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NORMAL TOLERANCE CHARACTERISTICS OF THE ANTIBODY-FORMING CELL PRECURSORS OF THE NZB MOUSE

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SUMMARY

Splenic PFC dose-response curves were measured in normal mice and in mice of the autoimmune NZB strain for the thymus-independent antigens pneumococcal polysaccharide type 3 (SIII) and bacterial levan. There were differences between the strains in the maximal response achieved to each antigen, but the level at which high dose tolerance occurred was the same in all the strains.

The splenic anti-sheep erythrocyte plaque-forming cell dose-response curve of lethally-irradiated, bone marrow restored NZB mice was compared with that of (C57/Bl × BALB/c)F_1 hybrid and (NZB × BALB/c)F_1 hybrid mice. Although the dose-response curves of the two hybrid stains were less sharp than that of the NZB, the peak response occurred at the same dose of SRBC (10^8, i.v.), and reached the same level, tailing off at higher doses.

These results indicate that the B cells of NZB mice display normal high-dose tolerance characteristics, at least to these three antigens.

INTRODUCTION

The NZB strain of mouse, first described by Bielschowsky, Helyer & Howie in 1959, has become well known in immunological circles for the autoimmune manifestations which spontaneously develop as it ages. Coombs' positive autoimmune haemolytic anaemia and renal disease due to glomerular deposition of antigen–antibody complexes develop in virtually all animals during the first year (Helyer & Howie, 1963) and antinuclear antibodies are found in many (Norins & Holmes, 1964). This disposition towards autoimmunity appears to be genetically governed (Ghaffar & Playfair, 1971) although not in a simple way, and certain hybrid strains, notably the NZB × NZW hybrid, show some of these features (Lambert & Dixon, 1968).

Although intensive work has been carried out on the immune system of these mice, the exact nature of their abnormality remains obscure. Immune responses have been measured

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to a variety of antigens with conflicting results in terms of the relative height of the antibody response, the mice giving higher than normal responses to some antigens (Baum, 1969; Staples & Talal, 1969; Weir, McBride & Naysmith, 1968), and lower than normal responses to others (Cerottini, Lambert & Dixon, 1969).

Perhaps of more relevance to the development of autoimmunity is the relatively poor susceptibility to high dose tolerance induction which these mice show. This has been described for two soluble protein antigens, bovine serum albumin (in the NZB mouse) (Weir *et al.*, 1969), and ultracentrifuged bovine gamma globulin (in the NZB × NZW hybrid) (Staples & Talal, 1969). Playfair (1971) has demonstrated a raised tolerance threshold of NZB thymocytes to SRBC in an irradiation reconstitution system, and Jacobs, Gordon & Talal (1971) have described the failure of SRBC and cyclophosphamide to tolerize NZB hybrid thymocytes where those of a control strain did become tolerant.

Less work has been carried out on their bone-marrow-derived, antibody-forming cell precursors, or B cells, although it was found by Staples, Steinberg & Talal (1969), that adult (NZB × NZW)F_1 bone-marrow cells could transfer resistance to tolerance induced by large doses of soluble bovine gamma globulin, where bone-marrow from young mice did not. This perhaps suggests that the B cells show a defect in tolerance induction also. However, the possibilities of some influence from the thymus or other tissues on the bone-marrow before transfer or of transmission of the resistance by thymocyte precursors were not excluded.

The present study was designed to answer this question: Do NZB B cells have an abnormally high tolerance threshold corresponding to that of their thymus-derived cells? To measure such a threshold for SRBC, bone-marrow cells and antigen were transferred into X-irradiated syngeneic hosts and the 8-day splenic plaque-forming cell response assayed. Since it is not unlikely that there are differences in the way B cells react to different types of antigen, two other studies were undertaken. The immune responses to pnemococcal polysaccharide type 3 (SIII) and to the bacterial polysaccharide levan require no help from T cells, although the characteristics of the high dose tolerance which can be induced by them appear to be different (Howard, 1972). Dose-response studies in intact mice should therefore reveal the response characteristics of the B cells on their own.

The BALB/c and its hybrid with the C57/Bl mouse were used as normal mice with which to compare the responses of the NZB and the (NZB × BALB/c)F_1 hybrid to these antigens. No abnormal tolerance threshold to any of these antigens was detected in either the NZB mouse or its hybrid.

MATERIALS AND METHODS

Mice

NZB mice were originally obtained from Dr M. Bielschowsky at the 57th inbred generation. After four generations at the Laboratory Animals Centre, Carshalton, they were maintained in the Middlesex Hospital Medical School Immunology Department animal colony by brother–sister mating. Other mouse strains were originally obtained from Carshalton, and hybrids were bred in the colony. Male mice 3–6 months old were used in all experiments except those involving irradiation.

