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PRODUCTION OF ANAPHYLACTIC ANTIBODIES TO DRUGS IN EXPERIMENTAL ANIMALS

I. Benzylpenicillin

By

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Drugs implicated in hypersensitivity reactions are numerous, but in most cases neither the form or forms of the drug that induce hypersensitivity reactions are known, nor have anaphylactic antibodies with specificity directed to drug determinants been demonstrated in sera of patients suspected of having undergone such reactions (4). One of the notable exceptions is benzylpenicillin (PG). Both the identity of the active forms of the drug and the production of antibodies with specificity towards the drug have been established (6).

Drugs, mostly in a conjugated form, have been used to produce antibodies to the drug moiety in experimental animals. This, however, is not a natural exposure and, in addition, one is actually selective in inducing antibody production to certain determinants. Antibodies produced in this manner belong, in the majority of the cases, to the IgG class and are not associated with hypersensitivity reactions. Such antibodies have found extensive use in radioimmunoassays (3, 12) for bioavailability studies.

We were interested to find out if animals could produce anaphylactic antibodies to drug determinants when they were exposed to a drug in as natural a way as possible. Thus, animals were either fed or immunized with the free drug.

In the initial phase of this study we looked for drugs that

fulfilled two main criteria: 1) their association with hypersensitivity reactions in man had to be well established; and 2) some of the allergenic determinants should be known, in order to facilitate specific antibody detection. As PG fulfilled these criteria, we started our investigation with this drug. The present paper describes our findings with this antibiotic, and indicates that anaphylactic antibodies to drug determinants can be produced in animals as a result of oral feeding or immunization with the free drug.

MATERIALS AND METHODS

Albino Wistar male rats (Woodlyn Farms, Guelph, Ontario, Canada), weighing between 200–250 g, were used for immunization. Similar rats were used for the collection of peritoneal mast cells, as well as as a source of normal rat serum (NRS). Female guinea-pigs (High Oak Ranch, Goodwood, Ontario, Canada), weighing between 300–350 g, were used to obtain the ileum for histamine bioassay.

Benzylpenicillin, sodium salt, (PG), penicilloylpolylysine (PPL) and bovine serum albumin (BSA) were obtained from Sigma Chemical Company (St. Louis, Mo., U.S.A.).

Atropine sulfate was a product of Merck, Sharp and Dome Canada Ltd. (Kirtland, Québec, Canada) and tripelenamine hydrochloride was obtained from CIBA Pharmaceuticals (Dorval, Québec, Canada). p-Chloromercuribenzoic acid was a product of BDH Chemicals (Montreal, Canada).

Medium 199-10X was purchased from Grand Island Biological Company (Grand Island, N.Y., U.S.A.), and pertussis vaccine from Connaught Medical Research Laboratories (Toronto, Canada). Sephadex G-25, coarse, was a product of Pharmacia Fine Chemicals (Uppsala, Sweden).

All buffers were made from reagent grade compounds.

Immunization: Groups of six rats were immunized with PG. Animals in Group I were injected with 1 mg PG (solution in saline) plus 7×10^9 *B. pertussis* organisms intraperitoneally. Animals in Group II were injected with 1 mg PG and 10 mg alum subcutaneously. Twelve days later, the animals were bled and their sera were pooled per group. The sera were kept in the refrigerator for initial testing, then aliquotted in 1 ml samples and stored at −70° C for further tests.

Feeding schedule: Groups of six rats were fed PG for 30 days in addition to their normal diet. PG was added in their drinking water at the required concentration. Animals in Group III had access to drinking water containing 10 mg PG per liter. For animals in Group IV the concentration of PG in their drinking water was 1 mg per liter. The consumption of PG per rat per day averaged 380 μg in Group III and 36.5 μg in Group IV. Sera obtained from

these animals were pooled per group and kept as indicated under 'Immunization'.

Collection of peritoneal rat mast cells: Peritoneal rat mast cells were collected as previously described (7) from either normal rats or rats injected intraperitoneally 24 hours earlier with the test sera. The cells were finally suspended in Tris-ACM buffer (8). The cell suspension used for a test consisted of cells that were obtained from two rats and were brought to 1.5 ml with buffer.

