# IMMUNOLOGICAL PROPERTIES OF CONJUGATES OF RAGWEED POLLEN ANTIGEN E WITH METHOXYPOLYETHYLENE GLYCOL OR A COPOLYMER OF D-GLUTAMIC ACID AND D-LYSINE*

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Chemically modified allergens may be useful immunotherapeutic reagents in man. What is required of such reagents is that the modified allergens show reduced allergenic activities yet retain the immunosuppressive properties of the native molecule. It is with this objective that we have prepared conjugates of antigen E with two polymers of large molecular weights. Antigen E is the major allergen of ragweed pollen and it is an acidic protein of ≈38,000 daltons (1). The two polymers are methoxypolyethylene glycol (MPEG)[1] of ≈5,000 daltons and a copolymer of D-glutamic acid and D-lysine (DGL) of ≈34,000 daltons. MPEG was chosen because MPEG protein conjugates are known to have reduced allergenic and immunogenic activities (2, 3). DGL was chosen because DGL conjugates with haptens of low molecular weight are known to possess hapten-specific immunosuppressive properties (4, 5). To study the influence of the bulky MPEG groups on the immunochemical properties of the conjugate, we also prepared a conjugate using methanol in place of MPEG.

In this paper we report comparative studies on the immunological properties of antigen E and its conjugates in the mouse as the experimental animal. The mouse system was chosen since it is generally accepted that the IgE response in this system is the best model for reaginic antibody formation in man (6). MPEG and methoxy antigen E conjugates were prepared by coupling the protein with cyanuric chloride activated MPEG (2) or methanol (7) as shown in Fig. 1. DGL antigen E conjugate was prepared via an intermolecular disulfide bond formation (8). As shown in Fig. 2, antigen E and DGL were first modified to contain reactive thiol and 4-dithiopyridyl groups, then they were coupled via these groups.

## Materials and Methods

*Materials.* Antigen E was isolated from ragweed pollen as described (9); its isoelectric forms B and C were used interchangeably in this study. MPEG of average mol wt of 5,000 was a gift of Union Carbide Corp., New York. A random copolymer of DGL in a molar ratio of 6:4, having an average mol wt of 34,000, was a gift from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.

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[1] *Abbreviations used in this paper:* BPO, benzyl-penicilloyl; DGL, D-glutamic acid and D lysine; HA, passive agglutination; MPEG, methoxypolyethylene glycol; PCA, passive cutaneous anaphylaxis, PEG, polyethylene glycol.

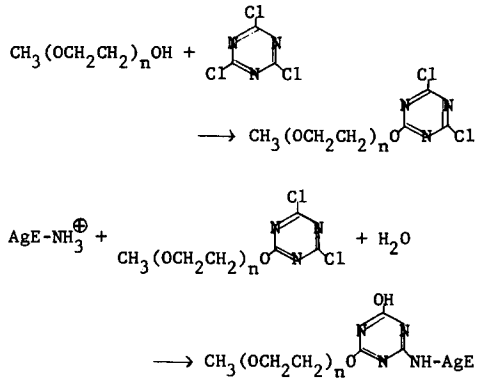

FIG. 1. Scheme for preparation of methoxypolyethylene glycol or methoxy conjugate of antigen E. For the sample of MPEG of 5,000 daltons used, n is equal to 114. For the methoxy conjugate n is zero. Antigen E has a mol wt of ≈38,000 daltons. The conjugates prepared contained about 6 MPEG or methoxy groups per mole of antigen E.

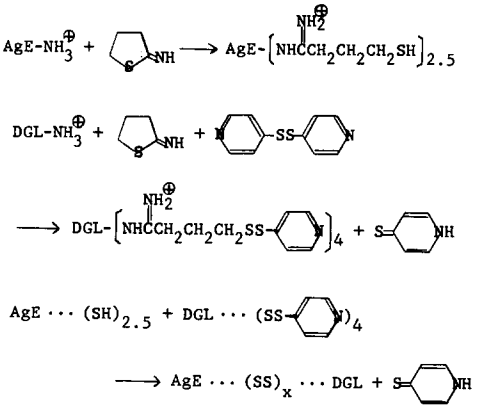

FIG. 2. Scheme for preparation of conjugate of antigen E with a copolymer of DGL of 34,000 daltons. The conjugate prepared is believed to have the composition of $[DGL_{1.2}\text{-}AgE_1]_n$ when n is in the range of 1–3. The number x of intermolecular disulfide bonds in the conjugate was not determined.

