Proc. Natl. Acad. Sci. USA
Vol. 73, No. 6, pp. 2091–2095, June 1976
Immunology

Induction of immunological tolerance to the major antigenic determinant of penicillin: A therapeutic approach to penicillin allergy

[benzylpenicilloyl-poly(DGlu,Lys)/IgE reaginic antibodies/bone-marrow-derived lymphocytes/human allergies/immunotherapy]

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Communicated by Albert H. Coons, March 8, 1976

ABSTRACT **High titered IgE, IgG, and IgM antibody responses to the major antigenic determinant of penicillin, the benzylpenicilloyl hapten, were elicited by the intraperitoneal injection of the hapten coupled to keyhole limpet hemocyanin mixed with the appropriate adjuvant. However, treatment of such mice with the benzylpenicilloyl derivatized synthetic copolymer of D-glutamic acid and D-lysine, either before or after primary immunization, resulted in significant suppression of the subsequent anti-benzylpenicilloyl antibody responses of the IgE and IgG classes, as measured at the humoral and cellular levels. The state of tolerance induced by benzylpenicilloyl-poly(DGlu,Lys) was highly specific, of long duration, and could be induced in a manner that would be appropriate for clinical use. These results provide a direct demonstration of the potential application of the poly(DGlu,Lys) immunotherapeutic approach to penicillin allergy in humans.**

The pathogenesis of several types of human autoimmune and allergic disorders is related to the production of antibodies to either "self" or environmental antigens. In such conditions, the ability to induce a selective, long-lasting state of immunological tolerance restricted to the bone-marrow-derived (B) lymphocyte population responsible for the production of these antibodies would be an invaluable therapeutic tool.

Over the past few years, studies from this laboratory have demonstrated (1, 2) and characterized (refs. 1–6; also reviewed in 7 and 8) a system of prolonged hapten-specific B cell tolerance using the synthetic random copolymer of D-glutamic acid and D-lysine (D-GL) to which the appropriate hapten has been linked. This method has proven to be extremely effective in several animal species (7–9) in inducing tolerance in the B lymphocyte precursors of antibody-forming cells of the IgM, IgG, and IgE (9, 10) antibody classes specific for the 2,4-dinitrophenyl (Dnp) hapten. Recent studies in our laboratory have shown that tolerance to nucleoside determinants can be obtained in mice injected with nucleoside conjugates of D-GL (11).

The present study was undertaken to ascertain the efficacy of this approach for inducing tolerance in mice to the benzylpenicilloyl (BPO) hapten, which is the major antigenic determinant of penicillin (12) and, therefore, the responsible sensitizing determinant in most cases of allergy to penicillin and its analogues. The experiments reported here demonstrate that administration of BPO-D-GL induces BPO-specific tolerance resulting in comparably effective depression of both IgG and IgE B cell function. Moreover, tolerance can be induced by administration of BPO-D-GL in a relatively safe manner that could be used in humans, thereby providing direct demonstration of the potential application of the D-GL immunotherapeutic approach to penicillin sensitivity.

Abbreviations: B lymphocytes, bone-marrow-derived lymphocytes; BPO, benzylpenicilloyl; D-GL, copolymer of D-glutamic acid and D-lysine; Dnp, 2,4-dinitrophenyl; HACA, heterologous adoptive cutaneous anaphylaxis; i.p., intraperitoneally; KLH, keyhole limpet hemocyanin; PCA, passive cutaneous anaphylaxis; PFC, plaque-forming cells; s.c., subcutaneously; SRBC, sheep erythrocytes.

MATERIALS AND METHODS

Proteins and Chemical Reagents. The copolymer of D-glutamic acid and D-lysine (D-GL) was obtained from Pilot Chemicals, Inc., Watertown, Mass. The polymer had an average molecular weight of 50,000 and a ratio of glutamic acid to lysine residues of 60:40. Keyhole limpet hemocyanin (KLH) was purchased from Pacific Bio-Marine Supply Co., Venice, Calif. Crystallized bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Mo. Crystalline potassium benzylpenicillin G was generously supplied by Dr. William Wheatley of the Bristol Laboratories, Syracuse, N.Y.

