INDUCTION OF TOLERANCE TO HETEROLOGOUS PROTEINS AND THEIR CATABOLISM IN C57BL/6 MICE*, ‡

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It has been shown by different authors (1–3) that mice can be made immunologically unresponsive to serum protein antigens by various experimental procedures. However, these studies were carried out only with bovine serum albumin and bovine gamma globulin, and the duration of tolerance achieved was relatively short. In the present study, a number of different antigens were employed to see if some of them induced a more complete and longer lasting state of unresponsiveness. Newborn C57BL/6 mice were injected with a single large dose of one of 13 different heterologous proteins. The duration of tolerance was followed in appropriate experimental groups until the animals lost the unresponsive state. Attempts were made to correlate the duration of tolerance with the "catabolism" of these proteins. By performing these experiments, it was hoped to get some answer to the still poorly understood mechanisms underlying the phenomenon of tolerance as well as that of antibody formation.

For the purpose of this report, "catabolism" is used to denote the disappearance of I^{131}-labeled proteins from the host, although it is recognized that unlabeled fractions of these proteins may persist intracellularly. However, this technique is the best at hand and data obtained by others (4) with it have been used to make certain postulations which are not supported by the present data.

Materials and Methods

Mice.—C57BL/6 obtained from the Roscoe B. Jackson Memorial Laboratory at Bar Harbor, Maine were used in all experiments. Breeding animals were caged with 3 to 4 females and 1 male, and pregnant females separated in single plastic cages. With careful handling of

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pregnant mice and their offspring, the over-all surviving rate was somewhat more than 50 per cent. The offspring were usually kept until about 25 to 28 days, and at times, even longer with their mother and then caged separately. Several days prior to the injection of I^{131}-labeled proteins KI was added to the drinking water in order to saturate iodine-utilizing tissues.

Antigens.—Proteins were obtained from the following sources: Rabbit serum albumin (RSA), Pentex, Inc., Lot 7501; human serum albumin (HSA), Squibb, batch 1186-IRR; sheep serum albumin (SSA), Pentex, Inc., Kankakee, Illinois, Lot 140G01; bovine serum albumin (BSA), Armour and Co., Lot W69012; equine serum albumin (ESA), Pentex, Inc., Lot E52; rabbit gamma globulin (RGG), Pentex, Inc., Lot R214; human gamma globulin (HGG), Squibb, batch 1895; sheep gamma globulin (SGG), Pentex, Inc., Lot 523; bovine gamma globulin (BGG), Pentex, Inc., Lot A061 and Armour and Co., Lot C-904; equine gamma globulin (EGG), Pentex, Inc., Lot E23; turkey gamma globulin (TGG), Pentex, Inc.; chicken serum albumin (CSA), prepared in our own laboratory by fractionation of whole serum (5); hemocyanin (HC) from giant keyhole limpet (*Megathura crenulata*) was prepared by differential centrifugation.[1]

Labeling of Proteins.—Proteins were trace-labeled with I^{131} (I*) as described previously (6). The amount of I*-protein injected was kept as low as possible in order to increase the sensitivity in assaying small amounts of antibodies (7). Therefore, relatively dilute protein solutions had to be labeled, which easily became denatured. As a consequence, all these preparations were used either immediately after labeling, or were stabilized by addition of mouse serum. However, it was found that the elimination of these preparations in untreated animals was quite inconsistent from one experiment to another. Therefore, the half-lives as given in Table II were obtained by injecting more concentrated proteins which contained less radioactivity per unit weight. The total amount of radioactivity per injection was in all experiments approximately 2 μc.

Induction of Immunological Unresponsiveness to Various Proteins.—Newborn mice were injected with a single dose of 20 mg protein within 24 hours after birth. In the hemocyanin experiment, only 5 and 16 mg, respectively, were given. The antigens were dissolved in phosphate-buffered 0.15 M NaCl (PBS) and volumes of 0.05 to 0.1 ml were injected subcutaneously in the neck region. Several of the protein preparations used were highly toxic for neonatal mice. However, extensive dialysis against PBS in the cold usually removed the toxicity.

