The Effect of Carrier Tolerance on the Antibody Response of Guinea Pigs to the Azobenzenearsonate Hapten[1] (35658)

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(Introduced by Oscar D. Ratnoff)

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Recently several investigators have shown that the immune response of an animal immunized with hapten-carrier conjugates is significantly influenced by the nature of the carrier molecule. Siskind *et al.* (1) demonstrated in guinea pigs that the carrier to which DNP is coupled influences the amount, type, and functional activity (*i.e.*, affinity) of anti-DNP antibodies, and Fronstin, Sage, and Vazquez (2) have shown that the degree of anti-DNP responsiveness elicited in mice is directly related to the antigenicity of the carrier.

The present study examines the antihapten antibody response of guinea pigs immunized with arsanilic acid conjugates of homologous and heterologous serum protein carriers, and compares the effect of natural and acquired tolerance to the carrier on these responses.

Materials and Methods. Random bred, adult male albino guinea pigs (300–360 g) were used in these experiments.

Reagents. Azobenzenearsonate (ABA) conjugates were made as before (3) by diazotization and coupling in the ratio of 10^{-5} moles arsanilic acid to 10 mg protein. Human, bovine, rabbit, and guinea pig serum albumins (HSA, BSA, RSA, GSA) and gamma globulins (HGG, BGG, RGG, GGG), obtained from Pentex Inc., Kankakee, Ind., were utilized as carrier proteins. In addition, arsanilic acid conujgated to egg lysozyme (Worthington Lab.) was used as the test antigen in studies of the antihapten antibody response. All conjugates were dialyzed against 0.01 *M* borate buffer pH 8.5 before use.

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Immunization procedure. Guinea pigs were immunized with 0.1 ml of complete Freund's adjuvant containing 100 μg of ABA-protein distributed equally among the four footpads. The complete Freund's adjuvant was prepared with 8.5 parts light mineral oil: 1.5 parts Arlacel A: 5 mg/ml killed, dried *M. tuberculosis.*

Production of carrier tolerance. In several experiments, guinea pigs were rendered tolerant to the protein carriers by the intravenous (iv) injection of saline solutions of the native protein at the time immunization was carried out (4).

Antibody determinations. Three weeks after immunization, guinea pigs were bled by cardiac puncture and the sera harvested. The 7S γ_1 antibody determinations were made by passive cutaneous anaphylaxis (5). One mg of test antigen in 1 ml of 0.5% Evan's blue dye in saline was administered intravenously 3 hr after intradermal injection of saline dilutions of the test sera. Reactions were read 20 min after intravenous injections of the dye.

Hemolytic antibody titers were measured by a modification of the technique of Bloch *et al.* (6) using the appropriate antigen in a 1 mg/ml concentration to coat the tanned sheep erythrocytes. The highest dilution giving complete hemolysis was taken as the end point.

Results. The results of immunization of guinea pigs with 100 μg of ABA conjugates of a variety of serum proteins are seen in Table I.

Of 19 guinea pigs immunized with 100 μg of ABA–BSA in Freund's complete adjuvant, 18 produced significant titers of anti-ABA hemolytic antibody and 10 produced 7S γ_1

TABLE I. Antihapten Antibody Response in Guinea Pigs Immunized with ABA Conjugates of Serum Proteins.

Immunized with (100 μg)	Antibody to ABA group		
	Hemolytic (γ_2)		Anaphylactic (γ_1)
ABA–BSA	18/19[a]	(80)[b]	10/19[c]
ABA–RSA	4/5	(40)	5/5
ABA–HSA	2/5	(10)	4/5
ABA–GSA	1/12	(0)	1/12
ABA–GSA (1 mg)	14/16	(80)	13/16
ABA–BGG	7/7	(320)	6/7
ABA–RGG	4/5	(80)	2/5
ABA–HGG	4/4	(40)	4/4
ABA–GGG	2/9	(0)	5/9
ABA–GGG (1 mg)	3/8	(20)	5/8

[a] When animals positive per number tested.
[b] Average titer of all sera.
[c] All sera were tested at a dilution of 1:5.

antibody. Similar results were observed with ABA–RSA and ABA–HSA although hemolytic titers tended to be lower than with the BSA conjugate. These results stand in marked contrast to the response seen after immunization with ABA–GSA, following which only 1 of 12 animals produced detectable γ_2 or γ_1 antibody to the ABA group.

