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Antibodies against Polyethylene Glycol Produced in Animals by Immunization with Monomethoxy Polyethylene Glycol Modified Proteins

Ary Wolfgang Richter, Eva Åkerblom

Departments of Biomedical Research, Pharmacia AB, and Experimental Allergy Research, Pharmacia Diagnostics AB, Uppsala, Sweden

Abstract. Antibodies to polyethylene glycol (PEG) were raised in rabbits by immunization with monomethoxy polyethylene glycol modified ovalbumin (OA), bovine superoxide dismutase (SOD), and ragweed pollen extract (Rag), given in Freund's complete adjuvant (FCA). Immunogenicity depended on the nature of the protein and the degree of modification. With modified OA, in the presence of FCA, the majority of animals showed an anti-PEG response. With modified SOD and Rag only a small proportion of animals responded. In the absence of FCA, modified OA, given s.c., did not elicit any anti-PEG antibody response in rabbits and only a weak response in mice. PEG of $\overline{M}_W$ 10,000 and 100,000 given in FCA was found nonimmunogenic in rabbits, and PEG of $\overline{M}_W$ 5.9×10^6, given s.c. to mice, showed no or very poor immunogenic properties. Gel diffusion, heterologous passive anaphylaxis and passive hemagglutination were used to demonstrate anti-PEG antibodies raised to PEG-modified proteins. Specificity was confirmed by hapten inhibition of precipitation, inhibition of passive hemagglutination and cross-reactivity tests. PEG of $\overline{M}_W \geq 4{,}000$ produced specific precipitates, smaller molecules acted as monovalent haptens. From hapten inhibition of precipitation by PEG of $\overline{M}_W$ 300 it appears that the antigenic determinant of PEG may be a sequence of 6–7 $-CH_2CH_2O-$units. Anti-PEG antibodies can be used analytically. By gel diffusion, Peg was detected in minimal concentrations of 0.1–1 μg/ml. The clinical relevance of these findings with regard to therapy with PEG-modified enzymes and allergens in humans remains to be established.

Introduction

In recent years, modification of proteins by polyethylene glycol (PEG) has been used to reduce the immunogenicity of various enzymes [1–3, 17]. The concomitant prolonged persistence of such modified enzymes in the circulation offers new possibilities for therapeutic applications. PEG modification was also applied to allergens with the aim to transform such modified allergens into tolerogens [10–12]. Animal experiments showed that specific IgE suppression by PEG-modified allergens is due to the generation of T-suppressor cells [13]. It is known that covalent binding of repetitive polymers such as polysaccharides to proteins yields conjugates, which upon immunization of animals are capable of inducing precipitating anti-polysaccharide antibodies [6, 20]. Since PEG-modified enzymes and allergens appear to have interesting clinical applications, it was considered of interest to study whether PEG-modified proteins could induce formation of antibodies against PEG.

Materials and Methods

Polyethylene Glycol

Preparations of PEG of $\overline{M}_W$ 300 to 5.9×10^6 and monomethoxy polyethylene glycol (MeOPEG) of $\overline{M}_W$ 3,000 and 10,000 were gifts from Union Carbide. Polyoxypropylene-polyoxyethylene glycol polymer (Pluronic F68) of $\overline{M}_W$ 8,750 is a commercially available preparation. This compound contained about 80% polyoxyethylene moiety.

