STUDIES ON ARTIFICIAL ANTIGENS*

II. The Antigenicity in Guinea Pigs of Arsanilic Acid Conjugates of Copolymers of D- or L-α-Amino Acids

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Studies on the antigenicity of synthetic polymers of amino acids and of hapten conjugates of these polymers have provided important information concerning the complex steps involved in the induction of antibody synthesis by protein antigens. Polyamino acids of a single α-amino acid such as poly-L-lysine or poly-L-glutamic acid are not recognized as antigens (1, 2). In contrast, random copolymers containing comparable amounts of 3 or 4 L-amino acids, such as glutamic acid, lysine, and alanine (GLA),[1] or glutamic acid, alanine, and tyrosine (GAT), or GLAT, have been found to be antigenic in rabbits (2, 3), guinea pigs (4), and man (5), in nearly every subject tested. Random copolymers of 2 L-amino acids GA, GL, or LA, induce also an immune response in guinea pigs (4, 6) but only in some animals and not in others. The ability to recognize these polyamino acids as antigens may depend on the ability of the animals to recognize specific antigenic determinants and also on their capacity to metabolize the molecule. Experiments with hapten conjugates of poly-L-lysine suggest that the failure of some of the guinea pigs to be immunized by certain copolymers of α-amino acids is not caused by the absence of antigenic determinants on these polymers but stems from the genetic incapacity of some guinea pigs to process these substances in the precise way required to induce antibody synthesis. Thus only about 30 per cent of random-bred, Hartley strain guinea pigs produce antidinitrophenyl (DNP) antibodies, when immunized with DNP conjugated with polylysine (7) although this hapten is recognized as an antigenic determinant by 100 per cent of guinea

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[1] In this article G, L, T, A, and AS used alone or in combination with each other stand for glutamic acid, lysine, tyrosine, alanine, and arsanilic acid respectively.

pigs if conjugated to foreign or guinea pig proteins. It should also be stressed that guinea pigs capable of recognizing DNP-polylysine as an antigen are also able to produce antibodies against other immunologically unrelated haptens on polylysine such as benzyl penicilloyl or paratoluenesulfonyl (8), and also against the copolymer of L-glutamic acid and L-lysine ($G_{60}L_{40}$) (7). Guinea pigs unable to produce antibodies to DNP-polylysine fail also to recognize GL or other hapten polylysine conjugates as antigens. Contrasting with the antigenicity of GL and DNP polylysine, the ability of guinea pigs to recognize copolymers of L-glutamic acid and L-alanine ($G_{60}A_{40}$) and DNP-polylysine does not appear to be related (6).

The observations with the polylysine hapten conjugates and the copolymers of GA and GL strongly suggest that besides the recognition of the antigenic determinant capable of inducing specific antibody synthesis, an earlier step of metabolism of the antigen molecule must take place, presumably at the level of the macrophages, for the inducer to be made (9, 10). Recent observations of Maurer (11) and Gill *et al.* (12) that copolymers of D-amino acids, are not antigenic in rabbits and guinea pigs while the corresponding conjugates of L-amino acids with the same relative composition behave as antigens in these species, are compatible with this interpretation.

To confirm and extend these observations use was made of the fact that paraarsanilic acid (AS), a hapten which is universally recognized as antigenic by random-bred guinea pigs, can be conjugated by diazotization on the tyrosines of both L-GAT or D-GAT. ASL-GAT[2] was found to be antigenic in 100 per cent of guinea pigs immunized; the specificity of the antibodies produced was directed predominantly against the arsanilic acid determinant. Under identical conditions of immunization and dosage, equal amounts of ASD-GAT[2] failed to induce antibody synthesis in any of the guinea pigs tested, in spite of the fact that the antiarsanilic antibodies produced by guinea pigs immunized with ASL-GAT could be shown to react with ASD-GAT, thus verifying the effectiveness of the arsanilic acid determinant on the non-antigenic arsanilic acid conjugate of the D-amino acid polymer.

