THE ROLE OF THYMUS DEPENDENT AND BONE MARROW DERIVED LYMPHOCYTES IN ACQUIRED TOLERANCE

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ABSTRACT

In this thesis evidence is presented of the existence in tolerant mice of a factor produced by thymus-dependent lymphocytes (T cells) that blocks receptors on bone marrow derived lymphocytes (B cells), thus preventing their functioning and leading to the expression of tolerance.

The production of antibody during early induction of tolerance is discussed, and the surprising finding of reduced splenic uptake of aggregated human gamma globulin (Agg HGG) labelled with ^{125}I (^{125}I-Agg HGG) in mice tolerant to HGG is explored in detail. The possibility that a serum factor is responsible for this reduction is ruled out, and the cellular implications examined. Tolerant T and B cells were investigated separately for their role in reducing the splenic localization of ^{125}I-Agg HGG.

Using the uptake of Agg HGG as a tool for studying the B cell function, the idea that tolerant T cells produce a blocking factor was tested and substantiated. The mechanism of action of this factor was then investigated, and it was shown to be responsible for partially blocking the uptake of ^{125}I-Agg HGG by B cells.

The role of T and B cells in transferring tolerance in chimaeric mice was analysed and evidence presented that after elimination of antigen, maintenance of tolerance to HGG in B cells is dependent on the presence of tolerant T cells.

The effect of the factor produced by tolerant T cells on the handling of homologous soluble immune complexes by B cells was also investigated in an attempt to further understanding of its mechanism of action and its nature.

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PART I: INTRODUCTION

PART I

INTRODUCTION

Immunological tolerance - that is to say, specific unresponsiveness of the immune system to a particular immunogen - has been shown to be primarily a phenomenon mediated through lymphoid cells, and recently the need for positive interaction between these cells for the final expression of tolerance has been demonstrated for some antigens. A consequence of the concept of tolerance has been that the cellular mechanisms involved in the achievement of this state have been studied exclusively in the context of an absence of immune response to the antigen used as tolerogen. Studying only the negative aspect of this positive phenomenon has inevitably restricted complete understanding of the cellular events involved.

In this thesis a testing system was devised whereby the tolerant state of an animal could be evaluated and the cellular events involved in the induction of tolerance could be studied in terms of positive cell function.

The testing system was devised from earlier work in this Laboratory in which germinal centre localization of an altered immunoglobulin (HGG) was found to be independent of immune response, i.e. of the presence or absence of antibody to

the same antigen, and to be a specific function of a sub-population of lymphocytes (bone marrow derived lymphocytes). By measuring the localization of this material in the germinal centres of mice tolerant to HGG, a function of bone marrow derived lymphocytes could be specifically assessed independently of interaction with thymus dependent lymphocytes and by using different combinations of these two types of cells from tolerant or normal animals to reconstitute thymectomized lethally irradiated mice, it proved possible to get further insight into the cellular mechanisms of tolerance.

A) General Features of Immunological Tolerance

Immunological tolerance represents a condition in which the capacity of an animal to react to a normally effective antigenic stimulus has been specifically abolished. It is a fascinating subject of great practical and theoretical interest to both the laboratory investigator and the clinician. It is important for the latter in transplantation surgery and for understanding of autoimmune diseases, and for the former in the comprehension of the working of immunological mechanisms.

At one time tolerance and immunological responsiveness were regarded as opposite, mutually exclusive phenomena, but in recent years a radical change in thinking has taken place as a result of experiments showing that tolerance is not a lack of response, but is a positive response on the part of the immune system. It has thus been recognized as providing an exceedingly versatile tool for the study of the immune response.

1. Immaturity of the Immune System

The observations of Traub (1938) and Owen (1945) that living foreign virus or cells present in an animal from early embryonic life onward enjoyed a privileged existence even after the animal had matured, led to Burnet and Fenner (1949) predicting the experimental demonstration of tolerance. They suggested that if an

antigen were introduced into an animal before its immune apparatus had properly developed, this antigen would be identified as a "self" antigen and the animal would fail to form antibody to it at a later stage, when immunological competence was acquired, and the antigen was reintroduced in a form or dose adequate for immunization of normal controls. The experiments of Billingham, Brent and Medawar (1953) showed that, with respect to immune responses to homografts, subsequent tolerance could indeed be achieved by injection of living allogeneic lymphoid cells into mouse embryos. Soon afterwards a similar principle was used to induce tolerance to defined protein antigens (Hanan & Oyama, 1954; Smith & Bridges, 1958). It seemed that the best example of immunological tolerance is the unresponsive state occuring in all animals to their own body constituents where the same principles can be invoked (Burnet, 1959).

The results of experiments with embryonic and neonatal animals suggested a susceptible period during which paralysis but not immunity could be induced. However, later work has shown that young animals or even embryos can sometimes produce an immune response. Thus, Howard and Michie (1962) showed that transplantation immunity could be induced in newborn mice with smaller doses of allogeneic cells than were required to paralyze, and Silverstein et al (1963) found that foetal sheep can respond to bacteriophage, ferritin and ovalbumin after 70 days of gestation (total gestation period, 150 days). Wegmann, Hellström and Hellström (1971) and Phillips et al (1971) also showed that even when immunocompetent cells are exposed to allogeneic cells at the time of their differentiation in the embryo they are still able to mount an immune response. The concept of a period of unique susceptibility to paralysis had therefore to be modified.

It has since been shown that an unresponsive state can be induced in adult, immunocompetent animals, when the antigen is injected under conditions which fail

to result in an immune response. Thus tolerance can be achieved in adults either when the immune response is temporarily abolished by X-irradiation (reviewed by Schwartz and Dameshek, 1963), or when the antigen is injected in a non-immunogenic form (Dresser, 1961a,1962a,b). In the former case, adults animals may be made tolerant with doses and forms of antigen comparable to those effective in embryonic or newborn animals while in the latter case it was thought necessary to administer the antigen in soluble form (Claman, 1963; Dietrich & Weigle, 1964).

Nevertheless it is well established that tolerance is more easily and more conveniently induced in newborn than in adult animals (for references see Smith, 1961) presumably due to their relative deficiency in immunologically competent cells.

2. Immunogenic Capacity of the Antigen

As a rule it can be said that the less the immunogenic capacity of an antigen the greater its ability to induce tolerance. Thus if the denatured or aggregated fraction of a protein preparation is removed by either biofiltration (Dresser, 1963) or ultracentrifugation (Dresser, 1962b) its immunogenic capacity is lost and its tolerogenic effect is increased. In the biofiltration phenomenon the antigen (e.g. BSA injected i.v. into rabbits) is screened "in vivo" by removal of the material susceptible to phagocytosis (Frei, Benacerraf & Thorbecke, 1965) and the fraction left in circulation two days after injection has only tolerogenic capacity when injected into a second recipient. Attempts to demonstrate the same effect in mice with BSA have nevertheless failed on some occasions (Mitchison, 1964).

In respect of immunogenic capacity, Dresser and Mitchison (1968) in their review on the mechanism of immunological paralysis, have considered two groups of antigen: non-immunogenic and weakly immunogenic.

Weakly immunogenic antigens are those that immunize only in doses higher than those needed for paralysis. This is the case with bovine serum albumin (BSA)

in mice (Mitchison, 1964); when injected alone a dose may be found which induces paralysis but which is below the minimum level required for immunization, although when injected with adjuvant the same dose elicits antibody to a titre as high as is obtained with a potent immunogen (such as lysozyme or diphtheria toxoid).

Non-immunogenic antigens are incapable of inducing an immune response, unless combined with adjuvant, or denatured or aggregated, but when given alone are able to paralyze. Good examples of this group are the immunoglobulins of various different species (Dresser, 1961a, b, 1962a,b; Weigle, 1962; Claman, 1963; Dietrich and Weigle, 1964; Biro and Guadalupe, 1965). Another example of this group is poly-D-aminoacids in mice (Janeway and Sela, 1967). These polypeptides are immunogenic when injected with Freund's adjuvant, but are tolerogenic when injected without adjuvant. They are resistant to degradation in the body (Gill et al, 1965) and recirculated for long time, in contrast to the corresponding poly-L-aminoacids, which can immunize when used in high doses, even without adjuvant. A similar phenomenon occurs with poly-DL-alanine in rabbits (Schechter, Bauminger & Sela, 1964), which is unable to immunize even when incorporated with Freund's adjuvant, although it does immunize when bound either to HSA or ribonuclease. In contrast to untreated rabbits, rabbits pretreated with poly-DL-alanine alone failed to respond to subsequent injection of poly-D-alanylated proteins.

All these results suggest an inverse relationship between antigenic and tolerogenic capacities in an antigen. The results of Schechter et al (1964) referred to above have been confirmed by similar experiments with polypeptides reported by Maurer, Pinchuck and Gerulat (1965) and Roelants and Goodman (1970), and point to the conclusion that a substance does not need to be immunogenic in order to induce tolerance. It has also been found that dinitrophenyl (DNP), a hapten that is able

to immunize when combined with keyhole limpet haemocyanin in complete Freund's adjuvant, is nevertheless able to induce tolerance in adult mice when bound to a non-immunogenic carrier (isogeneic serum or homopolymers of lysine) (Golan & Borel, 1971). There is thus a considerable amount of evidence that antigenicity is not a necessary feature of a tolerogenic substance.

It has been thought that in an adult animal an antigen that diffuses freely in the body fluids because of its physicochemical properties is more tolerogenic than immunogenic (Benacerraf, 1969). In contrast, antigens that do not have this property and are easily taken up by macrophages or cells of the reticuloendothelial system are often good immunogens (Benacerraf, 1969). In section B) of this introduction which deals with the fate of tolerogenic antigen, it will be seen that this is not invariably true.

3. Antigen Dose : High and Low Dose Tolerance

It was first noted by Glenny and Hopkins (1924) that the balance of immunological response between paralysis and immunity is determined by antigen dosage. They injected equine gamma globulin (antibody to diphtheria toxoid) into adult rabbits and followed its elimination from the circulation. It was demonstrated that continuous administration of the antigen decreased markedly its immune elimination. Interest in dosage was later revived by Dixon and Maurer (1955) who showed that adult rabbits could be rendered tolerant to a variety of foreign plasma proteins by repeated large doses of the antigen, and this was subsequently demonstrated by other authors (Smith, 1961; Dresser, 1962a; Sercarz & Coons, 1963; Mitchison, 1964). Similar results were obtained with transplantation antigens (Rubin, 1959; Shapiro, Martinez, Smith & Good, 1961; Brent & Gowland, 1963) by administration of doses of cells or extract that were massive as compared with those necessary either for inducing tolerance in newborns or for immunizing adults.

It was also found (Dresser, 1962b) that small doses of bovine gamma globulin can induce immunological tolerance, and this observation was later confirmed (Battisto & Miller, 1962; Claman, 1963). The concept that antigen can cause tolerance in two distinct zones of dosage was developed by Mitchison (1964), and was followed by similar findings with serum proteins (Dresser & Gowland, 1964; Biro & Guadalupe, 1965; Lance & Dresser, 1967; Thorbecke & Benacerraf, 1967) and with flagellin (Shellam & Nossal, 1968). The degree and duration of the immunological unresponsive state is nevertheless dose dependent, being in general more complete and of longer duration with larger dose of tolerogen injected (Weigle, Chiller & Habicht, 1972).

To explain the mechanism of high and low dose tolerance Nossal (1969) postulated that a cell will be rendered immune if during a given time it encounters doses of antigen of magnitude between "a" and "b" (where $b > a$), but will be rendered tolerant if it is subjected to less than "a" or to more than "b" over the same time. The dose response is of particular interest when the cellular implications of tolerance induction are considered and the effect on thymus and bone marrow cells are analyzed separately. Chiller, Habicht and Weigle (1971), found that bone marrow cells require more tolerogen to become unresponsive than thymus cells and suggested therefore that while high zone tolerance involves both thymus and bone marrow cells, in unresponsiveness induced with low levels of tolerogen only thymus cells become unresponsive. This idea is further strengthened by the finding of Mitchison (1971) that injection of primed (pre-immune) thymus-derived cells converted the unresponsive state produced by periodic injections of small doses of BSA into a responsive one, implying that reactive immunocompetent B cells were still present in these animals, whereas the same primed thymus derived cells did not modify the unresponsive state induced by repeated high doses of tolerogen.

It has been repeatedly noted also that tolerance induced by large doses of antigen may be subsequently maintained by injection of smaller doses at intervals. This is the case with strongly immunogenic antigens like mouse erythrocytes in rats (Makela & Nossal, 1962) and allogeneic erythrocytes in chickens (Mitchison, 1962). In the case of weak immunogens, like BSA or lysozyme in the mouse, the dose necessary to maintain tolerance is not significantly different from that required to induce paralysis in the low zone of dosage (Dresser & Mitchison, 1968).

4. Suppression of the Immune Response

It is not only the immunogenic capacity of antigens that is important in induction of immunological tolerance. The reactivity of the immune system is also relevant, and any means of diminishing the immune response can be used as a helper in the achievement of tolerance. The relative natural deficiency of immunocompetent cells in newborn animals has already been dealt with in this Introduction, and widely used treatments to achieve the same effect include irradiation and immunosuppressive drugs, and have been extensively reviewed (see Dresser & Mitchison, 1968, for references). Other immunosuppressive treatments which facilitate induction of paralysis include chronic draining from the thoraric duct (McGregor & Gowans, 1963, 1964) and administration of antilymphocyte serum (Woodruff & Anderson, 1963; Levey & Medawar, 1966) and of serum alpha$_2$globulin. This fraction of bovine serum, first described by Kamrin (1959), prolongs skin grafts in rats, and was further found to have immunosuppressive effects in other animals when administered before a variety of antigens(Mowbray, 1963; Mowbray & Hargrave, 1966; Mowbray & Scholand, 1966).

B) Antigen Distribution and Tolerance Induction

1. The Fate of Tolerogenic Antigen

The distribution of labelled antigen molecules during induction of tolerance

fails to reveal a single common pattern which covers all experimental circumstances. In many models, for instance BSA labelled with ^{125}I (Ada, Nossal & Austin, 1964) autoradiography shows a wide diffusion and prolonged extracellular persistence of the tolerogenic antigen and all areas of all lymphoid tissues show diffuse spread of antigen (Clark, 1966). Such a distribution of the antigen is consistent with the hypothesis of Frey et al (1965) that the molecules that are left in circulation for a long time after "biological filtration" are tolerogenic, and that lymphocytes are rendered tolerant by coming in contact with free nonprocessed antigen (Nossal & Ada, 1971). In other cases when essentially poorly digestible substances which persist inside the macrophages for a long time (such as pneunmococcal polysaccharide (Howard & Siskind, 1969) and the linear D-amino acid copolymer of tyrosine, glutamic acid and alanine (Janeway & Humphrey, 1969)) are used, it is possible that macrophages act as a reservoir for the slow, constant release of antigenic fragments and thus maintain the concentration of antigen or its fragments at tolerogenic levels.

Finally, in some tolerance models, for example that using "Salmonella" flagellar antigen, the antigen is chiefly detected in the lymphoid follicles during tolerance induction, while in the medullary macrophages its uptake is poor (Ada & Parish, 1968); this has been considered to be due to the presence of naturally occurring antibody to flagella. These results suggest that in this system antigen becomes firmly attached to, and concentrated at, the surface of dendritic follicular cells, and thus provides an extracellular deposit, in reasonably concentrated form, that is responsible for induction of tolerance in cells migrating through the white pulp.

2. The Localization of Antigen in already Tolerant Animals

When antigen is reinjected in animals showing full tolerance with no trace of antibody to any determinant of the antigen, it circulates widely and is distributed

as if it were an autologous constituent (Humphrey & Frank, 1967). If, however, during the induction of tolerance some antibody is produced or if some naturally occurring cross reacting antibody is present, this will cause rapid follicular localization of at least some of the antigen injected (Ada, Nossal & Pye, 1965).

C) Cellular Events during Tolerance Induction

Until recently little was known of the underlying cellular mechanisms involved in immunological unresponsiveness. It has been established that interactions between bone marrow (B cell) and thymus derived (T cell) lymphocytes is required for an immune response (Gowans & McGregor, 1965; Claman, Chaperon & Triplett, 1966; Davies et al, 1967; Miller & Mitchell, 1967; Mitchell & Miller, 1968), and it is also known that macrophages may participate in antibody production (Mosier, 1967). These findings have directed investigations towards study of cellular events in order to elucidate the mechanisms involved in immunological unresponsiveness.

1. Cellular Cooperation in the Induction of Tolerance

Immunological specificity and memory can readily be demonstrated in both T and B cells (Miller et al, 1971) but several investigators have attempted to determine the cellular site of specific immunological unresponsiveness with inconsistent results. Thus while tolerance is readily induced in and transferred by T cells (Miller & Mitchell, 1970; Chiller et al, 1971; Mitchison, 1971), the existence of tolerance in B cells is more controversial. Depending on the antigen and experimental conditions, both cell types have been reported to become unresponsive (Chiller, Habicht & Weigle, 1970, 1971; Mitchison, 1971; Gershon & Kondo, 1970, 1971) while others have claimed that unresponsiveness resides in either thymus cells, on the one hand (Taylor, 1968) or bone marrow cells on the other (Playfair, 1969; Miller et al, 1971). This difference of opinion may reflect differences in the cellular kinetics of the different experimental systems employed, as discussed in the

next section (p.19). Playfair's conclusion that tolerance could be induced in bone marrow cells was based on experiments with NZBx BALB/c F_1 hybrids treated with SRBC and cyclophosphamide. The immune response in NZB mice however is abnormal (Staples & Talal, 1969) and T cell deficiency has repeatedly been observed in this strain (Gazdar, Beitzel & Talal, 1971).

Chiller et al (1970) made normal mice tolerant to HGG and then transferred thymus or bone marrow cells from these tolerant animals to lethally irradiated syngeneic recipients, along with normal bone marrow or thymus cells respectively. In addition a third group of recipients were reconstituted with thymus and bone marrow cells from tolerant donors. All three groups of recipient mice proved to be tolerant on challenge. These results suggested that either thymus derived or bone marrow derived cells, but not necessarily both, need to be unresponsive for an unresponsive state to exist in the intact animal.

Gershon and Kondo (1970, 1971) provided further insight into the cellular relationships involved in tolerance. They showed that to achieve tolerance in mice to SRBC, the presence of T cells as well as B cells is necessary, because if only B cells are present during induction the resulting tolerance is abrogated by the injection of normal T cells. If, however, T cells as well as B cells were also present during the induction of tolerance, then the tolerant state induced was not reversed by a further injection of normal T cells accompanying the antigenic challenge. These important experiments, which are discussed in greater details under "Experimental Work and Discussion of Results" (p.72) showed that not only was cooperation of the two cell types necessary to induce tolerance in B lymphocytes but also that the milieu created by the presence of tolerant T cells prevented collaboration between normal T cells and tolerant B cells. It was postulated that T cells made tolerant by repeated high doses of antigen produce a factor, "IgY", (or induce the B cells to produce it)

that prevents the B cell population responding to antigen in the presence of normal T cells. This interpretation of Gershon and Kondo's results will later be discussed in detail in the light of the experiments to be reported in the present thesis.

The idea that long lasting tolerance to SRBC is primarily dependent on production of a specific factor by T cells, derived further support from the following elegant experiments of Miller (1971). Thoracic duct lymphocytes (TDL) of mice specifically made tolerant to fowl immunoglobulin G (FGG) (a thymus-dependent antigen) were coated with this antigen in the form of fowl anti-mouse lymphocyte globulin (FALG) and transferred to thymectomized, lethally X-irradiated,bone marrow reconstituted recipients. The antibody response to FGG was not enhanced; but when normal TDL similarly treated with FALG were injected, the antibody response to FGG in similar recipients was significantly enhanced.

It should be noted that FALG is not immunosuppressive in mice, presumably because it is non-complement fixing. Both populations of TDL, i.e. from normal mice or from mice tolerant to FGG, were agglutinated by FALG and must therefore have carried FGG on their surface for presentation to B cells. The failure of FALG-coated tolerant TDL to enhance the antibody response to FGG could not be attributed to their inability to recirculate normally and penetrate the correct sites in the spleen, since both normal and tolerant FALG-coated TDL populations were distributed in an identical way in the recipients, as judged by the distribution of ^{51}Cr-labelled cells. It might be suggested that the FALG-coated tolerant TDL could not proliferate in response to the tolerated antigen and that such proliferation is necessary for effective cooperation with the B cell. In the experiments described by Miller, however, proliferation was not necessary to provide enough T cells to transport and focus the antigen onto the B cells, since the tolerant FALG-coated TDL injected would have carried enough of the antigen on their surfaces. Thus

Miller postulates that the role of T cells is an active one that involves not only interaction with the antigen but also further differentiation with production of some factor, pharmacological or immunological, as suggested by the experiments of Gershon and Kondo (1971) discussed above.

In addition to the thymus and bone marrow cells, macrophages are undoubtedly involved in the immune response to some antigens. Mosier (1967) demonstrated that in vitro cultures of mouse spleen cells depleted of macrophages (adherent cells) did not respond to SRBC. These cultures recovered their immunological potency when macrophages were reintroduced (Mosier, 1967) or by addition of supernatants from peritoneal macrophage cultures (Hoffman & Dutton, 1971). Gallily and Feldmann (1967) showed that peritoneal macrophages incubated in vitro with Shigella were able to induce antibody response in mice exposed to 550 r, which did not respond to the injection of antigen alone or of lymph node cells treated in the same way. The immunogenicity of macrophages containing antigen depended on interaction with immunocompetent lymphoid cells since heavily irradiated hosts were unresponsive (Gallily & Feldmann, 1967; Unanue & Askonas, 1968), whereas when normal lymphoid cells were also supplied to these animals normal immune responses developed (Unanue & Askonas, 1968).

Martin (1966) observed that when newborn rabbits were injected with a tolerogenic dose of BSA and adult macrophages an immune response was observed instead of tolerance. Similarly newborn mice acquired immunological competence when injected with adult macrophages (Argyris, 1963; Bendinelli, Senesi & Falcone, 1971). "It is tempting to postulate that the macrophages, via antigen fixed on their surfaces, may stabilize the interaction between thymus and bone marrow cells" (Weigle et al, 1972). Whatever role the macrophages play in the immune response it is not specific, since it was shown by Kolsch and Mitchison (1968) that macrophages

from tolerant mice are just as effective in fixing antigen on their surface and priming mice, as macrophages from normal animals.

2. Kinetics of Cellular Induction of Tolerance

Immunological unresponsiveness to deaggregated HGG took four days to be completely established in mice (Golub & Weigle, 1967), although at 6 hours 70% of the spleen cells were found to be tolerant as judged by spleen cell transfer and plaquing tests (Chiller & Weigle, 1971). By briefly exposing mice to tolerogenic doses of BSA prior to collection of cells from their spleen and lymph nodes, which were then transferred into syngeneic unresponsive mice, Mitchison (1968) showed an induction period of 24 hours for the paralysing effect to occur. Recipient unresponsive mice received 600 r followed by 10 weekly injections of 5 mg of BSA, were then rested for 10 days, X-irradiated with a further 600 r, and then used as hosts for cell transfer on next day. One day after the cell transfer they were challenged with antigen in adjuvant, bled 20 and 40 days after the challenge, and the antigen binding capacity of their sera determined. The discrepancy between the speed and efficiency of high concentrations of antigen in inducing paralysis in cells about to be transferred and the effects observed in the whole animal, where induction takes far longer (Mitchison, 1964), can "perhaps be accounted for by the operation of the Sercarz and Coons (1962) effect," where under these conditions of transfer, cells which had been recently committed to an immune response tended to be selectively inactivated. A 4-5 day induction period for "in vivo" unresponsiveness to pneumococcal polysaccharide was observed by Matangkasombut and Seastone (1968). The induction period therefore appears to vary with experimental circumstances and with different antigens.

The kinetics of the induction of tolerance to HGG in mice was studied by Chiller, Habicht and Weigle (1971). They injected mice with 2.5mg of deaggregated

HGG and used either their thymus or their bone marrow cells, in combination with the normal cell counterpart, to reconstitute irradiated mice which were afterwards challenged with the immunogen. Their spleens were then assayed for plaque forming cells. Unresponsiveness in thymus cells was found to be complete by the second day and remained complete for 120 days; by day 155 thymus cells from tolerant mice were still able to induce tolerance in 50% of the recipient mice. The induction of tolerance in bone marrow cells was much slower, requiring 15-21 days to become complete. By day 49 it was completely lost and cells had returned to a responsive state.

Mitchison (1971) reported similar results. By transferring mice from a multiple high dose tolerogenic regime of injections of BSA to a low dose maintenance regime, which would maintain tolerance only in T cells, he was able to study the time taken by the B lymphocytes to recover from their state of tolerance. He found that the B cells of these mice took 10 weeks to regain appreciable reactivity.

From his reconstitution experiments with thymus and bone marrow cells from animals pretreated with BSA, Taylor (1968) concluded that tolerance could be induced in thymus cells but not in bone marrow cells, but his findings can be explained by the kinetics of induction of tolerance. In Taylor's experiments the immunocompetence of both T and B cells was tested 24 hours after a tolerogenic injection of BSA, at which time the majority of T cells had been rendered tolerant, but the whole B cell population was still responsive (Chiller et al, 1971). The same differences in the kinetics of tolerance induction between the two lymphocyte populations could perhaps be invoked to explain the conclusion that tolerance is present only in T cells in experiments reported by Miller et al (1971), in which cells were harvested three weeks after a high dose of fowl gamma globulin. Because the cells were not taken at different time intervals, the period of tolerance of B cells may well have been missed.

Weigle et al (1972) suggested that the differences between the two type of cells in the time necessary for induction of tolerance could be explained in two ways. Thymus cells became unresponsive by direct interaction with the tolerogen but bone marrow cells may either have to undergo an active process of antigen-directed differentiation during maturation from precursor cells, or induction of their tolerance may be dependent on pre-existing tolerance in thymus cells. The interaction between tolerant T cells and B cells would either be through direct contact of B cells with a small number of tolerant thymus cells migrating through the bone marrow, or would be through a factor secreted by unresponsive T lymphocytes, as suggested by Gershon and Kondo (1971).

The kinetic differences between thymus and bone marrow cells in recovery from tolerance are more difficult to explain, but are probably related to decrease in the concentration of tolerogen (Weigle et al, 1972). Lower levels of tolerogen could be enough to influence the T lymphocytes, which appear to carry fewer receptors than B cells and are more easily made tolerant by low concentration of antigen (Mitchison, 1971). The importance of maintaining antigen concentration for B cell tolerance is shown by the fact that "in vitro" induction of unresponsiveness with thymus independent antigens such as the lipopolysaccharide of E.coli (Britton, 1969) and flagellin (Diener & Armstrong, 1969) takes only a few hours.

3. The Role of Cellular Receptors in the Induction of Tolerance

The work of Naor and Sulitzeanu (1967) with ^{125}I-labelled BSA, designed to test part of the clonal theory (Burnet, 1959), showed that for any one antigen only a small proportion of the total lymphocytes population possessed receptors of the required specificity.

Following this, reports have accumulated to suggest that both thymus derived and bone marrow derived cells have specific receptors sites for antigen that are important in the induction of either immunological unresponsiveness or the immune response (review by Ada, 1970). The receptors on B cells are thought to be of a nature similar to the immunoglobulin made by the cells for secretion (Pernis, Forni & Amante, 1970; Rabellino et al, 1971; Greaves, 1970; Raff, 1970). There is no such agreement however on the nature of the receptor sites on the T cells. Using ^{125}I labelled rabbit antibody to mouse L chain, Jones, Torrigiani and Roitt (1971) were able to detect L chains on 4 per cent of thymus cells, but other workers were not able to localize antibody directed against IgG or its fractions on the surface of either thymus cells or thymus-derived cells using fluorescein-labelled antibody (Pernis et al, 1970; Unanue et al, 1971). However, following treatment with anti-light chain sera, the immune function of thymus-derived cells is inactivated (Greaves, Torrigiani & Roitt, 1969), and specific irradiation suicide of T cells caused by uptake of heavily labelled ^{125}I fowl gamma globulin is blocked by pretreating the cells with anti-kappa $F(ab')_2$ (Basten et al, 1971). Therefore the available evidence is not sufficient to indicate whether the effective receptor site on T cells is an intact immunoglobulin or an immunoglobulin fraction. It is reasonable to expect that differences in the nature of the receptors on T and B cells might dictate the pattern of the kinetics of induction and spontaneous termination of immunological unresponsiveness (Weigle et al, 1972). Whatever the nature of these receptors it appears that in any particular antigen the antigenic determinants that react with T cells are different from those that react with B cells (Mitchison, 1969; Rajewsky et al, 1969) and also that T cells have fewer receptors than B cells (Mitchison, 1971), as shown by the fluorescence experiments of Raff (1971) where B lymphocytes have easily demonstrable surface immunoglobulin, while T lymphocytes do not.