Proteins Modified with PEG

Four MeOPEG-modified ovalbumin (OA) derivatives, OA $(MeOPEG)_n$, two MeOPEG-modified bovine superoxide dismutase (SOD) derivatives, SOD $(MeOPEG)_n$ and three MeOPEG-modified ragweed pollen extracts (Rag), Rag$(MeOPEG)_n$, were prepared by modifying OA, SOD and Rag, respectively, with MeOPEG monosuccinate using a mixed anhydride method [26]. Mainly the primary amino groups of the proteins were modified with

Table I. Chemical data of MeOPEG-modified proteins

Compound	Formula	$\overline{M}_w$ of MeOPEG	Protein %	PEG[1] %	Degree of modification	
					MeOPEG/OA	NH_2/OA[2]
Ia	OA (MeOPEG)$_n$	10,700	34.2	45	6	9
Ib	OA (MeOPEG)$_n$	11,500	28.5	62	9	13
Ic	OA (MeOPEG)$_n$	11,500	23.4	79	13	6
Id	OA (MeOPEG)$_n$	11,500	16.5	86	20	1
					MeOPEG/SOD	NH_2/SOD[2]
IIa	SOD (MeOPEG)$_n$	11,500	36.6	62	5	-
IIb	SOD (MeOPEG)$_n$	11,500	22.5	80	11	11
					MeOPEG groups % of lysine residues	NH_2 groups[2] % of lysine residues
IIIa	Rag (MeOPEG)$_n$	3,000	21.0	38	73	17
IIIb	Rag (MeOPEG)$_n$	3,000	18.3	55	127	13
IIIc	Rag (MeOPEG)$_n$	10,700	14.4	64	48	46

[1] That the protein + PEG contents do not give 100% reflects varying water content, and in compound Ia also contamination with NaCl and in compounds IIIa–c a high content of carbohydrates.
[2] Number of unsubstituted primary amino groups.

MeOPEG. The degree of modification was determined by two methods: (1) the content of MeOPEG was determined by NMR analysis; (2) the unmodified primary amino groups were measured with the *o*-phthalaldehyde method [24]. The protein and lysine content was determined by amino acid assay. Since Rag is a mixture of proteins of different molecular weights the degree of modification was calculated as number of MeOPEG groups in percent of number of lysine residues in the protein mixture. Values over 100% could be obtained since in addition to lysine both primary α-amino groups and phenol and mercapto groups in the proteins may have been modified with PEG. Similarly the number of the unsubstituted primary amino groups were calculated as number of NH_2 groups in percent of number of lysine residues in the protein mixture. The chemical data of the modified derivatives are given in table I.

Immunizations

Rabbits. New Zealand white rabbits in groups of 3–10 were immunized intramuscularly with protein, MeOPEG modified protein, or PEG of $\overline{M}_w$ 10,000 and 100,000. Rabbits received the test compounds in Freund's complete adjuvant (FCA). The protein and the PEG-modified protein were given so that each dose contained 100 μg protein per animal.

The PEG was given in doses of 300 μg/animal. The immunization course comprised 1–4 injections. When several injections were given, the interval was 4 weeks. In one series, compound Ib was given subcutaneously, dissolved in saline, in doses of 100 μg protein per animal; totally, three doses were administered.

Mice. NMRI mice in groups of 4–15 were immunized once, using a subcutaneous dose of a MeOPEG-modified OA (compound Ib) and PEG of MW 5.9×10^6. The following doses were used: 0.001, 0.1, 10, and 1,000 μg of PEG and 30 μg of compound Ib (corresponding to 20 μg PEG).

Passive Hemagglutination

Preparation of Activated MeOPEG Monosuccinate. MeOPEG monosuccinate $\overline{M}_w$ 6,000 (990 mg, 0.19 mmol) was dissolved in 18 ml of dry dioxane at 50–60°C. The solution was cooled to room temperature and thereafter 26.6 μl (0.19 mmol) of isobutyl chloroformate were added. The reaction solution was stirred for 30 min and then evaporated in vacuum to dryness. The residue was washed with 7×10 ml of petroleum ether and then dried in vacuum.

Sensitization of Homologous Erythrocytes. After washing twice in saline, 200 μl of packed red cells were suspended in 9.8 ml of a 0.2 *M* borate buffer of pH 8.0, containing 0.12 *M* NaCl. 10 mg of activated MeOPEG monosuccinate were added to the mixture, which was gently agitated and kept for 1 h at room temperature. Thereafter, the cells were spun down and washed three times with saline and were resuspended in 0.9% saline to a 2% suspension.