Methods

Animals.—Male and female Hartley strain guinea pigs, weighing 300 to 450 gm. were used

Antigens.—Random copolymers of D- or L-glutamic acid (G), tyrosine (T), and alanine (A) were prepared by random polymerization of the *N*-carboxy anhydrides of the α-amino acids. The methods of preparation of the polymers and the techniques employed in determining the average molecular weights have been described (13). Two preparations have been used in these experiments: a copolymer of L-glutamic acid, L-alanine, and L-tyrosine (L-GAT) with a mole per cent composition 60 per cent glutamic acid, 30 per cent alanine, 10 per cent tyrosine, and an average molecular weight of 25,000, and a copolymer of D-glutamic acid, D-alanine,

[2] ASL-GAT, arsanilic acid, L-glutamic acid, L-alanine, and L-tyrosine; ASD-GAT, arsanilic acid, D-glutamic acid, D-alanine, and D-tyrosine.

and D-tyrosine (D-GAT) with the same relative mole per cent composition as the L-amino acids and an average molecular weight of 33,000.

Guinea pig serum albumin (GPA) was prepared by starch block electrophoresis of normal guinea pig serum.

Arsanilic acid conjugates (AS) of these preparations were made by diazotization (14). The number of arsanilic acid groups in the conjugates was estimated from the optical density of the preparations at 330 mμ using as reference the absorption coefficient of chloracetyltyrosine azo arsanilic acid at that wave length. The following conjugates were used: ASD-GAT preparation I; 16 groups/mole, preparation II; 7 groups/mole, preparation III; 13 groups/mole, ASL-GAT preparation I; 11 groups/mole, preparation II; 10 groups/mole, preparation III; 5 groups/mole, preparation IV; 8 groups/mole, ASGPA preparation I; 31 groups/mole, preparation II; 24 groups/mole.

Immunization.—The antigens were dissolved in 0.15 M NaCl and emulsified with an equal volume of complete adjuvants (Difco Laboratories, Inc., Detroit). The immunizing dose contained in 0.4 ml was distributed into the four foot-pads.

Skin Testing.—Intradermal injections of 10 or 50 μg of the antigens in 0.1 ml of 0.15 M NaCl were made at 1 week and again at 2 weeks after immunization. The reactions were observed at 2, 4, and 24 hours. The early reactions were graded + to ++++ according to the severity of the edema and hemorrhage (15). The diameters of the induration or erythema of the delayed reactions were recorded. 3 to 7 days after the last skin test, the animals were bled and the sera tested for antibodies against the arsanilic acid hapten.

Precipitin Test.—Capillary precipitin tests were carried out using undiluted sera and AS bovine fibrinogen at a concentration of 0.1 mg/ml, as described (16).

Passive Cutaneous Anaphylaxis (PCA).—PCA was carried out according to the technique of Ovary (17) using as antigen 0.5 mg of ASGPA 31 groups/mole, unless otherwise specified in Table III.

Systemic Anaphylaxis.—Some of the immunized guinea pigs were injected intravenously with ASL-GAT, ASD-GAT, or ASGPA, and observed for symptoms of anaphylaxis.

Passive Immune Lysis.—Tannic acid-treated sheep red blood cells were prepared by the method of Stavitsky (18) and were coated with highly conjugated AS bovine fibrinogen. After washing, the cells were resuspended in phosphate-buffered saline, pH 7.2. The titrations of antisera were performed after decomplementation at 57°C and absorption twice with sheep red blood cells. Starting with 1/10 dilutions, serial dilutions were made in 0.5 ml veronal buffer (19). To these were added 6 100 per cent units of guinea pig complement and 0.1 ml of the antigen coated cells. After incubation at 37°C for 30 minutes, the highest dilutions showing about 50 per cent lysis were considered the lytic antibody titers.

RESULTS

The immune response of guinea pigs immunized with ASD-GAT or ASL-GAT are presented in Table I. None of the 40 animals immunized with the arsanilic conjugates of the copolymer of D-amino acids showed any evidence of an immune response, either to the copolymer or to the arsanilic acid hapten. They were uniformly negative to skin tests with 10 or 50 μg of ASD-GAT. They showed no signs of anaphylaxis when challenged intravenously with ASD-GAT. The sera of 13 animals (Experiment 4), examined for antiarsanilic acid antibodies by precipitin test and passive hemolysis, were all negative. Considering the possibility that copolymers of D-amino acids might behave like pneumococcal polysaccharides and be immunogenic only at very low dose

TABLE I

Immune Response of Guinea Pigs to Arsanilic Acid Conjugates of Copolymers of D-GAT and L-GAT