However, as pointed out by Mitchison (1971), there is an apparent paradox in the fact that T lymphocytes which appear to carry fewer receptors than B lymphocytes, are nevertheless more easily rendered tolerant. He suggested that although T lymphocytes because of the small number of their receptors bind less antigen at a given concentration of antigen than B lymphocytes, they are nevertheless more easily triggered. He also suggested that there is a higher affinity for antigen on the part of the T lymphocyte receptor and thus, at a given concentration of antigen, T lymphocytes bind more antigen than B lymphocytes, even though they have fewer receptors.

The Clonal Selection Theory put forward by Burnet (1959), predicted that tolerance to self components is due to deletion of self-reacting clones of cells during foetal life. This same concept has been invoked to explain acquired tolerance, whether induced neonatally or in adult life. The antigen binding reaction has been used to test whether a particular cell clone has been specifically deleted in tolerant animals. Several such studies carried out with autoradiography of ^{125}I-labelled antigens have given equivocal answers.

Naor and Sulitzeanu (1969) observed either no or very few antigen-reactive cells in the lymph nodes of mice made tolerant neonatally with high doses of BSA. This suggests a deletion of antigen binding cells, or alternatively it could be due to saturation of binding sites on specific cells preventing further reaction with labelled antigen. Humphrey and Keller (1970) made mice tolerant from birth to haemocyanin (M.squinado) and found 47-170 per cent (mean 80 per cent) of antigen-reactive cells in their spleens and lymph nodes as compared with non-tolerant mice (100%). In contrast, Ada et al (1970) found a normal complement of antigen-binding cells in the spleens of rats made tolerant to fragments of flagellin, either with low doses from the day of birth or with high doses in adult life.

Thus, tolerance may not necessarily mean the destruction of the competent lymphocyte, but simply an inactivation, and the experiments already described where tolerance was achieved by transference of cells from tolerant mice to immunologically deficient mice (thymectomized and X-irradiated) support this concept.

D) The Role of Antibody in Immunological Tolerance

The role of antibody in immunological tolerance has been variously interpreted according to the nature of the antigen involved. For "non-replicating" antigens, such as BSA or SRBS, it has been suggested that any antibody produced could be the result of exhaustive cell differentiation; it has also been regarded as a tool for feedback control. In the case of "replicating" antigens such as allografts and self tissues, there is some evidence to suggest that antibody can also play a protective role by blocking antigen and preventing lymphocyte attack. These aspects will be discussed separately.

1. Antibody as a Result of Exhaustive Cell Differentiation or as a Tool for Feedback Control.

As Sterzl and Trnka (1957) first noted, antibody can be produced during the induction of tolerance. They detected a transient production of antibody in newborn rabbits injected with large amounts of S.paratyphi B which was followed by a tolerant state. Plaque forming cells (PFC) to SRBC were found during the induction of tolerance in newborn rabbits and piglets by Sterzl (1966), who suggested that the presence of antigen in excess caused an exhaustive differentiation of short-lived antibody producing cells, leading to transient production of antibody early in the induction of tolerance.

A similar transient appearance of antibody was also found in adult rabbits receiving repeated large doses of BSA by Dixon and Maurer (1955), and Weigle et al (1972) detected transient PFC during early induction of tolerance to BSA in

adult rabbits also.

Weigle et al (1972) stated that exhaustive differentiation during induction of tolerance occurs only in adult animals or when a poor tolerogen is used, since no antibody producing cells were detected in neonatal rabbits nor in adult mice receiving respectively BSA or a tolerogenic form of HGG.

These results suggest that during the induction of immunological tolerance some cells may become immune, but the majority of lymphoid cells able to respond become unresponsive. Tolerance would thus be a dynamic state, produced despite antibody production and not because of antibody production. However, Rowley and Fitch (1965) reported finding transient antibody in rats receiving repeated injections of SRBC from birth, but suggested that the role played by early antibody during induction of tolerance was that of feedback inhibition, resulting in a direct suppression of immunocompetent cells.

It must also be noted that in some cases (Crowle & Hu, 1969; Tong & Boose, 1970) tolerance to protein antigens (HSA or chicken ovalbumin and BGG, respectively) has been passively transferred with serum to normal recipient mice. This indicates that at least in some systems circulating antibody plays an active role in the manifestation of tolerance.

2. Antibody Blocking Lymphocyte Access to the Target Cell.

In some recently reported investigations of tolerance to histocompatibility antigens, where there was a failure of the host to reject allografts or tumours, antibody seemed to play an important role. Hellström and Hellström (1969) demonstrated that in mice with Moloney-virus-induced sarcoma, although the growth of the tumour was accompanied by a cell-mediated anti-tumour immunity, a serum factor was also produced that prevented tumour cell destruction by sensitized lymphocytes. Similarly it has been claimed that mice made neonatally tolerant

to allografts and later accepting skin-grafts from the donor strain, develop cell-mediated immunity to the target cell as shown by cytotoxicity in vitro, but also have circulating antibody that inhibits in vitro target cell destruction by sensitized lymphocytes (Hellström, Hellström & Allison, 1971). In the same way, lymphocytes from tetraparental mice (produced by fusing blastocytes of two strains of mice at the 8-cell stage) have been found to be cytotoxic for cells from both parental strains; and again their serum specifically blocks this effect (Wegmann, Hellström & Hellström, 1971). Lymphocytes from tetraparental mice also show reactivity in mixed culture with lymphocytes of either parental strain, and this reaction is blocked by serum from the tetraparental strain but not by other sera (Philips, Martin, Shaw & Wegmann, 1971). Similarly Micklem (1971) observed that when mouse lymphocytes are washed and then resuspended in saline with autologous erythrocytes, they form "rosettes" and that these rosettes are abolished if autologous serum is added. Thus in these cases "tolerance" appears to be due to the presence of blocking serum antibody rather than to specific unresponsiveness. In the case of failure to reject experimental tumours, antigen-antibody complexes have also been implicated as serum factors (Sjögren et al, 1971), binding to either the target cell or to the sensitized lymphocyte, in either case blocking the lymphocyte reaction.

E) Recovery from Immunological Tolerance

As has already been discussed in this "Introduction", both thymus and bone marrow cells are involved in the induction and maintenance of immunological unresponsiveness. In the termination of tolerance, whether spontaneously occurring or following immunization with cross-reacting antigens, co-operation between the two lymphoid cells also has an important role to play.

Thus rabbits made tolerant to native BSA at birth are completely unresponsive

when tested at 90 days. Their unresponsiveness can nevertheless be abrogated,as judged by immune elimination, by injection of aqueous solutions of heterologous albumins (Weigle, 1961) or of altered BSA molecules (Weigle, 1962). Furthermore such rabbits produce antibody which is qualitatively the same as the antibody produced in normal animals after injection of the cross-reacting antigen (Benjamin & Weigle, 1970a). Two months after the termination of unresponsiveness in this way, further antibody production can be stimulated by injection of native BSA (Benjamin & Weigle, 1970b). Nevertheless the antibody thus produced is directed only at the determinants on the BSA molecule which are related to those on the cross-reacting albumin used to terminate the tolerant state; but after several injections of BSA the unresponsive state is again expressed (Weigle, 1964).

These results are in perfect accordance with those of the experiments on the kinetics of induction of tolerance to deaggregated HGG in T and B cells already described. Thus when the rabbits made tolerant at birth were reinjected at 90 days of age with cross-reacting albumin, the tolerant state of the B cells was already lost and only the thymus cells remained tolerant. The unrelated determinants on the cross-reacting antigen could however react with non-tolerant thymus-derived cells with specific receptors for them, and these T cells could then stimulate the bone marrow derived cells which had by now recovered responsiveness to the related determinants. Therefore the thymus and bone marrow derived lymphocytes were reacting with different antigenic determinants as suggested by Rajewsky et al (1969) and Mitchison (1969). A humoral response to determinants common to BSA and the cross-reacting albumin was therefore produced and a new population of bone marrow derived cells responsive to BSA (memory cells) was recruited. It was these cells which were responsible for the subsequent production of antibody to an injection of native BSA. As the thymus-derived cells were still tolerant to native BSA, however,

further injections of BSA could not lead to recruitment of new B cell precursors reactive to determinants on native BSA shared with the cross-reacting albumin, with which the initial tolerance had been terminated, so that the supply of bone marrow derived cells carrying memory for shared determinants eventually became exhausted, and a state of full tolerance was restored.

Therefore ability of cross-reactive antigens to terminate tolerance depends on whether reactive B lymphocytes are present. These are present throughout in low dose tolerance, but in high dose tolerance only re-appear a long time after the tolerogenic injection, when the B cells have lost their unresponsiveness (Weigle, 1961, 1962; Benjamin & Weigle, 1970a,b). A similar explanation accounts for the results of experiments reported by Paul et al (1969) and Mitchison (1971), in which a low dose tolerance induced to BSA was abrogated by injection of DNP-BSA, whereas high dose tolerance was not. Unresponsiveness in T cells with responsiveness in B cells would also explain the normal response to DNP following immunization with DNP-horse gamma globulin of adult rabbits rendered tolerant by neonatal injections of DNP-BSA (Weigle, 1965a).

F) Unresponsiveness to Self Antigens

The experiments on acquired tolerance which have been discussed in the foregoing pages, lead to conclusions about the nature of tolerance which enable postulation of the probable mechanisms involved in unresponsiveness to self-antigens.

Due to the multitude of different self-antigens, whose physicochemical characteristics and concentrations are so enormously varied, more than one mechanism has to be invoked.

Thymus-dependent lymphocytes probably play a key role in prevention of autoimmunity, not only because being easily made tolerant they do not readily collaborate with responsive bone marrow derived cells that may be present, but also

because it appears from the experiments of Gershon and Kondo already discussed that they can exert specific "feedback" control of immune responses. This argument has been developed by Allison, Denman and Barnes (1971) and Allison (1971). When this controlling function of thymus-dependent lymphocytes is bypassed by exposure to virus infection, haptens and drugs, adjuvants or allogeneic cells (Allison et al, 1971) bone marrow cells can be stimulated. The presence of B-cells that can respond to self antigens when properly stimulated has been demonstrated by the formation of autoantibodies following immunization by host tissues with complete Freund's adjuvant (Glynn & Holborow, 1965).

Four postulates arising from the results of different workers have been formulated by Allison (1971):

1. "A lymphocyte cannot mount an immune response against antigenic determinants on its own membranes". Thus if immunoglobulins, which react against such determinants are formed during generation of diversity, the clones that possess these determinants are either eliminated or become unable to react to the corresponding antigenic stimulus. This postulate will not be further considered here.
2. "Soluble self-antigens presented to lymphocytes continuously in low dose induce unresponsiveness in T cells but not in B cells". Thus if stimulation of the T cells can be bypassed, the existing reactive B cells will embark on an immune response. A self-antigen which provides an example is thyroglobulin to which antibody can be easily produced by injections of either heterologous (Weigle & Nakamura, 1967) or altered homologous thyroglobulin (Weigle, 1965b), and both processes can lead to production of autoimmune thyroiditis (Nakamura & Weigle, 1969). It may be that the ease with which experimental autoimmune thyroiditis, aspermatogenesis, uveitis, encephalomyelitis and certain types of glomerulonephritis,

are induced, is also a result of lack of unresponsiveness in the bone marrow cells to soluble self-antigens present in insufficient concentrations in the body fluids to render B cells tolerant (Weigle, 1971). This could be the case in rheumatic fever, where antibody to heart antigens cross-reacting with streptococcal antigens is found. Peripheral blood lymphocytes of these patients are stimulated by streptococcal but not by heart antigens (McLaughlin, Paterson & Wilkes, quoted by Weigle, 1971), suggesting that despite antibody production the T lymphocytes of these patients are tolerant to heart antigens, and that it is the cross reactivity of streptococcal antigen that bypasses the tolerance of these cells, and leads to stimulation of the B cells.

3. "Soluble self-antigens presented to lymphocytes in high dose induce unresponsiveness in both T and B cells". This will be the case with most serum proteins as is suggested by the high dose tolerance induction experiments of Chiller et al (1971) and Mitchison (1971). Therefore individuals congenitally lacking one serum protein should have lymphocytes capable of reacting with that protein whereas normal individuals should not, and in fact mice lacking the fifth component of complement are able to make antibody against it (Herzenberg et al, 1963).

The ease with which autoantibodies to immunoglobulins can be induced may be due to the fact that there are so many individual immunoglobulin molecular variants that some of them may fall into the low dose range, so that potentially reactive B cell are still present and can react if the T cell tolerance is bypassed, or T cell function impaired. Either of these explanations could be proposed to account for the enhancement of antiglobulin antibody production in rheumatoid arthritis.

4. "Organ-specific cell membrane antigens not present in lymphocytes are tolerated because of blocking factors or selective feedback control". This postulate has strong support from the target-cell blocking experiments already referred to (Hellström et al, 1971; Philips et al, 1971; Wegmann et al, 1971) in the section "The Role of Antibody in Immunological Tolerance" (p.25) and also from the observation of Micklem (1971) that the "rosettes" formed when washed lymphocytes of mice are resuspended with autologous erythrocytes in saline, are abolished by the addition of autologous serum.

PART II

A NEW APPROACH TO THE STUDY OF B-CELL FUNCTION

The Localization of Aggregated HGG in Splenic Germinal Centres as a Model for Studying the Function of the Bone-Marrow-Derived Lymphocyte.

Localization of injected antigen in germinal centres of lymphoid tissue is associated and coincides with the appearance of specific antibody in the serum (Humphrey & Frank, 1967; Hanna, Francis & Peters, 1968). In the germinal centres, antigen assumes a characteristic dendritic intercellular distribution in close relation with the reticular cells in these areas (Mitchell & Abbot, 1965). It has been shown that in normal unprimed animals injected soluble immune-complexes are similarly localized within a few hours of injection (Lang & Ada, 1967; Herd & Ada, 1969) and that antigen uncomplexed with antibody does not localize in unprimed animals (Lang & Ada, 1967) or in tolerant ones (Humphrey & Frank, 1967). Therefore the localization of antigen in germinal centres of lymphoid tissue is antibody dependent. In the case of flagellin, which localizes in germinal centres of normal rats Lang and Ada (1967) invoked naturally occurring opsonizing antibody as an explanation.

Earlier work in this Laboratory (Brown, Schwab & Holborow, 1970b) showed that HGG altered by mild heat aggregation (Agg HGG) localizes in the same way, within 6 hours of its intradermal injection into normal guinea pigs, in the germinal

centre areas of the draining lymphatic nodes,and immunofluorescence showed the same dendritic pattern as that seen with antigen-antibody complexes. Nevertheless the localization of Agg HGG in germinal centres was not dependent on the presence of antibody, since it was seen in guinea pigs rendered specifically tolerant to HGG. Such localization in the germinal centres was not seen in animals given native monomeric heterologous IgG, for example, native human gamma globulin, neither was it seen after injection of other macromolecular or particulate substances such as aggregated HSA, colloidal carbon or streptococcal cell walls (Brown et al, 1970b). Monomeric rabbit IgG anti-HSA alone did not localize, but when combined with HSA in antigen excess, the resulting soluble immune complexes localized in germinal centres (Brown et al, 1970b).

It was therefore concluded that mild heat aggregation of HGG, or combination of antigen with heterologous antibody (HSA + rabbit anti HSA) caused an alteration of the immunoglobulin molecules, exposing sites hitherto unexposed in the same way as autologous IgG when complexed with antigen. As Fc fragments of IgG have been shown to localize similarly in the germinal centres (Herd & Ada, 1969) it was suggested that the sites exposed by heat aggregation or antigen combination are on the Fc portion of the molecule.

It was later shown that similar localization of Agg HGG occurred almost immediately in the splenic germinal centres of mice after i.v. injection (Brown, de Jesus, Holborow & Harris, 1970a).

When normal mouse spleen sections are treated with fluorescein labelled anti-mouse Ig, the following areas are identifiable (Fig. 1): The red pulp where only plasma cells are stained; the mantle zone of the white pulp, where the lymphoid cells show surface Ig staining; a dark unstained periarteriolar area within the

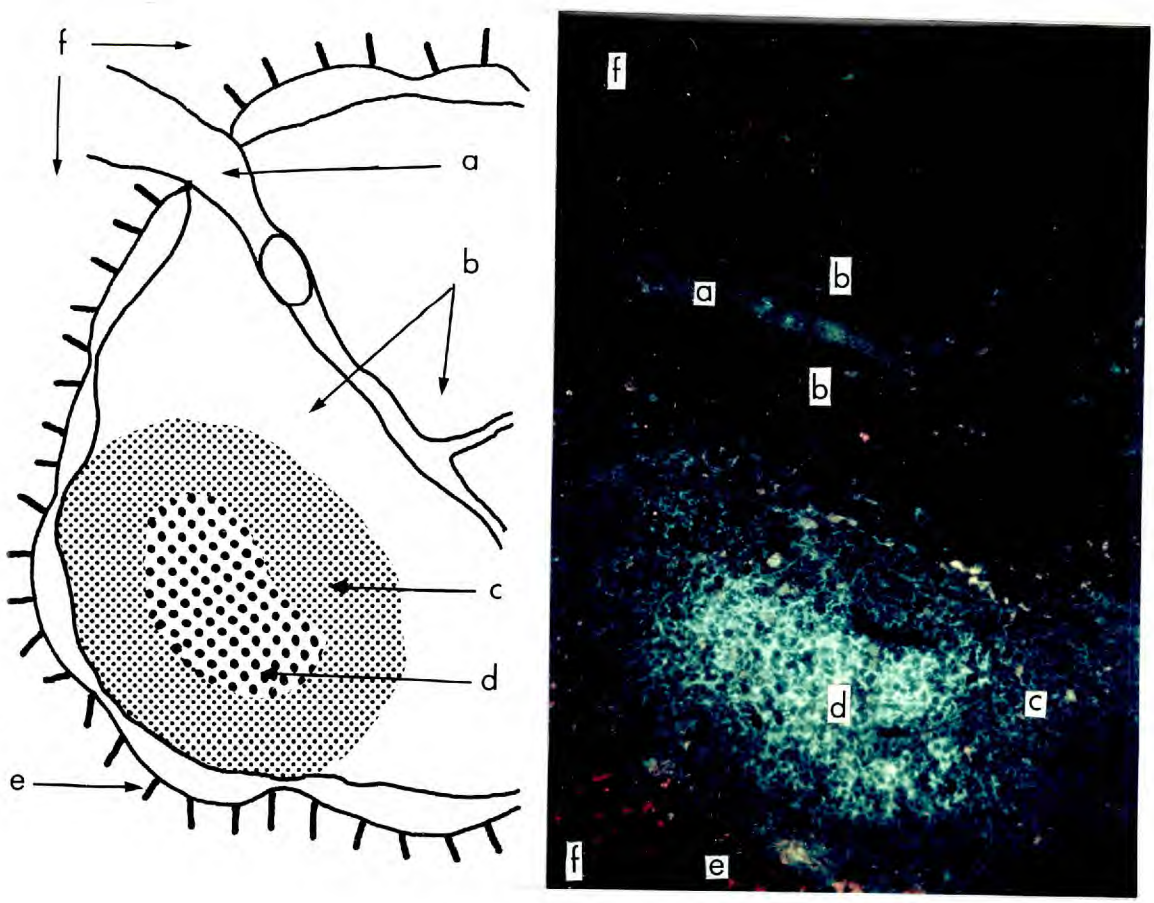


Fig. 1: Cryostat sections of normal mouse spleen stained with fluorescein labelled anti-mouse IgG. x40

a - Central arteriola

b - Periarteriolar lymphocyte sheath (thymus-dependent area)

c - Mantle zone

d - Germinal centre

e - Marginal zone

f - Red pulp

white pulp (the area populated by thymus dependent lymphocytes - Parrot, de Sousa & East, 1966), and the germinal centre with a dendritic pattern of staining. It is in this last area that immune complexes and aggregated IgG localize (Fig. 2).

Injected Agg HGG localizes in the spleen in two distinct ways. Studies to be reported (Brown, de Jesus, Holborow) have shown that within minutes of intravenous injection HGG can be seen within macrophages in the red pulp and in the marginal sinuses (Fig. 3,4). The macrophages are identifiable by uptake of previously i.v. injected carbon (Fig. 4). HGG is also seen outside the carbon-laden cells on the surface of other cells (Fig. 4). As time progresses the macrophage-ingested aggregated is catabolized and is no longer stainable, and a second pattern of staining gradually condenses to form the dendritic pattern within the germinal centres (Fig. 5,6,7). This pattern is almost completed by 6 hr and persists intact for several weeks after injection.

Because lymphocytes are cells that migrate from the blood to the splenic white pulp (Ford & Gowans, 1967) it was thought that these cells might be involved in the mechanism whereby Agg HGG reached germinal centres. Supporting evidence was provided by the fact that mouse lymphocytes from peripheral blood and spleen cells incubated "in vitro" with Agg HGG, retained HGG on their surface as judged by immunofluorescence, and when cells thus treated were i.v. injected into normal mice a dendritic pattern of localization was detected by immunofluorescence in the splenic germinal centres(Brown et al, 1970a). Furthermore when spleens were teased into single cell suspension 24 hr after i.v. injection of Agg HGG, and stained for HGG,fluorescent staining of the membranes of some cells was seen. In order to quantitate localization, Agg HGG was labelled with ^{125}I and spleens of mice receiving this material i.v. were counted in a scintillation counter. A good correlation was found between spleen counts and intensity of fluorescent staining of germinal centres for HGG. When mice were treated with a potent gamma G

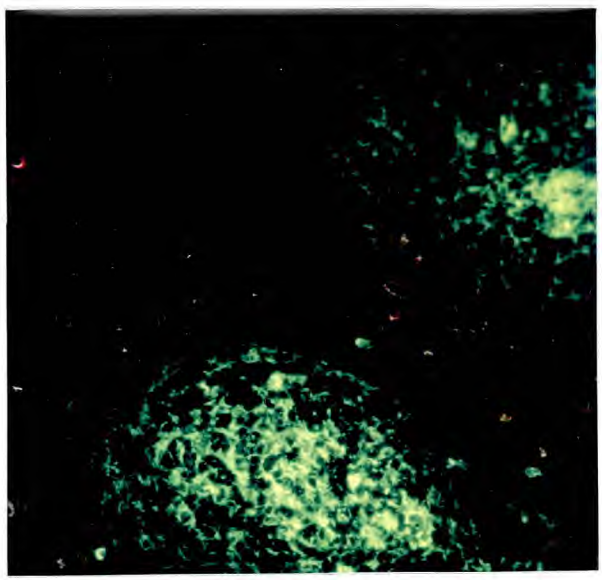


Fig. 2: Cryostat section of mouse spleen, 24 hr after i.v. injection of 75 µg Agg HGG, stained with fluorescein conjugated anti-human gamma globulin and showing typical dendritic pattern in two germinal centres in the white pulp. x40

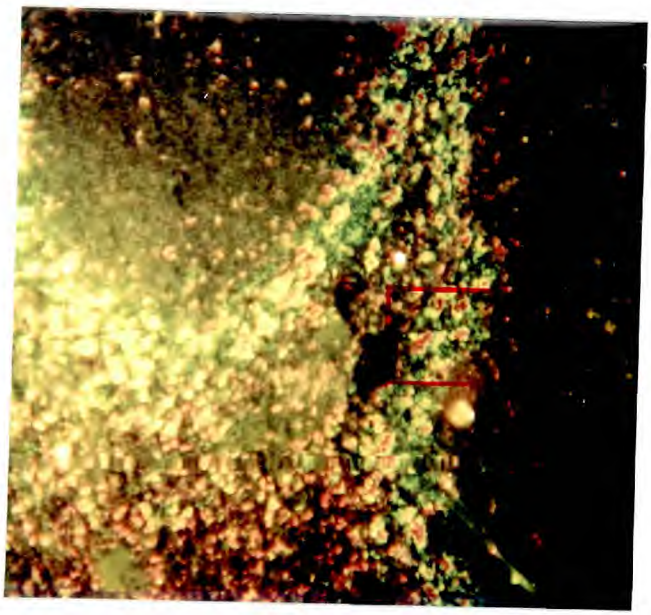


Fig. 3: Cryostat section of spleen of a mouse preinjected with carbon, removed 5 min after i.v. injection of 50 µg Agg HGG, and stained with fluorescein conjugated anti-HGG. Many macrophages are present in the red pulp and in the marginal sinuses and are identifiable by the red autofluorescence of carbon. Specific staining of HGG is seen within macrophages and around other cells in the same areas. x40

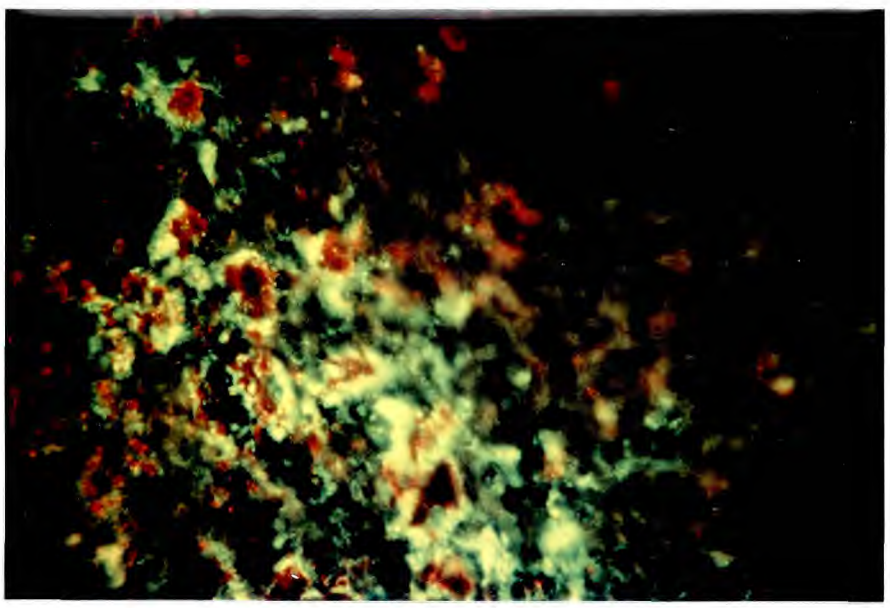


Fig. 4: Enlargement of the marginal zone area outlined in Fig. 3. On the right, the unstained white pulp; in the centre and to the left, HGG can be seen within macrophages and also apparently on the surface of other cells. x200

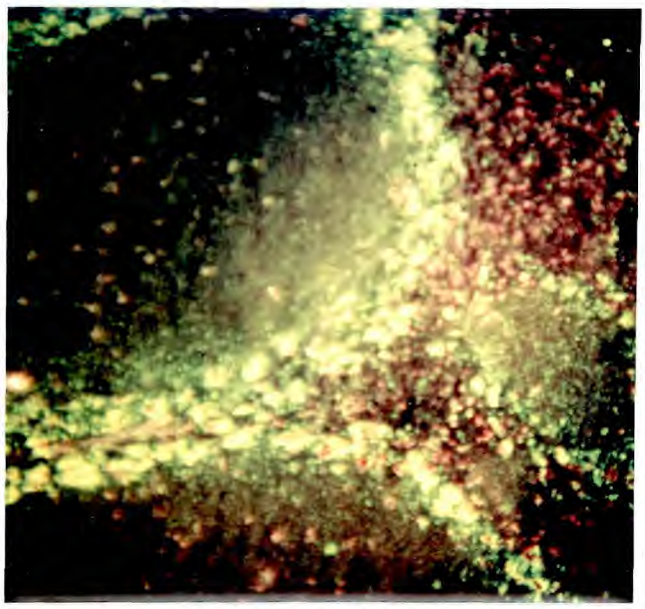


Fig. 5

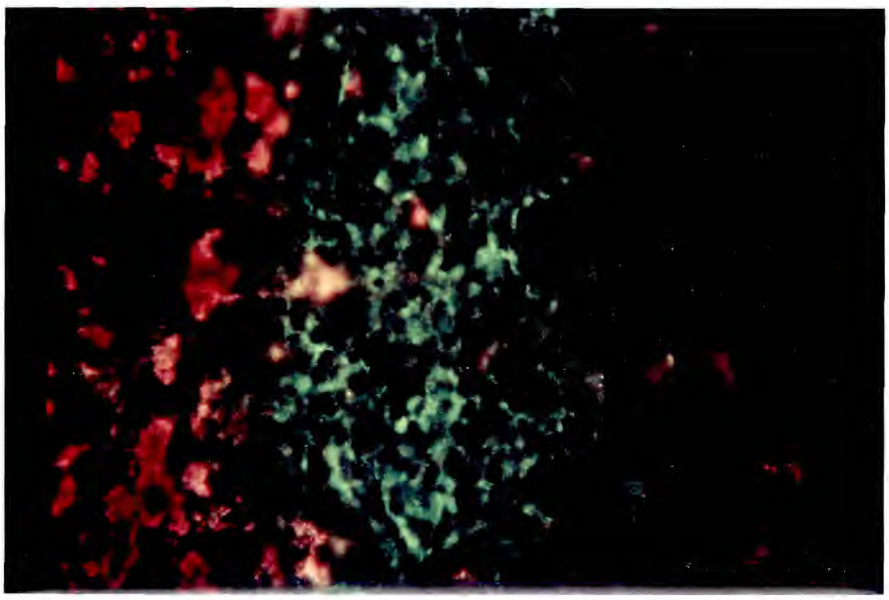


Fig. 6

Fig. 5.6: Cryostat sectionsof spleens of carbon preinjected mice, removed at various times after the i.v. of 50 µg Agg HGG and stained with fluorescein conjugated anti-HGG. Fig. 5: 30 min after the Agg HGG injection. Red pulp almost free of HGG, which is now concentrated in the marginal zone and just beginning to move into the white pulp. x40. Fig. 6: 6 hr after the Agg HGG injection; red pulp completely free of HGG, which is now only seen inside the white pulp forming a dendritic intercellular pattern in the mantle zone. x200

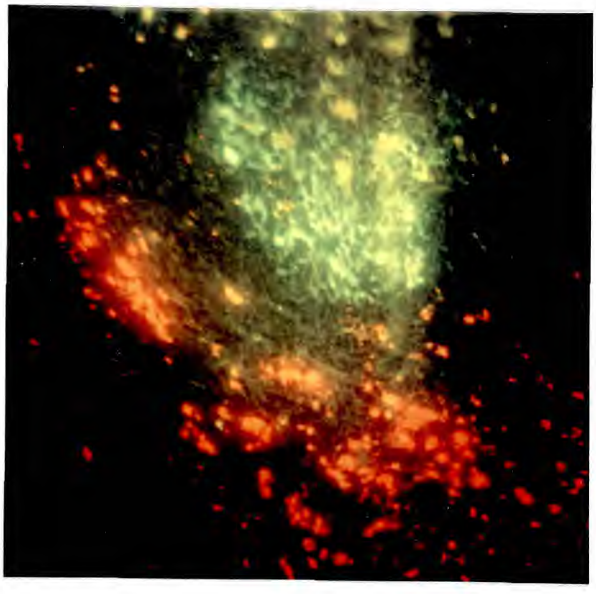


Fig. 7: Cryostat section of spleen of carbon preinjected mouse, removed at 24 hr after the i.v. of 50 µg Agg HGG and stained with fluorescein conjugated anti-HGG; typical dendritic pattern of staining of the HGG in the germinal centre of the white pulp. x40

fraction of a rabbit antimouse lymphocyte serum (ALG) 3 hrs before an i.v. dose of ^{125}I-Agg HGG, a marked reduction of uptake was found (Brown et al, 1970a). Impairment of ability to localize HGG in the spleen was dependent on the ALG dose injected and lasted for about 48 hrs after administration of 5 mg. All these results indicate that the localization of Agg HGG is dependant on lymphocytes that have receptors for altered HGG on their surface.