Procedure. Tests were performed in plastic microhemagglutination plates by mixing 50-μl volumes of serial twofold dilutions of test serum with 50-μl volumes of 2% sensitized red cells. Reading of settling patterns were made 2 h later at room temperature. Titers were defined as reciprocals of the highest serum dilution giving complete hemagglutination.

Inhibition Tests. Using rabbit anti-PEG sera, inhibition of passive hemagglutination was estimated by mixing 50-μl volumes of the inhibitor (PEG of $\overline{M}_w$ 4,000, 15,000 and 5.9×10^6) at concentrations between 0.001 and 100 μg/ml with 50-μl volumes of anti-

Table II. Precipitating antibodies to OA and PEG produced in rabbits repeatedly immunized with OA $(MeOPEG)_n$ derivatives Ib and Ic

Antigen concentration µg/12 ml gel		Immunizing antigens															
		compound Ib								compound Ic							
		ovalbumin				PEG 40,000				ovalbumin				PEG 40,000			
		35[1]	36	37	38	35	36	37	38	43	44	45	46	43	44	45	46
4 weeks[2]	320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	10	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	(+)	(+)	0	0	0	0	0	0	0	0	0	0	0	0
	2.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8 weeks	320	+	+	+	+	0	0	0	0	+	+	+	+	0	0	0	0
	160	+	+	+	+	0	0	0	0	+	+	+	+	0	0	0	0
	80	+	+	+	+	0	+	+	+	+	+	+	+	+	0	+	0
	40	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+
	20	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+
	10	(+)	(+)	(+)	(+)	(+)	+	+	+	0	0	0	0	+	+	+	+
	5	0	0	0	0	(+)	+	(+)	+	0	0	0	0	+	+	+	+
	2.5	0	0	0	0	0	(+)	0	0	0	0	0	0	(+)	(+)	(+)	(+)
	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12 weeks	320	+	+	+	+	0	0	0	0	+	+	+	+	0	0	0	0
	160	+	+	+	+	0	0	0	0	+	+	+	+	0	0	0	0
	80	+	+	+	+	0	0	0	0	+	+	+	+	+	0	0	(+)
	40	+	+	+	+	0	0	0	+	+	+	+	+	+	0	0	(+)
	20	+	+	+	+	0	+	+	+	+	+	+	+	+	0	+	(+)
	10	+	(+)	+	+	0	+	+	+	+	+	+	+	+	0	+	+
	5	0	0	0	0	+	+	+	+	0	0	0	0	+	(+)	+	+
	2.5	0	0	0	0	+	+	+	+	0	0	0	0	+	(+)	+	+
	1.3	0	0	0	0	0	(+)	(+)	(+)	0	0	0	0	0	(+)	(+)	0
	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16 weeks	320	+	+	+	+	0	0	0	0	+	+	+	+	0	0	0	0
	160	+	+	+	+	0	0	0	0	+	+	+	+	0	0	0	0
	80	+	+	+	+	0	0	0	+	+	+	+	+	0	0	0	0
	40	+	+	+	+	0	0	0	+	+	+	+	+	0	0	0	0
	20	+	+	+	+	(+)	+	+	+	+	+	+	+	0	0	(+)	(+)
	10	(+)	(+)	(+)	(+)	+	+	+	+	(+)	(+)	(+)	(+)	+	0	+	+
	5	0	0	0	0	+	+	+	+	0	0	0	0	+	0	+	+
	2.5	0	0	0	0	+	+	+	+	0	0	0	0	+	0	+	+
	1.3	0	0	0	0	0	(+)	+	+	0	0	0	0	(+)	0	0	0
	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Antibody determination was performed by RSRI.

[1] Rabbits No. 35–38 and 43–46.

