Effect of Fetal or Neonatal Immunization on Antibody Response of the Adult Rat.*† (22590)

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(Introduced by Z. Ovary.)
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It has been reported that animals exposed to an antigen during prenatal or early postnatal life may fail to produce antibodies to the same antigen administered in later life. Traub(1) observed that in a mouse colony carrying the lymphocytic choriomeningitis virus, an intra-uterine infection resulted in offspring harboring the virus for many months without evidence of immunologic response. Owen(2) described the natural occurrence of reciprocal antigenic tolerance in genetically dissimilar dizygotic bovine twins having a common placental circulation and has more recently described experimental reproduction of this phenomenon in rats(3). Dunsford *et al.*(4) also described the existence of a blood group chimera in a human twin having circulating erythrocytes of both types O and A, though genotypically blood group O. Such observations are in accord with the theory of antibody production proposed by Burnet and Fenner(5), who introduced the concept of "self-markers," and postulated that at some time during prenatal or neonatal life, animals "take stock" of their own "antigens" and henceforth can recognize an antigen as either autogenous, by not reacting to it, or as foreign and responding with antibody production. This theory would imply that a foreign antigen introduced into an animal prior to this stage of self-recognition will thereafter be treated as autogenous, so that later exposure provokes no immunological response. Early attempts by these authors to provide an experimental basis for this concept were unsuccessful(6). Support for this hypothesis was given by Billingham, Brent and Medawar(7) who demonstrated that the breakdown of skin homografts between 2 incompatible strains of mice could be prevented or greatly postponed by injection of splenic or other living cells from the donor strain into recipient fetuses. Following this observation—termed "actively acquired tolerance"—numerous investigators attacked this problem with a variety of soluble and particulate antigens or their derivatives in several animal species. Some workers did not observe acquired tolerance following prenatal exposure to the antigen(6,8,9). Where tolerance was observed, relatively massive doses of antigen were injected into young animals over prolonged periods of time(8,10,11).

The present study was designed to parallel the work of Billingham, Brent and Medawar in attempting to induce actively acquired tolerance in rats through prenatal or neonatal exposure to a cellular antigen. However, our experiments differed from the British study in that: 1) Unlike the homologous species relationship in the latter study, an antigen from a zoologically distant species was used; 2) The antigen consisted of cells not capable of multiplication, *i.e.* sheep erythrocytes; 3) Immunological response was measured by an *in vitro* technic inherently capable of greater precision than observations on the survival of skin homografts. This approach was selected with the rationale that if tolerance resulting in prolonged skin homograft survival is based on depressed antibody response, such a depression should be demonstrable in a more clearly defined immune system susceptible to accurate antibody titration.

Materials and methods. Animals. Mature albino rats of the Wistar strain were used for breeding purposes. *Antigen.* Sheep blood, drawn aseptically from a single animal was preserved at 2 to 5°C in sterile modified Alsever's solution(12). It was considered advisable to use erythrocytes from a single

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TABLE I. Weights and Ages of Rats at Time of Experiment.

Group	No. of fetuses at laparotomy	No. of survivors at time of challenge	Age at time of challenge, wk	Wt, g	
				Range	Avg
A	20	5*	10	155–240	188
B	10	4	10	165–184	175
C	8	7	10	134–167	147
D	13	9	9	97–169	125

* 1 rat died during first bleeding. Difference in numbers between second and third columns is accounted for by perinatal mortality.