X-irradiation-reconstitution procedure

Three to 4-month-old female mice weighing 24–30 g received 850 rads (of 250 KVP X-rays,

with 1 mm Al and 1 mm Al added filtration) in individual perforated lucite tubes in groups of twenty. Donor marrow was obtained from syngeneic male mice. Cell plugs were gently expressed from the femurs with ice cold Eagle's MEM via a 23-gauge needle, and cell clumps dispersed by gentle syringing through a 25-gauge needle. After washing, the cells were counted and 15×10^6 cells injected with varying numbers of SRBC into the lateral tail vein of each mouse. All mice received an intraperitoneal boost of 4×10^8 SRBC four days later. They were killed and spleens assayed for anti-SRBC PFCs on the eighth day.

Reagents

Purified SIII was obtained from Wellcome Reagents Ltd, as were SRBC in Alsever's solution and freeze-dried guinea-pig serum. Levan, isolated from *Enterobacter levanicum* according to the technique of Hehre *et al.* (1945) and O-stearoyl levan prepared by the method of Hämmerling & Westphal (1967) were kindly donated by Dr J. Howard.

Antigen coating of SRBC

SIII. The $CrCl_3$ method of Baker, Stashak & Prescott (1969) was used. Optimal coating of the SRBC was found to depend critically on the final pH of the reaction mixture which consisted of four-times-washed, 2-week-old SRBC, $CrCl_3$ solution and SIII. Consistent results were finally obtained by dissolving the SIII in 0·25 M piperazine buffer, pH 6·2, at a concentration of 1·2 mg/ml. After a 5-min incubation at room temperature the red cells were washed four more times in saline and were ready for use.

Levan

Three-times-washed 3–7-day-old SRBC were incubated with 100 μg of O-stearoyl levan per ml of packed red cells in phosphate-buffered saline, for 35 min at 37°C, and washed three times before use.

PFC assay

A slight modification of the plaque assay of Jerne & Nordin (1963) was used for the measurement of anti-SRBC PFCs. It was found unnecessary to wash spleen cells for the anti-SRBC assay, but those from mice which had received SIII or more than 100 μg of levan were washed three times to remove non-metabolized antigen. Each cell suspension was assayed on both control and sensitized SRBC, and the anti-SRBC PFCs subtracted to give the number of antigen-specific PFCs. Spleens assayed for anti-levan activity were incubated for 2 hr before, and 1 hr after the addition of 2 ml of complement to each plate and were gassed with a mixture of 5% CO_2 in air. Anti-SIII PFCs were fully developed after a total incubation time of $1\frac{1}{2}$ hr in air. Pooled, SRBC-absorbed, fresh-frozen guinea-pig serum diluted 1 in 10 with phosphate-buffered saline was found to be necessary for the maximal development of both the anti-levan and the anti-SIII plaques, whereas preserved guinea-pig serum was adequate for developing the anti-SRBC plaques.

Anti-θ serum

C3H anti-θ AKR serum was raised by the method of Raff (1969) and had a cytotoxic titre of 1 in 32 against thymocytes. It always killed more than 92% of thymocytes included as a control for each experiment. After treatment with anti-θ serum and preserved guinea-pig serum which had been absorbed with agarose, to remove cytotoxic activity against

mouse lymphocytes (Cohn & Schlesinger, 1970), aliquots of cells were taken for the measurement of cytotoxicity. In no case was there any increase in the proportion of dead BM cells after antiserum treatment.

RESULTS

Anti-SRBC response

Fig. 1 shows the splenic anti-SRBC responses of NZB, (NZB × BALB/c) and (C57/Bl × BALB/c) mice 8 days after irradiation, BM reconstitution, and immunization with a range of SRBC doses. There were fifteen to twenty mice in each group. In all strains the peak response, about 1000 PFC per spleen, followed the same amount of antigen, 10^7 SRBC. Although the shapes of the dose-response curves differ, there is no suggestion that the NZB mouse responds better to the higher SRBC doses and, indeed, it gave the poorest response of all the mice to 10^7 and 10^8 SRBC.