Experiment	No. of guinea pigs	Immunizing antigen	14 days 24 hrs. skin reactivity*				Anti-AS-Ab		Anaphylactic reactivity to:		
			D-GAT	ASD-GAT	L-GAT	ASL-GAT	Ppt.	PCA	ASD-GAT, 200 μg	ASL-GAT, 200 μg	ASGPA, 250 to 500 μg
		ASD-GAT									
1	10‡	16 group/mole 100 μg	0/10	0/10					0/10		
2	7	7 group/mole 100 μg		0/7							0/7
3	10	7 group/mole 0.01 μg		0/10							0/10
4	13§	13 group/mole 100 μg	0/13	0/13‖			0/13				0/13
		ASL-GAT									
5	9	11 group/mole 100 μg			8/9	9/9	5/9	4/4			
6	9	10 group/mole 100 μg			4/9	9/9	3/9	6/6			
7	9	10 group/mole 100 μg			5/9	8/9			5 dead/5	4 dead/4	
8	10	5 group/mole 100 μg				10/10					9 dead/10 1 severe shock
9	8	8 group/mole 50 μg				8/8	0/6	6/6			
10	14¶	8 group/mole 100 μg			8/14	14/14					12 dead/14 2 severe shock

* All skin tests: 10 μg of antigens.

‡ Eight of the animals were immunized 7 days later with 100 μg ASL-GAT, 11 g/M. All guinea pigs developed skin sensitivity and anaphylactic reactivity to this antigen at 2 weeks.

§ The sera of these animals were tested for γ_2 complement-fixing antiarsanilic antibodies by passive lysis of tanned SRBC coated with AS bovine fibrinogen, no lysis was observed at a dilution of 1/10.

‖ These guinea pigs were also negative when tested intradermally with 50 μg of ASD-GAT.

¶ The sera of 13 of these animals were tested for γ_2 complement-fixing antiarsanilic antibodies by passive lysis of tanned SRBC coated with AS bovine fibrinogen, 5 of these sera contained lytic antibodies in a titer of up to 1/80.

levels, a group of 10 guinea pigs (Experiment 3) was immunized with 0.01 μg of ASD-GAT without evidence of an immune response. 8 of the animals from Experiment 1, which did not respond to immunization with ASD-GAT, were immunized with 100 μg of ASL-GAT in complete adjuvants to verify that they

TABLE II

Skin Reactivity to ASD-GAT of Guinea Pigs Sensitized to ASL-GAT

Experiment No.	Immunizing antigen	Guinea pig No.	Skin reactivity to 10 μg				
			ASL-GAT, 8 or 10 g/M		ASD-GAT, 13 or 16 g/M		
			2 hrs.	24 hrs.	2 hrs.	24 hrs.	24 hrs.
7	ASL-GAT,	1	±	15 × 15	±	−	
	10 group/mole,	2	++	15 × 15	++	±	
	100 μg	3	+	20 × 20	−	−	
		4	++	15 × 15	++	−	
		5	+	20 × 20	+	−	
		6	++	40 × 40	++	−	
		7	±	8 × 8	±	−	
		8	+	Trace	+	−	
		9	−	±	−	−	
							50 μg
9	ASL-GAT,	1-0		21 × 21		±	8 × 8
	8 group/mole,	1-1		11 × 11		−	
	50 μg	1-2		13 × 13		±	
		1-3		14 × 14		−	−
		1-4		20 × 20		±	10 × 10
		1-5		14 × 14		±	7 × 7
		1-6		14 × 14		−	
		1-7		12 × 12		−	
10	ASL-GAT,	1-8	+	20 × 20			10 × 10
	8 group/mole,	1-9	+	30 × 30			±
	100 μg	2-0	+	10 × 10			±
		2-1	+	20 × 20			−

were capable of an immune response to ASL conjugates. All developed anaphylactic reactivity to ASL-GAT.

Contrasting with the behavior of the animals immunized with ASD-GAT, all 59 guinea pigs immunized with the arsanilic acid conjugates of the copolymer of the L-amino acids produced antibodies against the arsanilic hapten, demonstrated either by anaphylactic reactivity of the animals to ASGPA, ASD-GAT, or ASL-GAT, or by examination of their sera by precipitin test or PCA. The sera of the guinea pigs from Experiment 10, were also assayed for γ_2 hemolytic

(20) antiarsanilic acid antibodies which were found to be present in 5 of the 13 sera studied.

It is interesting to note (Experiment 7) that the arsanilic acid conjugate of D-GAT could provoke lethal anaphylactic shock in guinea pigs immunized with ASL-GAT in spite of the fact that it could not sensitize the animals.