bleeding for both the primary exposure and subsequent challenge, so as to minimize untoward effects due to possible minor antigenic variations in erythrocytes taken from different sheep. The cells were washed 3 times with 0.85% sterile saline, and a 5% suspension in 0.85% saline was prepared for the injections. This suspension contained approximately 8.0 x 10^8 erythrocytes, per ml as determined by direct count. *Procedure for intrafetal injection.* Pregnant animals were anesthetized with ether and the peritoneum was entered through a low midline incision. The gravid uterus was gently exteriorized, one horn at a time, and kept moist with warm saline sponges. The fetuses could be clearly visualized through the distended, transparent uterine wall by oblique illumination. The RBC suspension was injected into the peritoneal cavity of each fetus by careful penetration through the uterus and the lateral abdominal wall of the fetus with a 0.25 cc tuberculin syringe and #27 hypodermic needle. After all fetuses in each uterine horn were injected, the uterus was carefully replaced in the abdomen, and the abdominal incision was closed in 2 layers with continuous #0000 chromic catgut sutures. *Determination of hemolysin titers.* Titer estimations were performed by means of a spectrophotometric procedure currently used in this laboratory. The titer is expressed as the dilution of hemolytic antibody required to lyse exactly 50.0% of a carefully standardized sheep RBC suspension in 15.0 minutes, in the presence of an excess of guinea pig complement. Determinations are generally reproducible to within 10% (13).

Experimental. Animals were mated by placing 2 to 4 females with 1 male for 3 to 5 days. It was thus possible to approximate the fetal age at the time of antigen injection. The pregnant animals were divided into 4 groups, and when the fetuses were about 15 days old (*i.e.*, 7 days prenatal), intrafetal injections were made as follows:

Group A—Intrafetal RBC: 0.03 cc of a 5% suspension of sheep RBC, injected into the peritoneal cavities of 15-day-old fetuses (7 days prenatal). *Group B—Saline controls*: 0.03 cc of 0.85% saline injected into the peritoneal cavities of 15-day-old fetuses. *Group C—Noninjected controls*: Pregnant animals were laparotomized but the fetuses were not injected. *Group D—Postnatal RBC*: 0.05 cc of a 5% suspension of sheep RBC injected intraperitoneally into newborn fetuses, within 24 hours after birth. The animals in Groups A and D received only a single inoculation of antigen. Those in Group A received approximately 2.4 x 10^7 erythrocytes while those in Group D received 4 x

TABLE II. Individual Rat Hemolysin Titers.*

Rat No.	6th day post-challenge titer	Rat No.	6th day post-challenge titer
A 1†	30	B 1	980
A 2	350	B 2	490
A 3	1260	B 3	1020
A 4	1450	B 4	1000
A 5	825		
C 1	655	D 1	1000
C 2	1850	D 2	490
C 3	1070	D 3	550
C 4	1130	D 4	1040
C 5	920	D 5	615
C 6	1620	D 6	290
C 7	610	D 7	220
		D 8	330
		D 9	800

* Pre-challenge baseline titers were less than 20 for all rat sera except A 4 which had a titer of 34.
† This rat was pregnant at time of challenge and was not included in statistical evaluation of results.

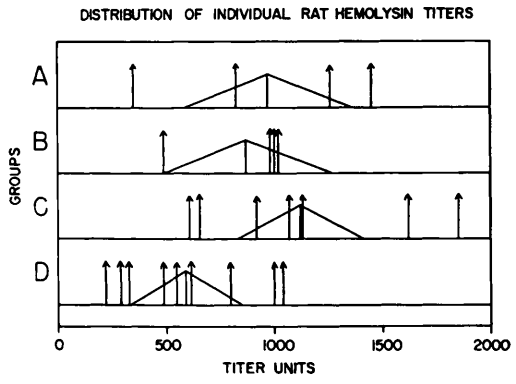

FIG. 1.

10^7 cells to compensate for increased body weight. The young were weaned at 20 to 25 days of age and maintained on the standard laboratory diet. At 9 or 10 weeks of age, each rat was bled from the ophthalmic venous plexus(14) to obtain 0.5 to 1.0 cc of blood for baseline determinations of hemolytic antibody. On the same day all animals were challenged with an intravenous injection of 1 cc of a 0.25% suspension of sheep RBC. On the 6th post-injection day, when antisheep RBC hemolysin titers are maximal(15), a second serum sample was obtained to determine the hemolytic antibody response.

