T cells and the humoral immune system

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aan Cisca,
Wineke en Ernst Michiel

ERRATUM

Page 47: '1 to 5 x 10^6 DRT' should be
'1 to 5 x 10^6 NT or 2 x 10^6 DRT'

Contents

What nature hides in one life form
she reveals in another
William Harvey (1578-1657)

Introduction

The discovery of T and B cells

More than forteen years have passed since the discovery that the thymus is essential for the development of cellular and humoral immunity in mammals. The notion that the thymus has a very important function in the development of the immune system was initiated with the discovery of Miller (1961) that neonatal thymectomy of mice depressed the rejection of allogeneic skin grafts. Subsequently it was observed that the production of antibodies to influenza virus (Miller, Marshall and White, 1962) or sheep erythrocytes (Humphrey, Parrott and East, 1964) was impaired after neonatal thymectomy. A few years later new important findings were added by the studies of Claman, Chaperon and Triplett (1966); Davies, Leuchars, Wallis, Marchant and Elliott (1967) and Mitchell and Miller (1968). The authors have shown that the addition of thymus cells to a bone marrow cell suspension which was injected into lethally irradiated recipients greatly enhanced the humoral response to sheep red blood cells (SRBC). When bone marrow cells alone or thymus cells alone were injected only very low responses were observed. It was concluded that there is a synergistic interaction between lymphoid cells from thymic and bone marrow origin. The thymus-derived lymphocytes (T cells) did not produce antibodies, but assisted the non thymus-derived cell to do so. Therefore the thymus-derived cell was called helper cell. The non-thymus derived lymphocyte, which turned out to be the precursor of the antibody-forming plasma cell, was called B cell. These observations on the co-operation between T cells and B cells were confirmed and extended by Mitchison and co-workers using hapten-carrier conjugates instead of SRBC (Mitchison, 1971a; Mitchison 1971b; Britton, Mitchison and Rajewski, 1971 and Mitchison, 1971c). In their studies different haptens, which are

in itself not immunogenic, were conjugated with different proteins as carrier molecules. In one of their experiments cells derived from animals primed with the hapten-carrier complex NIP-ovalbumin were mixed with cells derived from animals primed with another carrier bovine serum albumin (BSA), transferred to irradiated recipients and challenged with the hapten conjugated to the second carrier (NIP-BSA). In comparison with controls lacking the BSA-primed cells, the mixture regularly showed a higher response. Tests with allotype-marked carrier-primed cells showed that these cells did not participate in the production of the anti-hapten antibody and could therefore properly be regarded as helpers. They concluded that there was specific stimulation of the T cells by antigenic determinants on the carrier followed by the development of B cells into anti-hapten antibody-producing cells (plasma cells).

The name B cell originated from studies in birds in which was shown that the bursa of Fabricius is the main organ for the development of this cell type (Warner and Szenberg, 1964). Although mammals lack a bursa of Fabricius it is generally accepted that there is a mammalian equivalent of the bursa. It has been suggested that foetal liver (Owen, Cooper and Raff, 1974) or germinal centers in the peripheral lymphoid organs in the adult (Nieuwenhuis and Keuning, 1974) play this role in mammals.

The role of the macrophage

Mosier and Coppleson (1968) concluded from results of limiting dilution assays that a third cell was essential for the induction of the humoral immune response *in vitro*. This cell was defined as a radioresistant and adherent cell (Tan and Gordon, 1971). At present most authors agree that this cell belongs to the group of mononuclear phagocytes or macrophages. Both T and B cells may be optimally stimulated by antigen when it is concentrated by the macrophage. A part of the antigen is not metabolized but presented in a persisting immunogenic form on the cell membrane of the macrophage (Unanue, 1972).

Factors which play a role in the differentiation of hemopoietic stem cells into T and B cells

Several factors which play a role in the differentiation of hemopoietic stem cells into T and B cells are schematically presented in figure 1. It is known that during the ontogeny of birds multipotential hemopoietic stem cells (S) migrate from the yolk sac to the thymus and bursa (Moore and Owen, 1967a; 1967b) where they differentiate into T and B lymphocytes. Studies on mouse thymic rudiments *in vitro* also suggest that thymic lymphocytes were derived from blood-borne stem cells (Owen and Ritter, 1969). The factors in the thymic micro-environment which turned out to be rather important for the differentiation from stem cells into immature thymocytes (T_0) and from T_0 cells into a subpopulation of mature thymus cells called T_1 cells are the thymic hormones (TH) (White, 1975; Bach, Bach, Charriere, Dardenne, Fournier, Papiernik and Pleau, 1975; Trainin, Small, Zipori, Umiel, Kook and Rotter, 1975; Goldstein, Thurman, Cohen and Hooper, 1975). Antigens (AG) probably do not play an important role during the differentiation from T_0 to T_1 cell (Cantor and Boyse, 1975). These hormones which are produced by thymic epithelial cells are not only active within the thymus but also outside this organ. For example the number of colony forming units (CFU) in bone marrow of neonatally thymectomized mice is lower than in bone marrow of normal mice (Resnitzky, Zipori and Trainin, 1971). In these studies the number of CFU, which is a measure for the number of hemopoietic stem cells, was determined with the spleen colony assay of Till and McCulloch (1961). Further investigations of Zipori (1975) have shown that only 7 percent of the bone marrow CFU in neonatally thymectomized mice were in the S-phase of their cell cycle whereas 25 percent of the bone marrow CFU in normal mice were found in the same phase of the cell cycle. Addition of a thymic hormone either *in vivo* or *in vitro* reversed the suppressive effect of thymectomy on DNA synthesis in CFU. So it is obvious that thymic hormones have a direct effect on hemopoietic stem cells. Therefore thymic hormones might have - in our opinion - an indirect

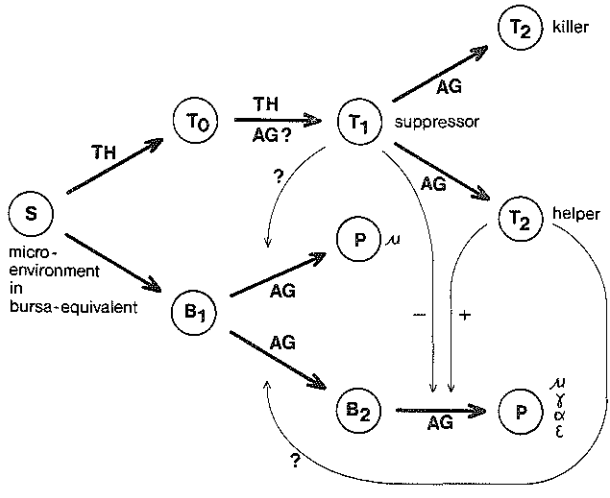

Fig. 1. A schematic diagram presenting several factors which play a role in the differentiation of hemopoietic stem cells (S) into T and B cells in the mouse. Stem cells migrate to the thymus where they differentiate under the influence of thymic hormones (TH) into immature thymocytes (T_0) and from T_0 into a subpopulation of T cells (T_1). The T_1 cells migrate to the peripheral lymphoid organs where they probably act as suppressor cells or differentiate under the influence of antigens (AG) into another subpopulation of T cells (T_2). Within the T_2 population 2 types of cells are distinguished: 'killer' cells which play a role in cellular immunity and 'helper' cells which are important in humoral immunity. Differentiation from stem cells into a subpopulation of B cells (B_1) is probably regulated by certain factors in the micro-environment of the bursa-equivalent. Upon antigenic stimulation B_1 cells differentiate into IgM-producing plasma cells (P) or into another subpopulation of B cells (B_2) which are the precursors of IgM-, IgG-, IgA- and IgE-producing cells. The differentiation of B_2 cells into plasma cells is regulated by antigens and substances produced by suppressor T cells (T_1) or helper T cells (T_2). The influence of the T cell population (T_1 or T_2) on the differentiation of B_1 cells into IgM-producing cells or into B_2 cells is not clear at present (?).

⟶ = differentiation from one cell type into another

⟶ = regulatory influence of the T cell population on the differentiation from B cells into plasma cells

The papers added to this thesis are dealing with several aspects of the regulatory influence of the T cells on the B cell system. Paper I is dealing with the supposed influence of T cells on the differentiation of stem cells into B_1 and B_2 cells. Paper II is dealing with quantitative aspects of T cell help (T_2) and T cell suppression (T_1) during in vivo or in vitro immune responses. Paper III is dealing with the period in which T cell help (T_2) is needed during the differentiation from B_2 cell into plasma cell. Paper IV is dealing with the effect of T helper cells (T_2) on the precursors of the IgA-producing plasma cells. Paper V is dealing with the effect of suppressor cells (T_1) and helper cells (T_2) on the number of B cell clones which will develop into plasma cells.

effect on the cell populations which are derived from stem cells e.g. T cells, B cells and cells of the myeloid series. There are no clear indications that thymic hormones play a role in the further differentiation from T_1 cell into another type of T cell (T_2), which takes place in the peripheral lymphoid organs (e.g. spleen and lymph nodes) under the influence of antigens (Raff and Cantor, 1971) and in direct contact with a special type of mononuclear phagocytes, the interdigitating cell (Veldman, 1970; Veerman, 1974; van Ewijk, Verzijden, van der Kwast and Luijcx-Meijer, 1974). The subdivision of the T cell population into T_1 and T_2 cells as mentioned above is based upon different properties of these cells (Raff and Cantor, 1971): the T_1 cell is present in higher concentrations in thymus and spleen than in lymph node, blood or thoracic duct and is relatively insensitive to anti-lymphocyte serum (ALS) *in vivo;* the T_2 cell is present in high concentrations in lymph nodes, blood and thoracic duct and is very sensitive to ALS *in vivo*. The number of T_1 cells decreases shortly after adult thymectomy, whereas the number of T_2 cells is not affected until very long after adult thymectomy. Rotter and Trainin (1975) observed an increase in the immune response to polyvinylpyrrolidone (PVP) at 6 weeks after adult thymectomy. This increase in the response to PVP is probably due to a decrease in the suppressor T cell population. Therefore it is tempting to say that the suppressor T cells are a part of the T_1 population. Within the T_2 cell population 2 groups of cells are present: (1) 'killer' cells e.g. cells committed to cytotoxic responses to alloantigens (cellular immunity) and (2) 'helper' cells which have a stimulating effect on antibody production (humoral immunity). Elegant studies of Cantor and Boyse (1975) have shown that these cells can be distinguished not only with functional tests but also using specific antisera for Ly antigens present on their cell membrane. These observations of Cantor and Boyse (1975) are not in agreement with the results of Kerckhaert (1974). He observed that the influence of antigen presentation was always in accordance with an inverse relationship between the humoral and the cellular response. This inverse relationship was explained by the assumption

that there is competition for a cell common to the humoral and the cellular response. In other words: Kerckhaert (1974) suggests that the 'killer' cell and the 'helper' cell are identical.

Humoral factors which regulate the development of B cells from hemopoietic stem cells are not clearly demonstrated at present. Most authors agree that the micro-environment in the bursa or the bursa-equivalent is an important factor in the development of B cells from stem cells. There are no indications that antigens play a role in the first steps of the B cell differentiation. Goidl and Siskind (1974) have shown that the heterogeneity of the antihapten response during the ontogeny of normal mice is the same as in germ-free mice. The subdivision of the B cell population into B_1 and B_2 cells is more or less hypothetical. Gershon (1974) suggests that there are two B cell classes: B_1 cells which require no help from T cells to be triggered to IgM production and B_2 cells which will not respond to antigen without T cell help. Not only precursors of IgM-producing cells, but also precursors of IgG-, IgA- and IgE- producing cells are present within the B_2 population. Another hypothesis is proposed by Nieuwenhuis and Keuning (1974). According to their ideas B_1 cells are directly bone marrow-derived cells which are involved in the formation of germinal centres. The germinal centre reaction, which is antigen-driven, produces B_2 cells. Cell proliferation in the germinal centre seems to be partly specific, i.e. directly related to the antigen which induced the germinal centre and therefore producing specific B_2 'memory' cells, and partly non-specific, leading to the production of B_2 cells capable of mediating a primary immune response to unrelated antigens. In agreement with these ideas of Nieuwenhuis and Keuning are the results of experiments performed by Cunningham and Pilarski (Cunningham and Pilarski, 1974; Pilarski and Cunningham, 1974 and Cunningham and Pilarski, 1975). They have shown that antigen stimulates the proliferation of an initial population of B cells with low affinity receptors from which it generates cells producing entirely new specificities for unrelated erythrocyte antigens.

The interaction between T and B cells

The interaction between T cells and B cells is rather complex. This complexity is even enlarged by recent findings concerning subpopulations of T cells (Raff and Cantor, 1971) and B cells (Gershon, 1974; Nieuwenhuis and Keuning, 1974). Moreover, it has been discovered that T cells not only act as helper cells but also as regulator or suppressor cells (Barthold, Kysela and Steinberg, 1974; Gershon, 1974). The first evidence for the existance of suppressor cells came from experiments of Baker, Barth, Stashak and Amsbaugh (1970) in mice. They observed an enhanced response to pneumococcal polysaccharide type III after removal of the T cell population by means of anti-lymphocyte serum.

The original theories concerning the co-operation between T and B cells are based on the idea that a direct cellular contact between T and B cells is needed in order to stimulate the B cells. According to the ideas of Bretcher and Cohn (1970) the formation of an antigen bridge between T and B cells is an essential step in this process. Antigen presented on the membrane of the T cell in a multivalent form induced plasma cell formation, whereas soluble antigen induced tolerance. The present theories concerning the co-operation between T and B cells are based on the idea that direct contact between T and B cells is not essential for the induction of plasma cell formation. It has been shown by several authors that T cells can be replaced by non-specific or by specific humoral factors produced by antigen-stimulated T cells. An antigen-specific T cell factor is demonstrated by the experiments of Feldmann and co-workers (Feldmann and Basten 1972; Feldmann, 1972). They have shown that antigen-stimulated T cells release immunoglobulin-like 'receptor' molecules from their cell membrane which are specific for the carrier determinants of an antigen. These 'receptor' molecules -called IgT by Feldmann- are absorbed on macrophages, presumably because of their cytophilic nature. In this macrophage-bound form IgT stimulates the B cell by presenting the antigen in a multivalent form. Another example of antigen-specific T cell factor is recently described by Taussig and Munro (1975). Here the factor is able to replace

T cells in the thymus-dependent antibody response to a synthetic polypeptide. Using specific antisera they were able to demonstrate that it is not an immunoglobulin but a product of the I-A subregion of the H-2 complex. The factor is not absorbed by macrophages but can be absorbed by purified peripheral B cells. This absorption is blocked if the B cells are pretreated with anti-H-2 sera. Thus B cells carry an H-2 coded 'acceptor'-site for the factor in addition to the well-known antigen-receptor, which has the properties of an immunoglobulin.

Non-specific T cell factors which have a stimulating effect on the humoral immune response are described by many authors (Schimpl and Wecker, 1972; Gorczynski, Miller and Philips, 1973; Hunter and Kettman, 1974 and Waldmann, 1975). These non-specific factors are in general produced by T cells on contact with foreign histocompatility antigens *in vitro*. If these factors are added in sufficient quantities at the right time they can fully substitute for T helper cells in the *in vitro* response of B cells to unrelated antigens such as SRBC. These factors can only stimulate B cells which carry their specific antigen. Because of the low concentration of these factors only the closest B cells will be stimulated e.g. B cells linked to the T cells by an antigen bridge on the surface of a macrophage. Therefore it might be that these non-specific factors act more or less like specific factors under normal *in vivo* conditions. At present it is not clear if all T cell factors, which have been described during the last 3 to 4 years, have the same effect on the humoral immune response. It is possible that they act at different moments and at different stages of the proliferation and differentiation of antigen-stimulated B-cells.