Qualitatively similar responses were obtained following immunization with 100 μg of ABA conjugates of heterologous globulins although in general these turned out to be better immunogens. All animals immunized with ABA–BGG produced high titers of hemolytic antibody to ABA; four of five immunized with ABA–RGG and four of four immunized with ABA–HGG did so as well. The 7S γ_1 antibody production was seen in similar proportions of these animals as well (Table I). Once again a conjugate of the homologous proteins ABA–GGG produced hemolytic antibody responses in only two of nine animals; five of nine animals produced 7S γ_1 antibody.

This lack of immunogenicity of conjugates of the naturally tolerated homologous serum proteins could in part be overcome by immunizing guinea pigs with larger doses of the ABA conjugates. Thus, in 16 animals immunized with 1 mg of ABA–GSA, 14 produced anti-ABA hemolytic antibodies, and 13 produced 7S γ_1 antibody. Less striking results were obtained with 1 mg ABA–GGG.

To extend these findings, 17 adult New Zealand white rabbits, weighing 2.5 kg were divided into four groups and immunized in eight toepads of four feet with 100 μg of either ABA–RSA, ABA–BSA, ABA–RGG, or ABA–BGG in Freund's complete adjuvant. Three weeks later, bleedings were obtained and antibody to the ABA group assayed by hemolytic assay (6). Results shown in Table II indicate that immunizations with conjugates of the heterologous proteins BSA and BGG gave high titers of antibody in all animals. Once again the homologous carriers RSA and RGG proved to be poorer immunogens, and although all animals responded, titers were significantly lower.

In an effort to compare the immunosuppressive effects of natural and acquired tolerance, adult guinea pigs were rendered tolerant to BSA or BGG, according to the method of Dvorak *et al.* (4) by iv injection of the protein in saline prior to immunization. Little if any suppression of the anti-ABA response was obtained in animals tolerant to the heterologous carrier. Thus in Table III, of 22 animals rendered unresponsive with 20 mg of BSA and immunized with 100 μg of ABA–BSA, 16 produced hemolytic anti-ABA of only slightly lower titer antibody, compared to 18 of 19 nontolerant animals. A larger immunizing dose of ABA–BSA (1 mg) did not significantly alter these differences in that 10 of 15 treated animals produced anti-ABA antibody compare to eight of eight controls. Since it seemed possible that the process of diazotization introduced significant changes in the basic antigenic deter-

TABLE II. Antihapten Antibody Response in Rabbits Immunized with ABA Conjugates of Serum Proteins.

Immunized with (100 μg)	Hemolytic antibody to ABA group	
ABA–BSA	4/4[a]	(2560)[b]
ABA–RSA	4/4	(120)
ABA–BGG	4/4	(2560)
ABA–RGG	5/5	(160)

[a] Number of animals positive per number tested.
[b] Average titer of all sera.

TABLE III. Effect of Acquired Tolerance to Carrier on Antihapten Antibody Response in Guinea Pigs Immunized with ABA–BSA and ABA–BGG.

Immunized with	Treatment	Antibody to ABA		Antibody to carrier	
		Hemolytic (γ_2)	Anaphylactic (γ_1)	(Hemolytic (γ_2)	Anaphylactic (γ_1)
ABA–BSA (100 μg)	None	18/19 (80)	10/19	13/15 (160)	5/6
ABA–BSA (100 μg)	20 mg BSA iv	16/22 (40)	12/22	0/17 (0)	3/6
ABA–BSA (1 mg)	None	8/8 (80)	8/8 (160)	7/8 (320)	8/8
ABA–BSA (1 mg)	20 mg BSA iv	10/15 (30)	10/15 (15)	0/15 (0)	0/15
ABA–BGG (1 mg)	None	7/7 (320)	6/7	5/7 (640)	5/5
ABA–BGG (1 mg)	30 mg BGG iv	5/5 (320)	5/5	5/5 (640)	5/5
ABA–BGG (1 mg)	100 mg BGG iv	4/5 (80)	4/5	0/5 (0)	0/5
ABA–BSA (100 μg)	10 mg PABA–BSA iv	5/6 (40)	5/6	6/6 (320)	N.D.
ABA–BSA (100 μg)	20 mg PABA–BSA iv	3/7 (40)	3/7	3/7 (120)	

minants of the protein carriers, ip injection of *p*-aminobenzoic acid conjugates of BSA (PABA–BSA) was used to induce tolerance to the carrier. This resulted in less suppression of the antibody response to unconjugated BSA, suggesting that some determinants are indeed destroyed by diazotization. However, despite this apparent closer approximation of the tolerogen to the immunogen, hemolytic and PCA antibody responses to ABA were only slightly suppressed, with 8 of 13 treated animals producing antibody as compared to 18 of 19 controls.