[2] Sera have been tested 4, 8, 12 and 16 weeks after starting immunization.

serum (containing 8 agglutinating doses) and adding 50-μl aliquots of 2% sensitized red cells. Readings of settling patterns were made 2 and 24 h later and the lowest concentration of inhibitor-preventing agglutination was registered.

Gel Diffusion Techniques

Gel double diffusion [18], single radial immunodiffusion (SRI) [15] and reversed single radial immunodiffusion (RSRI) [25] were used with the following modifications. The gel medium contained 1% agarose, 1% dextran B 512 of $\overline{M}_w$ 60,000, and 0.1 *M* phosphate buffer, pH 8.0. Plastic Petri dishes were used for double diffusion and RSRI. In the latter technique, the antigen was incorporated into 12-ml gel medium. For hapten inhibition in RSRI, precipitates were produced by applying anti-PEG antiserum (rabbit 38; table II) to agarose gel, containing 0.4 μg/ml of PEG $\overline{M}_w$ 40,000. 48 h later, solutions of PEG $\overline{M}_w$ 300 or 2,000 in saline, in a volume of 10 ml/plate, were poured on top of the gel plates and left for 24 h at 4°C. Thereafter solutions were poured off and the plates were inspected for absence or presence of precipitates.

Passive Anaphylaxis

Guinea pigs were sensitized intravenously with 1 ml of rabbit antiserum to compound Ib, collected after the fourth immunizing injection. Challenge was made via an intravenous catheter introduced into an ear vein. Symptoms of anaphylaxis were registered during 30 min following challenge.

Results

No or Very Poor Immunogenicity of PEG

PEG of $\overline{M}_w$ 10,000 and 100,000 given repeatedly to rabbits intramuscularly in FCA in doses of 300 μg/animal did not elicit specific antibody formation. Reversed single radial immunodiffusion and passive hemagglutination were used for antibody estimation. Out of a groupe of 15 mice, given PEG of $\overline{M}_w$ 5.9×10^6 as a single subcutaneous dose of 10 μg, only 2 animals mounted a weak and transitory response at day 5 (titers 2 and 4) and 1 at day 15 (titer 2). A lower dose of 0.1 μg given to a group of 5 animals elicited a response in 3 mice at day 5 (titers 8–4–8) and in 2 at day 15 (titers 2 and 8). In the latter animals, the response persisted at 30 days. Doses of 0.001 and 1,000 μ did not induce any response detectable by passive hemagglutination.

Immunogenicity of PEG-Modified Proteins

Precipitating antibodies against PEG were induced in rabbits in four immunization series with OA $(MeOPEG)_n$, SOD $(MeOPEG)_n$, and Rag $(MeOPEG)_n$ in the presence of FCA. The proportion of animals producing anti-PEG antibodies varied. In a preliminary experiment with OA $(MeOPEG)_n$ derivative Ia, 2 of 4 animals produced precipitating anti-PEG antibodies already after one immunizing injection. In a subsequent larger series with three other OA $(MeOPEG)_n$ preparations, Ib and Ic (table II) and Id, all animals produced precipitating anti-PEG antibodies. The response was strong and persistent following compound Ib. Compound Ic induced a strong anti-PEG antibody response at 8 weeks after starting the immunization course with a gradual decline of antibody levels at 12 and 16 weeks. With compound Id, a transient and weak anti-PEG antibody response was seen only at 8 weeks after initiation of immunization. With regard to the production of anti-OA antibodies, a strong and persistent response with precipitating antibodies was induced by compounds Ib and Ic. No precipitating anti-OA antibodies were induced by immunization with compound Id.

In another experiment with SOD $(MeOPEG)_n$ derivatives IIa and b, only 2 of 6 animals produced precipitating anti-PEG antibodies after the third immunizing injection. No precipitating anti-SOD antibodies were elicited by compound IIb, and the anti-SOD response to compound IIa was markedly reduced in comparison to the response to SOD in control animals. In similar immunizations with Rag $(MeOPEG)_n$ derivatives IIIa–c, 2 of 12 animals showed a transient response with precipitating anti-PEG antibodies.

