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## IMMUNOLOGICAL PROPERTIES OF DIALYSIS RETENTATES FROM PENICILLIN

By

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A number of papers have been published during the last two decades suggesting that penicillin allergy is partly due to compounds other than penicillin present in penicillin formulations (1, 2, 4, 14, 17). This implies that further purification of penicillin to eliminate impurities will reduce a number of adverse reactions attributed to this drug.

High molecular weight impurities of protein or polymeric character in penicillin preparations are considered by some to be capable of inducing anaphylactic antibody production in patients or eliciting anaphylactic reactions in already sensitized individuals (1, 2, 4, 14, 17). However, some controversy exists over the occurrence of such compounds (1, 2, 3, 17) and the role they play in anaphylactic reactions (1, 2, 17).

In addition to these human studies, animal studies have indicated that penicilloylated proteins are immunogenic in rabbits and guinea pigs (1, 2). Furthermore, such studies have shown that commercially available penicillin preparations can elicit cutaneous anaphylaxis in guinea pigs passively sensitized with homologous anti-benzylpenicilloyl (BPO) reagenic sera (11). Similarly, sensitized rat mast cells obtained from animals fed or immunized with penicillin are able to release histamine on challenge with either penicilloyl-polylysine (PPL) or penicillin (8). How penicillin, being a small molecule, could induce passive cutaneous anaphylaxis (PCA) reactions in

guinea pigs or release histamine from sensitized mast cells, is open to speculation. The possibility that impurities in penicillin are responsible for such reactions cannot be excluded.

In view of the controversy in the literature concerning the nature and the extent of contamination of penicillin preparations, and the role of such impurities in anaphylaxis, we have investigated the bulk drug penicillin preparations, currently marketed in Canada, for impurities. Dialysis retentates obtained from these preparations were then tested for their immunogenicity with respect to anaphylactic antibody production in experimental animals and were compared with synthetic polycondensation products, benzylpenicilloyl-bovine serum albumin (BPO-BSA) and PPL. The results on composition and extent of contamination by these high molecular weight impurities have been reported in detail elsewhere (19).

## MATERIALS AND METHODS

*Animals:* Albino Wistar male rats (Woodlyn Farms, Guelph, Ontario) and Wistar Furth rats (ARS-Sprague Dawley, Madison, Wisconsin, U.S.A.) weighing 200–250 g and Hartley guinea pigs (High Oak Ranch, Goodwood, Ontario) weighing 300–350 g were used.

*Chemicals and biochemicals:* Samples of benzylpenicillin were supplied from the following companies: Novopharm Ltd. (Scarborough, Ontario), Ayerst Laboratories Ltd. (Dorval, Québec) and Wyeth Ltd. (Windsor, Ontario). Bovine serum albumin (BSA), PPL and p-hydroxy mercuribenzoate were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.); O-phthal-dialdehyde from Calbiochem (San Diego, California, U.S.A.); medium 199–10X from Grand Island Biological Company (Grand Island, New York, U.S.A.) and pertussis vaccine from Connaught Medical Research Laboratories (Toronto, Ontario).

Buffers were made from reagent grade chemicals.

*Dialysis retentates and penicillin polymers:* Samples were obtained and characterized as described elsewhere (19). Briefly, 40–100 mg of benzylpenicillin (sodium or potassium salt) were dissolved in distilled water to give a 50 per cent solution. The solution was then placed in a Visking dialysis sac and dialysed to exhaustion against running distilled water for 10 days. Non-difusible residue, the retentate, was then obtained by lyophilization of the solution retained inside the dialysis sac. For penicillin polymers, an aqueous solution (25 per cent w/v) of benzylpenicillin (purified by dialysis to remove high molecular weight impurities) was left to stand for 14 days prior to

dialysis against distilled water for 9 days. The non-dialysable material was recovered by lyophilization.

*Benzylpenicilloyl-bovine serum albumin (BPO-BSA) conjugate:* BPO-BSA was prepared by the method as described by Vickers & Assem (18) and its composition was established to be BPO<sub>18</sub>-BSA.

*Immunization:* Groups of twelve rats (Albino Wistar or Wistar Furth) were immunized by intraperitoneal injection of 1 mg of the antigen (BPO-BSA, penicillin retentates or penicillin polymers) plus  $7 \times 10^9$  killed *B. pertussis* organisms. Twelve days later the animals were either bled and sera collected or sensitized peritoneal mast cells were harvested. The sera were kept refrigerated for initial testing, then divided into 1 ml samples and stored at  $-70^\circ\text{C}$  for further testing.

