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Selective depression of thymus-independent anti-DNP antibody responses induced by adult but not neonatal splenectomy

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SUMMARY

The effect of splenectomy was assessed on rats' capacity to respond to 2,4-dinitrophenol (DNP) conjugated to spider crab haemocyanin (MSH) or hydroxyethyl starch (HES). The response to DNP–HES was profoundly suppressed in all immunoglobulin classes and subclasses by adult splenectomy. This loss of responsiveness increased with time after removal of the spleen. By contrast no significant effect on the anti-DNP–MSH response was caused by splenectomy. Experiments in congenitally athymic rats confirmed the thymus dependency of anti-DNP antibody responses of all classes and subclasses induced by DNP–MSH. DNP–HES responses, however, were elevated in athymic animals. The effect of adult splenectomy in these animals was considerably less than that produced in euthymic rats. Neonatal splenectomy in euthymic rats only resulted in minor impairment of these rats' capacity to respond to DNP–HES as adults, indicating the ability of rats to compensate after neonatal but not adult splenectomy. These data are taken to show that the spleen plays an important role in responses against this thymus-independent type II antigen which is only partially replaceable by other compartments of the immune system.

Keywords marginal zone B cells splenectomy thymus-independent antigen

INTRODUCTION

The marginal zones of rat spleens contain a population of B lymphocytes which appears to develop as a distinct lineage from the recirculating B cells found in the follicles of all secondary lymphoid organs (Gray *et al.*, 1982; MacLennan *et al.*, 1982). A number of lines of indirect evidence suggest that these cells may participate in antibody responses against certain carbohydrate antigens. (1) Injected neutral polysaccharides selectively localise in dendritic cells situated in the marginal zones of mice (Humphrey & Grennan, 1981) and rats (Gray *et al.*, 1984). (2) IgG2c is a common surface membrane immunoglobulin on rat marginal zone B cells which also characteristically bear surface IgM but not IgD (Bazin *et al.*, 1982). IgG2c is particularly associated with antibody responses against thymus-independent type II antigens which include antigens such as 2,4-dinitrophenol (DNP)-Ficoll and pneumoccoccal polysaccharide (Der Balian *et al.*, 1980). (3) Selective suppression of the development of recirculating follicular B cells but not marginal zone B cells can be achieved in rats by administration of rabbit anti–rat IgD antibody from birth. These animals show augmented levels of IgG2c but lack IgG2a (Bazin *et al.*, 1982). The latter is particularly associated with thymus-dependent antibody responses in rats (Der Balian *et al.*, 1980). (4) Splenectomy in man is

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associated with an increased risk of developing fulminating pneumococcal septicaemia (King & Schumacker, 1952; Singer, 1973; Dickerman, 1976; Krivit, Giebink & Leonard, 1979, Francke & Neu, 1981).

There is no convincing evidence for the presence of anatomical structures equivalent to the marginal zones in secondary lymphoid organs other than the spleen. This report describes a study which investigates the effect of splenectomy in antibody responses against the thymus-independent antigen DNP–hydroxyethyl starch (DNP–HES). It is shown that adult splenectomy profoundly depresses production of anti-DNP antibody of all classes and subclasses induced by this antigen. However, antibody responses to the thymus-dependent antigen DNP–haemocyanin (DNP–MSH) are not obviously affected. Neonatal splenectomy and splenectomy in adult athymic rats by contrast has comparatively little effect on anti-DNP–HES responses.

MATERIALS AND METHODS

Animals. (LOU × DA)F_1 hybrid rats, derived from highly inbred parental strains were used in the majority of immunizations described in this paper. These animals were bred and maintained under standard laboratory conditions in the animal house of the Department of Immunology, University of Birmingham. Experiments in athymic rats were carried out at the Institut de Recherches Scientifiques sur le Cancer using outbred rats having the rnu mutation. Groups of rnu/rnu athymic rats and rnu/+ rats with a thymus were kept under specific pathogen free (SPF) conditions. Within any one experiment rats were age and sex matched.