As far as the suppressor cell activity of T cells is concerned also examples of specific and non-specific T cell activities can be given. Herzenberg and Herzenberg (1974) have shown that spleen cells from an allotype suppressed mouse will prevent the production of immunoglobulins of that allotype by spleen cells from normal mice. The suppressive activity was abolished by treatment with anti-θ serum and was allotype specific. Another example is tolerance to a specific antigen. It has

been shown by Basten, Miller, Sprent and Cheers (1974) that spleen cells from a tolerant mouse will suppress the response of syngeneic cells from a non-tolerant mouse. This specific suppression was again dependent on the presence of T cells from the tolerant mouse. In other studies the suppressive activity of T cells is non-specific. Rich and Pierce (1974) have shown that a supernatant from mitogen-stimulated T cell cultures suppresses the humoral immune response by normal spleen cells to any antigen tested.

Introduction to the papers

Various aspects of the interaction between the T cell population and the humoral immune system are described in the papers added to this thesis. Our first paper (appendix publication I) is dealing with the supposed influence of the T cell on the development of B lymphocytes from stem cells. The development of the B cell population in adult thymectomized, irradiated and bone marrow-reconstituted mice is compared with the same process in sham-thymectomized, irradiated and reconstituted animals. No stimulating or suppressive effect of the T cells on the generation of B cells was observed in our experiments. Thus the generation of 'virgin' B cells (B_1) or 'memory' B cells (B_2) is independent of the presence of T cells (figure 1). Our results were in agreement with the results of Owen, Cooper and Raff (1974), who have shown that there is a sequential development of IgM-, IgG- and IgA-B cells in organ cultures of mouse foetal liver in the absence of T cells.

Appendix publication II deals with the helper cell activity of T cells derived from different sources. The helper cell activity of normal thymocytes, dexamethasone-resistant thymocytes (DRT) and educated T cells (Ed.T) was studied *in vivo* and *in vitro*. It was observed that Ed.T were both *in vivo* and *in vitro* active as helper cells. Normal thymocytes and DRT were active as helper cells *in vivo* but showed a suppressive effect *in vitro*. Possibly the normal thymocytes and the DRT contain a high number of relatively undifferentiated T cells which act as suppressor cells (T_1) *in vitro*. The helper cell activity of the same cells *in vivo* might be explained by the fact that the

lymphoid organs in the animal (e.g. the spleen) provide a more suitable micro-environment for the differentiation from relatively undifferentiated T cells (T_1) into an other T cell type (T_2) than the culture dish.

Appendix publication III deals with the T cell-dependent period in the immune response to SRBC. It is quite obvious from the literature that the T cell plays an important role during the humoral immune response (Gershon, 1974), but it is not well established in which period the presence of the T helper cell (T_2) is essential. The results of our experiments indicate that helper cells play an important role during the first 3 days of the response *in vivo*. Comparable results were obtained by Britton, Mitchison and Rajewsky (1971). They incubated a mixture of allogeneic helper cells and syngeneic B cells from primed donors under *in vitro* conditions likely to support co-operative induction of the secondary response and then transferred the cells into hosts alloimmune to the helper cells. Under these conditions the response did not proceed after the transfer. Their conclusion was that under *in vivo* conditions a continuous co-operation between T and B cells is required in order to obtain a full response. However, our experiments concerning the T cell-dependent period of the immune response *in vitro* provide evidence for the notion that the presence of T helper cells is required for less than 1 or 2 days. The difference between the response *in vivo* and the response *in vitro* is probably due to a synchronous start of the plasma cell development *in vitro* and a more asynchronous start of this process *in vivo*.

Appendix publication IV deals with the thymus-dependence of of the IgA response to SRBC. There is circumstantial evidence in mice that B cells committed to IgG synthesis require a greater degree of interaction with T helper cells for triggering than do B cells committed to IgM synthesis (Taylor and Wortis, 1968; Wortis, Dresser and Anderson, 1969; Anderson, Dresser and Wortis, 1974; Bankhurst, Lambert and Miescher, 1975). The thymus-dependence of the IgA response is less clear-cut. Our experiments with mice provide evidence that the IgA response to SRBC is T cell-dependent.

Appendix publication V deals with the regulatory influence of the thymus or T cells on the heterogeneity of serum immunoglobulins. The transient appearance of homogeneous immunoglobulins, 'paraproteins', in serum is a frequent finding during the reconstitution of the immune system after bone marrow transplantation in children with severe combined immunodeficiency (Radl and van den Berg, 1973). The simplest explanation of this finding is that only a limited number of B cells repopulates the immune system during its early development. Later, more B cell clones arise and a gradual transition from homogeneous to heterogeneous responses will occur. However, the question arose whether cell populations other than B cells (e.g. T cells) were also important in the regulation of antibody heterogeneity. Therefore we studied the role played by the thymus and/or the T cells in the regulation of the heterogeneity of serum immunoglobulins in thymectomized or sham-thymectomized mice. The animals were lethally irradiated and reconstituted with bone marrow cells or foetal liver cells. It was observed that a delay in the maturation of the T cell population or the absence of the T-system contributed substantially to the appearance of homogeneous immunoglobulins during the reconstitution period. It is concluded that the T cell plays an important role in the regulation of immunoglobulin heterogeneity. Additional evidence for the idea that T cells are important for the maintenance of a normal heterogeneous spectrum of serum immunoglobulins can be derived from the observation of an increasing frequency of homogeneous immunoglobulins under conditions of decreasing thymic activity. An example of this phenomenon is the increasing number of homogeneous immunoglobulins during aging in man (Hällen, 1963) and in mice (Radl and Hollander, 1974).

Discussion

The central theme of this thesis is the regulatory influence of T cells on the course of life of B cells. Even during the early life of the B cell, i.e. during the development of the B cell from a hemopoietic stem cell T cells are supposed to play a role. For example, it was suggested by Mitchell, Pye, Holmes and Nossal (197? that T cells play a stimulatory role in germinal centre formation in mice. They observed that in 'nude' mice, which have been shown to display congenital thymic aplasia (Pantelouris, 1968), germinal centres are absent or very rare. Additional evidence for the idea that T cells play a role in germinal centre formation came from experiments of Gutman and Weissman (1972) who have shown that T antigen positive lymphocytes are present in germinal centres of normal mice. Moreover, studies by Nieuwenhuis and Keuning (1974) on germinal centres in the rabbit spleen and popliteal lymph nodes provided strong evidence for an important role of germinal centres in the generation of B cells. In our opinion it is questionable whether T cells play a direct role in the formation of germinal centres and thus in the generation of B cells from hemopoietic stem cells. This idea is based upon the observation that in the adult thymectomized, lethally irradiated and bone marrow-reconstituted mice (TxBM mice) which were used in our experiments (appendix publication I) considerable numbers of germinal centres were present in the absence of a normal T cell population (own observations; van Ewijk, Brons and Rozing, 1975). Moreover, there was no significant difference in the development of the B cell population from hemopoietic stem cells in sham-thymectomized and thymectomized mice (appendix publication I). Therefore we conclude, that T cells do not play an essential role in the generation of B cells. The only possibility for a T cell influence on B cell development is an indirect effect on antigen trapping. As helper cells T cells stimulate the production of antibodies to environ-

mental antigens. Complexes of these antibodies with their corresponding antigens are trapped in the follicles and thus stimulate the germinal centre reaction (van Rooijen, 1974).

Although the influence of the T cell on the development of the B cell from hemopoietic stem cells may be questionable, the role of the T cells in the subsequent differentiation of B cells into plasma cells is clearly demonstrated in the literature as well as in the papers added to this thesis (appendix publication II, III, IV and V). The T cells fulfil a regulatory function by either stimulating or suppressing the antigen-induced development of the plasma cells. In our experiments (appendix publication II) the stimulatory or suppressing effect of T cells obtained from the thymus of normal animals or dexamethasone-treated animals before contact with a specific antigen was compared with the same effect of T cells obtained from a spleen of an irradiated recipient 7 days after injection with thymocytes and a specific antigen ('educated' T cells). It was observed that the relatively undifferentiated cells from the thymus act as suppressor cells during an immune response *in vitro* but as helper cells during an immune response *in vivo*, whereas the more differentiated 'educated' T cells (T_2) act as helper cells both *in vivo* and *in vitro*. It is concluded that in our experiments the differentiation of suppressor (T_1) cells into helper (T_2) cells only occurred *in vivo*. Factors which play a role in this process are not only specific antigens but probably also thymic hormones produced by epithelial cells in the thymus or factors secreted by interdigitating cells in the peripheral lymphoid organs. The literature dealing with the differentiation from T_1 to T_2 cell *in vitro* is rather scarce. Mosier and Pierce (1972) have shown that the 'education' of T cells *in vitro* only occurs in the presence of thymic epithelial cells, splenic adherent cells or kidney fibroblasts. The best results were obtained with thymic epithelial cells.

During the antigen-induced differentiation of B cells into plasma cells the presence of T cells is needed only for a limited period of time. It is concluded from the results of our *in vitro* experiments described in appendix paper III that the B cell needs the T cell help only during the first one or two days after antigenic stimulation. During the following days the B cell differen-

tiates into an antibody-secreting cell. Cell proliferation occurs during the whole period between day 1 and day 4 but not during the first 24 hours. This has been shown by Dutton and Mishell (1967) using the ^{3}H-TdR suicide technique, which kills cells with an active DNA synthesis. In our experiments (appendix publication III) the antigen was present during the whole period from day 0 till day 4 or day 5 (peak of the response). It is conceivable that antigen is a proliferation-stimulus during the whole period but that the factor produced by T cells is a differentiation-stimulus, which is needed for only one or two days. The way in which this humoral factor produced by T cells regulates the B cell differentiation is unknown at present. It might act on the permeability of the B cell membrane or on the transcription process from DNA into m-RNA or the translation process from m-RNA into proteins.

There are many indications that the production of antibodies belonging to the different immunoglobulin classes in mice (IgM, IgG, IgA and IgE) is regulated by T cells. This T cell-dependency may be different when comparing the different classes and subclasses. From our experiments with SRBC (appendix publication IV) the conclusion was drawn that B cells committed to IgA synthesis require the same degree of interaction with T cells as B cells committed to IgM synthesis, but a lower degree of interaction with T cells than B cells committed to IgG synthesis. Experiments of Hamaoka, Katz and Benacerraf (1973) indicate that the production of IgE antibodies is also T cell-dependent. The fact that the production of antibodies belonging to the different classes has a variable degree of T cell-dependency suggests that some B cells need a higher concentration of the humoral factors produced by T helper cells than other B cells which might have more or better 'receptors' for the same T cell factors.

From the results of the experiments mentioned above (appendix publication II, III, and IV) the conclusion can be drawn that the T cell plays an important role in the regulation of the humoral response to SRBC in the mouse. This conclusion was confirmed by the experiments described in our last paper (appendix publication V). Here the influence of the T cell population was studied on the humoral responses to enviromental antigens. Under normal

conditions large numbers of B cells are triggered by these antigens and will develop into plasma cells which produce a very heterogeneous spectrum of serum immunoglobulins. In our experiments the heterogeneity of serum immunoglobulins was studied in thymectomized or sham-thymectomized mice after lethal irradiation and reconstitution with a small number of bone marrow or foetal liver cells. A few interesting observations were made: (1) in the animals which lack T cells (TxBM mice) the highest number of homogeneous immunoglobulins (H-Ig) were present; (2) in the animals in which both T and B cells were developing from stem cells (sham-TxBM mice) lower numbers of H-Ig were present; (3) in the animals which received additional T cells a nearly normal heterogeneous spectrum of immunoglobulins was observed. It is worthwhile to mention that it was shown in appendix publication I that the B cell population in sham-TxBM and TxBM mice increased during the first month after the bone marrow transplantation and reached a normal and constant level approximately one month after the transplantation. Therefore the occurence of H-Ig observed in the period between one and four months after the transplantation (appendix publication V) cannot be explained by a limited number of B cells but must be due to the absence or delayed maturation of the T cell population.

The persistence of H-Ig in sham-TxBM mice during 4 months or even longer after the transplantation (unpublished observation) is amazing. An explanation for this finding might be a deficient development of the T cell population in irradiated mice. Probably the thymus of these irradiated animals is not always capable of rebuilding a normal T cell population. Rotter and Trainin (1975) recently provided evidence that the number of helper T cells in these mice is normal but that the number of suppressor T cells. is lower than normal. Based on these data and our own observations (appendix publication V) the conclusion can be drawn that irradiated mice are far from normal even 4 or more months after irradiation and reconstitution.

Summary

Lymphoid cells and macrophages play an important role in the development and maintance of humoral and cellular immunity in mammals. The lymphoid cells in the peripheral lymphoid organs are divided into two major classes: (1) thymus-derived lymphocytes or T cells and (2) bursa-equivalent-derived lymphocytes or B cells. Humoral immune responses to most antigens require interaction between macrophages, T cells and B cells. Antigen-stimulated T cells do not produce antibodies but secrete regulatory substances which have a stimulating or a suppressive effect on the differentiation of antigen-stimulated B cells into antibody-producing plasma cells. The macrophage plays an important role in this T-B interaction by concentrating the antigen by endocytosis and presenting a part of it in a persisting immunogenic form on its cell membrane. Several aspects of the regulatory influence of the T cell on the humoral (B) immune system were investigated in our laboratory and the results of our experiments are presented in the papers added to this thesis.

In appendix publication I the supposed influence of the T cell population on the development of B cells from stem cells was investigated. The development of the B cell population in adult thymectomized, lethally irradiated and bone marrow-reconstituted mice was compared with the same process in sham-thymectomized animals which were treated in the same way. Using a functional test the number of B cells present in the spleen of the animals was assayed at various moments after bone marrow transplantation. A sequential appearance of the precursors of IgM-, IgG- and IgA-producing cells was observed. The number of B cells reached a constant and normal level at 30 days after transplantation. No significant difference between the thymectomized and the sham-

thymectomized animals was observed indicating that there was no influence of the thymus or the T cell population on the development of the B cell population from hemopoietic stem cells.

Appendix publication II deals with the helper cell activity of T cells derived from different sources. The helper cell activity of normal thymocytes, dexamethasone-resistant thymocytes (DRT) and educated T cells (Ed.T) was studied *in vivo* and *in vitro*. The helper cell activity of the different T cells was studied *in vivo* by means of cell transfer experiments. Lethally irradiated mice were injected intraveneously (i.v.) with sheep red blood cells (SRBC), B cells and various numbers of T cells. The spleen of the recipients was removed 7 days later and the number of plaque-forming cells (PFC) was determined. The helper cell activity of the different T cells was studied *in vitro* by culturing various numbers of T cells mixed with normal spleen cells and SRBC. The number of PFC per dish was determined 5 days later. The number of PFC per recipient spleen in the *in vivo* experiments or per dish in the *in vitro* experiments was considered as a measure for the helper cell activity of the T cells. It was observed that Ed.T were both *in vivo* and *in vitro* the most active type of helper cells. DRT and to a lesser extent normal thymocytes showed a stimulating effect *in vivo*, but a suppressive effect *in vitro*. Possibly normal thymocytes and DRT contain a high number of relatively undifferentiated suppressor cells (T_1). The helper cell activity of the same cells *in vivo* may be explained by the notion that the lymphoid organs in the animal (e.g. the spleen) probably provide a more suitable micro-environment for the differentiation from suppressor (T_1) to helper (T_2) cell than the culture dish.

Appendix publication III deals with the T cell dependent period in the immune response to SRBC. It is quite obvious from the literature that the T cell plays an important role during the humoral immune response, but it is not well established in which period the presence of the T helper cell is essential. To study the T cell dependent period in the immune response

of mouse spleen cells to SRBC the co-operation between T and B cells was abrogated at different times during the *in vivo* or the *in vitro* response. The abrogation was performed by killing the T cells with anti-θ serum or anti-H-2 serum. The surviving cells were subsequently cultured *in vitro* in the presence of SRBC and the number of PFC was determined each day. The results of our experiments indicate that T cells play an important role during the first 3 days of the response *in vivo*. However, in case of the *in vitro* response the presence of the T cells is only required during the first 2 days. The difference between the response *in vivo* and the response *in vitro* is probably due to a synchronous start of the plasma cell development *in vitro* and a more asynchronous start of this process *in vivo*. This asynchronous start of the response *in vivo* might be due to an influx of T- and B-cells in the spleen during the first days after antigen injection. As a consequence of this phenomenon a part of the B cells will start to differentiate into plasma cells at a later stage of the response.