Discussion. A number of approaches have been utilized over the past several years in an effort to understand the interaction between, and individual contributions of, hapten and carriers to the immune response. Several lines of investigation have suggested that the basic specificity of a hapten-carrier conjugate in the immune response is related to the protein carrier. This perhaps helps to explain the reduced capacity of immunologically tolerant animals to respond on immunization with a conjugate of a protein carrier to which they are tolerant (2, 7–10). If tolerant animals are immunized with a cross-reacting antigen, antibodies are formed which are primarily directed against those determinants in which the cross-reacting antigen differs from the tolerance inducing antigen (11). Indeed, Cinader *et al.* (8, 11–13) and Schechter *et al.* (14) have shown, using several different systems in rabbits, that immune responses to substituted carriers in carrier tolerant rabbits were dependent on the chemical nature of the hapten and the degree of hapten substitution (*i.e.*, molecular alteration) of the carrier. Dietrich (15, 16) has shown much the same with arsanilic and sulfanilic azo-HGG conjugates in HGG tolerant mice; the specificity of the antibody formed was directed against determinants comprising the haptenic group and part of the protein carrier. Thus, as Paul *et al.* (17) have pointed out, tolerance is not abrogated by immunizing with cross-reacting antigens, but antibodies with new specificities are produced.

Several groups have shown that a number of carrier characteristics influence the antihapten response and that using carriers to

which animals are tolerant reduces the antihapten response. Our studies utilizing the azobenzenearsonate system confirm these results in large part. Only minimal ABA specific antibody response is seen in guinea pigs immunized with ABA conjugates of homologous serum protein in Freund's complete adjuvant, whereas good antihapten responses follow immunization with ABA conjugate of heterologous carrier proteins in normal animals (Table I).

It is noteworthy that this immunosuppressive effect of natural tolerance to native proteins is overcome by using a higher dose of hapten for immunization, without introducing more hapten groups into the carrier molecule with its subsequent distortion of the protein structure. Thus, immunizing with 1 mg of ABA–GSA resulted in good antihapten antibody responses, whereas 100 μg failed to do so (Table II).

However, our results clearly show that the immunosuppressive effect of natural tolerance to homologous protein carriers is more profound than the effect of inducing carrier tolerance to heterologous proteins. In the latter case, antihapten antibody responses are somewhat less frequent, and titers are only slightly reduced, if at all (Table III). Since significant alterations in the antigenic nature of a carrier protein may result from the process of diazotization, we felt that the poor suppression of the antihapten antibody response we obtained might indeed be attributable to the presence of new determinants on the conjugated carrier molecule. Green *et al.* (18), using electrostatically aggregated DNP–PLL and BSA showed the foreign albumin must be capable of eliciting immune responses in order to provoke a response to DNP–PLL in genetic nonresponder guinea pigs. Thus, we studied the antihapten response to ABA–BSA in PABA–BSA tolerant animals, thereby using a BSA altered by diazotization, and more closely approximating the ABA substituted BSA, to induce tolerance. Despite this, we were unable to obtain more than slight suppression of the anti-ABA antibody response. In this sense, tolerance to PABA–BSA gave results more akin to acquired tolerance than to native tolerance.

Our data support the previous proposals that carrier function is essential for the development of antihapten immune responses, and further, that the recognition of carrier antigenicity is followed by a later stage of the immune response when individual determinants on the carrier molecule are recognized and more highly specific antibodies are produced.

The failure to closely approximate the effect of natural carrier tolerance by artificial means suggests several possibilities. First, it may be, as proposed by Boyden and Sorkin (10), that unresponsiveness induced by injection of an antigen (at birth, in their study) is basically different from the natural tolerance to homologous proteins. However, as Nachtigal, Eschel-Zussman, and Feldman have pointed out (19), the continued presence of the tolerogen stabilizes the existence of tolerance, and this could explain the apparent discrepancy between the effects of homologous and heterologous (but "tolerated") carriers in our studies. The other possibility is that the difference between natural and acquired tolerance is quantitative. This gains support from several lines of evidence. Boyden and Sorkin obtained antihapten responses to sulfanilic acid after injections with sulfanilic azo-RSA; we did not obtain such in guinea pigs with 100 μg of ABA–GSA or 100 μg ABA–GGG, but did begin to see such responses with 1 mg doses. Also, ABA–RSA in rabbits did elicit a small, but discernible antibody response to ABA. It may be that the antihapten responses in animals with acquired tolerance would approach those with native tolerance if a more profound type of tolerance was induced. Such experiments are being planned in an effort to investigate further some quantitative aspects of tolerance and immune responsiveness.

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