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Effect of Serum from Tolerant Mice on Immunity and Tolerance to a Bacterial Antigen

THE mechanism of immunological tolerance is still poorly understood. Numerous model systems have been used in attempts to resolve some of the important questions concerning the fundamental role of both antigen and antibody in the establishment and maintenance of immunological tolerance to various antigens, in a variety of conditions in mammalian or avian species[1–3]. It has been observed recently that establishment of immunological tolerance to certain antigens, such as serum proteins, foreign erythrocytes or bacterial extracts, can be prevented by specific antiserum[4–9]. The suppressive effect of antibody on the induction of tolerance seems to be similar in many respects to the effect of specific antiserum on antibody formation.

A number of investigators have found that serum antibody is important in the regulation of antibody at the cellular as well as the humoral level[10–13]. A relationship between serum antibody and induction, maintenance or termination of immunological tolerance is not yet clear.

Previous studies in our laboratory have shown that passive administration of antiserum to *Shigella* antigens suppresses induction of specific tolerance in newborn mice[4,14]. The time of serum inoculation in relation to antigen injection was important because tolerance could be blocked only when antibody was administered simultaneously or within 2 days after antigen had been given. Further studies indicated that injection of antiserum later in life, when tolerance was already established, had little or no demonstrable effect on maintenance of unresponsiveness. Several other investigators have observed that tolerance to protein antigens can be blocked either in neonatal or adult mice by simultaneous administration of antiserum to the specific antigen. Passive transfer of hyperimmune homologous or heterologous serum following establishment of tolerance has usually had little effect on unresponsiveness[1,5,15].

The biological effect of *de novo* antibody synthesis in animals "escaping" from tolerance, especially as related to maintenance of tolerance after additional injections of antigen, is not known. The relation of antibody activity in partially tolerant animals, as well as in immune animals, to establishment or maintenance of tolerance is also not clear. The experiments described here are concerned with effects of serum antibody obtained from mice "escaping" from immunological tolerance to *Shigella* antigen on induction of either immunity or tolerance in other recipient animals. The results obtained suggest that, despite similar titres, there may be both quantitative and qualitative differences in antibody activity to *Shigella* antigen in sera of mice recovering from tolerance, as compared with antibody in sera of actively immunized animals.

For these experiments newborn NIH albino A mice were inoculated with 20 μg of *Shigella* soluble antigen (SSA), as described previously, to induce specific immunological tolerance[4,16]. Such mice were unresponsive to challenge immunization with a concentration of *Shigella* antigen capable of inducing relatively high agglutinin titres in control mice. As Table 1 shows, mice injected with SSA as neonates rarely had titres greater than 1 : 40 when subsequently challenged with the same antigen. Control, non-tolerant animals responded with much higher agglutinin titres when challenged with *Shigella* antigen. Both groups of animals formed relatively similar amounts of circulating antibody to unrelated antigens such as sheep erythrocytes or bovine serum albumin (Table 2).

Table 1. EFFECT OF SERA FROM TOLERANT, IMMUNE OR NORMAL MICE ON ESTABLISHMENT OF TOLERANCE OR INDUCTION OF AGGLUTININ FORMATION IN EITHER NEONATAL OR ADULT MICE

Mouse serum injected	Peak agglutinin titres after challenge immunization* Tolerant mice (age in weeks)† 3	5	8	10	Normal adult mice‡
None (saline)	1 : 25	1 : 29	1 : 15	1 : 43	1 : 405
Normal	1 : 18	1 : 32	1 : 27	1 : 48	1 : 460
Early tolerant§	1 : 30	1 : 23	1 : 16	1 : 40	1 : 415
Late tolerant‖	1 : 14	1 : 32	1 : 22	1 : 35	1 : 493
Early immune¶	1 : 138	1 : 343	1 : 392	1 : 316	1 : 64
Hyperimmune**	1 : 248	1 : 365	1 : 330	1 : 410	1 : 10

* Mean titres of five or more mice per group per day were tested.
† Mice injected at birth with SSA and serum indicated; challenged with SSA at age indicated and serum titres determined 7–15 days later.
‡ Groups of 7–10 week old untreated adult mice injected with serum indicated, followed by challenge immunization with SSA; serum titres determined 7–15 days later.
§ Sera obtained 5–7 days after challenge immunization of 2–3 month old "early" tolerant mice; agglutinin titre = 1 : 32.
‖ Sera obtained 5–7 days after challenge immunization of 5–8 month old "late" tolerant mice; agglutinin titre = 1 : 256.
¶ Sera obtained 1 week after primary injection of 5–7 week old mice with 20 μg of SSA; agglutinin titre = 1 : 384.
** Sera obtained 5–7 days after last of four monthly injections of mice with 20 μg of SSA; agglutinin titre = 1 : 5,120.