Appendix publication IV deals with the thymus-dependence of the IgA response to SRBC. The thymus-dependence of the IgA response to SRBC was studied by means of cell transfer experiments in mice. Only low numbers of IgM-, IgG- and IgA-plaque-forming cells (PFC) were observed in those recipients which received only spleen cells from adult thymectomized, irradiated and bone marrow-reconstituted mice (B cells). High numbers of IgM-, IgG- and IgA-PFC were observed when B cells and educated T cells were transferred to the recipients. Evidence is provided that B cells committed to IgA synthesis require the same degree of interaction with T cells as B cells committed to IgM synthesis, but a lower degree of interaction than B cells committed to IgG synthesis. It is concluded that the IgA response to SRBC in mice is T cell-dependent.

Appendix publication V deals with the regulatory influence of the thymus or T cells on the heterogeneity of serum immunoglobulins. The transient appearance of homogeneous immunoglobulins, 'paraproteins', in serum is a frequent finding during the reconstitution of the immune system after bone

marrow transplantation in children with severe combined immunodeficiency. The simplest explanation of this finding is that a small number of B cells will first arise during the development of the immune system. Later, more B cell clones will develop and a gradual transition from homogeneous to heterogeneous responses will occur. We studied the role played by the thymus and/or the T cells in the regulation of the heterogeneity of serum immunoglobulins in thymectomized or sham-thymectomized mice. The animals were lethally irradiated and reconstituted with bone marrow or foetal liver cells. Blood samples were obtained in the period between 3 and 13 weeks after transplantation. The serum samples were tested for the presence of homogeneous immunoglobulins using electrophoretic techniques. It was observed that a delay in the maturation of the T cell population or the absence of the T-system contributed substantially to the appearance of homogeneous immunoglobulins during the reconstitution period. It is concluded that not only the number of B cell clones but also the T cell population is important in the regulation of immunoglobulin heterogeneity. The role of the T cells may be twofold; (a) as helper cells, they can promote a response of multiple B cell clones towards thymus-dependent antigens; (b) as suppressor cells, they may prevent an overshoot reaction of a restricted number of B cell clones towards thymus-dependent or 'thymus-independent' antigens.

Samenvatting

Bij het ontstaan van humorale en cellulaire immuniteit bij zoogdieren spelen zowel lymfoïde cellen als macrofagen een belangrijke rol. In de perifere lymfoïde organen kan men 2 verschillende typen lymfocyten aantreffen: (1) T-cellen, die een belangrijk ontwikkelingsstadium in de thymus hebben doorgemaakt en (2) B-cellen, die voor hun ontwikkeling afhankelijk zijn van de aanwezigheid van een bursa-equivalent. Voor het ontstaan van een humorale immunologische reaktie tegen de meeste antigenen is samenwerking nodig tussen macrofagen, T-cellen en B-cellen. Na kontakt met een antigeen produceren T-cellen zelf geen antilichamen maar wel oplosbare stoffen, die een stimulerend of een remmend effekt kunnen hebben op de differentiatie van B-cel tot plasmacel. De macrofaag is van belang, omdat deze cel in staat is het antigeen te concentreren en een deel van het antigeen in een zeer immunogene vorm te presenteren op zijn celmembraan. Verschillende aspekten, die betrekking hebben op de regulerende invloed van de T-cel op het humorale immuunsysteem werden op ons laboratorium onderzocht en de resultaten van onze experimenten worden weergegeven in de artikelen, die bij dit proefschrift gevoegd zijn.

In publikatie I van de appendix werd onderzocht in hoeverre de T-cel een invloed heeft op de differentiatie van hemopoietische stamcellen in B-cellen. De ontwikkeling van de B-cel populatie in adult-gethymectomeerde, lethaal bestraalde en met beenmergcellen gereconstitueerde muizen werd vergeleken met hetzelfde proces in dieren, die op dezelfde wijze behandeld waren, maar waarbij de thymus niet verwijderd werd. Op verschillende momenten na de beenmergtransplantatie werd het aantal B-cellen in de milt van deze dieren bepaald, waarbij gebruik gemaakt werd van een funktionele test. Waargenomen werd, dat de cellen, die de voorlopers zijn van de IgM-, IgG- en IgA-producerende cellen, na elkaar verschijnen. Ongeveer 30 dagen na de beenmergtransplantatie werd een normaal

en konstant aantal B-cellen aangetroffen. Er bleek geen significant verschil te bestaan tussen de dieren met een thymus en de dieren zonder thymus. Deze waarneming maakt het waarschijnlijk, dat er geen invloed is van de thymus of de T-cel populatie op de ontwikkeling van de B-cel populatie uit hemopoietische stamcellen.

Publikatie II van de appendix heeft betrekking op de helpercel-aktiviteit van verschillende T-cellen. De helpercel-aktiviteit van normale thymocyten, dexamethason-resistente thymocyten (DRT) en "opgevoede" T-cellen werd zowel *in vivo* als *in vitro* bestudeerd. Met behulp van celtransfer-experimenten werd de *in vivo* situatie onderzocht. Daarbij werden lethaal bestraalde muizen intraveneus ingespoten met schape-rode-bloed-cellen (SRBC), B-cellen en verschillende hoeveelheden T-cellen. Na 7 dagen werd de milt van de ontvangers verwijderd en het aantal plaque-vormende cellen (PVC) bepaald. De *in vitro* situatie werd onderzocht door verschillende hoeveelheden T-cellen te mengen met normale miltcellen en SRBC en vervolgens te kweken in petrischaaltjes. Het aantal PVC per bakje werd 5 dagen later bepaald. Het aantal PVC per milt in de *in vivo* experimenten of per bakje in de *in vitro* experimenten werd beschouwd als maat voor de helpercel-aktiviteit van de T-cellen. Uit experimenten bleek, dat "opgevoede" T-cellen de meest aktieve helpercellen waren zowel *in vivo* als *in vitro*. De DRT en in mindere mate ook de normale thymocyten gaven een stimulerend effekt te zien in de *in vivo* situatie, maar een remmend effekt in de *in vitro* experimenten. Dit remmende effekt *in vitro* kan wellicht verklaard worden door de aanwezigheid van relatief ongedifferentieerde remmende T-cellen (T_1) onder de normale thymocyten en de DRT. Het is mogelijk, dat deze relatief ongedifferentieerde remmende T-cellen onder *in vivo* omstandigheden wel het geschikte micro-milieu vinden, waarin zij tot helper T-cel (T_2) kunnen differentieren, maar onder *in vitro* omstandigheden niet.

Publikatie III van de appendix heeft betrekking op de periode, waarin helper T-cellen van belang zijn tijdens de immunologische reaktie tegen SRBC. Uit de literatuur blijkt namelijk wèl, dat T-cellen een belangrijke rol spelen tijdens de humorale immunologische reaktie, maar niet gedurende welke periode de aanwezigheid van de T-cel essentieel is. Dit laatste probleem werd experimenteel aangepakt door het samenwerkingsproces tussen T- en B-cellen op verschillende momenten tijdens de immunologische reaktie *in vivo*

of *in vitro* te stoppen. Het afbreken van de samenwerking tussen T-en B-cellen kon worden bereikt door de T-cellen te doden met behulp van anti-θ serum of anti-H-2 serum. Na de serum-behandeling werden de cellen gekweekt in petrischaaltjes en werd op achtereenvolgende dagen het aantal PVC in deze schaaltjes bepaald. De resultaten van onze experimenten geven aan, dat T-cellen een belangrijke rol spelen tijdens de eerste 3 dagen van de immunologische reaktie *in vivo*. Tijdens de reaktie *in vitro* zijn de T-cellen echter alleen gedurende de eerste 2 dagen nodig. Het waargenomen verschil tussen de reaktie *in vivo* en de reaktie *in vitro* kan worden verklaard door aan te nemen, dat de ontwikkeling van de plasmacellen *in vitro* synchroon start, terwijl ditzelfde proces *in vivo* asynchroon verloopt. Deze asynchrone start van de reaktie *in vivo* wordt naar alle waarschijnlijkheid veroorzaakt door een migratie van T- en B-lymfocyten naar de milt gedurende de eerste dagen na toediening van het antigeen. De B-lymfocyten, die op deze manier in de milt arriveren, zullen dan ook op een later tijdstip differentiëren tot plasmacel.

In publikatie IV van de appendix wordt de rol van de T-cellen bij de vorming van IgA besproken. De T-cel-afhankelijkheid van de IgA reaktie tegen SRBC werd bestudeerd met behulp van celtransfer-experimenten. Wanneer miltcellen van adult gethymectomeerde, lethaal bestraalde en met beenmergcellen gereconstitueerde muizen (B-cellen) werden ingespoten in bestraalde ontvangers, werden slechts lage IgM, IgG en IgA reakties waargenomen. Hoge aantallen IgM-, IgG- en IgA-PVC konden worden aangetoond in ontvangers, die zowel B-cellen als "opgevoede" T-cellen ontvingen. De mate, waarin de T-cellen de IgA reaktie stimuleerden, was vergelijkbaar met de mate, waarin de IgM reaktie werd gestimuleerd. De vorming van IgG werd echter nog sterker door de T cellen bevorderd. Uit deze experimenten kan de conclusie worden getrokken, dat de IgA reaktie tegen SRBC -bij muizen althans- T cel-afhankelijk is.

Publikatie V van de appendix heeft betrekking op de regulerende invloed van de thymus of de T-cellen op de heterogeniteit van serum-immunoglobulines. Het is bekend, dat er na beenmergtransplantaties bij kinderen met een ernstige

immuun-deficientie tijdelijk homogene immunoglobulines, "paraproteïnes", kunnen verschijnen in het serum. De meest voor de hand liggende verklaring voor dit verschijnsel is, dat er tijdens de ontwikkeling van het immuunsysteem eerst een gering aantal B-cellen verschijnt. In een latere fase ontwikkelen zich meer B-cel klonen en als gevolg daarvan veranderen de homogene reakties geleidelijk in heterogene reakties. In onze experimenten werd de heterogeniteit van de serum immunoglobulines in gethymectomeerde en sham-gethymectomeerde muizen bestudeerd. De dieren werden lethaal bestraald en vervolgens gereconstitueerd met cellen afkomstig uit beenmerg of foetale lever. Geringe hoeveelheden bloed werden afgenomen in de periode tussen 3 en 13 weken na de transplantatie. De sera werden geanalyseerd met behulp van electroforetische technieken en het aantal homogene immuno-globulines werd vastgesteld. De hoogste aantallen homogene immunoglobulines werden gevonden in die dieren, waarin de T-cel populatie ontbrak of waarin de T-cel populatie nog niet goed ontwikkeld was. Uit deze waarnemingen werd de conclusie getrokken, dat niet alleen het aantal B-cellen maar ook de T-cel populatie belangrijk is bij de regulatie van de heterogeniteit van immunoglobulines. De rol, die de T-cellen in dit regulatieproces vervullen, kan tweeledig zijn: (a) als helper cellen kunnen zij de reaktie van een groot aantal B-cel klonen tegen thymus-afhankelijke antigenen stimuleren; (b) als remmende cellen kunnen zij een buiten-sporige reaktie van een beperkt aantal B-cel klonen tegen thymus-afhankelijke of "thymus-onafhankelijke" antigenen voorkomen.

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Curriculum vitae

Na het behalen van het diploma gymnasium-β aan het Christelijk Streeklyceum "Buitenveldert" te Amsterdam werd door mij in 1960 begonnen met de studie biologie aan de Vrije Universiteit te Amsterdam. Het doctoraalexamen met hoofdvak Biochemie (Prof. Dr. R.J. Planta en Prof. Dr. L. Bosch) en de bijvakken Microbiologie (Prof. Dr. A.H. Stouthamer) en Dierfysiologie (Prof. Dr. J. Lever) werd in 1967 afgelegd. Van 1963 tot 1967 ben ik als student-assistent verbonden geweest aan de afdeling Dierfysiologie van de Vrije Universiteit (Dr. N. Spronk). Van 1967 tot 1969 ben ik ter vervulling van mijn militaire dienstplicht gedetacheerd geweest op het Medisch-Biologisch Laboratorium TNO te Rijswijk (Dr. O.B. Zaalberg).
Van 1969 tot begin 1975 ben ik als wetenschappelijk medewerker verbonden geweest aan de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam, alwaar het onderzoek werd verricht dat in dit proefschrift wordt beschreven.
Vanaf begin 1975 ben ik werkzaam binnen de afdeling Experimentele Diermorfologie en Celbiologie van de Landbouwhogeschool te Wageningen.

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The Recovery of the B-cell Population in Adult Thymectomized, Lethally Irradiated and Bone Marrow-Reconstituted Mice

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Summary. The recovery of the B-cell population in adult thymectomized, irradiated and bone marrow-reconstituted mice (T × BM mice) was studied. The number of B cells in the spleen of T × BM mice was estimated at various times after bone marrow transplantation. The spleen cells to be tested were mixed with dexamethasone-resistant thymocytes (DRT) and sheep red blood cells (SRBC) and transferred to irradiated recipients. The number of plaque-forming cells (PFC) in the spleen of the recipients was determined 7 days later. Using this functional B-cell assay a sequential appearance of the precursors of IgM–, IgG– and IgA–PFC in the spleen of T × BM mice was observed. The precursors of IgM–PFC (IgM–B cells) were present immediately after transplantation. The first IgG–B cells could be detected at 13–16 days after transplantation and the IgA–B cells finally appeared at 22 days after transplantation. The number of B cells reached a constant and normal level at 30 days after transplantation. The IgM–, IgG– and IgA–B cell development in sham-thymectomized, irradiated and bone marrow-reconstituted mice (ST × BM mice) was virtually the same as in T × BM mice.

INTRODUCTION

The recovery of immunological responsiveness after sublethal irradiation or lethal irradiation and bone marrow reconstitution is dependent on the development of at least two populations of cells: T and B cells. It has been shown by several authors that the development of the T-cell population is relatively slow (Vos, 1968; Nettesheim, Williams and Hammons, 1969; Gregory and Lajtha, 1970; Lafleur, Miller and Phillips, 1972). If the development of the B-cell population is compared with the development of the T-cell population, it is important to differentiate between IgM–B cells, IgG–B cells and IgA–B cells. Gregory and Lajtha (1970) have shown that, during the recovery period after irradiation and bone marrow reconstitution of mice, the IgM response to sheep red blood cells (SRBC) is limited by the number of T helper cells, which means that the recovery

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rate of IgM–B cells is higher than that of T cells. Data on the development of IgG– and IgA–B cells after bone marrow transplantation are not available at this moment.

According to the two-stage model for the development of antibody-producing cells as proposed by Cooper, Lawton and Kincade (1972) a sequential appearance of IgM–, IgG– and IgA–B cells is expected. This model divides plasma cell differentiation into two developmental stages. In the first stage, which occurs in the bursa of Fabricius or a bursa-equivalent, B-cell clones are generated from haemopoietic stem cells. During this process B cells with μ, γ or α chains on their cell surface appear sequentially. This development does not depend on specific antigenic stimulation, but is induced by the microenvironment in the bursa or in the bursa-equivalent. The second stage begins with the entry of the B cells into the circulation and their arrival in the peripheral lymphoid organs. In the second stage antigens do play a role in the further development of the B cells. Antigens stimulate the B cells to proliferate and differentiate into memory cells or plasma cells.

