Strain Differences in the Liver Autoantibody Response of Inbred Mice (33818)

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The measurement of antibody responses on inbred strains of mice has revealed significant interstrain differences, apparently under genetic control (1). Furthermore, responsiveness can develop at a different rate in different strains, even when the adult responses are similar. For example, the ability to make hemolytic antibody to sheep red blood cells (SRBC) appears earlier in NZB, and later in C57Bl, than in Balb/c or several other strains (2). The NZB mice are particularly interesting in that they consistently develop hemolytic anemia with positive direct Coombs tests (DCT) (3), and renal lesions similar to those of immune-complex glomerulonephritis (4).

Since a system has been described in rats for studying the autoantibody response to liver damage (5), it seemed worth applying it to inbred mice, with special reference to the NZB strain. The main question was whether the numerous spontaneous autoantibodies found in NZB mice represent a specific reactivity towards autoantigens, or simply a general immunological hyperreactivity to all antigens. A second question concerned the role of the thymus, since neonatal thymectomy, though depressing the response to many exogenous antigens (6) has also been reported to cause the appearance of autoantibodies (7).

Materials and Methods. Mice. Three strains were used, all supplied by the Laboratory Animals Center, Carshalton: NZB (originally obtained from Dr. M. Bielschowsky), Balb/c, and C57Bl. All mice were from brother–sister matings or the two succeeding random-mated generations.

Liver damage. After preliminary attempts at cryosurgery and the injection of liver homogenate, the method of Weir (5), which gave the most consistent results, was used. Carbon tetrachloride (CCl_4) was injected subcutaneously as a 10% solution in olive oil. The highest dose that did not cause unacceptable mortality in the youngest mice was found to be 0.1 ml/10 g of body weight, and this dose was used in all experiments. Mice were injected at the age of 4, 6, or 12–16 weeks, and bled from the retro-orbital plexus 5, 10, and 14 days later. At 14 days they were given a second injection of CCl_4 and bled 3 and 6 days later. The blood was allowed to clot at 37° for 30 min or overnight at 4°, and the serum was separated by centrifugation and inactivated at 56° for 30 min.

Preparation of liver homogenate. A modification of the method of Pinckard and Weir (8) was used. Mice from an unrelated strain (CBA) were killed and their livers were removed into cold 0.25 *M* sucrose and washed three times. The following fractionation was carried out at 4°. After the connective tissue had been removed, the liver was minced, weighed, added to 3 vol of 0.25 *M* sucrose, and homogenized in a Potter–Elvehjem glass homogenizer with a Teflon pestle. Two strokes up and down were applied three times with subsequent centrifugation at a speed of 1000, 1100, and 1100 rpm, for 10 min each. After every centrifugation the pellet was resuspended in 0.25 *M* sucrose and rehomogenized. Finally the three supernatants were pooled, centrifuged at 1700 rpm for 10 min, and the pellet was discarded. The resulting suspension, adjusted to 10% (w/v), was divided into aliquots of 5 ml and stored at −20° for use within 10 days.

Complement fixation tests. These were performed in a Takatsy microtitration apparatus. Complement was titrated in the presence of the 10% liver homogenate, which was not itself anticomplementary if used within 10

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days; 2 MHD of complement were used in all tests. One drop of serum (twofold dilutions starting with 1:4) in CFT buffer (Oxoid) containing 0.1% BSA, 1 drop of liver homogenate, and 1 drop of complement were incubated at 37° for 1 hr. In the controls the liver homogenate was excluded. After incubation, 1 drop of sensitized sheep erthrocytes was added and the trays were incubated for a further 0.5 hr at 37°, shaken, and left for 1 hr at 4°. The lowest serum dilution not showing complete lysis was taken as the complement-fixing (CF) antibody titer.

Thymectomy. This was carried out by the method of Miller (6) during the first day of life. Unfortunately the postoperative mortality in NZB and C57Bl mice made these strains unsuitable for the present experiments, but survival of Balb/c was good, with no wasting during the period under study.

Renal immunofluorescence. Ten 4-week-old mice (5 NZB, 5 C57Bl), and 19 6 weeks old (9 NZB, 10 Balb/c), were tested 1–2 weeks after their second injection of CCl_4. Immediately after sacrifice, frozen sections of kidney were cut and stained with fluorescein-conjugated rabbit antimouse gamma-globulin serum, prepared by hyperimmunizing rabbits with mouse gamma-globulin (mouse antirabbit RBC antibody) coated on rabbit RBC. The priming injection was given in Freund's adjuvant and the subsequent ones intravenously. The conjugation was carried out according to the method of Marshall *et al.* (9). Strong fluorescence of glomerulus or Bowman's capsule was scored as positive. Eight and 10-week-old NZB, Balb/c, and C57Bl kidneys from uninjected mice were used as controls.