Greenwood, Brown, de Jesus and Holborow (1971a) found that mice infected with murine malaria are, at the height of their parasitaemia, unable to localize Agg HGG or their own immune complexes in their splenic germinal centres,and that during this period they also show humoral immunosuppression. Their cell mediated immune responses remain unaffected (Greenwood, Playfair & Torrigiani, 1971b). These findings suggest that the lymphocytes implicated in Agg HGG uptake are bone marrow derived. This was also indicated by the fact that despite big doses of ALG injected, the germinal centre uptake of ^{125}I-labelled Agg HGG, as measured by splenic retention, was never completely abolished and reduction in uptake lasted only a short time, returning completely to normal 48 hrs after the last ALG injection (Brown, de Jesus, Holborow, unpublished observations). This would be expected if the cells involved are rapidly replaced, as B cells are known to be. It was later shown that thymectomized adult mice chronically treated with horse antilymphocytic serum (ALS) and tested ten days after the last ALS injection showed localization and uptake of Agg HGG to a similar degree as thymectomized mice chronically treated with normal horse serum, or normal mice (de Jesus, Holborow & Brown, 1972). In thymectomized, lethally X-irradiated, bone marrow reconstituted mice there was no defect in localization of Agg HGG in splenic germinal centres as compared with sham-thymectomized, lethally X-irradiated

bone marrow reconstituted mice (de Jesus, et al 1972). Indeed in both cases the thymus deprived mice had a slightly higher retention in their spleens. It was shown by Davies (1969) that thymectomized lethally X-irradiated bone marrow reconstituted mice have only the thymus independent zones of their lymphoid tissues repopulated, and by chromosome marker studies the repopulating cells were proved to be descendants of the donor bone marrow cells.

Therefore the results with thymus cell deficient mice point to the conclusion that the localization of Agg HGG and of autologous immune complexes in germinal centres depends upon uptake by bone-marrow-derived lymphocytes. Thus the uptake of Agg HGG into germinal centres provides a very useful tool for the study of the function and movement of B lymphocytes.

PART III

OBJECT OF THE PRESENT STUDY

It is obvious from the work described in the Introduction that T cells play a highly important role in the induction of tolerance, and there is evidence to suggest that so far as suppression of the humoral response is concerned, their action is mediated through production of a factor which prevents the necessary co-operation between T and B cells. Where this factor acts at the cellular level has not been defined.

It was hoped in the present work to obtain further evidence of the existence of this T cell factor, and to gain insight into its mechanism of action by studying the effect of tolerance upon a function of B cells which is independent of the immune response, i.e. the uptake of Agg HGG into germinal centres. To achieve this, several experiments were planned as follows.

Firstly to study quantitatively the uptake and persistence of Agg HGG in splenic germinal centres of i.v. injected normal mice subsequently developing an immune response. Secondly to carry out a similar study in mice tolerant to HGG, and to test whether absence of an immune response alters the splenic uptake of Agg HGG and its subsequent handling. Subsequent experiments were designed to analyse the implications of the findings for humoral and cellular mechanisms of tolerance.

PART IV: MATERIALS AND METHODS

PART IV

MATERIALS AND METHODS

Mice

In most of the experiments inbred BALB/c mice from the MRC Taplow colony were used; however, in a few experiments, inbred CBA mice from the same colony were used. Mice of both sexes and a wide range of ages were examined.

Phosphate buffer saline, pH 8.0 (PBS)

This buffer was obtained by adding 4.72 litres of 0.15M Na_2PO_4 in 0.85% NaCl to 0.28 litres of 0.15M KH_2PO_4 in 0.85% NaCl, and the pH adjusted by dropwise addition of either the alkaline or acid phosphate solution as necessary, under constant pH measurement.

Preparation of aggregated human gamma globulin (Agg HGG)

Heat aggregated complexes of human gamma globulin (HGG) were prepared as previously described (Greenwood et al, 1971a). A 2% solution of Cohn fraction II HGG (Koch-Light Ltd., Colnbrook, Bucks) in PBS was heated at 63°C for 15 minutes, cooled to room temperature and centrifuged at 3500 rev/min (2300 g) for 15 min to remove large aggregates and insoluble particles. The supernatant solution was then ultracentrifuged at 40,000 rev/min (143,000g) at 20°C for 90 min in a Spinco Model L. No. 40 rotor, the supernatant discarded and the pellet redissolved in the minimum amount of PBS. The protein concentration

of this solution was read by absorption spectrophotometry at 280 mμ. This Agg HGG thus prepared was used for immunizing mice to HGG.

Preparation of monomeric human gamma globulin (Mon HGG)

A 10% solution of HGG in PBS pH 8.0 was ultracentrifuged as above. The top two-thirds of the supernatant was carefully aspirated and the protein concentration read as indicated.

Electrophoresis on cellulose acetate membrane showed that the Mon HGG preparation was predominantly a population of molecules with the same mobility (Fig. 8). An immunoelectrophoresis developed with either sheep anti-whole human serum (Wellcome Reagents Ltd.) or sheep anti-human IgG (Wellcome Reagents Ltd.) a single arc of precipitation was seen (Fig. 9). This Mon HGG solution was used for the induction of tolerance.

Iodination of aggregated and monomeric human gamma globulin (^{125}I-Agg HGG; ^{125}I-Mon HGG)

The proteins prepared as above were labelled with ^{125}I using the method of McFarlane (1958) as follows:

Reagents:

- Carrier free ^{125}I (sodium iodide in dilute NaOH solution, pH 8-11, free from reducing agent = IMS.30; the Radiochemical Centre, Amersham).
- 8:2 alkaline glycine buffer (8 ml M glycine in M/4 NaCl + 2 ml N NaOH).
- 9:1 alkaline glycine buffer (9 ml M glycine in M/4 NaCl + 1 ml N NaOH).
- Iodine monochloride solution containing 0.42 mg I/ml

Stock solution: 150 mg NaI and 108 mg $NaIO_3, H_2O$ was dissolved in 2.5 ml of water, to which 2.4 ml of concentrated HCl (specific gravity 1.18) was added dropwise shaking continuously; 1 ml CCl_4 and one drop (0.035 ml) M/10 NaI was then added to produce a faint pink colour, and water added to

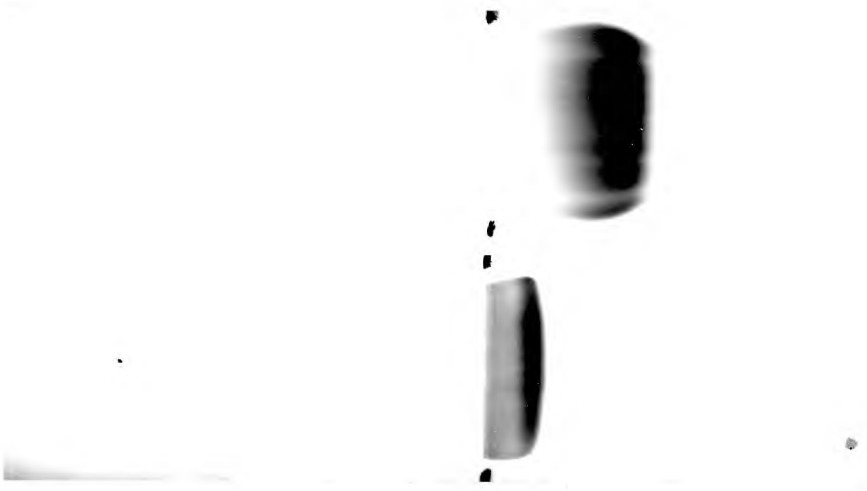


Fig. 8: Electrophoresis on cellulose acetate membrane of Mon HGG above, and Agg HGG below, stained with Ponceau S. The Mon HGG shows uniform mobility towards the cathode; the Agg HGG has hardly moved from the origin.

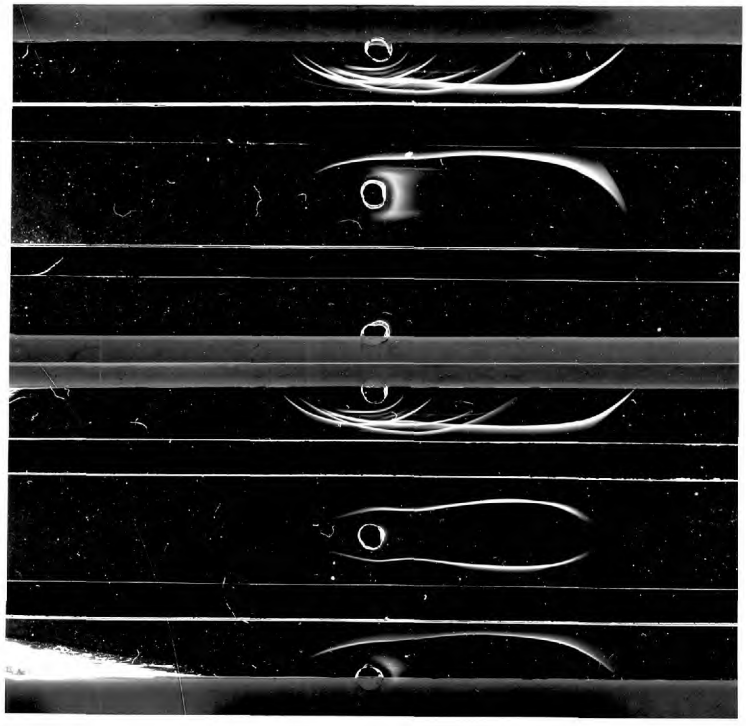


Fig. 9: Immunoelectrophoresis in agar

Troughs (from the top)
1. Sheep anti-whole human serum
2. Sheep anti-human IgM antiserum
3. Sheep anti-whole human serum
4. Sheep anti-human IgG antiserum

Wells (from the top)
1. Normal human serum (neat)
2. Monomeric HGG (8 mg/ml)
3. Human IgG (Cohn fraction II; 20 mg/ml)
4. Normal human serum (neat)
5. Human IgG (Cohn fraction II; 20 mg/ml)
6. Monomeric HGG (8 mg/ml)

The Monomeric HGG gives a single line only with anti-whole human serum and with anti-human IgG

bring the total volume to 10 ml; 1 ml CCl_4 was further added.

Working solution: 1 ml of the stock solution was mixed with 22.85 ml of 2M NaCl and 2.06 ml N NaOH and water added to a total volume of 48.75 ml.

Method:

The protein solution was adjusted to pH 9-9.5 with 8:2 alkaline glycine buffer. The amount of ^{125}I required to give a final activity of approximately 33 μCi/mg of protein was buffered at pH 9.0 by the addition of 9:1 alkaline glycine buffer and then a volume of the iodine monochloride working solution was added to give approximately one atom of iodine per molecule of protein, assuming 100% incorporation. This solution was shaken, and the protein solution rapidly squirted into it. After standing for a few minutes, the mixture was passed through a deacidite column ("Isopor" resin, deacidite FF-IP (SRA 65), anion exchange resin, the Permutit Company Ltd, London W.4.) of about 5 cm x 0.5 cm equilibrated in PBS, to remove non-protein bound iodine. The effluent iodinated protein solution was pooled according with the radioactivity counts and fractionated with sodium sulphate.

A solution of 2.18M sodium sulphate was added dropwise to the solution of HGG at room temperature with constant stirring, to achieve a molarity of 0.2M and then stirred for a further 30 min. The precipitate formed was removed by centrifugation at 3500 rev/min (2300g) for 30 min and discarded. The supernatant was then treated in the same way to bring the molarity to 0.4M sodium sulphate and the precipitate formed was collected after centrifugation in the same way, and dissolved in the smallest possible volume of PBS and dialysed against PBS until free of sodium sulphate. Studies to be reported (Brown, de Jesus & Holborow) showed that the best localization of Agg HGG in splenic germinal centres, as shown both by radioactivity measurements and

immunofluorescent staining, was achieved with the 0.4M sodium sulphate fraction. This fraction will be referred to as ^{125}I-Agg HGG.

In the case of monomeric human IgG (Mon HGG), the solution after labelling with ^{125}I was treated by dropwise addition of 2.18M sodium sulphate under continuous stirring and at room temperature, to bring the molarity to 0.62M sodium sulphate. This mixture was stirred for a further 30 min, centrifuged at 3500 rev/min (2300g) for 30 min and the precipitate discarded. The supernatant solution was dialysed against PBS until sulphate free. The fraction thus obtained was shown not to localize in the splenic germinal centres and will be referred to as ^{125}I-Mon HGG.

Potassium iodide drinking water

When whole body counts of the mice were required after injection of iodinated protein, thyroid uptake of the ^{125}I freed during catabolism was prevented by administration of drinking water containing 2% potassium iodide (KI drinking water) for at least 7 days before the injection of the radioactive protein and during the remaining time of the experiment (Sweet et al, 1965).

Radioactivity estimation

In experiments where whole body counts were necessary, the measurements of radioactivity were done on living mice in a modified Panax gamma one-sixty scintillation counter modified for this purpose. The apparatus incorporates a 4" x $4\frac{1}{2}$" NaI well crystal, and has provision for automatic counting of 20 mice. Where whole body counts were not required the organs and blood were counted in a crystal well scintillation detector used in conjunction with a Tracerlab spectometer.

Collection of blood and organs

Twenty four hours after injection of ^{125}I-Agg HGG mice were anaesthetized with ether, killed by exsanguination following cardiac puncture, and the spleen

and a sample of liver removed and weighed. Studies to be reported (Brown, de Jesus & Holborow) have shown that at doses up to 100 µg catabolic events are largely completed by 24 hrs, and that maximal uptake in the splenic germinal centres of normal mice has occurred by this time. The spleens were then snap-frozen in isopentane (2-Methylbutane, BDH Chemicals Ltd, Poole) at the temperature of liquid nitrogen and kept at -70°C for further studies by immunofluorescence, on cryostat section obtained from the snap-frozen blocks.

Immunofluorescent spleen staining

Cryostat sections of mouse spleens of 6 µ thickness were dried under a fan and stained with specific fluorescein conjugated sheep or rabbit antisera raised against HGG (Fig. 2) (Wellcome Reagents Ltd.) or against mouse gamma globulin (Fig. 1) (Nordic Pharmaceuticals and Diagnostics).

The anti-HGG conjugate was used at a dilution which gave no staining of normal uninjected spleen sections, and the anti-mouse IgG at dilution that gave no background staining in the red pulp of the spleen.

Staining for both human and mouse IgG was inhibited by absorbing the conjugates with purified HGG or mouse IgG, respectively. The specificity of these conjugates was further checked by showing that pretreating mouse spleen sections with unconjugated anti-HGG or anti-mouse IgG antisera blocked subsequent staining by the conjugates. Pretreatment with antisera of other specificities did not block.

The sections were examined in a Reichert Fluorpan binocular microscope, using an iodine quartz IZV lamp, a primary filter of interference (Balzer FITC-3) with maximum transmission at 495 nm and cut off at 500 nm, a barrier filter passing wavelengths above 505 nm, a dark-ground high power cardioid condenser, a 10 x and 40 x objectives (the latter with NA 0.95) and 6 x compensated eyepieces.

Induction of tolerance to HGG

Tolerance was achieved in BALB/c and CBA mice that received i.p. 7 mg of Mon HGG on the day of birth, and again at 1, 3 and 6 weeks, and subsequently at monthly intervals to a total of seven injections. Two to four weeks after the last tolerogenic injection, mice were used either as donors of cells or for study of the localization of ^{125}I-Agg HGG. Both BALB/c and CBA mice were used. Tolerance was demonstrated for a sample of each group thus treated by the absence of immune elimination of ^{125}I-Mon HGG as follows. Ten days to six weeks after the last tolerogenic injection mice of each strain were thyroid blocked and given i.p.an immunogenic dose of 500 µg Agg HGG. One week later a 10-50 µg i.v. tracer dose of ^{125}I-Mon HGG was given. Whole body counts were done immediately, and during the five following days and compared with the counts obtained in normal mice of the same strain and age that were given the same immunizing treatment. All mice treated with Mon HGG in this way proved to be tolerant.

Thymectomy

This operation was carried out on mice 6-8 weeks old under Nembutal anaesthesia (60 µg/gr of weight) (Abbot Laboratories Ltd, Agro-Vet Division, Queenborough, Kent) by sucking out the thymus through a midline incision just above the manubrium. When the mice were killed at the end of the experiments, they were checked macroscopically for absence of thymus.

Irradiation

Ten days to one month after thymectomy whole body X-irradiation was done in a Siemens 240 Kv, 10mA X-ray machine. Each BALB/c mouse received 850 r in about 22 min, 39.4 r/min, focal distance 50 cm, 1 mm Cu filter. CBA mice received 1000 r in about 26 min.

Reconstitution of irradiated mice

Within 6 hr of irradiation mice were reconstituted with bone marrow cells, either alone or with thymus cells, from tolerant syngeneic or from normal syngeneic donors according to the plan of each experiment.

Bone marrow cells were obtained by flushing out the femoral marrow with medium 199 (BDH Chemicals Ltd) containing 10% of foetal calf serum (FCS) (Tissue Culture Service) and added penicillin (20,000 U/100 ml) and streptomycin sulphate (10 mg/100 ml). The bone marrow cells were washed once and counted in a haemocytometer.

The thymuses were squeezed through a metal mesh (mesh, 0.5 mm) using the same medium, washed twice and counted.

Viability of the cells to be injected was tested by the trypan blue method.

To prevent infections all mice to be irradiated were given antibiotic in their drinking water (Neomycin sulphate 1.8 mg/ml and Polymixin B sulphate 0.15 mg/ml) for five days before irradiation, and continued on it for 10 days after irradiation. They also received i.p. 1 mg of ampicillin 3 hr before and 6 hr after irradiation. The ampicillin injection was repeated once a day for the 7 following days. This antibiotic regime was instituted because of the high number of deaths occurred in the earlier experiments where it was omitted, although no specific pathogens were found in cultures of blood, spleen, gut and lung obtained from moribund unprotected mice.

PART V: EXPERIMENTAL WORK AND DISCUSSION OF RESULTS

PART V

EXPERIMENTAL WORK AND DISCUSSION OF RESULTS

1) Quantitative Localization of ^{125}I-Agg HGG in the Mouse Spleen over a 4-Week Period after a Single i.v. Injection

The first experiments were designed to determine how long Agg HGG persisted in germinal centres and what part, if any, the immune response plays in its removal.

A. Localization in Normal Mice

36 BALB/c mice of both sexes, and 6 months old were given 2% KI in their drinking water for 10 days before the experiment was begun and for its duration, in order to prevent uptake by the thyroid of the radioactive iodine freed by catabolism of the injected labelled HGG (Sweet et al, 1965). This was necessary as whole body counts as well as organ counts were to be measured.

The mice were then injected in the tail vein with 50 µg ^{125}I-Agg HGG (equivalent to 15 x 10^3 c.p.s.) on day 0 and whole body counts were done immediately in the Panax gamma well counter. Subsequent counts were done daily for the first 9 days and then at 2, 3 and 4 weeks. Three mice were killed at each time interval. Blood, spleen, liver, thymus and lymph nodes were counted, and organs snap frozen for immunofluorescence studies.

It has previously been shown that there is a good correlation between the intensity of immunofluorescent staining for HGG in germinal centres and the number of counts in the spleen (Brown et al, 1970a).

Results: From the whole body counts and the counts of the daily samples of blood, it was found that immune elimination of HGG remaining in the circulation started on the 4th day (Fig. 10). After correction of the splenic counts for natural decay of ^{125}I it was found that a small but significant portion of the ^{125}I-Agg HGG injected, localized in the splenic germinal centres, where it persisted during the whole four weeks of the experiment, although there was a gradual decline of radioactivity and of corresponding intensity of the immunofluorescence as seen with specific anti-HGG conjugate (Fig. 11). It is interesting to note also that there was a marked fall in the splenic counts from 180 c.p.s./100 mg of tissue to 90 c.p.s./100 mg of tissue (Fig. 11) between the 3rd and 4th day after the i.v. injection. This halving of the spleen counts antedated the onset of the maximum immune elimination rate by one day.

Comments: The marked decrease in the splenic retention that occurred between the 3rd and 4th day could be due to local production of antibody in the white pulp and elimination of the deposited HGG through immune complex formation and phagocytosis. Such local production is suggested by the experiments of Sordat et al (1970) who demonstrated antibody within the lymphoid cells of developing germinal centres by using high-resolution electron microscopy to study horse radish peroxidase (HRP)-anti-HRP reactions.

The next experiment was therefore designed to assess effects due to antibody by using mice previously rendered tolerant to HGG. This was expected to provide additional evidence that localization of Agg HGG in germinal centres is independent of the immune response.

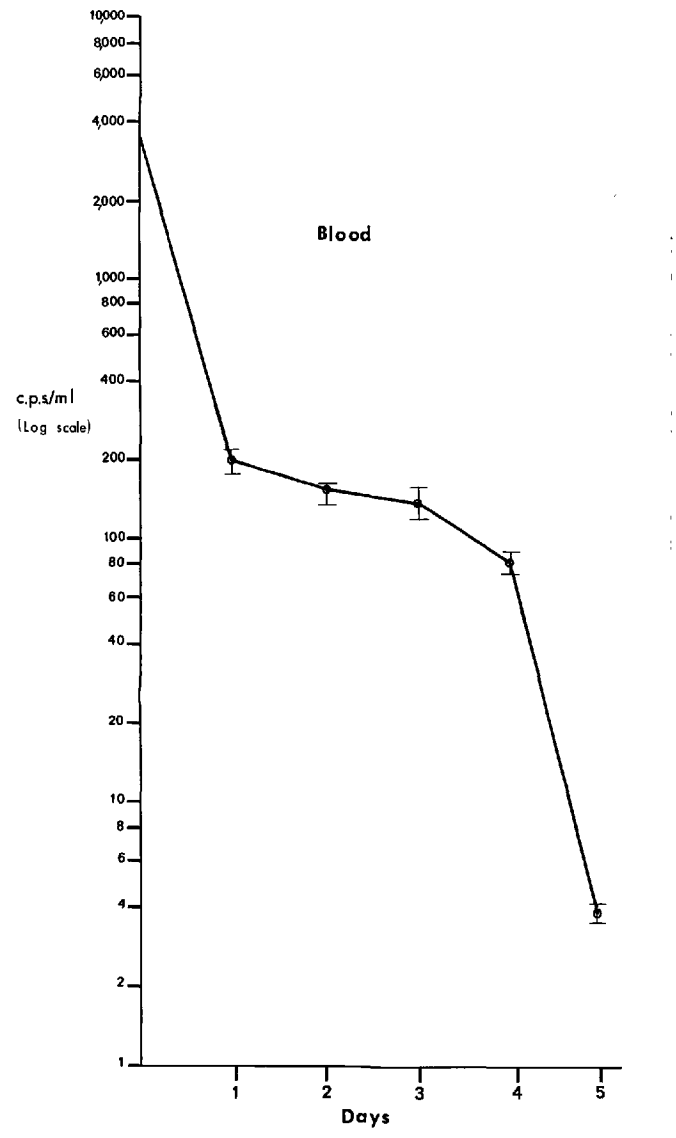


Fig. 10: Mice injected i.v. with 50 µg of ^{125}I-Agg HGG. Each point represents the mean count in blood samples from three mice taken at daily intervals. After the marked initial fall due to catabolism elimination and uptake in the splenic germinal centres, immune elimination of the remaining HGG in the circulation begins on days 3-4.

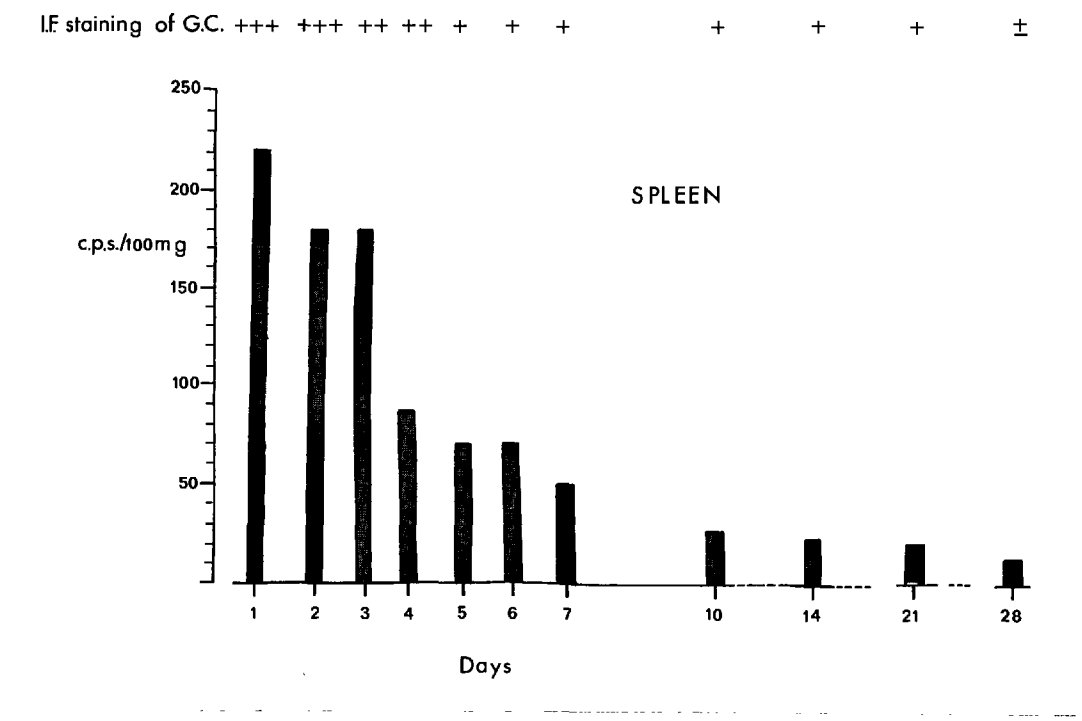


Fig. 11: Mean uptake of Agg HGG in spleens taken at various times after i.v. injection of 50 µg ^{125}I-Agg HGG. Results expressed as c.p.s./100 mg of tissue and the corresponding intensity of immunofluorescent staining of germinal centres with anti-HGG. Note that halving of the c.p.s./100 mg of tissue occurs between day 3 and 4, antedating the onset of maximum immune elimination in the blood on day 4 (Fig. 10)

B. Localization in Mice Tolerant to HGG

BALB/c mice were injected with Mon HGG from birth as described in Materials and Methods, and at 3 months they were tested for tolerance to HGG as follows: Thirteen mice that had received injections of Mon HGG and 6 normal BALB/c mice of the same age were given 500 µg of Agg HGG i.p. On the same day they were started on KI in their drinking water for thyroid blockade. Seven days later (Day 0) they received i.p. 10 µg of ^{125}I-Mon HGG as a tracer dose and whole body counts were done immediately and then daily for nine days. The daily counts were expressed as percentages of the individual whole body counts recorded on day 0 immediately after the i.p. injection of ^{125}I-Mon HGG. Table 1 and Fig. 12 show that mice that received Mon HGG from birth in this way were tolerant as judged by absence of an immune pattern of elimination of antigen.

