic response is found with similar kinetics as compared to the primary response (Fig. 1b).

In mice primed with TNP-LPS and boosted with either TNP-LPS or TNP-dLPS a significant IgG-PFC response was found in the spleen. This might be due to the generation of only a small number of memory B cells committed to IgG synthesis after immunization with TNP-LPS. This view would be supported by the observation that unprimed and TNP-LPS-primed donor spleen cells give rise to an equally high adoptive IgG antibody response to DNP-KLH (Table III). Previous results of others also indicate that spleen cells from mice primed with TNP-LPS do not produce secondary type adoptive IgG memory responses to DNP-KLH (14).

Primary immunization with TNP-BA induces neither an IgM- nor an IgG-PFC response in the bone marrow, but does so in the spleen. After secondary immunization, however, both IgM- and IgG-PFC appear in the bone marrow (Fig. 2). Although the bone marrow response is feeble, particularly the IgG-PFC response has the same kinetics as the secondary type responses to TD antigens.

In the case of DNP-Ficoll the secondary PFC response in the spleen and the bone marrow is as high as the primary response (Table I). This is not due to the failure of DNP-Ficoll to induce memory B cells. This is apparent from the observation that pre-immunization with DNP-Ficoll enhances the IgM- and IgG-PFC response to DNP-KLH (Table III). Furthermore, the adoptive IgM- and IgG-PFC response to DNP-KLH after transfer of DNP-Ficoll-primed donor spleen cells is enhanced as compared to the adoptive response by unprimed spleen cells (Table III).

No marrow PFC response is found after primary and secondary immunization with pneumococci (Pn). The secondary IgM-PFC response in the spleen, however, is greatly enhanced (Fig. 3). This indicates that IgM-memory B cells are formed during the primary response to Pn.

The failure of certain TI antigens (e.g., TNP-dLPS, DNP-Ficoll, Pn) to induce substantial secondary BM antibody formation is not due to their inability to (re)activate memory B cells. This can be concluded from our observation that the IgG memory B cells induced during the primary response to the TD antigen DNP-KLH can be (re)activated by TNP-LPS and DNP-Ficoll. The activation of these memory B cells leads to IgG-responses in both the spleen and the bone marrow (Table IV). Furthermore, the enhanced IgM-PFC responses found in the spleen after secondary immunization with TNP-LPS and Pn, is indicative for both the induction and activation of memory B cells committed to IgM synthesis (Figs. 1 and 3). The conclusion, that TI antigens are able to activate memory B cells, has also been reached by others (15-21).

Pre-immunization with either TNP-LPS or TNP-BA suppresses the PFC response to DNP-KLH (Table II). Similarly, the IgM-PFC response to TNP-LPS and DNP-Ficoll is suppressed by pre-immunization with DNP-KLH. These findings can be better explained by humoral factors present in the serum of these mice than by suppressor T cells, because we could not demonstrate such suppressive effects of pre-immunization in adoptive transfer experiments (Table III). Suppression of the secondary type response to TI antigens has also been reported by others (21-23). The humoral factors are probably anti-hapten antibodies (21,22), although suppressive anti-idiotypic antibodies induced by DNP-Ficoll have been

described as well (23). Such regulating factors may render interpretation of the secondary responses to TI antigens more difficult, and might account for the differential capacity of TI antigens to evoke antibody formation in the bone marrow after secondary immunization, as mentioned above.

Alternatively, there might be a heterogeneity among memory B cells of the same specificity induced by different antigens. This possibility is suggested by experiments which show that B lymphocytes of a certain specificity activated by TI antigens are qualitatively different from those activated by TD antigens (24-29). In accordance with these data two distinct IgG memory B cell populations have been described: B2 cells responsive to TD antigens and TI 1 antigens (e.g., TNP-LPS, TNP-BA) and B1 cells responsive to TI 2 antigens (e.g., DNP-Ficoll, Pn) (30). The experiments reported in this paper might support this distinction and, furthermore, would suggest that both subpopulations differ in migratory behaviour, at least with respect to their entrance of the BM.