Results. A total of 43 NZB, 32 Balb/c, and 14 C57Bl were tested at the age of 1 month. Most of the mice gave a brisk CF antibody response, with a peak at 5 days and titers as high as 2^9. However there were consistent strain differences—the average titer being highest in NZB and lowest in C57Bl at all stages of the response (Fig. 1, top). After a second injection of CCl_4 the titers rose again, with the same strain differences. When the distribution of individual titers during the first and second response is plotted (Fig. 2), it can be seen that 40/43 NZB, but only 1/11 C57Bl produced detectable antibody after the first injection, and 30/30 NZB and 4/11 C57Bl after the second. Balb/c results were intermediate in both cases.

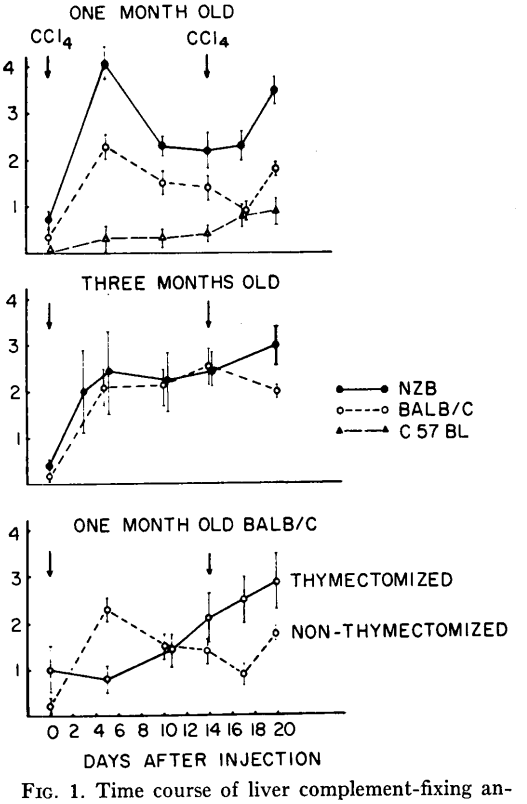

FIG. 1. Time course of liver complement-fixing antibody response following CCl_4 injection: arrows indicate times of injection; the values shown are logarithmic means ± 1 SE.

In NZB and Balb/c mice injected at 6 weeks, the differences were no longer significant, and in 3 month olds (Fig. 1, center) the titers were virtually identical in the two strains. The effect of neonatal thymectomy in 11 Balb/c was to reduce the 5-day response, but apparently to increase the later titers, particularly after the second CCl_4 injection (Fig. 1, bottom). In 8 thymectomized NZB tested at day 5 only, the average titer was 2.5, that is, about the same as in intact Balb/c. Renal glomerular fluroescence was found in 3/5 of the NZB injected at 4 weeks

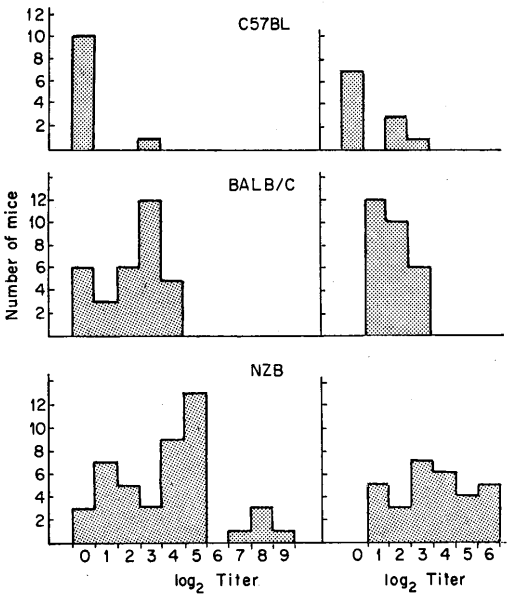

FIG. 2. Distribution of liver complement fixing antibody titers in 1-month-old mice, 5 days after a first (left) and second (right) injection of CCl_4.

(and tested at 8 weeks) but in 0/5 of the C57Bl. In the mice injected at 6 weeks, 9/9 NZB and 4/10 Balb/c were positive (Table I).