Thyroid blocked BALB/c mice, thirty three tolerant and 33 normal,were given 50 µg of ^{125}I-Agg HGG (equivalent to 15 x 10^3c.p.s.) i.v. on day 0 and whole body counts done immediately. Subsequent counts were done daily for the first 7 days and then at 10, 14, 21 and 28 days. Three mice were killed at each time interval, blood, spleen and liver were counted, and spleens were snap-frozen for immunofluorescence study.

Results and comments

As before, mice pretreated with repeated Mon HGG injections were in fact tolerant as shown by the absence of an immune elimination in whole body or blood counts (Fig. 13, 14).

a) Presence of HGG in germinal centres of mice made tolerant by injection of Mon HGG

Tolerant mice not injected with Agg HGG showed weak but definite staining of splenic germinal centres for HGG and they also appeared to show increased intensity

Table I: Tests of tolerance in mice receiving Mon HGG from birth.*

No. of mice per group	Treatment	MEAN WHOLE BODY COUNTS**								
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
13	Mon HGG from birth	93.28 (82.5/100)	77.57 (70.38/91.32)	66.48 (60.66/81.80)	59.25 (52.54/72.59)	53.45 (47.02/67.11)	44.55 (37.56/58.26)	39.64 (32.53/47.88)	33.41 (26.91/46.34)	21.45 (17.49/26.16)
6	None	1.40 (0.89/1.87)	0.50 (0.35/0.58)	0.40 (0.33/0.48)	0.36 (0.32/0.41)	0.14 (0.07/0.23)	-	-	-	-

*All mice were thyroid blocked and received i.p. 500 µg of Agg HGG as an immunogenic dose. Seven days later (Day 0) they were injected i.v. with 10 µg of ^{125}I-Mon HGG as a tracer dose for immune elimination.

**Mean (and range) of daily whole body counts expressed as % of initial individual whole body counts.

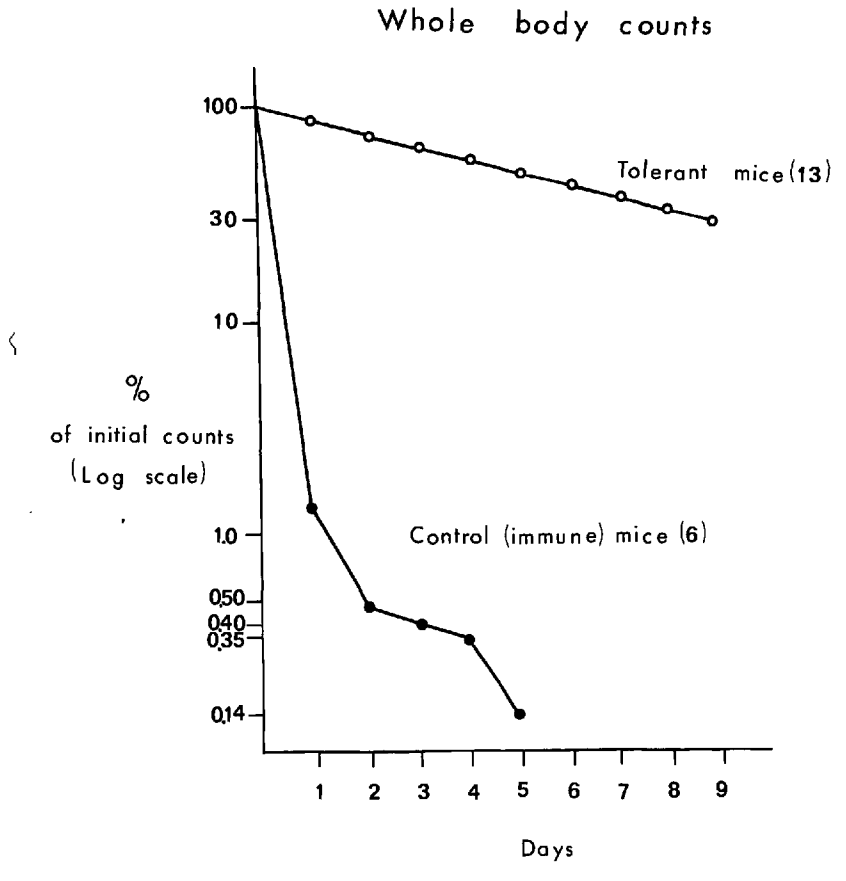


Fig. 12: Tolerant and immune mice injected i.p. with 10 µg ^{125}I-Mon HGG. Mean whole body counts expressed as percentages of counts on day 0 immediately after the ^{125}I-Mon HGG injection. Thirteen tolerant and six control mice received i.p. an immunogenic dose of 500 µg of Agg HGG seven days before the tracer dose of ^{125}I-Mon HGG. Immune elimination present in the control mice but not in the tolerant ones.

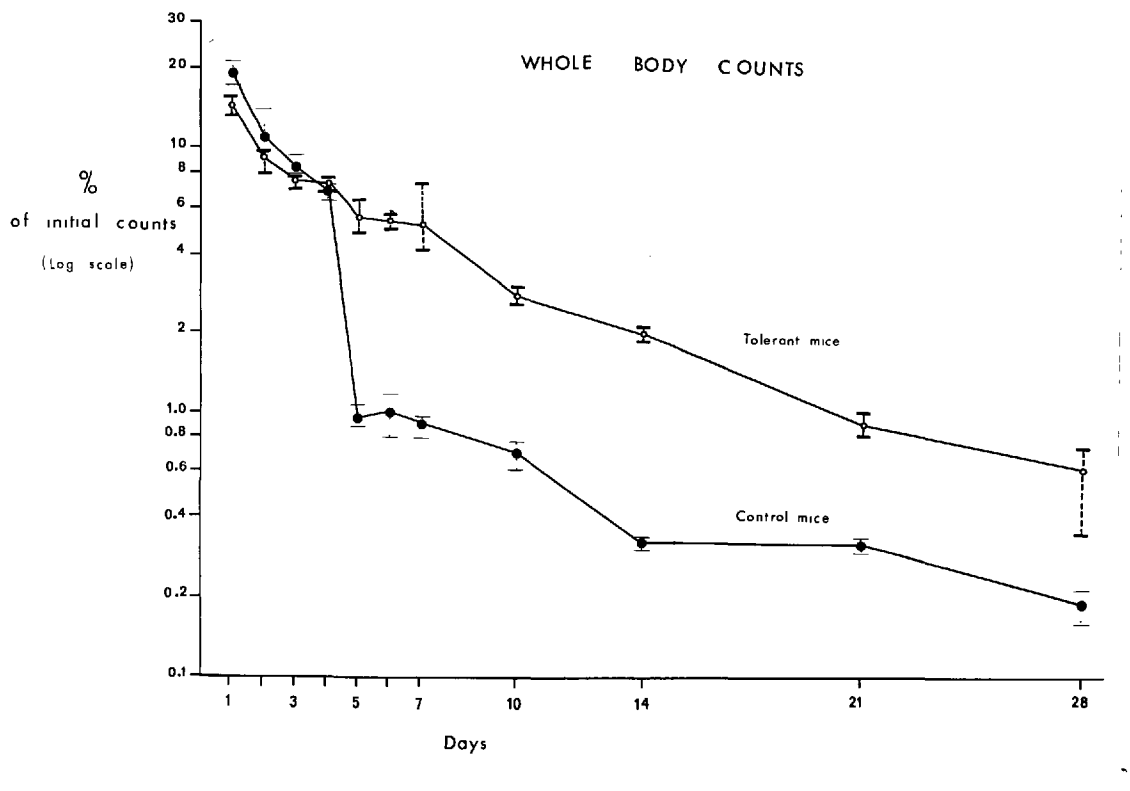


Fig. 13: Whole body counts in tolerant and control mice at various intervals after i.v. single injection of 50 µg ^{125}I-Agg HGG. Each point (mean of 3 mice) is expressed as a percentage of the mean initial count on day 0. Note between days 4-5 a marked fall in the whole body counts of control mice coinciding with the peak of immune elimination from the blood in these mice (Fig. 14)

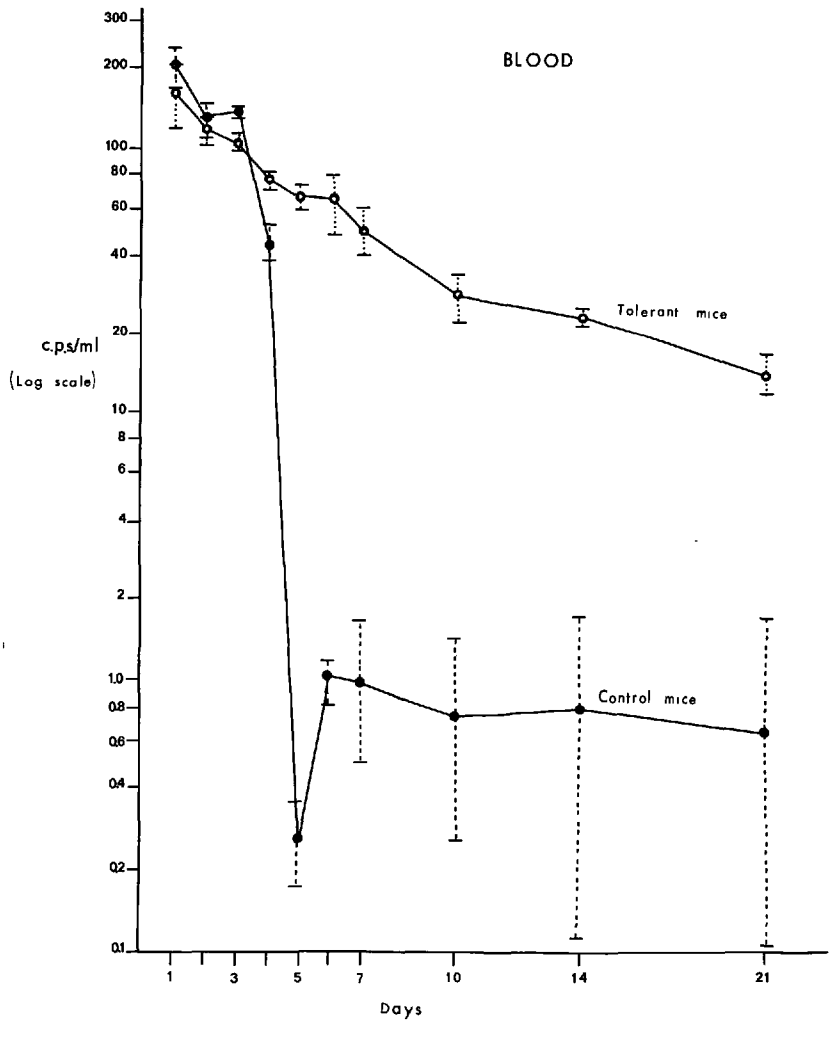


Fig. 14: Counts (c.p.s./ml) in blood from tolerant and control mice at various times after an i.v. injection of 50 µg ^{125}I-Agg HGG. Immune elimination started between days 3-4 in control mice, reaching its maximum on the following day.

of germinal centres staining for mouse IgG. It had to be concluded that although they had received only Mon HGG, a very small amount of this HGG had found its way to germinal centre areas even in tolerant mice.

It is known from previous work that localization of antigen in germinal centres occurs only in the presence of specific antibody (Humphrey & Frank, 1967; Lang & Ada, 1967; Hanna, Francis & Peters, 1968; Herd & Ada, 1969), with the exception of immunoglobulin antigens, such as HGG, which when altered by aggregation localize in germinal centres in normal animals (Brown et al, 1970b). There are thus three possible explanations for the appearance of HGG in germinal centres in the tolerant mice in this experiment before they had received Agg HGG:

1) There was some contamination of the Mon preparation with Agg. This seems unlikely since on electrophoresis on cellulose acetate the Mon HGG used for induction of tolerance showed only one component which ran cathodally, indicating a degree of homogeneity of the HGG molecules. Nevertheless very minute amounts of aggregated HGG, undetectable by this method, could have been present in the Mon HGG preparation and the additive effect of repeated injections could have led to depositions of a detectable amount in the germinal centres.

2) Some aggregated HGG was produced "in vivo" from the injected Mon HGG during the process of catabolism, and it is this aggregated material that is transported into germinal centres. This possibility seems to be remote since in other experiments not reported here no mouse IgG was found in the germinal centres of adult C_3H germ free mice, as judged by immunofluorescence. If some aggregated (denatured) IgG were formed during normal catabolic processing, these mice also should have some detectable mouse IgG in their germinal centres.

Nonetheless catabolic handling of autologous IgG could be different from that of heterologous native IgG (Mon HGG) and some alteration of the molecule might occur in the latter.

3) The third possibility is the in vivo formation of immune complexes. As Humphrey has said (1969), folicular localization is probably the most sensitive test available for the presence of antibody in an animal, and as already discussed (p. 24, 55) some authors have been able to detect antibody production at an early stage of induction of tolerance, and in fact the tolerant mice in the present experiment showed increased staining for mouse IgG in their splenic germinal centres. The antigenic stimulus for this production of antibody could be aggregated HGG originated as in 1) and 2) above or the Mon HGG itself. However formed, this antibody would have been only transiently produced by a few cells at an early stage of tolerance induction, since there was no immune elimination when the mice were tested.

The lack of persistence of any antibody produced early in induction of tolerance is also shown by the following experiment. Two tolerant and two normal mice were injected i.v. with 200 µg ^{125}I-Mon HGG and immune elimination from the blood and raised retention in the spleen were looked for. As Table II shows, neither was found, and it was concluded that in tolerant mice at the time of testing there was no detectable antibody reactive either with Agg HGG or with Mon HGG.

Table II:	24 hrs after injection	
Mice	BLOOD cps/ml	SPLEEN cps
Tolerant	2 501.5	106.5
	2 943.5	113.8
Normal	3 010.4	90.4
	2 837.7	110.2

Thus it is difficult to decide which is the most likely explanation for the appearance of small amounts of HGG in splenic germinal centres in tolerant mice, and perhaps all three mechanisms suggested may play a part.

However it may be inferred that in the presence of circulating monomeric IgG,altered IgG in germinal centres does not stimulate sustained antibody production. In the same way, in normal animals the presence of altered IgG in the germinal centres of the lymphoid tissues does not, in the presence of circulating native IgG, lead to production of antiglobulin auto-antibodies.

b) Measurement of splenic uptake of injected ^{125}I-Agg HGG by tolerant mice

Splenic uptake of ^{125}I-Agg HGG in tolerant mice was consistently only 50-60% of that in normal mice (Table III), although on immunofluorescence, staining of the splenic germinal centres for HGG appeared to be of the same intensity as in normal mice receiving Agg HGG. This lack of correspondence between radioactivity and immunofluorescent intensity is probably accounted for by the fact that, as described above, some HGG was already deposited in the splenic germinal centres of tolerant mice before any labelled Agg HGG was injected. This difference between normal and tolerant mice persisted; when splenic retention was measured for a period of 4 weeks from initial injection, the difference between normal and tolerant mice was consistently maintained from the beginning (Fig. 15).

It should be noted in Table III that a halving of spleen counts occurred between days 4 and 5 in the tolerant as well as in the normal mice. A similar fall was observed in normal mouse in the previous experiment (p. 55) between the 3rd and 4th day, and local production of antibody was invoked to explain it. It must therefore be concluded that in tolerant mice there is also local production of a small amount of antibody in germinal centres, after localization of the Agg HGG, which nevertheless does not interfere with the systemic expression of tolerance (compare

Table III: Retention of ^{125}I-Agg HGG in blood and spleens of tolerant and normal mice measured during a period of 4 weeks from injection.

No. of days after injection	SPLEEN retention*		Blood retention*	
	Tolerant mice	Normal mice	Tolerant mice	Normal mice
1	38.8(34.42/43.09)	64.94(49.01/73.16)	163.80(118.06/213.02)	205.95(174.83/235.92)
2	22.93(18.62/25.51)	45.72(44.22/46.71)	122.11(111.16/131.42)	132.49(108.99/147.38)
3	16.83(15.65/17.65)	40.74(36.98/43.36)	105.98(97.95/111.36)	139.65(135.30/142.96)
4	18.70(18.0/19.72)	43.36(37.43/50.0)	77.37(72.50/83.50)	47.09(39.43/52.96)
5	7.31(5.03/8.87)	21.38(18.06/25.04)	67.18(59.81/71.88)	1.26(1.17/1.35)
6	7.48(6.56/7.96)	16.79(12.08/20.21)	66.61(46.98/80.21)	1.74(0.09/2.60)
7	5.76(4.60/7.70)	20.68(17.23/26.07)	48.25(41.57/59.90)	1.06(0.96/1.69)
10	3.12(2.60/3.44)	10.39(6.08/13.32)	29.38(20.27/34.19)	0.76(0.26/1.49)
14	2.32(1.98/2.65)	5.54(3.66/7.05)	23.54(22.93/24.00)	0.80(0.65/1.76)
21	0.89(0.09/2.59)	3.30(2.44/3.88)	14.73(13.07/17.18)	0.65(0.25/1.71)
28	0.78(0.52/1.14)	2.79(1.96/3.32)	5.42(4.14/6.16)	0.34(0.16/0.52)

* Mean and range ( ) of three animals/day expressed as cps/spleen or ml of blood

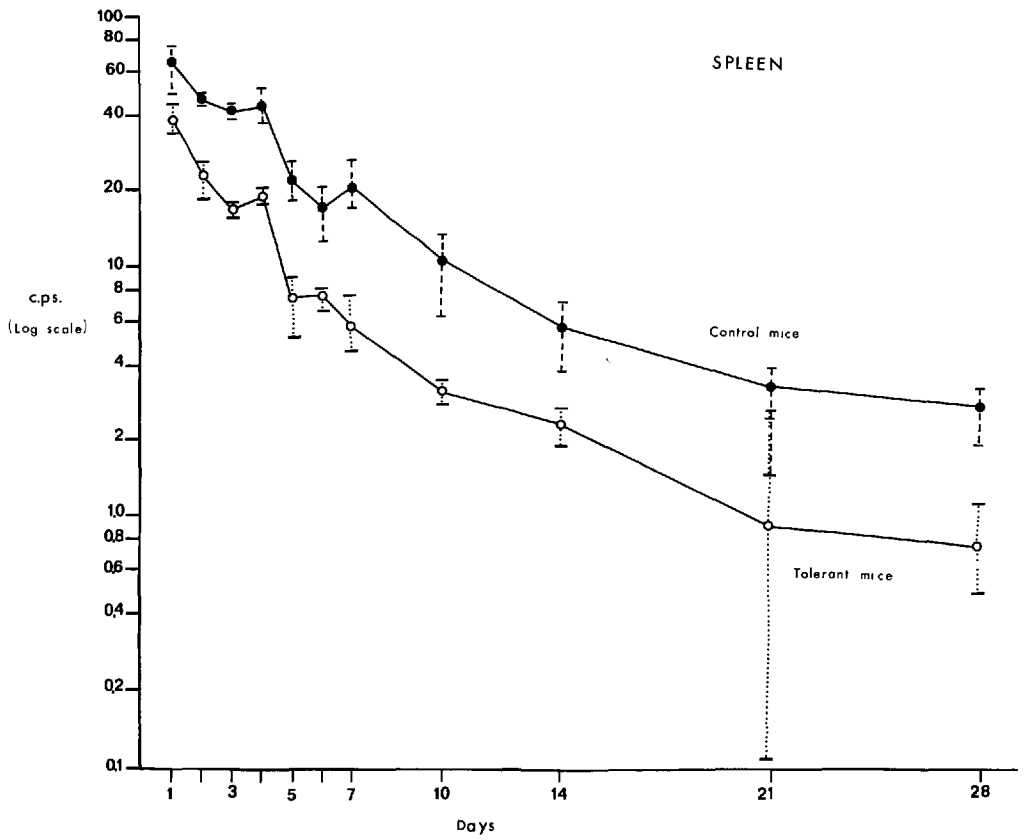


Fig. 15: Splenic retention of ^{125}I-Agg HGG in tolerant and control mice at various intervals after one i.v. injection of 50 µg on day 0, expressed as c.p.s. per spleen. Each point is the mean of three spleens. Note that the initial reduction of splenic uptake in the tolerant mice is consistently maintained from the beginning.

blood and spleen counts of tolerant and normal mice on days 4 and 5 in Table III).

2) Decreased Splenic Uptake of ^{125}I-Agg HGG in Tolerant Mice

Since it is known that the localization of Agg HGG in splenic germinal centres results from a cellular mode of transport (Brown et al, 1970; Greenwood et al, 1971a) the results of this experiment suggest that in tolerant mice this cellular function is in some way impaired. The crucial question that arises is whether this impairment reflects an alteration in the transporting cells themselves or the appearance of a humoral factor. If the latter, does it act upon the injected Agg HGG or upon the cells that transport it to germinal centres?

A. Is a humoral factor responsible for decreased germinal centres localization of ^{125}I-Agg HGG in tolerant mice?

A humoral factor reactive with Agg HGG might make it, or part of it, unrecognisable by the receptors on the lymphoid cell that are responsible for its uptake or it could act directly on the cells impairing their function. In either cases the final result would be a decrease in the splenic retention of Agg HGG or immune complexes in tolerant mice.

To test for the presence of such a humoral factor responsible for this impairment in tolerant mice the following experiment was carried out.

0.7 ml of pooled serum obtained from either normal or tolerant BALB/c mice, that had received their last tolerogenic dose of Mon HGG two weeks before the collection of serum, was incubated at room temperature for $\frac{1}{2}$ hr with 75 µg ^{125}I-Agg HGG. This mixture was then injected i.v. by the tail vein into normal BALB/c mice. Three 5 months old BALB/c mice received the mixture prepared with tolerant serum and three other mice of the same age the one prepared with normal serum. A third group of BALB/c mice received i.v. 0.7 ml of a solution of PBS containing 75 µg ^{125}I-Agg HGG. The mice were killed 24 hours after injection.

Blood, spleen and liver were counted for retention of ^{125}I-Agg HGG and spleens were snap-frozen for immunofluorescent studies.

Results: (See Table IV) No difference in the splenic localization of ^{125}I-Agg HGG was found between groups, and when cryostat sections were stained with anti-HGG conjugate a uniform strong staining (+++) was seen in all of them.

Table IV: Effect of tolerant serum on the splenic localization of 75 µg ^{125}I-Agg HGG in normal mice.

Mouse No.	Treatment	Spleen*		
		cps	mean	G.C.staining for HGG
1	0.7 ml PBS	56.1		+++
2	"	60.6	(59.6)	+++
3	"	62.1		+++
4	0.7 ml normal mouse serum	79.4		+++
5	"	72.3	(77.2)	+++
6	"	80.0		+++
7	0.7 ml serum from tolerant mouse	62.0		+++
8	"	64.3	(62.0)	+++
9	"	59.0		+++

*Individual spleen counts/second and mean ( ). G.C. = germinal centre

Comments: If in tolerant mice there is a factor reactive with Agg HGG which is responsible for the reduction of localization of ^{125}I-Agg HGG in the splenic germinal centres it is not readily demonstrable by serum transfer. However it could be that this factor is present in serum only in extremely small amounts or is only produced during stimulation by antigen. Thus if serum were taken from tolerant mice at 24 hrs after ^{125}I-Agg HGG injection and transferred to normal mice, it might be possible to demonstrate the factor by producing a decrease in

splenic localization of ^{125}I-Agg HGG. To test this possibility the next experiment was carried out.

Six tolerant and six normal CBA mice, 3 months old, were injected i.v. with 50 µg of ^{125}I-Agg HGG and killed 24 hrs later. Blood and spleen were counted and the serum separated from the pooled bloods. The tolerant spleens showed a retention of 72% of the normal retention of ^{125}I-Agg HGG (mean normal splenic retention = 78.63 c.p.s.; mean tolerant splenic retention = 58.16 c.p.s.). 1 ml of pooled serum from the tolerant mice was injected i.p. into 2 normal CBA mice, 3 months old. Two other CBA mice received 1 ml of pooled serum from normal mice. Twenty four hrs after these serum injection, the mice received i.v. 50 µg of ^{125}I-Agg HGG, were killed the following day and their bloods, spleens and livers counted.

Results: (Table V) No difference in the splenic localization of ^{125}I-Agg HGG was found between the two groups

Table V: The effect of serum from tolerant mice 24 hrs after injection of ^{125}I-Agg HGG on the splenic localization of 50 µg ^{125}I-Agg HGG injected i.v. into normal mice.

Mice received	Spleen in c.p.s.
1 ml of normal mouse serum	84.6 83.8
1 ml of tolerant mouse serum	82.4 84.1

Comment: No humoral factor detectable by reduction of splenic localization of ^{125}I-Agg HGG is produced after injection of Agg HGG into tolerant mice.

The results of these two experiments show that it is not possible to transfer

with serum from tolerant mice any impairment of splenic localization of ^{125}I-Agg HGG, even with serum obtained 24 hrs after administration of antigen.

B. Cellular implications of decreased splenic localization of ^{125}I-Agg HGG in tolerant mice

Therefore, attempts to transfer by serum the factor responsible for decreased germinal centre localization of Agg HGG in tolerant mice failed, but it could be that this factor is not free in the serum since it might act on the lymphoid cells that transport Agg HGG into the splenic germinal centres by sticking on their surfaces, and thus interfering with their function. If its avidity for the cells were high, it might not be readily detectable in the serum.

It therefore had to be considered whether some inhibition of uptake of Agg HGG and immune complexes in germinal centres is occurring in tolerant mice at the cellular level. It is known from previous work (Brown et al, 1970a; Greenwood et al, 1971a) that this uptake is due to lymphocytes which have on their surfaces receptors for Agg HGG, and the results of these experiments in tolerant mice indicate clearly that HGG acting as antigen to induce tolerance, affects these receptors in some way. It has recently been shown that the cells responsible for the transport of ^{125}I-Agg HGG and immune complexes into splenic germinal centres are bone marrow dependent lymphocytes or B cells (de Jesus et al, 1972). It seems likely, therefore, that in mice tolerant to HGG, there is impairment of this function in B cells.

As already discussed in the Introduction, there is now considerable evidence that in tolerance (Chiller et al, 1970; Gershon & Kondo, 1971; Mitchison, 1971; Weigle, 1971; Weigle et al, 1972), as in the immune response (Claman et al, 1966; Miller & Mitchell, 1967) co-operation between thymus-dependent and bone marrow derived lymphocytes plays an important part (review in Miller et al, 1971). The question thus arises from the foregoing results whether the impairment of B cell

function demonstrated is due to some control exercised by T cells rather,than to a primary defect in the B cells themselves.

Gershon and Kondo (1970) have shown that if thymectomized lethally irradiated mice, reconstituted with syngeneic bone marrow and also given thymocytes are treated with repeated large doses of sheep red blood cells (SRBC), they are unable to respond to a subsequent immunizing injection of SRBC, even after inoculation of normal thymocytes. However mice pretreated in the same way, but not receiving thymocytes before the tolerising injections of SRBC,were able to respond almost as well as untreated controls to this antigen when it was subsequently given with normal thymocytes. Thus the presence of thymus-derived lymphocytes during the induction of tolerance had created a milieu in the experimental mice in which normal thymocytes were unable to overcome tolerance by cooperating with the pretreated bone marrow derived cells.