Benzylpenicilloyl (BPO)–Carrier Conjugates. Benzylpenicillin was coupled to KLH and bovine serum albumin and to the D-GL copolymer according to Levine *et al.* (13, 14). The conjugates were assayed for BPO content by the penamaldate assay (15) and for protein concentration by the method of Lowry (16) or Kjeldahl nitrogen analysis (with a correction for the amount of nitrogen contributed by the BPO groups), yielding the following BPO conjugates: BPO_{35}-bovine serum albumin, BPO_{10}KLH, and BPO_{40}D-GL. The univalent ligand, BPO-*n*-propylamine was prepared as described (17).

Animals and Immunizations. CD rats were purchased from Charles River Breeding Laboratories, Wilmington, Mass. Eight- to 12-week-old BALB/c mice and (BALB/c × A/J)F_1 hybrids (CAF_1) were obtained from the Jackson Laboratories, Bar Harbor, Me., and were immunized and challenged intraperitoneally (i.p.) with BPO-KLH adsorbed on $Al(OH)_3$ gel (alum) as described previously (9) and according to experimental protocols as given in *Results*.

Measurement of Anti-BPO Antibodies. *(a) Serum IgE Antibodies.* The concentration of reaginic (IgE) anti-BPO antibodies in pools of sera from groups of mice was determined by passive cutaneous anaphylaxis (PCA) reactions in rats as described (18). The PCA titer is expressed as the reciprocal of the highest dilution of serum yielding a 5 mm diameter blueing reaction.

(b) Heterologous Adoptive Cutaneous Anaphylaxis (HACA). HACA reactions were performed according to the technique of Kind and Macedo-Sobrinho (19) and as modified by us to delineate, semiquantitatively, the number of IgE anti-BPO antibody-forming cells in mouse spleens.

(c) Serum IgM and IgG Antibodies. Serum BPO-specific IgM and IgG antibodies in heat-inactivated (56°, 30 min) individual mouse sera were determined by passive hemagglutination, using BPO coupled to sheep erythrocytes (SRBC, Colorado Serum Co., Denver, Colo.) as described (17). The assay was

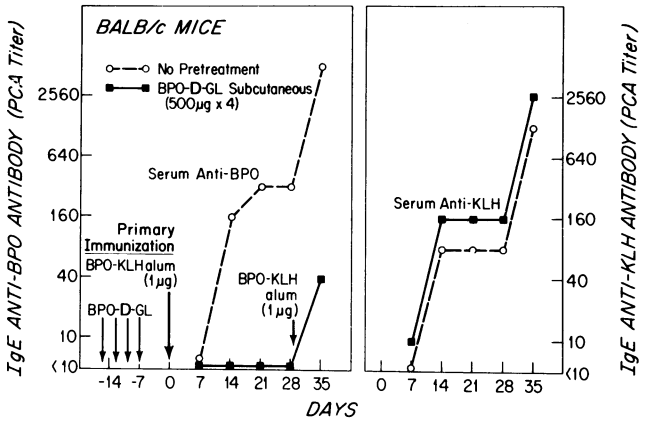

FIG. 1. Induction of BPO-specific tolerance with BPO-D-GL when treatment precedes primary immunization. The protocol is depicted at the bottom of the left-hand panel. Serum IgE anti-BPO (left panel) and anti-KLH (right panel) antibody responses of groups of five mice bled on the various days indicated after primary immunization are shown. Secondary challenge was administered on day 28 as illustrated.

performed in microtiter plates, and BPO specificity was confirmed by complete inhibition of hemagglutination by the univalent hapten, BPO-*n*-propylamine, at a final concentration of 7.5 mM and by the inability of sera that agglutinated BPO-SRBC to agglutinate uncoupled SRBC. IgM and IgG antibodies were distinguished by their susceptibility or resistance, respectively, to treatment with 0.1 M 2-mercaptoethanol at 25° for 2 hr.