Table 2. ANTIBODY TITRES OF TOLERANT AND CONTROL MICE TREATED WITH SERA FROM TOLERANT OR IMMUNE MICE AND INJECTED WITH EITHER SHIGELLA OR CONTROL ANTIGENS

Mouse group	Serum injected	Antibody titres after immunization with:§ SSA	S–RBC	BSA
Tolerant*	None	1 : 31	1 : 712	1 : 480
	Late tolerant†	1 : 23	1 : 824	1 : 554
	Hyperimmune‡	1 : 480	1 : 768	1 : 425
Normal control	None	1 : 415	1 : 928	1 : 470
	Late tolerant†	1 : 392	1 : 845	1 : 515
	Hyperimmune‡	1 : 12	1 : 910	1 : 538

* Mice injected at birth with SSA; treated when 4–6 weeks old.
† Sera obtained 5–7 days after challenge immunization of 5–8 month old tolerant mice; agglutinin titre = 1 : 256.
‡ Sera obtained 5–7 days after fourth monthly injection of normal adult mice with 20 μg of SSA; agglutinin titre = 1 : 10,240.
§ Groups of five to eight mice each immunized with either 20 μg of SSA, 2×10^8 sheep erythrocytes, or 5 mg of bovine serum albumin (BSA) in complete Freund's adjuvant; antibody titres determined 8–15 days later.

Sera were pooled from mice of various ages after neonatal establishment of tolerance for passive transfer into other mice. Sera from tolerant mice less than 3 months old rarely had significant agglutinin titres. Also, there was no detectable *Shigella* antigen in these sera, as shown by several serological inhibition procedures. Serum specimens were also obtained from older mice, usually 5 to 8 months old, emerging spontaneously from the tolerant state. Such mice often had specific agglutinins to *Shigella*, usually with titres of 1 : 100–1 : 200 or more, within 1–2 weeks after challenge immunization with SSA. Control negative sera were prepared from normal, non-injected mice, while control positive sera were obtained from mice which had received three or more monthly injections of SSA. Such sera usually had titres of 1 : 5,120 or more. For some experiments, gamma globulin fractions derived from these pooled sera were prepared by precipitation with 30 per cent ammonium sulphate. Results obtained either with the gamma globulin or the whole sera were equivalent.

Small quantities of these serum preparations were injected into recipient mice, either adult or neonates. Injection of 0·1 ml. of normal mouse serum into adult mice had no effect on their immune response to a simultaneous injection of SSA (Table 1). On the other hand, injection of the same quantity of hyperimmune anti-*Shigella* mouse serum, with an agglutinin titre of 1 : 5,120, markedly suppressed the appearance of expected antibody to *Shigella* (Table 1). A 1 : 100 and 1 : 200 dilution of this serum also markedly suppressed immunization to SSA. There was no effect on the response of these mice to other antigens such as sheep erythrocytes or bovine serum albumin.

Injection of 0·1 ml. of serum pool from young tolerant mice had no detectable effect on the appearance of agglutinins to SSA in normal adult animals (Table 2). Such "early" tolerant sera usually had titres about 1 : 30 or 1 : 40. Injection of 0·1 ml. of undiluted serum from older partially tolerant mice into other adult animals, together with SSA, also had no effect on the expected agglutinin response (Table 1). Such "late" tolerant sera had agglutinin titres of 1 : 256, similar to the antibody content of a 1 : 200 dilution of hyperimmune serum which could effectively block induction of agglutinin formation.