*MPEG-Antigen E Conjugate.* Two samples, a and b, of this conjugate were prepared with MPEG activated with cyanuric chloride under different conditions. One condition used was a modification of that reported (2), namely, the reaction medium benzene contained 1.3% water. Microanalysis showed the activated MPEG to contain 0.58% chlorine. MPEG-antigen E conjugate (sample a) was prepared by coupling antigen E (0.1 mM, 3.8 mg per ml) and activated MPEG (20 mM, 100 mg per ml) in 25 mM sodium borax buffer (pH 9; 20 ml) for 18 h at room temperature (r.t.). The conjugate was isolated by chromatography on a column (195 x 2.6 cm) of Sephacryl (Pharmacia Fine Chemicals Inc., Piscataway, N. J.), which was eluted with 0.1 M $NH_4HCO_3$ at a flow rate of ≈34 ml per hour. The conjugate was eluted near the column void volume (380–490 ml) and the unreacted MPEG and the antigen E were eluted together as a broad peak with its maximum at ≈650 ml. Amino acid analysis of the conjugate after acid hydrolysis (10) showed that its antigen E content was 55 mg (72% yield). Its lysine content was 12.3 ± 0.7 residues per mole as compared to that of 18 residues per mole for native antigen (11). The lysine content suggests a minimum of six MPEG groups per mole of conjugate because N,ε-(2 hydroxy-4-MPEG-6-triazinyl)-lysine is partially converted back to lysine during acid hydrolysis (3). On electrophoresis of the conjugate in SDS-4% polyacrylamide gel (12, 13), two broad bands were observed. A minor band was located near the origin of the gel and its

mobility would correspond to a molecule of a size > that of bovine plasma albumin decamer (670,000 daltons). A major band migrated as a broad zone in the mobility range of albumin dimer to pentamer, but the majority was in the range of trimer and tetramer (201,000–268,000 daltons).

Sample b of MPEG-antigen E conjugate was obtained by using MPEG activated by a different method. MPEG (10 g, 2 mmol) and cyanuric chloride (8.6 g, 46 mmol) were allowed to react in a mixture of anhydrous benzene (300 ml) and sodium hydride (0.29 g, 12 mmol) for 18 h at r.t. Microanalysis of the activated MPEG showed its chlorine content to be 0.29% (calculated for $C_{232}H_{459}O_{114}N_3Cl_2$:Cl, 1.37%). Sample b conjugate prepared with MPEG activated by the sodium hydride method contained 14 residues of lysine per mole and it was eluted slightly later from Sephacryl column than the sample a conjugate described above. On SDS gel electrophoresis, this conjugate migrated as a broad zone in the mobility region of albumin monomer to tetramer, but the majority was in the dimer to trimer region.

*2-Hydroxy-4-Methoxy-6-Triazinyl-Antigen E.* The conjugate was prepared on treatment of antigen E (0.12 mM) with 2,4-dichloro-6-methoxy-triazine (12 mM) (7) in sodium borax buffer (25 mM, pH 9) containing 2.5% dimethoxyethane at r.t. for 18 h. One of the two chloro groups of the triazine compound reacted with the amino groups of antigen E, while the other was hydrolyzed during the reaction, as indicated by the consumption of NaOH to keep pH constant. The conjugate was isolated by chromatography of reaction mxiture (3 ml) on a column (200 × 0.9 cm) of Sephadex G-100, which was eluted with 0.1 M $NH_4HCO_3$. The conjugate was obtained in polymeric and monomeric forms having a similar lysine content of 13.2 ± 1.2 residues per mole. The polymeric conjugate was eluted at the void volume of the column and the monomeric conjugate eluted at the same position as that of native antigen E in yields of 45 and 40%, respectively. On electrophoresis in 7.5% polyacrylamide gel in Tris-glycine buffer (14), the monomeric conjugate migrated as a broad diffuse zone having average mobility about twice that of native antigen E. Only the monomeric conjugate was studied for its immunological properties.

*DGL-Antigen E Conjugate.* DGL was converted to 4-(4′-dithiopyridyl)-butyrimidinyl-DGL as described (8) and the derivative contained about four reactive mixed disulfide groups per mole of DGL. Antigen E (0.18 mM) was converted into its 4-mercaptobutyrimidinyl derivative by reaction with 2-iminothiolane (190 mM) in sodium borax buffer (25 mM; pH 9.0) at r.t. for 2 h; the amidinated antigen E (80% yield) contained about 2.5 sulfhydryl groups per mole on titration with 4,4′-dithiodipyridine (8).

DGL-antigen E conjugate was prepared by reaction of 4-mercaptobutyrimidinyl antigen E (63 μM) with 4-(4′-dithiopyridyl)-butyrimidinyl DGL (124 μM) in sodium phosphate buffer (0.1 M; pH 7.2) containing EDTA (1 mM). After 18 h at r.t., the reaction mixture (3 ml) was passed through a 200 x 0.9 cm column of Sephadex G-100 which was eluted with the same phosphate buffer. The conjugate (75% yield) was eluted at the void volume of the column (50 ml), while unreacted antigen E was eluted at ≈82 ml vol. Chromatography on CL-6B Sepharose showed the conjugate to be free of unreacted DGL. The conjugate was found to contain antigen E and DGL in a molar ratio of about 1:1.2 on amino acid analysis. On electrophoresis in SDS-4% polyacrylamide gel, the conjugate migrated as a very broad zone extending from the origin of the gel to the position of monomeric bovine plasma albumin.

