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Antigenicity of Polypeptides (Poly Alpha Amino Acids). XIV. Studies on Immunological Tolerance with Structurally Related Synthetic Polymers.* (30058)

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For the past 10 years, synthetic polymers of amino acids have been used to study some of the molecular parameters of immunogenicity and antigenic specificity (Reviews in 1,2, 3). By altering the nature and amounts of the amino acids in the polymers, it is possible to study the effect of such variables as charge, conformation, optical configuration of the amino acids, helix content, etc. on immunogenicity. From studies of cross reactions with antisera and related polymers, it is becoming possible to learn the nature of the immunogenic determinants in these polymers(4). Although the structures of synthetic random copolymers of amino acids are not known, they are not as complicated as those present in natural proteins made up of many amino acids which contribute to secondary and tertiary structures. Therefore, in addition to studies on the immunogenicity of polymers, investigations in our laboratory, as well as in others(5,6) were undertaken on the production of acquired specific immunological tolerance towards a variety of synthetic antigens. A preliminary account of some of this work appeared several years ago (7). The present report deals with the effect of neonatal injections of synthetic polymers on the subsequent response of the adult rabbits against related cross reacting polymers.

Materials and methods. Table I lists the polymers employed in this study. The random copolymers and the homopolymer of glutamic acid were prepared by the polymerization of the appropriate N-carboxy-α-L-amino acid anhydride by methods referred to previously(8,9). The following abbreviations are used for the amino acids: G—glutamic acid, L—lysine, A—alanine, T—tyrosine. Subscripts refer to the mol percent composition of the amino acid. All rabbits employed were New Zealand white and were injected according to the following schemes:

TABLE I. Synthetic Polymers Studied for Immunological Unresponsiveness.

Polymer*	Nomenclature	Approx avg molecular wt
$Glu_{60}ala_{40}$	$G_{60}A_{40}$	33,000
$Glu_{42}lys_{28}ala_{30}$	$G_{42}L_{28}A_{30}$	65,000
$Glu_{60}lys_{40}$	$G_{60}L_{40}$	64,000
Glu_{100}	G_{100}	60,000

* Subscript refers to mol % of amino acid in polymer.

Experiment 1. ($G_{60}A_{40}$). The rabbits were injected intraperitoneally within 24 hours after birth with 100 mg of the antigen. At day 75, the rabbits were divided into 2 groups. Animals in Group A (1A) were injected in the toe pad with 20 mg of the polymer in complete Freund's adjuvant, and then bled weekly for 3 weeks (Course I). The following week (day 103), the rabbits were boosted with another injection of the polymer in adjuvant and bled again after 2 and 3 weeks (Course II). The rabbits in the second group (1B) received 3 intravenous injections of 20 mg of the polymer per injection on days 75, 82 and 96. One week later, the animals were injected with 20 mg of the $G_{60}A_{40}$ in adjuvant and bled and injected

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repeatedly as described above for Group 1A.

Experiment 2. ($G_{42}L_{28}A_{30}$). The same procedure was employed with the $G_{42}L_{28}A_{30}$ polymer for Groups 2A and 2B. Both of the above groups (1A and 1B; 2A and 2B) had controls consisting of litter mates that were first injected with the polymer in adjuvant on day 75.

Experiment 3. Neonatal rabbits were injected intraperitoneally with a solution of polyglutamic acid as follows: Group 3A received 25 mg on the day of birth; Group 3B received 50 mg on the day of birth; Group 3C received 100 mg on the day of birth; Group 3D received 50 mg on day 1 and 50 mg on day 3. On day 75, the above animals, as well as a control group (Group E), received an intravenous injection of 20 mg of polyglutamic acid. Two weeks later (day 89), all of the above rabbits, as well as a group of non-injected littermate rabbits (Group F), were immunized with the $G_{60}A_{40}$ polymer in complete adjuvant, and then bled weekly for 3 weeks (Course I). They then received another adjuvant injection (10 mg) of the polymer (day 117), and were bled weekly for 3 weeks (Course II).

