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Hyper-responsiveness in NZB mice to the experimental induction of anti-red cell autoantibody

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SUMMARY

Strain differences in ease of induction of autoantibody production were observed when mice were injected with rat RBC. Responsiveness was not linked to the H-2 locus. NZB and (NZB × BALB/c)F_1 mice were hyper-responsive both in terms of the induction of autoantibody and in the production of agglutinating antibody to rat RBC. C57BL, BALB/c and (C57BL × BALB/c)F_1 were poor responders. Injection of the rat RBC in FCA converted a poor responder into a good responder. Adult thymectomy and ALS treatment did not significantly enhance autoantibody production.

INTRODUCTION

It has been shown that immunization of mice with rat erythrocytes elicits anti-erythrocyte autoantibodies (Playfair & Marshall-Clarke, 1973; Cox & Keast, 1973, 1974). In this report some of the parameters involved in the induction of autoantibody—the effects of adjuvants, the dose of antigen and the effects of thymectomy and of anti-lymphocyte serum treatment have been investigated.

Different strains of laboratory animals exhibit significant variation in their susceptibility to the induction of autoimmune disease (Boehme, 1965; Hughes & Stedronska, 1973; Penhale *et al.*, 1975). Cross-breeding studies have established the involvement of genetic influences in these variations (Lee *et al.*, 1954; Vladutiu & Rose, 1971; Gasser, Newlin, Palm & Gonatas, 1973; Williams & Moore, 1973). The susceptibility of mice to the induction of autoimmune thyroiditis has been linked to the H-2 locus (Vladutiu & Rose, 1971) and that of rats to experimental allergic encephalomyelitis to their major histocompatibility locus (Williams & Moore, 1973; Gasser *et al.*, 1973). Therefore it seemed worth investigating strain differences in responsiveness to rat erythrocytes and the ease of induction of erythrocyte autoantibodies.

NZB mice spontaneously develop autoantibodies to various antigens, in particular autoantibodies against red cells are detectable from the age of about 4 months (Bielschowsky, Helyer & Howie, 1959; Helyer & Howie, 1963). Autoimmune haemolytic anaemia also develops in (NZB × BALB/c)F_1 hybrids although with a later age of onset (East, Harvey & Tilly, 1976). It was of particular interest to study and compare the susceptibility of the NZB and the (NZB × BALB/c)F_1 hybrid to the experimental induction of erythrocyte autoantibody.

MATERIALS AND METHODS

Mice. BALB/c, (C57BL × BALB/c)F_1 and (NZB × BALB/c)F_1 mice were bred in our laboratory from inbred parental strains originally obtained from the Laboratory Animals Centre, Carshalton. NZB mice were obtained directly from the Laboratory Animals Centre, Carshalton.

Antigen. Fresh rat RBC were obtained by cardiac puncture of Wistar rats maintained in our laboratory. RBC were washed three times in PBS prior to immunization.

Antisera. Anti-lymphocyte serum (ALS) was prepared in rabbits as described by Greaves *et al.* (1969). Rabbits received

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two i.v. injections of 10^9 mouse thymus cells in saline on days 0 and 14 and were bled on day 21. The ALS was absorbed before use, twice with mouse liver and twice with mouse RBC. This ALS had a cytotoxic titre of 1:128 against thymocytes using dye exclusion as an index of cell viability.

Thymectomy. Thymectomy was performed by Miller's method (Miller, 1962).

Direct antiglobulin (Coombs') test: DCT. Mice were bled from the retro-orbital sinus and the red cells washed four times in isotonic saline before testing. A single batch of rabbit anti mouse gammaglobulin (shown by immunoelectrophoresis to react against IgG and IgM) was stored in small aliquots at −20°C and used at a standard final dilution of 1:80. Agglutination was scored microscopically on a scale ranging from microscopic positive (1) to massive agglutination involving all the cells (4) after 30-min incubation with antiserum at room temperature. Positive and negative controls were included in all determinations.

Detection of rat agglutinins. Mice were bled from the retro-orbital sinus and doubling dilutions of the heat-inactivated serum tested in microwells against freshly washed rat RBC (2%) in 1% BSA in PBS. All dilutions of the sera were in 1% BSA in PBS. Agglutination was read after 1 hr at 37°C.

RESULTS

Effect of adjuvant

(C57BL × BALB/c)F_1 mice were immunized either with two weekly injections of 2×10^8 rat RBC in Freund's complete adjuvant (FCA) or four weekly injections of 2×10^8 rat RBC in PBS. Fig. 1 illustrates the effect of FCA on the subsequent induction of Coombs' positivity. Immunization using FCA resulted in a rapid induction of a visible Coombs' positivity. The amount of autoantibody produced by this method was far greater than that elicited by injection of rat RBC alone (using the Wilcoxon ranking test for differences between two samples, $2\alpha = 0{\cdot}01$ at 3 weeks and $2\alpha = 0{\cdot}02$ at 6 weeks). Two injections of 2×10^8 rat RBC with 2×10^9 *B. pertussis* also enhanced the autoantibody production significantly above that induced with the rat red cells alone.