*Methods.* Immunogenicity or immunosuppression studies were made in (BALB/c × A/J)$F_1$ hybrid mice which are known to be high responders to ragweed antigens (16). Mice were immunized intraperitoneally with 1 μg antigen E and 1 mg alum (17) in 0.2 ml of 0.15 N NaCl containing 5 mM sodium phosphate (pH 6.6). For immunosuppression studies, mice were treated subcutaneously with antigen E or its conjugate in 0.2–0.4 ml of 0.15 NaCl containing 5 mM phosphate (pH 7.2); the amounts used and the times of treatment are given in Tables I and II. Mice were bled weekly or biweekly and the collected sera were usually assayed immediately or stored frozen until assayed.

For antibody assay, twofold serial dilutions of the sera with phosphate-buffered saline (pH 7.2) containing 0.05% bovine plasma albumin were made. For antigen E-specific IgG antibodies, assays were made with passive agglutination (HA) of antigen E coated erythrocytes (15). To determine the contribution of specific IgM antibodies, agglutination assays were also made with sera dilutions which had been treated with 0.1 M mercaptoethanol at r.t. for 1 h (18). The contribution of IgM antibodies was found to be significant only in the first 2 wk after primary immunization.

TABLE I

*IgE Antibody Levels of AgE Sensitized Mice on Treatment with AgE or its Conjugates*‡*

| Group | | | Time of PCA titer | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 28 |
| | *μg* | *days* | *wk* | | | | | | | | | | | |
| Treated with AgE: | | | | | | | | | | | | | | |
| A | 0 | | 640 | 320 | 760 | 640 | 1,280 | 1,520 | 640 | 900 | 900 | 1,280 | 1,280 | 320 |
| B | 20 | 3 and 4 | 40 | 40 | 900 | 320 | 1,280 | 640 | | | | | | |
| C | 200 | 3 and 4 | 10 | <5 | 230 | 80 | 230 | 160 | | | | | | |
| D | 140 | 21 and 23 | 640 | 270 | 160 | 230 | 320 | 160 | 160 | | 130 | | 320 | 160 |
| E | 200 | 21 | 450 | 160 | 160 | 80 | 320 | 230 | | | | | | |
| F | 1,400 | 21 and 23 | | 80 | 80 | 80 | 130 | 80 | 80 | | 40 | | 200 | 80 |
| G | 140 | 49 and 51 | | | | 1,280 | 1,280 | 320 | 640 | | 640 | 640 | 640 | 160 |
| H | 1,400 | 49 and 51 | | | | 450 | 640 | 640 | 320 | 450 | 320 | 320 | 640 | 80 |
| Treated with MPEG-AgE: | | | | | | | | | | | | | | |
| I | 20 | 3 and 4 | <5 | <5 | 60 | 30 | 640 | 320 | 110 | 80 | | | | |
| J | 40 | 3 and 4 | <5 | <5 | <5 | <5 | 110 | 80 | 80 | 40 | | | | |
| K | 140 | 4 and 6 | <5 | <5 | 20 | 10 | 110 | 80 | 40 | 160 | 160 | 160 | 160 | 80 |
| L | 200 | 3 and 4 | <5 | <5 | 40 | 10 | 80 | 80 | 80 | 80 | | | | |
| M | 140 | 21 and 23 | 640 | 110 | 20 | 40 | 230 | 80 | 40 | 110 | 90 | 320 | 160 | 80 |
| N | 200 | 21 | | 320 | 110 | 160 | 160 | 320 | 160 | 320 | | | | |
| O | 1,400 | 21 and 23 | | 110 | 40 | 40 | 160 | 160 | 60 | 40 | 70 | 320 | 160 | 80 |
| P | 140 | 49 and 51 | | | | 1,500 | 1,280 | 1,280 | 900 | 900 | 1,280 | 640 | 640 | 160 |
| Q | 1,400 | 49 and 51 | | | | 760 | 320 | 640 | 450 | 450 | 450 | 640 | 320 | 80 |
| Treated with DGL-AgE: | | | | | | | | | | | | | | |
| R | 100 | 4 and 6 | <5 | 20 | 40 | 40 | 230 | 320 | 80 | 160 | 230 | 640 | 230 | 160 |
| S | 100 | 21 and 23 | 450 | 320 | 80 | 80 | 450 | 640 | 320 | 320 | 320 | 320 | 320 | 160 |
| T | 1,000 | 21 and 23 | 640 | 80 | 40 | 80 | 230 | 160 | 40 | 110 | 110 | 320 | 230 | 80 |

* Mice (BALB/c × A/J)$F_1$ hybrids were immunized intraperitoneally with 1 μg antigen E and 0.2 mg alum each time at weeks 0, 4, 8, and 18. The amounts of conjugate used for treatment refer only to the protein portion of the molecule; the doses were given subcutaneously.

