IMMUNOLOGICAL UNRESPONSIVENESS IN RABBITS PRODUCED BY NEONATAL INJECTION OF DEFINED ANTIGENS*

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Previous studies on the ontogeny of the specific immune response shortly after birth in several species (1–10) have indicated that newborn chicks, guinea pigs, rabbits, and human beings form little or no detectable antibody in response to antigenic stimulation, but develop this ability between 2 and 4 weeks of age. In several known situations, however, such antigenic stimulation is not lost upon the newborn animal but results in a highly specific hiatus in his immunological capacity which persists after maturity. The studies establishing this last concept are reviewed exhaustively by Billingham, Brent, and Medawar (11) particularly in reference to "acquired tolerance" to homografts. In respect to less complex antigens, the investigations of Hanan and Oyama (12), Dixon and Maurer (13), and Cinader and Dubert (14) have indicated that multiple injections of heterologous plasma proteins during the neonatal period result in prolonged and specific inability to respond immunologically to the injected protein.

This investigation has been concerned with the mechanisms which govern the early development of specific immunity in mammals and particularly the unresponsive state resulting from neonatal exposure to antigen. The present paper describes the production of a prolonged state of immunological unresponsiveness in rabbits by a single injection of defined protein antigens at birth, and defines the amount of antigen required, the duration of the unresponsive state, the degradation of antigen in unresponsive animals, and also describes a number of attempts to produce an analogous state with a variety of bacterial antigens. A preliminary report of these studies has appeared else-

where (15).[1] A subsequent paper will be concerned with the definition of immunological response of the rabbit from birth to maturity in terms of antibody formation as well as the unresponsive state.

Experimental Procedures and Methods

The experimental design employed throughout most of this investigation consisted of giving litters of newborn New Zealand albino rabbits a single intraperitoneal injection of a defined antigen, allowing the animals to stay with the doe until weaned, and then at 3 to 4 months of age, or in some experiments at various other intervals, attempting to immunize them with the antigen injected at birth. In experiments in which it was required to immunize the animals before 90 days of age, half of each litter were used as a non-injected control group and identified by India ink tattooing on the ear. However, the uniform response at 3 to 4 months of age of this stock of animals to the immunizing schedule employed soon made this step unnecessary. When initial immunization was to be attempted at more than 90 days of age, whole litters born within 1 to 3 days of the injected litters were set aside as controls. The mortality from handling and injecting the newborn animals was not different in injected and control groups similarly handled, and appeared to be significantly reduced when the person who fed, watered, and handled the rabbits regularly, handled and held the newborn animals for injection. The animals were fed a standard antibiotic-supplemented ration except in the experiments in which the effect of streptococcal infection was assessed, when a ration not supplemented with antibiotic was supplied.

Protein antigens.—Various lots of crystalline bovine albumin (BSA) obtained from Armour and Company, crystalline ovalbumin (EA) obtained from Worthington Chemical Company, and pooled human gamma globulin (HGG) from recently outdated lots prepared by Squibb Laboratories, were dissolved at 4°C. in pyrogen-free 0.145 M NaCl, and sterilized by Sietz filtration. The macroglobulin employed was isolated from the plasma of a patient with macroglobulinemia by repeated precipitation at low ionic strength, employing aseptic technique, and 1:10,000 final concentration of merthiolate was added to the solution as a preservative. This material was apparently homogeneous electrophoretically and in the ultracentrifuge. After determination of the protein content of the various antigens by the biuret method, the concentration was adjusted so that the desired amount of antigen could be administered in 0.1 to 1.0 ml.

Immunization procedures.—In the earlier experiments the initial attempt to immunize the rabbits injected at birth consisted of two intravenous injections, 10 days apart, of 10 mg. of the antigen, and was started when the animals were 90 to 120 days of age. Bleedings were then taken at weekly intervals, or more frequently, until antigen was no longer detectable in the serum, when at least two subsequent bleedings were made for antibody determination.

The duration of the unresponsive state induced by neonatal injection of BSA in groups of rabbits which had failed to respond to antigenic challenge at 3 to 4 months of age, was assessed by rechallenge at approximately 4 months intervals. These subsequent attempts at immunization were made by one of two methods. Most animals received 15 to 25 mg. per kilo BSA, or appropriate antigen, intravenously, following a prebleeding, and were bled weekly or more often, until the antigen disappeared from the serum. Some groups of animals received an intradermal injection of 10 to 80 mg. BSA incorporated in a water-in-oil emulsion with an equal volume of adjuvant mixture (bayol F, containing one per cent lano-

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[1] These experiments were also presented in part before the Central Society for Clinical Research, Chicago, November 6, 1956; other portions were presented before the American Association of Immunologists, in Chicago, April, 1957.

lin and 1 mg. per ml. heat-killed *Mycobacterium butyricum*) and were bled weekly for antibody determination.

In all the studies blood specimens were taken aseptically from the marginal ear vein or by heart puncture, and allowed to clot at room temperature for 1 hour and then kept at 4°C. until processed. Within 24 hours after bleeding the sera were quickly frozen at −70°C. in rubber-stoppered pyrex tubes and stored at −20°C. until used.

Qualitative determination of antigen disappearance from the circulating blood was made by a capillary tube precipitin method. 0.2 × 100 mm. capillary tubes containing the unknown serum and a potent rabbit antiserum were incubated at 37°C. for 1 hour, then at 4°C. for 48 hours. Final readings for the presence or absence of precipitate were made at 48 hours. Normal serum was always included as a control. By employing known concentrations of antigen in normal rabbit serum it was determined that as little as 0.3 microgram antigen N per ml. was detectable with the available rabbit antisera.

Quantitative immunochemical determination of the amount of antigen present in the serum at intervals after challenge was made, employing standardized rabbit antisera, the precipitate nitrogen being estimated by a micromodification of the Heidelberger-MacPherson (16) method using the Folin-Ciocalteu reagent, or the microKjeldahl method, depending upon the amount of precipitate nitrogen, and the resulting data are expressed as micrograms antigen nitrogen per milliliter of serum.

Determination of antibody.—In most of the experiments reported the presence or absence of antibody was determined by the capillary tube precipitin technique. The antibody concentrations given in one of the tables were determined quantitatively by the same techniques as antigen concentration and expressed as micrograms of antibody nitrogen per milliliter of serum. In some experiments the Ouchterlony agar diffusion plate method (17) was employed, placing 0.5 ml. antigen (5 to 50 μg. antigen N per ml.) in the center well and the 0.5 ml. of undiluted sera in surrounding wells.