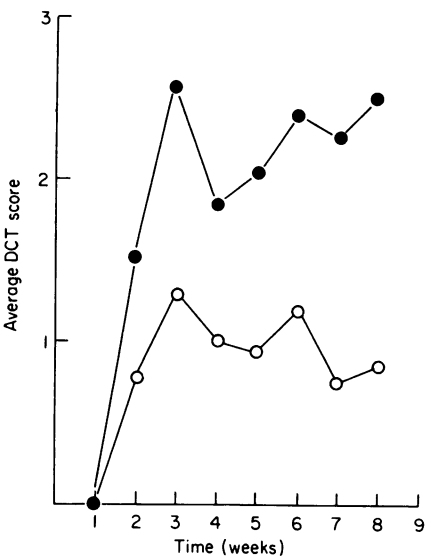

FIG. 1. The effect of FCA on the induction of anti red cell autoantibody in (C57BL × BALB/c)F_1 mice. (●——●) Mice injected with rat RBC+FCA (fourteen mice); (○——○) mice injected with rat RBC alone (twenty mice).

Effect of ALS and thymectomy followed by ALS on the induction of autoantibody by rat RBC+FCA

0·5 ml ALS was injected i.p. either once (1 week before) or twice (1 and 2 weeks) before rat RBC+CFA. It can be seen from Fig. 2 that this treatment did not enhance autoantibody production. The combination of thymectomy and ALS delayed onset and diminished the amount of antibody produced suggesting that severe depletion of T cells affects the resulting response. The ALS presumably did not

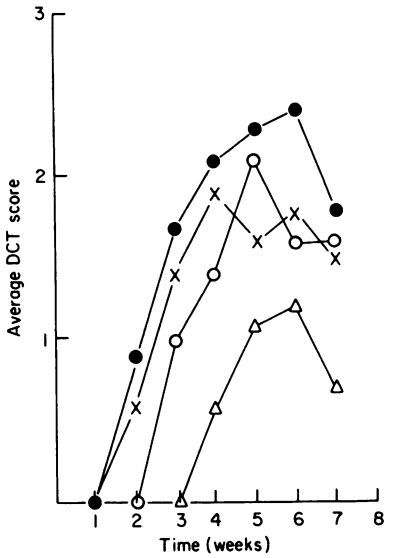

FIG. 2. The effect of thymectomy and ALS on the induction of anti red cell autoantibody in (C57BL × BALB/c)F_1 mice injected with rat RBC+FCA. (●——●) Mice injected with rat RBC+FCA; (×——×) mice injected with rat RBC+FCA, one injection of ALS at week −1; (○——○) mice injected with rat RBC+FCA, pretreated with ALS, injected 1 and 2 weeks prior to rat RBC injections; (△——△) thymectomized mice injected with rat RBC+FCA, pretreated with ALS, injected 1 and 2 weeks prior to rat RBC injections, five mice in each group.

significantly affect B-cell function as two injections of ALS did not diminish the production of autoantibody.

Effect of adult thymectomy on the induction of autoantibody

Because of the possibility that the FCA was affecting the T cells controlling the autoimmune response

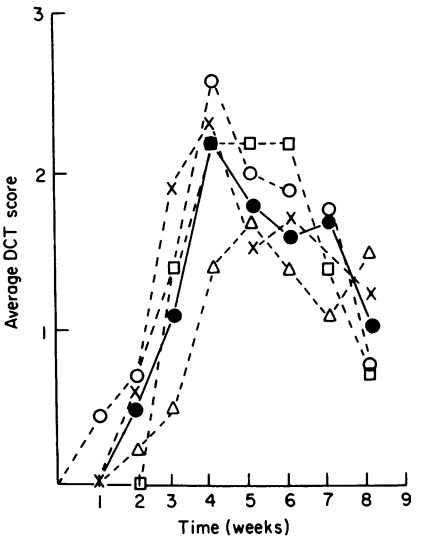

FIG. 3. The effect of thymectomy on the induction of anti-red cell autoantibody in (C57BL × BALB/c)F_1 mice injected with rat RBC alone. All mice were injected with rat RBC alone, five mice in each group. (●——●) Sham-thymectomized. Injections of rat RBC commenced on the following days post thymectomy (○- - -○) 6 days; (△- - -△) 9 days; (□- - -□) 15 days; (×- - -×) 27 days.

to RBC, the effect of adult thymectomy on the capacity of four weekly injections of rat RBC to elicit autoantibody was investigated. The course of rat RBC injections was initiated at 6, 9, 15 or 27 days after thymectomy. Control mice were sham-thymectomized. The result of this is shown in Fig. 3.