Results. All animals were weighed at the time of weaning and at regular intervals thereafter until termination of the experiment. The growth rates in the 4 groups were entirely comparable. Final weights and ages of the rats at the time of challenge are shown in Table I. The baseline antisheep RBC hemolysin titers, determined in serum specimens drawn just prior to the challenge antigen injections, were less than 20 in all but one of the animals (see Table II). With respect to the post-challenge titers, there is no statistical difference between the intrafetal antigen injection group (A), the saline control group (B), and the non-injected rats (C). The titer of the postnatal antigen injection group (D) does not differ significantly from either A or B, although there is a significant difference ($0.02>P>0.01$) from non-injected rats (C) (Table III). Fig. 1 illustrates the distribution of individual rat hemolysin titers (arrows), as well as the mean titers (vertical lines) and 95% confidence limits of the mean (triangles).

TABLE III. Mean Titers on Sixth Post-Challenge Day.

Group	Mean titer	Stand. error of mean†	95% confidence limits of mean†
A	971 ± 490	192	587–1355
B	872 ± 254	192	488–1256
C	1122 ± 466	145	832–1412
D	591 ± 300	128	335– 847
	Pooled 383*		

* As there is no significant difference in stand. dev. of the 4 groups, the 4 values were pooled to obtain a more accurate stand. dev. based on a larger sample of animals.

† Calculated from this pooled stand. dev.

Discussion. These experiments demonstrate that rats exposed to a foreign antigen during the perinatal period retain the capacity for an unaltered antibody response to this antigen when mature. The findings are thus at variance with those of Billingham, Brent and Medawar, in that "actively acquired tolerance" was not observed. On the sixth post-challenge day, all animals but one exhibited a pronounced rise in hemolysin titer from baseline levels. The only rat which failed to respond proved to be pregnant during the challenge period. The mean post-challenge titers of the 4 groups, except those of groups C and D, are statistically similar. The mean titer of the postnatal RBC injected group (D) is significantly lower than that of the non-injected rats. Conceivably this difference may be due to the younger age of the group D animals, to their postnatal exposure to the amount of antigen injected, or to a combination of both factors. Even if the difference in titers is the specific result of neonatal exposure to the antigen, it seems unlikely that such a slight antibody depression might be expected to account for tolerance to homografts. The diminution of antibody response observed in the group D rats is far less pronounced than that reported by others in postnatally exposed animals(8,10,11).

In view of the disparity between the results of this and previous studies on immunologic unresponsiveness, an analysis of the differences in experimental conditions is indicated. As outlined above the perinatal

exposure to antigen in the present work consisted of a single inoculation of a relatively small amount of cells incapable of multiplication. By contrast, tolerance was achieved in the previous studies when immature animals were subjected to a prolonged contact with relatively massive doses of antigen. In some instances the antigenic dosage was of sufficient intensity and duration so that prolonged persistence of circulating antigen was demonstrated(8,10). In other instances antigen administration consisted of daily injections for several months(11). A suppression of mean agglutinin titers was recently reported in groups of chickens exposed as embryos, to a single injection of dead *Salmonella pullorum* (16). Where individual titers were given by the author, the overlap in antibody response between the experimental and control chickens was essentially similar to that observed in the present work. Furthermore, the conclusions drawn in(16) were based on serum dilution titers and are not in accord with those derived from similar experiments in chickens performed by Cohn(9), who estimated the antibody response precisely in absolute weight units. In another recent study, also in accord with our findings, the intra-abdominal injection of 0.2 ml of rabbit blood into 6-day-old chick embryos, failed to suppress formation of natural heterohemagglutinins(17). Even tolerance conferred by living cells(7) may be a reflection of massive doses of antigen, in that surviving transplanted cells may continue to liberate antigen and thus saturate the recipient. This situation would obtain only if the cells are introduced into young animals prior to the appearance of the faculty of immunologic response. Moreover the animal's immaturity appears to be a facilitating rather than an essential condition, in that immunological capacity of the young is more easily overwhelmed than that of the mature animal. But even in the latter, massive antigen injection plus the superimposition of irradiation injury induces immunologic unresponsiveness to protein antigens(11). With pneumococcal polysaccharide, which persists in tissues for long periods(18), the administration of 5-50 μg to mice suffices to suppress antibody formation as shown in Felton's studies of "immune paralysis"(19). Indeed this author reports that there may be a quantitative relationship between the amount of pneumococcal polysaccharide required to induce immunologic paralysis and the specific immune state of the animal. Animals immunized when mature required a larger dose of antigen for the induction of immune paralysis. Finally, recent evidence indicates that even in "immune paralysis" antibody may be formed but is not detectable because of its interaction with antigen present in excess(20).