Adult and neonatal splenectomy. Newborn rats (within 3 days of birth) were anaesthetized by placing on a block of ice, for about 5 min before the operation. A small incision was made through the skin and abdominal wall. The stomach is held gently and pulled to the exterior, the spleen followed and was easily accessible. This procedure reduced the risk of disruption of the spleen. A hot needle was used to burn through the splenic blood vessels and membranes holding it in place. Cauterization reduced the bleeding and reduced the risk of seeding of fragments of spleen into the peritoneum. The abdominal wall was closed with two or three stitches and the skin glued together with isobutyl-2-cyanoacrylate monomer tissue adhesive (Ethicon Ltd, Edinburgh, UK). Finally Nobecutane plastic skin (Astra Chemicals Ltd, Watford, UK) was sprayed over the wound. Sham operated animals had their spleens mobilised in the same way before closing the wound. Animals were left to recover under a 100 W light bulb. The risk of rejection by the mother was reduced by spraying Nobecutane into the cage prior to reunion with offspring. These rats were subsequently immunized at 3–4 months of age.

Adult splenectomy was carried out by a standard technique. The blood vessels in the splenic peduncle were tied off with catgut and the spleen removed. The wound was stitched and skin clips applied. These animals were used at various times after operation.

Antisera. Rabbit antibodies to rat IgM, IgD, IgG1, IgG2a, IgG2b, IgG2c and IgA were prepared and their specificity tested as described by Bazin, Beckers & Querinjean (1974) and Bazin *et al.* (1978). A sheep anti-rabbit immunoglobulin antiserum used as a final layer in the radioimmunoassay was prepared in the Immunodiagnostic Research Laboratory, Department of Immunology, University of Birmingham and labelled with ^{125}I using the chloramine-T method (Hunter & Greenwood, 1962).

Antigens. Dinitrophenylated hydroxyethyl starch (DNP–HES) was a kind gift from Professor J. H. Humphrey, Royal Postgraduate Medical School, London and was prepared as described in Humphrey (1981). Spider crab (*Maia sqinada*) haemocyanin (MSH) was also provided by Professor J. H. Humphrey and was dinitrophenylated using a technique to obtain high substitution ratios. Fifty micrograms of MSH was diluted in 10 ml of 0·1 M borate buffer pH 8·5. This solution is made 10% by weight in 2,4-dinitrofluorobenzene (DNFB, Sigma, UK). The DNFB was supplied dissolved in oil, thus the mixture was warmed at 37°C for 3 min to encourage it to go into aqueous solution. It is then stirred, at room temperature for 1 h. The solution was then extensively dialysed at 4°C against saline to remove free DNFB. Substitution ratio of 150 DNP:1 MSH was obtained, assuming a mol. wt of 10^6 for MSH.

DNP-ovalbumin (DNP–OA) used for coating radioimmunoassay plates was prepared in the same manner except that the mixture was made 25% by weight in DNFB. This produced a substitution ratio of 9 DNP:1OA.

Immunizations. DNP–HES was injected i.v. in aqueous solution. Dose–response and time course experiments showed the optimum dose to be 5 μg per rat and the peak primary response to occur at 10 days (Gray, 1983) after which this level of antibody was sustained for 6 weeks without further immunization. This dose and the 10 day response was used in all experiments. Boosting with this antigen produces little augmentation of responses.

The primary injection of DNP–MSH was given i.p. in the form of an alum precipitate together with 5×10^9 chemically killed *Bortatella pertussis* organisms as adjuvant (Pertussis vaccine, Wellcome, UK). Secondary immmunization with 50 μg of DNP–MSH was in aqueous solution via the i.v. route 3 weeks after priming. Dose–response and time course experiments revealed an optimum primary dose of 50 μg DNP–MSH and a peak response at approx 15 days after the boost.

Radioimmunoassay. Fifty microlitres per well of DNP–OVA diluted to 50 μg/ml in 0·1 M carbonate buffer, pH 9·5, was used to coat polystyrene multiwell plates (Linbro, Flow Laboratories) at 4°C overnight. The plates were washed five times in PBS+0·2% bovine serum albumin (BSA). Dilutions of test serum in duplicate, were placed in the top row of wells and then serial dilutions made, the final volume per well was 50 μl. After 1 h incubation, at room temperature, the plates were washed (five times) and the second layer antibody added (rabbit anti-rat IgM, IgG1, IgG2a, IgG2, IgG2c or IgA) again for 1 h. Washing, as before, was followed by addition of the final layer (^{125}I-sheep anti-rabbit IgG) for similar period at room temperature. After a final washing step the plates were dried and cut up and wells counted on an LKB Mini Gamma Counter.

Titres we calculated by taking the dilution of antiserum giving 30% of maximum binding obtained with a positive control antiserum. Pre-immunization titres were also measured. Their arithmetic titre was subtracted from the arithmetic post-immunization titre if the increase in titre was less than four-fold. Student's *t*-test was used as a measure of statistical significance of the difference between groups throughout this study.