Challenge Procedure and Tests for Tolerance.—It is very difficult to obtain by usual methods an immune response to protein antigens in C57BL/6 mice. From all the various procedures and experiments, it seems that the incorporation of the antigen into Freund's adjuvant is the method of choice, and regularly an immune response can be observed. The animals were separated into groups, and the groups challenged 4, 6, 8, 12, or 18 weeks after birth by injecting into the foot-pads 0.1 mg antigen in 0.05 ml of incomplete Freund's adjuvant (8). Thus, once an animal had been challenged with the antigen and the tolerance examined, it was discarded.

Antibody formation was ascertained by following the elimination of circulating trace-labeled antigen (9). This method has been shown to detect as little as 0.0012 to 0.0033 μg antibody nitrogen per ml of serum (7). In a large number of experiments it was found that antibodies began to appear around the 5th day after immunization. Therefore, 1 week after the mice had been injected with antigen in Freund's adjuvant, a small dose of labeled antigen (8 to 15 μg, approximately 2 μc) was injected intraperitoneally (i.p.). Whole body counts were determined in a well type Geiger-Müller counter right after injection of the I*-protein and repeated at constant intervals. The counts were corrected for coincidence, background, and decay of I*. Control groups consisting of normal animals of approximately the same age, either untreated or immunized at the same time as experimental animals, were included in every experiment. The experimental procedure is outlined in Table I.

[1] The procedure was obtained through the courtesy of Dr. D. H. Campbell.

Experimental animals (A) were assumed to be fully tolerant (+) if the trace-labeled protein was eliminated at the same rate as in the non-immunized controls (D), partially tolerant (±) if the rate of elimination was significantly shorter than in this control group, and non-tolerant (−) if the rate of elimination followed the same pattern as in the immunized control group (C) (Fig. 1). A typical experimental finding is shown in Fig. 2 where all the animals (A) injected with RGG were only partially (±) tolerant when challenged 12 weeks after birth.

Individual sera were tested for antibodies by hemagglutination tests (HA) 2 to 3 weeks after the injection of the trace-labeled protein. A modified Takatsy technique (10) and sheep erythrocytes prepared according to a method described by Csizmas (11) were used. Furthermore, the sera were analyzed by double diffusion in agar (O) (12) and immune electrophoresis (IE) (13).

RESULTS

1. Elimination Rates of Different Serum Proteins.—Elimination rates were determined graphically by plotting log remaining I*-activity *versus* time following injection. From these plots half-lives were calculated. The results given in Table II show that the albumins had a half-life ranging from 11 to 26 hours. These proteins were eliminated more or less exponentially. On the other hand, mammalian gamma globulins showed a different behavior. They were eliminated much slower than the albumins and the elimination rates were not constant during a given time period of several days, being always greater during the 1st day than 2 or more days later. This might be the result of heterogeneity, denaturation, or to a lesser degree, to impurities inherent in commercial gamma globulin preparations. For our purposes, only the rates of the slower phase were taken into consideration. Half-lives of mammalian gamma globulins thus ranged from 4 to 8 days. TGG was eliminated like an albumin with an average half-life of 11 hours, whereas HC was cleared at an even faster rate.

TABLE I

Outline of Experimental Procedure

Group	No. of Mice	Protein at birth*	Subsequent injection of protein‡	I^{131}-protein§
A	‖	Yes	Yes	Yes
C	5	No	Yes	Yes
D	5	No	No	Yes

* 20 mg protein injected within 24 hours after birth.

‡ Injected 4, 6, 8, 12, or 18 weeks after birth with 0.1 mg protein in incomplete Freund's adjuvant.

§ Injected with I^{131}-labeled protein 6 to 7 days after challenge injection of antigen in adjuvant.

‖ As listed in Fig. 4 and 5.