Twelve male guinea pigs (Hartley) were immunized by repeated injections of penicillin polymers (1  $\mu\text{g}$  per animal) in aluminum hydroxide gel (1 mg per animal) at 0, 4, 9, 14, 17, 20 and 25 weeks (6). The animals were bled at 5, 10, 15, 18, 21 and 26 weeks by cardiac puncture. Individual sera were stored at  $4^\circ\text{C}$  and tested within 2 days or kept frozen at  $-70^\circ\text{C}$ .

*Collection of peritoneal rat mast cells:* These were collected as described previously (7) from normal or immunized rats. The cell suspensions consisted of cells obtained from six rats and were brought to 6.0 ml volume with Tris-ACM buffer (9).

*Passive cutaneous anaphylaxis (PCA) assays:* PCA tests were performed according to the method described by Ovary (13). Serum was injected intradermally in 0.1 ml volumes. In rats, the 4- and 24-h PCA reactions to assess IgG<sub>a</sub> and IgE antibodies respectively were employed (10, 20). In guinea pigs a sensitization period of 4 h was used for  $\gamma\text{S}\gamma_1$ -mediated PCA reactions and 7 days for antibodies persisting longer in the skin (15). Animals were challenged with 0.5 to 1.0 mg of the antigen in 1.0 ml of 1 per cent Evans Blue dye. In the case of PPL the challenging dose was  $5 \times 10^{-5}$  M. Reactions were read in 30 min. Two to four recipients were used for each PCA assay.

*In vitro histamine release from rat mast cells:* Rat mast cell suspensions (0.2 ml) and antigen at various concentrations, indicated in the Tables, in 0.2 ml of Tris-ACM buffer were incubated for 15 min at  $37^\circ\text{C}$  with gentle shaking. The incubation mixtures were brought to 2.0 ml with buffer and then centrifuged at  $4^\circ\text{C}$  for 5 min at 300 g. The supernatants were deproteinized with 70 per cent perchloric acid (0.07 ml/2.0 ml sample volume) and were kept frozen overnight for histamine release determination. The histamine content of the samples was measured according to a published modification (9) of the spectrophotometric method by Shore et al. (16). All samples were run in duplicate and the standard curve method was used to determine histamine levels in samples and controls.

TABLE 1

*Analytical and Immunological Data of Retentates and Derivatives of Various Benzylpenicillin Samples.*

| Sample              | Yield of retentate* | Protein detected by DGE† | Anaphylactic antibody production in the rat |      |
|---------------------|---------------------|--------------------------|---------------------------------------------|------|
|                     |                     |                          | IgE                                         | IgGa |
| 1                   | 58.0                | +                        | —                                           | ND § |
| 2                   | 40.7                | +                        | —                                           | ND   |
| 3                   | 60.6                | +                        | —                                           | —    |
| BPO-BSA             | NA "                | +                        | +                                           | +    |
| Penicillin polymers | NA                  | —                        | —                                           | —    |

\* Expressed as mg of retentate per 100 g of bulk material.

† DGE = Disc gel electrophoresis.

§ ND = Not done.

NA = Not applicable.

## RESULTS

Samples employed as antigens in the present study were dialysis retentates from benzylpenicillin preparations. Yields of dialysis retentates and the presence or absence of protein impurities in the retentates are shown in Table 1. Visible protein bands were obtained on polyacrylamide gels after electrophoresis and staining from two of the samples used in this study. However, the bands were seen only after using 1 mg of

TABLE 2

*Comparison of the Composition of Penicillin Retentates and Penicillin Polymers.*

|                                                                 | Penicillin retentates | Penicillin polymers |
|-----------------------------------------------------------------|-----------------------|---------------------|
| Protein                                                         | Trace or Absent       | Absent              |
| Penicilloyl structure                                           | Present               | Present             |
| Infrared absorption at $1780\text{ cm}^{-1}$ ( $\beta$ -lactam) | Absent                | Absent              |
| Penamaldate                                                     | Trace                 | Trace               |
| Penicillenate                                                   | Trace                 | Trace               |
| Penicilloic                                                     | Trace                 | Trace               |