Diphtheria antitoxin determination.—1.0 ml. fluid diphtheria toxoid (lot 1867-28A), kindly supplied by the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, New York, and containing 136 L$_f$ toxoid, was given to newborn rabbits and again as a subsequent immunizing dose. The presence of circulating antitoxin was inferred from reversal of a positive Shick reaction induced by giving 0.1 ml. of a dilution of diphtheria toxin (Lederle, lot 2286 4733A) containing 0.01 unit toxin per ml. (standardized against National Bureau of Standards horse antitoxin) intradermally, and examining the area 72 hours later for presence or absence of a reaction.

Group A streptococcal antibody determination.—A Group A, type 30, rabbit virulent streptococcus, obtained from Dr. Gene Stollerman of Northwestern University, Chicago, was used for all streptococcal experiments. Vaccines of heat-killed organisms were prepared and administered according to a schedule suggested by Lancefield (18). The presence of anti-M and anti-C in the rabbits' sera was detected by Lancefield's method, using appropriately absorbed hot acid extracts. Experimental infections were induced by giving 0.2 ml. of an 18 hour tryptose phosphate broth culture of the microorganism intradermally. Sensitivity to streptokinase was determined by injecting 0.2 ml. of saline dilution of commercial streptokinase-streptodornase (varidase) containing 500 units of streptokinase per ml. The skin tests were considered positive if an area of erythema and induration 1 cm. in diameter or greater was present at 48 hours.

Typhoid antibody determination.—1.0 ml. typhoid-paratyphoid vaccine (Wyeth TAB vaccine) was given intraperitoneally to newborn rabbits, and again as immunizing injection at 3 months of age. Bleedings were taken 4 and 10 days later, and antibody titers against the O and H antigens were determined by tube agglutination. The test antigens were kindly supplied by Dr. Henry Bauer of the Minnesota Department of Health.

Tuberculin sensitivity.-A culture of the Phipps strain of BCG was obtained from Dr. Gardner Middlebrook of Denver, Colorado, and maintained on Petroff agar slants. The live organisms were harvested by scraping the agar surface, suspended in 0.145 M NaCl as a heavy opaque suspension, and injected in a 1.0 ml. volume in newborn rabbits. The subsequent immunization attempts were made by giving weekly intradermal injections of 0.2 ml. of the living BCG suspension, and tuberculin sensitivity was detected by weekly testing, using 0.1 ml. of a 1:100 dilution of old tuberculin intradermally. A positive reaction consisted of 1.0 cm. or greater area of erythema and induration 48 hours after the test was applied. The groups of rabbits which had received 1.0 or 2.0 ml. of undiluted old tuberculin intraperitoneally at birth were sensitized and tested in the same manner as the BCG group.

Meningococcal endotoxin.- −0.2 ml. of a 1:10 dilution of the crude agar washings (19) was given to newborn rabbits, representing the maximum amount tolerated by newborn rabbits without significant mortality resulting from the endotoxin alone. The subsequent challenge in these experiments consisted in determining: (a) the febrile response to an intravenous administration of 1.0 ml. or a 1:10,000 dilution of the endotoxin; and (b) the incidence of the local Shwartzman reaction after an intradermal injection of 0.2 ml. of a 1:10 dilution of meningococcal endotoxin followed in 18 hours by an intravenous injection of 1.0 ml. of a 1:80 dilution.

Cell transfer studies were performed by a modification of the methods of Roberts and Dixon (20), and Harris, Harris, and Farber (21), employing donor animals which had been immunized by intravenous injections of alum-precipitated BSA. In each experiment the time of the last injection before transfer is given. The spleen, mesenteric, popliteal, and axillary lymph nodes were obtained under aseptic conditions immediately after sacrificing the donor by exsanguination, and were kept in standard Tyrode solution containing 0.125 per cent Knox gelatin while the cells were teased out with needle and forceps. The preparations were then strained through 50 mesh stainless steel gauze, and 100 units of penicillin and 1 mg. of streptomycin per ml. were added. The number of cells was counted in a standard hemocytometer chamber, viability estimated by trypan blue staining, and the cell suspension was then injected intramuscularly into the recipients. Siliconized glassware was employed throughout the procedure. Estimated viability of the cells in the experiments reported varied from 70 to 95 per cent, and the time elapsing from sacrifice of the donor to injection of cells did not exceed 2 hours in any experiment. In experiments in which the recipients also received BSA, this was given intravenously at the time of cell transfer. Bleedings of the recipients were made at 2 to 3 day intervals for 11 to 18 days and the presence of antigen or antibody determined by the methods described above. Recipients designated as x-irradiated received 400 roentgens total body radiation in a 100 kv. General Electric x-ray machine, 24 hours prior to cell transfer.

EXPERIMENTAL

Relation of Neonatal Antigen Dose to Incidence of Unresponsive State

Preliminary experiments indicated that prolonged failure of antibody production followed a single injection of 100 mg. BSA on the day of birth in rabbits as compared with uninjected litter mates. On this basis groups of three to five litters of rabbits were given a single intraperitoneal injection within 12 hours of birth of amounts of BSA ranging between 0.001 and 100 mg. Four litters of rabbits born within the same 2 week period were kept as controls. Between 87 and 120 days later the survivors of the groups injected at birth and the control litters received two intravenous injections of 10 mg. BSA 10 days apart, and the presence of BSA or anti-BSA in serum specimens was determined weekly for up to 6 weeks. The result

of this attempted immunization in relation to the amount of BSA received at birth is given in Table I.

In substantially all the animals given 100, 50, or 20 mg. BSA at birth, BSA was present in the sera longer than 2 weeks and no precipitating antibody was produced as a result of this stimulus. Approximately one-fourth of the rabbits which received 10 mg. at birth produced detectable antibody and nearly all the rabbits which re-

TABLE I

Influence of a Single Neonatal Injection of BSA upon the Immune Response to BSA in Rabbits between 87 to 120 Days of Age

Amount BSA injected during neonatal period*	No. in group	No. with detectable BSA in serum within given interval after second immunizing injection of BSA			No. with detectable anti-BSA in serum
		7–11	14–18	21–34	
mg.					
100 at birth.............	24	22	22	6	2
50 at birth.............	12	11	11	1	0
20 at birth.............	12	11	9	5	1
10 at birth.............	14	13	9	5	3
1 at birth.............	7	5	1	0	6
0.8 at birth.............	4	1	0	0	3
0.1 at birth.............	3	0	0	0	3
0.01 at birth............	2	0	0	0	2
0.001 at birth...........	4	0	0	0	4
100 at day 3............	47	44	41	0	3
100 at day 9............	4	3	2	2	1
100 at day 15...........	16	6	4	2	7
100 at day 17...........	16	4	4	0	12
None..................	18	3	1	0	16

* Litters of rabbits were given the indicated amounts of BSA intraperitoneally on the designated day of life. After 87 to 120 days the survivors were challenged with two intravenous injections of BSA, 10 days apart, and bled weekly or more often thereafter until antigen had disappeared or antibody had appeared in the serum.

ceived 1 mg. or less at birth responded at this age by developing detectable antibody, as did 16 of the 18 control animals.

