

Hypersensitivity to phenylglycine acid chloride

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Twenty-four industrial workers, some of whom had respiratory symptoms on exposure to phenylglycine acid chloride (PG-AC), were evaluated. A univalent and several polyvalent conjugates of PG-AC were synthesized for skin testing. Based on history, skin testing, Prausnitz-Küstner (P-K) studies, and inhalation challenge, evidence is presented that in some instances the respiratory symptoms elicited by PG-AC are immunologically mediated, apparently via a Type I immediate hypersensitivity reaction involving reaginic antibody.

There are relatively few reports in the literature of respiratory allergy precipitated by exposure to a chemically defined low molecular weight substance. Moreover, only rarely have such compounds been shown to produce asthma on an immunologic basis, and to evoke a positive immediate-type wheal and erythema skin test reaction that could be passively transferred.¹⁻⁸ Therefore, a group of workers at a chemical plant, who reportedly developed asthmatic symptoms on exposure to phenylglycine acid chloride hydrochloride (PG-AC), was studied in an attempt to determine if this represented an allergic response. Moderate exposures to PG-AC are known to produce irritation of mucous membranes. By history, however, some of these workers developed asthma on exposure even to minute amounts of this substance. Allergic sensitivity to PG-AC appears not to have been previously reported in the literature.

PG-AC is a white powder that is highly reactive chemically, combining readily with proteins and other organic compounds. Only in recent years has it been produced commercially in bulk, since its main commercial use is in the synthesis of ampicillin, where it constitutes the side chain attached to the 6-aminopenicillenic acid nucleus (Fig. 1).

MATERIALS AND METHODS

Twenty-four plant workers, all of whom had been exposed to PG-AC for a period of several years, volunteered for evaluation. This group included most of the workers who had experienced respiratory difficulty on exposure to PG-AC as well as co-workers who had manifested no symptoms on exposure, to serve as controls.

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Supported in part by United States Public Health Service Grants 5T01-AI00044-14 and 5M01-RB42.

Received for publication Oct. 16, 1972.

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Special abbreviations used

AC:	acid chloride
CM:	carboxymethyl
EACA:	epsilon-aminocaproic acid
HBr:	hydrobromide
HGG:	human gamma globulin
NCA:	N-carboxyanhydride
PG:	phenylglycine
PLL:	poly-L-lysine
TLC:	thin-layer chromatography

Each worker first answered a four-page medical questionnaire that stressed his allergic and cardiopulmonary history. Medical records kept by the plant and by each man's own physician were examined and abstracted. Following this each worker underwent a personal interview and physical examination. In addition, the following studies were performed on each subject: (1) a smear of nasal secretions stained with Wright-Giemsa stain was examined for percentage of eosinophils; (2) a white blood cell count and a quantitative blood eosinophil count were done using the Unopette system (Becton-Dickinson); and (3) vital capacity and FEV₁ were measured by a McKesson Vitalometer.

Each worker was skin-tested, both by prick and intracutaneous methods, to a battery of materials coded by number only. This skin test battery included: (1) one phenylglycine epsilon-aminocaproic acid (PG-EACA) conjugate; (2) two phenylglycine poly-L-lysine (PG-PLL) conjugates; (3) two phenylglycine human gamma globulin (PG-HGG) conjugates; (4) normal saline, phosphate buffer, tris buffer, and PLL controls; and (5) common aeroallergens: mixed grasses, ragweed, house dust, and a mold mixture (*Alternaria*, *Hormodendrum*, *Aspergillus sp.*, *Penicillium sp.*, and *Fusarium*).

Serum samples were obtained from each worker for passive transfer studies.

In developing conjugates of PG for skin testing, the compounds chosen were EACA, PLL, and HGG in order to have both univalent and polyvalent conjugates (Fig. 1). The EACA conjugate as well as one PLL and one HGG conjugate were synthesized using the N-carboxyanhydride (NCA) of PG and reacting it in an alkaline aqueous solution (pH 10.2) for 5 to 10 minutes followed by acidification, as described by Hirschmann and associates.^{9, 10} These syntheses and the purification of the resulting products are described briefly in the following paragraphs.

