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Tolerance in Adult Rabbits by Repeated Non-Immunogenic Doses of Bovine Serum Albumin

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Summary. A series of multiple intravenous injections of bovine serum albumin, starting with doses too small to be immunogenic, has been used for the induction of tolerance in adult rabbits. With normal outbred animals, approximately 50 per cent of the rabbits were rendered completely tolerant by an injection schedule with 0·1 mg as the initial dose and a total dose of 9 mg given over a period of 5–7 weeks. The mechanism of this 'low-dose tolerance' is discussed.

INTRODUCTION

Studies with soluble serum protein antigens such as bovine γ-globulin (Dresser, 1962; Claman, 1963) and human γ-globulin (Biro and Guadalupe, 1965) have shown that these antigens, when freed of aggregated material by ultracentrifugation, can induce immunological unresponsiveness in adult animals with doses which otherwise are immunogenic. Similarly, bovine serum albumin (BSA) which had been cleared of aggregated or denatured material by previous intravenous injection in a rabbit whose reticulo-endothelial system was used as a filter, proved to be capable of inducing tolerance in adult rabbits (Frei, Benacerraf and Thorbecke, 1965). These results indicate that commercial serum protein preparations consist of two types of materials with respect to their capacity to induce tolerance or immunity. The native non-aggregated fraction which represents the major portion of these preparations induces tolerance more readily, while the denatured fraction is a more efficient antigen, probably because of its greater ability to be phagocytosed (Thorbecke, Maurer and Benacerraf, 1960) or arrested in special locations in lymphoid tissue (Nossal, Ada and Austin, 1964; Cohen, Vassalli, Benacerraf and McCluskey, 1966).

Since the immunogenic fraction represents a minor portion of these preparations, it was reasoned that doses too small to elicit antibody formation might still suffice to induce tolerance provided a critical concentration could be maintained over a sufficient period to ensure effective exposure of all susceptible cells. It has, indeed, been reported by Mitchison (1964) that low doses of BSA as well as very large doses, could induce tolerance in adult mice, while moderate doses were immunogenic.

The present experiments illustrate that repeated intravenous injections of BSA, starting with doses insufficient to immunize and followed by increasing doses over a period of weeks, are capable of inducing a state of complete or partial tolerance in about 50 per cent of adult rabbits. Furthermore, the offspring of the mating of rabbits which had been rendered tolerant by this method were more susceptible to the tolerance-inducing effects of low doses of this antigen.

MATERIALS AND METHODS

Male rabbits weighing 2–3 kg were used in most of these experiments, but a few females were also included in Group IV. Crystalline bovine serum albumin (Armour & Co., Kankakee, Illinois) was used. It was trace labelled with ^{131}I (McConahey and Dixon, 1966). To establish the minimal immunogenic dose of the BSA preparation, 0·1 mg (Group I) or 1 mg (Group II) of [^{131}I]BSA was injected intravenously in each of four rabbits per group and the antigen disappearance curve determined for each animal. None of the rabbits injected with 0·1 mg of [^{131}I]BSA showed an immune elimination, whereas, two of the four rabbits injected with 1 mg of [^{131}I]BSA did show evidence of an immune response. In a previous study it was shown that seven out of seven rabbits injected with 8 mg of [^{131}I]BSA formed antibodies (Frei *et al.*, 1965).

Two groups of nine rabbits each, Groups III and IV, were then injected at regular intervals with increasing doses of BSA according to the schedule below. The BSA was administered in 2 ml of 0·15 N NaCl slowly over a 1–2-minute period in the marginal ear vein, taking care not to infiltrate the tissues. Group III received 0·25 mg on days 1, 8, 10 and 12; 0·5 mg on days 15, 17, 19; and 1 mg on days 22, 24, 26, 29, 31 and 33. This group was bled and challenged with alum precipitated BSA 7 days later. Group IV received 0·1 mg on day 1; then 0·25 mg on days 8, 15, 17, 19, 22, 24 and 26; 0·5 mg on days 29, 31 and 33; and 1 mg on days 36, 38, 40, 43, 45 and 47. A fifth group (Group V) consisted of the eleven offspring of the mating four rabbits from Groups III and IV which had been rendered tolerant. These animals when they reached 2·5–3 kg of body weight received the same dose schedule of BSA as Group IV. Both Groups IV and V were challenged 3 days after the last injection. The challenge consisted of 15 mg of alum precipitated BSA intravenously. The animals were bled after 10 days, re-injected with 10 mg of BSA intravenously and bled again 1–2 weeks later. An uninjected control group consisting of fourteen rabbits (Group VI) was similarly challenged. The sera were assayed for anti-BSA antibodies by the passive haemagglutination technique (Stavitsky, 1954).

RESULTS

The results of these experiments are found in Table 1. In both Groups III and IV, four out of nine rabbits proved to be completely tolerant to challenge with BSA. In addition, two more animals from Group IV showed a greatly reduced response (partial tolerance). Eight out of eighteen animals showed immune responses indistinguishable from control animals. The presence of antibodies previous to challenge was a fair indication whether or not tolerance had been achieved. It should be noted that more animals were made tolerant by the course of injections given to animals in Group IV, which received initially only 0·1 mg BSA, than by that given to Group III, initiated by 0·25 mg BSA.