From these data it would appear that the minimal amount of BSA given at birth which is required to induce unresponsiveness lasting 3 to 4 months in a high percentage of rabbits, is between 10 and 20 mg.

Relation between Age at Neonatal Injection and Incidence of Unresponsive State

As a part of the first experiment, litters of rabbits were given 100 mg. of BSA intraperitoneally on the 3rd, 9th, 15th, or 17th day of life, and their response to

antigenic challenge tested at 87 to 120 days as above. The results (also shown in Table I) indicate that while the majority of the 3 day group did not respond to stimulation, one-fourth of the 9 day group, one-half of the 15 day group, and three-fourths of the 17 day group were able to form antibody to BSA at 3 to 4 months of age.

These data indicate that unresponsiveness lasting 3 to 4 months can be induced, with the relatively large amounts of BSA employed, only if the antigen was given during the first 2 weeks of life, unresponsiveness occurred in a small but significant proportion of animals when the antigen was given at 15 to 17 days of age.

Duration of the Unresponsive State

Groups of animals given 10 to 100 mg. BSA during the neonatal period, and found to be unresponsive at 87 to 120 days as described in the preceding sections, were rechallenged at 90 to 120 day intervals (exceptions noted) for up to 620 days. The challenge in each instance consisted of a single intravenous injection of BSA, usually 15 mg. per kilo. Eighty-five to 95 per cent of normal rabbits of comparable weight, which were included in each challenge group as controls for the antigenicity of the BSA, cleared antigen within 14 days and developed circulating antibody. Table II gives the results of attempts to challenge the unresponsive groups in terms of the occurrence or failure of precipitating antibody production.

These data indicate that most of the animals which received 20 to 100 mg. of BSA at birth failed to produce antibody over the 620 day period of study, and as will be shown, they continued to show prolonged antigen clearance. Three of the seven animals of the 10 mg. group produced antibody on the third challenge at 380 days, but four were unresponsive on each challenge. The three rabbits which produced antibody at 380 days had cleared antigen rapidly (half-life = 1.9, 2.6, 4.0 days) after the 194 day challenge in contrast to their initial challenge, but failed to produce detectable circulating antibody. Of the group which received 100 mg. at 3 days of age, three animals were persistently unresponsive through the fifth challenge. All but one of the 15 day group which were unresponsive at the initial challenge showed evidence of an immune response by the third immunization attempt.

The data appeared to support the concept that when the unresponsive state is induced sufficiently early in the neonatal period with threshold amounts of BSA, it is essentially permanent. The data also suggested that when it is produced with smaller amounts of antigen (e.g. 10 mg.), or later in the neonatal period, the unresponsive state is either incomplete or not permanent. However, the interpretation that the unresponsive state is permanent is based upon the assumption that the amounts of antigen employed for challenge on each occasion had no effect upon its duration. The experiments summarized in Tables II and IV, however, offered several observations casting doubt upon the validity of this assumption. For example, over 270 days had elapsed since the antigenic

challenge prior to which five animals which had received 100 mg. at 3 days of age, produced antibody. By contrast the interval between injections was always less than 200 days, and averaged 120 days in the persistently unresponsive group. Data to be presented in another section also hinted strongly that repeated attempts at immunization were possibly responsible for the observed persistence of unresponsiveness.

TABLE II

Results of Repeated Intravenous Injections of BSA in Rabbits Which Received Various Amounts of BSA in the Neonatal Period and Were Unresponsive to BSA Challenge at 87 to 120 Days of Age

No. of challenge*	Age range of group at time of challenge	Anti-BSA production						
		Injected at birth				100 mg. injected		
		100 mg.	50 mg.	20 mg.	10 mg.	3 days	9 days	15 days
	days							
1 (see also Table I)	87–120	2/24‡	0/12	1/12	3/14	3/47	1/4	7/16
2	154–211	0/22	0/3		0/7	1/15	1/3	2/8
3	267–411	1/14	0/2	1/5	3/7§	6/9‖	0/2	2/6¶
4	472–531	0/8	0/1		0/4	0/3	0/1	0/1
5	532–620	0/8	0/1		0/4	0/3	0/1	0/1

* Antigenic challenge on each occasion other than the original challenge (see Table I) consisted of an intravenous injection of 15 mg. per kilo BSA, followed by weekly bleedings until antigen was no longer detected or maximal antibody titer had been achieved. Animals which produced circulating antibody were discarded before the succeeding challenge.

‡ Numerator, those in the challenged group which produced detectable antibody; denominator, number in group challenged within age interval.

§ Three animals producing antibody showed rapid clearance of antigen on prior challenge, but no detectable antibody.

‖ Five of the 6 animals which produced antibody in this group had not received BSA since the first challenge (270 to 300 day interval).

¶ Three animals which did not produce antibody showed rapid clearance after this challenge, but died before any subsequent challenge.

As a result of these earlier studies, experiments were designed to obtain data on the duration of the unresponsive state in the absence of repeated antigenic challenge.

Litters of rabbits were given 100, 10, 1.0, or 0.1 mg. BSA intraperitoneally at birth; groups receiving each amount were challenged with litter mate controls only once, at intervals which depended upon the amount given at birth. The results of these attempted immunizations, given in Table III, show clearly that the unresponsive state is of a finite duration, related generally to the amount of antigen administered at birth. Sufficient numbers of groups were not included to define exactly the duration of unresponsiveness in each dosage group; however, it appears that the duration is

longer than 135 and less than 189 days in the 100 mg. group, between 103 and 189 days in the 10 mg. group, between 62 and 100 days in the 1 mg. group, and between 47 and 62 days in the 0.1 mg. group.