(d) Measurement of BPO-Specific Plaque-Forming Cells (PFC). Anti-BPO antibody-forming spleen cells were assayed by the Jerne-Nordin PFC technique (20) using BPO-SRBC as indicator. Indirect, or IgG, PFC were developed with a polyvalent rabbit anti-mouse immunoglobulin antiserum (21). BPO-specific PFC are reported as the difference obtained after subtracting PFC against SRBC from those against BPO-SRBC. BPO specificity of the assay was corroborated by specific inhibition with 2.5 mM BPO-propylamine.

Measurement of Serum Anti-KLH Antibodies. Levels of serum IgE anti-KLH antibody were determined by PCA reactions; IgG anti-KLH antibodies were determined by radioimmunoassay using ^{125}I-labeled monomeric KLH (11).

Spleen Cell Cultures for *In Vitro* Anti-BPO Antibody Responses. The Mishell–Dutton system (22) was used with modification for culturing cells in microcultures (23).

Statistical Analysis. For hemagglutination, individual titers within a given group were analyzed for means and standard errors arithmetically. PFC values and serum anti-KLH antibody titers were logarithmically transformed, and means and standard errors were calculated. All group comparisons were made with Student's *t*-test.

RESULTS

Induction of BPO-specific tolerance with BPO-D-GL when treatment precedes primary immunization

(*a*) Analysis of Humoral Immune Responses. Two groups of normal BALB/c mice were injected subcutaneously (s.c.) with four doses of either saline or 500 μg of BPO-D-GL at 3-day intervals. One week after the last dose, animals were primarily immunized with 1 μg of BPO-KLH mixed with 4 mg of alum, and then bled at weekly intervals. On day 28 after primary immunization, all mice were secondarily challenged with 1 μg of BPO-KLH mixed with 2 mg of alum. Seven days later they were bled and sacrificed. Spleen cells were removed, analyzed for BPO-specific PFC and HACA activity, and tested for PFC responses to BPO-KLH *in vitro*.

Table 1. Effect of BPO-D-GL pretreatment on primary IgM and IgG anti-BPO antibody responses of BALB/c mice to BPO-KLH

Protocol			Serum anti-BPO antibody*	
Group	Pretreatment	Days after priming	IgM	IgG
I	Saline s.c.	7	5.4 ± 0.24	0
		14	4.2 ± 0.20	4.0 ± 0.32
		21	3.4 ± 0.24	4.8 ± 0.37
		28	1.2 ± 0.20	3.4 ± 0.25
		35	4.0 ± 0.32	2.4 ± 0.24
	BPO-specific PFC†	35	67(1.15)	768(1.04)
II	BPO-D-GL s.c.‡	7	4.3 ± 0.25	0
		14	3.8 ± 0.48	0
		21	2.8 ± 0.95	0
		28	1.3 ± 0.63	0.5 ± 0.50
		35	1.5 ± 0.28	1.0 ± 0.41
	BPO-specific PFC†	35	55(1.20)	262(1.05)

The protocol is the same as is illustrated in Fig. 1. All groups contain five mice.

* Arithmetic means ± standard errors of $\log_2$ of the reciprocal of the highest dilution of serum from individual mice in each group which caused agglutination of BPO-SRBC. Statistical comparisons of anti-BPO responses between groups I and II were significant ($0.05 > P$) in the following cases: Day 7—IgM: $P = 0.014$; day 14—IgG: $P = 0.00001$; day 21—IgG: $P = 0.000009$; day 28—IgG: $P = 0.0008$; day 35—IgM: $P = 0.0176$; IgG: $P = 0.00074$.

† Geometric mean of BPO-specific PFC per 10^6 spleen cells. Numbers in parentheses represent standard errors. Statistical comparison of IgG PFC of Group I with II yielded $P = 0.00009$.

‡ 500 μg four times.

As shown in the left panel of Fig. 1, control animals developed good primary IgE anti-BPO antibody responses which peaked at day 21 and manifested sharp anamnestic responses on day 35 after secondary challenge on day 28. In contrast, mice pretreated with BPO-D-GL failed to produce detectable primary IgE anti-BPO responses and produced only low amounts of antibody after secondary challenge. Specificity of tolerance induced by BPO-D-GL is evidenced by the comparable IgE anti-KLH antibody titers between treated and control mice over the entire observation period (Fig. 1, right panel). The data in Table 1 illustrate similar findings in serum anti-BPO antibody responses of the IgG class. This was corroborated by the substantially fewer BPO-specific PFC of the IgG class detected in treated mice on day 35. No significant differences were observed between the two groups in levels of BPO-specific IgM serum antibody or IgM PFC.