Experiment 4. Neonatal rabbits were injected on days 1, 4, and 11 with 25 mg per injection of $G_{42}L_{28}A_{30}$ (Group 4A). On day 75, they, as well as a control group (Group 4B), received 10 mg of the same polymer intravenously. On day 90, all rabbits were immunized with the $G_{60}A_{40}$ polymer in complete adjuvant. Bleedings were taken weekly for 3 weeks following the immunization (Course I). The immunization and bleedings were repeated as in the previous groups for the second course.

Experiments 5-7. Neonatal rabbits were injected intraperitoneally as above with $G_{60}A_{40}$ (Group 5), G_{100} (Group 6), or $G_{60}L_{40}$ (Group 7) as follows: 50 mg—day 1 and 50 mg—day 4. On day 75, these animals, as well as the controls (5B), received an intravenous injection of 10 mg of the same polymer. On day 90, all the rabbits were immunized with the $G_{42}L_{28}A_{30}$ polymer in complete adjuvant followed by weekly bleedings and reimmunization as discussed above for the other groups. Another control group (6 & 7 control) did not receive the day 75 intravenous injection, but was immunized on day 90. Table II summarizes the experiments and the number of animals in each experiment.

Testing of sera. Passive cutaneous anaphylaxis (PCA). The PCA test was performed on all sera (0.1 ml undiluted serum or dilutions thereof) as described by Ovary, using a challenging injection of 250 μg of polymer(10). Blueing reactions were recorded 20 minutes later.

Passive hemagglutination. The Takatsy micro modification for passive hemagglutination (HA) of tannic acid treated coated sheep red blood cells was employed (11,12). It was possible to detect antibody against $G_{42}L_{28}A_{30}$ with tannic acid and polymer coated cells. Antibody against $G_{60}A_{40}$ was detected with sheep cells coated with $G_{54}A_{36}L_{10}$ or sheep cells coupled with $G_{60}A_{30}T_{10}$ by the bis diazo benzidine (BDB) method.

Quantitative precipitin studies. Pools of sera that gave positive PCA reactions were analyzed for antibody by a micro modification(4) of the micro precipitin technique as originally developed by Heidelberger and MacPherson(13). Aliquots of the sera from the weekly bleedings following each course of immunization were pooled and analyzed quantitatively.

Fluorescence microscopy. Hyperimmune rabbit antisera against the polymers $G_{42}L_{28}A_{30}$ and $G_{60}A_{40}$ were fractionated with 50% ammonium sulfate (5°C for 2 hours). The globulin precipitates were dialyzed free of sulfate and conjugated with fluorescein isothiocyanate according to the procedure of Marshall *et al.* Unbound fluor was removed by dialysis and absorption with rabbit liver powder. Brush imprints on microscopic slides were made of spleens. To detect antigen, the spleens were stained directly with the appropriate fluorescent conjugate, and to detect antibody, the indirect method of overlaying first with polymer, removing the unbound polymer, and staining with the appropriate conjugated antisera was employed(15). Slides were examined with a Leitz Ortholux microscope using as light source an Osram HBO 200 mercury vapor lamp.

Results. The results of the antibody responses in the various experiments are sum-

marized in Table II. Data obtained with HA are not included as, in all instances, they parallel the PCA results.

Experiments 1 and 2. Neonatal injections of either $G_{60}A_{40}$ or $G_{42}L_{28}A_{30}$ resulted in establishment of the unresponsive state against the homologous polymer. After the first injection of the antigen in adjuvant, none of the experimental animals produced detectable antibody whereas the sera from the control rabbits in the $G_{60}A_{40}$ and $G_{42}L_{28}A_{30}$ groups had PCA titers of 500 and 200 respectively. After the second injection of adjuvant, 7/11 (1A) and 9/12 (2A) rabbits had antibody detectable by PCA. However, the average titers were much lower than the controls, *i.e.*, in Experiment 1: ($G_{60}A_{40}$) 30 *vs* 1500; Experiment 2: ($G_{42}L_{28}A_{30}$) 100 *vs* 1200. The additional intravenous injections of polymer on days 75, 82 and 96 extended the tolerant state in both groups so that even after the second course of injection of polymer in adjuvant, none of the experimental rabbits produced detectable antibody.