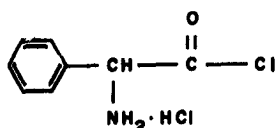
PG-EACA conjugate

To a solution of 5.6 Gm. of boric acid in 200 ml. water was added 5.2 Gm. (0.04M) of EACA. The solution was cooled to 0° C. and the pH adjusted to 10.2 with 20 per cent NaOH. To this solution 7.1 Gm. (0.04M) of PG-NCA was added with rapid stirring. The pH was maintained at 10.2 by the addition of NaOH as required. After 5 minutes, the pH was adjusted to 5.5 with concentrated HCl, and stirring was continued until CO₂ evolution ceased. The mixture was filtered and the filtrate freeze dried. The residue was extracted with 200 ml. methanol, which was concentrated under negative pressure to a volume of 100 ml. The concentrated extract was treated with ether until the cloud point was reached. After a few minutes, the derivative crystallized and was isolated by filtration. The material was recrystallized from water-isopropanol, and the resulting product gave a single spot by TLC analysis. A standard 10⁻²M solution of the PG-EACA conjugate was made using 0.01M phosphate buffer (pH 7.4) in normal saline.

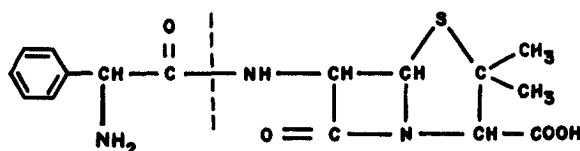
PG-PLL Conjugate 1

To 50 ml. water cooled to 0° C. was added 4 Gm. of PLL HBr (Pilot Laboratories, Watertown, Massachusetts) with a degree of polymerization of 35 (MW 7,400), thus making

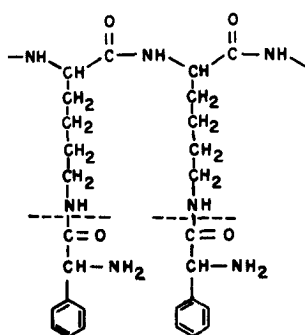
STRUCTURE OF
PHENYLGLYCINE ACID CHLORIDE HYDROCHLORIDE



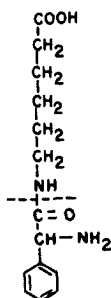
STRUCTURE OF AMPICILLIN



PHENYLGLYCINE
POLY-L-LYSINE
CONJUGATE



PHENYLGLYCINE
EPSILON-AMINOCAPROIC
ACID CONJUGATE



PHENYLGLYCINE
HUMAN GAMMA
GLOBULIN
CONJUGATE

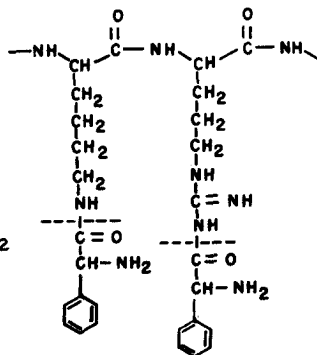


FIG. 1. Structural formulas of PG-AC and ampicillin; and probable structures of PG conjugates with PLL, EACA, and HGG. (In the PG-HGG conjugates, the PG undoubtedly is linked to the carrier largely through lysine residues, but additional coupling through arginine also is a possibility.)

a $1.1 \times 10^{-3}M$ solution with 0.02M of $\epsilon-NH_2$ groups in the 50 ml. The pH was adjusted to 10.2 with NaOH. With rapid stirring, 1.77 Gm. (0.01M) of PG-NCA was added and the pH maintained at 10.2 with NaOH. (The molar ratio of PG: $\epsilon-NH_2$ groups was 1:2.) After 10 minutes the mixture was acidified with HCl to a pH of 5.5 and stirred for another 10 minutes. The pH was then adjusted to 6.5 and the mixture filtered. The final concentration of the product was approximately 4 Gm. in 400 ml. or $1.35 \times 10^{-3}M$.