A similar experiment performed with CAF_1 mice demonstrated that, 5 months after the administration of BPO-D-GL, pretreated mice manifested quite significant suppression of the IgE anti-BPO antibody response (8% of control) after primary immunization with BPO-KLH as compared to mice that were either not pretreated or pretreated with BPO coupled to the L-stereoisomeric form of GL (BPO-L-GL).

(*b*) Analysis of Tolerance at the Cellular Level. It is possible that the PCA and hemagglutination values obtained in treated mice reflect neutralization of secreted antibody by circulating nonmetabolizable BPO-D-GL. This possibility has been eliminated by demonstrating the diminished ability of IgE B cells

Table 2. Effect of BPO-D-GL pretreatment on formation of IgE anti-BPO antibody assayed by HACA reactivity

Protocol*		Magnitude of HACA reaction†			
		Spleen cells injected ($\times 10^6$) per site			
Group	Pretreatment	0.5	1.0	2.5	5.0
I	Saline s.c.	2+	4+	4+	4+
II	BPO-D-GL s.c. (500 μg × 4)	0	0	0	2+

* The protocol is the same as is depicted in Fig. 1. Spleens from five mice in each group were removed on Day 35 (7 days after boost), washed three times, and, after being resuspended to equal concentrations of viable cells, injected into the dorsal skin of CD rats at the cell numbers listed. The HACA reaction was elicited 36 hr later with BPO-BSA and Evans' blue dye.

† The reaction was scored on a +1–+4 scale based on the intensity of blueing and degree of edema of at least 5 mm in diameter found at the injection site.

from mice treated with BPO-D-GL to elaborate BPO-specific antibodies, as measured in the HACA assay. In this assay, BPO-specific IgE antibody-secreting cells are removed from the tolerant milieu, adoptively transferred intracutaneously in varying numbers to rats and thereby freed to elaborate, if possible, specific IgE antibody which can no longer be affected by circulating BPO-D-GL. With this assay as a semiquantitative measurement of BPO-specific IgE antibody-forming cells, it was found that as few as 5×10^5 spleen cells from the untreated control group produced a positive HACA reaction, whereas ten times as many spleen cells from mice treated with BPO-D-GL were required to elicit a reaction of the same magnitude (Table 2). Moreover, at all cell numbers tested, the magnitude of the HACA reaction was at least 2-fold more intense in controls than in treated mice. These results corroborate, therefore, at the cellular level the suppression of IgE anti-BPO antibody responses as determined by PCA reactivity.

A similar approach was used to eliminate the possibility of neutralization as an explanation for diminished IgG anti-BPO antibody responses. Thus, spleen cells (1×10^6/per well) from the untreated control and BPO-D-GL-treated mice were cultured either in the absence or presence of BPO-KLH (0.1 μg/per well), this being the third exposure of such cells to the immunizing antigen. As shown in Fig. 2, cells from untreated mice displayed very high *in vitro* IgG and moderate IgM anti-BPO responses, whereas responses of cells from mice pretreated with BPO-D-GL were significantly lower in both classes.

Induction of BPO-specific tolerance in previously immunized mice by administration of BPO-D-GL

Three groups of BALB/c mice were immunized i.p. with 1 μg of BPO-KLH mixed with 4 mg of alum. Two weeks later, the groups were bled and then treated with saline, 500 μg of BPO-D-GL i.p., or 500 μg of BPO-D-GL s.c. on 2 alternating days (days 14 and 16). On day 18, all animals were secondarily challenged with 1 μg of BPO-KLH mixed with 2 mg of alum and bled at 7-day intervals over the ensuing 3 weeks. Twenty-one days after secondary immunization, the animals were sacrificed and their spleen cells assayed for BPO-specific PFC.