RESULTS

Immunoglobulin isotypes of anti-DNP antibodies induced by DNP–HES and DNP–MSH

This is illustrated in all the experiments described in this report (Figs 1, 2, 3 & 4). Both antigens induce anti-DNP antibodies of IgM and IgA class and all IgG subclasses. The ranking of antibodies with the various heavy chain isotypes is different for the two antigens. For DNP–HES anti-DNP IgG2c > IgM > IgA = IgG1 = IgG2b > IgG2a. There is some variation between experiments in the level of IgG2a anti-DNP induced by DNP–HES. By contrast anti-DNP antibodies induced by DNP–MSH when ranked by isotype were IgG2a > IgG1 = IgG2b > IgM = IgA = IgG2c.

DNP–HES primary responses were little increased after boosting, while DNP–MSH responses were greatly augmented by secondary immunization. After boosting the DNP–MSH responses were higher in terms of anti-DNP titre than those induced by DNP–HES. IgG2c anti-DNP titres induced by DNP–MSH (the lowest isotype induced by DNP on this carrier) were equivalent to those induced by DNP–HES, where IgG2c is the highest ranking isotype.

Thymus dependency of anti-DNP antibody induced by DNP–HES compared with DNP–MSH

A series of athymic animals, homozygous for the mutation rnu, were used to detect thymus dependancy of these antigens. Controls were euthymic, heterozygous (rnu/+) littermates. No rise above background anti-DNP antibody titres was seen after either primary or secondary immunization with DNP–MSH (10 rats, two experiments; data not shown), i.e. the induction of antibody production to this antigen appeared to be strictly thymus-dependent. By contrast the euthymic litter mate controls produced a normal response to DNP–MSH. Athymic animals gave augmented responses to DNP–HES compared to those in euthymic controls rats (Fig. 2; nine rats, two experiments). The levels of anti-DNP IgG2c were significantly higher in nude rats than euthymic controls for both experiments ($P < 0.05$).

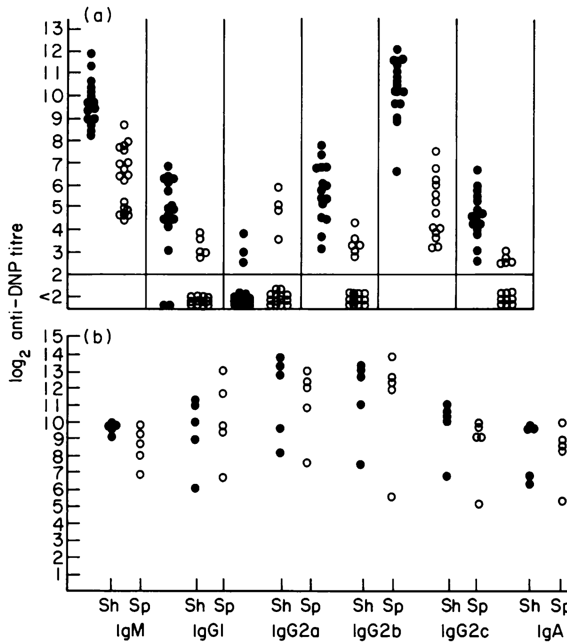

Fig. 1. (a) Serum anti-DNP antibody response to DNP–HES in normal and adult splenectomized rats. Sh = sham operated animals; Sp = splenectomized animals. Each dot represents one animal. All differences between subclass responses in splenectomized and sham operated groups are significant at the 0·1% level, except IgG2a which shows no difference and IgA ($P < 0{\cdot}01$). (b) Serum responses to DNP–MSH following adult splenectomy.

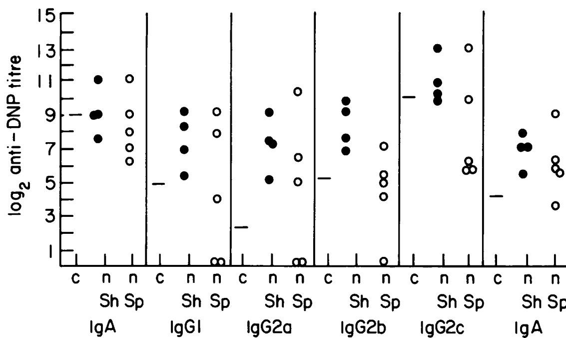

Fig. 2. Serum anti-DNP antibody responses to DNP–HES in intact and adult splenectomized athymic (nude) rats. Immunization was 2 weeks after operation. C = rnu/+ euthymic rats which had been sham operated median values for nine rats shown in this group for clarity; nsh = rnu/rnu athymic rats which had been sham operated; nsp = rnu/rnu athymic rats which had been splenectomized. Each dot represents one animal. Significant differences: IgG2b nsh > nsp $P < 0{\cdot}05$. IgG2b c < IgG2bnsh $P < 0{\cdot}05$, IgG2c c < nsh $P < 0{\cdot}05$.