TABLE 3

*Elicitation of Cutaneous Anaphylaxis in Rats Passively Sensitized with Rat Anti-BPO-BSA Serum (Pool).*

| Serum dilution | PCA reaction in millimeters of diameter<br>Challenging antigen |         |     |                     |
|----------------|----------------------------------------------------------------|---------|-----|---------------------|
|                | BPO-BSA                                                        | PPL     | BSA | Penicillin polymers |
| 1:1            | 20 × 25                                                        | 15 × 15 | N*  | N                   |
| 1:5            | 15 × 15                                                        | ND†     | N   | N                   |
| 1:10           | 13 × 13                                                        | ND      | N   | N                   |

\* N = Negative.

† ND = Not done.

the retentates for electrophoresis. It was estimated (19) that the amount of protein in samples giving visible bands was of the order of 1–5 per cent of the retentate or 0.001 to 0.005 per cent of the original penicillin preparation. Neither the penicillin polymers nor the retentate from sample # 2 gave any visible protein bands, indicating that if protein was present in sample #2, it was in amounts less than 0.001 per cent. In Table 2 other analytical data and a comparison between retentates and polymers are included. Penicilloyl groups were detected in all retentates and penicillin polymers using the penamaldate method (5). Traces of penicillenate and penamaldate based on ultraviolet spectrum and penicilloate from thin layer chromatography were also found. Both retentates and penicillin polymers, however, lacked  $\beta$ -lactam absorption at  $1780\text{ cm}^{-1}$  in their infrared spectra. It was concluded that the retentates consisted mainly of penicillin polycondensation products.

In order to assess the possible role of retentates and penicillin polymers in penicillin allergy, the study of their immunological properties with respect to anaphylactic antibody production in Wistar rats were undertaken. The results are shown in Table 1. IgE antibody production was measured using 24-h sensitization period for PCA reactions. Of the animals immunized with BPO-BSA, 66 per cent responded

TABLE 4  
*Histamine Release from Rat Mast Cells on Antigen Challenge.*

| Challenging ag.*<br>μg/ml | Immunizing ag.<br>Challenging ag.<br>BPO-BSA | Histamine release per cent                               |                                            |
|---------------------------|----------------------------------------------|----------------------------------------------------------|--------------------------------------------|
|                           |                                              | Immunizing ag.<br>Challenging ag.<br>Penicillin polymers | Normal cells<br>Challenging ag.<br>BPO-BSA |
| $5 \times 10^2$           | $3.80 \pm 0.20$                              | $2.50 \pm 0.10$                                          | $1.50 \pm 0.10$                            |
| $5 \times 10^1$           | $4.20 \pm 0.30$                              | $2.00 \pm 0.10$                                          | $1.30 \pm 0.05$                            |
| $5 \times 10^0$           | $6.50 \pm 0.20$                              | $1.50 \pm 0.10$                                          | $1.50 \pm 0.10$                            |
| $5 \times 10^{-1}$        | $8.80 \pm 0.50$                              | $1.60 \pm 0.10$                                          | $1.50 \pm 0.10$                            |
| $5 \times 10^{-2}$        | $4.80 \pm 0.10$                              | $1.00 \pm 0.05$                                          | $1.90 \pm 0.10$                            |
| $5 \times 10^{-3}$        | $3.20 \pm 0.50$                              | $1.00 \pm 0.05$                                          | $1.50 \pm 0.05$                            |
| 0.0                       | $0.80 \pm 0.10$                              | $1.60 \pm 0.05$                                          | $1.50 \pm 0.10$                            |

\* Ag. = Antigen.

with IgE antibody production. None of the animals immunized with either the retentates or the penicillin polymers produced detectable levels of IgE antibody. Similar results were obtained when sera from the immunized animals were tested using the 4-h sensitization period for PCA reactions. The retentates and the penicillin polymers were therefore not immunogenic. However, under the immunization procedure used in this study low levels of IgGa antibody were produced in rats immunized with BPO-BSA.

The specificity of the antibody induced in animals immunized with BPO-BSA was directed towards the penicilloyl group and not towards the carrier molecule. In Table 3, it can be seen that PPL acted as a good elicitor of PCA reactions in animals passively sensitized with these sera, while BSA did not elicit any reaction at all.