It can be seen that adult thymectomy neither significantly enhanced nor suppressed the subsequent induction or the rate of decline of Coombs' positivity implying that cells of recent thymic origin ('T_1') (Kappler *et al.*, 1974) are not involved either in the production or in the regulation of the amount of autoantibody produced in this system.

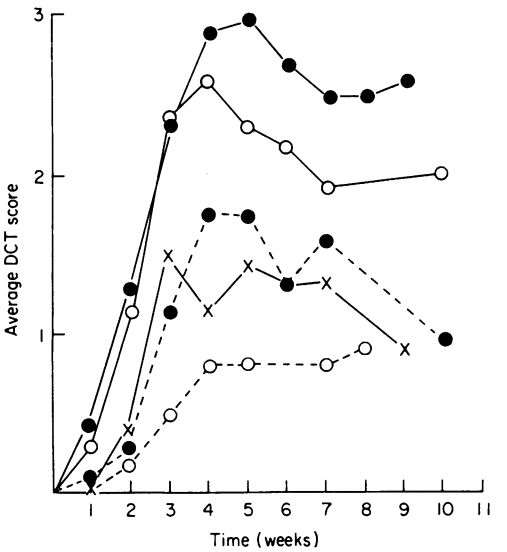

FIG. 4. Strain differences in the induction of anti-red cell autoantibody. (●——●) NZB; (○——○) (NZB×BALB/c)F_1; (●- - -●) (C57BL×BALB/c)F_1; (×——×) BALB/c; (○- - -○) C57BL. Not less than ten mice in each group.

Strain differences in the induction of Coombs' positivity

NZB, (NZB×BALB/c)F_1, BALB/c, C57BL and (C57BL×BALB/c)F_1 mice were subjected to a regime of four weekly injections of rat RBC to elicit autoantibody production. It can be seen from Fig. 4 that the NZB and the (NZB×BALB/c)F_1 hybrid are higher responders than the other three strains of mice, the C57BL strain being the poorest responder. The differences between the high responders, the NZB and its hybrid, and the other mice was highly statistically significant (weeks 4+5, $2\alpha=0{\cdot}01$). The differences between the NZB and its hybrid was not statistically significant at 4 or 5 weeks.

The relationship between the susceptibility of a strain of mice to induction of erythrocyte autoantibody and its ability to form agglutinating antibody to rat RBC was next investigated. It can be seen from Table 1 that the NZB mouse not only is a high responder in terms of autoantibody production but also gives a high non cross reacting response to rat RBC. There was no agglutinating antibody activity detectable against mouse RBC; presumably all autoantibody is absorbed *in vivo*. The low responder (C57BL×BALB/c)F_1 hybrid gives a comparatively low response to rat RBC. Thus there appears to be a correlation between the strain responsiveness to induction of autoantibody and the agglutinin response to the inducing agent although within a group the individual Coombs' scores do not always correlate well with the rat agglutinin titre.

Dose response to rat RBC

The effect of varying the dose of rat RBC on the subsequently induced autoantibody was investigated. It can be seen from Fig. 5a, b that 100-fold reduction in the dose of the antigen did not significantly

TABLE 1. Rat agglutinins: relationship to subsequent induction of Coombs' positivity by rat RBC

Strain	Week 1		Week 3		Week 7	
	DCT	Rat titre	DCT	Rat titre	DCT	Rat titre
NZB	0	256	3	4096	3	2048
	0	0	2	1024	3	1024
	0	256	4	1024	2	1024
	0	256	3	4096	2	1024
	1	256	4	4096	3	4096
C57BL × BALB/c	0	16	1	64	1	128
	0	8	2	128	1	16
	0	256	2	256	1	256
	0	8	1	32	1	256
	0	4	3	64	3	256