The effect of neonatal splenectomy on the ability of rats to respond as adults to DNP–HES and DNP–MSH

Groups of rats were subjected to splenectomy or sham splenectomy on their day of birth as described in the methods. Four months later they were immunized with either DNP–HES or DNP–MSH. The results are shown in Fig. 3. In respect to DNP–HES there is a slight trend to reduction of anti-DNP antibody response. In one experiment there was significant ($P < 0{\cdot}05$)

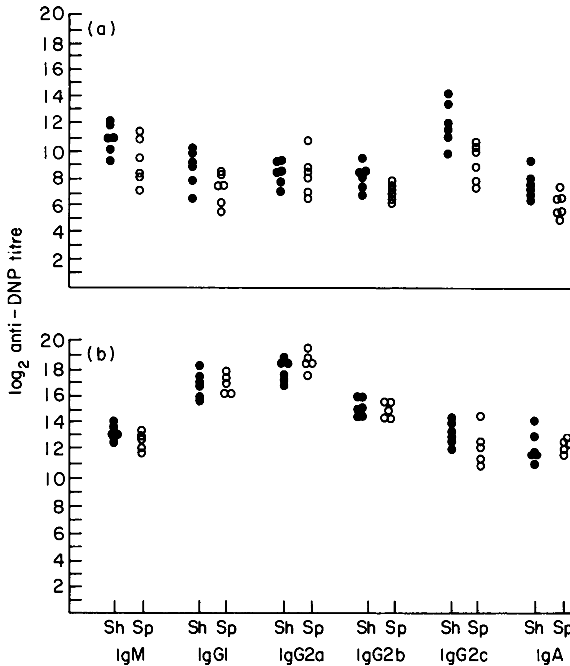

Fig. 3. (a) Serum anti-DNP response to DNP–HES in neonatally splenectomized (Sp) and sham operated (Sh) inbred rats [(Lou/DA) F_1 hybrids], 10 days after primary immunization. Differences between sham and splenectomized groups are significant in IgM ($P<0{\cdot}05$), IgG2b ($P<0{\cdot}02$), IgG2c ($P<0{\cdot}01$) and IgA ($P<0{\cdot}05$) subclass responses. (b) Serum response to DNP–MSH in neonatally splenectomized (Sp) and sham operated (Sh) rats, 15 days after secondary immunization. There are no differences between splenectomized and sham operated groups. Each dot represents one animal.

diminution of the IgG2c anti-DNP antibody levels but this was not confirmed in the second experiment.

Little or no effect of splenectomy was seen on the rats immunized with DNP–MSH on the levels of anti-DNP antibody produced (Fig. 3b).

The effect of adult splenectomy on the anti-DNP response of rats to DNP–HES and DNP–MSH
Adult splenectomy, in contrast to neonatal splenectomy produced a profound reduction in anti-DNP-HES responses in euthymic rats (Figs. 1a & 4). This depression was most marked for IgG2c but significant depression was seen for all isotypes except IgG2a where control levels of antibody response were already low. In comparison to the effect of adult splenectomy on the response to DNP–HES in euthmyic rats, splenectomy in nude rats appeared to cause relatively less suppression (Fig. 2).

DNP–MSH responses were not influenced following adult splenectomy (Fig. 1b).

The inability of rats to recover their responsiveness to DNP–HES with time after adult splenectomy
Neonatally splenectomized rats produce near normal anti-DNP-HES responses 4 months after splenectomy. To see if rats, subjected to adult splenectomy, were also able to recover, with time, their capacity to respond to DNP–HES, groups of rats were immunized at various intervals after splenectomy. The loss of DNP–HES responsiveness was found to be more profound 3 months after adult splenectomy than 14 days post-splenectomy (Fig. 4).