Altogether, 18 of 34 rabbits immunized with PEG-modified proteins in FCA produced precipitating anti-PEG antibodies. In a further series in which 4 rabbits were exposed to weaker immunogenic stimulation by three subcutaneous doses of compound Ib, OA $(MeOPEG)_n$, dissolved in saline only, no anti-PEG antibodies were detected. In a group of 10 mice

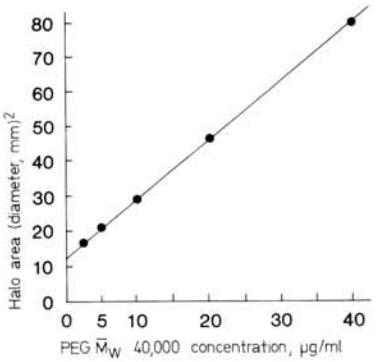

Fig. 1. Estimation of PEG by SRI. Linear regression of precipitate halo area on concentration of PEG ($\overline{M}_w$ 40,000) is obtained. 2 ml of anti-PEG antiserum (anti-Ia) was admixed to 8 ml 2% agarose gel. Measuring range: 2.5–40 μg PEG/ml.

Table III. Specific precipitate formation between anti-PEG antiserum[1] and fractions of PEG of varying $\overline{M}_w$ in Ouchterlony gel double diffusion

Molecular weight of PEG	Concentration of PEG, μg/ml											
	1,000	500	250	125	63	32	16	8	4	2	1	0.5
300	0	0	0	0	0	0	0	0	0	0	0	0
2,000	0	0	0	0	0	0	0	0	0	0	0	0
4,000	++	++	+++	+++	++	++	+	+	0	0	0	0
6,000	+++	+++	+++	+++	++	++	+	+	+	0	0	0
15,000	+++	+++	+++	++	++	+	+	+	+	0	0	0
40,000	+++	+++	++	++	++	+	+	+	+	+	+	0
6,000,000	++	++	++	++	++	+	+	+	+	0	0	0
Rag	0	0	0	0	0	0	0	0	0	0	0	0
Compound IIIa	+	+	+	+	+	+	+	+	+	(+)	0	0
Compound IIIb	+++	+++	+++	+++	+++	+++	++	+	+	+	(+)	(+)

[1] Antiserum from rabbit 38 taken after the 2nd immunizing injection with Ib.

Table IV. Specificity of rabbit anti-PEG antibodies, evidenced by inhibition of homologous passive hemagglutination

Inhibitor	Inhibitor, μg/ml						
	0 (NaCl)	0.001	0.01	0.1	1	10	100
PEG, $\overline{M}_w$ 4,000	0	0	0	0	0	0	+
PEG, $\overline{M}_w$ 15,000	0	0	0	0	±	+	+
PEG, $\overline{M}_w$ 5.9×10^6	0	0	0	0	+	+	+

Hyperimmune rabbit anti-PEG antiserum (rabbit 38; table II) and sensitized rabbit red cells were used. Titer of this antiserum by passive hemagglutination was 1,024.

0 = No inhibition, ± = partial inhibition, + = inhibition of agglutinate.

given a single subcutaneous dose of 10 μg of compound Ib, a transient and weak anti-PEG antibody response was demonstrable by passive hemagglutination. 5 days after immunization, 3 animals showed a humoral response, and at 14 days 6 animals had the following titers: 4–4–8–8–64–64. At 30 days the response had subsided.

Passive Anaphylaxis

4 guinea pigs were specifically sensitized with rabbit anti-PEG antiserum and 24 h later they were challenged intravenously with 0.5 mg of PEG of $\overline{M}_W$ 40,000. All animals rapidly developed typical anaphylactic shock with sneezing, dyspnea, loss of righting reflexes, asphyctic convulsions, and death from 3–5 min after challenge. 4 nonsensitized animals injected with the same dose of PEG did not show any adverse signs.