Because of its relatively low molecular weight, this conjugate could not be purified by dialysis, and therefore carboxymethyl (CM)-cellulose was employed as an exchange resin. After pre-cycling, 5 Gm. of CM 23 resin (Whatman) were suspended in 0.005M phosphate buffer at pH 6. Using a batch technique, 1 Gm. (100 ml.) of the PG-PLL conjugate was mixed with the suspended CM 23 resin and allowed to stand for several hours. Following centrifugation the resin was resuspended in 0.005M tris buffer (pH 8.75) in 0.5M saline. After a second

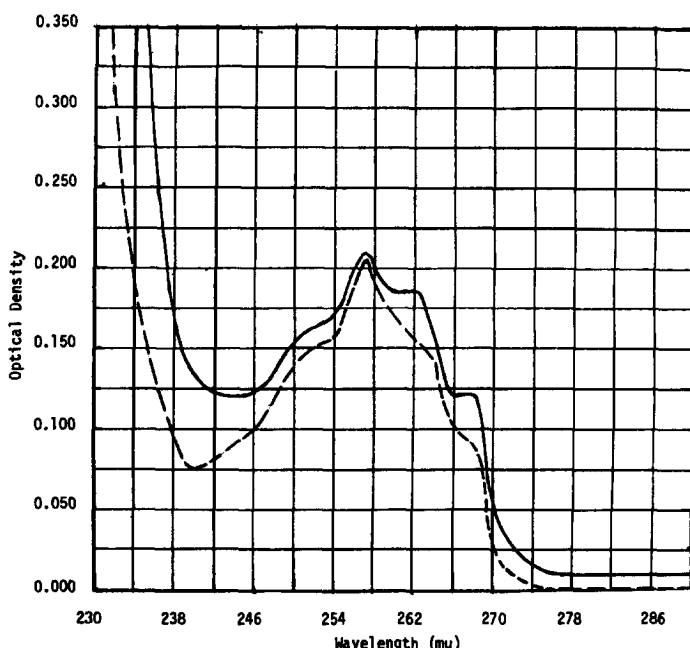


FIG. 2. Absorption spectra of 10^{-3} M PG-AC (—) and 10^{-3} M PG-EACA conjugate (---) in phosphate buffer.

centrifugation, the supernatant containing the eluted PG-PLL conjugate was diluted threefold with distilled, deionized water and the pH adjusted to 8.2.

PG-HGG Conjugate 1

A 30 ml. sample of a 15% solution of HGG (Parke, Davis & Co.) was diluted with 50 ml. of water and cooled to 0° C. The pH was adjusted to 10.2 with 20% NaOH, and 2.6 Gm. (0.0146M) of PG-NCA was added with rapid stirring. The pH was maintained at 10.2 with NaOH. After 10 minutes the mixture was acidified with HCl to a pH of 5.5 and stirred for another 10 minutes. The pH was then adjusted to 6.5 and the mixture filtered. This conjugate was purified by three dialyses using 0.005M phosphate buffer (pH 8.2) in normal saline. Based on absorption at $280\text{ m}\mu$, the final concentration of the PG-HGG conjugate was approximately 4×10^{-4} M.

A second PLL and a second HGG conjugate were synthesized by reacting them directly with PG-AC in an alkaline aqueous solution using a slight modification of Levine's method of preparing penicilloyl conjugates.¹¹ The conjugates were not treated with succinic anhydride because of the possibility of blocking the amino group on the PG moiety and thus altering antigenicity of the conjugate. These syntheses and purification of the resulting products are discussed briefly in the paragraphs that follow.