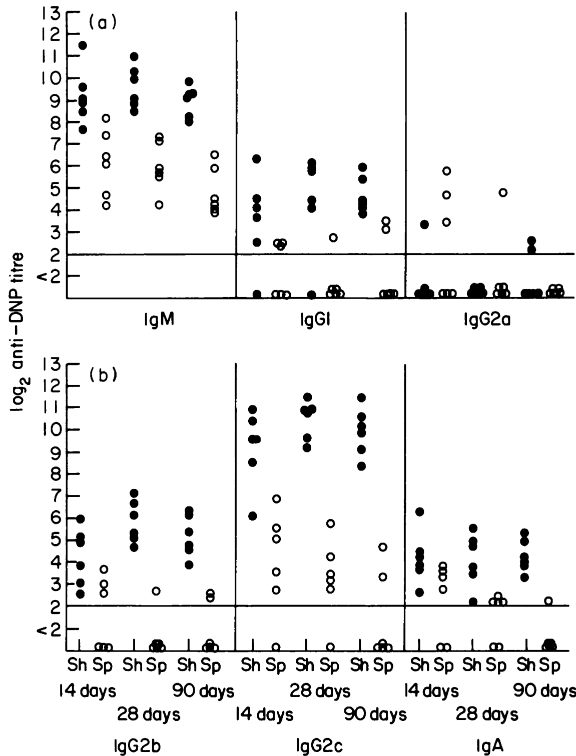

Fig. 4. (a & b). Serum anti-DNP response to DNP–HES with immunization 14, 28 and 90 days after splenectomy. Differences between responses in splenectomized (Sp) and sham operated (Sh) groups showed the following levels of significance: 14 days; IgM $P<0{\cdot}01$, IgG1 $P<0{\cdot}05$, IgG2a NS, IgG2b $P<0{\cdot}02$, IgG2c $P<0{\cdot}001$, IgA $P<0{\cdot}05$. 28 days; IgM $P<0{\cdot}001$, IgG1 $P<0{\cdot}001$, IgG2a NS, IgG2b $P<0{\cdot}001$, IgG2c $P<0{\cdot}001$, IgA $P<0{\cdot}01$. 90 days; IgM $P<0{\cdot}001$, IgG $P<0{\cdot}001$, IgG2a NS, IgG2b $P<0{\cdot}001$, IgG2c $P<0{\cdot}001$, IgA $P<0{\cdot}001$. NS = not significant. Each dot represents one animal.

DISCUSSION

This study shows that adult splenectomy causes marked and lasting impairment to the capacity of rats to produce anti-DNP antibodies, when immunized with DNP on a neutral polysaccharide. Conversely adult splenectomy does not impair rats' ability to respond to DNP on a protein carrier (MSH). Among possible reasons for this effect are: (1) loss of cells which can present antigens on a neutral polysaccharide carrier; (2) elimination of the splenic marginal zone B cell compartment and (3) loss of a subset of B cells that is dependent on the lymphopoietic capacity of the red pulp for its maintenance in adult rats. There is some evidence that all three of these factors may be of importance.

Humphrey & Grennan (1981) in their studies of carbohydrate localization in secondary lymphoid organs were first to describe the selective uptake of neutral polysaccharides in the marginal zones of mouse spleen. Subsequently cells with the same capacity have been demonstrated in the marginal zones of rats (Gray *et al.*, 1984). This localization is in dendritic cells which do not express class II histocompatibility antigens (Humphrey & Grennan, 1981). Although there is no direct evidence that these are antigen presenting cells, the selective effect of adult splenectomy on antibody responses to DNP–HES is compatible with this possiblity. Sporadically, small numbers of cells localizing neutral polysaccharides are found in the area immediately deep to the subcapsular sinus of lymph nodes (Humphrey & Grennan, 1981). A systematic search for these cells in secondary lymphoid organs is called for in neonatally splenectomized and nude rats compared to adult

splenectomized and normal rats. The current study has shown that nude rats and neonatally splenectomized rats retain substantial capacity to respond to DNP–HES in the absence of a spleen. Careful search for splenic regrowth was made in all splenectomized animals at sacrifice. This was not the explanation for the relatively trivial effect of neonatal splenectomy on subsequent response to DNP–HES. Consequently, in these rats the cells and microenvironment necessary for anti-DNP–HES must have developed outside the spleen. The ability of athymic splenectomized and neonatally splenectomized rats to respond to DNP–HES indicates that i.v. immunization, as used in this study, was not a bar to this antigen gaining access to extra-splenic sites of responsiveness.