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APPENDIX PAPER VII

THE PROLIFERATIVE ACTIVITY OF ANTIBODY FORMING CELLS IN THE MOUSE BONE MARROW

G. Koch, B.D. Lok and R. Benner

Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands

SUMMARY

The proliferative activity of antibody-forming cells was studied in the bone marrow of mice immunized with either sheep erythrocytes (SRBC), TNP-LPS or DNP-Ficoll. Peak proliferative activity was found during the first few days of the response. Elimination of the proliferating cells in this period caused a profound and long-lasting suppression of the antibody formation in the marrow.

INTRODUCTION

After secondary, but not after primary, immunization with T-dependent (TD) antigens antibody-producing plaque-forming cells (PFC) appear in the bone marrow. The occurrence of these PFC in the bone marrow is strictly dependent on the availability of B memory cells. The B memory cells are activated in the peripheral lymphoid organs by the booster immunization and then emigrate via the blood stream toward the bone marrow (1). In contrast to TD antigens, primary immunization with certain T-independent (TI) antigens (TNP-LPS, DNP-Ficoll) does lead to antibody formation in the marrow.

Antibody formation to TNP-LPS is dependent on the local activation of newly formed B cells by the mitogenic moiety of LPS. These activated B cells then differentiate into PFC *in situ* (2). The precursors of PFC which appear in the bone marrow after primary immunization with DNP-Ficoll, on the other hand, mainly belong to a slowly renewing, long-lived B cell population. These B cells most likely are activated in the peripheral lymphoid organs and then emigrate toward the bone marrow (2).

So far it is unclear whether the appearance of PFC in the bone marrow after immunization with TD antigens and DNP-Ficoll merely represents the immigration of end-stage cells which do no longer proliferate, or that these cells can still proliferate *in situ*. Similarly it is unclear whether bone marrow antibody formation induced by TNP-LPS is associated with a local clonal expansion of the activated B cells. To investigate the proliferative activities of the bone marrow antibody-forming cells and their precursors we investigated the effect of elimination of the proliferating cells upon the marrow PFC response. This was done by means of injection of hydroxyurea (HU), a drug that kills cells which are in the S-phase of the cell cycle.

MATERIALS AND METHODS

Mice. Female (C57BL/Rij x CBA/Rij)F1 mice, 14-20 weeks of age, were

used. They were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Antigen and immunization. SRBC from a single donor were used. They were obtained from the Central Veterinary Institute, Schiedam, The Netherlands. Lipopolysaccharide (LPS) from *E.coli* 055:B5 (Difco Laboratories, Detroit MI, USA), prepared according to the phenol extraction method, was conjugated with 2,4,6 trinitrobenzenesulfonic acid (TNBS) as described previously (2). Ficoll with a molecular weight of 400,000 daltons was obtained from Pharmacia (Uppsala, Sweden). It was derived to N-(2-amino-ethyl)carbamylmethylated Ficoll (AECM-Ficoll) and conjugated with dinitrobenzenesulfonic acid as described by Eisen (3). Secondary responses to SRBC were induced by primary immunization with 10^7 SRBC and secondary immunization with 2×10^6 SRBC. Primary responses to TNP-LPS and DNP-Ficoll were induced by injection of 10 µg of the antigen. All immunizations were done by iv injection in a tail vein.

Preparation of cell suspensions. Cell suspensions of spleen and femoral bone marrow were prepared in a balanced salt solution as described previously (4).

Assay for PFC. Cell suspensions were assayed for IgM- and IgG-PFC as described previously (5). Anti-TNP PFC responses were assayed with SRBC conjugated with TNBS (6) as targets.

Hydroxyurea treatment. Mice were exposed to hydroxyurea (HU) according to the schedule devised by Hodgson et al. (7). Mice were given two intraperitoneal injections of the drug at a dose of 1g/kg body weight. The second injection was given 7 hr after the first.

Splenectomy. Splenectomy (Sx) and sham-splenectomy (ShSx) were done under Avertin anaesthesia as described previously (2,4).

