THE IMMUNOGENICITY OF VINYL POLYMERS*

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Studies with synthetic polypeptide antigens have provided much information about the chemical basis of immunogenicity and about the structure of antigenic sites.¹⁻⁸ Experiments reported from this laboratory³ suggested that the properties of a molecule minimally necessary to induce antibody formation are those quite general to macromolecules. Certain features, such as the presence of tyrosine or phenylalanine, enhance immunogenicity. Other properties, such as D-optical activity or high charge, depress immunogenicity but do not abolish it. Those compounds which induce only small amounts of antibody apparently do no better, because the rate of their degradation is so slow that only minute doses must be administered lest the threshold level for the initiation of immunological paralysis be exceeded. It may be that a poor immunogen is poor only because it is efficient at inducing paralysis; but this proposition is a difficult one to test, since the mechanism of paralysis is not understood.

The present study was undertaken to test further these generalizations by investigating the immunogenicity of nonbiological macromolecules, i.e., vinyl polymers. Each of the four vinyl polymers chosen for study possessed particular properties of interest. Polyvinylpyrrolidone is the most peptide-like of the vinyl polymers because it contains a substituted amide group; it also contains a heterocyclic ring which is a model for aromatic amino acids. Poly(methacrylic acid-2-dimethylaminoethyl methacrylate) is a polyampholite; thus it resembles proteins in respect to charge distribution. Lastly, polymethacrylic acid and polyvinylamine are completely charged, vinyl homopolymers—a combination of characteristics that should produce the most unfavorable type of immunogen.³ Therefore, their immunogenic activity should provide a stringent test of the generalizations stated above.

Materials and Methods.—The polyvinylamine (PVAm), polymethacrylic acid (PMA), and poly(methacrylic acid-2-dimethylaminoethyl methacrylate) (PMD)⁹ were gifts of Professor Paul Doty. The polyvinylpyrrolidone (PVP) was obtained from Sigma Chemical Co., St. Louis, Mo. The physical chemical characterizations of the polymers are summarized in Table 1, and their structures are shown in Figure 1. The molecular weight was determined by the high-speed equilibrium technique of Yphantis.¹⁴ The degree of polymerization was calculated from the molecular weight and the mean residue weight. The partial specific volume was calculated from the data of Cohn and Edsall¹⁰ and of Fajans and Johnson.¹¹ In the latter calculations, the partial specific volume of the $-\text{NH}_3^+$ group was obtained by subtracting the partial specific volume of hydrogen from that of the NH_4^+ group.

The immunogenicity studies were carried out on randomly bred New Zealand white rabbits weighing 2.5–3 kg. Two schedules of immunization were utilized, and in both cases the antigen was incorporated into Difco Freund's complete adjuvant. The first schedule (Table 2) utilized three doses of antigen (100, 10, 1 μg) in each of three different groups of animals. The rabbits were bled 4 weeks after each injection of antigen (bleedings I–III), and bleeding IV was taken 1 month after bleeding III, but without any further injection of antigen. The second schedule (Table 3) was designed to confirm the

finding of an antibody response to PMA and PVAm. It utilized two doses of antigen of 1 μ g each given 15 weeks apart. Bleeding A was taken 4 weeks after the first dose of antigen, and bleeding B, 3 weeks after the second dose.

The precipitin reactions were carried out as described previously:¹⁵ 0.5 ml of antigen solution was added to 0.5 ml of serum, and the mixture was kept at 4° for 7 days. Following incubation, the reaction mixtures were centrifuged, and the precipitates were washed twice and then dissolved in 0.25 *M* acetic acid. The tyrosine absorbance was read at 277 m μ . In the experiments utilizing the γ -globulin fraction of serum, the γ -globulin was prepared by precipitation with 18% sodium sulfate (final concentration). The passive cutaneous anaphylaxis tests were carried out as described by Ovary.¹⁶

TABLE 1. *Polymers tested for immunogenicity.*

Polymer	Molecular weight	Residue weight	Degree of polymerization	Partial specific volume ^a
PVP	180,000	111	1620	0.85
PMD ^b	350,000 ^c	123 ^d	2845	0.88 ^d
PMA	15,000 ^e	108 ^f	140	0.61 ^f
PVAm	43,000 ^g	79 ^h	540	0.78 ^h
Poly Glu ⁵² Lys ³² Tyr ¹⁵ (no. 4)	86,000	158 ^{f,h}	545	0.67 ^{f,h}

^a Calculated from the data of Cohn and Edsall¹⁰ and Fajans and Johnson.¹¹

^b Preparation no. 9 of ref. 9.