All but 1 of the 59 guinea pigs immunized with ASL-GAT showed, at 2 weeks, skin reactions to 10 μg of ASL-GAT. The intensity of the immediate and delayed reactions observed to ASL-GAT in Experiments 7, 9, and 10, are presented in Table II and are representative of all the animals studied. Delayed reactions to L-GAT were less frequent and less intense. No immediate reactions

TABLE III

*Passive Cutaneous Anaphylaxis Titers of Sera from Guinea Pigs Immunized with ASL-GAT against: ASL-GAT, L-GAT, ASD-GAT, and ASGPA**

Experiment	Sera from guinea pig	ASL-GAT 8 g/*M*	L-GAT	ASD-GAT 7 g/*M*	ASGPA 31 g/*M*
9 (see tables I and II)	1-0	1/1000‡	1/50	1/1000	1/500
	1-2	1/50	Neg.§	1/50	Trace
	1-4	1/50	Neg.	1/50	1/50
	1-5	1/100	Neg.	1/100	1/50
10	2-2	1/1000		1/1000	
	2-3	1/1000		1/1000	

* Dose of challenging antigens 250 μg of L-GAT, ASL-GAT, ASD-GAT; 500 μg ASGPA.

‡ Titers refer to highest dilution of sera giving positive PCA reactions in an average of 3 to 6 guinea pigs.

§ Neg. refers to absence of reactions with lowest dilution used, 1/50.

to L-GAT were observed. A comparison of the skin reactivity of guinea pigs immunized with ASL-GAT, to ASL-GAT and ASD-GAT is also found in Table II. Immediate hypersensitivity reactions of comparable intensity could be elicited by either 10 μg of ASD-GAT or ASL-GAT. Severe delayed reactions could only be provoked by the immunizing antigen and not by equal amounts of ASD-GAT; weak reactions were observed to 50 μg of ASD-GAT, illustrating the extensive immunological specificity of delayed reactions previously described (15). Nevertheless, the ASD-GAT conjugates could effectively combine with the antibodies produced against ASL-GAT as illustrated in Table III. The PCA titers of several sera from guinea pigs immunized with ASL-GAT towards GAT, ASL-GAT, ASD-GAT, and ASGPA, were examined. The GAT antibody titer was negligible, but ASD-GAT and ASL-GAT with comparable number of AS groups were equally effective in eliciting PCA reactions and behaved in this respect a little better than ASGPA. Similar crossreactions, involving glutamyl specificity, between D-GA, D-GAT and poly-D-glutamic

acid and antibodies produced to L-GA and L-GAT, was also observed by PCA (11).

DISCUSSION

The data presented show that an immunogenic hapten arsanilic acid, capable of inducing an antibody response, specific for the hapten, when bound to a copolymer of 3L-α-amino acids, is not antigenic in guinea pigs when conjugated to a copolymer of the corresponding D-amino acids with the same relative composition and comparable molecular weight. These observations lend support to the concept (21, 22, 11, 7), which postulates the metabolism of the antigen previous to the formation of the immunogenic fragment carrying the antigenic determinant and capable of inducing antibody synthesis. The nature of these metabolic steps have not been investigated, but it is reasonable to assume in keeping with the evidence of Fishman and Adler (9, 10), that they involve first the cleavage of the antigen in the macrophages by proteolytic enzymes. This process may be blocked in the case of polymers containing D-amino acids. Fishman and Adler reported also that small molecular weight RNA extracted from macrophages incubated with antigen was capable of inducing antibody production by rat lymph node cells (10). The problem arises whether this RNA is acting as an acceptor and transporter for the immunogenic fragment as is suggested by the observations of Garvey and Campbell who were able to extract fragments of antigen bound to nucleic acid from liver of immunized animals (22). Such RNA acceptors would be expected to be specific for the terminal amino acids of the immunogenic fragment.

The study of the immunogenic fragments produced by macrophages is essential to a better understanding of the early steps of induction of antibody synthesis. The use of well-defined synthetic polyamino acids antigens to which trace labeled antigenic haptens are conjugated, may prove to be highly suitable for such an investigation.

SUMMARY

An effort was made to immunize guinea pigs with arsanilic acid conjugates of copolymers of D- and L-α-amino acids; ASD-GAT and ASL-GAT. An immune response was observed only in the case of ASL-GAT. Antibodies specific for the arsanilic acid hapten were produced which could also react with ASD-GAT or ASGPA. These findings indicate that the proper metabolism of the antigen may be essential to the induction of the immune response.

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