These data, while establishing that unresponsiveness is not permanent, also indicate that it was greatly prolonged in the prior experiments by the increments of antigen required to elicit a specific immune response.

TABLE III

Duration of Unresponsiveness in Rabbits Given 0.1, 1.0, 10.0, or 100.0 Mg. BSA at Birth, and Challenged only Once

Amount injected at birth	Age at time of challenge*	No. producing detectable antibody No. in group
mg.	*days*	
100	100	0/4
	135	0/3
	163	3/7
	189	4/5
	230	4/5
	250	3/3
10	89	0/3
	103	3/6
	180	5/5
1.0	64	0/3 (5/5)‡
	122	3/4
0.1	31	1/8 (5/8)‡
	47	0/8 (5/5)
	62	4/5 (8/9)
	109	5/5 (6/6)

* Litters of rabbits were given the indicated amount of BSA at birth, and challenged only once at the indicated age. At that time they were given 15 mg. per kilo BSA intravenously, and bled serially for detection of precipitating antibody.

‡ Results of simultaneous immunization in litter mates which had received no antigen at birth are shown in parentheses.

Effect of Intradermal Injections of BSA in Unresponsive Rabbits

It seemed possible that more intensive and sustained antigenic stimulation might induce a specific immune response in unresponsive animals, which failed to respond when challenged intravenously with similar amounts of antigen. Accordingly, groups of rabbits which had received from 50 to 200 mg. BSA at birth, and which had been demonstrated on one or more occasions to be unresponsive, were given intradermal injections of BSA, incorporated in complete Freund's adjuvants in amounts ranging in various attempts from 10 to 80 mg. Table IV shows the result of these immunization attempts. Animals getting BSA—adjuvant mixture failed to respond with anti-

body production when less than 100 days had passed since intravenous challenge. However, in a group challenged after a 145 day interval, half produced antibody and all the rabbits stimulated at 209 or 226 day intervals responded. Animals with identical prior injection histories were not available as controls to receive intravenous injections of plain BSA in identical amounts. However, comparable data on animals with similar, but not identical injection histories, which were injected intravenously with plain BSA, indicate that the intradermal administration of BSA in adjuvants apparently provided a greater antigenic stimulus than plain BSA given intravenously. This interpretation must be made cautiously, however, since large sterile abscesses

TABLE IV

Results of Intradermal Injections of BSA in Previously Unresponsive Rabbits of Various Ages and Intervals Since Prior Antigenic Challenge *

Interval since prior injection	Age	Amount of intradermal BSA	No. producing anti-BSA ⁄ No. in group
days	*days*	*mg.*	
60	284	40	0/4
90	190	80	0/2
90	288	10	0/3
145	265	35	1/2
209	312	80	3/3
226	330	80	1/1
235–279‡	335–389	10–45 (intravenous)	1/7

* Rabbits which had received 50 to 200 mg. BSA at birth, and which had been found unresponsive to one or two prior BSA injections, were given BSA intradermally incorporated in complete Freund's adjuvants, and serum specimens taken at weekly intervals thereafter tested for the presence or absence of BSA or anti-BSA.

‡ This group, consisting of rabbits which had received 50 to 100 mg. BSA at birth and which had been unresponsive on one prior occasion, was challenged within the stated intervals by intravenous injection of plain BSA, and is shown here for comparison with intradermally injected animals with a similar injection history. Note that this group was not run concurrently with the intradermal groups, and therefore cannot be considered a true control.

produced by administering adjuvant mixtures were often associated with loss of weight in the animals and, in a few observations, with extremely rapid non-immune disappearance of intravenously administered BSA, suggesting a high rate of protein turnover such as occurs in dogs with turpentine abscesses (22).

These results add to the evidence that unresponsiveness is not a permanent state, and that its duration is related to the interval since prior antigen administration as well as to the original dose received.

To complete the evidence that the precipitating antibody which appeared in the serum of previously unresponsive rabbits after those challenges described in the previous two sections was indeed anti-BSA, and not antibody against a minor component of the antigen, the agar diffusion technique was employed. The serum of each animal which produced anti-BSA represented in Tables I,

II, and IV, when allowed to diffuse and interact with crystalline BSA, showed a single band varying somewhat in width, which "cornered" cleanly with anti-BSA of known homogeneity. This "reaction of identity" provides evidence that the antibody produced was not directed against trace components of the antigen (23).

Unresponsiveness to Other Heterologous Proteins Induced by Neonatal Injection

A single injection of ovalbumin (100 mg., 10 mg., and 1 mg.), human gamma globulin (166 mg.), or a human macroglobulin (105 mg.) was given intraperitoneally to litters of rabbits on the day of birth. The results of these experiments, summarized in Table V, show that neonatal injection of soluble proteins from three animal species, varying in estimated molecular weight from 40,000 (EA) to approximately 1,000,000 (macroglobulin), inhibited the immune response to the same antigenic challenge at 100 to 167 days.

As with BSA, the effectiveness of EA in inducing unresponsiveness appeared to be related to the amount given at birth, requiring more than 10 mg. for unresponsiveness of 167 days' duration. EA unresponsiveness also appears not to be permanent, since by 262 days all but two of the rabbits receiving the largest amount of EA had produced antibody.

In other experiments which are not presented here in detail, unresponsiveness was induced to human serum albumin (fraction V) and bovine gamma globulin (Armour). It is of interest that since both of these antigens were relatively inhomogeneous, precipitating antibody formed in response to unidentified minor components at the time of the second antigenic challenge while the major component was cleared slowly and no precipitating antibody was detected. Similarly, a small amount of precipitating antibody to a minor component of HGG was observed in the two animals challenged the second time at 280 days (Table V).

