Antigenicity of Polypeptides (Poly α Amino Acids): Immunogenicity of Chemically Modified Polymers. II[1] (34856)

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In continuing studies aimed at describing some of the parameters of immunogenicity in several species, it was of interest to investigate the immunogenic properties of a few neutral derivatives of polymers of amino acids. The neutral polymers considered here are the propanol amide derivatives of the carboxyl groups of poly (L-glu^{60}-L-ala^{30}-L-tyr^{10})n, poly (L-glu^{90}-L-tyr^{10})n and poly (L-glu^{60}-L-ala^{40})n[2]. A previous report has dealt with this latter polymer (1). The newer derivatives were prepared to extend previous observations which indicated that a neutral modification of the free carboxyl groups of immunogenic polymers reduces immunogenicity, and in addition, to learn whether incorporation of tyrosyl residues could enhance the poor immunogenicity of the poly GA propanol amide and poly G propanol amide polymers.

The present report will discuss findings on immunogenicity in rabbit, guinea pig, mouse, sheep, and man, and subsequent reports will deal with the specificity of the antisera in several species. Aside from the basic information gained, these studies which were aimed at trying to develop a nonimmunogenic plasma expander, offer a modification which tends to reduce immunogenic properties.

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[2] The nomenclature is based upon recommendation of IUPAC-IUB Commission on Biochemical Nomenclature, October, 1967. Biochem. J. 106, 577 (1968).

Materials and Methods. The polymers included in this study are listed in Table I.

The hydroxy propanol glutamide (propanol amine) derivatives of the various polymers were prepared employing the technique of Lupu *et al.* (2). The benzyl glutamate polymers previously described were reacted with propanol amine which converted the esters to hydroxypropylglutamide residues. All polymers were dialyzed and lyophilized before use.

Immunization procedures. Rabbits. Poly GAT amide. Immunization was undertaken at three different times as follows:

(a) R No. 988-992 were injected in the footpad with 5 mg of the polymer in complete adjuvants. Bleedings were taken weekly beginning 2 weeks after the injection and continued for 3 months. At that time the three surviving rabbits were boosted with another 5 mg of polymer in complete adjuvants and exsanguinated 2 weeks later.

(b) R No. 1005-1010 were injected and bled as above. The second injection of polymer in adjuvant was given 2 months after the first. The two best reactors received another injection 2 months later and were exsanguinated 2 weeks after the last injection.

(c) R No. 1078-1087. Immunization was as above. However, this group was boosted with 3 mg of polymer 6 weeks later, and again 10 weeks after the last injection. Bleedings were taken weekly for several months.

Poly G amide. Although the nonimmunogenicity of this polymer in rabbits was reported previously, high doses of polymer were used for immunization (20 mg × three courses). The present protocol employed injections of either 10 μg or 1 mg of polymer in complete adjuvants. Three rabbits were in-

TABLE I. Polymers Employed in Present Study.

Polymer	Nomenclature employed in text	Average molecular weight
Poly (L-glu^{60}-L-ala^{40})$_n$	GA	43,000
Poly (γ -N- 3 hydroxypropyl α-L glutamide)$_n$	G amide	75,000
Poly (γ -N- 3 hydroxypropyl α-L glutamide^{90}-L-tyr^{10})$_n$	GT amide	50,000
Poly (γ -N- 3 hydroxypropyl α-L glutamide^{60}-L-ala^{40})$_n$	GA amide	50,000
Poly (γ -N- 3 hydroxypropyl α-L glutamide^{60}-L-ala^{30}-L-tyr^{10})$_n$	GAT amide	22,000

jected with each dose and bled weekly for 6 weeks. All were then reinjected with 100 μg of the polymer and bleedings taken for another month.

Poly GT amide. Three rabbits were injected with 2 mg of polymer in complete adjuvants. Bleedings were taken weekly for a month at which time the rabbits were "boosted" with 100 γ of polymer and additional bleedings taken for another month.