Discussion. The study establishes two points: the applicability to mice of the CCl_4 method for stimulating liver autoantibodies, and the existence of strain differences in the ensuing response. Without conclusively answering the question posed in the introduction, the results favor the idea that general immunological reactivity, rather than a special autoimmune tendency, underlies the high NZB response. In the first place, the strain difference, at least as regards the NZB and Balb/c, is present at 1 month but absent in adult mice, recalling the finding (2) that the hemolytic plaque-forming cell response to SRBC appeared earlier in NZB than Balb/c, but that both reached the same adult levels. Thus there is no reason to consider the CF antibody response as unduly high in the NZB, relative to other humoral responses, but merely as part of a general process by which antibody responsiveness develops unusually early in this strain. Secondly, the very low CF antibody response in the C57Bl, again reminiscent of their late-developing SRBC response (2), makes it probable that there is a spectrum of reactivity from strain to strain, so that if these results were to be taken to show that NZB is a "more autoimmune" strain than Balb/c, then Balb/c ought to be considered "more autoimmune" than C57Bl. However, no evidence of spontaneous autoimmunity has yet been recorded in Balb/c mice. The simplest interpretation seems to be that mice whose humoral antibody responses to exogenous antigens are higher than usual, for whatever reason, will also respond more virogously after damage to their own tissues. The effect of thymectomy—a depression of the early CF antibody, but a later increase is very similar to its effect on the response to SRBC (10), again suggesting that the autoimmune CF antibody response is much like any other antibody response. (This is not to say that absence or malfunction of the thymus may not play a part in perpetuating otherwise transient autoimmune responses.) The kidney data, though statistically inadequate, also fit in with this concept: NZB, with the most CF antibodies, also developed the most renal immunofluorescence, Balb/c much less, and C57Bl hardly any. This suggests that the spontaneous renal disease, with positive immunofluorescence, of NZB mice may be due to excessive or prolonged tissue breakdown and autoimmune elimination, representing once again one extreme of a spectrum, and perhaps a clue to the understanding of the

TABLE I. Renal Immunofluorescence in Mice Injected with CCl_4 and Control (uninjected) Mice.[a]

Mice	Treatment	Tested at 8 weeks	Tested at 10 weeks
NZB	CCl_4	3/5	9/9
	Control	3/20	6/20
Balb/c	CCl_4		4/10
	Control		0/10
C57Bl	CCl_4	0/5	
	Control	0/10	

[a] The number positive and the number injected are shown.

very similar lesions in human SLE. What originally initiates such a process in the intact animal remains to be elucidated.

Summary. Autoimmune responses to liver damage by CCl_4 were induced in mice of three inbred strains, and tested for by complement fixation with liver homogenate. In 1-month-old mice, but not in adults, large strain differences were found: NZB having the highest titers, Balb/c intermediate, and C57Bl the lowest; NZB mice also developed more immunofluorescent staining of the kidneys after CCl_4. These results may have some bearing on the pathogenesis of the spontaneous autoimmune lesions in the NZB strain.

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Hematological Effects of Apholate on Channel Catfish *(Ictalurus punctatus)* (33819)

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There have been many reports concerning the toxicological effects of the alkylating agent, apholate (2,2,4,4,6,6-hexahydro-2,2,4,4,6,6-hexakis (1-aziridinyl)-1,3,5,2,4,-6-triazatriphosphorine) in insects. Toxicological studies have been conducted in cattle (1), sheep (2), rats (3), mice (4), and fowls (5,6), but work with fish has been limited (7).

The delayed lethal syndrome resulting from treatment of animals with alkylating agents was reported by several workers, but the actual cause of death is not known (8). The present paper describes the hematological effects of oral administration of apholate to channel catfish (*Ictalurus punctatus*), and the delay in lethality, even with massive doses.

Materials and Methods. Channel catfish fingerlings, weighing 5–20 g, were maintained at 22° in 5 or 10-gal aquariums 10–14 days before, and during, experimentation. Single oral doses of apholate (crystallized from ethyl acetate) were administered in no. 5 gelatin capsules.

In the study of hematological changes produced by apholate, total erythrocyte and leukocyte counts were made by the method of Hesser (9), and differential leukocyte counts were made on thin smears stained with Wright's stain, using the classification of Jakowska (10). The blood was obtained by cardiac puncture using heparin as anticoagulant. Preliminary (normal) counts were made on 90 animals, and 2 weeks were allowed for recovery from cardiac puncture before treatment. Individual fish were marked for identification by small notches in the caudal fin, so that cell counts on each animal could be compared. The animals were then divided into three groups at random; 30 controls received empty capsules, 30 animals received 200 mg of apholate/kg; and 30 animals re-