Characteristics of Antigen Disappearance in Unresponsive Rabbits

As is shown in Table I, antigen clearance as compared with controls was greatly prolonged in those animals failing to produce anti-BSA. Since the rate and character of antigen disappearance are related closely to antibody formation to heterologous proteins (24, 25), it was of interest to examine quantitatively the rate of antigen disappearance in rabbits which were unable to form precipitating antibody. Data from quantitative determinations of the amount of BSA in the serum of representative animals, which in retrospect had failed to produce precipitating antibody and cleared antigen slowly, were plotted semilogarithmically, and half-life estimations taken from those plots in which three points or more were available. As will be seen from data summarized in Table VI, and exemplified by the plots on three unresponsive rabbits challenged at 8 months of age (Text-fig. 1), the disappearance rate is linear as long

as measurable antigen is present in the serum. No phase of accelerated disappearance such as characterizes the normal immune response, is observed. Half-life values for BSA in the unresponsive animal were all between 5.1 and 8.5 days (mean value = 6.86 days), and did not vary significantly on successive challenges in the same rabbit. Presumably the progressive decrease in serum concentration is related to the rate of catabolic breakdown (26) of protein in the animal, although the possibility of bowel or urinary excretion has not been excluded by critical studies. The half-life values in unresponsive rabbits are

TABLE V

Response to Human Gamma Globulin, Human Macroglobulin, and Ovalbumin at Various Ages in Rabbits Which Received the Antigen at Birth

Antigen given at birth and amount	Results of initial antigenic challenge*		Results of second antigenic challenge	
	Age at challenge	Antibody production	Age at challenge	Antibody production
	days		*days*	
HGG 166 mg.................	100	0/5‡	280	0/2
None.......................	100	5/5	—	—
Macroglobulin 105 mg..........	120	0/9	—	—
None.......................	120	5/6	—	—
Ovalbumin				
100 mg.....................	167	0/4	262	1/3
10 mg.....................	167	2/6	262	3/4
1 mg.....................	167	2/4	262	2/2
None.......................	167	7/7	—	—

* Rabbits which received the indicated amounts of antigen at birth were challenged at the stated intervals by a single intravenous injection of 15 mg. per kilo of the antigen given at birth, and bled serially for antigen or antibody determination.

‡ Numerator, number developing detectable antibody; denominator, number of survivors in group.

similar to those measured during the non-immune phase of antigen disappearance in normal animals in our laboratory, but are somewhat longer than those found by Dixon, Maurer, and Deichmiller (27) for homologous I^{131}-labelled albumin. The values, however, approximate very closely those obtained by immunochemical methods by Johnson, Watson, and Cromartie (28), and by Dixon and Maurer (29) for adult rabbits whose immune response was inhibited by massive administration of BSA, neonatal injection, or x-irradiation.

Specificity of the Unresponsive State

The highly specific character of the unresponsive state produced by neonatal exposure to protein antigen has been shown previously (12, 14). Table VII

gives the results of an experiment showing a normal immune response to oval-bumin in rabbits unresponsive to BSA because of a single injection of 100 mg. given at birth. These data confirm the previous studies in showing that the unresponsive state is in a sense, as immunologically specific as the normal immune response.

TABLE VI

Rates of Disappearance of Intravenously Injected BSA from Serum of Unresponsive Rabbits*

Animal No.	Amount at birth	Half-life of BSA at varying age		
		Age range, *days*		
		100–113	187–201	322–329
		days	*days*	*days*
41–72	100 mg.	7.3	6.8	
41–74		8.4	6.2	
41–75		6.0	6.6	
41–76		5.1	6.0	6.0, 6.9
164				8.3
156	50 mg.			7.4
41–38				5.3
41–37				7.1
159	10 mg.	.	8.6	
202			4.7	
41–69			5.9	6.4
41–58			5.9	7.9
41–66			8.5	7.8
151	100 (9 days)			7.0
183	100 (15 days)			8.0
192	100 (15 days)			5.9

* Half-lives measured on serum of animals which in retrospect were repeatedly unresponsive to BSA because of neonatal injection of that antigen.

Unresponsiveness to BSA Induced by Oral Administration at Birth

Absorption of intact proteins from the gastrointestinal tract occurs during the neonatal period in several species (30–32), and following diarrheal states in human infants (33). In preliminary experiments 260 mg. of BSA was given to rabbits by polyethylene gavage tube on the day of birth; BSA was consistently detected in the sera at 1 to 3 days of age in concentration as high as 13.5 mg. per ml. serum. Significant absorption of this protein could be demonstrated up until 14 days of age, but after that absorption was irregular and never more than a trace in amount. Litters of rabbits were then fed varying amounts of BSA at birth by gavage tube, and challenged at 54 to 140 days of age to determine their response to BSA. The results of three such

experiments, given in Table VIII, indicate that an unresponsive state was induced in a significant number of rabbits in some litters; however, much greater variation was encountered than in with those rabbits to which similar amounts of this antigen
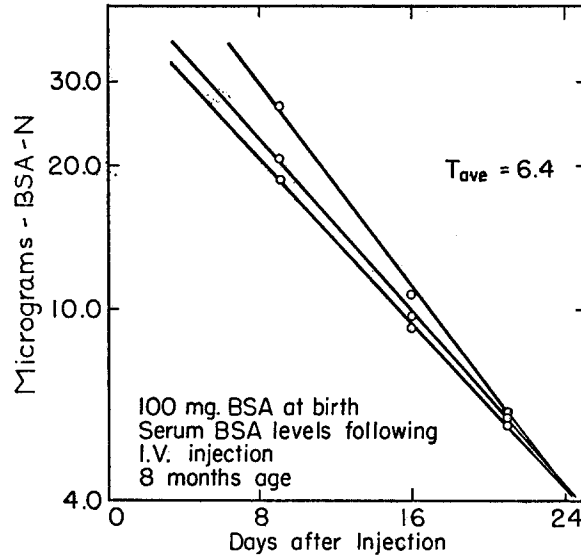
TEXT-FIG. 1. Rate of disappearance of BSA in three unresponsive rabbits at 8 months o age, following intravenous injection of 25 mg. BSA per kilo. The average half-life was 6.4 days.

TABLE VII

Immune Response to Ovalbumin in BSA-Unresponsive Rabbits

Group	No. in group	No. with detectable anti-EA*	No. with Arthus reaction
Received BSA at birth, no response at 100 and 230 days..............	5	5	5
Controls, same age, no BSA..........	5	5	4

* Results of serial bleedings following each intradermal injection of 1 mg. ovalbumin every other day for 21 days.

were given parenterally. Again a relationship between the age at challenge and the outcome of attempted immunization is seen.

These results appear to be accounted for entirely by the absorption, intact, of amounts of immunologically active protein comparable to those which in other experiments induced unresponsiveness when given intraperitoneally. The only similar experiments found in the literature are those of Chase (34), who

by administering large amounts of 2,4-dinitrochlorobenzene in oil to adult guinea pigs orally, was able to induce a prolonged state of specific unresponsiveness, whereas hypersensitivity to this substance always resulted from intradermal injection in unfed controls. Inasmuch as 2,4-dinitrochlorobenzene is highly irritating, it seems doubtful that this phenomenon could be accounted for by direct absorption of the intact chemical, but it does seem possible that absorption could occur after conjugation with some substance within the lumen of the intestine.