Antigen presenting cells by themselves are clearly not enough to achieve antibody production. What are the B cells activated by this antigen? If DNP–HES is presented on dendritic cells in the marginal zone both recirculating B cells (Nieuwenhuis & Ford, 1976) and marginal zone B cells will have access to these antigens. If it is the recirculating B cells which respond to DNP–MSH, it is clear from this study that they require T help as well as sight of DNP to be activated. However, the dendritic cells of the marginal zone, which localise HES, lack class II histocompatibility antigens and as such may not be able to stimulate T cell help for recirculating B cells. The splenic marginal zone B cells, by contrast, may have the capacity to respond to antigen on marginal zone dendritic cells in the absence of T cell help. Certainly the results of this study using DNP–HES and those of other workers using similar antigens (DNP-Ficoll) show that the induction of this response is not thymus-dependent (Sharon *et al.*, 1975; Mongini, Stein & Paul, 1981; Zinkernagel & Blanden, 1975).

As marginal zone B cells do not recirculate (Gray *et al.*, 1982; Kumararatne, Bazin & MacLennan, 1981) they do not have access *in vivo* to antigen presenting cells which carry class II antigens. Characteristically these interdigitating cells are located in the paracortex and interfollicular areas of lymph nodes, afferent lymph, body surfaces and the periarteriolar lymphocytic sheath of the spleen. In spleen cell suspensions *in vitro* marginal zone B cells would come into contact with class II positive interdigitating cells. *In vitro* several workers have demonstrated that both IgM^{+}, IgD^{+} and IgM^{+}, IgD^{-} cell populations respond to DNP-Ficoll (Layton *et al.*, 1979; Layton, Teale & Nossal, 1979; Marshall-Clarke, Keeler & Parkhouse, 1983). Similarly, the T cell dependence of *in vitro* responses to TI-2 antigens (Mond *et al.*, 1980, 1983; Nordin & Schreier, 1982) may only reflect the requirement of IgM^{+}, IgD^{+} recirculating, follicular B lymphocytes.

A further indirect line of evidence linking marginal zone B cells with the DNP–HES response is the dominance of IgG2c in this response and the relative lack of IgG2a. This isotype association has previously been reported by Der Balian *et al.* (1980) and Slack *et al.* (1980). A significant minority of marginal zone B cells express IgG2c (Bazin *et al.*, 1982). Also, rats treated from birth with anti-IgD antibodies develop marginal zone B cells but are deficient in recirculating B cells. These rats have very low levels of IgG2a but elevated levels of IgG2c (Bazin *et al.*, 1982).

The progressive loss of DNP–HES responsiveness with time following adult splenectomy was an unexpected finding. It also contrasts with the capacity of neonatally splenectomized animals to respond to DNP–HES. This finding raises the possibility that the spleen might be the source, in adults, of B cells of the marginal zone and/or marginal zone dendritic cells. Recent unpublished experiments with irradiation bone marrow chimaeras using genetically marked bone marrow donor cells have shown that marginal zone dendritic cells are derived from bone marrow (J. H. Humphrey, personal communication). Our own preliminary, unpublished experiments with short term bone marrow chimaeras, between congenic strains of rats differing at the kappa light chain allotype, indicate that adult bone marrow is not readily capable of generating marginal zone B cells. However, studies of chimaeras for periods of greater than 14 days are required before any firm conclusions can be drawn.

There is evidence, on the other hand, that B cell lymphopoiesis in the spleen is associated with a different sequence of surface membrane immunoglobulin expression from that of bone marrow. Aspinall & Owen (1983) showed that newly formed B cells in adult bone marrow expressed first IgM and then IgD before leaving their site of generation. We have shown, in rats deprived of B cells by neonatal and continued administration of anti-rat IgM and IgD, that B cells produced in the spleen, after stopping suppression, express IgM and IgM plus IgA. They do not express surface IgD while in the splenic red pulp. Further evidence indicates that marginal zone B cells and recirculating B

cells represent different B cell lineages: (1) suppression from birth with anti-IgD allows the development of marginal zone but not follicular B cells (Bazin *et al.*, 1982) and (2) rats treated with rabbit anti-rat IgM antibodies from birth have suppressed B cell development. However, in some animals after some weeks treatment follicular B cells do develop but we have not seen marginal zone B cells in these animals (Chassoux *et al.*, 1983).

In conclusion, the present experiments link the spleen with antibody responses to DNP presented on a neutral polysaccharide. Evidence cited in the discussion indicates that this may reflect a distinct antigen presenting and antigen responsive system located in the microenvironment of the splenic marginal zone.

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