2. Clearance Rates in Newborn Animals.—20 mg I*-BGG or I*-BSA were injected into mice of several litters within 24 hours after birth and the elimination followed. Both BGG and BSA behaved similarly. During the first 2 days of life,

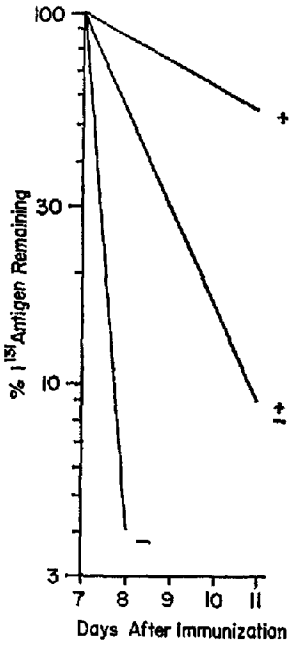

FIG. 1

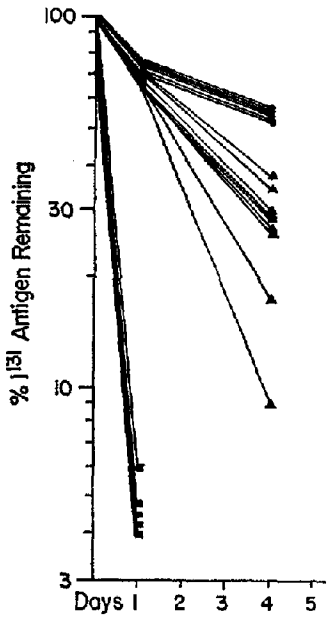

FIG. 2

FIG. 1. Elimination of soluble antigen in normal and tolerant mice. + = tolerant, ± = partially tolerant, − = non-tolerant.

FIG. 2. Elimination of I^{131}-RGG. ●——●, normal, non-immunized controls (D); ▲——▲, partially tolerant animals (A); ■——■, immunized controls (C).

TABLE II

Half-Lives of I^{131}-Labeled Proteins in C57BL/6

Protein	Average Half-Life*		Days‡
	Days	*Hrs.*	
RSA		23	0 to 4
HSA		26	0 to 4
CSA		26	0 to 4
SSA		11	0 to 2
BSA		20	0 to 3
ESA		22	0 to 4
RGG	6	6	4 to 6
HGG	6	16	1 to 6
SGG	5		1 to 6
BGG	4	18	4 to 6
EGG	8	8	2 to 6
TGG		11	0 to 2

* Ten mice per group.

‡ Period for half-life determination.

the injected proteins were metabolized very slowly. After the 2nd day, the rates of clearance greatly increased and reached normal adult values on approximately the fourth day of life (Fig. 3).

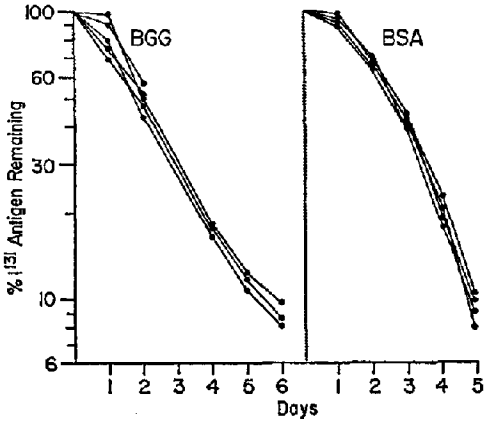

FIG. 3. Elimination of I^{131}-BGG and I^{131}-BSA in newborn C57BL/6.

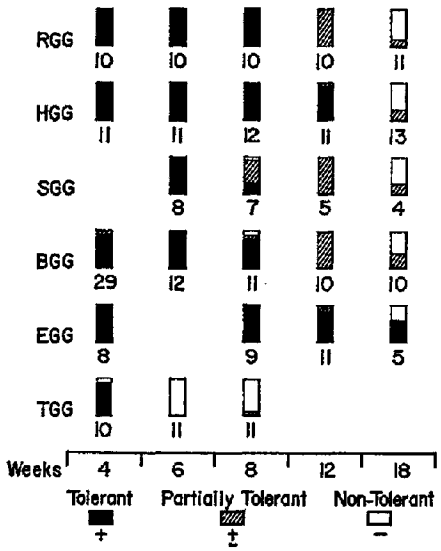

FIG. 4. Duration of tolerance following neonatal injections of gamma globulin. Figure under each bar represents number of mice (A) used per experiment.