PG-PLL Conjugate 2

To 20 ml. of water at 25° C. was added 60 mg. of PLL HBr (Pilot Laboratories, Watertown, Massachusetts) with an average degree of polymerization of 478 (M. W. 100,000), thus making a 3×10^{-4} M solution with 0.015M of $\epsilon\text{-NH}_2$ groups in the 20 ml. The pH was adjusted to 11.0 with 1N NaOH, and 95 mg. (0.023M) of PG-AC was added with rapid stirring. The pH was maintained at 11.0 with NaOH. (The molar ratio of PG: $\epsilon\text{-NH}_2$ groups was 3:2.) After stirring for one hour, the pH of the resulting mixture was titrated down to 7.4 with 1N HCl. The final concentration of the product was approximately 1.5×10^{-4} M. The

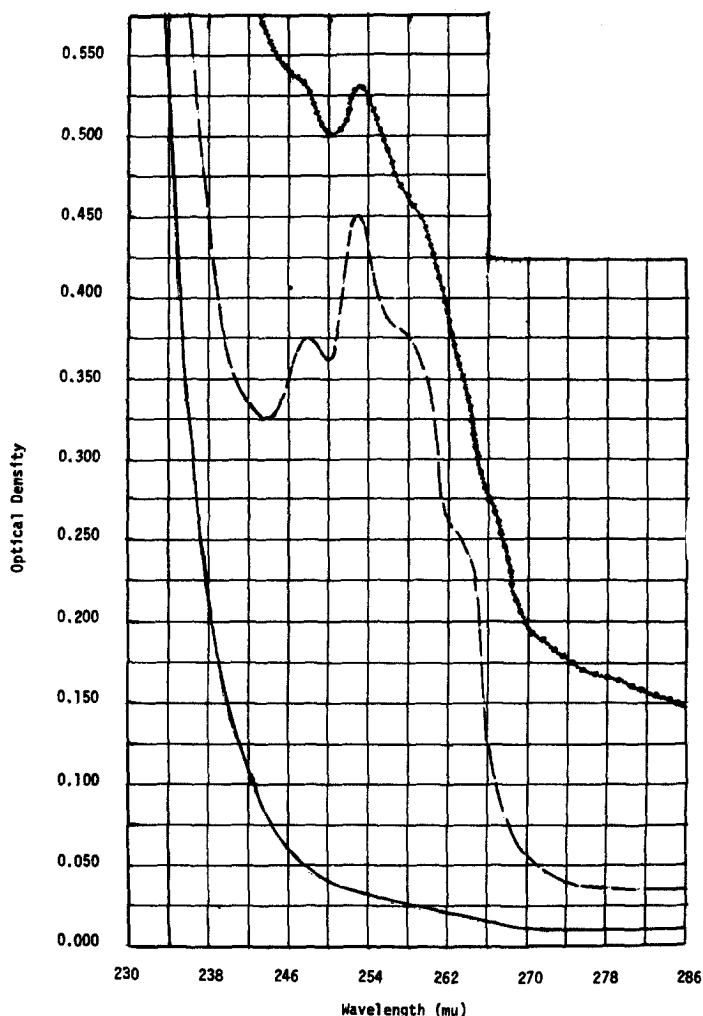


FIG. 3. Absorption spectra of 0.3 per cent PLL (—), PG-PLL Conjugate 1 (before purification on CM-cellulose) (---), and PG-PLL Conjugate 2 (•••) in phosphate buffer.

PG-PLL conjugate was then dialyzed against 0.005M tris buffer (pH 8.2) in normal saline 6 times for 4 hours each. A seventh and final dialysis was carried out against 0.005M phosphate buffer (pH 8.2) in normal saline for 8 hours.

PG-HGG Conjugate 2

To make a 4×10^{-3} M solution, 120 mg. of human gamma globulin was dissolved in 20 ml. of water and the pH adjusted to 11.0 with 1N NaOH. Since there are approximately 8 Gm. lysine per 100 Gm. gamma globulin,¹² the above solution contained approximately 3.3×10^{-3} M of ϵ -NH₂ groups. Then 13.6 mg. (3.3×10^{-3} M) of PG-AC was added with rapid stirring and the pH maintained at 11.0 with NaOH. After stirring for one hour, the pH of the resulting mixture was titrated down to 7.4 with 1N HCl. Following this the PG-HGG conjugate was dialyzed in the same manner as the PG-PLL Conjugate 2 (see above).