Experiment 3. In these experiments, the effect of the neonatal injection of different amounts of polyglutamic acid (25-100 mg) on the subsequent response against $G_{60}A_{40}$ is seen. The neonatal injection of 25 mg of polyglutamic acid (3A) caused a depression in level of antibody produced and in time of appearance of antibody. The 3 reactors in this group reacted by the third week in contrast to the control rabbits that usually reacted after the first week. However, after a second injection of the antigen in complete adjuvants, the 3 reactors produced levels of antibody approaching those of control rabbits.

With the small number of animals in the experiment, it appears that the neonatal injection of 100 mg G_{100} is more effective than 25 mg (Group 3A) and that 2 injections of 50 mg each (Group 3D) is more effective than a single 100 mg dose (Group 3C) in depressing the immune response against $G_{60}A_{40}$. An intravenous injection of G_{100} into control animals, as adults, had little depressing effect on the subsequent response to $G_{60}A_{40}$ (Group 3E) and differed little from the response of the Group 3F control rabbits.

Experiment 4. The neonatal injection of $G_{42}L_{28}A_{30}$ caused a definite depression in the subsequent response against $G_{60}A_{40}$. Only 1/6 rabbits reacted in the first course of immunization and its serum gave a PCA titer of 10. Even after the second injection, the antibody level in this one reactor, although definite (18 μg antibody N/ml serum) was considerably below the average of the control rabbits in this group (75 μg antibody N/ml serum).

Experiment 5. The neonatal injection of $G_{60}A_{40}$ caused a definite decrease in the first course response against $G_{42}L_{28}A_{30}$. Whereas 0/4 experimental rabbits reacted, 3/4 of the control rabbits reacted. However, after the second injection of the homologous polymer, 3/4 experimental rabbits reacted (70 μg Ab N/ml serum) in contrast to 4/4 of the control rabbits (95 μg Ab N/ml serum) indicating a waning of this depressing effect.

Experiments 6 & 7. The data here indicate that neonatal injections of G_{100} or $G_{60}L_{40}$ did not lead to reduction in the subsequent responses against the $G_{42}L_{28}A_{30}$ polymer.

Fluorescent microscopy. The spleens from almost all animals were obtained at the end of experiment and checked for the presence of antigen and antibody forming cells. The general pattern observed was that animals that were immunologically unresponsive against the specific polymer, as judged by the testing of sera also showed the absence of detectable antigen and specific antibody forming cells. On the other hand, the spleens of control animals, as well as of those in the experimental groups with antibody detectable by PCA, showed good to strong immunofluorescence by indirect staining indicative of the presence of antibody forming cells.

Discussion. Of great interest to immunologists is to learn more about the molecular basis of immunological tolerance. It has been shown that tolerance can be produced against serum proteins(16) and "broken" by injections of a *distantly* related albumin. Rabbits tolerant to BSA, after injection of HSA, lost their tolerance against the original BSA(17). The tolerant state could not be broken if there was a large cross reaction between the serum proteins employed. In another study,

TABLE II. Summary of Immune Response in Experiments Dealing with Acquired Immunological Tolerance in Rabbits.