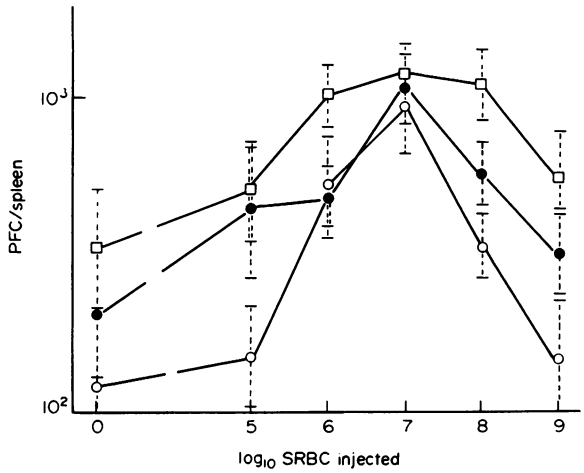

FIG. 1. Splenic 8-day anti-SRBC PFCs produced by irradiated NZB, (NZB × BALB/c)F_1 and (C57/Bl × BALB/c)F_1 mice injected with 15×10^6 syngeneic BM cells and various numbers of SRBC. All mice received SRBC 4×10^8 i.p. on day +4. Log mean and standard error given. □, (NZB × BALB/c)F_1; ●, (C57/Bl × BALB/c)F_1; ○, NZB.

Table 1 shows the effect of pretreatment of the BM cells with anti-θ serum or normal AKR serum and complement. Although all the groups gave reduced responses when compared with untreated BM, there was no significant difference between those of cells treated with anti-θ or with normal serum.

Anti-SIII response

The time-course of the anti-SIII splenic PFC response was first measured in the (C57/Bl × BALB/c) hybrid to discover the day of peak response. Fig. 2 shows that there was little difference between the response elicited by 0·5 μg given i.p. and that following 2·5 μg except that the peaks occurred on days 4 and 6 respectively. The time courses of the response of NZB, (NZB × BALB/c)F_1 and BALB/c mice to 0·5 μg are shown in Fig. 3, and here all the responses were maximal at day 4. There is a ten-fold difference in the total number of PFCs

TABLE 1. Effect of anti-θ treatment of BM on the 8-day splenic SRBC PFC response of lethally irradiated mice receiving 4×10^7 SRBC i.v. and BM cells which had been treated with antiserum and complement

Mouse strain	BM cell serum treatment	Splenic PFCs (log mean ± SE)
NZB	Normal AKR	585 (376–910)
NZB	Anti-θ	338 (233–514)
(C57/Bl × BALB/c)F_1	Normal AKR	419 (210–870)
(C57/Bl × BALB/c)F_1	Anti-θ	526 (330–820)
(NZB × BALB/c)F_1	Normal AKR	652 (338–1258)
(NZB × BALB/c)F_1	Anti-θ	945 (514–1738)

produced by the different strains, BALB/c mice responding the best, and the NZBs giving the lowest response.

Groups of mice of all four strains were immunized i.p. with doses of SIII ranging from 0·1 to 250 μg. Eight to ten mice in each group were killed 4 and 6 days later, and their spleens assayed for anti-SIII PFCs. The results for each strain are presented in Fig. 4a–d. Although, again, there are differences in the total number of PFCs produced by the different strains, immunization with 0·1 μg was always followed by a good PFC response, the response to 100 μg was considerably reduced in all strains tested, and none of the groups of mice developed a response to 250 μg SIII. The demonstration of true tolerance requires that the tolerant animal be unable to respond to subsequent injection of antigen. Five NZB and 5

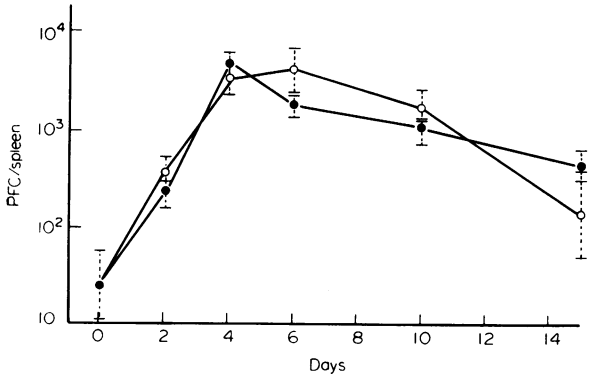

FIG. 2. Time course of the splenic anti-SIII PFC response of (C57/Bl × BALB/c)F_1 mice after i.p. injections of 0·5 μg and 2·5 μg SIII. Log mean and standard error given. ○, 0·5 μg SIII; ●, 2·5 μg SIII.