The possibility of penicillin polymers being elicitors of anaphylactic reactions in previously sensitized animals, while not being immunogenic, was also considered. For this purpose, animals were passively sensitized with the rat anti-penicilloyl sera and challenged in PCA tests with BPO-BSA, and penicillin polymers. The penicillin polymers could not elicit any PCA

reactions in animals sensitized in this manner towards the penicilloyl group. Individual sera as well as a pool of all positive sera were used to test this hypothesis. The results obtained with the pooled serum are shown in Table 3.

Another attempt was made to induce anaphylactic antibody production to penicillin polymers by using inbred Wistar-Furth rats. Histamine release from actively sensitized peritoneal mast cells on antigen challenge was used in these experiments as an indicator of antibody production (10, 20). Results are shown in Table 4; again only BPO-BSA released histamine from mast cells taken from animals immunized with BPO-BSA. The histamine release was antigen-concentration dependent, giving maximum release at  $0.5 \mu\text{g/ml}$ . Mast cells from animals immunized with penicillin polymers did not release histamine in excess of control values. Control experiments, in which normal rat mast cells were challenged with the antigen utilized for immunization consistently gave histamine release of the order of 1–2 per cent.

Penicillin polymers were further tested for their immunogenicity with respect to anaphylactic antibody production, using guinea pigs. Serial immunizations and bleedings were performed according to the schedule outlined under "Materials and Methods". Individual sera and bleedings were tested using the PCA test, two sensitization periods (4 h to detect  $7S\gamma_1$  antibody and 7 days to detect antibodies persisting longer in the skin) being employed. None of the animals responded with production of either type of antibodies even at the end of 25 weeks of immunization.

## DISCUSSION

In the present study the immunogenicity of high molecular weight impurities present in benzylpenicillin preparations marketed in Canada was investigated. The composition of dialysis retentates was similar in all samples studied (19) and we chose three for immunological studies. Furthermore, since the main component of the penicillin retentates was penicillin

polymers some of the immunological studies were done only with the latter compounds.

BPO-BSA was shown, in the present study, to be immunogenic in rats with respect to anaphylactic antibody production. Of the animals immunized with this compound 66 per cent produced both IgE and IgGa antibodies. The specificity of the antibodies was directed towards the haptenic group and not towards the carrier molecule. The specificity was examined by using PPL, which acted as a good elicitor of PCA reactions in animals sensitized with homologous anti-BPO-BSA serum, and BSA, which did not elicit any reactions at all in similar experiments.

Penicillin polymers and retentates were non-immunogenic in Albino Wistar rats with respect to anaphylactic antibody production. Penicillin polymers were similarly non-immunogenic in both Wistar Furth rats and Hartley guinea pigs. Furthermore, penicillin polymers were non-elicitors of PCA reactions in Albino Wistar rats passively sensitized with rat anti-BPO-BSA sera (Table 3).

Penicillin preparations currently marketed in Canada are purified to the extent that they contain insignificant or non-detectable amounts of protein. On the other hand, all penicillins tested contained significant amounts of penicillin polymers. Penicilloylated proteins have been clearly established to be capable of producing hypersensitivity reactions in man and also of inducing the production of anaphylactic antibodies in experimental animals. Therefore because penicillin polymers were unable to produce anaphylactic antibodies in experimental animals, a similar non-immunogenic effect in humans would also be expected.

#### SUMMARY

Retentates from benzylpenicillin preparations marketed in Canada were used in immunological studies in order to determine if they were immunogenic in experimental animals with respect to anaphylactic antibody production. The retentates obtained consisted mainly of penicillin polymers. Im-

purities of protein nature were either insignificant or absent. The penicillin polymers were found to be non-immunogenic in Albino Wistar rats, Wistar Furth rats and Hartley guinea pigs. Furthermore, they were unable to effect positive PCA tests in rats passively sensitized to the penicilloyl determinant.

*Note added in proof:* We have just noted the report by Munro et al. (Int. Arch. Allergy 50, 192-205, 1976) in which they find that purified polymers isolated from 6-aminopenicillanic acid and from semisynthetic penicillins and cephalosporins could not induce the formation of specific antibodies in baboons, guinea pigs and rabbits. They showed, however, that such polymers could elicit reactions when given intravenously to passively sensitized guinea pigs. In this respect polymers isolated from benzylpenicillin were the least reactive. In our study polymers from benzylpenicillin could not elicit such reactions when given to passively sensitized rats.

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