Exp No.	Group No.	Polymer injected			Immune response					
					1st course			2nd course		
		Neonatally	Adult I.V.	Adult Adj	No. of* reactors	PCA† titer	Antibody† per ml serum (μgN)	No. of* reactors	PCA† titer	Antibody† per ml serum (μgN)
1	1A	$G_{60}A_{40}$		$G_{60}A_{40}$	0/12	—‡	—	7/11	30	—
	1A (cont'l)			$G_{60}A_{40}$	5/7	500	30	4/6	1500	95
	1B	$G_{60}A_{40}$	$G_{60}A_{40}$§	$G_{60}A_{40}$	0/6	—	—	0/6	—	—
	1B (cont'l)		$G_{60}A_{40}$§	$G_{60}A_{40}$	0/3	—	—	3/3	1000	70
2	2A	$G_{42}L_{28}A_{30}$		$G_{42}L_{28}A_{30}$	0/12	—	—	9/12	100	—
	2A (cont'l)			$G_{42}L_{28}A_{30}$	5/6	200	—	5/6	1200	75
	2B	$G_{42}L_{28}A_{30}$	$G_{42}L_{28}A_{30}$§		0/6	—	—	0/6	—	—
	2B (cont'l)		$G_{42}L_{28}A_{30}$§		1/4	250	—	2/4	750	65
3	3A	G_{100}	G_{100}‖	$G_{60}A_{40}$	3/5	75	8.0	3/5	1500	98
	3B	G_{100}	G_{100}‖	$G_{60}A_{40}$	0/3	—	—	0/1	—	—
	3C	G_{100}	G_{100}‖	$G_{60}A_{40}$	0/2	—	—	0/2	—	—
	3D	G_{100}	G_{100}‖	$G_{60}A_{40}$	0/3	—	—	1/2	500	40
	3E		G_{100}‖	$G_{60}A_{40}$	3/3	150	50	3/3	2000	127
	3F			$G_{60}A_{40}$	5/6	150	40	5/5	2000	170
4	4A	$G_{42}L_{28}A_{30}$	$G_{42}L_{28}A_{30}$‖	$G_{60}A_{40}$	1/6	10	—	1/6	—	18
	4B (cont'l)		$G_{42}L_{28}A_{30}$‖	$G_{60}A_{40}$	4/6	500	29	4/6	1000	75
5	5A	$G_{60}A_{40}$	$G_{60}A_{40}$‖	$G_{42}L_{28}A_{30}$	0/4	—	—	3/4	750	70
	5B (cont'l)		$G_{60}A_{40}$	$G_{42}L_{28}A_{30}$	3/4	500	22	4/4	1500	95
6	6	G_{100}	G_{100}‖	$G_{42}L_{28}A_{30}$	5/5	750	67	5/5	2000	170
7	7	$G_{60}L_{40}$	$G_{60}L_{40}$‖	$G_{42}L_{28}A_{30}$	4/5	750	56	5/5	1500	139
6 & 7	Control			$G_{42}L_{28}A_{30}$	5/5	300	50	5/5	1500	145

* Number of reactors based on PCA reactions in guinea pigs.
† Average includes only rabbits having antibody detectable by PCA.
‡ Indicates analysis not performed.
§ Polymer injected intravenously on days 75, 82 and 96.
‖ Polymer injected intravenously on day 75.

Weigle demonstrated that BSA tolerant rabbits injected with cross reacting acetyl BSA, picryl BSA or arsanil BSA failed to lose their tolerant state against BSA. However, sulfanil BSA injections could break the tolerance, as could azo derivatives of BGG break tolerance in rabbits tolerant against BGG(18). Cinader *et al* have approached the problem by producing immunological tolerance against HSA and then injecting azo HSA in adult life(19,20). Most of the rabbits remained tolerant. However, some rabbits produced antibody which reacted weakly only with the azo HSA. From these, as well as other investigations(21,23), it appears that when an animal tolerant against antigen A produces antibody against antigen B, the antibody is directed against determinants which are present in the second antigen but not in the first.