To investigate further why cooperation between these two types of cells was prevented in these circumstances, Gershon and Kondo (1971) transferred spleen cells of mice pretreated as above into thymectomized lethally irradiated recipients, and tested the ability of the transferred cells to cooperate with normal thymocytes in a new environment free of antigen or other potential immunosuppressive factors. They found that the spleen cells of mice made tolerant in the presence of thymocytes were not able to cooperate with normal thymocytes even in this new environment, and in fact prevented the collaboration of normal thymocytes and normal bone marrow derived cells injected into the recipients. To explain these results they favour the hypothesis that thymus derived cells of tolerant mice produce a substance, which they call IgY, that is responsible for the shut-off effect on collaboration between T and B cells which is necessary for an immune response.

The following experiments were therefore devised to test whether impairment

of localization of Agg HGG in germinal centres in tolerant mice could also be due to a factor produced by T cells affecting B cell function.

i) The role of "tolerant" T cells in decreasing splenic localization of ^{125}I-Agg HGG

Thymectomized lethally irradiated BALB/c mice were reconstituted within 6 hours of irradiation with 30×10^6 syngeneic thymus cells and 30×10^6 syngeneic bone marrow cells. Three groups of five mice received mixtures of cells as follows:

Group A - Received T & B cells from normal mice

" B - " T & B " " tolerant mice

" C - " T cells from tolerant mice (tolerant T cells) & normal B cells.

Eleven days after reconstitution the survivors were used to study the splenic localization of 50 µg ^{125}I-Agg HGG (equivalent to 15×10^3 c.p.s.) injected i.v. There were three survivors in group C and only one in group B. Since there were no survivors in group A three normal BALB/c mice of the same age had to be used as controls for splenic localization of the injected Agg HGG. The mice were killed 24 hrs after injection of ^{125}I-Agg HGG, blood, spleen and liver were counted for radioactivity and the spleens snap-frozen for immunofluorescent studies.

Results: In mice of group C, which received tolerant T cells and normal B cells, a mean reduction to 71% localization in the spleen was found (Table VI). The single mouse that received both T and B cells from tolerant donors showed a greater reduction (to 45% of normal).

The immunofluorescent staining with anti-HGG in the spleens was in accordance with the radioactivity counts.

Comment: It was previously found (p. 65) that tolerant mice showed a reduction to 50-60% in the capacity of their B cells to transport ^{125}I-Agg HGG into splenic germinal centres and although the results shown in Table VI lacked proper controls

Table VI: Effect of tolerant T cells on the B cell function of transporting ^{125}I-Agg HGG into the spleen.

Mice	Cells used in reconstitution	Spleen				
		Weight mg	cps*	cps/100 mg	%**	G.C. staining for HGG
Control	Normal mouse	165	82.7	50.1	-	+++
		145	77.7	53.6	-	+++
		135	80.1	59.3	-	+++
				(54.3)	100.00	
Group B	Tolerant T + tolerant B	130	32.0	24.6	45.30	Neg
Group C	Tolerant T + normal B	150	37.9	25.3	46.59	Neg
		120	54.0	45.0	82.87	+
		60	27.7	46.1	84.71	Neg
				(38.8)	(71.45)	++

*counts per second

**cps/100mg of tissue expressed as a percentage of the mean normal cps/100mg of tissue; G.C.: germinal centres; in brackets the mean values.

and included only a few mice, nevertheless mice that had received tolerant T cells and normal B cells showed a reduction of localization of ^{125}I-Agg HGG to 71% of normal. The reduction of splenic uptake of ^{125}I-Agg HGG in mice reconstituted with tolerant T cells and normal B cells supports the idea that T cells in tolerant animals produce a factor mediating tolerance (Gershon & Kondo, 1971). Since localization of Agg HGG in splenic germinal centres is a function of B cells (de Jesus et al, 1972), this result strongly suggests that the factor produced by tolerant T cells acts upon the B cells.

To increase the number of these observations and to test the role of tolerant B cells, the following experiment was performed.

ii) The role of tolerant B cells

Donors of cells were tolerant BALB/c mice as described, or normal BALB/c of the same age. Recipients were BALB/c thymectomized, lethally irradiated (as indicated in material and methods), and reconstituted not later than 6 hrs after irradiation with either 20×10^6 B cells alone or 17×10^6 T cells and 10×10^6 B cells obtained from normal or tolerant mice. The experimental groups were as follows (5 mice in each group):

Group A - Received normal B cells only

" B - " " T & normal B cells

" C - " tolerant T & normal B cells

" D - " " B cells only

" E - " " T & tolerant B cells

" F - " normal spleen cells

Three weeks after reconstitution the mice received i.v. 50 µg ^{125}I-Agg HGG (equivalent to 15×10^3 c.p.s.) and were killed 24 hrs later; the spleens were counted for retention of radioactivity and snap-frozen for immunofluorescent study. Controls were BALB/c mice of the same age, thymectomized, lethally irradiated and reconstituted with 100×10^6 spleen cells from normal BALB/c (Group F). All the mice in groups A, B and E died between the 5th and 7th day after reconstitution.

Results: (Table VII) Three weeks after reconstitution, mice that received T cells from tolerant donors with B cells from normal mice (Group C) showed as before a definite reduction of splenic localization of ^{125}I-Agg HGG as compared with recipients of normal spleen cells (Group F). Mice that had received only B cells from tolerant donors (Group D) showed a greater reduction. Again, immunofluorescent results (staining of germinal centres for HGG) agreed with the spleen radioactive counts.

Table VII: The effects of tolerant T and B cells on splenic localization of ^{125}I-Agg HGG.

Group	Cells used in reconstitution	Spleen			
		Weight in mg	cps*	%**	G.C.staining for HGG
F	100×10^6 normal spleen cells	145	56.0	-	+++
		115	44.6	-	+++
		130	49.4	-	+++
			mean(50.0)	100	
D	20×10^6 tolerant B cells	150	10.73	21.46	Neg
		140	8.20	16.40	Neg
		125	33.68	67.36	++
			mean(17.53)	mean(35.0)	
C	17×10^6 tolerant T cells + 10×10^6 normal B cells	145	35.95	71.9	++
		120	12.87	25.74	Neg
		145	38.50	77.0	++
		105	14.87	29.74	Neg
			mean(25.55)	mean(51.1)	

*counts/second

**c.p.s. in the spleen expressed as a percentage of the mean c.p.s. in the control group, i.e. that received normal spleen cells after lethal irradiation; G.C.: germinal centres.

Comment: The results in group C (mice reconstituted with tolerant T cells and normal B cells) again points to the conclusion that tolerant T cells are indeed responsible for producing a blocking factor that prevents normal B cells injected at the same time from performing normally their function of transporting ^{125}I-Agg HGG into splenic germinal centres.

The group D results (mice reconstituted only with tolerant B cells) show that B cells once made tolerant and thus deficient in their function of transporting ^{125}I-Agg

HGG into germinal centres, remain deficient even in the absence of tolerant T cells, for at least 3 weeks. The reason why these group D mice showed an even greater reduction of uptake than the group C mice may be that in their case the B cells they received were already deficient, while in group C the normal B cells transferred had to be subjected to the influence of the tolerant T cells.

3) The Role of T and B Cells in Transferring Tolerance

In the foregoing experiments the decreased splenic uptake of ^{125}I-Agg HGG in mice receiving cells from tolerant donors was attributed to the transfer of tolerance. The recipients however were not tested for absence of immune elimination or other immune response. The next experiment was therefore designed to test the state of tolerance of mice receiving different combination of tolerant and normal cells.

BALB/c mice were thymectomized, lethally irradiated and reconstituted, in groups of five mice each, according with the following scheme:

Group	Cells transferred	No. of cells
A	Normal B cells	18×10^6
B	" T & normal B cells	20×10^6T & 18×10^6B
C	Normal spleen cells	70×10^6
D	" T & tolerant B cells	20×10^6T & 24×10^6B
E	Tolerant T & normal B cells	14×10^6T & 18×10^6B
F	Tolerant T & tolerant B cells	14×10^6T & 24×10^6B
G	Tolerant B cells	24×10^6

Donors were tolerant BALB/c or normal BALB/c about 3 months old.

Three weeks after reconstitution all mice were injected i.p. with an immunogenic dose of 100 µg of Agg HGG and started on KI drinking water. One

week later they received i.p. 50 μg ^{125}I-Mon HGG (equivalent to 6.5 x 10^3 c.p.s.) as a tracer dose for immune elimination studies. Whole body counts were done immediately after injection of ^{125}I-Mon HGG and during the following 6 days. Three normal BALB/c of the same age and similarly given KI were included as controls for elimination of ^{125}I-Mon HGG, and received the same dose i.p. All mice continued to receive KI for the whole remaining time of the experiment, for thyroid blockade.

Results: (Table VIII and Fig. 16) As judged by the absence of immune elimination, tolerance was achieved in the recipient mice of either tolerant T + tolerant B cells (group F) or tolerant T cells + normal B cells (group E) or tolerant B cells alone (group G).

Mice that received normal T cells + tolerant B cells (group D) showed definite immune elimination although its onset was delayed by 3 or 4 days as compared with control groups (A, B and C) that received combination of normal cells.

Comment: Since HGG is known to be a thymus-dependent antigen (Davies, 1969), the fact that mice in group A (reconstituted with normal bone marrow cells) showed immune elimination is evidence that some T cells were present in the normal bone marrow transferred, despite the statement of Miller et al (1971) that the lymphocytes in thymectomized, lethally irradiated recipients of syngeneic bone marrow constitute a relatively pure population of B cells.

The eventual appearance of immune elimination in mice of group D (reconstituted with normal T cells and tolerant B cells) is evidence that new non-tolerant B cells were regenerated from the transferred bone marrow. The reason for the delayed onset of immune elimination in these mice could be that the mature

Table VIII: The role of tolerant T and B cells in transferring tolerance to thymectomized, lethally irradiated recipients as tested by the absence of immune elimination of ^{125}I-Mon HGG.

Group*	No. of mice	Cells transferred**	Whole body counts*** Day 1****	Day 2	Day 3	Day 4	Day 5	Day 6	Immune Elimination
Normal	3	None	89.53 (86.51/91.19)	67.66 (58.99/74.26)	47.64 (44.36/50.39)	40.04 (33.14/46.32)	31.55 (23.77/37.19)	24.76 (15.82/29.28)	Absent
C	2	nSpleen	15.65 (12.46/18.84)	7.33 (5.14/9.53)	1.87 (1.78/1.96)	0.67 (0.58/0.76)	0.50 (0.42/0.58)	0.38 (0.32/0.44)	Present
B	4	n T + n B	55.40 (46.23/64.04)	37.41 (25.02/48.53)	23.70 (9.62/38.85)	12.27 (1.52/22.28)	4.57 (0.54/8.78)	1.87 (0.33/4.48)	"
A	3	n B	44.24 (5.17/81.86)	31.55 (1.20/68.72)	19.79 (0.75/52.15)	10.62 (0.43/29.48)	4.87 (0.28/13.52)	2.08 (0.26/5.36)	"
D	3	n T + t B	79.96 (77.86/83.04)	60.92 (54.29/72.31)	43.62 (33.76/58.47)	25.11 (13.64/41.07)	12.27 (4.45/25.56)	6.84 (1.85/15.51)	"
E	5	t T + n B	87.32 (81.13/92.26)	75.97 (61.48/85.81)	63.86 (48.68/72.54)	53.41 (40.30/61.27)	40.63 (24.45/51.30)	31.68 (16.9/42.44)	Absent
F	3	t T + t B	84.47 (83.02/85.26)	73.43 (69.74/76.37)	60.34 (57.40/64.65)	53.57 (47.09/63.92)	40.07 (32.20/51.59)	31.56 (16.32/44.78)	"
G	3	t B	89.65 (86.99/91.56)	75.30 (71.36/81.86)	65.44 (61.43/70.28)	55.54 (52.92/59.63)	44.95 (42.32/48.56)	38.58 (35.44/43.04)	"

*Mice of all groups, except the normal group received 100 µg Agg HGG as an immunogenic dose 3 weeks after reconstitution, and 1 week later received 50µg ^{125}I-Mon HGG as a tracer dose. The normal mice received only the tracer dose of ^{125}I-Mon HGG. **n, cells from normal donors; t, cells from tolerant donors; T, thymus cells; B, bone marrow cells. *** Whole body counts expressed as percentage of the individual initial counts on day 0, immediately after injection of 50µg ^{125}I-Mon HGG. ****Mean percentage value and range in brackets.

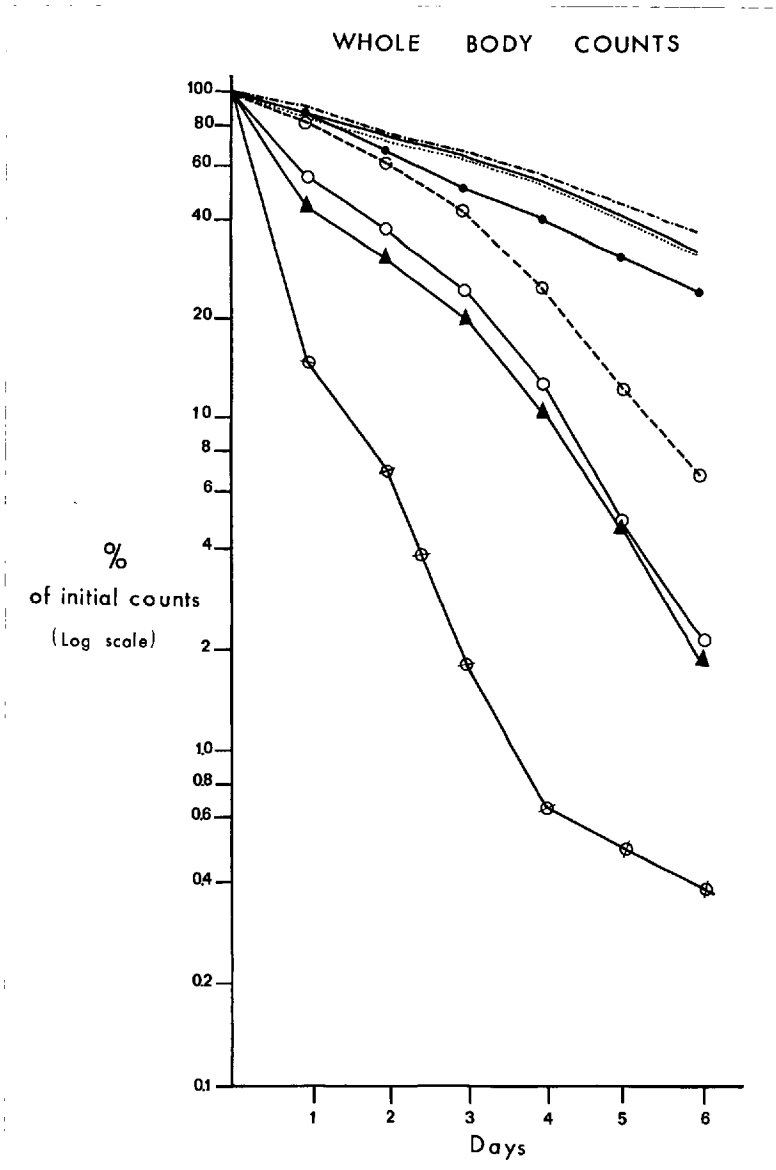


Fig. 16: Elimination of 50 µg of ^{125}I-Mon HGG in mice thymectomized, lethally X-irradiated and reconstituted with different combinations of cells as follows:

- Tolerant B cells (group G)
- Tolerant T + normal B cells (group E)
- Tolerant T + tolerant B cells (group F)
- Normal mice
- Normal T + tolerant B cells (group D)
- Normal T + normal B cells (group B)
- Normal B cells (group A)
- Normal spleen (group C)

B cells injected were tolerant, and the normal T cells injected with them had to wait for the regeneration of new B cells, which has not been subjected to the action of either tolerogen itself or the tolerogenic factor of T cells.

Therefore the absence of immune elimination in group G mice, which received bone marrow cells from tolerant mice, must be attributed to the presence of some tolerant T cells in the bone marrow inoculum, which were able to induce tolerance in the newly-regenerating B cells. This is further evidence not only of the existence of a factor produced by tolerant T cells, but also that this factor acts on B cells.

The absence of immune elimination in group E mice (reconstituted with tolerant T and normal B cells) is further evidence again. When a large dose of normal T cells (20×10^6) is injected with the tolerant bone marrow (group D), however, the relatively few tolerant T cells in the latter do not produce enough of the factor to overcome the action of the former.

4) The Effect of Tolerance to HGG on the Localization of Homologous Soluble Immune Complexes

The foregoing experiments present evidence to strengthen the hypothesis that in tolerant mice T cells produce a factor that blocks the B cells, thus preventing them from reacting to antigen. This blocking factor is primarily directed at specific antigen receptors on B cells but in the special case of HG G it might be responsible also for partially blocking receptors on B cells which take up Agg HGG as an altered immunoglobulin. Thus the existence of two types of receptors may be postulated; one type directed at the antigenic determinants of HGG as a foreign antigen, the other directed at determinants of autologous antibody altered by complexing with antigen and hence cross-reacting with heat aggregated HGG. The B cell receptors that are also blocked in mice tolerant to HGG, and whose blocking is

manifested as decreased uptake of Agg HGG, must be those of the second type which share specificity with the first type. That is to say, the tolerising factor produced by T cells is directed to receptors on B cells for HGG as antigen, but because HGG is also an immunoglobulin which when altered shares determinants with altered mouse immunoglobulin, the factor is able partially, though not completely, to block these receptors for altered immunoglobulin.

This postulate predicts that in mice tolerant to HGG the uptake of homologous immune complexes would be impaired. The next experiment tested this prediction.

Mouse anti-HGG was raised in BALB/c mice by one i.p. injection of 500 μg of Agg HGG followed 10 days later by another of 100 μg. Mice were bled 5 days after the boosting injection and serum separated. This serum proved to contain anti HGG precipitins by immunodiffusion. 150 μg (equivalent to 38.5×10^3 c.p.s.) of ^{125}I-Mon HGG in 50 μl of PBS were mixed with 0.5 ml of the mouse anti-HGG antiserum, and allowed to react at room temperature for 2 hrs and then centrifuged at 3,500 r.p.m. (2,300g) for 15 min. Under these conditions of antigen excess no precipitate was formed. This mixture was injected i.v. by the tail vein into each of 4 tolerant BALB/c mice and 3 normals. Twenty four hrs after this injection, the mice were killed and spleens, blood and livers counted for HGG retention.

As controls for radioactivity due to free monomeric HGG uncombined with antibody 3 normal BALB/c mice were injected i.v. with a mixture of 0.5 ml of normal mouse serum and 150 μg of ^{125}I-Mon HGG, incubated at room temperature for at least 2 hours.

Results: (Table IX) The mean splenic localization of immune complexes in tolerant mice was 72.5% that found in normal mice. This figure underestimates the true difference in splenic uptake between normal and tolerant mice receiving homologous immune complexes because as the complexes were prepared in antigen excess, both groups received the same amount of excess unbound antigen (Mon HGG), which increased the spleen counts equally above their respective counts due solely to germinal centre uptake. If the counts due to unbound ^{125}I-Mon HGG in the spleen could be calculated and deducted, the difference between the uptake in tolerant and normal mice would be even greater.

Table IX: Spleen localization in tolerant BALB/c mice of immune complexes formed in vitro

Mice	Injection received	Spleen retention	
		c.p.s.	%
Normal	NMS + Mon	160.6	-
		189.8	-
		234.9	-
		mean(195.1)	-
Normal	Mouse a.HGG + Mon	719.8	-
		787.0	-
		645.0	-
		mean(717.27)	100
Tolerant	Mouse a.HGG + Mon	449.0	62.60
		606.7	84.58
		587.1	81.85
		437.6	61.00
		mean(520.1)	mean(72.50)

NMS = normal mouse serum; mouse a.HGG - mouse anti-HGG; c.p.s. = counts/second in the whole organ ; % = counts expressed as % of the mean retention in spleen of normal mice receiving the immune complexes.

In a further experiment (Table X) serum from mice made tolerant to HGG failed to affect the localization of immune complexes in the spleens of normal mice. This result was in agreement with the results previously obtained (p 69, Table IV), where the localization of Agg HGG was unaffected by the transfer of "tolerant" serum.

Table X: Effect in normal mice of "tolerant" serum on the splenic localization of injected immune complexes made from mouse a. HGG with Mon HGG

Mice received	Spleen c.p.s.
Normal mouse serum	837.2
	1 241.3
	852.3
Tolerant mouse serum	963.3
	1 226.1
	1 131.6

Each mouse received 1.3 ml (half i.p. and half i.v.) of either "tolerant" or normal mouse serum 3.5 hrs before the i.v. of 0.5 ml of the mixture containing the immune complexes, prepared as before.

Comment: The results of these experiments with homologous immune complexes therefore confirmed the prediction made above, and substantiate that the factor produced by T cells in mice tolerant to HGG blocks not only the antigen receptors on B cells, but also partially blocks their receptors for altered mouse immunoglobulin. This dual effect is attributable to the fact that in this system, the antigen is itself an immunoglobulin which in the altered state (e.g. by heating) present some antigenic determinants which are the same as those exposed on altered mouse immunoglobulin.

PART VI

GENERAL DISCUSSION

It is a well established fact that localization of antigen in germinal centres is antibody dependent, that is to say, it only occurs when the animal is immune or when it receives antibody passively (Mitchell & Abbot, 1965; Lang & Ada, 1967; Herd & Ada, 1969). The ability to localize antigen has been considered by Humphrey (1969) as being probably the most sensitive test available for the presence of antibody.

The mechanism of germinal centre localization of Agg HGG in normal animals is thought to be different. Published work has shown that it is dependent on the presence of lymphoid cells which have receptors for altered IgG (Brown et al, 1970a; Greenwood et al, 1971a); these cells were later identified as belonging to the B cell population (de Jesus et al, 1972), since normal localization and uptake of ^{125}I-Agg HGG were found in the germinal centres of thymus deficient mice.

Since the localization and uptake of ^{125}I-Agg HGG in germinal centres is rapid and independent of antibody, and since similar localization is achieved when soluble immune complexes, although not antigen or antibody alone, are injected

(Brown et al, 1970b) it seemed likely that the receptors on B cells directed to autologous altered antibody are the receptors responsible for uptake of ^{125}I-Agg HGG into germinal centres. That is to say, B cell lymphocytes have receptors for determinants of autologous IgG when complexed with antigen, and alteration of the HGG molecule by heat leads to exposure of similar determinants not exposed in the native state. It is with these determinants that the B cell receptors interact, since they are similar to those exposed on the autologous antibody molecule when combined with antigen (Diagram I, p.90). Thus as there are plenty of B cells available with receptors for its exposed determinants, Agg HGG localizes in the germinal centres soon after injection. However, when the native form of an immunoglobulin (e.g. Mon HGG) is injected into normal animals, no germinal centre localization occurs, because the determinants exposed during aggregation are hidden. The injected immunoglobulin therefore recirculates and is catabolized in the same way as autologous immunoglobulins.

In the experiments reported here, HGG was found in the splenic germinal centres of mice made tolerant with Mon HGG before any Agg HGG had been injected. Since no antibody was detected at the time of testing and only Mon HGG had been injected, the localization of HGG in the germinal centres of these mice must therefore have been due to antibody production during the early stages of induction of tolerance. This explanation is in accordance with the findings of early antibody production during induction of tolerance by other authors (Dixon & Maurer, 1955; Sterzl & Trnka, 1957; Rowley & Fitch, 1965; Sterzl, 1966; Chiller et al, 1971) and is also supported by the increase in mouse IgG found in the germinal centres of these tolerant mice in the present work.

The function of antigen deposited in germinal centres is nevertheless obscure. Since during induction of tolerance by injection of minute amounts of flagellin,

this antigen is rapidly localized in germinal centres, it has been suggested that its localization here may play a role in the induction of tolerance (Ada & Parish, 1968; Parish & Ada, 1969). Thus the lymphoid follicles, where antigen is attached to the surface of the dendritic cells, would provide a unique source of antigen concentrated to the extent necessary to induce tolerance in cells migrating through the white pulp.

The experiments reported here, however, (p. 55) indicate that antigen deposited in germinal centres can in fact be used to induce an immune response (i.e. production of antibody), at least locally in the germinal centres. Following a single i.v. injection of Agg HGG in normal mice, immune elimination started on the 4th day (Table III, Fig. 10) and was either concomitant with, or antedated by one day a halving of the amount of aggregate persisting in the germinal centres (Fig. 11). This halving does not seem to be a consequence of systemic antibody production, i.e. removal by circulating antibody, since it also occurred in tolerant mice, where there was no circulating antibody (Table III). This seems to point to local antibody production in the germinal centres. Whether in normal mice this may act as a switching-on mechanism in antibody production is not clear. In tolerant mice, however, this local antibody production is restricted to the germinal centre and has no systemic effect, and therefore produces no modification of the tolerant state. This may reflect the state of affairs in normal animals in which autologous altered (i.e. immunogenic) IgG is continually deposited in the germinal centres after complexing with antigen, without leading to abrogation of tolerance and consequent autoantibody production.

As shown by Brown et al (1970b) localization of Agg HGG in germinal centres of lymphoid tissue is independent of the immune status of the animal, and therefore

occurs in normal animals as well as in tolerant ones. It is reported in this thesis that when splenic retention of Agg HGG was quantitated by using ^{125}I labelled aggregate, it was surprisingly found that in mice tolerant to HGG,splenic uptake was consistently reduced to 60% of the normal retention (Table III and Fig. 15). Since the uptake of Agg HGG in germinal centres is a B cell function (de Jesus et al, 1972) this reduction must be interpreted as a B cell defect, i.e. in mice tolerant to HGG either some of the B cells with receptors for altered IgG are completely prevented from functioning normally, or the whole population of B cell with these receptors is partially blocked (Diagram II,p.90). This impairment of this B cell function could be mediated either by a serum factor in the tolerant mice, or the B cells themselves might somehow have become partially incompetent in these animals. It was not possible to transfer deficient germinal centre localization of Agg HGG to normal animals with serum from tolerant mice (Table IV). However, a serum factor might not be present in a transferable amount, or might only be produced after antigenic stimulation. The latter possibility was excluded when it was shown that preinjecting the tolerant mice with Agg HGG before collecting serum for transfer into normal animals, had no effect in reducing the localization of Agg HGG in the germinal centres of normal mice receiving such serum (Table V).

In face of this, the defect has to be attributed to the B cells themselves, either as a primary fault, or as a result of T cell action.

It is a well established fact that co-operation between T and B cells plays an important role not only in the immune response to some antigens (Taylor, 1969; Miller et al, 1971) but also in tolerance (Chiller et al, 1970; Gershon & Kondo, 1970; Mitchison, 1971; Weigle, 1971). It has been postulated (Gershon & Kondo, 1971) that in tolerant mice, thymus derived cells produce a factor that is responsible for preventing the collaboration between T and B cells necessary for the immune

response, but evidence for the existence of this factor has not been provided.

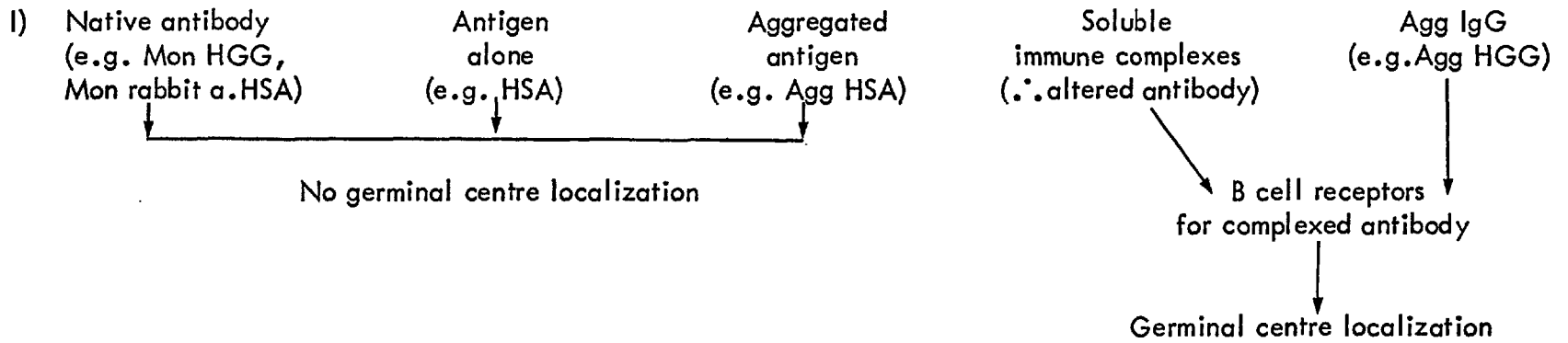
It was thought that, since the localization of Agg HGG in germinal centres is a function of B cells, the existence of a T cell factor influencing B cells in tolerant mice could be tested for by using different combinations of T and B cells from tolerant and normal mice to reconstitute thymectomized lethally X-irradiated recipients. When mice were reconstituted with tolerant T cells and tolerant B cells or with tolerant B cells alone the localization of ^{125}I-Agg HGG in the splenic germinal centres of the recipients was reduced to means of 45 and 35%, respectively, of that in normals (Table VI and VII). Similarly, in mice reconstituted with tolerant T cells and normal B cells a decreased localization was found (in two experiments, 71 and 51% of the normals), although not so marked as in the two previous groups (Tables VI and VII).