3. Duration of Tolerance as Determined by the Elimination Method.—The duration of tolerance induced in mice with different protein antigens is shown in Figs. 4 and 5. Mice injected with 20 mg of mammalian gamma globulin were usually tolerant for 8 to 12 weeks, except the animals injected with SGG. These

mice were tolerant for a somewhat shorter time. On the other hand, the avian gamma globulin (TGG) induced tolerance lasting only for 4 weeks. Most albumins induced a state of immunological unresponsiveness of 4 to 6 weeks. However, it will be noted that mice injected with CSA were not fully tolerant even 4 weeks after birth. Similar results were obtained with RSA. After 4 weeks, only 4 out of 8 animals were fully tolerant and the remaining 4 animals were not tolerant. On the other hand, SSA and BSA induced tolerance which lasted for a somewhat longer period of time.

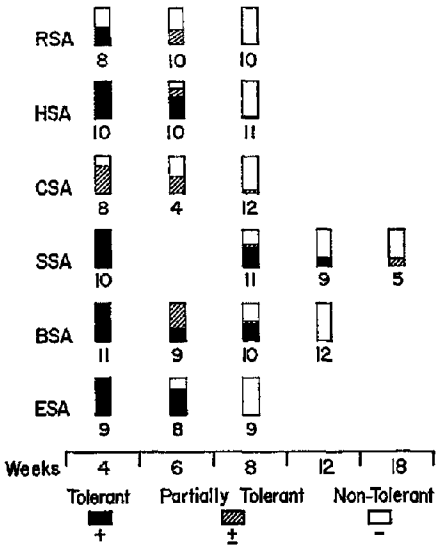

FIG. 5. Duration of tolerance following neonatal injection of albumins. Figure under each bar represents number of mice (A) used per experiment.

4. Hemagglutination and Gel Diffusion Tests.—In many instances, sera from experimental animals had HA titers which could not be correlated with the results obtained by the I*-antigen elimination method. These findings, however, became obvious when gel diffusion techniques were applied. Sera from mice considered to be fully tolerant to BGG gave in O-plates a weak single band, whereas sera from immunized control animals revealed either single or double bands. IE clearly demonstrated that all the mice injected at birth with BGG and later injected with Freund's adjuvant containing BGG formed antibodies to a beta globulin component present in the commercial BGG preparation. On the other hand, immunized control animals consistently formed antibodies to the gamma globulin as well as to the beta globulin impurity. Sera from mice which had lost the tolerant state, as judged by the elimination method, generally did not show precipitating antibodies against the gamma globulin. Only 1 serum

from a mouse found to be non-tolerant 8 weeks after birth gave 2 bands in O and IE tests.

Sera of some of the mice tolerant to EGG and RGG contained precipitating antibody to a beta globulin of EGG and RGG preparations, respectively, following injection with Freund's adjuvant containing the tolerated antigen. Sera from normal mice injected with adjuvant containing EGG usually showed 2 bands. In several of the sera, however, the gamma globulin band was absent, or in addition to a gamma and beta globulin band, there was a third band located in the vicinity of the gamma globulin band. This additional band occurred only in sera from immunized control mice. Sera from immunized animals which had lost unresponsiveness to RGG showed the gamma globulin band. IE of sera from normal mice injected with adjuvant containing RGG, inconsistently revealed a component that had the mobility of albumin. O and IE of sera from normal mice injected with HGG or SGG in Freund's adjuvant yielded only a single band. Sera from HGG-tolerant and SGG-tolerant mice showed no bands following injections with the respective antigen in adjuvant. Bands were also absent in the sera of immunized mice which had lost their tolerant state.