In our own experiments, we have been dealing with polymers showing different degrees of cross reactivity with each other. Neonatal injections of the non-immunogenic polyglutamic acid(9) were effective in producing tolerance against the $G_{60}A_{40}$, but not against the $G_{42}L_{28}A_{30}$. The extent of cross reaction of G_{100} with hyperimmune rabbit anti $G_{60}A_{40}$ and anti $G_{42}L_{28}A_{30}$ sera is 61% and 0%, respectively(4). Although it will be reported separately that polyglutamic acid and glutamic acid containing polymers may act as haptenes when precipitated by the appropriate protein carrier(24), the effect of neonatal injections of G_{100} reported here would be the first instance of tolerance being induced in rabbits against haptenic determinants by injections of a non-immunogenic haptene. Previous attempts to produce tolerance against haptenic determinants in rabbits have all been negative(20,25). Recently, Schechter *et al*(6) have reported production of tolerance against poly DL alanyl determinants present on poly DL alanyl HSA or RNase by neonatal injections into rabbits of the non-immunogenic multichain polymer, poly DL alanine. It may be that in order to produce tolerance in rabbits against haptenic determinant groups or against determinant groupings in general, these have to be present on the appropriate degradable carrier. Just as degradation of an antigen appears to be a necessary but not sufficient step for induction of antibody formation against determinants(26,29), the same might obtain for induction of tolerance against haptenic groups in rabbits. The absence of detectable antigen and antibody in the spleens from rabbits tolerant against the polymers is indicative of a true tolerant state and not the trapping of antibody by persisting antigens.

$G_{42}L_{28}A_{30}$, which cross reacted with anti $G_{60}A_{40}$ sera about 30%, when injected neonatally into rabbits, created a tolerant state against $G_{60}A_{40}$. However, the reverse situation did not obtain with equal efficiency with $G_{60}A_{40}$, which cross reacted 32% with an anti $G_{42}L_{28}A_{30}$ serum pool. Neonatal injections of $G_{60}A_{40}$ were effective in establishing a tolerant state during the first course of immunization with $G_{42}L_{28}A_{30}$. However, another adjuvant injection of $G_{42}L_{28}A_{30}$ led to the "breaking" of the tolerant state. Whether the antibody produced in this latter situation, or from any other rabbit exhibiting "breaking" of the tolerant state is different from the antibody produced in normal rabbits, is under investigation. Also, $G_{60}L_{40}$, which cross reacts about 20% with anti $G_{42}L_{28}A_{30}$ sera, was not effective in the amounts injected in establishing tolerance against $G_{42}L_{28}A_{30}$.

These experiments indicate that the extent of cross reaction of related polymers, as measured by *in vitro* tests, cannot always serve as an indicator whether cross tolerance may be established. It was easier to produce cross tolerance against $G_{60}A_{40}$ than against $G_{42}L_{28}A_{30}$. This may be because there are more different determinant groups, or a more complicated structure, in the latter polymer. That $G_{60}A_{40}$ has no α-helix content and $G_{42}L_{28}A_{30}$ about 40% α-helix(30), may indicate the presence, in this latter polymer, of immunogenic areas formed by 2° and 3° configurations which are absent in the truly straight chain non-helical polymers polygultamic acid, $G_{60}A_{40}$ and $G_{60}L_{40}$.

Summary. It has been shown that neonatal injections of the polymers $G_{60}A_{40}$ and $G_{42}L_{28}A_{30}$ can establish acquired immunological tolerance against these homologous polymers. Neonatal injections of polyglutamic acid are more effective than $G_{42}L_{28}A_{30}$

in establishing cross tolerance against $G_{60}A_{40}$. Neither polyglutamic acid nor $G_{60}L_{40}$ when injected neonatally, were effective in reducing an adult response against $G_{42}L_{28}A_{30}$. However, $G_{60}A_{40}$ did reduce the response against a first course of immunization with $G_{42}L_{28}A_{30}$. This tolerance was broken by a second immunization with polymer in complete adjuvants.

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Inhibition of Poliovirus Minute-Plaque Mutants and Echoviruses by Sulfated Polysaccharides.* (30059)

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Takemoto and Liebhaber(1) reported that dextran sulfate inhibited multiplication of encephalomyocarditis (EMC), echo-, coxsackie-A9 and herpes viruses and enhanced plaque-forming capacity of poliovirus type 1 Mahoney. Sulfated polysaccharide in agar has been shown to inhibit multiplication of herpes virus(2), dengue virus(3), EMC virus (4), and a *minute-plaque* (*m*) mutant of poliovirus(5).

The effects of natural and synthetic sulfated polysaccharides on growth of 4 *m* mutants of poliovirus and on plaque formation by these and certain prototype echo- and wild-type polioviruses are reported herein.

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