These results are evidence that in mice tolerant to HGG the T cells are producing a factor that interferes with a normal function of the B cells, i.e. localization of ^{125}I-Agg HGG in the germinal centres (Diagram III, p.90). That the factor produced by tolerant T cells needs to act upon the majority of B cells in order to interfere with splenic uptake is shown by the fact that mice receiving only tolerant B cells showed a greater reduction than the mice receiving tolerant T cells and normal B cells.

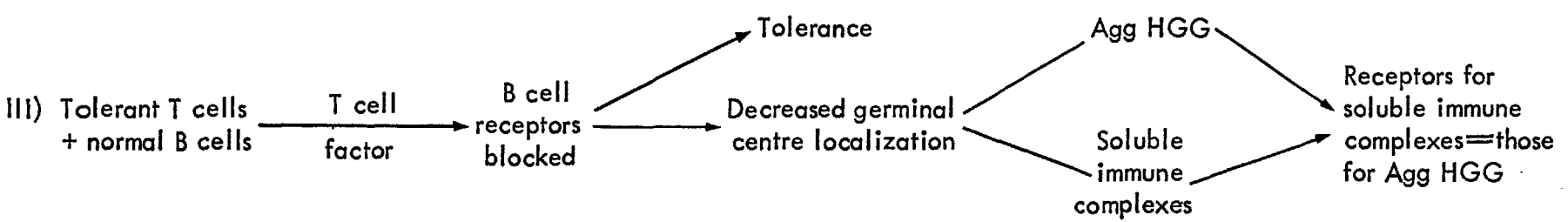
Mice reconstituted with these combinations of cells and manifesting decreased splenic retention of Agg HGG were in fact tolerant to HGG, since they did not respond to an immunogenic dose of Agg HGG as was shown by absence of immune elimination of a subsequent tracer dose of ^{125}I-Mon HGG (Table VIII and Fig. 16).

In contrast immune elimination of ^{125}I-Mon HGG was obtained in mice reconstituted with normal T and tolerant B cells, or with normal spleen cells or with normal bone marrow cells alone. The result in this last group must mean that the number of T cells transferred with the bone marrow preparation was enough to co-operate in the

DIAGRAMS


I)
Native antibody
(e.g. Mon HGG,
Mon rabbit a.HSA)
Antigen
alone
(e.g. HSA)
Aggregated
antigen
(e.g. Agg HSA)
No germinal centre localization
Soluble
immune complexes
(∴altered antibody)
Agg IgG
(e.g.Agg HGG)
B cell receptors
for complexed antibody
Germinal centre localization


II)
Tolerance
not transferable
with serum
Decreased germinal
centre localization
B cell blockade


III)
Tolerant T cells
+ normal B cells
T cell
factor
B cell
receptors
blocked
Tolerance
Decreased germinal
centre localization
Agg HGG
Soluble
immune
complexes
Receptors for
soluble immune
complexes=those
for Agg HGG

induction of the immune response to HGG, since this is a thymus dependent antigen (Davies, 1969). Thus the reduction of splenic retention of ^{125}I-Agg HGG in the group reconstituted with tolerant bone marrow cells is further evidence that tolerant T cells produce a factor that influences B cells and that even the small number of tolerant T cells transferred with the bone marrow preparation produce enough of the factor to act upon B cells newly regenerated after reconstitution. Without these T cells, tolerance would have been probably lost because the last tolerogenic injection had been given six weeks before. However, when normal T cells are supplied in greater numbers, they overcome the action of this small number of tolerant T cells supplied with the tolerant bone marrow and the tolerant state is lost (group D, Table VIII and Fig. 16). Nevertheless the fact that immune elimination did not occur in this group until four days after the injection of the tracer dose of ^{125}I-Agg HGG, is evidence that the normal T cells had to wait for maturation of newly regenerated B cells in order to produce the immune response, because the mature B cells injected with them had already been made tolerant and therefore unresponsive.

The fact that in these experiments recipients of normal T and tolerant B cells were not tolerant on challenge is in apparent contradiction with the findings of Chiller et al (1970) and Weigle (1971) who found that if either T cells or B cells were tolerant, tolerance was achieved in the recipients of such cells. The apparent contradiction could perhaps be explained by the kinetics of cellular induction and termination of tolerance. In the experiments presented here the cells were harvested three weeks after the last tolerogenic injection and the immunogenic state of the recipients was tested three weeks after reconstitution (six weeks after the last tolerogenic injection). As was shown by Chiller et al (1971) by day 49 the B cells from tolerogen-injected donors have regained their responsiveness; thus in the

present experiments by the time of testing the recipients of tolerant B and normal T cells, the tolerance of the B cells had already terminated.

Thus, evidence for the existence of the hypothetical factor produced by tolerant T cells has been presented in this thesis. The question thus arises why in the presence of this factor, splenic uptake of Agg HGG is significantly reduced.

This factor is primarily directed towards the receptors of B cells for the antigenic determinants of HGG; but because the antigen in this case is an IgG, the factor cross reacts with some of the receptors for altered autologous IgG considered to be also present on B cells and to be responsible for uptake of Agg HGG as altered immunoglobulin. This incomplete cross reactivity could be responsible for the reduction of splenic retention of Agg HGG in tolerant mice, since only those receptors for altered IgG that share specificity with the receptors for HGG as antigen are blocked. That is to say, all receptors on the B cells for the antigenic determinants of HGG are blocked in animals tolerant to HGG, but because this antigen is an immunoglobulin which when altered shares determinants with altered mouse immunoglobulin, the factor produced by tolerant T cells that is responsible for total blockade of the receptors for HGG as antigen, also blocks some of the receptors for altered immunoglobulin determinants. A prediction from this postulate is that the mice tolerant to HGG would also show a reduction in uptake of homologous IgG complexed with antigen to form soluble immune complexes, due to a partial blockade by the tolerant T cell factor of receptors for such altered IgG. As shown in Table IX, mice made tolerant to HGG had in fact a reduced capacity for localizing soluble homologous immune complexes, thus confirming the idea that it is the factor that is produced by

tolerant T cells and primarily directed at HGG antigenic determinants that is responsible for the partial blockade of the receptors for altered IgG also present on the B cells.

As long as tolerant T cells persist in the animal, this T cell factor is responsible for maintaining tolerance, even when the tolerogen has been eliminated and at a time when the B cells have recovered responsiveness. The existence of this factor explains how low dose tolerance is achieved, i.e. T cells, which are readily and easily made tolerant, produce the factor and thus the B cell receptors for the corresponding antigenic determinant are blocked and become unresponsive to the direct action of the antigen. Nevertheless the B cells remain reactive and able to respond when educated T cells (pre-immune) present the antigen to them, as is shown by the experiments of Mitchison (1971). In the case of high dose tolerance B cells are completely blocked not only by the T cell factor but also by the direct action of the antigen, and are therefore unresponsive even to presentation of antigen by educated T cells (Mitchison, 1971). After the antigen is eliminated, the new generation of B cells meet only the T cell factor which makes them unresponsive to direct antigenic stimulation. However if at this stage educated T cells are presented then the animal loses its tolerance (Mitchison, 1971). Thus the T cell factor prevents the reactive B cell "seeing" the antigen directly, but if antigen is presented indirectly through an educated T cell then the B cells are able to respond.

In the experiments of Gershon and Kondo (1971) where all the results are in favour of a T cell factor, as already discussed, normal T cells failed to abrogate the tolerance achieved when tolerant T cells were injected into mice reconstituted

with normal B cells. This result was attributed to the tolerant T cells influencing the normal T cells - "infectious tolerance". In Gershon and Kondo's experiment 30×10^6 normal T cells were injected with 100×10^6 tolerant cells. In the experiment reported in this thesis (group D in Table VIII and Fig. 16) these proportions were reversed, and tolerance was abrogated. Therefore it may be concluded that normal T cells in sufficient numbers can overcome the effect of the tolerant T cell factor and are not affected by it. That there is in fact competition between these two types of cells, is shown by the delay in achieving immunity in this group of animals, and the fact that normal T cells have to wait for normal B cells to be regenerated in order to manifest an immune response confirms this.

PART VII

CONCLUSIONS

Previous work has shown that localization of Agg HGG in lymphoid germinal centres is dependent on the existence of receptors on B cell lymphocytes and that 24 hr after an i.v. injection, the splenic retention of ^{125}I-Agg HGG is an expression of the localization of Agg HGG in the germinal centres.

From the work presented in this thesis it is concluded that:

1) Antibody is produced locally in germinal centres of both normal and tolerant mice 3-4 days after localization of Agg HGG in the germinal centres.

2) During the early stage of induction of tolerance to HGG antibody is also produced which can combine with Mon HGG and lead to its deposition in germinal centres.

3) Tolerance produced by repeated high dose injections of mice with Mon HGG from birth, is not affected by the local antibody production that occurs in the splenic germinal centres after an i.v. of Agg HGG.

4) Tolerance to HGG is adoptively transferred to thymectomized lethally X-irradiated mice reconstituted with either tolerant T cells and tolerant B cells,

or tolerant B cells alone, or tolerant T cells with normal B cells; but is not transferred by normal T cells and tolerant B cells. Therefore in this system, T cells play an important role in the expression of tolerance.

Tolerant B cells lose their tolerance when injected with normal T cells.

5) Tolerant mice, and the chimaeric mice referred to in 4), show a decreased splenic retention of ^{125}I-Agg HGG (reduced to about 60% of the retention in normal mice).

6) This is due to a partial B cell blockade, by a factor which must bind to the B cells, since it was not possible to reduce the splenic localization of Agg HGG in normal mice which had been pretreated with serum from tolerant mice.

7) The last three conclusions provide evidence for the existence in tolerant mice of a factor produced by T cells that blocks B cells.

8) Mice tolerant to HGG also show reduction of splenic retention of homologous soluble immune complexes to a degree similar to that found with Agg HGG. This is not only further evidence that tolerance is mediated through a factor that blocks B cells, but is also evidence that Agg HGG is taken up by the same receptors on B cells that take up autologous IgG complexed with antigen.

9) It is concluded that the factor produced by T cells in mice tolerant to HGG is directed primarily at the receptors for the antigenic determinants of HGG that are present on B cells; but because the antigen in this case is an immunoglobulin, the factor cross-reacts with, and blocks some, of the

receptors that B cells have for altered autologous IgG, which are responsible for uptake of Agg HGG.

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Lymphocyte-mediated Transport of Aggregated Human γ-Globulin into Germinal Centre Areas of Normal Mouse Spleen

GERMINAL centres develop early in the immune response, and although knowledge of these structures is far from complete, they are known to be intimately associated with the capacity to produce specific antibodies after antigenic stimulation[1]. The localization of antigen in these centres coincides with the appearance of specific serum antibody[2], which is found in a close relationship to reticular cells in these areas[3]. It produces a characteristic dendritic intercellular pattern of immunofluorescent staining or autoradiography when isotopically labelled. This localization of antigen in germinal centres did not occur in rabbits rendered tolerant to haemocyanin or human serum albumin[4], but injected immune complexes produced an identical pattern of localization of antigen in germinal centres in normal rat lymph nodes[5].

More recent studies[6] have established that alteration of heterologous IgG either by heat aggregation or by complexing as antibody with specific antigen is necessary for its localization in the germinal centres of auricular lymph nodes of normal guinea-pigs after injection into the ear. Such localization was not seen in animals given native, monomeric heterologous IgG. Nevertheless it does not depend on the antigenicity of the injected aggregate, for it was also seen in animals rendered specifically tolerant.

Thus germinal centre localization of antigen is mediated by specific antibody molecules through the configurational changes that they undergo on complexing with antigen, but the localization mechanism itself is obscure. With regard to localization, aggregated human IgG behaves identically to the antibody component of immune complexes[6]. It therefore provides a convenient substitute for complexes in further studies of the localization mechanism.

Preliminary experiments showed that heat aggregated[6] human IgG becomes localized in germinal centres in the white pulp of the mouse spleen within hours of intravenous injection. Normal mice were then studied using heat aggregated human IgG labelled with ^{125}I. A monospecific rabbit anti-human IgG fluorescein conjugate was used to produce immunofluorescence. Intravenous injection of less than 10 μg aggregated human IgG produced a dendritic

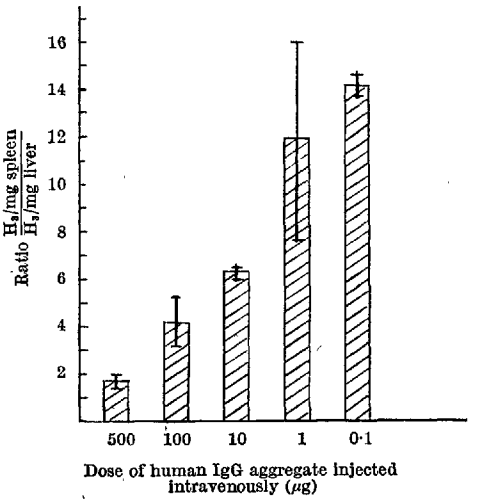

Fig. 1. Uptake at 24 h of human IgG aggregate by spleen and liver tissues in Balb/c strain adult mice. Vertical lines show range (500, 100, 0·1 μg, two mice; 10 μg, four mice; 1 μg, eight mice).

pattern of germinal centre staining in the spleen. There was no evidence of phagocytosis of this material by macrophages in the red pulp of this organ or by the Kupffer cells of the liver. With larger amounts of aggregate, extensive phagocytosis of IgG by macrophages in the liver and spleen was seen. This phagocytosed material was rapidly catabolized, as shown by loss of isotopic label and loss of fluorescence from the spleen and liver, both more or less completed by 24 h after injection. At this time there was always bright germinal centre staining of IgG.

In the guinea-pig experiments[6], this germinal centre localization persisted for at least 4 weeks. It therefore seemed that germinal centre localization of human IgG is one means of preserving molecules of this protein intact in the body. This preservation of aggregated IgG by the mouse spleen (Fig. 1) is shown by the increasing ratio of spleen/liver uptake 24 h after intravenous injection of decreasing doses of protein. Maximal incorporation of aggregated IgG into the germinal centre of mice spleens occurred 24 h after intravenous injection, when most of the phagocytosed material had been catabolized. Immunofluorescence a few hours after injection of aggregated IgG showed a diffuse pattern of localization in the white pulp marginal zones and after 24 h the typical dendritic pattern of distribution had developed. Mouse complement was not

detected by staining in these areas in sections treated with rabbit anti-mouse complement conjugate. Because lymphocytes are the cells which characteristically migrate from the blood to the splenic white pulp[7], cells of this type might be involved in the mechanism whereby aggregated IgG reached germinal centres. Two types of experiment were done.

In the first, peripheral blood leucocytes or spleen cell suspensions from normal C57Bl or Balb/c mice were incubated *in vitro* with ^{125}I-labelled aggregated human IgG in medium 199 containing normal mouse serum at 4° C. Uptake was measured by scintillation counting. In these conditions 10–20 per cent of the radioactivity was retained after exhaustive washing of the cells. Immunofluorescence revealed that at least 40 per cent of the cells had taken up the IgG on their surface membranes in a characteristic staining pattern. These had the morphological features of lymphocytes and not macrophages. The cell membrane staining was punctate and distributed uniformly around cells like a necklace. Occasional cells showed polar staining with concentration of IgG at one or two areas on the surface of the membrane. Table 1 shows that the intravenous injection of 40×10^6 peripheral white cells or 100×10^6 splenic cells, pre-incubated in this way with aggregated IgG, caused germinal centre localization of IgG in the spleens of normal syngeneic recipients. It should be noted that less than 1 per cent of the injected IgG was localized in the spleens in this way, the rest being catabolized. Thus many of the cells carrying human IgG were either removed by the phagocytic cells of the recipients or lost their labelled IgG to them. The amount of aggregated IgG on the cells if given alone would have resulted in some germinal centre staining. It was therefore possible that some IgG was transferred in some way from the donor cells and was subsequently localized in germinal centre areas of the spleens of recipients. Further studies are required to investigate this crucial point using lymphoid cells pre-labelled with isotopically labelled precursors of DNA and RNA. Because lymphocytes were postulated to be transporting the aggregated IgG into germinal centres it was considered possible that the protein demonstrated in these areas was on lymphoid cells closely

Table 1. SPLENIC UPTAKE AND GERMINAL CENTRE LOCALIZATION OF CELL BOUND AGGREGATED HUMAN IgG ON TRANSFER TO SYNGENEIC Balb/c MICE

No. of recipients	Cell source	No. of cells transferred	Protein bound to cells (μg)	Splenic uptake at 24 h (μg)	Germinal centre staining
1	Blood leucocytes	$2{\cdot}6 \times 10^6$	5·7	0·016	Negative
2		20×10^6	7·29	0·023	Negative
2		40×10^6	6·21	0·028	+
2	Spleen	100×10^6	8·0	0·096	++

Table 2. EFFECT OF ANTILYMPHOCYTIC IgG ON SPLENIC UPTAKE OF AGGREGATED ^{125}I-HUMAN IgG 24 H AFTER INTRAVENOUS INJECTION INTO Balb/c MICE

No. of mice	Treatment	Dose of human IgG (μg)	Mean splenic uptake*	Germinal centre staining
3	ALG, 2·5 mg × 2†	10	28	Negative
3	ALG, 2·5 mg × 2	1	41	Negative
3	Normal rabbit IgG, 2·5 mg × 2	10	100	++
3	Normal rabbit IgG, 2·5 mg × 2	1	100	++
2	Nil	10	100	++
2	Nil	1	100	Not done

* Expressed as percentage of uptake in untreated or normal rabbit IgG treated mice.

† Doses spaced 24 h apart; human IgG given 3 h after second dose.

apposed to the dendritic cells[3]. When spleens which would have shown staining confined to germinal centres were teased into single cell suspensions and stained for IgG with the anti-human IgG fluorescein conjugate, many lymphocytes with a characteristic membrane fluorescence were found. It can therefore be argued that at least part of the localization of aggregated IgG in germinal centres of normal mouse spleens was due to lymphocytes with this protein on their surfaces.

If localization of aggregated human IgG occurs by a lymphocyte mediated mechanism in which circulating lymphocytes transfer it into the white pulp of the spleen where it becomes concentrated in germinal centre areas, interference with the functions of these cells should prevent this characteristic localization. Antilymphocytic serum can be considered to possess this property[8]. Mice treated with the γ-globulin prepared from a potent antilymphocytic serum were found to be unable to localize aggregated human IgG in the germinal centre areas of their splenic white pulp.

Table 2 shows the results of such an experiment. Antilymphocytic IgG given in two spaced injections reduced the splenic retention of aggregated ^{125}I human IgG 24 h after injection to 28 per cent of that in mice given either human IgG alone or human IgG and the same quantity of normal rabbit IgG instead of antilymphocytic IgG. This reduction of uptake was correlated with negative immunofluorescence. Another experiment (Table 3) showed that this effect of antilymphocytic IgG was dose dependent. Results not shown here indicated that significant prevention of localization of aggregated human IgG in germinal centres lasted for about 48 h after administration of 5 mg of antilymphocytic IgG.

These results indicate that a large proportion of lymphocytes in the blood and spleens of normal mice can take up aggregated human IgG on to their surface membranes. The *in vitro* uptake is unlikely to involve binding of C1q because complement was not present in the medium.

Table 3. SPLENIC UPTAKE AND GERMINAL CENTRE LOCALIZATION OF AGGREGATED HUMAN IgG* IN PAIRS OF MICE PRE-TREATED WITH ALG

Antilymphocytic IgG dose (mg)	Splenic uptake†	Germinal centre staining
5	5·49 (4·96, 6·03)	Negative
2·5×2‡	5·09 (4·47, 5·71)	Negative
2·5	6·07 (5·43, 6·72)	±
1·0×2	8·65 (8·63, 8·68)	+
1·0	11·68 (11·58, 11·78)	++
0·1×2	11·80 (10·31, 13·30)	++
0·1	16·07 (15·50, 16·64)	+++
Nil	15·54 (15·24, 15·84)	+++

* 50 μg injected intravenously 3 h after last antilymphocytic IgG injection.
† μg protein $\times 10^{-2}$.
‡ Two intravenous doses spaced 24 h apart.

Presumably these cells possess specific receptors for altered gamma globulin. The nature of this receptor and the functional significance of these cells is being studied. Lymphocytes of normal mouse spleen possess receptors for specific antigen[9,12] and the present studies indicate that a large number of lymphocytes apparently possess receptors for altered human γ-globulin. It is therefore postulated that for every lymphoid cell possessing receptors specific for any given antigen there are many more with receptors for altered antibody, and it is possible that all antigen reactive lymphocytes possess receptors for altered γ-globulin. If this were so, a recruiting mechanism for induction of the immune response to an antigen would exist. Only a few antigen reacting cells need respond to their specific antigens by producing specific antibody. These antibodies would form complexes with their antigen which would in turn be the means of recruiting other cells carrying immunoglobulin receptors for altered γ-globulin. Lymphocytes carrying these complexes would become localized in the white pulp of the spleen or the cortex of lymph nodes, where they could migrate to the germinal centres. This would explain the mechanism of localization of autologous immunoglobulin in germinal centres. Thus antigen would be brought to areas where it could play a crucial, direct part in regulating the production of new cells capable of producing specific antibodies during their migration from germinal centres to the red pulp of the spleen and medullary areas of lymph nodes. The suppression of localization of aggregated human IgG by antilymphocytic IgG in these studies suggests that the cells involved form part of the recirculating pool of lymphocytes, and are either of thymic origin or are dependent on the presence of an intact thymus[13]. Such a population of cells functioning in the immune response to antigen in the manner suggested could provide a basis both for the mechanism of action of early antibody in enhancing the response to sheep erythrocytes[14] and also for the suppression of the response by antibody[15,16]. The importance

of these cells in immune responsiveness to antigens is being evaluated.

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IMMUNOSUPPRESSION IN MURINE MALARIA

II. THE EFFECT ON RETICULO-ENDOTHELIAL AND GERMINAL CENTRE FUNCTION

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IMMUNOSUPPRESSION IN MURINE MALARIA

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SUMMARY

The mechanism of the immune suppression of mice infected with the rodent malaria parasite *Plasmodium berghei yoelii* has been investigated.

The clearance from the peripheral blood of carbon and ^{51}Cr-labelled sheep erythrocytes was enhanced during the period of maximal parasitaemia and maximal immunosuppression, but the uptake of sheep erythrocytes by the spleens of infected mice did not differ significantly from the uptake by the spleens of healthy mice.

There was no uptake of aggregated human γ-globulin into germinal centre areas of the spleens of infected mice during the period of maximal immune suppression, but the ability to localize human γ-globulin returned at a time when the mice recovered immune competence.

It seems probable that acute malaria infections of mice induce a quantitative or qualitative defect in the cells responsible for transporting immune complexes into germinal centres. This defect may play a part in the immunosuppression induced by the malaria parasite.

INTRODUCTION

Mice infected with the rodent malaria parasite *Plasmodium berghei* show a markedly diminished immune response to sheep erythrocytes (SRBC) injected during the period of malaria parasitaemia (Salaman, Wedderburn & Bruce-Chwatt, 1969) but show normal rejection of skin homografts and other cell-mediated responses (Greenwood, Playfair & Torrigiani, 1971). When spleen cells from infected mice at the peak of parasitaemia are injected into irradiated syngeneic recipients together with sheep erythrocytes, their ability to mount an antibody response is partially restored, which suggests that a disturbance of

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the inductive phase of the antibody response may be one of the immunological defects induced by the malarial infection (Greenwood *et al.*, 1971).

The immune response of normal mice to sheep erythrocytes is greatly reduced by blockade of the reticulo-endothelial system with carbon (Sabet, Newlin & Friedman, 1968, 1969). Since large amounts of parasite material and red cell debris are released into the circulation during the course of a *P. berghei* infection, the possibility arises that the accompanying immunosuppression is due to an analogous reticulo-endothelial blockade.

It was also found that mice infected with *P. berghei* show a diminished antibody response to heat-aggregated human γ-globulin (HGG) injected during the period of malarial parasitaemia (Greenwood *et al.*, 1971). In normal mice, a small but constant proportion of aggregated HGG is transported to germinal centre areas of the spleen within hours of injection (Brown, Schwab & Holborow, 1970a) and further work has suggested that this transport is mediated by lymphoid cells (Brown, *et al.*, 1970b).

The studies reported here investigate two aspects of malarial infection in mice that may be related to its immunosuppressive effect: (a) reticulo-endothelial function, and (b) germinal centre localization of aggregated HGG given during the period of immunosuppression and on recovery.

MATERIALS AND METHODS

Mice

BALB/c mice aged 3–6 months were used in all experiments.

Malaria infection

Mice were infected with *P. berghei yoelii* and the course of their infection followed as previously described (Greenwood *et al.*, 1971). Maximal parasitaemia and maximal immunosuppression occurred at the tenth day of infection.

Pathology

Mice were killed at varying intervals after infection, the liver and spleen removed and sections prepared for routine histology.

Carbon clearance

The rate of carbon clearance by control and infected mice was determined by the method of Biozzi, Benacerraf & Halpern (1953). Mice were injected intravenously with a suspension of colloidal carbon (Pelikan, Gunther Wagner, Lot C11 1431A) in a dose of 16 mg/100 g body weight. Blood was taken by retro-orbital bleeding 1, 5, 15 and 30 min after injection, added to 2 ml of a 0·1% solution of sodium carbonate and the optical density read at 675 nm. The logarithms of the optical densities obtained were plotted against time and the phagocytic indices K and α determined (Biozzi *et al.*, 1953).

Clearance of sheep erythrocytes

Sheep erythrocytes were labelled with ^{51}Cr as described by Stern, Bartizal & Divshony (1967). One ml of washed, packed sheep erythrocytes was added to 0·5 ml of a solution of sodium radiochromate (Radiochemicals Centre, Amersham) diluted in phosphate buffered saline (PBS) to give a solution with an activity of 10–15 μCi/ml. After 30 min incubation at

room temperature the cells were washed 3–5 times in PBS until the supernatant was free of radioactivity.

Mice were injected with 0·5 ml of a 10% solution of labelled red cells and 25 μl of blood taken from the orbit 1 and 30 min later. The radioactivity of the 30 min sample, measured in a well γ-scintillation counter, was compared with the radioactivity of the 1 min sample and a clearance index determined as indicated below:

$$\text{Clearance index} = \frac{\text{cpm of blood taken at 30 min}}{\text{cpm of blood taken at 1 min}} \times 100$$

Mice were killed 24 hr after injection, and their liver and spleen removed, weighed and counted. The activity of the liver and spleen was compared with the activity of a 0·5 ml aliquot of the original solution of sheep erythrocytes and the percentage uptake of sheep erythrocytes by the liver and spleen calculated.

Handling of heat-aggregated HGG

Preparation of HGG. Heat-aggregated complexes of human γ-globulin were prepared in the following way. A 2% solution of Cohn fraction II human γ-globulin (Koch-Light Ltd, Colnbrook, Bucks.) in PBS at pH 8·0 was heated for 15 min at 63°C and then centrifuged at 3500 rev/min for 15 min to remove large particles. The supernatant was then centrifuged at 40,000 rev/min, 143,000 *g*, at room temperature for 90 min, the supernatant discarded and the pellet resuspended in PBS. The protein was then iodinated by the method of McFarlane (1958) using carrier free ^{125}I (Radiochemical Centre, Amersham) to give an activity of approximately 25 μCi/mg protein. The solution was then fractionated as follows. A solution of 2·18 M sodium sulphate was added drop by drop to the solution of HGG at room temperature with constant stirring until a molarity of 0·2 M was achieved. The solution was then stirred for a further 30 min and the precipitate formed removed by centrifugation at 3500 rev/min for 30 min. The supernatant was then treated in the same way to bring the solution to a final molarity of 0·4 M. The precipitate formed on this occasion was retained, dissolved in PBS and dialysed against PBS until free of sodium sulphate. This fraction of heat-aggregated HGG, the biological and physical characteristics of which will be reported in detail elsewhere, was used in all experiments.