PG-AC as well as the PG-EACA conjugate were found to have an absorption peak at 257 mμ by spectrophotometric analysis (Fig. 2). The absorbance at this wavelength was shown to

TABLE I. Results of skin test in study group

Agent	Worker										
	1 Q*	2 Q	3 Q	5 NA†	6 Q	7 A‡	8 NA	9 A	10 Q	11 A	12 C
Grass	—	—	—	—	2+ IC§	—	2+ P	4+ P	—¶	—	—
Ragweed	—	—	—	—	—	—	—	4+ IC	2+ IC	—	—
Mold	—	—	—	—	—	—	—	4+ IC	2+ P	—	—
House dust	3+ IC	—	3+ IC	—	—	—	3+ IC	3+ IC	2+ IC	—	—
Controls	—	—	—	—	—	—	—	—	—	—	—
PG-EACA	—	—	—	—	—	—	—	—	—	—	—
PG-PLL No. 1	—	—	—	—	—	4+ IC	—	3+ IC	—	3+ P	—
PG-PLL No. 2	—	—	3+ IC	—	—	2+ IC	—	—	—	3+ P	—
PG-HGG No. 1	—	—	4+ IC	—	—	4+ P	—	4+ P	—	4+ P	—
PG-HGG No. 2	—	—	3+ IC	—	—	4+ IC	—	3+ IC	—	3+ P	—

*Q = Questionable.

§IC = Intracutaneous.

†NA = Not allergic to PG-AC.

||P = Prick.

‡A = Allergic to PG-AC.

¶— = Negative.

be linearly related to the concentration of PG-AC with a molar extinction coefficient of 210. The two PLL conjugates of PG also both showed absorption peaks at 257 μ , while PLL by itself exhibited no such peak (Fig. 3). The PG molarity of the PLL conjugates could be calculated from the extinction coefficient of PG at 257 $m\mu$. Relating this to the amount of PLL used in making the conjugates, the average compositions of the two PLL conjugates were derived. Approximately 45 per cent of the ϵ -NH₂ groups per molecule of PLL were conjugated in the first PG-PLL conjugate, giving a formulation of PG₁₆PLL₃₅; and approximately 35 per cent of the ϵ -NH₂ groups per molecule of PLL were conjugated in the second PG-PLL conjugate, giving a formulation of PG₁₇₆PLL₄₇₈. Although there was a slight inflection at 257 $m\mu$ when the HGG conjugates were subjected to spectrophotometric analysis, no distinct peaks were obtained, and thus no quantitative statement can be made concerning the amount of PG that was conjugated per molecule of gamma globulin.

All conjugates were sterilized prior to skin testing by centrifugation at 20,000 r.p.m. for 45 minutes. By means of animal and human trials, concentrations of the conjugates were determined that were without any irritant effect upon prick testing or intracutaneous injection. For PG-PLL No. 1, these concentrations proved to be 1.35×10^{-6} M for prick tests and 1.35×10^{-7} M for intracutaneous tests. The respective concentrations were 1.5×10^{-4} M and 1.5×10^{-5} M for PG-PLL No. 2, 4×10^{-4} M and 4×10^{-6} M for PG-HGG No. 1, and 3×10^{-5} M and 3×10^{-7} M for PG-HGG No. 2. A concentration of 1×10^{-2} M PG-EACA was used for both prick and intracutaneous testing.

Inhalation challenges were performed in the same manner as described in a previous study.⁷ Total vital capacity, FEV₁, auscultatory findings, and subjective symptoms were monitored in single-blind fashion after inhalation of varying concentrations of conjugates or diluent. Prick, intracutaneous, and passive transfer (P-K) tests were done in standard fashion.¹³ A 0.02 ml. volume was used for intracutaneous injections and the tests scored for wheal and erythema responses after 20 minutes.

RESULTS

Based on the medical questionnaire, past medical records, and personal interview, the 25 workers were divided into three categories: those whose symptoms on exposure to PG-AC were felt probably to be on an allergic basis; those

history												
13 Q	14 A	15 NA	16 NA	17 NA	18 NA	19 A	20 A	21 Q	23 A	24 NA	25 NA	26 Q
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	2+ IC	—	3+ IC	—	—	—	—	3+ IC	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	3+ P	—	—	—
—	2+ IC	—	—	—	—	4+ P	4+ IC	3+ IC	4+ P	—	—	—
—	2+ IC	—	—	—	—	3+ IC	3+ IC	3+ IC	3+ IC	—	—	—

whose symptoms, if any, were felt to be on an irritant basis; and those in whom the history was equivocal. These evaluations were made independently of knowledge of the skin test results and were based primarily on the amount of PG-AC required to produce difficulty, the type of symptoms, documentation of wheezing by auscultation during an attack, and rapidity of onset of respiratory distress after exposure.