Sera from mice injected after birth with HSA, ESA, SSA, and CSA and challenged with antigen in adjuvant never showed precipitation bands in O and IE, regardless of whether the animals were tolerant or had lost the tolerant state. Sera from normal immunized mice sometimes revealed a single albumin band. On the other hand, a small number of the sera from mice which had lost tolerance against RSA or BSA showed precipitation bands. Several sera from normal mice immunized with RSA gave 2 bands in IE. The second band corresponded to a beta globulin. This band, however, was never present in tests carried out with sera from mice injected with RSA after birth and later challenged with antigen in Freund's adjuvant.

5. Induction of Tolerance to Hemocyanin.—Since HC is rapidly eliminated from normal mice, the immune elimination technique could not be applied. The results are based on HA tests and IE. In a group of 10 mice injected with 5 mg HC at birth, only 1 animal was tolerant 4 weeks later, as judged by both the HA test (titer 4) and the absence of a precipitation band. The remaining 9 mice showed titers ranging from 8 to 32 and IE revealed 1 to 2 preicpitation bands. Another group of 10 mice was injected with 16 mg HC within 24 hours after birth and 4 of these animals were tolerant as judged by the same criteria. These sera had titers of 2, and again no bands were present in IE. The results for the remaining 6 animals were identical with those described for the first group. In both groups precipitation bands were consistently very weak. The sera from immunized control animals gave HA titers ranging from 64 to 128 and 1 to 2 strong bands were present in IE tests. Sera from non-immunized controls were negative by both tests.

Another group of 9 mice injected at birth with 16 mg HC was challenged 6

weeks later with HC in incomplete Freund's adjuvant. At that time, 5 mice had lost their tolerant state (HA titers 32 to 128 and 2 strong precipitation bands). The sera of 4 animals which apparently had lost their tolerant state to a lesser degree had HA titers ranging from 8 to 16 and revealed weak bands in IE.

DISCUSSION

The phenomenon of immunological unresponsiveness has not been convincingly explained, and is not yet understood. Although many mechanisms on a cellular, subcellular, or even molecular level may be involved, several experimental data have been used to support the assumption that persistence of antigen plays the major role in the maintenance of the tolerant state. It has been shown in various animal species that the duration of tolerance is dose-dependent and may be indefinitely prolonged by injections of very large amounts of antigen during embryonic or early life. Furthermore, tolerance induced at birth with smaller doses may be maintained by single or repeated reinjections of the antigen in the adult animal (14). In the present study, another parameter was examined in order to evaluate the effect of antigen persistence on the duration of the tolerant state.

The present results fail to show a correlation between the persistence of labeled protein antigens in the mouse and the duration of tolerance. With several albumin antigens, there was an inverse relationship between the duration of tolerance and persistence of antigen. Tolerance induced to SSA lasted longer than tolerance induced to any other albumin, although SSA was catabolized twice as fast as any other albumin. Similarly, a complete tolerant state was not induced to CSA, and this albumin was catabolized as slowly as most of the other albumins. Also, the rate of catabolism of BSA was approximately the same as the rate of catabolism of RSA, HSA, and ESA, but the duration of tolerance to BSA was significantly longer. On the other hand, tolerance induced to mammalian gamma globulins usually persisted longer than tolerance induced to albumins, and the half-lives of these gamma globulins were longer than the half-lives of the albumins. However, the difference between the half-lives of albumins and gamma globulins was not proportional to the difference in the duration of tolerance. In most cases, the half-lives of the gamma globulins were 4 to 8 times as long as the half-lives of the albumins, while tolerance to the gamma globulins persisted only 2 to 3 times as long as did tolerance to the albumins. Furthermore, there was no significant difference between the duration of tolerance to SGG and SSA, but the half-life of SGG was 10 times as long as the half-life of SSA. The difference in the duration of tolerance to mammalian gamma globulins and albumins may be the result of differences in the physical-chemical nature of these two types of proteins, rather than a difference in their persistence in the mouse. Similarly, the short duration of the tolerant state induced to HC may be related to its physical-chemical properties, rather than its apparently rapid

clearance from the mouse. Among the gamma globulins, there appears to be some correlation between persistence of antigen and duration of tolerance. EGG had the longest half-life and the longest duration of the tolerant state, while TGG had the shortest half-life and the shortest duration of the tolerant state.