In vivo handling of HGG. Malaria-infected and control mice were injected with the ^{125}I HGG prepared as described above and the distribution of the antigen at varying times after injection determined. Thyroid uptake was blocked by the administration of drinking water containing 0·125 g KI/l for several days before injection. At set times after injection whole body counts were measured in a modified Panax gamma one–sixty counter. Mice were then anaesthetized, killed by exsanguination and the liver and spleen removed and weighed. The radioactivity of the blood, liver and spleen was measured. Samples of liver and spleen were snap-frozen in isopentane at the temperature of liquid nitrogen and cryostat sections were subsequently stained with a sheep antiHGG conjugate, a rabbit antimouse γ-globulin conjugate and an antimalaria antigen conjugate prepared from the sera of rats infected with *P. berghei*.

Uptake of HGG in vitro. The *in vitro* uptake of the HGG fraction obtained as described above by spleen, thymus and peripheral blood cells was determined as previously described (Brown *et al.*, 1970b). Cell preparations from malaria-infected and control mice were washed in medium 199 containing 15% foetal calf serum (Tissue Culture Services, Ltd, Slough)

and exposed to a solution of HGG at a concentration of 50 $\mu g/10^7$ cells for 30 min at 4°C. The cells were then washed three times in medium 199 and the cell bound radioactivity determined. The cells were stained to show membrane fluorescence by incubating 2×10^6 cells with 1 drop of antiHGG conjugate for 30 min at room temperature.

RESULTS

Pathological changes

Marked splenic enlargement occurred during the course of *P. berghei* infection of mice and 10 days after infection, the time of maximal parasitaemia, the mean spleen weight of thirty malaria-infected BALB/c mice was 1330 ± 48 mg in comparison with a mean spleen weight of 161 mg $\pm$ 4 mg in twenty-five normal BALB/c mice of the same age. Three weeks after infection the mean spleen weight was approximately one-half this value and 6 weeks after infection the spleen had returned to a normal size.

On histological examination of spleens from mice killed 10 days after infection, considerable disturbance of the normal architecture was seen with hyperplasia of both red and white pulp. The marginal zone became indistinct and the red pulp contained an increased number of nucleated cells. The white pulp areas were occupied predominantly with large pale cells. Spleens examined 17 days after infection showed a more normal arrangement of red and white pulp. The red pulp contained fewer nucleated red cells and now showed many plasma cells. The white pulp areas comprised a central area of pale-staining cells showing mitoses, with a peripheral rim of smaller cells with more darkly staining nuclei. Twenty-one days after infection the histological changes in the spleen were similar to those observed at 17 days but some of the pale cells in the central areas of the white pulp now showed pyknotic changes.

No significant enlargement of the liver was found in mice killed 10 days after malaria infection, but a number of histological changes were observed. Small areas of necrosis of the hepatic parenchyma cells were seen and a mononuclear cell infiltration was present. Mononuclear cells were seen predominantly in the portal tracts but some were also present around the central sinus of some liver lobules. 21 days after infection the cellular infiltrate had disappeared and regenerative changes were observed in the parenchyma.

Carbon clearance

The rate of carbon clearance was measured in ten mice 10 days after malaria infection, the time of maximal immunosuppression (Greenwood *et al.*, 1971) and in ten age matched controls. Malaria-infected mice showed more rapid carbon clearance ($K = 0{\cdot}29$) than the controls ($K = 0{\cdot}22$) ($P = 0{\cdot}01$). The range of K values obtained in the mice infected with malaria (0·016–0·048) was wider than that observed in the controls (0·018–0·029) and several malaria-infected mice showed very rapid clearance of carbon. Due to the large spleen weight of the malaria-infected mice the α constant was lower in the malaria-infected mice than in the controls ($\alpha = 6{\cdot}1$ for the malaria-infected mice compared with 8·7 for the controls).

On histological examination of the liver and spleen 24 hr after administration of carbon, a similar distribution of the carbon particles was observed in the malaria-infected animals and the controls. The Kuppfer cells of the liver were heavily pigmented in livers from each group and within the spleens maximal deposition of pigment was observed in the marginal zones. Smaller clumps of pigment were also present within the central area of the white pulp, probably within tingible-body macrophages.

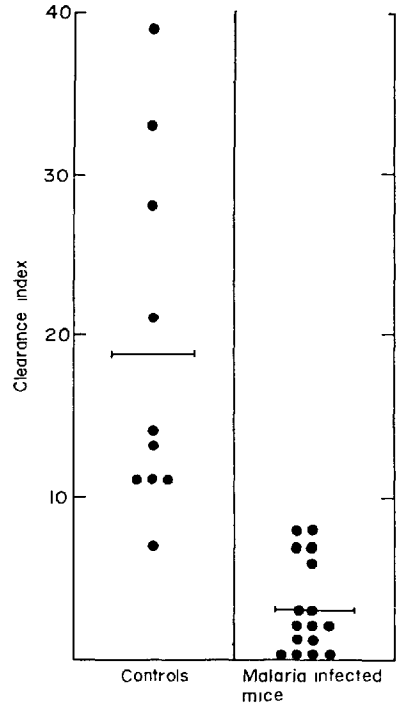

FIG. 1. Clearance of ^{51}Cr-labelled sheep erythrocytes from the peripheral blood of normal and malaria-infected mice measured 30 min after injection.

Clearance of ^{51}Cr labelled sheep erythrocytes

The rate of clearance from the peripheral blood of an intravenous injection of ^{51}Cr-labelled sheep erythrocytes was determined over a 30 min period in ten mice on day 10 of a malaria-infection and in ten control mice of the same age. The sheep erythrocytes were cleared much more rapidly by the malaria-infected mice than by the controls (Fig. 1).

The distribution of ^{51}Cr in the liver and spleen was determined 24 hr after the administration of labelled sheep erythrocytes by intravenous or by intraperitoneal injection. When the sheep erythrocytes were given intravenously, malaria infected mice showed a significantly increased uptake of radioactivity by the liver (Table 1). The proportion of ^{51}Cr localized within the spleen did not differ significantly between malaria-infected and control mice when sheep erythrocytes were given either intravenously or intraperitoneally.

The effect of malarial infection on the fate of injected heat-aggregated HGG

200 μg of aggregated HGG were injected intravenously into six normal mice and into six mice on the 10th day of malarial infection. Two mice from each group were killed and examined at 1, 6 and 24 hr following the injection of HGG.

Fluorescent staining patterns

(a) *Splenic localization.* In normal mice at 1 hr following injection HGG was seen in the periphery of the white pulp and marginal zones in an intercellular pattern. At the same

TABLE 1. Uptake of ^{51}Cr-labelled sheep erythrocytes by the liver and spleen of normal mice and malaria-infected mice 24 hr after intravenous or intraperitoneal injection of 0·5 ml of a 10% solution of labelled red cells. Results expressed as a mean percentage of the total dose given. Ten mice in each group

	Liver		Spleen	
	Malaria-infected	Controls	Malaria-infected	Controls
Intravenous erythrocytes	64·5±2·5	38·3±3·3	5·2±1·2	7·6±0·8
	$P<0{\cdot}01$		n.s	
Intraperitoneal erythrocytes	56·9±4·1	51·3±4·6	6·3±2·2	2·5±0·3
	n.s		n.s	

time after injection in malaria-infected mice only occasional faint flecks of staining could be seen in the marginal zone. In normal mice 6 hr after injection bright staining could be seen within the white pulp in a characteristic dendritic intercellular distribution. This pattern was still present at 24 hr after injection. In malaria-infected mice no staining of the white pulp was seen at 6 or 24 hr after injection.

By contrast, staining of white pulp for mouse γ-globulin showed staining in both groups although in malaria infected animals this pattern appeared more fragmented. An anti-malarial antigen conjugate did not show dendritic staining.

(b) *Hepatic localization.* 1 hr after injection HGG could be seen within the Kuppfer cells in both groups of animals although the staining was much less intense in the malaria-infected mice. No staining was seen at 6 and 24 hr in either group, presumably due to loss of antigenic determinants of the HGG on phagocytosis.

Isotope incorporation

These results correlated well with fluorescent staining patterns. Malarial spleens showed less incorporation than normal spleens at 6 and 24 hr after injection despite the greatly increased spleen size. However, the hepatic uptake by malaria-infected mice was greater at 6 and 24 hr.

The effect of duration of malaria-infection on the localization of HGG

A standard dose of 50 μg aggregated ^{125}I HGG was injected into eight malarial-infected mice and eight normal controls. Two mice were injected on days 5, 10, 17 and 23 post-malarial infection and, together with two controls, were killed and examined 24 hr later. This is at a time when most catabolic events are completed and maximal uptake in the lymphoid system is seen in normal animals. Blood, spleens and livers were examined as before, using fluorescent microscopy and scintillation counting.

The splenic uptake is shown in Table 2. At 5 and 10 days after infection (Fig. 2) no localization in the white pulp was observed in malaria-infected mice. At 17 and 23 days after infection, mice which still had parasitaemia and grossly enlarged spleens still showed diminished uptake, whereas the remaining mice in which parasites had disappeared from the peripheral blood and whose spleen weights were decreasing at these times, showed

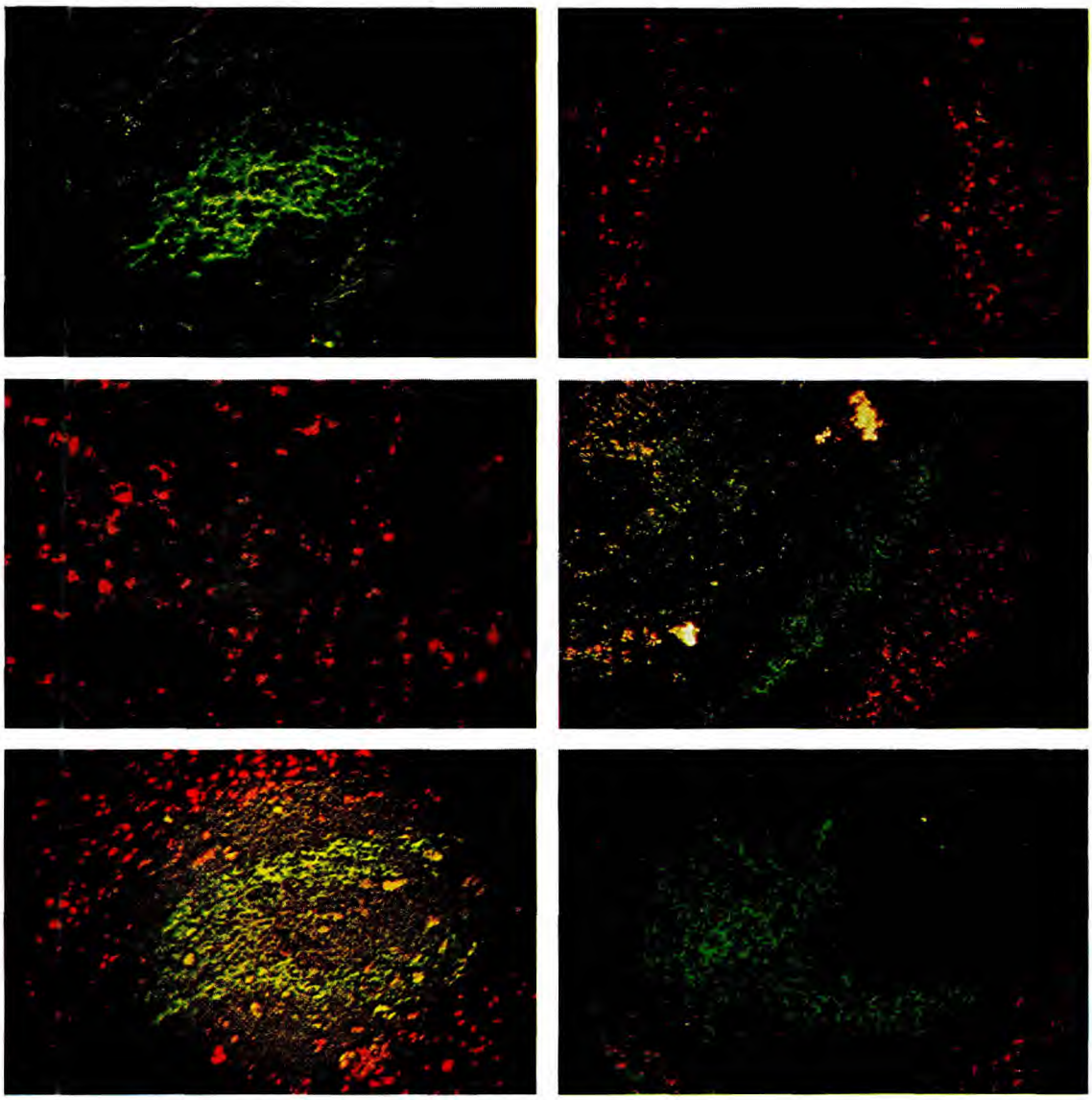

FIG. 2. Immunofluorescent staining of HGG or mouse IgG in mouse spleens at various stages of malarial infection. (a)–(e) stained with FITC-antiHGG 24 hr after intravenous injection of HGG; (f) stained with FITC-antimouse γ-globulin 24 hr after intravenous injection of HGG.

(a) Normal mouse spleen: Typical dendritic pattern in germinal centre in white pulp. (b) Fifth day of parasitaemia. No staining of white pulp. Red material pigment shows demarcation between red and white pulp. (c) Tenth day of parasitaemia. No staining; marginal zone now disorganized with pigment bearing cells in white pulp. (d) Seventeenth day after initial infection. Recovery stage. No parasitaemia. Uptake of HGG within white pulp. (e) Twenty-third day: dendritic staining pattern in germinal centre. (f) Twenty-third day: white pulp stained for mouse IgG; return of and enhancement of dendritic pattern.

(*Facing p.* 350)

TABLE 2. Splenic uptake of 50 μg aggregated HGG at different days of malaria-infection

Days of malarial infection	Malaria-infected mice				Control mice			
	Spleen weight (mg)	HGG retained (cps)	Germinal centre staining		Spleen weight (mg)	HGG retained (cps)	Germinal centre staining	
			HGG	Mouse IgG			HGG	Mouse IgG
5	240	120	±	−	115	82·9	+++	+
	350	57·6	−	−	115	93·5	+++	+
10	1000	25	−	±†	100	87·7	+++	+
	1160	11·5	−	±†	150	95·7	+++	+
17	718	48	++	±†	125	93·83	+++	+
	500	183·86	+++	+	115	96·67	+++	+
23	400	177·6	++	+	145	77·07	+++	+
	1630	19·7	−	±†	125	67·7	+++	+
	340	573·0*	+++	N.D.	152	169*	+++	+
	480	554·0*	+++	N.D.	125	137*	+++	+

* 500 μg HGG aggregate injected.

† Staining just present but dendritic pattern fragmented in white pulp.

enhanced uptake. Fluorescent microscopy of the spleens of mice showing an enhanced uptake at 17 and 23 days showed bright staining widely distributed within the periphery of enlarged malpighian bodies. With an antimouse IgG conjugate, normal mouse spleens showed mouse IgG in plasma cells in the red pulp and in typical dendritic pattern in many germinal centre areas. In malaria infected mice, autologous IgG was either absent from the white pulp or the dendritic pattern was very faint and fragmented.

The areas of maximal staining on serial sections stained with haematoxylin and eosin contained a high proportion of lymphocytes.

The effect of increasing the dose of injected HGG on localization patterns in malaria-infected mice

Ten malaria infected mice at the tenth day of infection and ten normal mice were injected in pairs with doses of aggregated HGG from 1 to 500 μg. The mice were killed 24 hr after injection, and blood, livers and spleens examined. Regardless of the dose injected, no germinal centre uptake occurred in malaria-infected mice (Table 3). Hepatic uptake was consistently higher in infected mice compared with controls.

Uptake of HGG in vitro

This was carried out as described in the Methods section. No significant difference was found between spleen cells, peripheral blood cells and thymus cells from mice on day 10 of malarial infection and from normal mice, either by isotope uptake or by membrane fluorescence.

TABLE 3. The splenic localization of different doses of aggregated HGG injected on the tenth day of malarial infection

Quantity injected (μg)	Malaria-infected mice				Normal mice			
	Spleen weight (mg)	HGG* retained (cps/spleen)	Germinal centre staining		Spleen weight (mg)	HGG* retained (cps/spleen)	Germinal centre staining	
			HGG	Mouse IgG†			HGG	Mouse IgG†
1	1115	2·21	–	±	115	9·65	–	+
	1110	2·23	–	±	100	5·54	–	+
	1145	1·72	–	±	100	7·99	–	+
10	1040	7·82	–	±	155	30·10	–	+
	1115	9·19	–	±	135	35·78	–	+
	1325	5·45	–	±	110	9·19	–	+
50	1000	5·76	–	±	100	85·41	+++	+
	1160	0·5	–	±	150	94·0	+++	+
200	1235	46·12	–	±	115	72·71	+++	+
	1210	229·53	N.D.	N.D.	120	85·97	+++	+
500	1000	‡	–	±	120	‡	+++	+
	1160	‡	–	±	110	‡	+++	
								+

* Protein retained measured in cps. This value is corrected for plasma radioactivity.
† Staining pattern of mouse IgG at this stage showed a faint fragmented dendritic pattern.
‡ Unlabelled protein.

DISCUSSION

No evidence of reticulo-endothelial blockade was found in these experiments. On the contrary, at the time of maximal immunosuppression, which corresponds with the height of malarial parasitaemia, the clearance from the peripheral blood of carbon and ^{51}Cr-labelled sheep erythrocytes was enhanced. This finding is in agreement with the previous observations of Cantrell & Elko (1966) and Lucia & Nussenzweig (1969) who showed enhanced clearance of carbon in rats infected with *P. berghei*, *P. chabaudi* and *P. vinckei* respectively.

24 hr after the intravenous injection of labelled sheep erythrocytes infected mice showed a significantly increased liver uptake as compared with normals, suggesting that the liver is largely responsible for the enhanced clearance in malaria infected mice. However, the percentage uptake of ^{51}Cr by the spleen was not significantly less in malarial than in healthy mice, so that their failure to produce antibody or plaque-forming spleen cells on challenge with SRBC can hardly be due to diversion of the SRBC away from the spleen. Furthermore, in other experiments not reported here, measurement of the plasma volume of the spleen using iodinated monomeric HGG confirmed adequate perfusion of the malarial spleen with injected antigen.

Contrasting with the normal reticulo-endothelial uptake of injected antigen in the spleens of malarial mice is the impairment of splenic uptake of aggregated HGG. We have already shown (Brown *et al.*, 1970a) that injected HGG altered by heat-aggregation, and also immune complexes, are within hours concentrated in germinal centre areas of lymphoid tissue

in a dendritic or reticular pattern, and remain intact there for long periods of time. Subsequently we have suggested (Brown *et al.*, 1970b) that aggregated HGG, and by inference immune complexes containing autologous antibody, are carried into germinal centres by lymphocytes having surface receptors for altered immunoglobulin, and since prior administration of antilymphocyte globulin prevents this localization, that these lymphocytes are mobile. The present experiments, which show a failure of transport of intravenously injected aggregated HGG into germinal centre areas in the malarial mouse spleen, suggest that malarial infection interferes with the function of this cell population, or a part of it.

It is not yet clear how malarial infection brings about this interference. One possibility is that the anatomical disruption of the red and white pulp of the spleen during the parasitaemia stage is so profound that access of lymphocytes to germinal centre areas via the marginal sinuses and the white pulp is prevented. The work of Hunter, Wissler & Fitch (1968) suggests that the integrity of the marginal zone is essential for the passage of cells or antigen into the white pulp. However, failure to localize HGG evidently precedes gross anatomical disorganization, as Fig. 2(b) shows. A second possibility suggested by the transfer experiments of Greenwood *et al.* (1970) is that the malarial infection disturbs the normal function of the dendritic cells themselves, but the experiments reported here provide no evidence on this point. We have found (unpublished observations), however, that while sublethal irradiation given after injection of aggregated HGG has no effect on the localization pattern, irradiation before injection impairs localization in germinal centres, suggesting that the cells transporting aggregated HGG are more sensitive to injury than the dendritic cells.

A third possibility, that germinal centre areas in malarial spleens are already saturated with immune complexes arising from an immune response to the malarial infection, appears to be ruled out by the disappearance of mouse IgG dendritic patterns as the parasitaemia reaches its peak, and the failure to demonstrate malarial antigen in the white pulp at this time.

Fourthly, it could be argued that since the presence of antibody is necessary for the localization of most antigens in germinal centres (Humphrey & Frank, 1967), the failure of HGG to localize in this way in malarial mouse spleens is due to the immuno-suppressive effect of the infection. It has already been shown (Brown *et al.*, 1970a), however, that aggregated HGG localizes in normal germinal centres without the assistance of antibody, presumably because it carries the same localizing determinants as the latter.

Finally, it might be postulated that the effect of the malarial parasitaemia is to deplete the lymphoid cells that carry immune complexes into germinal centres, and we suggest that this is the explanation of the findings. That these cells are not representative of the thymus dependent population of lymphocytes is suggested by the finding (Brown *et al.*, unpublished observation) that thymectomized, irradiated, bone marrow injected ('deprived') mice remain capable of localizing HGG in splenic germinal centres, and it may be noted again that Greenwood *et al.* (1970) found the cell mediated immune responses of malarial mice to be normal.

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A DEFECT OF B-LYMPHOCYTE TRANSPORT OF AGGREGATED HGG INTO GERMINAL CENTRES IN NZB AND NZBX NZW F_1 HYBRID MICE

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SUMMARY

Previous experiments have shown (i) that the localization of intravenously injected heat aggregated human γ-globulin (HGG) in the germinal centres of normal mice provides a model for studying the natural uptake of circulating immune complexes in these areas, and (ii) that the aggregated HGG is carried into germinal centres by lymphocytes which have receptors for altered γ-globlin.

Evidence from thymus-cell depletion experiments is now presented which suggests that the lymphocytes concerned are bone-marrow derived B cells. Defective localization was found in NZB and NZBX NZW F_1 hybrids at different ages and the onset of the defect antedates the onset of autoimmunity and the appearance of histological abnormalities in the spleen. As disease develops it progresses to a complete inability to localize complexes in germinal centres. It is concluded that a functional defect of the bone marrow-derived lymphocyte population exists in these mice.

INTRODUCTION

New Zealand Black mice (NZB) have been extensively studied as a model of autoimmune diseases (Bielschowky, Helyer & Howie, 1959; Howie & Helyer, 1968). During their first year of life, these mice develop a haemolytic anaemia with positive direct Coombs' test, splenomegaly, antinuclear antibodies and renal disease, due to glomerular deposition of antigen–antinuclear antibody complexes (Lambert & Dixon, 1968). Antinuclear antibodies are more pronounced and renal disease is manifested at an earlier stage in the NZBX NZW F_1 hybrid (B/W) mice.

As well as immunologically mediated lesions, these animals show several abnormalities of immune response. Thus an unduly early maturation of their immune system has been

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reported (Playfair, 1968; Evans, Williamson & Irvine, 1968) and a relative inability of young NZB and B/W mice to acquire and maintain tolerance to protein antigens (Weir, McBride, Naysmith, 1968; Staples & Talal, 1969) has been demonstrated. Both hyper-responsiveness (Weir et al, 1968; Morton & Siegel, 1969; Staples & Talal, 1969) and hypo-responsiveness (Stutman, Yunis & Good, 1968; Salomon & Benveniste, 1969) have been observed in these animals, but when their antibody production to a range of different antigens is compared with that of several normal strains it appears simply that they give a higher antibody response to some antigens and a lower antibody response to others (Cerottini, Lambert & Dixon, 1969).

So far as lymphocyte abnormalities are concerned, old NZB mice have a lymphopenia and their lymph nodes also are depleted of lymphocytes (East, de Sousa & Parrott, 1965). Labelling studies with tritiated thymidine (Denman & Denman, 1970) shown that old mice are depleted predominantly of long-lived lymphocytes and there is also evidence from cell-homing studies that in these mice there is a decrease with ageing in the recirculating cell content of lymph nodes and spleen that coincides with the onset and development of autoimmunity (Zatz, Mellors, Lance, 1971). Furthermore, Steinberg, Law & Talal (1970) have shown that the resistance of adult B/W mice to attempts to induce tolerance with ultracentrifuged BGG is overcome by transferring thymus cells from young B/W mice, suggesting that in the adult B/W mouse also the thymus cells are deficient. Thymus deficiency has been related to the development of autoimmunity in NZB by de Vries & Hijmans (1966) and by Holmes & Burnet in B/W (1966).

Previously we studied the transport of immune complexes or heat-aggregated human gamma globulin (HGG) into germinal centre areas of lymph nodes (Brown, Schwab & Holborow, 1970a) and spleen (Brown *et al.*, 1970b) and found that this transport is mediated by mobile lymphocytes that have receptors directed to sites exposed on IgG molecules either by complexing with antigen or by mild heat treatment (aggregation) (Brown *et al.*, 1970b). In view of the cellular deficiencies described in NZB and B/W mice, we have therefore now investigated their ability to localize aggregated HGG and immune complexes in the germinal centres of the spleen.

We also present data suggesting that the lymphoid cells responsible for the transport of aggregated HGG and immune complexes into germinal centre areas in normal mice are of bone marrow origin (B cells) rather than thymus dependent (T cells).

MATERIALS AND METHODS

Mice

NZB, CBA, BALB/C and C_{57} Bl inbred strains from the Taplow Colony were used, and F_1 hybrids were bred from NZB female mice crossed with NZW male also maintained at Taplow.

Mice of both sexes and a wide range of ages, from 5 weeks up to 20-months old, were examined.

*Preparation of heat-aggregated HGG labelled with ^{125}I (*Agg HGG)*

Heat aggregated complexes of HGG were prepared and iodinated as described previously (Greenwood *et al.*, 1971a).

*In vivo uptake of *Agg HGG*

*Agg HGG prepared as described above was injected intravenously into mice in various doses. Twenty-four hours after injection mice were anaesthetized with ether, killed by exsanguination and the spleen and a piece of liver removed and weighed. Studies to be reported (Brown, de Jesus & Holborow) have shown that at doses up to 100 μg catabolic events are largely completed and that maximal uptake in the splenic germinal centres of normal mice has occurred by 24 hr..

The radioactivity of the blood, liver and spleen was measured in a crystal well scintillation detector used in conjunction with a Tracerlab spectrometer. The spleen was then snap-frozen in isopentane at the temperature of liquid nitrogen, and cryostat sections were cut.

Direct Coombs' test

Rabbit antimouse γ-globulin obtained from Wellcome Reagents Ltd., was absorbed with an equal volume of packed washed normal mouse red blood cells (RBC), diluted 1:10 with phosphate buffered saline (pH 8·0), divided into small volumes and frozen.

Agglutination tests were done at room temperature, mixing one drop of diluted anti-globulin serum with one drop of 20% mouse RBC on a glass slide.

Normal mouse RBC were not agglutinated. Large agglutinates appearing rapidly were scored as +++, and weaker reactions were scored as ++; agglutination only visible under the microscope was scored as +.

Positive and negative control sera were always included.

Antinuclear factor

Sera from all mice were examined for the presence of antinuclear factor (ANF) by indirect immunofluorescence. Cryostat sections of rat liver were treated with the test serum diluted 1:10, washed and then treated with a 1:16 dilution of a specific rabbit anti-mouse γ-globulin conjugated with FITC (Nordic Pharmaceuticals and Diagnostics).

After washing, sections were mounted in 90% glycerol and examined microscopically with a dark ground condenser, iodine quartz illumination and a FITC-3 interference primary filter with a Wratten 12 secondary filter.

Proteinuria

Proteinuria was detected with 'Albustix' where a ++ reaction is equivalent to 100 mg albumin per 100 ml urine. Only ++ or stronger reactions were considered as evidence of renal disease.

Immunofluorescent spleen staining

Cryostat sections of 6 μm thickness were dried under a fan and stained with specific fluorescein conjugated sheep or rabbit antisera raised against HGG (Wellcome Reagents Ltd.) or against mouse γ-globulin (Nordic Pharmaceuticals and Diagnostics).

The anti HGG conjugate was used at a dilution which gave no staining of normal uninjected spleen sections and the antimouse immunoglobulin at dilution that gave no background staining. Staining of both human and mouse immunoglobulin was inhibited by absorbing the conjugates with purified HGG or mouse IgG, respectively.

Anti-mouse lymphocyte serum (ALS)

This serum was obtained from Wellcome Reagents Ltd. According to the manufacturers it had been raised in horses by repeated injections of mouse thymocytes without adjuvant, and had been inactivated at 56°C for 30 min. Activity had been tested by prolongation of graft survival when tail skin from A strain mice was grafted on to CBA strain recipients. Two doses of 0·5 ml of ALS given on days 2 and 5 after operation increased the mean survival time of the grafts from approximately 11 to over 20 days.