RESULTS

The bone marrow PFC response to an iv booster injection of 2×10^6 SRBC is dependent on the immigration of spleen-derived SRBC-activated B memory cells and/or their progeny (1,8). This immigration takes place during the first 4 days after the booster injection (8). Thus, splenectomy on day 4 no longer influences the marrow PFC response (c.f. Fig. 1). To investigate whether the immigrant B cells still proliferate within the (bone) marrow we treated such immunized, splenectomized mice with HU on different days after the booster injection and enumerated the number of anti-SRBC PFC at 14 days after the booster injection. Treatment with HU on day 4 and 5 reduced the number of a PFC in the marrow to 10-20% of the number found in non-treated control mice. Beyond day 6 HU treatment did not have a significant effect upon the bone marrow PFC response. Essentially the same results were found in similar experiments involving anti-SRBC PFC enumeration on day 10 and day 28 after the booster injection (data not shown).

In a comparable experimental setup it was investigated whether

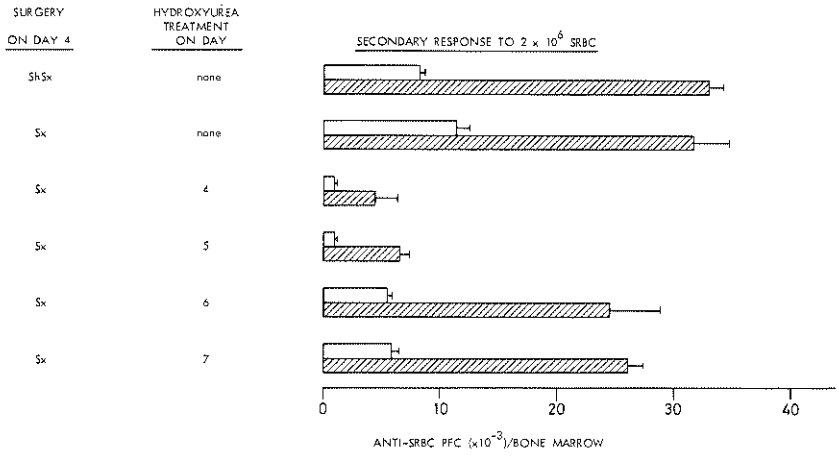

Figure 1. Influence of hydroxyurea treatment upon the secondary PFC response to SRBC in mouse bone marrow. Open columns represent the geometric mean of the IgM-PFC response, hatched columns of the IgG-PFC response. Bars represent 1 SEM. PFC were determined 14 days after the booster injection of 2 x 10^6 SRBC iv (n = 5).

bone marrow antibody formation induced by primary immunization with TNP-LPS and DNP-Ficoll is also associated with proliferation of the relevant B lineage cells within the bone marrow. In these experiments however, the mice were not splenectomized. Thus, mice immunized with 10 µg TNP-LPS or DNP-Ficoll were treated with HU on day 2, 4 or 6 after antigen injection, and anti-TNP PFC were determined in spleen and bone marrow on day 10. HU treatment on days 2 and 4 after immunization with TNP-LPS reduced the number of PFC in the bone marrow to 10 and 40% of the value found in non-treated controls, respectively. No effect on the bone marrow PFC response was found in case of HU treatment at 6 days after immunization (Fig. 2, upper part). In the spleen HU treatment had much less effect upon the PFC response. In this organ, a significant reduction of the number of PFC was only found on day 2 after immunization. The effect of HU treatment upon the PFC response in the spleen and the bone marrow of mice immunized with DNP-Ficoll was essentially the same as described for TNP-LPS. In this case, however, there was still a clear proliferative activity by day 6 after immunization (Fig. 2, lower part).

In all experiments HU appeared to cause a temporary decrease of the total nucleated cell count in the bone marrow, as has been reported by others (9). No correlation was found between the number of PFC and the nucleated cell count in the marrow (data not shown).

DISCUSSION

Antibody formation *in vivo* is largely dependent upon a clonal expansion of the antigen-activated B cells. This has been shown in various species and for a variety of antigens (10,11). These studies, however, were all restricted to spleen and lymph nodes. In recent