Specificity of Anti-PEG Antibodies

Evidence for specificity was obtained from (a) gel diffusion, (b) cross-reactivity and (c) passive hemagglutination studies.

(a) Very low concentrations of PEG of $\overline{M}_W$ 40,000 were capable of forming specific precipitates both in RSRI (final concentration 0.1–0.2 μg/ml; table II) and double diffusion (1 μg/ml; table III). Further evidence is the complete dissolution of precipitates between PEG of $\overline{M}_W$ 40,000 and anti-PEG antibodies by low molecular PEG preparations of $\overline{M}_w$ 300 and 2,000, illustrating the principle of hapten inhibition of precipitation. Unrelated OA anti-OA precipitates were not dissolved by application of solutions of low molecular weight PEG to gel plates.

(b) A polyoxypropylene-polyoxyethylene glycol block polymer of $\overline{M}_W$ 8,750 gave precipitate lines in gel double diffusion with anti-PEG antisera. These

lines fused completely with those produced by PEG of $\overline{M}_w$ 6,000.

(c) The new technique described for the coating of red blood cells with PEG for passive hemagglutination showed that sera of rabbits with precipitating anti-PEG antibodies gave high titers of up to 1,024, whereas pre-immunization sera gave titers of 0 or 2. Inhibition of passive hemagglutination by admixture of PEG to anti-PEG antiserum is another indicator of specificity. As seen from table IV the inhibition produced is $\overline{M}_W$ dependent. With PEG of MW 5.9×10^6 complete inhibition was achieved by a concentration of 1 μg/ml.

Influence of Antigen Size on Precipitate Formation

Using PEG of $\overline{M}_W$ between 300 and 5.9×10^6 in gel double diffusion, preparations with an $\overline{M}_W$ between 4,000 and 5.9×10^6 formed single precipitate lines with anti-PEG antiserum, raised against compound Ib, OA $(MeOPEG)_n$. In contrast, low molecular PEG of $\overline{M}_W$ 300 and 2,000 did not form precipitates over the concentration range tested (table III). PEG of $\overline{M}_W$ 40,000 proved the most efficient antigen, giving precipitates at concentrations of 1 μg/ml.

Analytical Applications of Anti-PEG Antibodies

PEG may be detected and semiquantitated in PEG-modified proteins by gel double diffusion (see bottom of table III). The presence of PEG and its approximate concentration in PEG-modified proteins can be estimated in lowest concentrations of about 1 μg/ml using antisera raised to a PEG-modified protein with a non-cross-reacting protein moiety. In SRI, a linear correlation was obtained between area of precipitate halo and concentration of PEG of $\overline{M}_W$ 40,000. The measuring range was from 2.5 to 40 μg/ml of PEG $\overline{M}_W$ 40,000.

Discussion

Our studies show that PEG of $\overline{M}_W$ 2,000–10,000 acquires immunogenic properties by covalent coupling to protein. However, the immunogenicity of the PEG moiety varied, depending on the nature of the protein. The anti-PEG antibody response induced in the large majority of rabbits immunized with PEG-modified OA and the much weaker anti-PEG response elicited in this species by PEG-modified SOD and Rag may be reflections of the varying immunogenicity of the carriers used for preparing the conjugates [for reviews on immunogenicity see references 7 and 22].

Further, the degree of modification was a crucial factor in determining the capacity of a PEG-protein conjugate to elicit an anti-PEG response. Modification of OA with 6 PEG of $\overline{M}_W$ 11,000 resulted in production of both precipitating anti-PEG and anti-OA antibodies, whereas introduction of 20 PEG conferred predominantly nonimmunogenic properties to the conjugate both with respect to the anti-OA and anti-PEG responses. Similar findings have been reported with various other carrier molecules and haptens, where an increasing degree of substitution resulted first in an increase and then in a decrease of immunogenicity [4, 14].