The present study on adult mice, thymectomized, irradiated and reconstituted with bone marrow (T × BM) and mice sham-thymectomized, irradiated and reconstituted with bone marrow (ST × BM) was performed to get information about the IgM–, IgG– and IgA–B cell development after bone marrow transplantation. Since the model of Cooper *et al.* (1972) is based on observations in the chicken (Kincade and Cooper, 1971) and in man (van Furth, Schuit and Hijmans, 1965; Lawton, Self, Royal and Cooper, 1972) an experimental study in the mouse provides additional information about B-cell development in mammals.

MATERIALS AND METHODS

Animals

(DBA/2 × C57Bl/Rij)F1 female mice were obtained from the Medical Biological Laboratory TNO, Rijswijk. Irradiated recipients were 15–25 weeks old.

Antigen

SRBC were obtained as a sterile suspension in Alsever's solution from the Department of Clinical Microbiology, Erasmus University, Rotterdam. The cells were washed twice with phosphate-buffered saline before use. For immunization the mice were injected intravenously (i.v.) with 4×10^8 SRBC.

Plaque assay for antibody-producing cells

A slight modification of the plaque technique as described by Zaalberg, van der Meul and van Twisk (1968) was used. A rabbit anti-mouse IgG serum was employed to facilitate the plaque formation by IgG-producing cells. The number of IgG–PFC was calculated by subtracting the number of direct plaques (IgM–PFC) from the number of indirect plaques (IgM–PFC + IgG–PFC).

The presence of IgG–PFC was considered as significant when there was no overlap between the upper 95 per cent confidence limit in the direct assay and the lower 95 per cent confidence limit in the indirect assay. The calculation of the 95 per cent confidence limits was performed as described previously (Benner, Meima, van der Meulen and van Muiswinkel, 1974). The number of IgA–PFC was calculated in a similar way using rabbit anti-mouse IgA serum in the indirect assay.

Cell suspensions

Cell suspensions were prepared as described by Vos (1967). A balanced salt solution (Mishell and Dutton, 1967) containing 5 per cent newborn calf serum was used.

Anti-θ serum

Anti-θ serum was obtained by immunizing AKR/FuRdA mice with C3H f/A thymocytes (Reif and Allen, 1966). The immunized animals were bled 4 or 5 days after the last intraperitoneal (i.p.) injection. The serum was not inactivated or absorbed before use. The cytotoxic titre of the serum was determined by using dexamethasone-resistant thymocytes (DRT) as target cells. It is known that DRT have a θ-antigen content more characteristic of peripheral T cells than of corticosteroid-sensitive thymocytes (Raff and Cantor, 1971). The cytotoxicity assay was a slight modification of the two-step procedure described by Chan, Mishell and Mitchell (1970).

Dexamethasone-resistant thymocytes (DRT)

A modification of the method of Andersson and Blomgren (1970) was used to obtain corticosteroid-resistant thymocytes. Mice (5–6 weeks old) were injected i.p. with 30 mg/kg of dexamethasone-21-phosphate (Merck & Company, Incorporated, Rahway, New Jersey) diluted 1 : 4 in saline. The thymuses were removed 48 hours later and a cell suspension was prepared. The cells were washed twice and coarse debris was removed from the suspension by the method of Shortman, Williams and Adams (1972).

X-irradiation

For lethal irradiation mice were exposed to 825 rads delivered at a rate of 32 rad/minute by a Philips Mueller MG 300 X-ray machine operating at 250 kV and 11 mA with a 1 mm Cu filter. The distance to target was 53 cm. Further details are described by Vos (1967).

T × BM and ST × BM mice

Cell suspensions to be tested for B-cell activity were obtained from the spleen of T × BM or ST × BM mice. Thymectomy or sham-thymectomy was performed at an age of 5 or 6 weeks according to the method of Miller (1960). The animals were irradiated and injected i.v. with 3×10^6 anti-θ serum-treated syngeneic bone marrow cells 2 or 3 weeks after surgery. Before the spleen of T × BM mice was used as a source of B cells the mediastinum was carefully inspected for thymic remnants.

B-cell assay

The B cells were tested for function by plaque-formation in irradiated recipients. The suspension to be tested for B-cell activity was mixed with $10–12 \times 10^6$ DRT and 4×10^8 SRBC and injected i.v. into the recipients. The recipients received no extra SRBC injection when B-cell assay type I was used (Fig. 1). In some experiments B-cell assay type II was used: in those cases a second injection (4×10^8 SRBC, i.p.) was administrated to the recipients on day 4. The spleen of the recipients was removed on day 7 and the number of IgM–, IgG– and IgA–PFC was determined with the plaque assay.

The number of PFC was considered as an estimate of the number of B cells that were present in the cell suspension 7 days before. Preliminary experiments have been done to exclude the possibility that the PFC response was limited by a lack of helper cells. It was observed that an amount of 5×10^7 normal thymocytes, which is often described in the

literature (Gregory and Lajtha, 1970; Lafleur *et al.*, 1972), is not enough for an optimal IgG response. However, an optimal IgM and IgG response could be achieved when $10–12 \times 10^6$ DRT were used as hèlper cells (van Muiswinkel, Zaalberg, Majoor, Lubbe, van Soest and van Beek, 1975).

RESULTS

THE B-CELL DEVELOPMENT IN THE SPLEEN OF T × BM MICE

The recovery of the B-cell population was primarily studied in T × BM mice. The pooled spleen cells of four or five T × BM mice were assayed for their B-cell content at various days after bone marrow transplantation. The amount of spleen cells injected into each irradiated recipient was the equivalent of a third of a spleen (Fig. 1). No fixed cell

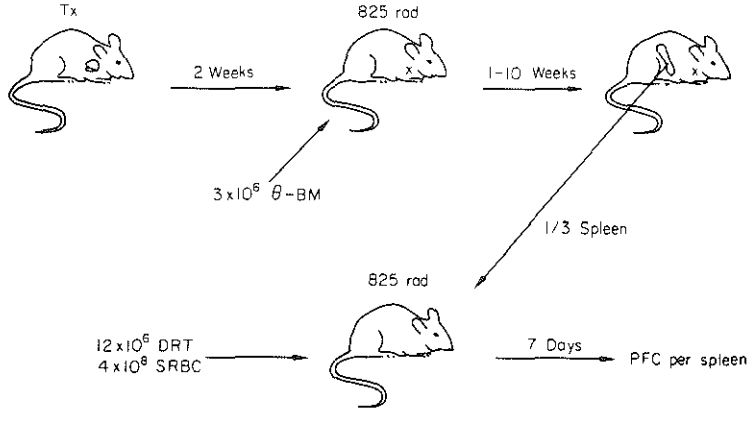

FIG. 1. The experimental procedure used for the study of the B-cell development in thymectomized (Tx) mice after irradiation (825 rad) and reconstitution with 3×10^6 anti-θ serum-treated bone marrow cells (θ-BM).

number was transferred because the number of spleen cells first increases and subsequently decreases before it reaches a constant and normal level at 3 weeks after irradiation and bone marrow reconstitution (Vos, 1964).

The results of the first experiment, using B-cell assay type I (1 × SRBC), are shown in Fig. 2. It was observed that the spleen of the T × BM mice contains only IgM–B cells and no significant numbers of IgG– or IgA–B cells at 8 days after transplantation. Significant numbers of IgG–B cells were detected for the first time 16 days after transplantation, while IgA–B cells could not be demonstrated at that time. The IgM–B cells showed a more than 10-fold increase in the period between days 8 and 16, but no further increase was observed in the period after day 16. The number of IgG–B cells showed a 6-fold increase in the period between days 16 and 39 and was more or less stable after day 39. The IgA–B cells finally appeared at day 22. In the period after day 22 the number of IgA–B cells did increase slightly, but was usually lower than the number of IgM–B cells.

In the second experiment, using B-cell assay type II (2 × SRBC), higher IgM and IgG responses could be obtained (Fig. 3). As in the first experiment a clear increase in the number of IgM–B cells was observed in the period between days 7 and 15, however,

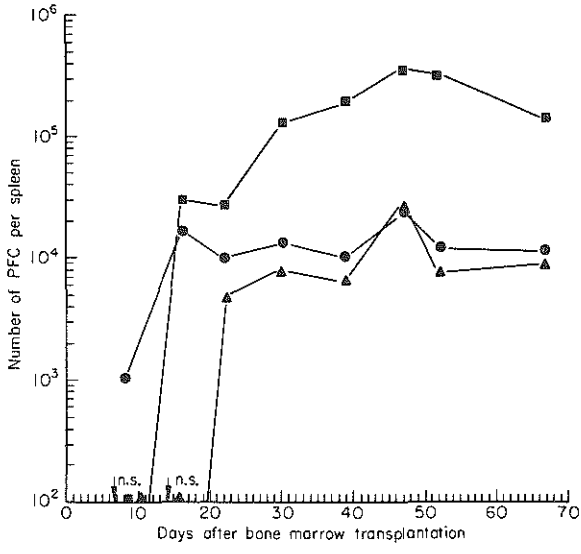

FIG. 2. B-cell development in the spleen of thymectomized mice after irradiation and bone marrow transplantation. The abscissa shows the number of days between transplantation and removal of the T × BM mouse spleens (four or five per point). The ordinate shows the number of IgM– (●), IgG– (■) and IgA–PFC (▲) detected in the pooled spleens of the irradiated recipients (four per point), which were used in the B-cell assay type I (1 × SRBC). The number of T × BM mouse spleen cells, which was injected into one recipient, was equivalent to the amount of cells present in a third of a spleen. n.s. = The number of indirect plaques (IgM + IgA or IgM + IgG) did not differ significantly from the number of direct plaques (IgM).

contrary to the data from the first experiment this increase did not level off after day 15, but continued until day 27. The appearance of significant numbers of IgG–B cells at day 15 and the subsequent development of the IgG–B-cell population are in agreement with observations in the first experiment (Fig. 2). No significant numbers (above the background) of IgA–PFC could be demonstrated in the second experiment, probably due to high numbers of IgM–PFC in the spleen of the recipient mice. The difference between the number of indirect plaques (IgM–PFC + IgA–PFC) and the number of direct plaques (IgM–PFC) was not significant.

In both experiments a clear increase in the number of IgM–, IgG– and IgA–B cells could be demonstrated during the first 30 days after transplantation, but in the period between day 30 and day 86 no considerable changes in the number of B cells were observed. It can be concluded that the development of the B-cell population in the spleen of T × BM mice was completed 30 days after bone marrow reconstitution.

THE B-CELL DEVELOPMENT IN THE SPLEEN OF ST × BM MICE

To investigate the possible influence of the thymus or T cells on the development of B cells from haemopoietic stem cells two experiments with T × BM and ST × BM mice were performed. Spleen cells of the animals were tested with B-cell assay type I (1 × SRBC) at various days after transplantation. The results are shown in Table 1. There was no significant difference in the development of IgM–, IgG– and IgA–B cells in T × BM and

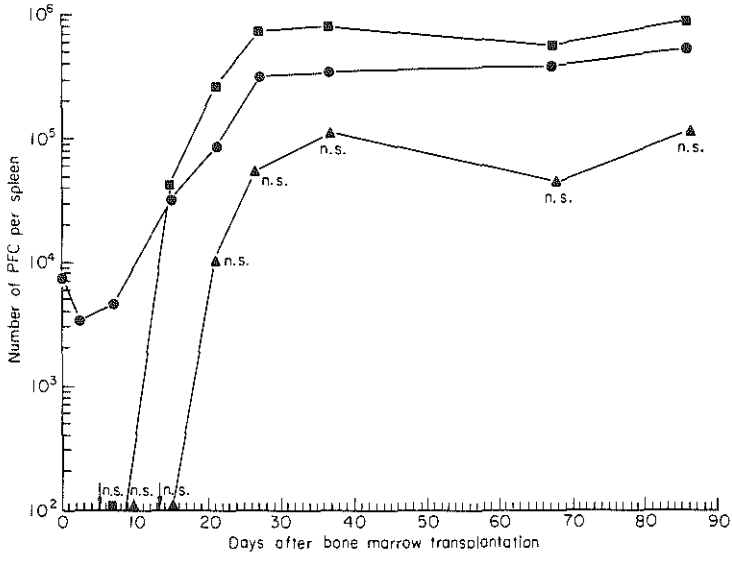

FIG. 3. B-cell development in the spleen of thymectomized mice after irradiation and bone marrow transplantation. The abscissa shows the number of days between transplantation and removal of the T × BM mouse spleens (four or five per point). The ordinate shows the number of IgM– (●), IgG– (■) and IgA–PFC (▲) detected in the pooled spleens of the irradiated recipients (four per point), which were used in the B-cell assay type II (2 × SRBC). The number of T × BM mouse spleen cells, which was injected into one recipient, was equivalent to the amount of cells present in a third of a spleen. n.s. = The number of indirect plaques (IgM + IgA or IgM + IgG) did not differ significantly from the number of direct plaques (IgM).

TABLE 1

THE B-CELL DEVELOPMENT IN THE SPLEEN OF T × BM AND ST × BM MICE

Experiment	Donors	Days after transplantation	PFC per recipient spleen (× 10^3) IgM	IgG	IgA
A	T × BM	15	6·67	18·8	0
	ST × BM	15	4·70	26·3	0·10 (n.s.)
	T × BM	44	19·6	188·4	10·2
	ST × BM	44	19·2	180·8	13·4
	T × BM	56	8·10	151·9	6·90
	ST × BM	56	8·60	288·4	8·70
B	T × BM	7	0·55 (0·34–0·88)	0·28 (n.s.)	n.d.
	ST × BM	7	0·65 (0·51–0·85)	0·13 (n.s.)	
	T × BM	13	7·24 (5·89–8·89)	8·19 (6·23–10·8)	n.d.
	ST × BM	13	6·63 (5·22–8·42)	11·1 (9·34–13·1	
	T × BM	126	9·04 (7·95–10·3)	53·7 (42·8–67·5)	n.d.
	ST × BM	126	6·30 (5·57–6·97)	89·8 (78·9–101·9)	

The irradiated recipients used in the B cell assay type I were injected with one-third of a spleen of the donor mice. In experiment A the PFC assay was done on pooled spleens of four or five recipients. In experiment B the PFC assay was done on individual spleens of the recipients (four to five mice per group). The results of experiment B are expressed as geometric mean (± 1 (log) s.e.).

n.s. = The number of indirect plaques (IgM + IgG or IgM + IgA) did not differ significantly from the number of direct plaques (IgM).

n.d. = Not determined.

ST×BM mice during the period from 7 to 126 days after the bone marrow transplantation. The conclusion can be drawn that there was no influence of the thymus or T cells on the B cell development in this experimental system.

THE B-CELL POPULATION IN THE SPLEEN OF NORMAL AND PRIMED MICE

To investigate whether the B-cell level reached in T×BM and ST×BM mice was comparable to that in normal mice, experiments were performed to estimate the number of B cells in the spleen of normal and primed mice.

It is shown in Table 2 that the number of IgM– and IgG–B cells in the spleen of normal mice is comparable to the number of IgM– and IgG–B cells in T×BM and ST×BM mice at 30 or more days after transplantation (Figs 2, 3 and Table 1). The number of IgA–B cells in normal mice is equal to (experiment A, Table 2) or lower than (experiment B, Table 2) the number of IgA–B cells in T×BM and ST×BM mice.

The number of IgM–, IgA– and IgG–B cells in primed mice was respectively 5–13, 13 and 9–33-times higher than in normal mice. From these experiments the conclusion can be drawn, that the B-cell population in T×BM and ST×BM mice at 30 or more days after transplantation is comparable to the B-cell population in normal mice, but different from the B-cell population in primed mice.