Heat inactivated normal horse serum (NHS) was given to the control mice.

Both ALS and NHS were tested for anti-mouse RBC haemagglutinins by titration against a 20% suspension of normal mouse RBC. The ALS agglutinated mouse RBC to a titre of 1/5 and the NHS only when used neat. Since the titres of both were so low, they were administered without absorption by mouse RBC.

Thymectomy

This operation was done in CBA mice 4-6-weeks old under Nembutal anaesthesia, by sucking out the thymus through a midline incision just above the manubrium. At the end of the experiment all thymectomized animals were checked macroscopically for absence of thymus.

Irradiation

Ten to 14 days after thymectomy whole body X-irradiation was done, each mouse receiving 850 rad in about 22 min.

Reconstitution with bone marrow cells

After irradiation mice were reconstituted on the same day with $5–10 \times 10^6$ bone marrow cells, obtained by flushing out femoral marrow from CBA with medium 199 (BDH Chemicals Ltd) containing 10% of foetal calf serum (FCS) and added penicillin and streptomycin. The bone marrow cells were washed once and counted. Viability was tested by the trypan blue method.

RESULTS

1. *Localization of *Agg HGG in splenic germinal centres of thymus deprived CBA and $C_{57}Bl$ mice.*

(a) *Thymectomized, X-irradiated and bone-marrow reconstituted mice.* Two separate experiments were carried out on different occasions on CBA mice. In each experiment the mice were divided into three groups of three, as follows: group A, thymectomized, X-irradiated and reconstituted with bone marrow cells (see Materials and Methods); group B, sham thymectomized, X-irradiated and reconstituted with bone marrow cells; group C, normal mice of the same age. In experiment 1 mice were tested 68 days after reconstitution and in experiment 2, 45 days after. All mice received intravenously 50 μg of *Agg HGG and were killed 24 hr later.

Although the normal mice (group C) had higher counts for radioactivity in their spleens than mice of groups A and B, no significant difference was seen between groups in the intensity of staining of the germinal centres for HGG (Table 1). The lower counts in the treated mice were probably due to incomplete recovery after irradiation and reconstitution.

TABLE 1. Failure of depletion of thymus-derived-lymphocytes to decrease splenic uptake of 50 μg *Agg HGG injected i.v. into CBA mice

Experiment	Group	Interval in days between reconstitution and injection of *Agg HGG	Spleen		
			Cps.	mean Cps	G.C. staining for HGG
I	A	68	115·91	95·43	+++
			80·09		++
			90·30		++
	B	68	84·73	84·39	++
			82·80		++
			85·65		++
	C	68	93·36	111·65	++
			122·74		+++
			118·87		+++
II	A	45	181·5	118·1	+++
			117·9		+++
			54·8		+
	B	45	106·1	109·0	+++
			133·5		+++
			87·4		++
	C	45	238·5	195·8	++++
			179·1		+++
			170·0		+++

A; Thymectomized mice, lethally X-irradiated, bone marrow reconstituted.
B; Sham-thymectomized mice, lethally X-irradiated, bone marrow reconstituted.
C; Normal mice of same age.
Cps = counts/second in the whole spleen.
G.C. = germinal centres.

(b) *Thymectomized mice chronically treated with ALS.* Four weeks after thymectomy twenty-four C_{57}Bl mice, divided into two groups of twelve, received intraperitoneally 0·5 ml of ALS or NHS daily on 5 consecutive days in each week, for a period of 2 months. Five of the twelve ALS treated and nine of the twelve NHS treated mice survived this treatment. Ten days after the last injection of either ALS or NHS, the mice were given 50 μg of *Agg HGG intravenously and killed after 24 hours. The radioactivity in spleen, liver and blood was measured, and cryostat sections of the spleen were examined by immunofluorescence for HGG, horse γ-globulin and mouse IgG. As controls, three C_{57}Bl mice thymectomized at the same time but receiving no further treatment, and four normal C_{57}Bl mice of the same age as the thymectomized mice were included.

Isotope intake (Table 2). No difference in the mean splenic uptake of *Agg HGG was found between the thymectomized ALS treated mice and the thymectomized controls, but the mean splenic count in the non-thymectomized mice was lower than in any of the thymectomized groups. This difference may not have been significant, but it may be noted that in the previous experiment (1a) also a similar difference was observed between the thymectomized and the sham-thymectomized groups (Table 1).

TABLE 2. Failure of thymectomy and ALS to prevent uptake of *Agg HGG in splenic germinal centres of $C_{57}Bl$ mice

Thymectomy	Treatment	Spleen					Antibody response†	
		HGG retention (Cps)	mean (Cps)	G.C. staining for HGG	G.C. staining for mouse γ	G.C. staining for horse γ	Anti-whole horse serum	Anti-horse γ
Yes	ALS	70·0	100·5	++	+	+	Neg	Neg
		133·1		++	Neg	+	Neg	Neg
		100·1		++	Neg	Neg	Neg	Neg
		108·8		++	Neg	Neg	Neg	Neg
		90·4		++	+	+	Neg	Neg
Yes	NHS	99·8	88·9	+++	++++	+++	+	+
		102·5		+++	++++	+++	+	+
		110·8		++	+++	++	Neg	Neg
		78·4		++	++	+++	+	+
		88·2		+++	++	++	Neg	Neg
		96·9		++	+++	+++	+	+
		60·7		++	+++	+++	+	+
		84·2		+++	++	+++	+	+
		78·3		++	+++	+++	+	+
Yes	None	111·5	100·0	++	+	Neg	N.T.	N.T.
		109·2		+++	+	Neg	N.T.	N.T.
		79·4		++	+	Neg	N.T.	N.T.
No	None	62·4	68·65	++	+	Neg	Neg	Neg
		68·3		++	+	Neg	Neg	Neg
		80·7		+++	+	Neg	N.T.	N.T.
		63·2		++	+	Neg	N.T.	N.T.

* Agg HGG = Heat-aggregated HGG labelled with ^{125}I.
† Detected by immunodiffusion.
Cps = counts/second in the whole spleen.
G.C. = germinal centre.
N.T. = not tested.

Immunofluorescence study (Table 2). There was no significant difference between the four groups in intensity of staining of splenic germinal centres for HGG. However, with anti-mouse IgG conjugate the ALS treated group showed no or only faint staining, whereas in the NHS treated group the staining of germinal centres for autologous IgG was bright.

Staining for horse γ-globulin in the splenic germinal centres was very bright in the NHS treated group, but negative or only faint in the ALS group. All controls were negative.

Antibody to horse serum and horse γ-globulin. Tested by immunodiffusion, the ALS treated mice had no detectable antibody to either horse serum or horse γ-globuin but 7 out of 9 of the NHS treated had.

2. *Localization of aggregated HGG in splenic germinal centres of NZB and B/W mice*

(a) *Immunofluorescence in young NZB and B/W* (Table 3). 50 μg doses of aggregated HGG were injected intravenously into fifty-five normal mice—BALB/C, $C_{57}Bl$ or CBA—

of various ages (2–10 months), seventeen B/W females (1·5–4 months) and thirteen NZB of both sexes (1·5–3 months).

All animals were tested for a direct Coombs' reaction, ANF and proteinuria, before injection of aggregated HGG. All had normal tests with the exception of seven older B/W mice which had ANF scored as +.

In spleen sections from mice of all the strains at this young age group killed 24 hr after injection, HGG was present in the germinal centres, as shown by immunofluorescence. The staining was bright, specific and well-defined, being distributed in the characteristic dendritic intercellular pattern already described (Brown *et al.*, 1970a; 1970b; Greenwood *et al.*, 1971a) and there was no apparent difference in intensity of staining between any of the strains examined. Staining for mouse γ-globulin revealed a similar but usually less bright staining pattern in the splenic germinal centres of all the mice. In four NZB mice this staining was as bright as that of the HGG.

TABLE 3. Relation between the ability of mice to localize intravenously injected aggregated HGG in splenic germinal centres and the presence of autoimmune disease

Strain	Number of mice tested	Age in months	Dose of Agg HGG (μg)	Coombs' test	ANF	Renal disease*	Splenic germinal centres stained for	
							HGG	mouse IgG
BALB/c	30	2–10	50	Neg	Neg	Neg	++	+
C_{57}Bl	15	2–10	50	Neg	Neg	Neg	++	+
CBA	10	3–10	50	Neg	Neg	Neg	++	+
B/W	17	1·5–4	50	Neg	Neg/+	Neg	++/+	+
NZB	13	1·5–3	50	Neg	Neg	Neg	++	+/++
NZB	9	10–20	50–500	+++	+/+++	+	Neg	Neg/+
B/W	15	6–8	50–500	+/++	+++	+	Neg	Neg

* Renal disease was considered to be positive when proteinuria tested by "Albustix" was ++ or stronger (correspondent to 100 mg/100 ml urine or more).

Anti-mouse IgG conjugate also gave a fainter but definite staining of the whole white pulp with the exception of the periarteriolar areas. This appeared to be due to the presence of immunoglobulins on and/or between the small lymphocytes which constitute the bulk of the white pulp.

(b) *Immunofluorescence in old NZB and B/W* (Table 3). Fifteen B/W females from 6 to 8-month old and nine from NZB 10 to 20-month old, all with features of well developed autoimmune disease—i.e. positive Coombs' and ANF tests and marked proteinuria—lacked the ability to localize aggregated HGG in the spleen at a detectable level.

Even when the dose of HGG was increased from 50 to 500 μg no localization was found in any area of the splenic white pulp of the old mice.

A similar lack of staining was apparent with anti-mouse IgG. The dendritic intercellular pattern of mouse IgG found in germinal centres of young animals had disappeared completely in the old B/W mice and in five of the oldest NZB mice. In four NZB aged 10 months faint germinal centre staining for mouse IgG was still present.

(c) *Splenic uptake of isotope-labelled aggregated HGG in old NZB and B/W mice.* When aggregated HGG labelled with ^{125}I (*Agg HGG) was used, the amount of isotope detected in the spleen correlated with the presence or absence of a fluorescent dendritic staining pattern for HGG in the germinal centres (Table 4). Thus, three old NZB, and five old female B/W mice with autoimmune disease, and three healthy adult BALB/c mice, were injected intravenously with a standard dose of 75 μg of *Agg HGG and killed 24 hr after this injection.

Splenic uptake in all but one of the diseased NZB and B/W mice was much lower than in the healthy control mice, coinciding with a lack of localization in the germinal centres by immunofluorescence. In two of the old NZB mice there was marked splenomegaly, and in one of these the whole spleen radioactivity count was normal and in the other 50% of normal. When the results were expressed as counts/second/100 mg of tissue however, the splenic uptake was shown to be depleted to the same extent in these as in the other diseased mice in this group (Table 4).

TABLE 4. Splenic uptake of 75 μg of *Agg HGG given intravenously to old healthy and diseased mice

Strain	Age in months	Autoimmune disease†	Splenic Weight (mg)	Splenic Uptake in Cps	Splenic Uptake in Cps/100 mg	Germinal centres staining for HGG
BALB/c	6	Absent	160	148·7	92·94	+++
		Absent	155	164·7	106·25	+++
		Absent	95	146·3	154·00	+++
NZB	10–14	Present	1095	163·2	14·90	Neg
		Present	1200	63·1	5·26	Neg
		Present	265	28·5	10·75	Neg
B/W	6–8	Present	240	16·6	6·91	Neg
		Present	295	44·6	15·12	Neg
		Present	295	23·2	7·86	Neg
		Present	165	20·2	12·24	Neg
		Present	155	14·3	9·22	Neg

* Agg HGG: Heat-aggregated HGG labelled with ^{125}I.

† Autoimmune disease was considered to be present when there was a positive direct Coombs' and ANF tests together with a proteinuria of ++ or stronger.

Cps = Isotope incorporation expressed as counts/second/spleen.

Cps/100 mg = Isotope incorporation expressed as counts/second/100 mg of spleen.

3. *Effect of age and disease on the splenic uptake of *Agg HGG*

(a) *NZB mice.* NZB mice were grouped according to age and tested for Coombs' and ANF positivity and presence of proteinuria. Eight mice aged between 1·5 and 2 months, twelve aged 3–7 months, two aged 8 months, nine aged 10–11 months, four aged 14 and six aged 20 months were examined. Each received intravenously 50 μg of *Agg HGG in PBS (pH 8·0) and were killed 24 hours later.

Equal numbers of mice of normal strains—BALB/c, $C_{57}Bl$ or CBA—were included as controls for each age group and tested in the same way. The mean (counts/sec) splenic uptake of *Agg HGG in the normal control mice in each age group was taken as 100% for that particular age, and individual splenic uptakes in the NZB mice in each age group were expressed as percentages of the normal. It was found that NZB mice 1·5–2-month old, although still apparently localizing HGG normally in their splenic germinal centres as judged by immunofluorescent staining, and still normal in other respects as judged by negative Coombs' and ANF tests, and the absence of proteinuria (Fig. 1), nevertheless were already by this age defective (mean uptake 66·5% of normal) in retaining *Agg HGG in their spleens (Fig. 2).

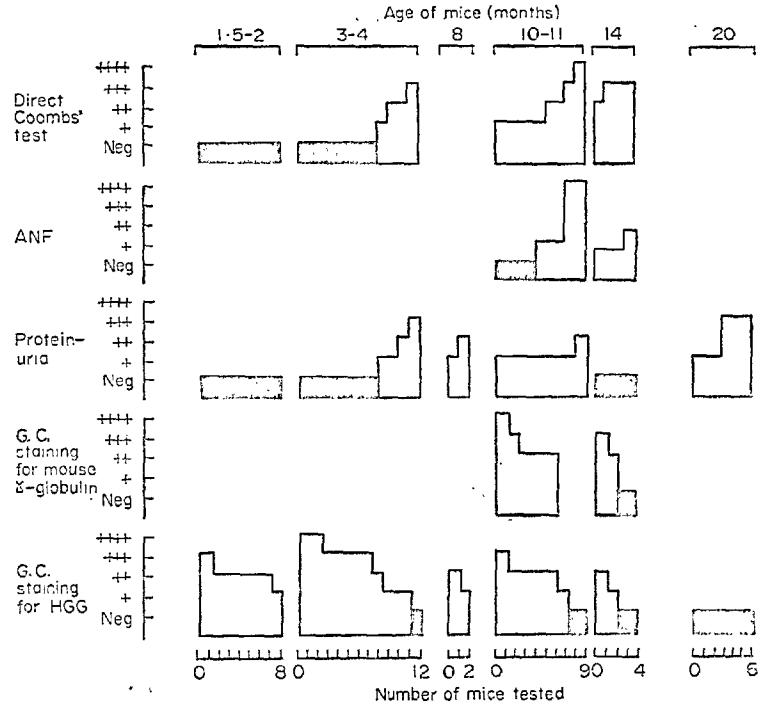

FIG. 1. The relation between direct Coombs' test, ANF, proteinuria and splenic (G.C.) germinal centre staining for HGG and mouse γ-globulin in NZB mice of different ages, injected intravenously with 50 μg Agg HGG labelled with ^{125}I. In control mice (BALB/c, $C_{57}Bl$ or CBA) germinal centre staining for mouse IgG was not more than + or ++ at any age; for HGG it was +++ or ++++ at all ages. Solid bars, negative results. Open bars, positive results.

This quantitative defect in splenic retention became steadily more marked with age and development of disease, falling to a mean of 20% of normal uptake when the mice reached 20 months of age (Fig. 2). Because old NZB mice develop marked splenomegaly this quantitative defect in their splenic retention of aggregated HGG with ageing and disease becomes more evident when the uptake for each 100 mg of splenic tissue is calculated. This falls from 65·5% of the normal uptake at 1·5–2 months to 3·5% at 20 months (Fig. 2). The fall

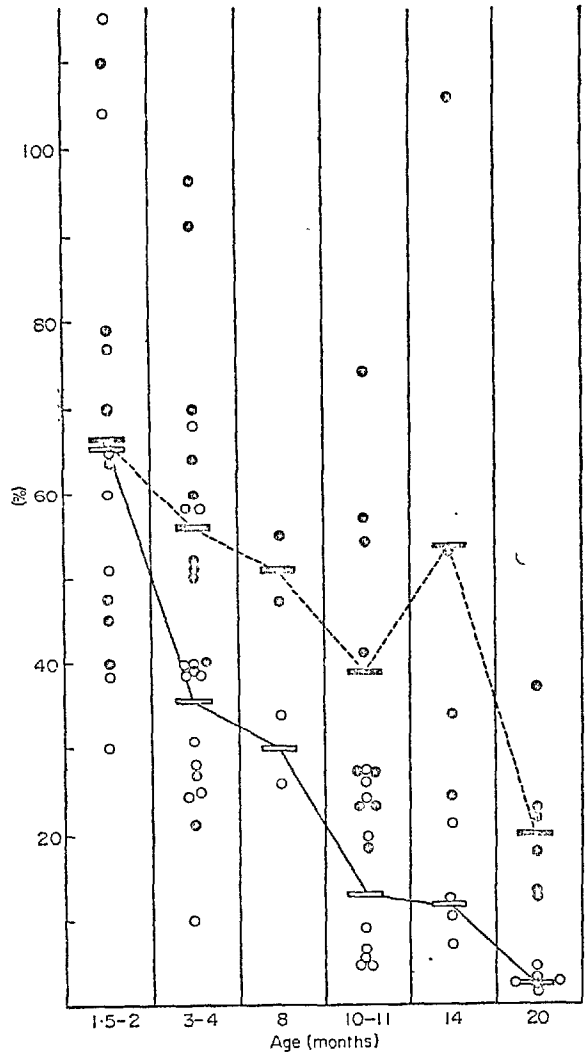

FIG. 2. 24-hr splenic uptake of aggregated HGG in NZB mice of different ages, expressed as percentages of the mean uptake of the same i.v. dose (50μg *Agg HGG) by the spleens of normal mice of the same ages. ⊙, Individual % uptake/whole spleen; solid bars, mean % uptake/whole spleen; ○, individual % uptake/100 mg of splenic tissue; open bars, mean % uptake/100 mg splenic tissue. Note that when uptake in the whole spleen (⊙) is corrected for spleen weight (○) the defective uptake is more clearly shown, i.e. the uptake curve falls off sharply with age. (—).

in uptake was accompanied by a decrease in the brightness of staining of the splenic germinal centres for HGG and mouse γ, and eventually neither was detectable (Fig. 1).

(b) *B/W female mice.* Three B/W females 1·5-month old and three groups of five females, 3–3·5, 5–6 and 7–8-month old respectively were tested and injected as above, and compared with normal control mice in the same way.

As before, the mean splenic uptake in the control mice at each age group was taken as 100% for that age, and the uptake in B/W mice was calculated as a percentage of this normal.

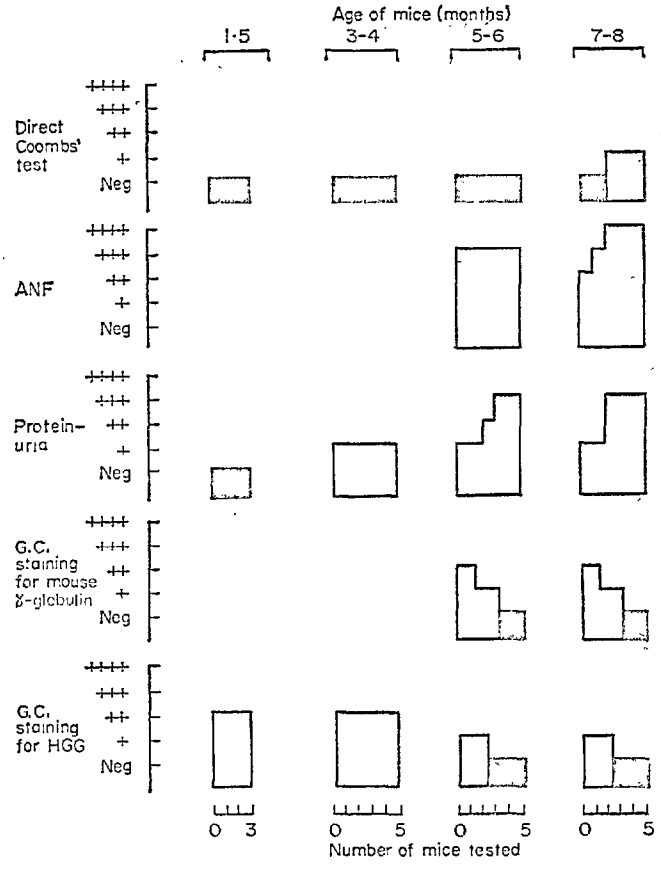

FIG. 3. The relation between direct Coombs' test, ANF, proteinuria and splenic (G.C) germinal centre staining for HGG and mouse γ-globulin in B/W female mice of different ages, injected intravenously with 50 μg Agg HGG labelled with ^{125}I. In control mice (BALB/c, $C_{57}Bl$ or CBA) germinal centre staining for IgG was not more than + or ++ at any age; for HGG it was +++ or ++++ at all ages. Solid bars, negative results; open bars, positive results.

Again we found that at an early age—1·5 month—and before any signs of disease were detected and while germinal centres staining for HGG was still apparently normal (Fig. 3), the ability to transport *Agg HGG to the spleen and to retain it there in germinal centres was diminished to 64·5% that of the normals (Fig. 4). As before the defect became more marked with age and disease, falling to 17% of the normal uptake when the mice were

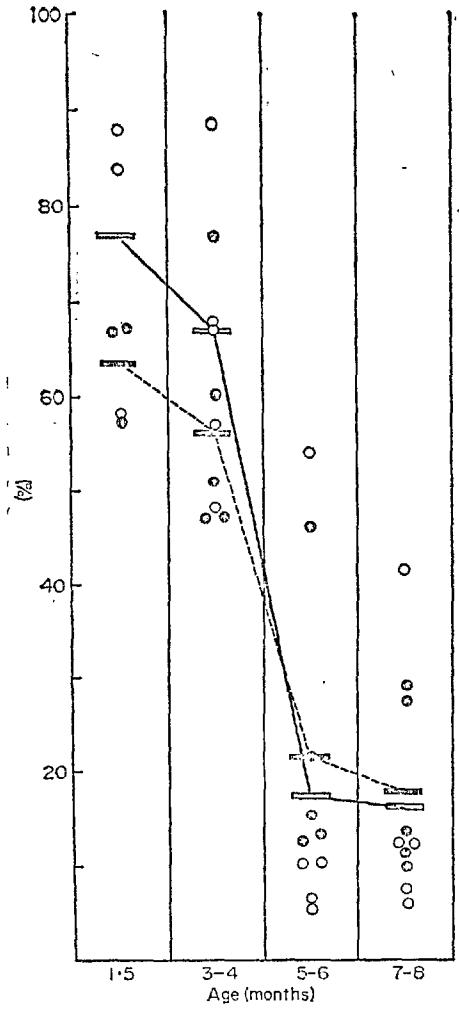

FIG. 4. 24-hr splenic uptake of aggregated HGG in B/W female mice of different ages, expressed as percentage of the mean uptake of the same i.v. dose (50 μg *Agg HGG) by the spleens of normal mice of the same ages. o, Individual % uptake/whole spleen; solid bars, mean % uptake/whole spleen; ○, individual % uptake/100 mg of splenic tissue; open bars, mean % uptake/100 mg splenic tissue. Note that because of the absence of gross splenomegaly in the B/W mice the curves for whole spleen uptake (- - -) and uptake/100 mg tissue (—) are similar.

7–8-month old. Because of the absence of gross splenomegaly in the B/W mice the curves for whole spleen uptake and uptake/100 mg of tissue were similar (Fig. 4). This decrease in splenic retention was accompanied by a decrease in the intensity of immunofluorescent staining for HGG and mouse γ in the splenic germinal centres and by the development of the signs of disease (Fig. 3).

DISCUSSION

In NZB mice histological abnormalities in the thymus at an early age have been demonstrated (DeVries & Hijmans, 1966; Holmes & Burnet, 1966) and in both NZB and B/W mice cell mediated immune responses are depressed. The data presented by various authors point to a deficiency of thymus-dependent cells increasing with age in these animals, e.g. increased survival of skin or tumour allografts (Teague *et al.*, 1970), hypo-responsiveness of spleen cells to mitogenic agents (Leventhal & Talal, 1970; Rodey, Good & Yunis, 1971), inability of the spleen cells of aged as compared with young NZB mice to induce a graft versus host reaction (Cantor, Asofsky, Talal, 1970). A depletion of long-lived lymphocytes in old NZB mice was demonstrated by autoradiographic analysis by Denman & Denman (1970) and a similar conclusion were reached by Zatz, Mellors & Lance (1971) who in a study of the migration patterns of injected ^{51}Cr-labelled lymphocytes into lymphoid organs found a decreased recirculating cell content in lymph nodes and spleens from ageing NZB as compared with CBA mice. Using the regression time of virus-induced murine sarcomas as an indication of immunological competence, Gazdar, Beitzel & Talal (1971) confirmed that cell mediated immune responses are depressed in aged NZB and B/W mice, and found moreover that this depression precedes the onset of detectable autoimmune disease. All these findings indicate that NZB and B/W mice become relatively deficient in thymus-dependent lymphocytes.

There is good evidence from animal experiments that thymectomy or depletion of thymus-derived lymphocytes may lead to the development of autoantibodies. Thus neonatally thymectomized rabbits (Sutherland *et al.*, 1965) and mice (Teague, 1967; Thivolet *et al.*, 1967) often develop positive Coombs' test and antinuclear antibodies, and the development of antinuclear antibodies that occurs naturally in ageing A/J mice (Teague & Friou, 1964, 1969) is reversed by transfer of thymus cells from young syngeneic mice. Furthermore, with chronic administration of ALG to C_{57}Bl mice antinuclear antibody appears (Denman, Denman & Holborow, 1966), and the transient induction of a positive Coombs' test in young NZB recipients of old NZB spleen cells is rendered permanent if the young recipient NZB have received ALG treatment (Holborow & Denman, 1968).

It has already been suggested that the bone marrow derived-lymphocyte population also is abnormal in these mice. Zatz *et al.* (1971) reported that after the age of 3 months and coinciding with the onset of autoimmunity, the number of non-recirculating cells in the spleens of NZB mice sharply increases as compared with normal mice, while the recirculating population falls. Staples, Steinberg & Talal (1970) however have produced evidence that by the same age the bone marrow-derived lymphocytes of NZB mice are already less easily made tolerant to BGG than those of normal mice, even in the presence of competent thymus-derived cells. Furthermore, Cerottini *et al.* (1969) have shown that B/W mice are poor responders to keyhole limpet haemocyanin, an antigen thought to be thymus independent (Taylor, 1969).

We have reported previously that the transport of Agg HGG and immune complexes into germinal centres of lymphoid tissue is mediated by mobile lymphocytes that have receptors for IgG altered either by mild heat aggregation or by complexing with antigen (Brown *et al.*, 1970a; 1970b). We also found that mice infected with malaria were unable to localize either Agg HGG or their own immune complexes into their germinal centres during the period of humoral immunosuppression that coincides with the peak of para-

sitaemia (Greenwood *et al.*, 1971a) although their cell mediated immune response remained unaffected (Greenwood, Playfair & Torrigiani, 1971b). This finding suggested to us that the cells responsible for the transport of Agg HGG or immune complexes into germinal centres are more likely to be cells of the bone marrow-derived than of the thymus-dependent population of lymphocytes.

The results presented here support this idea. Thus, thymus-deprived mice (thymectomized and chronically ALS injected, or thymectomized, lethally X-irradiated and bone marrow reconstituted) are able to transport and localize Agg HGG and immune complexes into their splenic germinal centres at least as well as normal mice. Indeed, the isotope experiments suggest (Tables 1 and 2) that thymectomy may even increase the uptake in germinal centres. Thymectomized lethally X-irradiated and bone marrow reconstituted mice have been shown by Davies (1969) to have only the thymus-independent zones of their lymphoid tissues repopulated, and by chromosome marker studies the repopulating cells were proved to be descendants of the bone marrow donor cells. Our experimental results thus point to the conclusion that the localization of Agg HGG and of autologous immune complexes in germinal centres depends upon transport by bone marrow-derived lymphocytes.

In the work reported here we have found that in NZB and B/W mice localization is defective from an early age, even before the onset of autoimmunity. Since in both types of mice germinal centres are present (East, 1970) and apparently normal, it must be concluded that there is a fault in the transport mechanism and therefore that a functional abnormality of the bone marrow cell population exists in these animals which antedates the onset of autoimmune disease.

This conclusion is in accordance with the suggestion of Staples *et al.* (1970) that during the process of immunological maturation in the NZB mice not only the thymus-dependent but also the bone marrow lymphocytes are affected, and our results in both NZB and B/W mice indicate that these defects combine to contribute to the ultimate emergence of the autoimmune disease.

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