Guinea pigs. Poly GAT amide. Groups of Hartley strain and Strain 13 guinea pigs weighing 350–450 g were immunized with 10 μg, 100 μg, or 1 mg of polymer in complete adjuvants. Each group consisted of 6–7 guinea pigs. The initial injection of polymer was given in the hind footpads (0.1 ml per footpad). Two to three weeks later, a booster injection of the same amount of polymer was given in the neck area. Three weeks after the last injection, the animals were bled from the retroorbital sinus or by cardiac puncture and tested by passive cutaneous anaphylaxis as indicated below. Exsanguination was performed within 2 weeks. Sera were kept frozen until tested.

Mice. Poly GAT amide. As mice have been shown not to respond to copolymers of two amino acids or derivatives analogous to copolymers (3, 4), immunization here was undertaken only with the poly GAT amide. Two groups of 20 Swiss-Webster mice per group were injected intraperitoneally with 1 mg or 10 μg of polymer in ml of complete adjuvant. The first booster injection was given 6 weeks later as above, but in 0.4 ml. Eye bleedings and "tapping" of ascites fluid were performed periodically.

Sheep. The basic protocol for immunization with the polymers poly GA, poly GA amide, and poly GAT amide was similar to the one employed for immunization with poly (GAT)$_n$ and other polymers (5). Three sheep were injected subcutaneously with 20 mg of each polymer in complete adjuvants three times at weekly intervals. Test bleedings were drawn before each injection and sera checked for antibody by precipitin reaction. After a 3-week interval, two more weekly injections were given. Bleedings were taken periodically, and "booster" injection was given when the levels of antibody were declining. As no responses were noted against the poly GA amide polymer after 3 months, the respective sheep were reimmunized with GA in incomplete adjuvant as above.

Humans. The procedures for injecting, bleeding, skin testing, and analyzing the sera were as reported previously (6).

Poly GA amide. Twelve human volunteers received four intramuscular injections (0.5 ml per injection) of 10 mg GA amide per injection given during a period of 2 weeks. Bleedings were obtained before, 10 days after, and 3 weeks after the last injection. Skin testing with 0.1 ml of mg/ml was performed at the same time.

Poly GAT amide. Eight volunteers received injections and were bled as above. Four received a total of 4 mg of poly GAT amide and four received 80 mg of polymer.

Testing of sera. Passive cutaneous anaphylaxis (PCA). For initial screening of the rabbit and guinea pig sera, the PCA test was performed as described by Ovary (7). Challenging injections consisted of 1 ml of a solution of 500 μg polymer and 0.5% Evans blue dye.

Quantitative precipitin reactions. The microprecipitin reaction was employed for de-

Table II. Species Response Against Polymers.[a]

Species	GA	GA amide	GAT	GAT amide
		Polymer		
Rabbit	60/100[b]	7/13[b]	50/60[b]	4/5; 6/6; 10/10
Guinea pig				
Hartley strain	6/21[b]	0/12[b]	3/7[b]	5/6;[c] 6/7;[d] 3/7[e]
Strain 13	0/4[b]		13/18;[b] 14/15[b]	3/6;[c] 5/7;[d] 2/6[e]
Mice (Swiss-Webster)	0/40[b]		20/20[b]	0/40
Sheep	3/3	0/3; (3/3)[f]	3/3[b]	2/3
Man		0/12	0/16	0/8

[a] Number responding/number tested.
[b] Data from previous publications.
[c] Immunization dose 10 μg.
[d] Immunization dose 100 μg.
[e] Immunization dose 1 mg.
[f] Response after reimmunization with Poly GA.

termining the amounts of antibody N per ml of serum (8). Precipitin rections with rabbit, sheep, and mouse sera used 0.2–2.0 ml of serum depending on the amounts of antibody N precipitated. With human sera, because of the low levels of antibody generally present, 3.0 ml of serum was employed.

Antimouse γ globulin reaction. As the mouse sera were negative by microprecipitin reaction, the "anti-globulin reaction" with ^{125}I-labeled poly GAT amide was employed to detect antibody capable of binding antigen. High-titered sheep anti-mouse γ globulin was used to precipitate the globulin in the presence of ^{125}I-labeled poly GAT amide (9). The washed precipitates were counted in a scintillation γ well counter.