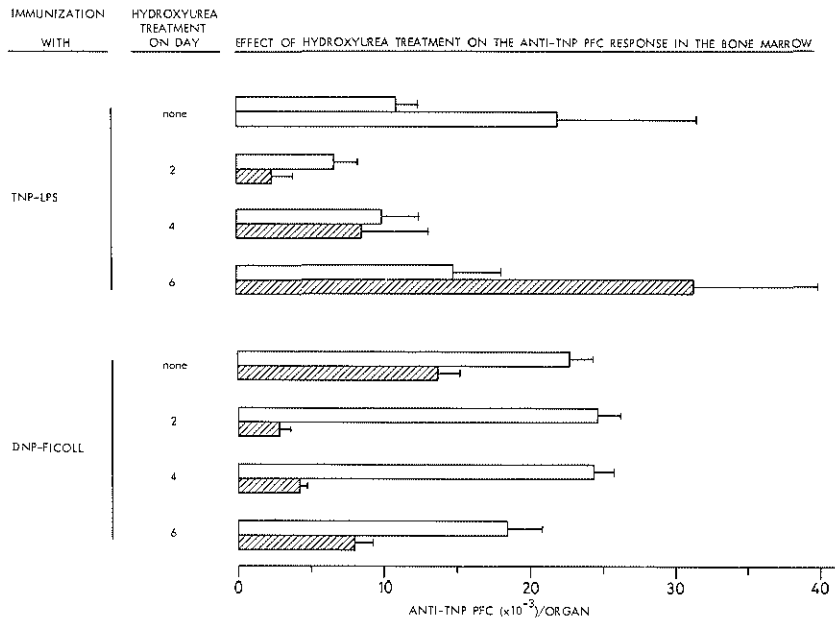

Figure 2. Influence of hydroxyurea upon the primary anti-TNP PFC response in mouse spleen and bone marrow after immunization with either TNP-LPS or DNP-Ficoll. Open columns represent the geometric mean of the response of the spleen, hatched columns the response of the bone marrow. Bars represent 1 SEM. PFC were determined 10 days after immunization (n = 5).

years it has become clear that the bone marrow is an important site of antibody formation as well. Antibody-forming cells which appear in the bone marrow are mostly derived from peripheral lymphoid organs (1). So far, only the antibody formation in the bone marrow to TNP-LPS has been shown to be dependent upon a local activation and differentiation of the antibody-forming cells (2).

Experiments that deal with the proliferative activity of antibody-forming cells in the bone marrow are difficult to design as to the immigration of cells from peripheral lymphoid organs. Therefore we made use of the data from our previous splenectomy experiments, which showed that the immigration of SRBC-activated B cells giving rise to PFC is restricted to the first 4 days after booster immunization (8). Thus, the reducing effect of HU treatment upon the bone marrow PFC response of splenectomized mice can be interpreted in terms of proliferative activity of the relevant cell compartment. Peak proliferative activity of the antibody-forming cell compartment in the marrow was found to occur on day 4 and 5 after secondary immunization with SRBC (Fig. 1). The HU-induced reduction of the bone marrow PFC was, in contrast to the HU-induced decrease of the cellularity of the marrow, not a temporary phenomenon, but still present 4 weeks after the booster injection. This indicates that the magnitude of the antibody formation in the bone marrow is highly dependent upon two events: the migration of antigen-

activated B cells toward the bone marrow (1,8), and the proliferative activity of these immigrant cells within the marrow. Both events are restricted to the first few days after immunization, in spite of the sustained character of the bone marrow PFC response (12).

The marrow PFC response induced by injection of DNP-Ficoll is also mainly caused by immigration into the marrow of B cells activated by the antigen in peripheral lymphoid organs (2). After antigenic stimulation of these cells they probably start to proliferate in the spleen and continue to proliferate in the marrow (Fig.2).

Antibody formation in the bone marrow to TNP-LPS is caused by a local activation and differentiation of B cells, and is dependent upon the mitogenic moiety of LPS (2). The differentiation of these activated B cells is clearly associated with proliferation, which is maximal about 3 days after immunization (Fig. 2).

Remarkably, the splenic PFC response induced by TNP-LPS and DNP-Ficoll was much less affected by HU treatment than the bone marrow PFC response. This might be due to a repeated induction of specific B cells in the spleen by the persisting antigen (13,14), so that at the moment of testing (day 10) the initial HU-induced decrease of the splenic PFC response has been compensated for by newly activated B cell clones. The induction of bone marrow antibody formation, on the other hand, seems to be restricted to the first few days after immunization (8), so that elimination of the proliferating cells in this period causes a sustained depression of the antibody formation in the marrow.

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