TABLE 2

THE B-CELL POPULATION IN THE SPLEEN OF NORMAL AND PRIMED MICE

Experiment	Donors	B-cell assay	PFC per recipient spleen ($\times 10^3$) IgM	IgG	IgA
A	Normal mice	I	8·42 (6·52–10·9)	318·1 (247·9–408·2)	3·98 (3·35–4·74)
		II	114·0 (80·7–151·2)	1026·5 (827·0–1274·1)	<25·0 }
B	Normal mice	I	16·6 (11·6–23·7)	92·8 (65·9–130·8)	<1·0 } (n.s.)
		II	287·3 (240·7–342·9)	309·6 (222·0–431·7)	<10·0 }
C	Primed mice	I	112·9 (94·9–134·2)	2739·1 (2407·9–3115·7)	52·5 (42·9–64·1)
		II	1348·4 (1156·7–1572·0)	10137·5 (8518·7–12504·0)	<300·0 (n.s.)

The irradiated recipients used in the B-cell assay were injected with a third of a spleen of the donor mice. The recipients were tested individually 7 days later (four to five mice per group). The results are expressed as geometric mean (± 1 (log) s.e.). Normal mice were 15–25 weeks old. Primed mice were injected i.v. with 4×10^6 SRBC at an age of 15 weeks and used 6 months later.

n.s. = The number of indirect plaques (IgM + IgA) did not differ significantly from the number of direct plaques (IgM).

DISCUSSION

A sequential development of the precursors of IgM–, IgG– and IgA–PFC in the spleen of T×BM mice is described. IgM–B cells are present immediately after the bone marrow transplantation, because bone marrow from normal mice contains some IgM–B cells (Mitchell and Miller, 1968; Lafleur *et al.*, 1972). The rapid development of the IgM–B cells during the first 2–4 weeks after transplantation is in agreement with the results in other publications, in which the recovery of the IgM response is studied in irradiated mice injected with bone marrow cells (Gregory and Lajtha, 1970), foetal liver cells (Nossal and Pike, 1973) or purified haemopoietic stem cells (Lafleur *et al.*, 1972). In the first experiment

(Fig. 2) no further increase in the number of IgM–B cells was observed in the period between 16 and 67 days after the transplantation. This may be a result of the indirect method used to estimate the number of B cells. The production of IgM is known to be suppressed by IgG molecules of the same specificity (Möller and Wigzell, 1965). If the same suppression takes place in the B-cell assay that was used in our experiments, possibly the number of IgM–B cells was still increasing in the period after day 16, which could not be followed in the first experiment. However, in the second experiment (Fig. 3) a further increase in the number of IgM–B cells could be demonstrated in the period between day 15 and day 27 after transplantation. The second SRBC injection on day 4 in the B-cell assay type II probably reduced the inhibitory effect of the IgG response on the IgM response. In this way a more precise idea of the IgM–B-cell development could be obtained.

The same sequence IgM→IgG→IgA was observed in the development of the B-cell population in the chicken embryo (Kincade and Cooper, 1971) and in the human foetus (van Furth *et al.*, 1965; Lawton *et al.*, 1972). Comparable results were obtained in recent studies by Rádl, Dooren, Eijsvoogel, van Went and Hijmans (1972) and Vossen, de Koning, van Bekkum, Dicke, Eijsvoogel, Hijmans, van Loghem, Rádl, van Rood, van der Waaij and Dooren (1973) on the development of the immune system after a successful thymus and bone marrow transplantation in patients suffering from a severe combined immunodeficiency. After transplantation the immunoglobulin-containing cells in bone marrow and the immunoglobulins in serum appeared in the following order: IgM; IgG; IgA; IgD. These data and the experimental results described in this paper agree very well with the two-stage model for the development of antibody-producing cells as proposed by Cooper *et al.* (1972). Our experimental approach provided an opportunity to separate the two stages in plasma cell development. The first stage occurs in the T×BM or ST×BM mice after bone marrow transplantation, while the second stage is started upon transfer of the spleen cells of the T×BM or ST×BM mice mixed with DRT and SRBC to irradiated recipients in the B-cell assay (see Introduction).

Whether specific antigens play a role during the B-cell development after bone marrow transplantation is a question that is difficult to answer. Experiments of Cunningham and Pilarski (1974) are very interesting in this respect. They provide some evidence for the view that antigen stimulates the proliferation of an initial population of B cells from which it generates B cells producing entirely new specificities. Therefore it would be of interest to compare the B-cell development in conventional animals with the B-cell development in germ-free animals in which the antigenic stimulation is relatively low.

There are some interesting data and ideas about the influence of T cells on B-cell development. It is proposed by Nieuwenhuis and Keuning (1974) that germinal centres function as an antigen-dependent amplification system for the B-cell population and by Mitchell, Pye, Holmes and Nossal (1972) that T cells play a stimulatory role in germinal centre formation. However, it has been shown by Unanue, Grey, Rabellino, Campbell and Schmidtke (1971) that injection of T cells into T×BM mice did not alter B-cell development. In their study immunofluorescent techniques were used to enumerate the B cells. Our experiments in which a functional B-cell assay was used indicate that there was no difference in the B-cell development in T×BM and ST×BM mice during the period between 1 and 18 weeks after transplantation (Table 1). Therefore we must conclude that our data do not provide evidence for the view that T cells play a stimulatory role in B-cell development.

In conclusion we can say that in our experiments with T×BM and ST×BM mice a

sequential appearance of the IgM–, IgG– and IgA–B cells was observed and that the number of B cells in the spleen reached a constant and normal level approximately 1 month after irradiation and bone marrow reconstitution.

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The helper cell activity of T cells from different sources[1]

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The helper cell activity of different T cells was studied both *in vivo* and *in vitro*. Three types of T cells were used:

a. Normal thymocytes (NT) derived from 5-6 weeks old (DBA/2 x C57BL/Rij)F1 mice.
b. Dexamethasone-resistant thymocytes (DRT) derived from mice 48 h after intraperitoneal (i.p.) injection of 30 mg/kg dexamethasone-21-phosphate (Merck and Company, Incorporated, Rahway, New Jersey).
c. Educated T cells (Ed.T) obtained from spleens of mice 6 or 7 days after X-irradiation (825 rads, 250 kV, 11 mA, 1 mm Cu) and intraveneous (i.v.) injection of 5×10^7 NT and 4×10^8 sheep red blood cells (SRBC) (Mitchell and Miller, 1968).

The helper cell activity of the different T cells was studied *in vivo* by means of cell transfer experiments. Lethally irradiated recipients were i.v. injected with 4×10^8 SRBC, 3×10^7 B cells and various numbers of T cells. The B cells were obtained from the spleens of adult thymectomized mice which were irradiated and reconstituted with anti-θ treated bone marrow cells (van Muiswinkel, van Beek and van Soest, 1975). The spleen of the recipients, which were used in the cell transfer experiments, was removed on day 7 (peak of the response) and the number of IgM- and IgG-plaque-forming cells (PFC) was determined with the plaque assay

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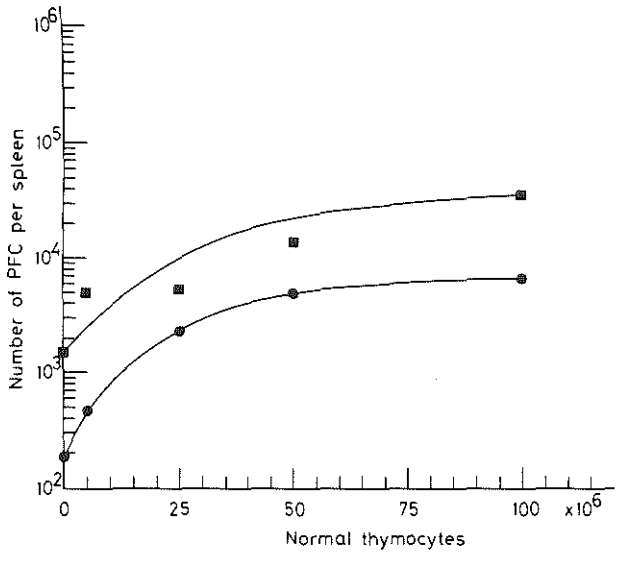

Fig. 1. The helper cell activity of normal thymocytes (NT) in vivo. Irradiated recipients were i.v. injected with 4 x 10^8 SRBC, 3 x 10^7 B cells and various numbers of NT. The abscissa shows the number of NT transferred. The ordinate shows the number of IgM- (●) and IgG-PFC (■) detected in the pooled spleens of irradiated recipients (four per point) 7 days after cell transfer.

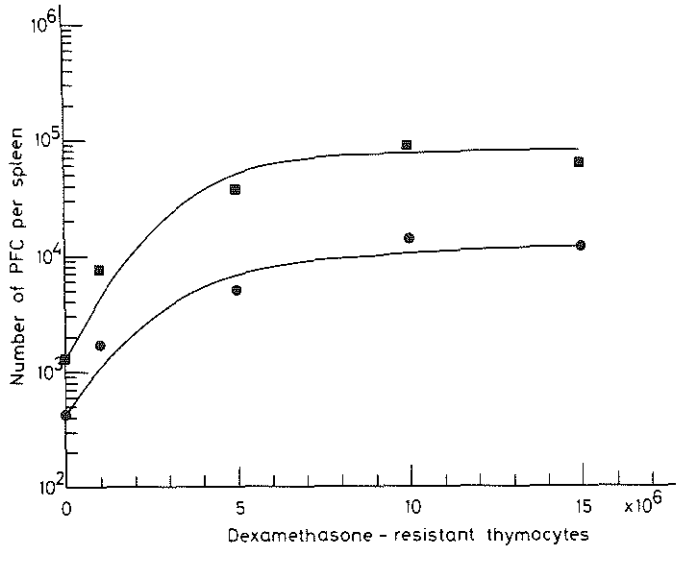

Fig. 2. The helper cell activity of dexamethasone-resistant thymocytes (DRT) in vivo. Irradiated recipients were i.v. injected with 4 x 10^8 SRBC, 3 x 10^7 B cells and various numbers of DRT. The abscissa shows the number of DRT transferred. The ordinate shows the number of IgM- (●) and IgG-PFC (■) detected in the pooled spleens of irradiated recipients (four per point) 7 days after cell transfer.

(Zaalberg, van der Meul and van Twisk, 1968; Benner, Meima, van der Meulen and van Muiswinkel, 1974). In some experiments the number of IgA-PFC was determined as well. The number of IgM-, IgG- and IgA-PFC per recipient spleen was considered as a measure of the helper cell activity of the T cells.

It was observed that only low numbers of IgM- (180-1540), IgG (650-3450) and IgA-PFC (220-950) were present in the spleens of those recipients which received only B cells (Figure 1, figure 2, figure 3 and table I). Higher PFC responses were obtained when a mixture of B cells and various numbers of T cells was transferred. Optimal IgM- (7000-19,000), IgG- (100,000-360,000) and IgA-PFC (10,000-21,000) responses were observed when 10^7 DRT or 3 x 10^6 Ed.T were transferred (Figure 2, figure 3 and table I). However, suboptimal IgG-PFC (14,000-50,000) responses were observed when a number of 0.5 to 1 x 10^8 NT was injected (Figure 1 and table I). It was striking that the helper activity of 10^8 NT was lower than that of 10^7 DRT. Due to the occurrence of embolism higher numbers of NT could not be injected into the recipients. These observations indicate that the use of DRT or Ed.T as helper cells should be preferred to obtain optimal PFC responses in an *in vivo* transfer system.

The helper cell activity of the different T cells was studied *in vitro* by using the culture system of Mishell and Dutton (1967). Normal mouse spleen cells were incubated *in vitro* with SRBC (10^7) in the presence of NT, DRT or Ed.T. The response in control cultures containing only normal spleen cells and SRBC was 1000 to 2000 IgM-PFC per dish on day 5. It is known that the T helper cell is the limiting cell type in mouse spleen cell cultures (Vann and Dotson, 1974). Therefore an enhanced response could be expected when T cells are added to the cultures. However, the number of IgM-PFC decreased when 1 to 5 x 10^6 DRT were added (Table II).
A clear increase in the PFC response was observed when 0.5 to 2 x 10^6 Ed.T were added as helper cells. This stimulating effect of the addition of Ed.T was reduced when 4 x 10^6 Ed.T were used (Table II).

It is obvious that high numbers of NT and DRT affect a suppressive influence on the humoral response *in vitro* and a stimulating influence on the response *in vivo*. Possibly the NT and DRT population contains only relatively undifferentiated T cells (T1) which have to differentiate to an other T cell type (T2) before they can act as helper cells. Our experiments are in agreement with the idea that T1 cells could affect a regulatory or suppressive influence on the humoral immune response (Cantor and Boyse, 1975). The lymphoid organs in the animal (e.g. the spleen) probably provide a more suitable micro-environment for the differentiation from T1 to T2 than the culture dish. Factors which play a role in the differentiation from T1 to T2 could be antigens and thymic hormones (Goldstein, Thurman, Cohen and Hooper, 1975).

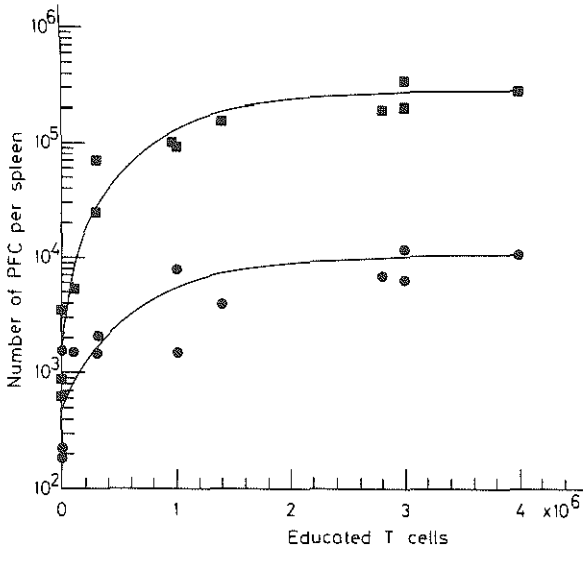

Fig. 3. The helper cell activity of educated T cells (Ed.T) in vivo. Irradiated recipients were i.v. injected with 4 x 10^8 SRBC, 3 x 10^7 B cells and various numbers of Ed.T. The abscissa shows the number of Ed.T. transferred. The ordinate shows the number of IgM- (●) and IgG-PFC (■) detected in the pooled spleens of irradiated recipients (four or five per point) 7 days after cell transfer. The data in this figure are derived from 3 separate experiments.

Table I

The helper cell activity of different T cells in vivo

Experiment	Cells transferred		PFC per recipient spleen			
	T cells ($x10^6$)	B cells ($x10^6$)	IgM	IgG	IgA	
A	-	30	1,540	3,450	950	
	100 NT	30	16,460	49,160	11,630	
	10 DRT	30	18,900	130,400	21,350	
B	-	30	220	600	220	(n.s.)
	50 NT	30	5,140	13,910	950	(n.s.)
	3 Ed.T	30	13,000	210,000	10,000	

Cells were transferred into irradiated recipients together with 4 x 10^8 SRBC. PFC assay was done on pooled spleens of four recipients 7 days after cell transfer.
NT = normal thymocytes; DRT = dexamethasone-resistant thymocytes; Ed.T = educated T cells; B cells = spleen cells from adult thymectomized, irradiated and bone marrow-reconstituted mice.
n.s. = Number in indirect plaques (IgM-PFC + IgA-PFC) did not differ significantly from the number of direct plaques (IgM-PFC).

Table II

The effect of T cells on the immune response of normal spleen cells cultured in vitro

T cells ($x10^6$)	Normal spleen cells ($x10^6$)	IgM-PFC per dish (Fraction of control)
1 NT	19	0.86
2 NT	18	0.50
5 NT	15	0.36
0.2 DRT	20	1.02
0.5 DRT	20	0.91
2 DRT	18	0.54
0.5 Ed.T	20	2.82
2 Ed.T	18	3.23
4 Ed.T	16	1.29

Cells were cultured in the presence of 10^7 SRBC. PFC assay was done on day 5 (4-5 dishes per group).
NT = normal thymocytes; DRT = dexamethasone-resistant thymocytes; Ed.T = educated T cells.