‡ Antigen E-specific IgE antibody titers were determined by PCA reactions as described in Materials and Methods. The majority (80%) of reported titers are geometric means of duplicate or triplicate analyses, and the titers seldom varied by more than one dilution on repeat assays.

TABLE II

*IgG Antibody Levels of AgE Sensitized Mice on Treatment with AgE or its Conjugates*‡*

| Group | | | Time of HA titer | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 28 |
| | *μg* | *days* | *wk* | | | | | | | | | | | |
| Treated with AgE: | | | | | | | | | | | | | | |
| A | 0 | | 40 | 250 | 6,400 | 5,100 | 11,000 | 8,910 | 4,500 | 6,400 | 6,400 | 6,400 | 3,700 | 2,600 |
| B | 20 | 3 and 4 | 30 | 110 | 2,500 | 2,300 | 4,500 | 3,200 | | | | | | |
| C | 200 | 3 and 4 | 40 | 160 | 3,200 | 2,500 | 1,600 | 1,600 | | | | | | |
| D | 140 | 21 and 23 | 40 | 6,400 | 6,400 | 6,400 | 6,400 | 3,200 | 3,200 | | | 930 | | 1,600 |
| E | 200 | 21 | 40 | 1,300 | 1,600 | 3,200 | 3,200 | 1,600 | | | | | | |
| F | 1,400 | 21 and 23 | | 2,900 | 5,080 | 4,500 | 4,500 | 3,200 | 1,600 | | | 1,600 | | 1,000 |
| G | 140 | 49 and 51 | | | | 12,800 | 12,800 | 9,000 | 5,700 | 3,200 | 3,200 | 3,200 | 3,200 | 3,200 |
| H | 1,400 | 49 and 51 | | | | 16,000 | 9,000 | 9,000 | 6,400 | 3,200 | 3,200 | 3,200 | 3,200 | 1,600 |
| Treated with MPEG-AgE: | | | | | | | | | | | | | | |
| I | 20 | 3 and 4 | 25 | 25 | 2,500 | 2,300 | 1,300 | 3,200 | 800 | 1,600 | | | | |
| J | 40 | 3 and 4 | 5 | 25 | 2,500 | 3,200 | 1,100 | 1,600 | 800 | 1,600 | | | | |
| K | 140 | 4 and 6 | 25 | 160 | 3,600 | 1,600 | 7,000 | 3,400 | 1,600 | 3,200 | 1,600 | 3,200 | 1,900 | 2,300 |
| L | 200 | 3 and 4 | 10 | 10 | 1,100 | 800 | 400 | 800 | 800 | 800 | | | | |
| M | 140 | 21 and 23 | 40 | | 5,100 | 3,200 | 3,200 | 5,100 | 3,200 | 1,100 | 1,600 | 1,600 | 1,600 | 1,600 |
| N | 200 | 21 | | | 5,400 | 5,400 | 3,200 | 1,600 | 1,600 | 1,600 | | | | |
| O | 1,400 | 21 and 23 | | 1,000 | 940 | 800 | 560 | 800 | 400 | 250 | 250 | 800 | 1,100 | 1,100 |
| P | 140 | 49 and 51 | | | | 6,400 | 12,800 | 10,100 | 5,100 | 6,400 | 6,400 | 3,200 | 3,200 | 1,600 |
| Q | 1,400 | 49 and 51 | | | | 8,100 | 17,400 | 10,000 | 10,100 | 6,400 | 6,400 | 3,200 | 2,300 | 3,200 |
| Treated with DGL-AgE: | | | | | | | | | | | | | | |
| R | 100 | 4 and 6 | 50 | 800 | 6,400 | 3,200 | 4,700 | 3,200 | 2,000 | 1,600 | 3,200 | 3,200 | 940 | 940 |
| S | 100 | 21 and 13 | 40 | | 6,400 | 6,400 | 7,100 | 9,000 | 4,000 | 3,200 | 800 | 1,610 | 2,300 | 1,610 |
| T | 1,000 | 21 and 23 | 80 | | 6,400 | 2,500 | 4,700 | 4,500 | 2,000 | 1,600 | 3,200 | 3,200 | 2,300 | 1,100 |

* Same as *, Table I.

‡ Antigen E-specific IgG antibodies were determined by agglutination tests as described in Materials and Methods. The majority of reported titers are geometric means of duplicate or triplicate analyses, and the titers seldom varied by greater than one dilution on repeat assays.