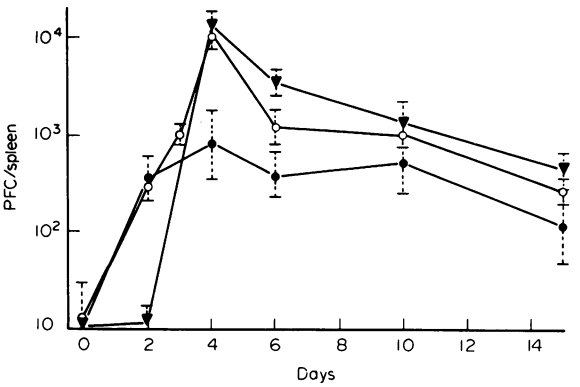

FIG. 3. Time course of the splenic PFC response of NZB, (NZB × BALB/c)F_1 and BALB/c mice, after i.p. immunization with 0·5 μg SIII. Log mean and standard error given. ▼, BALB/c; ○, (NZB × BALB/c)F_1; ●, NZB.

(C57/Bl × BALB/c)F_1 mice were therefore injected i.p. with 0·5 μg of SIII 10 days after they had received 250 μg SIII. Table 2 shows that none of these mice had responded with an increase in splenic anti-SIII PFCs 4 days later.

Anti-levan response

Fig. 5 shows the 5-day splenic anti-levan PFC responses of NZB and (C57/Bl × BALB/c) F_1 mice after receiving various doses of levan intravenously. Background PFC levels of the two groups were similar, but the peak response of hybrid mice was greater than that of the NZB. Both strains responded well to 250 μg, and poorly to 500 μg of levan, so that the threshold of high-dose tolerance induction was the same. Injection of more than 500 μg of levan killed the mice.

TABLE 2. Effect of i.p. injection of 250 μg SIII on the subsequent 4-day splenic PFC response to 0·5 μg SIII of NZB and (C57/Bl × BALB/c)F_1 male mice. The tolerizing injection was given 10 days before immunization. Five mice in each group

Mouse strain	Pretreatment	4-day splenic anti-SIII PFC (log mean ± SE)
(C57/Bl × BALB/c)F_1	Nil	4768 (2492–7018)
(C57/Bl × BALB/c)F_1	250 μg SIII	19 (11–33)
NZB	Nil	959 (368–1820)
NZB	250 μg SIII	15 (8–30)

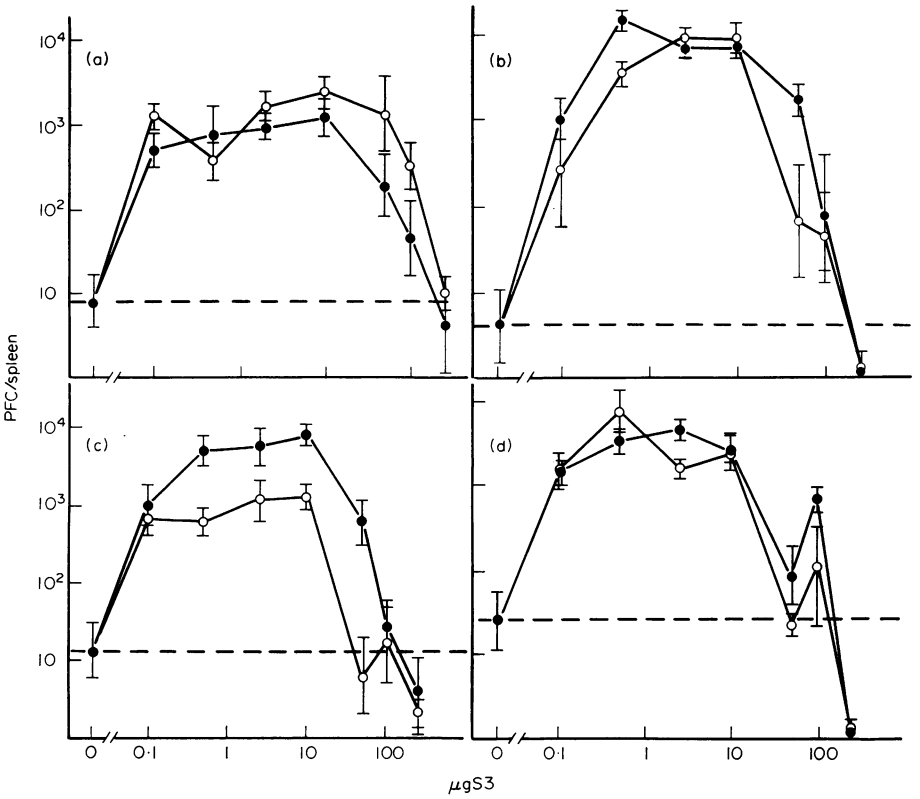

FIG. 4. Dose response curve for mice injected i.p. with various amounts of SIII. Splenic SIII PFCs measured on days 4 and 6. Log mean and standard error given. ●, day+4; ○, day+6; (a) NZB; (b) BALB/c; (c) (NZB × BALB/c)F_1; (d) (C57/Bl × BALB/c)F_1.