Results with the skin testing agents are shown in Table I. Intracutaneous tests were not done when the prick test result was 2+ or more, and all tests were performed in a double-blind manner. Correlating the skin test results and clinical evaluations (Table II), all 7 workers who were felt to be allergic to PG-AC by history had positive skin tests to the PG-PLL and/or PG-HGG conjugates, and the 8 who were felt not to be allergic by history all had negative results. Of the 9 whose histories were equivocal, 2 had positive skin tests to the PG-PLL and/or PG-HGG conjugates and 7 had negative results. All 24 workers manifested a negative skin test to the controls and to the PG-EACA conjugate. Moreover, 31 normal and atopic controls were negative when skin-tested by both prick and intracutaneous methods to all the PG conjugates.

The 9 workers having positive skin tests were compared with the 15 workers having negative reactions with respect to all other clinical and laboratory parameters measured (Table III). Although there was some variation between the two groups, using the chi-square test none of the differences was statistically significant, perhaps in part related to the small numbers involved.

Using the serum samples collected from the workers, passive transfer studies were done with sera from the three (7, 11, and 23) who manifested the most marked skin test reactions to the polyvalent PG conjugates. It was possible to passively transfer positive reactions with all three sera. Worker 7's serum gave a positive reaction to the PG-HGG Conjugate 1 at up to a 1:270 serum dilution

TABLE II. Relationship of historical evaluation to results of skin testing

Historical evaluation	Skin testing to PLL and HGG conjugates
7 "allergic" to PG-AC	7 with positive skin tests
9 "questionable"	2 with positive skin tests
	7 with negative skin tests
8 "not allergic" to PG-AC	8 with negative skin tests

TABLE III. Comparison of subjects reacting positively and negatively on skin testing with PLL and HGG conjugates of PG-AC

	Skin test positive to PLL or HGG conjugates (9)	Skin test negative to PLL and HGG conjugates (15)
Personal history of atopic disease	3 (33%)	3 (20%)
Positive inhalant skin tests*	4 (45%)	5 (33%)
Boggy nasal mucosa	1 (11%)	3 (20%)
Nasal smear positive for eosinophils	2 (22%)	1 (7%)
Wheezing with forced expiration	4 (45%)	2 (13%)
Abnormal pulmonary function studies	5 (55%)	5 (33%)
Blood eosinophilia	1 (11%)	1 (7%)

*One or more 2+ prick or 2+ intracutaneous tests.

and a positive reaction to the PG-PLL Conjugate 1 at up to a 1:90 serum dilution (Table IV). All three sera showed loss of P-K activity following heating at 56° C. for 4 hours prior to planting. Control injections of the PG-HGG and PG-PLL conjugates into unsensitized skin sites showed no reaction.

Further studies were done in an attempt to see if the positive P-K reactions to the polyvalent conjugates could be blocked by the PG-EACA conjugate. Serum from Worker 7 (0.1 ml.) was incubated with the PG-EACA conjugate (0.02 ml.) for one-half hour prior to planting at two sites. Other sites of inoculation with 0.1 ml. serum (1:1 and 1:10 dilutions) were injected with 0.02 ml. of the PG-EACA conjugate 48 hours, 24 hours, 6 hours, and one-half hour prior to challenge with the PG-HGG conjugate. With both the in vitro and in vivo neutralization tests, performed twice in each of 2 recipients, the univalent PG-EACA conjugate was unable to block the positive P-K reactions to the PG-HGG conjugate at all time intervals studied (Table IV). In additional, similar passive transfer neutralization experiments, the sites were challenged with PG-PLL conjugate. Most of the sites failed to react, but it was difficult to be certain that all of the negative responses indicated neutralization by PG-EACA rather than being due to technical artifact. Control sites, where saline was substituted for the PG-EACA conjugate in both the in vitro and in vivo neutralization methods, were consistently positive when challenged with the PG-HGG and PG-PLL conjugates.