Injection of these sera into neonatal mice receiving a tolerance-inducing inoculum of SSA had various effects (Tables 1 and 2). Hyperimmune anti-*Shigella* serum, either undiluted or diluted up to 1 : 200, markedly inhibited the establishment of tolerance. A 1 : 400 or greater dilution had no effect. Mice receiving this serum at birth, plus SSA, produced relatively normal concentrations of agglutinins when challenged with SSA 3 months later. On the other hand, animals injected at birth with SSA and either saline or normal serum were tolerant to SSA when subjected to challenge immunization (Tables 1 and 2). Similarly, mice injected at birth with SSA and sera from either "early" or "late" tolerant mice were unresponsive to challenge immunization when 3 months old. In other experiments, neonatal mice injected once with SSA at birth and several times during 1 week with 0·1 ml. of "late" tolerant serum were also unresponsive at challenge immunization. All mice, regardless of whether injected with SSA or antiserum at birth, responded similarly to challenge immunization with unrelated antigens such as sheep erythrocytes or bovine serum albumin later in life (Table 2).

These results indicate that antibody to *Shigella* in sera from partially tolerant mice may be markedly different from antibody in sera of normal immune animals. It seems plausible that the antibody specificity may be directed towards "minor" antigens of *Shigella* unrelated to immunogenesis and tolerogenesis. Also, it is probable that the affinity of antibodies in sera of "late" tolerant mice is also different from that in sera from hyperimmune mice. Antiserum obtained from normal mice shortly after a single injection of SSA can readily block establishment of either tolerance in neonates or immunity in adults, despite low titres ranging from 1 : 200 to 1 : 400. Thus differences in the biological effects of sera with low titres from tolerant and from immune mice do not seem to be a consequence merely of different agglutinin titres or even of possible differences in molecular form.

These observations also suggest that specific antibody in sera of mice "escaping" from tolerance may have little or no effect on continuation or maintenance of tolerance in the donors. Such antibody has little or no effect on induction of antibody-formation or immunological tolerance in other animals, either adults or neonates, injected with SSA. It seems probable that mice escaping from tolerance may respond to minor antigens in the obviously complex *Shigella* extracts, which contain several somatic lipopolysaccharide antigens. The mice are probably still unresponsive to major antigens of SSA, while they form small amounts of agglutinins to minor antigens. Such findings may be directly related to the phenomenon of "split tolerance" and to immunological tolerance to strongly immunogenic antigens associated with allografts[17]. Although in many cases it may seem that tolerance is abrogated to a tissue graft, as detected by a variety of immunological and serological parameters, it is possible that an individual may respond immunologically to "minor" antigenic determinants without loss of tolerance to major transplantation antigens.

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HERMAN FRIEDMAN

Department of Microbiology,
Albert Einstein Medical Center and
Temple University School of Medicine,
Philadelphia.

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Mechanism of Exchange of Inorganic Phosphate with Bone Mineral and its relation to the Mechanism of Calcification

INORGANIC phosphate exchange with bone mineral and with synthetic hydroxyapatites has been studied by the same methods as calcium exchange[1,2]. The two types of exchange differ, however. At a *p*H of about 7·4, the inorganic phosphate solutions contain HPO_4^{2-} as the predominant ion, the concentration of PO_4^{3-} being less than 1 per cent of the total phosphate[3], while the calcium solutions contain almost all the calcium as Ca^{2+}. Increasing the relative concentration of PO_4^{3-} by increasing the *p*H might be expected to increase the rate of phosphate exchange. Experiments on the uptake of ^{32}P-labelled inorganic phosphate by defatted bone powder and anorganic bone were carried out as described for calcium and other elements[4,5]. The results are shown in Table 1.

Uptake did not increase at the higher *p*H. In the absence of added carrier, the results differed little at the two *p*Hs. In the presence of phosphate carrier, the amount of exchange was greater at *p*H 7·45 than at *p*H 11·0.

Table 1. THE UPTAKE OF ^{32}P BY BONE POWDER AND ANORGANIC BONE AT DIFFERENT *p*Hs

	*p*H 7·45		*p*H 11·0	
Percentage uptake in 2 h Added carrier	Bone powder	Anorganic bone	Bone powder	Anorganic bone
None	37·2	75·8	39·7	66·5
2·5 mmole/l. of phosphate	10·1	20·8	5·2	11·9
Percentage uptake in 24 h Added carrier				
None	58·9	72·1	58·6	79·2
2·5 mmole/l. of phosphate	21·1	24·8	14·0	17·7

All experiments in triplicate, except for loss of 1 vial at *p*H 11. Standard deviation estimated (by grouping these and other experiments) to be 1·08. Standard error of groups of 3, 0·62.