As shown in the left panel of Fig. 3, all three groups of mice displayed comparable levels of IgE anti-BPO antibodies im-

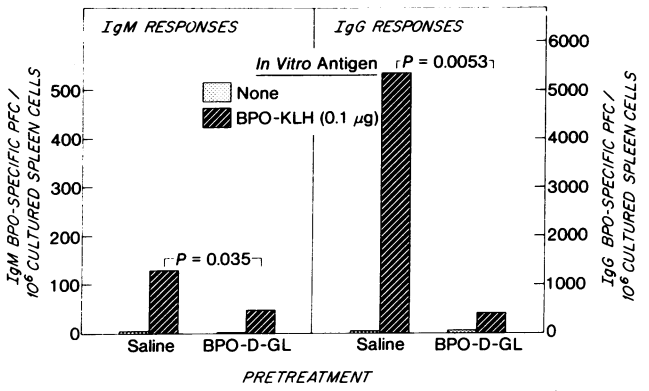

FIG. 2. *In vitro* anti-BPO antibody responses to BPO-KLH of spleen cells from untreated and BPO-D-GL-treated mice. Spleen cells obtained from mice in Groups I and II of the experiment depicted in Fig. 1 were removed on day 35 (7 days after secondary challenge) and cultured for 4 days in the absence or presence of BPO-KLH as indicated above. IgM (left panel) and IgG (right panel) BPO-specific PFC per 10^6 cultured cells are illustrated as geometric means of replicate cultures of respective groups. The corresponding P values of comparisons between responses of spleen cells of untreated and tolerant mice are depicted.

mediately preceding BPO-D-GL treatment. After secondary challenge with BPO-KLH, the untreated control mice manifested good anamnestic IgE responses, whereas the two groups treated with BPO-D-GL failed to respond to secondary immunization with BPO-KLH and, moreover, exhibited a decrease in circulating IgE anti-BPO antibody levels, this being most marked in the group treated with BPO-D-GL s.c. in which no IgE antibody was detectable by day 21. The effects were comparable in the IgG antibody class, as measured by both hemagglutination and PFC assays (Table 3). In contrast, both the IgE (Fig. 3, right panel) and the IgG (Table 3) anti-KLH antibody responses of the treated groups were not diminished by BPO-D-GL administration, but on the contrary, were somewhat augmented in comparison to the control group (Fig. 3, right panel). This sparing of the antibody responses to the linked carrier determinants demonstrates again the hapten-specific nature of the tolerance induced by BPO-D-GL.

The duration of such tolerance was demonstrated in a companion experiment in which CAF_1 mice, which had been initially primed with BPO-KLH and subsequently treated with BPO-D-GL, remained suppressed over at least a 6-month period, as manifested by their diminished IgE anti-BPO responses (12.5% of control) to three separate challenges with BPO-KLH.

DISCUSSION

These studies demonstrate that a state of specific immunological tolerance to the major allergenic determinant of penicillin, BPO, can be induced in mice by administration of BPO-D-GL. This tolerance is manifested in both the IgE and IgG antibody classes and can be established irrespective of the immune status of the animal at the time of treatment. Thus, BPO-specific tolerance could be induced in both unprimed mice and in mice previously primed and actively producing IgE and IgG anti-BPO antibodies, by treatment with BPO-D-GL administered in appropriate doses either i.p. or s.c. The high degree of BPO specificity of unresponsiveness induced by BPO-D-GL was evidenced by the capacity of treated mice to develop normal primary and secondary anti-KLH antibody responses of both IgE and IgG classes. Finally, the unresponsiveness after BPO-D-GL administration was shown to be due to a central inhibition of BPO-specific cells, as evidenced by the inability of spleen

Table 3. Effect of intervening treatment with BPO-D-GL on secondary IgM and IgG anti-BPO and anti-KLH antibody responses of BALB/c mice to BPO-KLH