Two workers, 7 and 23, were admitted to the Clinical Research Unit for more complete clinical studies and inhalation challenges to the PG-PLL Conjugate 1. Numerous laboratory studies done on both workers, all of which were

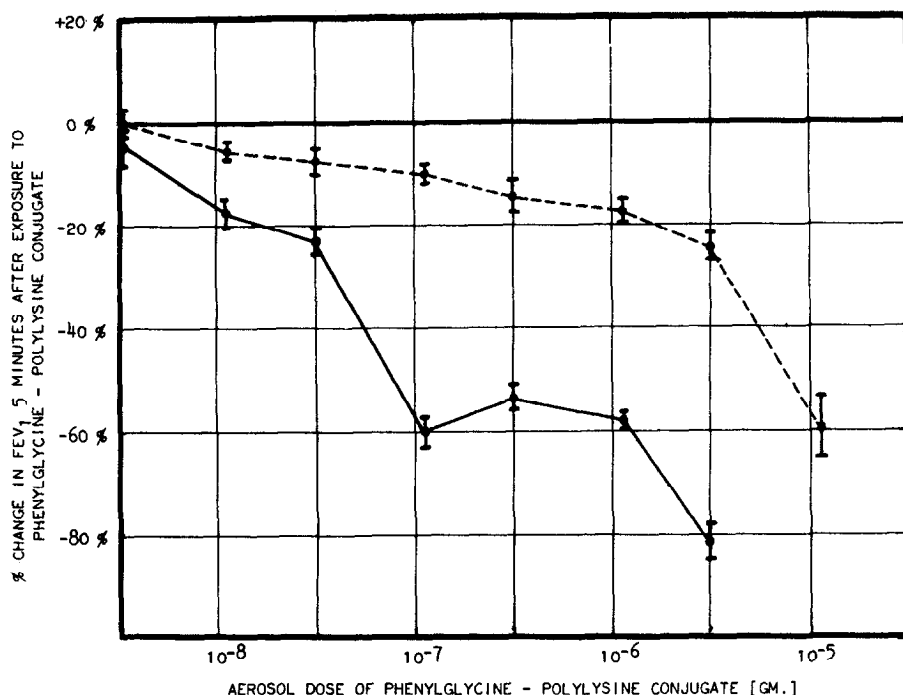


FIG. 4. Results of inhalation challenge tests. Vertical lines indicate one standard deviation of triplicate measurements (----, Worker 7; —, Worker 23).

TABLE IV. Results of passive transfer (P-K) tests

	Worker 7	Worker 11	Worker 23
Passive transfer (P-K) possible	Yes	Yes	Yes
Greatest dilution of serum giving a positive P-K with HGG conjugate	1:270	1:8	1:8
Greatest dilution of serum giving a positive P-K with PLL conjugate	1:90	1:4	1:1
Able to block HGG conjugate with EACA conjugate	No	N.D.	N.D.
Able to block PLL conjugate with EACA conjugate	Yes	N.D.	N.D.

N.D. = Not done.

normal, included: multiphasic screen, complete blood count, urinalysis, chest roentgenogram, complete pulmonary function tests, serum protein electrophoresis, IgG, IgA, and IgM measurements by radial immunodiffusion, and serum C3 levels. The 2 individuals' serum IgE levels were 600 and 36 ng. per milliliter, respectively, as measured by the double antibody technique, the former value being above the normal range for this laboratory.

Whereas 2 normal and 3 control asthmatic subjects have manifested no change in flow rates after inhalation of up to 130 μ g of the PLL derivative, both of these workers demonstrated positive inhalation challenges at much higher dilutions of the PG-PLL conjugate. In Fig. 4 is shown the per cent decrease

in FEV₁ within 5 minutes after a series of inhalation challenge tests done in a single-blind fashion and between which the flow rates returned to base line values. The pronounced falls in FEV₁ were accompanied by subjective dyspnea and auscultatory evidence of wheezing.