The lack of correlation between the persistence of labeled antigen and duration of tolerance does not necessarily mean that the presence of antigen in the host after neonatal life plays no role in the maintenance of tolerance. However, the interpretation that the duration of tolerance is directly dependent upon the persistence of antigen does not appear warranted. It is obvious that for any given antigen, larger or repeated injections of the antigen would prolong the tolerant state. On the other hand, if the duration of tolerance induced by different antigens is not related to the rate of catabolism of the antigens, some other factor(s) must be involved. It is possible that tolerance, once induced, could persist even after the elimination of antigen until mutations or other events gave rise to a significant number of competent cells. The frequency with which mutations would give rise to cells able to respond to different antigens would probably vary from antigen to antigen and could account for the present observations. The function of repeated injections of antigen in prolonging tolerance may be to suppress or eliminate competent cells arising from mutations after disappearance of antigen.

The results obtained in experiments to determine elimination patterns of heterologous proteins in neonatal mice revealed, during the first 2 days following injection, that neonatal mice eliminated proteins very slowly It was observed that this apparent slow elimination was in part the result of retention of non-protein-bound I*. Similar retention of non-protein-bound I* was observed in neonatal rabbits injected with I* protein (15, 16). However, after the 2nd day, the catabolism became increasingly faster and reached normal adult values the 3rd or 4th day following injection. Similar findings were obtained with neonatal rabbits (15, 16), but in these studies the period of very slow elimination lasted considerably longer (40 days after birth).

In using commercial protein preparations to study tolerance, one has to be cautious in selecting the criteria to evaluate the tolerant state. Impurities are frequently inherent in these preparations. This was well documented in this study with immune electrophoresis. BGG, EGG, and RGG contained beta globulin to which newborn animals were not rendered tolerant, presumably because the threshold dose to induce tolerance was not reached. When mice were challenged with antigen in incomplete Freund's adjuvant at a time when tolerance still existed to the gamma globulin, antibodies were produced to a beta globulin component. Sera from animals which had lost tolerance to the main component, as judged by the elimination method, usually did not reveal precipitating antibodies against gamma globulin. Similar findings were made with the albumins. These findings could mean either that the agar diffusion

techniques were not as sensitive as the antigen elimination technique, or that the animals which had lost their tolerant state only produced non-precipitating antibodies.

SUMMARY

C57BL/6 mice were rendered tolerant to one or another of 13 soluble protein antigens. Tolerance was induced by a single injection of 20 mg protein within 24 hours after birth. The duration of the unresponsive state was measured and compared with the rates of catabolism of the antigens as determined in adult and new born mice. The data presented fail to show a correlation between the persistence of labeled protein antigen and the duration of tolerance. In several occasions, even an inverse relationship between duration of the unresponsive state and persistence was demonstrated. The results, therefore, strongly indicate that the duration of tolerance is not dependent on the rates of catabolism of the antigens.

Several of the commercial protein preparations used in this study contained minor impurities to which the animals were generally not rendered tolerant. By means of diffusion in agar techniques, it was demonstrated that mice injected at birth with a tolerance-inducing dose of antigen would generally not reveal precipitating antibodies to this antigen after the tolerant state had been abolished. A speculative explanation was given in terms of quantitative or qualitative differences of antibodies found in such animals as compared to the immunized control mice.

After the 3rd or 4th day of life, newborn mice catabolized I^{131}-labeled heterologous proteins at the same rates as adult mice. The apparent slow elimination during the first days of life was, at least in part, the result of retention of non-protein-bound I^{131}.

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