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The T cell dependent period of the immune response to sheep erythrocytes[1]

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SUMMARY

To study the T cell dependent period of the immune response of mouse spleen cells to sheep erythrocytes the cooperation between T and B cells was abrogated at different times during the *in vivo* or the *in vitro* response. The abrogation was performed by killing the T cells with anti-θ serum or anti-H-2 serum. The surviving cells were subsequently cultured *in vitro* and the number of IgM- plaque-forming cells was determined each day. The results indicate that T cells play an important role during the first 3 days of the response *in vivo*. However, during the *in vitro* response the presence of the T cells is only required during the first 2 days. The difference between the response *in vivo* and *in vitro* is probably due to a synchronous start of the plasma cell development *in vitro* and a more asynchronous start of this process *in vivo*.

INTRODUCTION

Studies by Claman, Chaperon and Triplett (1966) and by Mitchell and Miller (1968) have established that two types of lymphoid cells are involved in the *in vivo* plaque-forming cell (PFC) response to sheep red blood cells (SRBC). One of these cells is thymus-dependent (T cell) and the other is thymus-independent (B cell). Both antigenic stimulation and T cell

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help are required for the development of anti-SRBC antibody-producing cells from B cells. Subsequent work with carrier-hapten systems provided additional insight into the nature of cell interactions in humoral immunity. Mitchison and co-workers demonstrated that there is a specific T cell stimulation by carrier components followed by antihapten antibody production by B cells (Mitchison, 1971). Mosier and Coppleson (1968) provided evidence that a third cell was essential for the induction of the primary immune response *in vitro*. These observations were confirmed by Tan and Gordon (1971). The third cell appeared to be a radioresistant and adherent cell. At present most authors agree that this cell is the macrophage, which concentrates the antigen by endocytosis and presents a part of it in persisting immunogenic form on his cell membrane (Unanue, 1972).

The mechanism underlying the interaction between macrophages, T cells and B cells turned out to be rather complex. Several theories have been postulated to explain the observations in different laboratories (Gershon, 1975). At least 2-3 stimuli are needed for the development of plasma cells from B cells: (1) a specific stimulation by macrophage-associated antigen (Unanue, 1972), (2) a non-specific stimulation by humoral factor(s) produced by antigen-stimulated T cells (Schimpl and Wecker, 1972; Gorczynski, Miller and Philips, 1973; Hunter and Kettman, 1974 and Waldmann, 1975) and/or (3) a specific stimulation by an immunoglobulin-like factor produced by antigen-stimulated T cells (Feldmann and Basten, 1972; Feldmann and Nossal, 1972). This factor, which is called IgT by Feldmann, is absorbed on macrophages, presumably because of its cytophilic nature, and is in this condition capable of enhancing the B cell response to antigen. Another antigen-specific T cell factor is recently described by Taussig and Munro (1975). This T cell factor is not absorbed on macrophages, has a molecular weight of about 50,000 and is not an immuglobulin like IgT of Feldmann. At present it is not clear if all T cell factors, which have been described during the last 3 years, have the same influence on the humoral immune response, but it is possible that they act at different moments and at different stages during the proliferation and differentiation of antigen-stimulated B cells.

It is quite obvious from the literature that the T cell plays an important regulatory role during the humoral immune response (Gershon, 1974), but it is not well established in which period the presence of the T helper cell is essential. The present study was performed to determine the T cell dependent period of the *in vivo* and *in vitro* response of mouse spleen cells to SRBC.

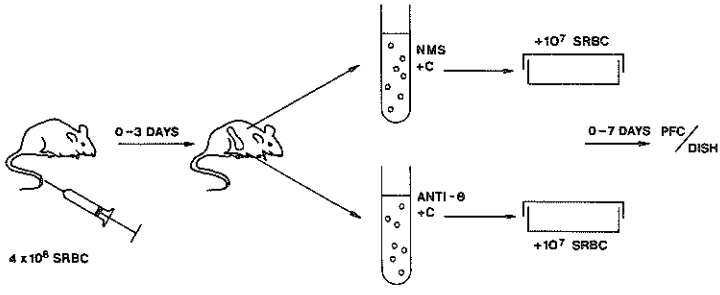


Fig. 1. The experimental procedure used for the study of the T cell dependent period in the immune response in vivo. Spleen cells from normal or primed mice were treated with normal mouse serum (NMS) and complement (C) or with anti-θ serum and C. After serum treatment the cells were cultured and at successive days the number of plaque-forming cells (PFC) per dish was determined.

MATERIALS AND METHODS

Animals

CBA/Rij, (C57BL/Rij x CBA/Rij)F1 and (DBA/2 x C57BL/Rij)F1 male and female mice were obtained from the Radiobiological Institute TNO and the Medical Biological Laboratory TNO (Rijswijk, The Netherlands).

Antigen

Sheep red blood cells (SRBC) were obtained as a sterile suspension in Alsever's solution from the Department of Clinical Microbiology, Erasmus University, Rotterdam. The cells were washed twice with phosphate buffered saline before use. For immunization the mice were injected intravenously (i.v.) with 4×10^8 SRBC.

Plaque assay for antibody-producing cells

A method for the determination of IgM-producing cells ('direct' plaque-test) in an agar-free medium was used as described by Zaalberg, van der Meul and van Twisk (1968). The monolayer plaque assay slides were produced according to the technique of Majoor, van 't Veer and Zaalberg (1975). The 'indirect' plaque test (IgM- + IgG-producing cells) was not generally used, because no significant numbers of IgG-producing cells appeared during the *in vitro* response of non-primed mouse spleen cells using SRBC as an antigen (Pierce, 1969; and own observations).

Culture method

A modification of the culture method of Mishell and Dutton (1967) was used. The spleen cell suspension was cultured in medium F-12 according to Ham (1965) without thymidine and linoleic acid but supplemented with 10 per cent foetal bovine serum (Flow lab. Ltd., Scotland), 50 I.U./ml penicillin G and 50 µg/ml streptomycin. The cultures were started with 1 ml volumes of spleen cells (2×10^7 cells/ml) mixed with SRBC (1 to 1.2×10^7 cells/ml) in 35 x 10 mm Petri dishes (Falcon Plastics, no. 3001, U.S.A.). One day later 2 ml of medium F-12 including foetal bovine serum and antibiotics was added to the dishes.

This modification of the original technique of Mishell and Dutton (1967) made daily addition of a nutritional mixture unnecessary (Rossi and Zaalberg, 1974). A normal *in vitro* response was observed with peak values of 500 - 2000 PFC per dish on day 4 or day 5.

Anti-sera

Anti-θ serum was obtained by immunizing AKR mice with C_3H thymocytes (Reif and Allen, 1966). The animals were bled 4 or 5 days after the last injection. The cytotoxic potency of the serum for corticosteroid-resistant thymocytes (CRT) was determined by using a trypan blue exclusion assay. It is known that CRT have a θ-antigen content more characteristic of peripheral T cells than of corticosteroid-sensitive thymocytes which constitute the majority of the thymocytes (Raff and Cantor, 1971). In a two-step procedure the cells (10^7/ml) were incubated with anti-θ serum or normal AKR serum in melting ice for 30 minutes, washed in a balanced salt solution (Mishell and Dutton, 1968), resuspended to the original concentration and incubated at 37° C for 15 minutes with guinea pig serum (1 : 4 dilution, Flow lab. Ltd., Scotland). The guinea pig serum was absorbed with mouse spleen cells prior to use. The percentage of dye excluding cells was determined after addition of trypan blue to the suspension. The amount of anti-θ serum used in the experiments was 2 or 3 times higher than needed to kill more than 95% of the CRT. The final dilution varied between 1 : 5 and 1 : 20.

Anti-H-2^b serum was produced in CBA/Rij mice by 6-10 weekly intraperitoneal (i.p.) injections of C57BL/Rij spleen cells. The cytotoxic potency of the serum for C57BL/Rij mesenteric lymph node cells was determined with the same assay as described for anti-θ serum.

X-irradiation

Mice were exposed to 825 rads delivered by a Philips Mueller MG 300 X-ray machine (physical constants : 250 kV, 11 mA, 1 mm Cu filter).

Educated T cells

Cell suspensions enriched for educated T cells were obtained from spleens of mice 6 or 7 days after irradiation and i.v. injection of 5 x 10^7 syngeneic thymocytes and 4 x 10^8 SRBC (Mitchell and Miller, 1968). An optimal *in vitro* anti- SRBC response is observed when 10^5 to 10^6 educated T cells are added to spleen cells from adult thymectomized, irradiated and bone marrow-reconstituted mice (Feldmann and Basten, 1972 a).

B cells

Cell suspensions enriched for B cells were obtained from spleens of adult thymectomized, irradiated mice reconstituted with 3 x 10^6 anti-θ serum treated bone marrow cells (van Muiswinkel, van Beek and van Soest, 1975). The spleen cells were harvested at 4-5 weeks after reconstitution.

RESULTS

The T cell dependent period of the immune response in vivo

The immune response *in vivo* was started by an i.v. injection of 4 x 10^8 SRBC into normal (DBA/2 x C57BL/Rij)F1 mice. At successive days during the response the spleen was removed and a cell suspension was prepared. The cooperation between the T and B cells was abrogated by killing the T cells by means of anti-θ serum an complement. Another part of the suspension was incubated with normal mouse serum (NMS) and complement. This NMS treatment had no effect on the T cells and allowed further interaction between T and B cells. The surviving cells after serum treatment were mixed with SRBC and transferred to petri dishes. The dishes were tested individually for the presence of IgM-plaque-forming cells (IgM-PFC) after 0-7 days of culturing. The experimental procedure is schematically presented in figure 1. A typical picture was obtained (figure 2A), when spleen cells of non-primed animals were cultured after serum treatment. Anti-θ treatment reduced the *in vitro* response to 5-10 per cent of the normal response indicating that the *in vitro* response was highly thymus-

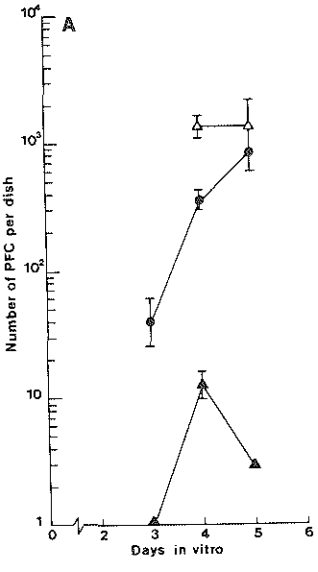
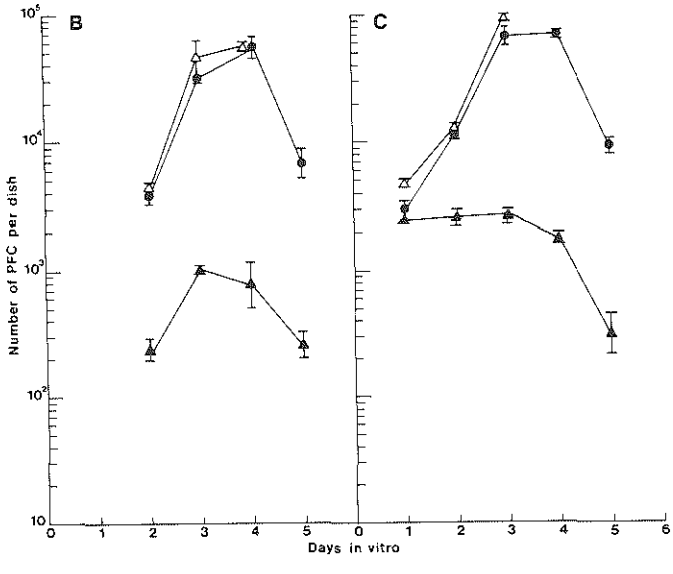


Fig. 2. The effect of serum treatment on the in vitro response of mouse spleen cells. The cells were obtained from normal mice (fig. 2A), mice primed with SRBC 2 days previously (fig. 2B) or 3 days previously (fig. 2C). The cells treated with normal mouse serum and complement (●) or anti-θ serum and complement (▲) and subsequently transferred to petri dishes. Additional educated T cells (10^6) were added to some dishes containing anti-θ treated cells (Δ). The dishes (4-5 per group) were tested for the presence of IgM-PFC at various days after starting the cultures. The results are expressed as geometric mean (± one (log) S.E.).



dependent. Addition of educated T cells to the anti-θ treated cells restored the response to normal indicating that the precursors of the antibody-forming cells (B cells) were not injured by the anti-θ treatment. When spleen cells from animals primed 2 or 3 days before were similarly treated with sera and subsequently cultured comparable results were obtained (figure 2B and 2C). Anti-θ treatment reduced the *in vitro* response again to 4-10 per cent of the normal response indicating that the response was still thymus-dependent. The conclusion can be drawn that T cells play an important role during the first 3 days of the immune response *in vivo* and that the majority of the B cells does not reach a T cell independent stage within this period. The level of the *in vitro* response of the primed cells is noteworthy. The normal response of primed cells (figure 2B and 2C) is 50-100 times higher than the normal response of unprimed cells (figure 2A). This observation is in agreement with the results of Dutton and Mishell (1967) and Pierce (1969).

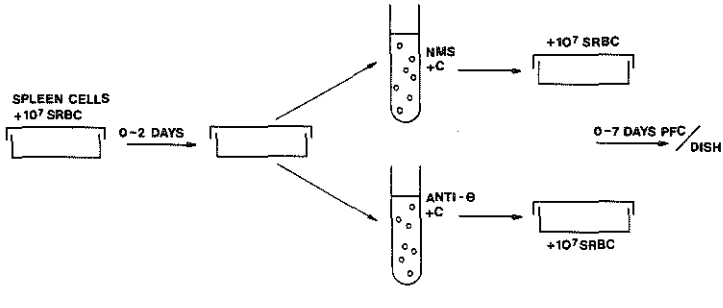


Fig. 3. The experimental procedure used for the study of the T cell dependent period in the immune response in vitro. Normal spleen cells or spleen cells cultured in the presence of SRBC were treated with normal mouse serum (NMS) and complement (C) or with anti-θ serum and C. After serum treatment the cells were transferred to new petri dishes and at successive days the number of plaque-forming cells (PFC) per dish was determined.

The T cell dependent period of the immune response in vitro

The immune response *in vitro* was started by culturing spleen cells from normal (DBA/2 x C57BL/Rij)F1 mice in the presence of SRBC. At successive days during the response the cells were harvested and incubated with anti-θ or NMS. The cultured cells were transferred to new petri dishes after serum treatment and the incidence of PFC was assessed at various days. The experimental set up in this experiment is shown in figure 3. It was observed that anti-θ treatment of spleen cells before culturing reduced the subsequent *in vitro* response to 1-10 per cent (figure 4A and table I, exp. A). However, the same anti-θ treatment after culturing spleen cells for 1 day reduced the *in vitro* response to 46 per cent (table I, exp. A). There was no reduction by anti-θ after culturing the cells for 2 days (figure 4B).

In another experiment using a mixture of B cells from CBA/Rij mice and educated T cells from (C57BL/Rij x CBA/Rij)F1 mice comparable results were obtained (table I, exp. B). It is worthwhile to mention that we were not able to obtain *in vitro* co-operation between B cells from DBA/2 or C57BL/Rij mice and educated T cells from (DBA/2 x C57BL/Rij)F1 mice indicating that semi-allogeneic T-B cell co-operation is only possible in certain combinations of parent and F1 hybrid. It is concluded from our experiments with normal spleen cells or combinations of B and T cells that the presence of T cells is only required during the first 1 or 2 days of the *in vitro* response of mouse spleen cells to SRBC. Apparently the majority of the B cells reached a T cell independent stage within 2 days of culturing.

In the experiments described above the co-operation between T and B cells was abrogated by means of anti-θ serum. However, there are indications that the amount of θ antigen on the T cell membrane decreases when these cells are incubated *in vitro* (Pierce, 1973). The T cell could become insensitive for anti-θ treatment as a result of the θ antigen reduction on its cell membrane. Therefore we used in one experiment anti-H-2 serum instead of anti-θ serum to kill the T cells. The procedure in our experiment (table II) was the same as used by Cosenza and Leserman (1972). In their experiments the role of the macrophage was studied.