For antigen E-specific IgE antibodies, assays were made using passive cutaneous anaphylaxis (PCA) in retired male breeder rats (19, 20). Twofold serial dilutions of mouse sera (0.10 ml per site) were injected intradermally into separate sites on the shaved dorsal skin of the rat. After a 4–16-h incubation period, the rat was challenged intravenously with 1.5 ml of phosphate-buffered saline containing antigen E (0.4 mg) and Evans blue (7.5 mg). The IgE antibody content was recorded as the reciprocal of the highest serum dilution giving a 5 mm bluing reaction 15 min after antigen reaction.

Under the conditions used, an agglutination titer of 100 is estimated to be equivalent to ≈10 μg per ml of IgG antibody (15), and a PCA titer of 100 is equivalent to ≈10 ng per ml of IgE antibody (21).

Allergenic activity of conjugates was studied as follows. Separate skin sites of a shaved rat were sensitized with 0.10-ml aliquots of 1/50 diluted mouse anti-antigen E sera, which was found to have a PCA titer of 640. 4 h later the rat was given 1.5 ml of Evans blue solution intravenously. The sensitized sites were then challenged locally with serial threefold dilutions of test antigen in a 0.05-ml vol. The lowest antigen dilution which gave a 5-mm bluing reaction 5 min after injection is taken as the end-point. Antigenic activity of conjugates was studied by inhibition of agglutination of antigen E-coated erythrocytes with specific rabbit antisera (3, 15).

## Results

The conjugates inhibited the agglutination of antigen E-coated erythrocytes with antigen E-specific rabbit antibodies. Under the conditions used, the concentrations required for inhibition were 0.25 μg per ml for antigen E, 25 and 2.5 μg per ml for samples a and b of MPEG-antigen E, 0.5 μg per ml for methoxy-antigen E, and 2.5 μg per ml for DGL-antigen E. Therefore, the antigenic activities of MPEG, methoxy, and DGL conjugates are, respecitvely, 10–100, 2 and 10-fold less active than antigen E. Samples a and b of MPEG-antigen E differ chemically in the number of MPEG groups introduced.

The conjugates also showed reduced activities when tested by heterologous PCA on rats sensitized with antigen E-specific mouse IgE antibodies (≈0.12 ng per site). Under the conditions used, PCA reactions were observed when the sensitized sites were challenged with 0.05 ml of 0.5 ng per ml of antigen E, 400 and 40 ng per ml of samples a and b of MPEG-antigen E, 0.5 ng per ml of methoxy-antigen E or 14 ng per ml of DGL-antigen E. On a relative basis, the allergenic activities of MPEG and DGL conjugates are, respectively, about 80–800 and nine times less active than antigen E, while the methoxy conjugate is equally as active as antigen E.

When mice, which are high responders to ragweed antigens (16), were immunized with antigen E (1 μg) with alum as adjuvant, high titers of antigen E-specific IgE and IgG antibodies were readily obtained by weeks 2 and 4, respectively, (group A in Tables I and II). Under identical conditions, mice immunized with MPEG-antigen E conjugate (1 μg of antigen E equivalent) gave no detectable antibody response. Also, the MPEG-antigen E conjugate did not prime the mice for a secondary response when they were challenged with antigen E and alum by week 8.

Comparative studies were made on the effectiveness of antigen E and its MPEG or DGL conjugates to suppress antigen E specific antibody responses in mice. Groups of three mice were primed intraperitoneally with antigen E (1 μg) and alum at week 0 and boosted at weeks 4, 8, and 18. They were treated subcutaneously with varying amounts of antigen E or conjugates (20–700 μg of antigen E equivalent), either as a single dose or two equal doses at different time periods. In Tables I and II are given, respectively, the antigen E-specific IgE and IgG antibody levels of the control and treated groups at biweekly intervals.