TABLE VIII

Effect of BSA Given Through a Gastric Tube at Birth on Subsequent Immune Response to BSA*

| Litter No.	Age at challenge	Antibody response
	days	
1	54	1/6‡
Controls	60	7/7
2	117	1/2
3	117	2/5
4	125	1/1
Controls	120	4/4
5	138	2/2
6	140	5/5
Controls	140	4/4

* Litters of rabbits were fed approximately 1.0 ml. of 10 per cent BSA by polyethylene gastric tube on the day of birth. At the indicated age these and control litters born at the same time were challenged with intravenous BSA, 15 mg. per kilo, and bled serially to detect anti-BSA production.

‡ Numerator, number producing detectable anti-BSA; denominator, number challenged.

Cell Transfer Studies

The techniques of transfer of lymphoid tissue from an immune to a nonimmune animal were employed in various attempts to overcome inhibition of antibody production in the unresponsive animal. The experiments summarized in Table IX show first, that transfer of cells from normal animals previously immunized to BSA to an unresponsive host, resulted in antibody production comparable to that of the normal recipients. The average antibody content of the sera at varying times after the cell transfer in one of the experiments is shown graphically in Text-fig. 2. These data indicate that no humoral inhibitor of specific antibody production was operating, since the degree of response was quantitatively similar to that of normal animals. The data also suggest that any antigen which might hypothetically persist from neonatal injection was either located in sites which prevented significant binding of the antibody pro-

duced by the immune cells, or was present in amounts too small to affect the antibody levels significantly. It is of considerable interest that all the unresponsive animals which had produced antibody vicariously, were still unresponsive when rechallenged 2 months later, and injected antigen was cleared at the same rate as in the last challenge prior to cell transfer. Experiments in which lymph nodes and spleen cells were transferred from unresponsive to normal or normal x-irradiated animals, or from normal rabbits to x-irradiated unresponsive recipients without detectable antibody production, suggest that the spleen and lymph node cells of the unresponsive rabbit were not actively
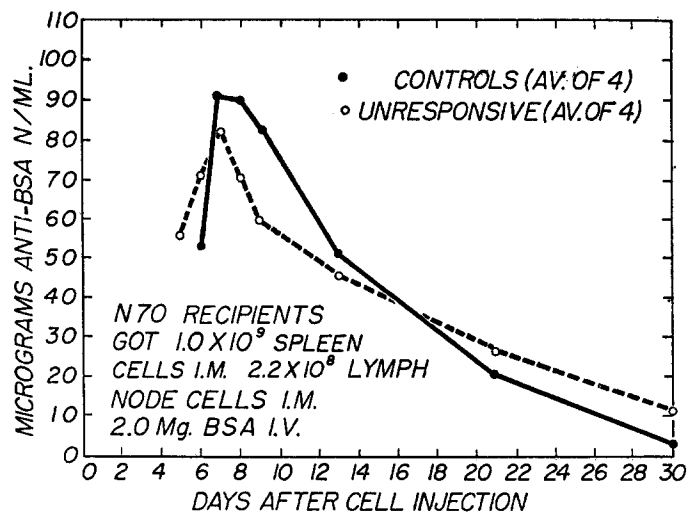
TEXT-FIG. 2. Anti-BSA titers in normal and BSA-unresponsive rabbits receiving immune cells.

engaged in antibody formation, and required more than a normal environment to initiate antibody synthesis. Since it has not been possible here or in other reported studies (20)[2] to induce in x-irradiated recipients detectable antibody production to heterologous proteins by transferred normal cells, these experiments must be regarded as inconclusive on this point. Examination of Wright-Giemsa–stained impression smears and methyl green–pyronine–stained sections of the depots of transferred cells during the height of antibody formation, revealed large numbers of what appeared to be plasma cells, particularly in the most peripheral areas of the depots. Similar observations in their studies of normal and normal x-irradiated animals are recorded by Roberts and Dixon (20).

[2] In a personal communication Dr. F. J. Dixon and Dr. William Weigle state that they have now been able to detect a primary immune response to BGG in unresponsive rabbits which have received large numbers of normal cells.

Attempts to Alter Response to Various Bacterial Antigens by Neonatal Exposure

Efforts were made to alter the subsequent specific response to a variety of bacterial products by injecting a relatively large amount of the antigen at birth.

TABLE IX

Production of Antibody in BSA-Unresponsive Rabbits Receiving BSA-Immune Cells

Donor			Recipient*				
Group	Mean anti-BSA titer	No. of donor cells injected into recipient	Group status	BSA given	No. in group	Mean day anti-gen clear-ed	Maximum antibody titer
	$\mu g./N/ml.$			$mg.$			$\mu g. N/ml.$
BSA-immune; 9 days poststimulation	125.5 (6)‡	1 × 10⁹ spleen 2.2 × 10⁸ lymph node	(a) Unresponsive (b) Normal	2.0 2.0	4 4	5 7	83.7 (4)‡ 94.5 (4)
BSA-immune; 41 days poststimulation	106.5 (5)	5.6 × 10⁸ spleen 9.6 × 10⁷ lymph node	(a) Unresponsive (b) Normal (c) Normal, x-irradiated	2.0 2.0 2.0	3 3 2	5 5 5	11.0 (3) 11.0 (3) 27.0 (2)
BSA-immune; 90 days poststimulation	12.5 (4)	1.4 × 10⁸ spleen 1.8 × 10⁸ lymph node	(a) Unresponsive (b) Normal	1.0 1.0	2 2	6 6	16.5 (2) 11.0 (2)
BSA-unresponsive	0 (3)	7.3 × 10⁸ spleen 5.6 × 10⁸ lymph node	(a) Normal, x-irradiated	2.0	3	12	0
BSA-unresponsive	0 (3)	7 × 10⁸ spleen 1.8 × 10⁸ lymph node	(b) Normal, x-irradiated	2.0	4	15	0
Normal	0 (5)	6.7 × 10⁸ spleen 1.8 × 10⁸ lymph node	(a) Normal, x-irradiated (b) Unresponsive x-irradiated	5.0 5.0	2 3	11 18	0 0

* Recipient rabbits were bled and the presence of antigen or titer of antibody determined at 1 to 3 day intervals after receiving the cells and BSA indicated. The mean day of antigen clearance is an average of the latest day antigen was detected in serum of each rabbit in the group.

‡ Number in parentheses refers to number of animals comprising the mean value.