Protocol					
Group	Intervening treatment	Days after secondary challenge	Serum anti-BPO antibody* IgM	Serum anti-BPO antibody* IgG	Serum anti-KLH antibody† IgG (ABC_{33})
I	Saline	−4	3.0 ± 0.71	4.2 ± 0.75	<5
		7	1.5 ± 0.87	9.2 ± 0.25	20.6 (1.212)
		14	0	11.0 ± 0.41	33.5 (1.195)
		21	0.50 ± 0.29	11.0 ± 0.71	27.4 (1.095)
	BPO-specific PFC‡	21	39(1.35)	467(1.27)	
II	BPO-D-GL i.p. (500 μg × 2)	−4	3.2 ± 0.48	4.8 ± 0.63	<5
		7	3.5 ± 0.29	1.2 ± 0.25	39.4 (1.193)
		14	1.2 ± 0.25	2.5 ± 0.29	34.1 (1.174)
		21	2.2 ± 0.48	2.2 ± 0.25	47.3 (1.183)
	BPO-specific PFC‡	21	22(1.29)	28(1.32)	
III	BPO-D-GL s.c. (500 μg × 2)	−4	4.0 ± 0.89	3.2 ± 0.74	<5
		7	3.5 ± 0.93	2.4 ± 0.51	39.5 (1.012)
		14	2.4 ± 0.40	3.4 ± 0.68	61.5 (1.024)
		21	0.8 ± 0.37	4.4 ± 0.24	59.6 (1.191)
	BPO-specific PFC‡	21	28(1.41)	41(1.21)	

The protocol is the same as is illustrated in Fig. 3. Groups II and III contained five mice; group I, four mice.

* Arithmetic means ± standard errors of $\log_2$ of the reciprocal of the highest dilution of sera from individual mice in each group which caused agglutination of BPO-SRBC. Statistical comparisons of anti-BPO responses between Groups I and II yielded significant ($0.05 > P$) P values in the following cases: Day 7—IgG: $P = 0.0000001$; day 14—IgM: $P = 0.002$; IgG: $P = 0.000001$; day 21—IgM: $P = 0.02$; IgG: $P = 0.00002$. Statistical comparisons between Groups I and III yielded significant P values in the following cases: Day 7—IgG: $P = 0.00001$; day 14—IgM: $P = 0.001$; IgG: $P = 0.00004$; day 21—IgG: $P = 0.00003$.

† Geometric mean of dilutions of individual antisera in each group capable of binding 33% of the ^{125}I-labeled KLH ligand (ABC_{33}). Numbers in parentheses represent standard errors. There were no statistically significant differences of ABC_{33} values between any of the groups.

‡ Geometric mean of BPO-specific PFC per 10^6 spleen cells. Numbers in parentheses represent standard errors. Statistical comparison of IgG PFC of Group I with II yielded $P = 0.00038$; similar comparison of IgG PFC of Group I with III yielded $P = 0.00009$.

cells from treated animals to secrete IgE or IgG antibodies or to develop *in vitro* BPO-specific PFC responses.

These results extend our previous observations utilizing the D-GL copolymer for induction of tolerance in guinea pigs and mice to the Dnp hapten in both IgG and IgE antibody classes (1–10) and in mice to nucleoside determinants (11). The efficacy of this approach in inducing BPO-specific tolerance, as shown here, provides another step toward eventual clinical application of the D-GL copolymer in human allergic disorders.

The successful induction of true hapten-specific tolerance has been accomplished *in vivo* and *in vitro* by several investigators using several different systems (24–40). Detailed evidence has been presented for the existence of a central mechanism of tolerance in the hapten-D-GL model, which is ex-