^c Isoelectric point: pH 8.60.⁹

^d Zwitterion form (ionized carboxylic acid and protonated tertiary amine group).

^e pK_i = 4.85.¹²

^f Sodium salt.

^g pK_i = 9.4.¹³

^h HCl salt.

FIG. 1.—Schematic representations of the four vinyl polymers used in this study. PMA exists in neutral and basic solutions as the sodium salt, and PVAm exists in neutral and acid solutions as the hydrochloride. PMD exists in neutral solutions as a zwitterion with a negative charge on the carboxyl group of the polymethacrylic acid moiety and a positive charge on the nitrogen of the 2-dimethylaminoethyl methacrylate portion of the molecule. The PMD molecule contains 29% of the former component and 71% of the latter component.⁹

TABLE 2. *The antibody response to vinyl polymers after different numbers of injections of antigen.*

Polymer	Antibody Response ($\mu\text{g AbN/ml}$) ^a					
	Bleeding I (immunizing dose)			Bleeding II (first booster dose)		
	100 μg	10 μg	1 μg	100 μg	10 μg	1 μg
PVP ^b	2,4,7,10	2,25,3,3,16	11,20,8,2,15	3,1,3,3	0,3,1,1,4	3,7,3,0,22
PMD ^b	8,0,1,2,3	30,8,8,8,12	0,0,0,0,0	17,8,17,7,12	16,2,3,19,0	0,0,0,0,0
PMA ^c	0,0,0,0	0,0,0,0	0,0,0,2	0,0,0,0	0,0,1,1	1,0,0,1
PVAm ^c	0,0,0,0,0	1,1,1,3,3	3,1,0,0,1	0,0,0,1,2	0,0,0,0,1	2,0,0,0,0
Polymer	Bleeding III (second booster dose)			Bleeding IV (no more antigen)		
	100 μg	10 μg	1 μg			
PVP ^b	0,0,0,0	0,0,0,0,0	0,2,0,0,7	...	...	...
PMD ^b	2,8,9,2,3	12,0,2,11,0	0,0,0,0,0	...	...	...
PMA ^c	0,0,0,0	0,0,1	0,0,0,0	3,2,3,3	3,2,5	1,5,4,4
PVAm ^c	0,0,0,0,0	0,1,0,0	2,1,1,0,0	2,3,1,1,0	2,0,0,0	0,0,0,0

^a The data are for individual rabbits; antibody concentrations are listed in the same order for each bleeding.

^b Four of five guinea pigs gave weak passive cutaneous anaphylaxis reactions (+) with a γ -globulin fraction of pooled sera containing 44 $\mu\text{g AbN/ml}$ of anti-PVP, and three of five guinea pigs gave only equivocal reactions ($\pm$) with a γ -globulin fraction containing 36 $\mu\text{g AbN/ml}$ of anti-PMD. The time interval between injection of antibody into the skin and challenge with antigen was 3 hr. In a second set of experiments, an interval of 24 hr did not yield stronger reactions.

^c The presence of antibody in these antisera was confirmed by repeating the precipitin reactions on the γ -globulin fractions of the pooled sera. Over the antigen dose ranges used, the γ -globulin fraction of pooled normal rabbit sera did not precipitate with either antigen.

Results.—All four vinyl polymers elicited antibody formation. The data obtained by the first schedule of immunization are summarized in Table 2, and representative precipitin curves are shown in Figure 2. The antibody response was moderately good with PVP and PMD, but it was low and variable with PMA and PVAm. There was no nonspecific precipitate with normal sera over the antigen dose ranges used in these studies. Low doses (10 and 1 μg) of PVP generally elicited the best antibody responses. The antibody concentrations declined and vanished as more antigen was given, especially with the higher doses (100 and 10 μg). The intermediate dose (10 μg) of PMD elicited the best antibody response, and the antibody concentration decreased slowly with continued administration of antigen. The highest dose was effective also, but the lowest dose (1 μg) was inadequate to stimulate antibody formation at all. The antibody