The consistently negative results of studies with a wide spectrum of bacterial antigens are given in Table X. In every experiment the results of the test challenge at 3 months of age were not significantly different from those in similar numbers of control animals or litter mates. These data cannot be taken as indicating more than failure under the given circumstances, however. It would appear, for example, that Buxton (35) and Kerr and Robertson (8) have succeeded in inducing partial inhibition of the immune response to *Salmonella pullorum* in

TABLE X

Response to Various Bacterial Antigens in Rabbits Which Received the Antigen as a Single Neonatal Injection

Antigen employed	Antigen injected on day of birth	Age at challenge	Antigen employed in challenge, route, and dose	Criteria of response to challenge	No. responding / No. in group
		days			
Diphtheria toxoid	Fluid toxoid 130 Lf/ ml., 2.0 ml. intraperitoneally	134–141	Fluid toxoid, 130 Lf per ml., 1.0 ml. i.m. weekly 3 doses	Reversal of positive Schick test (to 0.1 ml. 1:4000 toxin), 7 days after 3rd injection	8/9
	Controls, no toxoid	127	Same		4/4
Typhoid paratyphoid	Commercial heat-killed vaccine containing 10^8 typhoid organisms and 10^7 paratyphoid A and B organisms, per ml. 2.0 ml. i.p.	62	TAB vaccine i.v., 1.0 ml. single dose	Agglutinating antibody to O or H antigens at 4 or 10 days after challenge in 1:160 dilution or higher	9/9
	Controls—no antigen at birth	69	Same		8/8
Group A streptococcus	Heat-killed type 30 streptococcal vaccine 10^8 organisms per ml. 2.0 ml., i.p.	124–130	(*a*) Heat - killed type 30 streptococcal vaccine 1.0 ml. i.v. 3 times weekly for 4 weeks	(*a*) Development of anti-M (type 30). Development of anti-A	6/6 6/6
	Controls—no vaccine	124–130	Same	Anti-M Anti-A	5/5 5/5
	Heat-killed type 30 streptococcal vaccine 10^8 organisms per ml. 2.0 ml., i.p.	124–130	(*b*) Living 18 hour culture, type 30 streptococcus, 0.2 ml. i.d. weekly for 4 weeks	(*b*) Development of delayed hypersensitivity to intradermal injection of 100 units streptokinase-streptodornase	6/6
	Controls—no vaccine	124–130	Same	Same	7/7
Tuberculin and/or BCG	Living BCG (Phipps strain) in thick saline suspension 1.0 ml. i.p.	102	Living BCG (Phipps strain) thick saline suspension, 0.2 ml. i.d. weekly in different sites	Development of delayed sensitivity to i.d. injection of 1:100 old tuberculin, tested weekly	5/5
	Controls—none	116	Same	Same	8/8
Tuberculin and/or BCG	Old tuberculin, 1.0 ml. undiluted, i.p.	100	Same	Same	4/4
	Controls—none	100	Same	Same	8/8
Bacterial endotoxin	1:10 meningococcal culture filtrate 0.2 ml.-i.p.	90	Meningococcal filtrates	(*a*) Febrile response to 1.0 ml. 1:10,000 dil. i.v.	(*a*) 4/4 (*b*) 4/4
	Controls—none	86	Same	(*b*) Local Shwartzman reaction 0.2 ml. 1:10 i.d. preparing dose followed in 18 hours by 1.0 ml. 1:80 i.v.	(*a*) 4/4 (*b*) 4/4

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chicks and to *Trichomonas foetus* in cattle, respectively, by neonatal injection. Perhaps, in view of the demonstration that relatively large amounts of defined protein antigens are required to induce an unresponsive state lasting 3 to 4 months, this threshold was never achieved with respect to any single antigen by injection of the whole bacterial cell or crude extracts. Too, earlier challenge may have demonstrated differences in injected animals and controls, although in experiments to be reported, it has been found that an immune response occurs late in the neonatal period even when relatively large amounts of bacterial antigens are given at birth. On the other hand, the particulate or macromolecular state of these bacterial antigens differed significantly from the proteins, and might, as a result, elicit a different response mechanism. In spite of these negative results it appears that further work along these lines is warranted.

DISCUSSION

In these studies attempts have been made to define semiquantitatively, the state of specific immunological unresponsiveness, or tolerance, which is induced by injecting heterologous proteins into newborn rabbits. The data presented indicate that BSA in amounts exceeding 10 mg. given intraperitoneally or fed orally at birth, inhibits the normal immune response to this antigen for 90 to 120 days, and that 100 mg. given intraperitoneally as long as 17 days after birth results in some inhibition of the immune response tested after this period. Similarly, specific inhibition of immunity was induced with defined protein antigens of avian and human origin, ranging in estimated molecular weight from 40,000 to one million. The characteristics of the antigen disappearance in unresponsive animals are similar to the non-immune phase of antigen elimination in normal animals, but the loss from the serum continues exponentially so long as measurable amounts are present.

The data regarding the duration of the unresponsive state appear to be of particular interest in relation to the possible mechanisms involved. If the state of unresponsiveness once induced by a single threshold dose of the antigen given during a critical phase of early postnatal life was found to be permanent and independent of further antigenic challenge, such evidence might be interpreted as indicating that a permanent adaptive change had occurred during the neonatal period prior to development of the capacity to recognize the proteins as foreign (or "non-self"), and that the change had been passed on to the progeny of the cells originally making the adaptation. Such a mechanism, proposed by Burnet and Fenner (36), finds apparent support in the experiments showing induction of prolonged tolerance to homografted tissue by a single injection of viable reticuloendothelial cells at birth (11, 37–40) and in experiments demonstrating tolerance of tumors induced by neonatal implantation (41, 42). However, the data presented in this paper seem clearly to indicate

that unresponsiveness to defined protein antigens, at least, is finite, and related in duration to the amount of the antigen provided at birth. Further, the additional increments of antigen provided by repeated attempts to challenge the unresponsive animal at intervals up to 620 days, definitely prolonged the unresponsive state much longer than the expected duration for a given neonatal dose. It is therefore difficult to reconcile these data with the adaptation hypothesis as it has been stated.