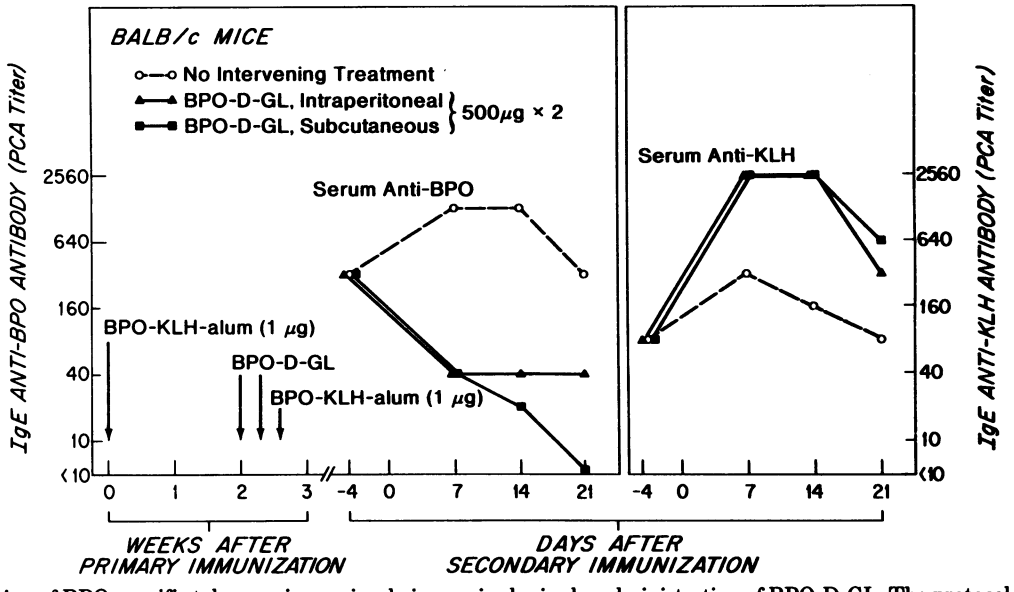

FIG. 3. Induction of BPO-specific tolerance in previously immunized mice by administration of BPO-D-GL. The protocol is depicted at the bottom of the left-hand panel. Serum IgE anti-BPO (left panel) and anti-KLH (right panel) antibody responses of three groups of five mice bled on the various days indicated after primary and secondary immunization with BPO-KLH are illustrated.

pressed predominantly in the population of hapten-specific antibody-forming cells and their precursors, i.e., B lymphocytes (reviewed in ref. 7, 8, and 10).

The precise mechanism(s) of the potent tolerogenic properties of D-GL derivatives has not been delineated, although recent studies of Ault *et al.* (6) demonstrated a persistence of Dnp-D-GL on the surface of Dnp-specific B cells and a marked inability of such cells to regenerate surface immunoglobulin receptors. These observations were in marked contrast to findings with Dnp derivatives of the L-isomeric form of GL, thereby suggesting interference by D-GL with a surface event—e.g., an enzymatic step—necessary for expression of B cell function. The question still remains whether or not the D-GL derivative on the cell surface generates a tolerogenic signal resulting in intra- or subcellular events that inhibit cell reactivity to antigen.

An important therapeutic consideration in any situation in which IgE antibodies are involved is the potential of the immunotherapeutic substance itself to cause degranulation of armed mast cells, thereby resulting, inadvertently, in an anaphylactic reaction. Since mast cells of primed animals will be stimulated to degranulate and secrete vasoactive amines by BPO-D-GL when the latter is administered intravenously (unpublished observations), the observation in the present studies that administration of BPO-D-GL s.c. is better than the systemic route of administration for inducing tolerance in BPO-specific B cells of the IgE class is quite important and suggests that such compounds may be used in allergic humans.

It seems, therefore, that the approach of inducing tolerance in IgE B cells with BPO-D-GL may be effective in the treatment of penicillin allergy, allowing sensitive patients to receive this antibiotic and its analogues should it be indicated, and to provide prophylaxis against inadvertent life-threatening exposure to the drug. In addition, the capacity to inactivate BPO-specific B cells of the IgG class may be of therapeutic value in cases of acute drug-induced, antibody-related hemolytic anemia, and possibly in cases of penicillin cogener-associated interstitial nephritis mediated by anti-tubular basement membrane antibodies (41).

We thank Mrs. Nomi Eshhar, Mrs. Mercy Koshy, and Miss Tova Waks for skilled technical assistance and Marsha Goldman and Mrs. Charlene Small for secretarial assistance in preparation of the manuscript. This investigation was supported by Grant AI-10630 from the National Institutes of Health. N.C. is supported by NIH National Research Service Award no. 1-F32-AI-01965 from the National Institute of Allergy and Infectious Diseases.

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