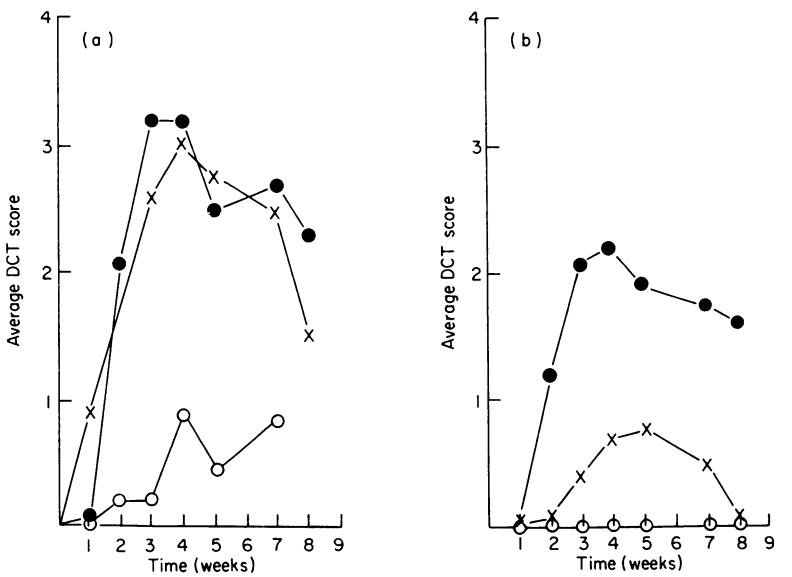

FIG. 5. Dose response to rat RBC. (a) NZB mice; (b) (C57BL × BALB/c)F_1 mice. Five mice in each group. (●——●) 2×10^8 rat RBC per injection; (×——×) 2×10^6 rat RBC per injection; (○——○) 2×10^4 rat RBC per injection.

diminish autoantibody production in the high responder strain but markedly reduced it in the low responder strain. As little as 2×10^4 rat RBC induced a positive DCT in 80% NZB mice but did not induce any autoantibody production in the (C57BL × BALB/c)F_1.

DISCUSSION

Immunological unresponsiveness to an antigen can be terminated by injection of cross reacting antigen (Weigle, 1973; Yamashita *et al.*, 1976). Playfair & Marshall-Clarke (1973) and Cox & Keast (1973, 1974) have demonstrated that repeated injections of rat RBC into mice results in a development of autoantibody against autologous erythrocytes.

The results presented in this paper demonstrate that there are significant strain differences in the response to rat RBC injections. Interestingly the NZB and its hybrid the (NZB × BALB/c)F_1 (H-2d) are high responders both in terms of the ease of induction of autoantibody and in their production) of haemagglutinating antibody to rat RBC. Both these strains of mice spontaneously develop with age a Coombs' positive haemolytic anaemia. The C57BL (H-2b), the BALB/c (H-2d) and the (C57BL × BALB/c)F_1 hybrid (H-2b, d) were, on the other hand, poor responders. Susceptibility to induction of autoantibody was not therefore directly related to the H-2 locus. This differs from the experimental induction of autoimmune thyroiditis in mice in which a close relationship exists between H-2 type and autoimmunity (Vladutiu & Rose, 1971).

These results are in accord with those of Cox & Keast (1974) who found that the C57BL mice were poorer responders than C3H to autoantibody induction. The preliminary observations of Playfair & Marshall-Clarke (1973) which indicated that NZB mice were poorer responders than C57BL have subsequently been shown to have been erroneous owing to contamination of the mouse strains used in those experiments.

In view of the high rat agglutinin response in the NZB it would seem possible that this strain and its hybrid might have a larger number of T cells reactive against rat RBC. This could be directly investigated by examining the number of T-rosetting cells against rat RBC in different strains of mice. An expanded clone of anti-rat RBC T cells in the NZB would allow a greater collaboration with self-reacting B cells and a subsequent more vigorous reaction against autologous RBC. Alternatively, this may be yet another example of increased antibody formation by NZB mice which is attributable to a generalized abnormal cooperative role of T cells (Playfair, 1968, 1972; Talal & Steinberg, 1974; Palmer *et al.*, 1976) or the presence of B cells which are more easily triggered.

Adjuvants appear to augment the response to autologous RBC, converting a poor responder into a good responder. This could theoretically occur by extensive lymphocyte stimulation (Unanue, Askonas & Allison, 1969; Allison & Davies, 1971), by removal of T-cell suppression or by activation of macrophages. It would be attractive to attribute this hyper-responsiveness of the NZB and its hybrid to a lack of suppressor T cells (Chused, Steinberg & Parker, 1973) however, the thymectomy data and the effects of ALS which we have presented in this paper do not support a significant modulating effect of a T cell in this system. NZB mice and the (NZB × BALB/c)F_1 hybrid harbour murine leukaemia virus (Mellors & Huang, 1966; East *et al.*, 1967; Prosser, 1968; Nowinski *et al.*, 1968; Mellors, Aoki & Huebner, 1969; Aoki *et al.*, 1970; Lerner *et al.*, 1972; East *et al.*, 1976). Endogenous viral nucleic acid could be acting as an adjuvant rendering the mice hyper-responsive to a range of antigens. In support of this Jacobs *et al.* (1972) have demonstrated that New Zealand mice respond very poorly to the adjuvant effects of poly I poly C, suggesting already maximal stimulation.

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