The data presented, on the other hand, appear to be consistent with an hypothesis that the persistence of the originally injected antigen in critical tissues is directly or indirectly responsible for inhibition of the immune response to this antigen at subsequent challenge. In support of this, the duration of the unresponsive state is directly related to the amount of antigen given at birth. Regardless of the amount of BSA given at birth, calculations based upon the assumption that the measured antigen disappearance rates in the serum can be extrapolated beyond measurable levels, yield the same value of from 10^{12} to 10^{13} molecules of BSA, as the total number remaining at a time estimated to mark the end of the unresponsive state (*i.e.*, midway between the latest age at which unresponsiveness was demonstrated, and the earliest age at which an immune response was observed). Although these figures are subject to revision with the availability of more refined data, the order of magnitude suggested is a sufficiently large number of molecules to conceivably play a role in suppression of the immune response. The site at which the hypothetically persisting antigen localizes would then be of considerable interest. The data presented would suggest that the critical antigen may be located intracellularly, unavailable to passively administered antibody (13), or to antibody produced by transferred cells, since such antibody has no effect upon the unresponsive state. Only the liver is a known site of prolonged persistence of protein antigen in rabbits, although in the reported studies this localization occurs in association with antibody formation (43) rather than with an unresponsive state. Studies in progress on the relative antigen concentration in various organs and cells of the young and unresponsive rabbit, compared with that of adult animals, and its association with various nucleoprotein fractions, may provide more precise information on the localization and significance of antigen in the unresponsive animal.

A review of the various experimental models of immunological tolerance reveals considerable data bearing upon the finding that a continuous supply of antigen is necessary to maintain a prolonged state of unresponsiveness. Germane to the present studies are recent investigations of Wolf and associates (44) demonstrating specific suppression of the precipitin response in chickens by intraperitoneal administration of BSA within 50 hours of hatching. Little or no precipitin formation occurred in these animals upon challenge at 6 weeks of age, but by 12 weeks, only a slight statistical difference from controls

in mean precipitins titers was found. In mice (45), a single neonatal injection of 18 to 36 mg. BSA induces a state of tolerance to anaphylatic sensitization present at 7 weeks, but not at 10 weeks of age. Similar results in mice have been obtained independently by Torres and Hughes (46).

Less clearly related to the present studies are those in which adult rabbits were injected with massive amounts of heterologous proteins (13) or BSA (28), resulting in prolonged antigen clearance and failure to form the corresponding precipitating antibody. Most of the animals in these studies could produce precipitating antibody to BSA after final clearance of the antigen, however. Sufficient data are not available to determine with finality whether the amount of antigen required at birth to induce unresponsiveness might be quantitatively similar, when related to body mass, to that required to suppress the immune response in adult animals. Such calculations as may be made from existing data suggest that a much larger amount per unit of weight, is required for suppressing the adult immune response to BSA, adding to other evidence that the production of unresponsiveness requires the special conditions present in the newborn period or perhaps in the x-irradiated rabbit (13), but not in the adult animal.

Simonsen (39) was able to suppress agglutinin formation to turkey and goose erythrocytes in chickens at 6 weeks of age by injection of the corresponding erythrocytes before hatching; later challenge, however, demonstrated no suppression of immunity. Hasek (38) partially suppressed both precipitin formation after goose serum protein injection and agglutinin formation to goose erythrocytes in 8 week old ducks by a single injection of goose erythrocytes after 15 days' incubation. By repeated injections starting at hatching, however, nearly complete suppression of agglutinin formation lasted at least until 25 weeks of age. By analogy, the foreign cell precursors persisting in both naturally occurring (47), and artificially induced (38) blood cell chimera, might provide a continuous source of antigen.

With respect to pneumococcal polysaccharide, clear-cut evidence is available that persistent antigen is associated with suppression of the specific precipitin response to this antigen (48). Like unresponsiveness to defined protein, "immunological paralysis" is highly specific, (49, 50) dose-dependent, and lasts at least as long as antigen persists (51, 52). However, apparently unlike the present or other similar models, it may be induced in adults with appropriate amounts of antigen, and passively administered antibody is bound by the persisting antigen. The relation of this phenomenon to the other examples of tolerance therefore remains moot until more data are available.

The data on tolerance of homografted tissue or tumors induced by neonatal or prenatal injection of viable cells also do not relate clearly to the present studies. It seems reasonable, however, to interpret the function of the viable cells injected at birth, whose progeny are known to persist (53), as a continuous

source of antigens which suppress transplantation immunity, analogous to the requirement of a continuous supply of antigen for prolonged unresponsiveness to defined proteins. The observation (11) that acquired tolerance to homografts is reversed by giving tolerant mice normal adult isologous cells, might then be interpreted as a means of bringing about rejection of the original cells at the same time as the homograft, thus destroying the source of transplantation antigens. Until more is known of the nature and number of tissue antigens involved in transplantation immunity, these suggestions remain highly speculative.

In conclusion, the present study and the available data from similar experimental models, indicate tentatively that the requirements for prolonged immunological tolerance include: (a) initial exposure to a threshold amount of the antigen around the time of birth, and (b) a continuous source of the antigen. The critical question of how this antigen inhibits rather than initiates a specific immune response remains unanswered. The answer to this question, however, appears fundamental to an understanding of immunological tolerance as well as of acquired immunity, and does not appear to be satisfactorily accounted for in any of the current theories of acquired immunity (54–56).

SUMMARY

The phenomenon of immunological unresponsiveness induced in the neonatal rabbit by a single injection of a defined protein antigen, has been characterized semiquantitatively, and studies bearing upon the mechanism of such unresponsiveness have been presented.

A single intraperitoneal injection at birth of 10 to 100 mg. BSA, HGG, ovalbumin, or a human macroglobulin, or an oral feeding of 100 mg. BSA, induced a state of unresponsiveness lasting at least 90 to 120 days. 100 mg. BSA given from birth to 17 days of age, but not later, produced unresponsiveness of 90 to 120 days' duration.

Data are presented which show that the duration of unresponsiveness is finite and related to the amount of antigen given at birth, and that it may be indefinitely prolonged by repeated injections of antigen.

Disappearance of injected antigen in the unresponsive animal was exponential with time, with no accelerated or immune phase. Administration of the antigen in adjuvants resulted in significant shortening of the duration of unresponsiveness. The transfer of immune cells to the unresponsive host while resulting in vicarious antibody formation, did not affect the underlying unresponsive state.

Negative results of attempts to produce unresponsiveness to a variety of bacterial antigens are presented. The implications of the data are discussed, particularly in reference to the other experimental models of immunological tolerance, and to the various theories of acquired immunity. It is clear that

any satisfactory theoretical explanation of acquired immunity will have to account simultaneously for the phenomena of immune tolerance.

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