Our results confirm the reported marked decrease of immunogenicity of the protein moiety of PEG-modified proteins [1, 2]. PEG is a linear polymer composed of repetitive units of ethylene oxide. In the literature, apparently no data on its immunogenicity are reported and PEG is assumed to be nonimmunogenic. Our experiments show that PEG of $\overline{M}_W$ 10,000 and 100,000 given repeatedly to rabbits in FCA did not induce specific antibodies and that PEG of $\overline{M}_W$ 5.9×10^6 is not or very poorly immunogenic in mice. From its immunobiological properties PEG therefore may be defined as a polyvalent hapten. Though high molecular weight often favors immunogenicity, this was not the case with PEG in the animal species and under the conditions tested here. Among macromolecules, which in spite of large molecular size have been found to be nonimmunogenic, are homopolymers of α-amino acids [16]. Other high MW synthetic polymers found to be poorly immunogenic include polyvinylpyrrolidone, polyvinylamine, and polymethacrylic acid [5].

Our finding that PEG acquired immunogenic properties by covalent coupling to protein is in accord with data on other polymers. For example, antibodies to the nonimmunogenic homopolymers of α-amino acids may be obtained by using the latter as haptens. This was achieved by covalent attachment of the homopolymer to protein [21]. *Goebel and Avery* [6] first used this principle for pneumococcal type III polysaccharide and comparable results have been obtained for hydroxyethyl starch [20] and other polysaccharides. Whereas hydroxyethyl starch was found nonimmunogenic in rabbits and man, pneumococcal

polysaccharide is a poor immunogen in rabbits. Both polymers acquire immunogenicity and induce precipitating anti-polysaccharide antibodies when coupled to protein. This change of immunologic properties is considered to reflect the partial or complete transformation of a thymus-independent antigen or a polyvalent hapten into a thymus-dependent antigen [for review see 19].

The chain-like structure of the PEG molecule, consisting of repetitive units of $-CH_2CH_2O-$, and the finding of hapten inhibition of precipitation in the PEG anti-PEG system by PEG of $\overline{M}_W$ 300, indicate that the antigen combining site of anti-PEG antibodies is complementary to 6–7 repeat units. Sequences of this size on the PEG molecule would therefore correspond to the size of an antigenic determinant. Comparable findings have been obtained, e.g. in the dextran anti-dextran system [9].

The fact that high molecular PEG produces specific precipitation at very low concentrations shows it to be an effective antigen. As with other linear polymers this is probably due to the large number of sterically accessible determinants. It is of interest in this connection that the unspecific precipitation of proteins in the presence of PEG, utilized as 'enhancing effect of PEG on immunochemical reactions' [8], requires PEG concentrations in the range of 2–6%. This range is several hundred times higher than that required for specific precipitate formation.

The availability of precipitating anti-PEG antibodies allows to estimate PEG by a variety of immunochemical methods, exemplified in this study by single radial immunodiffusion. Our method offers an alternative to existing chemical methods [23] and may have the advantage of greater specificity and sensitivity.

What is the significance of our experimental findings for the potential use of PEG-modified allergens and enzymes in humans? For clinical purposes PEG-modified proteins are administered in milligram doses subcutaneously. Under comparable conditions in rabbits, we did not obtain any demonstrable anti-PEG antibody response even with our most active anti-PEG antibody-inducing immunogen (compound Ib) and only a weak response was seen in mice. However, the possibility that PEG-modified proteins may induce an anti-PEG antibody response also in man should be kept in mind. The results of clinical trials will determine the usefulness of PEG-modified allergens and enzymes in humans.

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Correspondence to: Dr.A.W.Richter,
Department of Biomedical Research,
Pharmacia AB, Box 181,
751 04 Uppsala (Sweden)