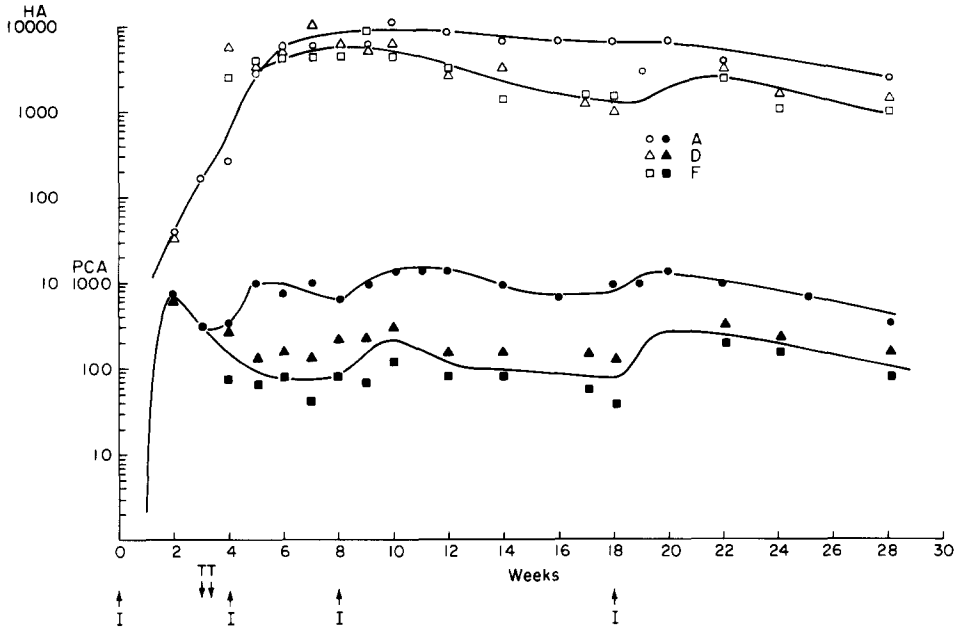


FIG. 3. Immunosuppression of antigen E sensitized mice on treatment with antigen E. Mice were sensitized intraperitoneally on day 0 with antigen E (0.026 nmol) and alum, then treated subcutaneously on days 21 and 24 with antigen E, a total of 3.6 and 36 nmol, respectively, for groups D and F. Group A is the untreated control group. All groups were rechallenged with antigen E on weeks 4, 8, and 18. HA and PCA denote titers of antigen E-specific IgG and IgE antibodies, respectively.

The data for control group A, groups D and F treated with 140 and 1,400 μg of antigen E, and groups M and O treated with 140 and 1,400 μg of MPEG-antigen E, are also shown in Figs. 3 and 4. The treated groups were given the immunosuppressive agent during week 3 when the mice had high levels of IgE antibodies but low levels of IgG antibodies from their primary response. After boosting at week 4, the treated groups did not show a secondary response of IgE antibodies, but the control group did. After further boostings at weeks 8 and 18, the treated groups did show secondary responses of IgE antibodies. Before boosting at weeks 8 and 18, the IgE antibody levels of treated groups were ≈one tenth of that of the control group. At the peak of secondary responses at weeks 10 and 20 the IgE antibody levels of treated groups were ≈one fifth of that of the control group. At week 28, the IgE antibody levels of treated groups were still ≈one fourth of that of the control group.

In addition to changes in IgE antibody levels, the treated groups also showed reduced IgG antibody levels when compared to the control group (Figs. 3 and 4). It is evident from the figures that both the kinetics and the magnitude of IgG antibody response in mice are different from those of the IgE antibody response. The control and the treated groups did not show any secondary IgG antibody responses after boosting at weeks 4 and 8. The treated groups did show a secondary IgG antibody response after the third boost at week 18, but the control group did not. Groups D and F, treated, respectively, with 140 and 1,400 μg of antigen E, showed similar degrees of suppression of IgG antibody response, their levels at weeks 18 and 22 being ≈one fourth and one fifth, respectively, of that of the control group. Group M, treated

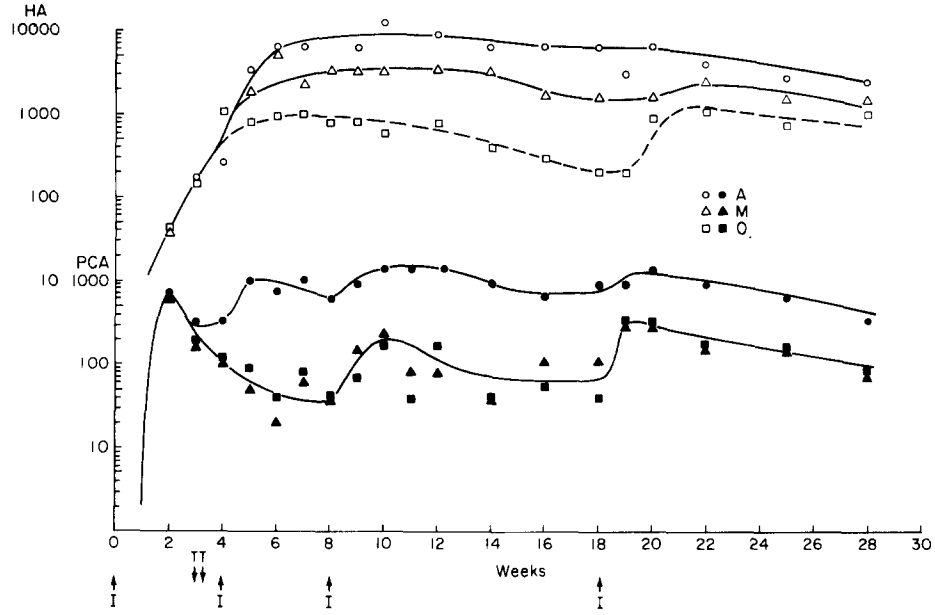


FIG. 4. Immunosuppression of antigen E sensitized mice upon treatment with MPEG antigen E. Group A is the same control group as in Fig. 3. Groups M and O were treated twice with a total of 3.6 and 36 nmol of conjugate, respectively. All other conditions are the same as those shown in Fig. 3.

with 140 μg of MPEG-antigen E conjugate, showed similar suppression to those of groups D and F. But group O, treated with 1,400 μg of the conjugate, showed a stronger suppression, its IgG antibody levels at weeks 18 and 22 being ≈one thirtieth and one fifth, respectively, of that of the control group.