They were able to abrogate the interaction between macrophages and lymphoid cells by adding an anti-H-2 serum (without complement) to the dishes at various times after starting the culture. In our experiment we used a CBA anti-C57BL serum to abrogate the co-operation between educated T cells from (C57BL/Rij x CBA/Rij)F1 mice and B cells from CBA/Rij mice (table II, exp. B). It is shown that the anti-H-2^b serum reduced the response to 24 per cent when the serum was added at the start of the culture but there was an increase in the response when it was added 1 day later. There was no effect of the addition of the same anti-H-2b serum to cultures of normal CBA spleen cells (table II, exp. A) indicating that our anti-H-2^b serum did not have a non-specific suppressing effect on mouse spleen cell cultures.

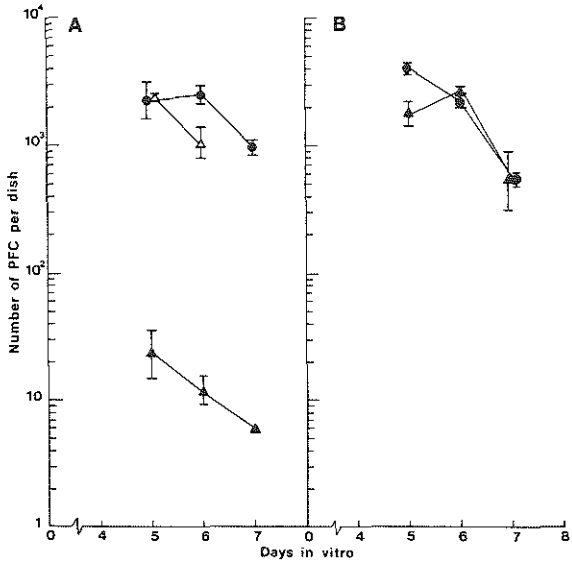


Fig. 4. The effect of serum treatment on the in vitro response of mouse spleen cells. The cells were treated with normal mouse serum and complement (●) or with anti-θ serum and complement (▲). Serum treatment of the cells was performed immediately before culturing (fig. 4A) or after culturing for 2 days (fig. 4B). Additional educated T cells (10^6) were added to some dishes containing anti-θ treated cells (Δ). The dishes (4-5 per group) were tested for the presence of IgM-PFC at various days after starting the cultures. The results are expressed as geometric mean (± one (log) S.E.).

Table I

The effect of serum treatment on the in vitro response of mouse spleen cells

Experiment	Cultured Cells	Interval between start of the culture and serum treatment (in days)	Serum treatment	PFC per dish
A	Normal spleen cells*	0	θ + C	3
		0	NMS + C	250 (157- 398)
		1	θ + C	902 (761-1069)
		1	NMS + C	1933 (1731-2160)
B	B cells + Ed. T cells **	0	θ + C	96 (80- 115)
		0	NMS + C	1308 (1155-1482)
		2	θ + C	3433 (3024-3899)
		2	NMS + C	2494 (1950-3191)

The cells were treated with anti-θ serum and complement (θ + C) or with normal mouse serum and complement (NMS + C) before culturing or after culturing for 1 or 2 days. After serum treatment the cells were cultured (4 dishes per group) and tested for the presence of IgM-PFC on day 5 (Peak of the response). The results are expressed as geometric mean (± 1 (log) S.E.).

* Normal spleen cells were obtained from (DBA/2 x C57BL/Rij)F1 mice.

**B cells = spleen cells from adult thymectomized, irradiated and bone marrow-reconstituted CBA/Rij mice; Ed.T cells were obtained from (C57BL/Rij x CBA/Rij) F1 mice.

Table II

The effect of serum treatment on the in vitro response of mouse spleen cells

Experiment	Cultured Cells	Interval between start of the culture and addition of serum (in days)	Serum added	PFC per dish
A	Normal spleen cells*	0	Anti-H-2^b	302 (267- 342)
		0	NMS	432 (355- 527)
		1	Anti-H-2^b	310 (274- 351)
		1	NMS	315 (256- 387)
B	B cells + Ed. T cells **	0	Anti-H-2^b	231 (204- 261)
		0	NMS	902 (767-1060)
		1	Anti-H-2^b	751 (713- 793)
		1	NMS	429 (380- 483)

One-tenth milliliter of CBA anti-C57BL (Anti-H-2^b) or normal CBA serum (NMS) was added to the dishes at the start of the culture or after 1 day of culturing. The dishes (4 per group) were tested for the presence of IgM-PFC at day 4 (Peak of the response). The results are expressed as geometric mean (± 1 (log) S.E.).

* Normal spleen cells were obtained from CBA/Rij mice.

**B cells = spleen cells from adult thymectomized, irradiated and bone marrow-reconstituted CBA/Rij mice; Ed.T cells were obtained from (C57BL/Rij x CBA/Rij) F1 mice.

DISCUSSION

It has been reported by Haskill, Marbrook and Elliot (1971) that a small but significant portion of the immune response by normal spleen cells takes place as a result of a dividing population of antibody forming cell precursors which respond to SRBC *in vitro* independent of the presence of θ-positive cells. This T cell-independent cell population was isolated by fractionating normal spleen cells on a density gradient. Our observation that anti-θ treatment of non-primed spleen cells did not eliminate, but only reduced the *in vitro* response to 1-10% of the normal response, is in agreement with the results of Haskill *et al.* (1971). There are two ways to explain this phenomenon of T cell independency: (1) a part of the B cells present in normal mouse spleen is stimulated by 'thymus-independent' antigenic determinants on the cell membrane of SRBC. These 'thymus-independent' antigens could be substances like lipopolysaccharide from E. coli, a well-known 'thymus-independent' antigen. (2) the B cells present in normal spleen are stimulated by thymus-dependent antigenic determinants on the cell membrane of SRBC and by factors produced by T helper cells. However, during the development of plaque-forming cells from B cells the B cell reaches a differentiation stage in which the presence of T cell factors is not required for its further development.

Our observations on the T cell-dependent period during the immune response *in vitro* are in favour of the last explanation. In our experiments a switch from a T cell-dependent stage to a T cell-independent stage was observed 1 or 2 days after starting the cultures. The small but always detectable *in vitro* response of anti-θ treated normal spleen cells could be explained by the presence of a small proportion of the B cell population which is continuously stimulated *in vivo* by enviromental antigens cross-reacting with SRBC (Cheng and Trentin, 1967).

Observations on the *in vivo* response do not fit with these ideas about a switch from a T cell-dependent stage to a T cell-independent stage during the anti-SRBC response. It has been shown by Chan, Mishell and Mitchell (1970) that anti-θ treatment reduced the *in vitro* response of unprimed and of *in vivo* primed

spleen cells to the shame extent. In their experiments the primed spleen cells were obtained from animals which were i.v. injected with SRBC 10 days previously. In our experiments the same reduction by anti-θ serum was observed when spleen cells were obtained from animals which were i.v. injected with SRBC 2 or 3 days before the start of the culture. However, the absolute number of PFC in the dishes containing NMS or anti-θ treated cells from primed donors is approximately 100 times higher than in dishes containing cells from normal mice. This indicates that both the number of B cells, which still need T cell help, and the number of B cells, which reached a T cell-independent stage, has increased in the spleen of primed mice. The conclusion seems to be justified that there is no switch from a thymus-dependent stage to a thymus-independent stage during the *in vivo* immune response as a whole, but that an increasing number of individual B cells reaches a thymus-independent stage during the first days of the *in vivo* response.

However, during the *in vitro* response a complete switch from a thymus-dependent stage to a thymus-independent stage was observed 1 or 2 days after the culture. Most of this work on the *in vitro* response was performed using an experimental system in which anti-θ serum was used. A critical point in this experimintal system is the sensitivity of cultured T cells for anti-θ treatment. It has been suggested by Pierce (1973) that the amount of θ antigen on the T cell membrane decreases in the period between 1 and 2 days of culturing. His conclusion was, that the T cell becomes insensitive for anti-θ treatment after *in vitro* incubation. The observations of Jones (1972) are in contradiction with this conclusion of Pierce. Jones could not demonstrate a difference in the concentration of antiserum required to demonstrate H-2 or θ on fresh lymphocytes or lymphocytes cultured for 3 to 4 days in the presence of phytohaemagglutinin, pokeweed mitogen, concanavalin A or allogeneic cells. In our experiments on the T cell-dependent period *in vitro* both anti-θ and anti-H-2 sera were used to kill the T cells. Unfortunately, our anti-H-2 serum was less active than our anti-θ serum in reducing the response when added on day 0 of the culture (table II). When the same anti-H-2

serum was added on day 1 no suppression but even a stimulation of the response was observed. This enhancing effect on day 1 might be explained by the elimination of suppressor T cells. Our experiments using anti-H-2 serum were not in contradiction with our experiments using anti-θ serum and, in fact, more or less in agreement with the latter experiments.

Our observations on the T cell-dependent period of the immune response *in vitro* are in agreement with the period that macrophages (Cosenza and Leserman, 1972) or intact erythrocyte antigens (Leserman, Cosenza and Roseman, 1972) are required during the immune response *in vitro*. These authors were able to eliminate the macrophages by means of an anti-H-2 serum and the erythrocytes by NH_4CL induced lysis. It was observed that the elimination of macrophages or erythrocyte antigens had a suppressive effect when performed before day 2 of the culture but no effect after day 2. These observations on the period that T cells, macrophages and antigens are needed during the *in vitro* response support the idea that the cooperation between T and B cells occurs on the membrane of the macrophage and is induced by membranebound antigen.

The difference between the T cell dependent period *in vivo* and the T cell dependent period *in vitro* is probably due to a synchronous start of the plasma cell development *in vitro* and a more asynchronous start of the same process *in vivo*. One of the reasons for this asynchronous start *in vivo* could be the antigen-induced recruitment of circulating lymphocytes as described by Sprent, Miller and Mitchell (1971) and Rowley, Gowans, Atkins, Ford and Smith (1972). These authors observed an influx of lymphocytes in the spleen during the first 2 to 3 days after i.v. immunization. It is obvious, that the response of the newly arrived cells starts later than the response of the cells present in the spleen before antigen injection. Sprent and co-workers suggest that the majority of the lymphoid cells which settle in the spleen during the first days after antigen injection, are T cells and it is not clear from their studies if B cells are recruited during the same period. Experiments in our laboratory using thymusless mice (nude or adult thymectomized, lethally irradiated and bone marrow-reconstituted mice) indicate that not only the number of T cells,

but also the number of specific B cells in the spleen increases during the first days after injection of SRBC (to be published). Therefore it is tempting to say that this increase of the number of SRBC-specific B cells during the *in vivo* response accounts - at least in part - for the asynchronous start of the *in vivo* response.

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Thymus Dependence of the IgA Response to Sheep Erythrocytes

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Summary. The thymus dependence of the IgA response to sheep red blood cells (SRBC) was studied by means of cell transfer experiments in mice. Only low numbers of IgM-, IgG- and IgA-plaque-forming cells (PFC) were observed in those recipients which received only spleen cells from adult thymectomized, irradiated and bone marrow-reconstituted mice (B cells). High numbers of IgM-, IgG- and IgA-PFC were observed when B cells and educated T cells were transferred to the recipients. Evidence is provided that B cells committed to IgA synthesis require the same degree of interaction with T cells as B cells committed to IgM synthesis, but a lower degree of interaction with T cells than B cells committed to IgG synthesis.

INTRODUCTION

There is circumstantial evidence that in mice bursa-equivalent-derived lymphocytes (B cells) committed to IgG synthesis require a greater degree of interaction with thymus-derived lymphocytes (T cells) for triggering than do B cells committed to IgM synthesis (Taylor and Wortis, 1968; Wortis, Dresser and Anderson, 1969; Anderson, Dresser and Wortis, 1974). Experiments concerning the thymus dependence of the IgG subclasses have shown that the synthesis of IgG1 is more thymus-dependent than the synthesis of IgG2a and IgG2b (Wortis *et al.*, 1969; Luzzati and Jacobson, 1972; Pritchard, Riddaway and Micklem, 1973).

The thymus dependence of the IgA response is, however, less clear-cut. Serum IgA levels in neonatally thymectomized and in adult thymectomized, irradiated mice are normal or even increased as compared with the serum IgA levels in normal mice (Humphrey, Parrott and East, 1964; Fahey, Barth and Law, 1965; Bazin and Duplan, 1966). The amount of IgA in serum of mice homozygous for mutation 'nude' which have been shown to display congenital thymic aplasia (Pantelouris, 1968) is, however, significantly reduced compared with the amount of IgA in the serum of mice heterozygous for the same mutation (Salomon and Bazin, 1972; Luzzati and Jacobson, 1972; Pritchard *et al.*, 1973). An analogous decrease in the serum IgA level was observed in adult thymectomized, irradiated and foetal liver reconstituted rabbits (Perey, Frommel, Hong and Good, 1970). Neonatal thymectomy in rabbits only slightly reduced the amount of rabbit serum IgA, but induced a markedly depressed IgA response to arsanil-azo–bovine serum albumin

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(AA–BSA) (Clough, Mims and Strober, 1971). However, the IgA response to human serum albumin in thymectomized mice was not significantly depressed (Torrigiani, 1972).

In view of these conflicting data we considered it worthwhile to perform a study on the immune response to sheep red blood cells (SRBC). The use of this antigen offered the opportunity to enumerate individual IgM-, IgG- and IgA-PFC instead of measuring IgM-, IgG- and IgA-levels in serum.

MATERIALS AND METHODS

Animals

(DBA/2 × C57Bl/Rij)F1 female mice were used. Irradiated recipients were 15–25 weeks old.

Antigen

For immunization mice were injected intravenously (i.v.) with 4×10^8 SRBC.

X-irradiation

Mice were exposed to 825 rads delivered by a Philips Mueller MG 300 X-ray machine (dose rate: 32 rad/minute; physical constants: 250 kV, 11 mA, 1 mm Cu filter).

Cell transfer

Irradiated recipients were injected i.v. with the appropriate cell suspension and SRBC. The spleen of the recipient mice was removed 7 days later and the number of IgM-, IgG- and IgA-PFC was determined with the plaque assay.

Plaque assay for antibody-producing cells

A method for the determination and the statistical analysis of the number of IgM-, IgG- and IgA-PFC was used as described before (Benner, Meima, Meulen and Muiswinkel, 1974).

B cells

Cell suspensions enriched for B cells were obtained from spleens of adult thymectomized, irradiated and bone marrow (3×10^6) reconstituted mice (T × BM mice). The bone marrow cells were treated with anti-θ antiserum and complement *in vitro* before transfer. The spleen cells were harvested at least 6 weeks after reconstitution.

Educated T cells

Cell suspensions enriched for educated T cells (ed. T cells) were obtained from spleens of mice 6 or 7 days after irradiation and i.v. injection with 5×10^7 syngeneic thymocytes and 4×10^8 SRBC (Mitchell and Miller, 1968).

RESULTS

THYMUS DEPENDENCE OF THE IgM, IgG AND IgA RESPONSE TO SRBC

To study the thymus dependence of the IgM, IgG and IgA response to SRBC irradiated recipients were divided into three groups. One group was injected i.v. with 3×10^7 B cells and SRBC. A second group was injected i.v. with 3×10^7 B cells, $3–4 \times 10^6$ ed. T

cells and SRBC. A third group was injected i.v. with $3–10 \times 10^6$ ed. T cells and SRBC. The spleen of the recipients was removed 7 days later and the number of IgM-, IgG- and IgA-PFC per recipient spleen was determined. Only low numbers of IgM- (220–230), IgG- (600–1920) and IgA-PFC (130–650) were observed in those recipients which received only B cells (Table 1). None, or very few (5–19) IgM-, IgG- and IgA-PFC were found in the spleen of recipients inoculated with ed. T cells only. However, when a mixture of B and ed. T cells was transferred, high numbers of IgM- (5500–13,000), IgG- (141,500–345,400), and IgA-PFC (3400–10,000) were observed. After injection of B and ed. T cells the IgM, IgG and IgA responses were respectively 24–59, 74–432 and 11–45 times higher than after administration of B cells only.