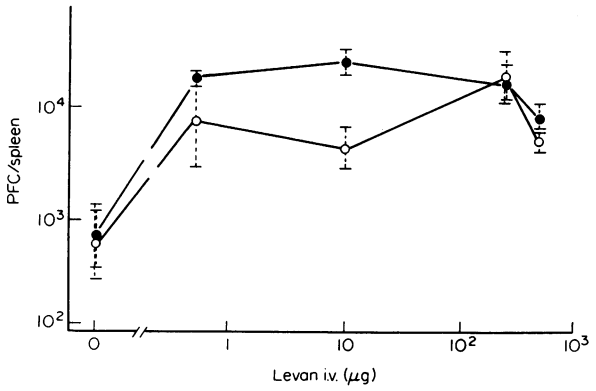

FIG. 5. Dose response curve for mice injected with various amounts of levan i.v. Splenic levan specific PFCs measured at day 5. Log mean and standard error given. ○, NZB; ●, (C57/Bl × BALB/c)F_1.

DISCUSSION

Before analysing these results one must first decide whether the antibody responses measured are, indeed, produced by B cells acting without the aid of T cells. The thymus-independence of immune responses to levan and SIII is well established for the mouse (Howard, 1972), but the anti-SRBC response of transferred bone-marrow cells merits more consideration. It has been shown that anti-SRBC antibodies can be produced both by congenitally thymusless (nu/nu) mice (Pantelouris & Flisch, 1972) and by thymectomized irradiated mice restored with foetal liver taken at an age before the development of the embryonic thymus (Tyan, Herzenberg & Gibbs, 1969), indicating that T cells are not essential to this response. Pretreatment with anti-θ serum of the transferred BM cells used in the present experiments had no more effect on the subsequent response than did normal mouse serum, which suggests that the contribution of donor T cells to the measured response was negligible. Evidence of the absence of a contribution by host radio-resistant T cells to the response of transferred marrow cells has been presented in a previous paper (Playfair & Purves, 1971a), where it was shown that thymectomy of host mice with or without injections of anti-thymocyte globulin had no effect on the subsequent anti-SRBC response of transferred syngeneic bone-marrow in the (NZB × BALB/c)F_1 mouse.

While is it impossible to be certain that a very small number of surviving T cells may not somehow be involved, there is no evidence to support this notion.

Since all the antibody responses measured were those of cells which are capable of antibody production without the aid of thymus-derived cells, they fall, by definition, into the category of the B1 cell defined by Playfair & Purves (1971b), and discussed by Gershon (1973). These results, then, show that such B1 antibody-producing cells of autoimmune NZB mice behave normally when exposed to high doses of three antigens, SRBC, SIII and levan, that is, the tolerance threshold of these cells is not abnormal. Incidentally it was shown that the maximal immune response is rather low when compared to that of normal mice.

The abnormal resistance to tolerance induction which the intact mice show to some antigens, including SRBC, is therefore not due to a defect of the B1 cell, and could be confined to either the T cell or to the B2 cell, defined by us as a B cell which can respond to antigen only with the help of a T cell. The finding of Staples *et al.* (1969) that BM from adult (NZB × NZW)F_1 hybrid mice was capable of transferring resistance to tolerance induction with soluble bovine gamma globulin could perhaps be interpreted as indicating that it is the B2 cell which is responsible, but this depends on the assumptions that the bone-marrow had not been influenced by the thymus before transfer and that the trait of resistance was not associated with thymocyte precursor cells. From two experiments in NZB hybrids (Playfair, 1969; Jacobs *et al.*, 1971) showing that cyclophosphamide-induced tolerance to SRBC could be obtained in bone-marrow but not in thymus cells, it would seem that the T cell is more likely to be at fault.

It is interesting to note that the NZB produced a rather low antibody response to all three of the antigens used, which contrasts with the previously described hyper-reactivity of NZB mice to several protein antigens (Staples & Talal, 1969; Weir *et al.*, 1968). Although it is tempting to speculate that it is again the T cells which are responsible for this high reactivity to antigens which are known to be thymus-dependent, and that the B1 cells are hyporeactive, extrapolation from results obtained with a small number of antigens is probably unjustified.

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