Treatment of mice with antigen E or the conjugates was also done within 1 wk after priming with antigen E at a time when the mice had no detectable levels of IgE or IgG antibodies. Under these conditions, MPEG-antigen E was found to be a more effective immunosuppressive agent than antigen E. This conclusion follows from the data in Tables I and II for groups B and I which had been treated with 20 μg of antigen E or its MPEG conjugate, respectively. At week 4 the IgE antibody levels of groups B and I were, respectively, one eighth and <one sixtieth of that of the control group A. At week 4 the IgG antibody levels of groups B and I were, respectively, ≈one fifth and one tenth of that of the control group A.

The data in Tables I and II also indicate the dose dependent nature of immunosuppression. This is seen by comparing the data for groups B and C treated with 20 and 200 μg of antigen E, and for groups I, J, and L treated with 20, 40, and 200 μg of MPEG-antigen E. The data would indicate that 20 μg of tolerogen per mouse is about the minimal amount required to attain near maximal suppression of IgE and IgG antibody responses.

Treatment of mice with antigen E or the conjugates was also done 7 wk after priming with antigen when the mice had high levels of both IgE and IgG antibodies. Under these conditions weak suppression of IgE and IgG antibodies was observed, probably at best half the levels of the control group. This is seen by comparing the data in Tables I and II for groups G and P, which were treated with 140 μg of antigen

E or its MPEG conjugate, respectively, or for groups H and Q which received 1,400 μg of the immunosuppressive agent, with those of control group A.

For the data given in the Tables, groups I, J, L, and N were treated with sample a of MPEG-antigen E, while groups K, M, O, P, and Q were treated with sample b of this conjugate. Groups K and L were treated under nearly identical conditions with samples a and b of MPEG-antigen E, which is also the case for groups M and N. Very similar degrees of suppression were obtained with these two samples of MPEG-antigen E.

DGL-antigen E appears to be slightly less effective in immunosuppression than MPEG-antigen E, as seen when comparing the data in Tables I and II. When both conjugates were administered under similar conditions, groups K, M, and O treated with MPEG-antigen E showed slightly stronger immunosuppression than groups R, S, and T treated with DGL-antigen E, and the difference is probably twofold.

## Discussion

As reported above, treatment of antigen E primed mice with antigen E, its MPEG or DGL conjugates is effective in suppressing antigen E-specific IgE and IgG antibody responses. The suppression is long lasting, but it is weakened after two or more challenges with the immunogen. The degree of suppression attained is dependent on the immune state of mice at the time of treatment, the particular immunosuppressive agent and the dose. The best suppression of antibody response was observed with mice which had no detectable antibodies at that time of treatment, while the weakest suppression was observed with mice which had high levels of antibodies at time of treatment. The data show that the IgE antibody response is more readily suppressed on treatment with antigen E or its conjugates than IgG antibody response. Under favorable conditions, a dose of treatment agent, which is equivalent to 20 times the immunizing dose, is sufficient to give observable suppression of both IgE and IgG antibodies. The data suggest that MPEG-antigen E is more effective as an immunosuppressive agent than antigen E, and that DGL-antigen E is probably less effective than MPEG-antigen E.

MPEG- or DGL-antigen E conjugates are not only more effective than antigen E as an immunosuppressive agent, but they are also less allergenic and less antigenic than antigen E. These changes in the allergenic and antigenic activities of the conjugates indicate a reduction in their antigenic valency. The allergenic activity of an antigen depends on the interaction of multivalent antigen with cell-bound IgE antibodies leading to the release of chemical mediators of immediate hypersensitivity, and univalent fragments are inactive in this regard (22).

MPEG-antigen E is apparently nonimmunogenic in mice, nor does it prime the animals for a secondary response to antigen E. The apparent loss of immunogenicity of MPEG-antigen E indicates a reduction in the antigenic valency as well as in the antigenic specificity. Immunogenicity of an antigen for IgE and IgG antibody responses is known to require collaboration of T and B cells, and T and B cells may have specificities for different antigenic determinants (23–25).