DISCUSSION

Phenylglycine acid chloride sensitivity appears to be a new Industrial Medicine problem since this compound has been produced in bulk only in recent years. As a highly reactive substance, this white, powdery material produces mucous membrane irritation in essentially all subjects exposed to a sufficiently high concentration. However, its avidity for amino groups also predisposes to the formation of hapten-protein conjugates and hence immunogenicity and allergenicity. The clinical problem is not whether it is an irritant *or* an allergen, but the more difficult question of whether it is an irritant *and* an allergen. Based on the results of the skin testing, the passive transfer experiments, and the inhalation challenges, the information accrued in this study substantiates the allergenicity of PG-AC. Moreover, the data indicate that Type I hypersensitivity¹⁴ is involved in the immune response, and this would appear to be relevant to the workers' clinical asthma.

The specificity of the immune response was not fully delineated. As shown in Table I, all 9 workers reacting positively by skin testing to the polyvalent conjugates reacted to the PG-HGG conjugates, but only 5 of the 9 reacted positively to the PG-PLL conjugates. In addition, the PG-EACA conjugate was unable to inhibit the cutaneous response of passive transfer sites to challenge with PG-HGG Conjugate 1. Both of these results suggest the possibility that some of the reaginic antibodies were reactive with carrier-determinant groups extending beyond, or other than, the lysyl-hapten moiety. It is of interest that the univalent conjugate, PG-EACA, gave uniformly negative skin test reactions. This was the expected result, since in order to produce mediator release in Type I hypersensitivity reactions, it is generally necessary to employ immunologically multivalent antigens that can bridge two or more reaginic antibody molecules on the surface of the reactive cells.¹⁵⁻¹⁷ Elicitation of immunologic responses by univalent haptens has been reported, however.¹⁸ Initially, consideration was given to determining whether inhalation of the PG-EACA conjugate might be able to inhibit respiratory responses to PG-AC in sensitized individuals, but the results of the passive transfer inhibition studies, as discussed above, did not encourage pursuing such observations.

A systematic study of the epidemiologic and industrial hygiene aspects of PG-AC asthma was not attempted, but it is obvious from the presence of a minimum of 9 cases of sensitization to this compound at one chemical plant that it is potentially a potent allergen. Although there are many employees at the factory who, due to their locale in the plant, have insignificant, if any, exposure to PG-AC, somewhere between 100 and 150 workers have had significant exposure to PG-AC since its production at the plant was first begun. Since the installation of equipment to handle PG-AC in a largely closed system, the development of new cases of allergy has essentially ceased. However, some of

the previously sensitized workers continue to have asthma at times after working in certain areas of the plant where they may be exposed to small amounts of PG-AC. The retrospective histories indicate that in a number of instances sensitization followed rather heavy exposure to PG-AC. This pattern has also been observed in workers developing toluene diisocyanate asthma.⁸

Although PG constitutes the side chain on ampicillin (Fig. 1), cephalixin, and cephaloglycin, any relationship between industrial PG-AC asthma and allergy to these antibiotics remains undetermined. None of the sensitized workers was known to have experienced an allergic reaction to any of these antibiotics before or after acquiring their PG-AC sensitization. Three other subjects who had had a typical delayed, erythematous, maculopapular eruption after ampicillin administration failed to react to the battery of skin testing materials employed in this study. However, there is uncertainty that such reactions are immunologically mediated.¹⁹ It has been shown that some patients exhibit skin test reactivity to ampicillin or ampicillin-polylysine and not to other penicillins.²⁰ Therefore, further studies are needed in patients with urticarial or other more typically allergic reactions to these antibiotics to determine whether or not they would react by skin testing to the PG conjugates.

The authors wish to acknowledge the assistance of Dr. Andrew G. Zeiler for the preparation of the PG-EACA, PG-PLL No. 1, and PG-HGG No. 1 conjugates. IgE measurements were made by Dr. Paul Steinberg.

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