Results. Table II presents a summary of the results obtained with the various polymers. Some comparative information from previous reports are also included. The new data are as follows:

Rabbits. No responses were noted against the poly G amide and poly GT amide polymers. However, with the poly GAT amide, significant reponses were noted in all three experiments. The response as measured by PCA reaction was 4/5, 6/6, and 10/10 rabbits. Precipitating antibody was eventually present in all latter positive sera. The range of antibody precipitated per ml of serum was 15–70 μg N.

Guinea pigs. Significant responses (PCA in guinea pigs) were produced against the poly GAT amide polymer in both Hartley strain and Strain 13 guinea pigs. With both strains of guinea pigs immunization with 100 μg polymer produced the best reaction. Of interest is the lack of uniform response among the inbred Strain 13 guinea pigs.

Mice and man. No detectable responses were noted.

Sheep. Although no responses were obtained against the poly GA amide in the three sheep, good levels of precipitating antibody were produced against the poly GA polymer (three of three), *i.e.*, 60–200 μg antibody N per ml of serum. Moreover, the sheep that did not respond to the poly GA amide polymer did produce precipitating antibody when reinjected with GA polymer. Only two of three sheep immunized with the poly GAT amide responded. They produced significant levels of precipitating antibody which was followed for several months. The levels of antibody in these two sheep varied from 20–170 μg N per ml of serum.

Discussion. The intent of this report is to discuss only the area of immunogenicity. It was shown previously that the chemical modification of the free carboxylate groups in poly GA reduced immunogenicity. The significant differences in responses against poly GA and poly GA amide in rabbit and guinea pig and now with sheep support the above contention.

Although the free glutamate and net negative charge may have contributed to the immunogenicity of poly GA and poly $(G^{60}A^{30}T^{10})_n$, neutral polymers can also be immunogenic (1, 10) but the responses vary from species to species. The poly GA amide was immunogenic only in rabbits and a response of 30–50 μg antibody N per ml serum was observed (1). When the analogous tyrosine containing polymer (poly GAT amide) was studied, enhanced immunogenicity was found in rabbits, guinea pigs, and sheep. It would appear that in these species two opposing phenomena might govern the outcome, *i.e.*, the chemical modification acting to reduce immunogenicity, and the presence of tyrosyl residues acting to counteract this effect. Although tyrosine has enhanced immunogenicity of the polymers, in subsequent publications, it will, indeed, be shown that most of the specificity of the anti-poly GAT amide is against poly GA amide. The findings with poly GT amide indicate that introduction of tyrosyl residues could counteract the "depressing" effect of neutralizing the glutamate residues when one is dealing with poorly immunogenic non modified polymers.

What mechanism might be responsible for the enhancing effect by tyrosyl residues and the depressing effect of the propanol amide modification is not known. The one species in which the above explanation does not obtain is with mice. In contrast to the other species, however, no copolymer of two amino acids has been found to be immunogenic (3, 4). Here the importance of both the free glutamyl and tyrosyl residues has been shown, *i.e.*, GT cross-reacts 80–125% with mouse anti-GAT (11). Possibly because of this, the modification of glutamyl residues abolishes immunogenicity.

These as well as the findings of others with neutral polymers indicate that charge *per se* is not important in contributing to immunogenicity. Nevertheless, the negatively charged polymers are better immunogens. On the practical side the findings in humans that under the conditions studied neither of the modified polymers are immunogenic indicate that this kind of modification might be considered as a means of reducing immunogenicity of protein and protein-like molecules.

Summary. It has been shown that chemical modification of the negatively charged carboxyl groups of synthetic polymers of amino acids via introduction of neutral determinants tends to reduce immunogenic properties of the respective polymer.

In some instances the presence of tyrosyl residues can, to a limited degree, reverse the above effect.

The possible implication of these findings for development of nonimmunogenic synthetic plasma expanders is presented.

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