The reduction in antigenic valency and specificity of the MPEG conjugate is probably a consequence of steric hindrance by the large polymer groups which reduce the accessibility of antigenic determinants of antigen E. Alternatively, this reduction

may be a consequence of conformational changes of antigen E in the conjugate, because the B-cell-specific antigenic determinants of antigen E are conformation dependent (26). However, conformational changes of MPEG-antigen E, if any are present, are unlikely to play an important role, because its chemically analogous methoxy conjugate has essentially the same antigenic or allergenic activity as the native antigen.

The large polymer groups can also reduce the susceptibility of the conjugates to proteolysis, because the polymer groups reduce the accessibility of susceptible peptide bonds. For example, MPEG conjugates of asparaginase show markedly increased resistance to tryptic digestion when compared to the native enzyme (27). Therefore, the antigen E conjugates may be processed differently from the native antigen by macrophages. Since macrophage processing of antigens is known to be of importance in immune responses (28), this possible difference of antigen E and its conjugates may be important in their different immunosuppressing activities.

It is of interest to compare the suppression of IgE antibody induced by DGL-antigen E with that induced by the benzylpenicilloyl DGL conjugate (BPO-DGL) (5). Mice, immunized with BPO coupled keyhole limpet hemocyanin and alum at weeks 0 and 3, were treated subcutaneously with DGL-BPO (20 nmol of conjugate equivalent to 800 nmol of BPO groups) at week 2. Compared to the untreated control group there was a greater than 32-fold suppression of anti-BPO IgE antibody at week 8. In Table I, group T treated with DGL-AgE (26 nmol of antigen E) under similar conditions showed an eightfold suppression of anti-antigen E IgE antibody at week 8 as compared to the control group A.

The different degrees of immunosuppression observed with the DGL-antigen E and DGL-BPO conjugates may be a consequence of their different immunochemical properties. One is the possible instability of DGL-antigen E conjugates under in vivo conditions owing to its labile disulfide linkages. Another is the difference in their antigenic structures. The DGL-BPO conjugate contains multiple antigenic determinants of identical specificity, but the DGL-antigen E conjugate contains multiple determinants of different specificities. In addition, the BPO conjugate has a higher epitope density than the antigen E conjugate. Although the epitope densities of antigen E or its DGL conjugate are not known, it is unlikely that a protein antigen of 38,000 daltons will have more than 10 determinants. These differences in the immunochemical properties of DGL-BPO and DGL-antigen E conjugates may be responsible for their different cellular mechanisms of induction of immunosuppression. Hapten-DGL conjugates have been reported to induce tolerance primarily at the B-cell level (29).

Another group of workers has recently reported the use of polyethylene glycol (PEG) conjugates of ragweed proteins for suppression of IgE antibody response in mice (30). Their findings on PEG-ragweed proteins are less extensive than the present data on MPEG-antigen E, but they are in general agreement with the present report. PEG has two terminal hydroxyl groups while MPEG has only one. Coupling of proteins with activated PEG can in theory give a more complex mixture of conjugates than coupling with activated MPEG.

Other workers have used formaldehyde or glutaraldehyde polymerization of pollen proteins to reduce their allergenic activity. These treatments have been applied to antigen E (31), ragweed pollen extract (32, 33), purified rye pollen allergen (34) and

Timothy pollen extract (35). Such treated pollen proteins showed 10 to 100-fold reduction in their allergenic activities. In experimental animals, such treated pollen proteins retained their ability to induce IgE and IgG antibodies specific for the native proteins. Desensitization of allergic patients with such treated pollen proteins induced significant increases of allergen-specific IgG antibodies in all cases, but only slight or no decrease in allergen specific IgE antibodies (33, 36, 37). The retention of immunogenicity by formaldehyde or glutaraldehyde-treated pollen antigens in experimental animals and man is in contrast to the apparent nonimmunogenicity of MPEG-antigen E in mice.

## Summary

The major allergen of ragweed pollen, antigen E, was modified by coupling its amino acid groups with either methanol, methoxypolyethylene glycol (MPEG) of 5,000 daltons, or a synthetic copolymer of D-glutamic acid and D-lysine (DGL) of 34,000 daltons, all appropriately activated. These conjugates were characterized chemically and immunologically. Compared to the native antigen, the methoxy conjugate showed little reduction in allergenic activity, but the other two conjugates showed strong reductions, as measured by heterologous passive cutaneous anaphylaxis in rats sensitized with murine anti-antigen E reaginic sera. The MPEG conjugate was apparently nonimmunogenic in mice known to be high responders to the native antigen. MPEG and DGL conjugates retained the immunosuppressive property of the native antigen as subcutaneous treatment of antigen E sensitized mice with these two conjugates led to significant long-lasting depression of their antigen E-specific IgE and IgG antibody levels. These immunological changes are believed to result from reduction of antigenic valency and specificity upon coupling the bulky molecules to the protein antigens.

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