In retrospect therefore, is it not possible that this entire range of phenomena characterized by adversely altered immunologic response, although induced by various procedures, are not simply expressions of immune paralysis? Such an interpretation would emphasize a quantitative paralyzing basis to immunologic unresponsiveness rather than a delicate qualitative alteration in the antigen-host relationship, as would follow from the theory of Burnet and Fenner.

Summary. Rats exposed to sheep erythrocytes before or at time of birth exhibit an undiminished capacity for hemolysin production in response to a subsequent challenge with the same antigen given 9 to 10 weeks later. A possible explanation to account for the disparity between these results and those obtained by other investigators is offered.

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Sulfhydryl Factors in Degradation of Insulin-I^{131} by Liver Extracts.* (22591)

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The degradation of insulin-I^{131} by both a heat stable and a heat labile component of rat liver homogenate has been reported by Mirsky *et al.*(1). Lehmann and Schlossmann(2) had previously observed that insulin could be inactivated by a dialyzable heat stable factor in rabbit muscle extracts. This factor was able to reduce the disulfide linkages of insulin, and the authors concluded that it was a sulfhydryl compound such as glutathione (GSH) or cysteine. As liver has a relatively high content of GSH, it was of interest to find whether this compound could produce changes in the trichloroacetic acid (TCA) solubility of insulin-I^{131}. The present studies on the degradation of insulin-I^{131} in the presence of GSH were carried out in the hope of acquiring better understanding of the processes that might be involved in the inactivation of insulin by rat liver extracts.

Materials and methods. Crystalline zinc insulin, highly purified amorphous insulin, and crystalline glucagon were gifts of Eli Lilly and Co. The crystalline insulin was iodinated by Abbott Laboratories, and glucagon was kindly iodinated by Dr. Alfred Staub. Crystalline bovine plasma albumin and iodinated human serum albumin were obtained from the Armour Laboratories. Reagent grade GSH was a product of the Fisher Scientific Co., and oxidized glutathione (GSSG) was purchased from the Nutritional Biochemicals Corp. *p*-Chloromercuribenzoic acid (PCMB) was obtained from the Bios Labs, Inc. *Preparation of liver extracts.* Livers of decapitated 3-month-old Sprague-Dawley rats were perfused *in situ* with ice cold isotonic saline. The livers were then homogenized in 1.15% KCl with a glass homogenizer. The homogenate was diluted to a 10% tissue concentration and centrifuged for 25 minutes at 110,000 x *g*. The resultant supernatant fraction was used. For certain experiments, an acetone powder was prepared from the supernatant fraction. Prior to the assay procedure each 50 mg of powder was extracted with 1 ml of cold distilled water and material which had not dissolved at the end of 1 hour was removed by centrifugation. An aliquot of supernatant fraction from rat liver was placed in a boiling water bath for 10 minutes in order to prepare the heat stable factor. It was found in preliminary experiments that heating for 2 minutes was sufficient to produce maximum inactivation of the heat labile system which degraded insulin-I^{131}. *Measurement of insulin-I^{131} degradation.* 1 ml of liver extract or GSH solution that had been adjusted to pH 7.5 was preincubated for 5 minutes at 37°C in a 12 ml centrifuge tube. Then 1 ml of 0.067 M potassium phosphate buffer of pH 7.5, containing a trace quantity (0.1-2 μg) of dialyzed insulin-I^{131}, 1 mg of bovine plasma albumin,

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