TABLE 1

THYMUS DEPENDENCE OF THE IgM-, IgA- AND IgG-PFC RESPONSE TO SRBC

Experiment number	Cells transferred		PFC/recipient spleen		
	B cells*	Ed. T cells	IgM	IgA	IgG
1	3×10^7	–	220	220 (n.s.)†	600
	3×10^7	3×10^6	13000	10000	201000
	–	1×10^7	6	0	0
2	3×10^7	–	230	650	800
	3×10^7	4×10^6	11600	7000	345400
	–	4×10^6	19	0	0
3	3×10^7	–	230	130 (n.s.)†	1920
	3×10^7	3×10^6	5500	3400	141500
	–	6×10^6	5	15 (n.s.)†	0

Cells were transferred into irradiated recipients together with 4×10^8 SRBC. PFC assay was done on pooled spleens of four or five recipients 7 days after cell transfer.

* B cells = spleen cells from adult thymectomized, irradiated and bone marrow-reconstituted mice.

† n.s. = Number of IgM-+IgA-PFC did not differ significantly from the number of IgM-PFC.

DISCUSSION

The results of the cell transfer experiments described in this paper lend further support to the idea that co-operation between T and B cells is required during the primary IgA response. The same conclusion can be drawn from a study of Clough *et al.* (1971) on the IgA response to AA-BSA in neonatally thymectomized and intact rabbits. In another study Benner, Meima and Meulen (1974) have shown that in the adoptive secondary response to SRBC anti-θ treatment of mouse bone marrow cells did decrease the IgA response to SRBC. From this experiment the conclusion was drawn that B memory cells still need the help of T cells to evoke an IgA-PFC response.

The results of studies on the IgM, IgG and IgA level in mouse serum are only partly in agreement with the previously described T-cell dependence of the IgA response. In nude mice the serum IgM level is normal but the IgG and IgA levels are subnormal (Salomon and Bazin, 1972; Luzzati and Jacobson, 1972; Pritchard *et al.*, 1973). These data are in agreement with the idea that the IgG and IgA responses are largely thymus-dependent. However, in neonatally or adult thymectomized mice the amount of IgM, IgG and IgA is normal or even increased (Humphrey *et al.*, 1964; Fahey *et al.*, 1965; Bazin and Duplan, 1966).

For the differences between nude mice and thymectomized mice a few explanations can be provided. In the first place a few T cells may be left in thymectomized mice and these T cells may operate as helper cells in responses of B cells to environmental antigens (Doenhoff, Davies, Leuchars and Wallis, 1970). A second possibility is a difference in the B-cell population in nude and thymectomized mice. Mitchell, Pye, Holmes and Nossal (1972) have shown that germinal centre formation in nude mice is deficient. Nieuwenhuis and Keuning (1974) recently published a hypothesis proposing that germinal centres function as an antigen-dependent amplification system for the B-cell population. This could indicate that both the T- and the B-cell population in nude mice is deficient. Preliminary experiments in our laboratory using the same transfer system as described in this paper are in agreement with the idea that in nude mice the number of precursors of IgG- and IgA-PFC is low compared with normal or thymectomized mice. A third explanation for the difference in IgA serum level between nude and thymectomized mice could be a difference in the intestinal flora or a difference in the transport speed of the intestinal content (van der Waaij, personal communication). It has been shown by Crabbé, Bazin, Eyssen and Heremans (1968) that the serum IgA concentration in germ-free mice is only 5 per cent of the serum IgA concentration in conventional mice. Another important observation is that local irradiation of the intestine of conventional mice caused a marked decrease in the serum IgA level but only a slight decrease in the serum IgM and IgG level (Bazin, Maldague and Heremans, 1970). It is concluded by Vaerman (1973) that serum IgA in mice is mainly synthesized by plasma cells present in the intestinal mucosa. The IgA synthesis in the intestine could be affected by the production of lipopolysaccharide (LPS) in Gram-negative bacteria. It is known that LPS can replace helper cells in response to thymus-dependent antigens (Sjöberg, Andersson and Möller, 1972; Watson, Trenkner and Cohn, 1973). This means that helper cells might not be required for the local IgA synthesis in the gut when enough LPS is produced by the intestinal bacteria. This mechanism could explain the normal or even increased IgA production in thymectomized mice.

Serum immunoglobulin levels are the results of stimulation by unknown environmental antigens which may be thymus-dependent or thymus-independent. This means that conclusions on the thymus dependence of the different immunoglobulin classes based on serum immunoglobulin levels have only a limited value and must be confirmed by studies on the antibody responses to well defined antigens.

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The regulatory influence of the thymus-dependent immune system on the heterogeneity of immunoglobulins in irradiated and reconstituted mice[1]

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The transient appearance of homogeneous immunoglobulins (H-Ig), 'paraproteins', in serum is a frequent finding during the reconstitution of the immune system after bone marrow transplantation in children with severe combined immunodeficiency (Radl and van den Berg, 1973) or in patients with aplastic anaemia or leukemia pretreated with an immuno-suppressive regimen (unplubished observation). Similar findings were obtained in lethally irradiated and bone marrow-reconstituted animals (Radl, van den Berg, Voormolen, Schaefer and Hendriks, 1974). Moreover, it was shown in rhesus monkeys that these H-Ig represent a specific antibody response towards antigenic stimulation (van den Berg, Radl, Löwenberg and Swart, 1975).

In the present work the role played by the T-system in the regulation of the heterogeneity of serum immunoglobulins was investigated in thymectomized or sham-thymectomized mice. The animals were lethally irradiated and reconstituted with bone marrow cells or foetal liver cells. It was observed that a delay in maturation of the T cell population or the absence of the T-system contributed substantially to the appearance of H-Ig during the reconstitution period.

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Figure 1 and figure 2 were not published in the original paper.

EXPERIMENTAL DESIGN AND METHODS

(DBA/2 x C57BL/Rij)F1 male and female mice were thymectomized (Tx) or sham-thymectomized (STx) at 5-6 weeks of age (van Muiswinkel, van Beek and van Soest, 1975).

In the first experiment (exp. A, table I) the animals were lethally irradiated (825 rad of X-rays, 250 kV, 1 mm Cu filter) and injected intraveneously (i.v.) with 3×10^6 anti-θ serum treated syngeneic bone marrow cells 2-4 weeks after surgery (van Muiswinkel *et al.*, 1975). The STx mice were subdivided: one group of STx mice received 3 intraperitoneal (i.p.) injections of 10^7 corticosteroid-resistant thymocytes (CRT) during the first week after transplantation (STx + T). The CRT were obtained from 6 weeks old mice 48 hours after i.p. injection with 30 mg/kg dexamethasone-21-phosphate (Merck & Company, Incorporated, Rahway, New Jersey). The other group of STx mice received no extra CRT. Blood samples were obtained by heart puncture under nembutal anaesthesia in the period between 3 and 13 weeks after transplantation. The criterion for a designation of H-Ig in the serum sample was the occurrence of a narrow, homogeneous extra band in the β-γ region when tested by agar electrophoresis according to Wieme (1959) (Fig. 1) and/or of a symmetric deviation of a precipitin line in the same region by immunoelectrophoretic examination using polyvalent and monospecific antisera for mouse immuno-globulin classes and subclasses (Fig. 2).

In the second experiment (exp. B, table I) the STx and Tx mice were reconstituted with 1.5×10^6 foetal liver cells. These liver cells were derived from embryos at 14-15 days gestation. At that time foetal liver does not contain B lymphocytes (Nossal and Pike, 1973; Rozing and Benner, 1975).

As a control the sera of 50 normal mice of comparable age were investigated in the same way.

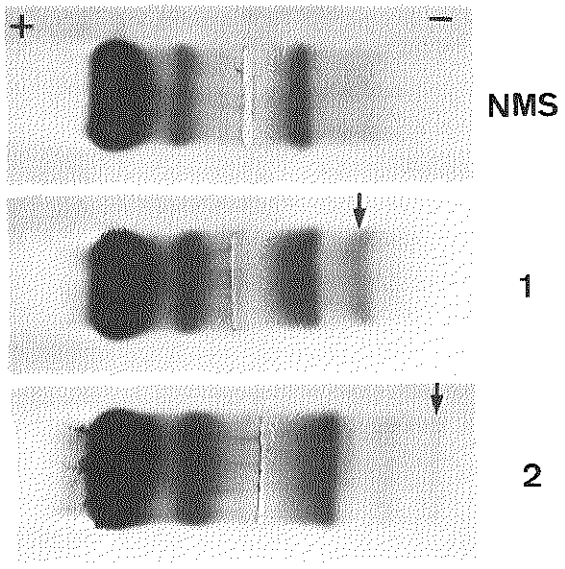

Fig. 1. Wieme's agar electrophoresis of pooled normal mouse serum (NMS) and of sera from irradiated and foetal liver-reconstituted mice (1 and 2). Note the clear homogeneous Ig component in serum 1 and the faint but distinct band in serum 2 indicated by arrows. The anode is to the left.

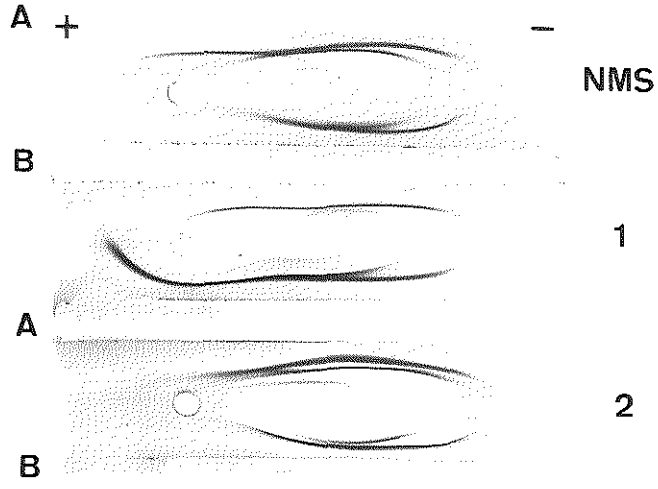

Fig. 2. Immunoelectrophoresis of pooled normal mouse serum (NMS) and of sera from irradiated and bone marrow-reconstituted mice (1 and 2). Rabbit antiserum against mouse immunoglobulins (A) or against mouse IgG-2a and IgG-2b (B) were placed in the throughs. Note the homogeneous component in the IgG-2a and the IgG-1 subclass in serum 1 and the homogeneous component in the IgG-2a subclass in serum 2. The anode is to the left.

RESULTS

No H-Ig were found in any of the serum samples from 50 normal untreated mice aged 5-6 months.

However, a number of H-Ig were detected in the sera of irradiated and bone marrow-reconstituted mice. The frequency at which the H-Ig appeared in the various groups of mice is given in table I, experiment A. Only 3-7% of the STx + T mice developed H-Ig during the period of 3 months after transplantation. The STx mice showed a higher frequency of H-Ig (15-19%). Even higher numbers of H-Ig were detected in the sera of Tx mice (33-45%). Most of the animals could be followed during the whole period of 3 months or even longer (details of this follow-up study will be published elsewhere). The H-Ig were usually transient, but they persisted in the sera of some animals for one or even two months. On several occasions, they gradually changed into a more heterogeneous population of immunoglobulins. One homogeneous component usually appeared at a time, but two or more H-Ig were observed in some cases. Practically all immunoglobulin classes and subclasses were represented among the H-Ig observed in the immunoelectrophoresis.

TABLE I

Homogeneous immunoglobulins (H-Ig) in the sera of irradiated and reconstituted mice.

Experiment	Group	Age in months	Percentage of mice with H-Ig 3-5 weeks after transplantation	7-9 weeks after transplantation	12-13 weeks after transplantation
A	STx + T*	3-6	7 (28)**	3 (32)	3 (28)
	STx	3-6	16 (25)	19 (36)	15 (27)
	Tx	3-6	33 (60)	45 (60)	37 (57)
B	STx	4-6	9 (23)	13 (31)	23 (30)
	Tx	4-6	20 (25)	28 (32)	33 (30)

*STx + T = sham-thymectomized mice that received 3×10^7 corticosteroid-resistant thymocytes after bone marrow transplantation; STx = sham-thymectomized mice; Tx = thymectomized mice. All mice were irradiated and injected with 3×10^6 anti-θ serum treated bone marrow cells (Experiment A) or 1.5×10^6 foetal liver cells (Experiment B).
**Number of animals tested in parentheses.

In the second experiment, where STx and Tx mice were reconstituted with foetal liver cells, a similar picture was seen (Table I, Exp. B), with one exception where the maximum changes appeared later. The agreement of the results of the two experiments indicates that the H-Ig observed in the first experiment were also produced by B cell clones newly developed from hemopoietic stem cells and not from committed B cells present in the bone marrow graft.

DISCUSSION

Radl and co-workers have shown that transient H-Ig appear frequently in the serum of man (Radl and van den Berg, 1973) and monkeys (Radl *et al.*, 1974) during reconstitution of the immune system after bone marrow transplantation. The simplest explanation of this finding is that a limited number of B cells will first arise during the development of the immune system. Later, more B cell clones will develop and a gradual transition from homogeneous to heterogeneous responses will occur. However, the question arises whether cell populations other than B cells are also important in the regulation of antibody heterogeneity. It was shown that B cells in mice reach their normal values 3-4 weeks after irradiation and reconstitution (Nossal and Pike, 1973; van Muiswinkel *et al.*, 1975 and Rozing and Benner, 1975). It is interesting, therefore, that in our experiments, H-Ig were observed in the period between 7 and 13 weeks after transplantation. The greatest numbers of H-Ig were observed in Tx mice, but they were also frequently observed in STx mice. It is important in this respect to realize that the recovery rate of the T cells in normal or STx mice after irradiation and reconstitution is slower than that of the B cells. It has been shown that the T cell population is still below its normal level at 15 or even 30 weeks after reconstitution (Vos, 1968 and Rozing and Benner, 1975). The difference in development of the T and B cells is schematically presented in figure 3. These data, together with the observation that the addition of T cells during the recovery period (STx + T mice) favours a heterogeneous immunoglobulin spectrum, strongly suggest an important role of the T-system in the regulation of immunoglobulin heterogeneity. The role of the

T cells may be twofold; (a) as helper cells, they can promote a response of multiple B cell clones towards thymus-dependent antigens; (b) as suppressor cells, they may prevent an overshoot reaction of a restricted number of B cell clones towards thymus-dependent (Gershon, 1974) or 'thymus-independent' antigens (Barthold, Kysela and Steinberg, 1974).

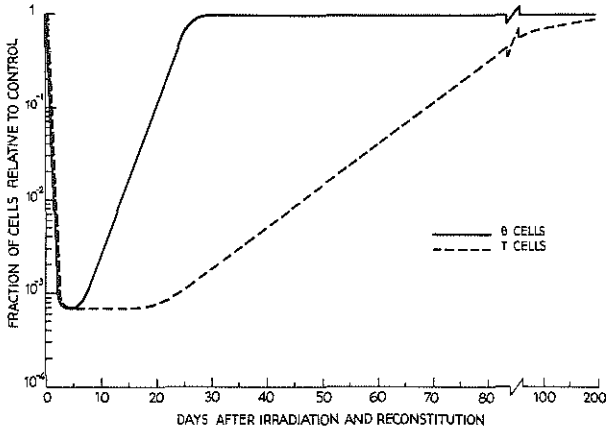

Fig. 3. A schematic diagram of the development of the B and T cell population in the spleen of normal mice after irradiation and reconstitution with hemopoietic stem cells.

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