The finding that eight out of eighteen animals in Groups III and IV showed an immune response after challenge which was indistinguishable from that of the controls, could be interpreted as the result of fortuitous events, or as reflecting genetic differences in the ability of random-bred rabbits to recognize antigenic determinants on BSA. The response of the offspring of the mating of rabbits which could be made tolerant was, therefore, studied. Only one out of twelve such animals showed a normal immune response after the standard tolerance-inducing treatment used in these experiments and repeated challenge. Four rabbits were partially tolerant and the remaining seven animals did not

TABLE 1

INDUCTION OF SPECIFIC IMMUNOLOGICAL TOLERANCE IN ADULT RABBITS BY REPEATED INJECTIONS OF SMALL DOSES OF BOVINE SERUM ALBUMIN

Animals	Initial dose BSA (mg)	Total dose BSA (mg)	Total period of injections (days)	Immune response before challenge	Reactions upon challenge*		
					Tolerant haem titre < 1:20	Partially tolerant haem titre 1:40 to 1:160	Immune haem titre > 1:5000
Group I 4 rabbits	0·1	0·1	—	0/4			
Group II 4 rabbits	1·0	1·0	—	2/4			
Group III 9 rabbits	0·25	8·5	33	5/9	4/9	0/9	5/9
Group IV 9 rabbits	0·1	9·35	47	3/9	4/9	2/9	3/9
Group V 12 rabbits	0·1	9·35	47	2/12	7/12	4/12†	1/12
Group VI Controls 14 rabbits	—	—	—	—	0/14	0/14	14/14

* Challenge consisted of 15 mg alum precipitated BSA intravenously, bleeding after 10 days, re-injection with 10 mg BSA and bleeding again 1–2 weeks later. All bleedings were assayed for anti-BSA antibodies by the passive haemagglutination technique with BSA-coated tanned sheep erythrocytes.

† Two of these partially tolerant animals showed passive haemagglutination titres of 1:320 and 1:640, respectively.

show any anti-BSA antibodies detectable by passive haemagglutination. There appears to be a slightly higher incidence of tolerant animals in this group than in the other two experimental groups of animals.

DISCUSSION

These experiments demonstrate that, as was shown for mice by Mitchison (1964), adult rabbits can also be rendered immunologically tolerant to a soluble foreign serum protein, BSA, if this antigen is administered intravenously in doses too low to induce an immune response and is re-injected at regular intervals for a sufficient period of time for immunological unresponsiveness to be established. The dose level at which this phenomenon is observed is very similar on a weight basis in adult mice and rabbits. This tolerance was established in rabbits with doses of BSA injected several times a week, starting with 0·1 mg and increasing up to 1 mg. In mice the best results in the low dose range were obtained with repeated injections of 0·01 mg (Mitchison, 1964). A striking feature of the induction of tolerance with low doses of BSA is that the success of the experiment depends clearly on early events and that great care has to be taken that the initial injections be of doses sufficiently low and adequately spaced so as not to induce immunization while the cells are exposed to the tolerance-inducing antigen.

The induction of immunological unresponsiveness with low doses of a foreign serum protein results, probably, from the fact that this preparation consists of a heterogeneous population of molecules with respect to its capacity to induce an immune response. Only a small fraction of aggregated or denatured material was shown in similar serum protein antigens to be capable of inducing antibody synthesis, while the rest of these preparations was very effective in inducing tolerance (Dresser, 1962; Biro and Guadalupe 1965;

Frei *et al.*, 1965). Thus, if cells capable of becoming tolerant are exposed to soluble antigens in adequate amounts and for a sufficient period of time, in the absence of an immune response, tolerance is observed. This result is achieved in the case of 'low dose tolerance' by injecting an insufficient dose of the immunogenic fraction. Tolerance can also be obtained, even with larger doses of antigen, if the primary immune response is interfered with by immunosuppressive agents (Schwartz, 1967).

How can these results be explained at the level of the immunocompetent cells? Two of the various possible mechanisms appear to deserve further study. Tolerance may result from the effect of antigen on immunologically immature cells. This effect may be achieved in the absence of an immune response by the mature cells, if the soluble antigen is administered for a sufficient length of time to allow all the immunocompetent cells originally present to be replaced by such tolerant cells. This hypothesis assumes that: (a) tolerant cells exist, (b) immunocompetent cells have a relatively short life, and (c) mature immunocompetent cells are not made tolerant by this technique. The other hypothesis postulates that immunocompetent cells can be made tolerant, and that tolerance results from the exposure of these cells to native antigens which have not been previously processed by cells of the reticuloendothelial system (Frei *et al.*, 1965).

The extent and depth of tolerance achieved following the repeated injections of low doses of BSA was probably limited because of the level of doses used. However, the animals in which specific immunological unresponsiveness was achieved, were clearly tolerant as also shown by the results of studies of the specificity of the antibodies produced by these animals when breaking of tolerance was attempted with a cross-reacting antigen (Paul, Siskind and Benacerraf, 1967).

A somewhat higher incidence of tolerance was achieved in the animals which were the offspring of rabbits that had been made tolerant by this technique. This suggests that the ability of an animal to become immune or tolerant to a given antigen may be under some genetic control and may depend upon the ability of the animal to recognize major antigenic determinants on the molecule. This, in turn, may be intimately related to the number of immunocompetent cells capable of reacting with this antigen.

ADDENDUM

Since submission of this manuscript another group of rabbits, consisting of offsprings from rabbits which could not be made tolerant by the injection schedule used with groups IV and V (Table 1), were also similarly studied. The does were tested for tolerance induction after raising the young. Among seven of these offsprings only two showed a high degree of tolerance, a significantly lower incidence than observed in offsprings from tolerant rabbits (nine out of twelve). The recent experiments of Sobey, Magrath and Reisner (1966) demonstrating genetically determined differences between rabbits in their immunological responsiveness to BSA are consistent with the observations presented in this paper.

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