TABLE 3. *The antibody response to polymethacrylic acid (PMA) and polyvinylamine (PVAm) elicited by the second immunization schedule.**

Antibody Response ($\mu\text{g AbN/ml}$)			
PMA		PVAm	
Bleeding A	Bleeding B	Bleeding A	Bleeding B
3	5	0	0
3	4	0	2
2	4	0	0
2	4	0	0
4	5	3	4
3	11	0	0
3	13	3	1
		2	2
		2	1

* The data are for individual rabbits; antibody concentrations are listed in the same order for each bleeding. Antigen injections are discussed in the text (see *Materials and Methods*).

FIG. 2.—Representative precipitin curves of the antibody-antigen reactions of the four vinyl polymers.

response to PMA and PVAm was generally best following intermediate and low doses of antigen. After antigen injection had ceased, the antibody response was better (bleeding IV); this suggests that the amounts of antigen given previously had induced partial immunological paralysis in the rabbits. Thus, with the vinyl polymers, the polyampholyte was a better immunogen than either of the charged homopolymers. The same was true with the polypeptides, in which case a copolymer of glutamic acid and lysine was a better immunogen than either polyglutamic acid or polylysine.³

The passive cutaneous anaphylaxis test in guinea pigs (Table 2) showed weak reactions with PVP and equivocal reactions with PMD. The amounts of antibody used would ordinarily give very strong reactions with protein or polypeptide antigens.³ Therefore, the antibody to the vinyl polymers does not have a similar biological capability in this regard.

The results on the immunogenicity of PMA and PVAm were confirmed by a second set of experiments in which less antigen was used for immunization (schedule 2, Table 3). These data again demonstrate the presence of antibody following immunization with these two vinyl polymers: All seven animals responded to PMA, and five of nine animals responded to PVAm. Following the second injection of antigen, the amount of antibody formed remained unchanged or was somewhat increased.

The cross-reactivity among the various vinyl polymers, and between the vinyl polymers and a synthetic polypeptide, was examined in order to demonstrate the

immunological specificity of the antibodies involved. The results are shown in Table 4. The only cross-reactions were those predictable on the basis of structural similarities. PVP cross-reacts with poly Glu⁵²Lys³³Tyr¹⁵ (no. 4), because of the structural similarity between the heterocyclic ring in the PVP and the tyrosine in the polypeptide. The heterocyclic ring of PVP is a prominent antigenic determinant,^{17, 18} and the antibody directed at this determinant would be spatially compatible, at least in part, with tyrosine, which forms an important part of the polypeptide's antigenic sites.^{3, 5, 15} The lack of a reverse reaction, that is, between the antibody to the polypeptide and the PVP, is not surprising, since the phenolic hydroxyl group in tyrosine is a very important component of the tyrosine antigenic determinant. This is illustrated by the previous finding¹⁵ of a relatively low level of cross-reactivity between phenylalanine-containing polymers and antisera to tyrosine-containing polymers. On the other hand, antisera to phenylalanine-containing polypeptides cross-reacted well with tyro-

TABLE 4. *The cross-reactivity among vinyl polymers and a synthetic polypeptide.*

Antibody*	Antibody Reaction (μ g AbN/ml)				
	PVP	PMD	PVAm	PMA	Poly Glu ⁵² Lys ³³ Tyr ¹⁵ (no. 4)
PVP	44	0	0	0	9
PMD	0	36	0	14	0
Poly Glu ⁵² Lys ³³ Tyr ¹⁵ (no. 4)	0	0	0	0	275

* γ -Globulin fraction of pooled antisera.

sine containing polypeptides. Thus, the cross-reactivity between the PVP and poly Glu⁵²Lys³³Tyr¹⁵ (no. 4) systems follows the same type of pattern. The other cross-reaction was that between anti-PMD and PMA. This finding was expected, since PMA is one component of the copolymer PMD (Fig. 1).

Discussion.—A number of previous studies on the immunogenicity of vinyl polymers (reviewed in ref. 1)—for example, on PVP,^{17–20} PVP with amino acid or sugar determinants added,²¹ or on polystyrene^{19, 20, 22}—failed to demonstrate antibody formation in the rabbit or in any other species, with one exception. High-molecular-weight PVP (mol wt 1,000,000) elicited antibody formation in 25 of 66 humans, but a lower-molecular-weight PVP (mol wt 250,000) did not.¹⁷ In addition, the anti-PVP sera did not yield a passive cutaneous anaphylaxis reaction in guinea pigs. The apparent lack of immunogenicity of PVP in rabbits and of the lower-molecular-weight PVP in humans is probably due to the fact that too much antigen was administered; hence, it induced immunological paralysis. Maurer¹⁷ gave approximately 3000–7000 μ g antigen/kg to the rabbits (estimated from his paper), whereas in the present study we gave only 0.3–100 μ g/kg.²³ In the studies with the higher-molecular-weight PVP in humans, Maurer gave approximately 1200 μ g/kg, and antibody formation ensued. This dose is high,²⁴ but its effectiveness may have been due to the fact that PVP accumulates in the liver and remains there for a very long period of time. In this way the amount of material available at the sites of immunization may have been reduced and in some cases fallen below the level for immunological paralysis. In long-term studies of humans immunized with PVP,¹⁸ the anti-PVP concentra-

tions fell from a range of 8–26 μg AbN/ml to a range of 0–3 μg AbN/ml in one year following the primary course of immunization (100 mg of PVP). In one person who received only one injection of 20 mg of PVP, the antibody level remained relatively high (9.5 μg AbN/ml).²⁵ In addition, this person had one of the highest initial antibody responses, and the level of antibody declined quite gradually, whereas it fell rapidly in those who received 100 mg of the antigen. A second course of immunization (60 mg PVP) 13 months after the first one failed to elicit any antibody at all. The findings in humans can all be explained by the induction of partial immunological paralysis by the first course of immunization, and complete paralysis by the second course. Thus, the data on both humans and rabbits are quite compatible with our postulate concerning the relationship between the structure of the antigen and the dose required for immunization.

There are two other cases in which the size of the dose can influence the induction of an immune response. Studies on the effect of adjuvants in mice²⁶ indicated that their mode of action was the slow release of small amounts of antigen over a prolonged period of time. Secondly, the high efficiency of antigen combined in an antibody-antigen precipitate for eliciting delayed hypersensitivity in guinea pigs without eliciting antibody formation²⁷ could be due to the very slow release of antigen from the precipitate.

The results of the studies on the immunogenicity of vinyl polymers confirm the postulate involving the relationship between the structure of the antigen and the dose needed to stimulate antibody formation. The interplay between the dose of antigen, including its mode of administration, and the rate of antigen degradation regulates the amount of antigen available to initiate an immunological reaction. This regulation adjusts the balance between immunological stimulation and immunological paralysis. Small doses of poor immunogens stimulate antibody formation and larger doses induce immunological paralysis. The critical antigen levels are evidently determined by the genetic background of the animal, because they vary among species and among the various strains of a given species.^{28, 29}

There are, in addition, three implications to be drawn from the demonstration that vinyl polymers are immunogenic: First, the antigen does not have to be fragmented in order to induce antibody formation, since there is no evidence that vinyl polymers can be enzymatically or in any other way physiologically attacked. Secondly, the surface topography of the molecule is probably the crucial structural parameter for interaction with the immunocompetent cell, regardless of how the surface is constructed. Thirdly, immunogenicity is probably a general property of most if not all macromolecules, since the vinyl polymers which elicited an antibody response are biologically quite alien substances.

Summary.—A representative spectrum of vinyl polymers is immunogenic in rabbits. Polyvinylpyrrolidone and poly(methacrylic acid-2-dimethylaminoethyl methacrylate) consistently elicited a moderate amount of antibody, and polymethacrylic acid and polyvinylamine elicited a low and variable antibody response. These studies are consistent with the hypothesis that there is a balance between immunological stimulation and paralysis which is determined by the structure of the antigen. The latter sets the level and the range of dosage that can be used in immunizing an animal. The critical role of antigen catabo-

lism in immunogenicity appears to be that of adjusting the amount of antigen left intact. In this manner, it acts in concert with the original dose to regulate the amount available to stimulate antibody formation.

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Abbreviations: PVAm, polyvinylamine; PMA, polymethacrylic acid; PMD, poly(methacrylic acid-2-dimethylaminoethyl methacrylate); and PVP, polyvinylpyrrolidone.

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