Detection of IgGa antibody: Rat mast cell suspension (0.1 ml), PPL at a concentration of penicilloyl groups ranging from 1×10^{-8} to 5×10^{-4} M (0.1 ml) in Tris-ACM buffer and test serum (0.1 ml) were incubated for 15 min at 37° C with mild shaking. The incubation mixtures were brought to 0.5 ml with buffer and then centrifuged in the cold for 5 min at 300 *g*. The supernatants were kept frozen overnight for histamine determination. Two controls were run simultaneously: 1) the test sera were replaced by NRS (to determine if antigen alone released any histamine); and 2) the antigen was replaced by buffer (to determine if the test sera alone released any histamine). Both controls gave comparable results. For the determination of total histamine, 0.1 ml of the cell suspension was brought to 0.5 ml with buffer, and the suspension boiled in a water bath for 5 min. After centrifugation at 750 *g* for 30 min, the supernatant was frozen overnight before determining histamine content. When PG was used as antigen for challenge, its concentration varied between 0.1–6.0 mg/ml.

Detection of IgE antibodies: For the detection of IgE antibodies, rat mast cells sensitized passively *in vivo* were used. *In vivo* passive sensitization was accomplished by injecting rats intraperitoneally with the test sera (0.3 to 1.0 ml) and collecting the sensitized cells 24 hours later. The cells were challenged with the antigen in the presence of NRS (1/3 in volume). A number of experiments were run in the absence of NRS in order to determine the effect of NRS on histamine release. Controls consisted of 1) sensitized cells treated as the experimentals but the antigen was replaced by buffer to determine spontaneous histamine release; and 2) non-sensitized cells which were challenged with the highest concentration of antigen, in order to determine if antigen alone released any histamine from cells. All other conditions were similar to those described under 'Detection of IgGa antibodies'.

Histamine determination: Histamine was determined using the atropinized guinea-pig ileum (1). Animals were killed by head trauma. Intestinal sections about 27 mm in length were bathed in Krebs-Henseleit solution at 38° C in a bioassay apparatus. Atropine sulfate was routinely added to the bathing solution at a final concentration of 5×10^{-7} M. Appropriate concentrations of histamine standards were added to the bath, the capacity of which was 10 ml. Contractions elicited by standards and experimentals were recorded with MFE Angle to voltage transducer (John DeGroot Associates, Scarborough, Ontario, Canada). The histamine contents of tests and controls were calculated from a standard curve. Contractions due to the material released from sensitized rat mast cells upon specific challenge were inhibited by the addition of tripelennamine hydrochloride (pyribenzamine), confirming its identity with histamine.

Isolation of high molecular weight contaminants from PG: PG (25 g) was dissolved in double-distilled water, and dialysed at 4° C against water for 7 days, with changes of water every 24 hours. The retentate was then freeze-dried. The dry material was dissolved in 5 ml of buffer (Tris-HCl, 0.01 M, pH 7.5) and chromatographed on a Sephadex G-25 column (2.5 × 45 cm) at 4° C. Fractions eluted from this column were freeze-dried. Before analysis and testing, they were dissolved in 4 ml of the same buffer. Fractions were analysed for penicilloyl content using the penamaldate test (5).

RESULTS

Histamine release from rat mast cells was used throughout this study for the detection of anaphylactic antibodies. Fig. 1 shows a typical histamine bioassay using the guinea-pig ileum. In the extreme right, histamine standards are depicted, followed by total histamine (T.H.) in 1 :4 dil., controls (C) and experimentals (IgE antibody, PPL antigen). The numbers in brackets correspond to the percent histamine release upon antigen challenge (at the molar concentrations indicated).

Detection of penicilloyl-specific IgE antibody: Our first attempt was aimed at determining if sera obtained from rats exposed to PG contained IgE-type antibodies with specificity directed towards the penicilloyl group. Fig. 2 indicates the results. Control values have been subtracted from the experimental values. All sera had easily-detectable levels of IgE antibody to the penicilloyl determinant.

Sera from Group I animals (i.p. immunization) and from Group III animals (receiving 380 μg PG per day orally) appeared to give the best results, followed by sera from Group II (s.c. immunization) and Group IV (receiving 36.5 μg per day orally) animals.

The histamine release in all these experiments was antigen concentration dependent, and the maximum histamine release was obtained at penicilloyl concentration of 1×10^{-6} to 1×10^{-5} M; with the exception of the serum from Group IV animals which gave maximum histamine release at higher penicilloyl molarity.

Table 1 shows the reproducibility of our results. The same

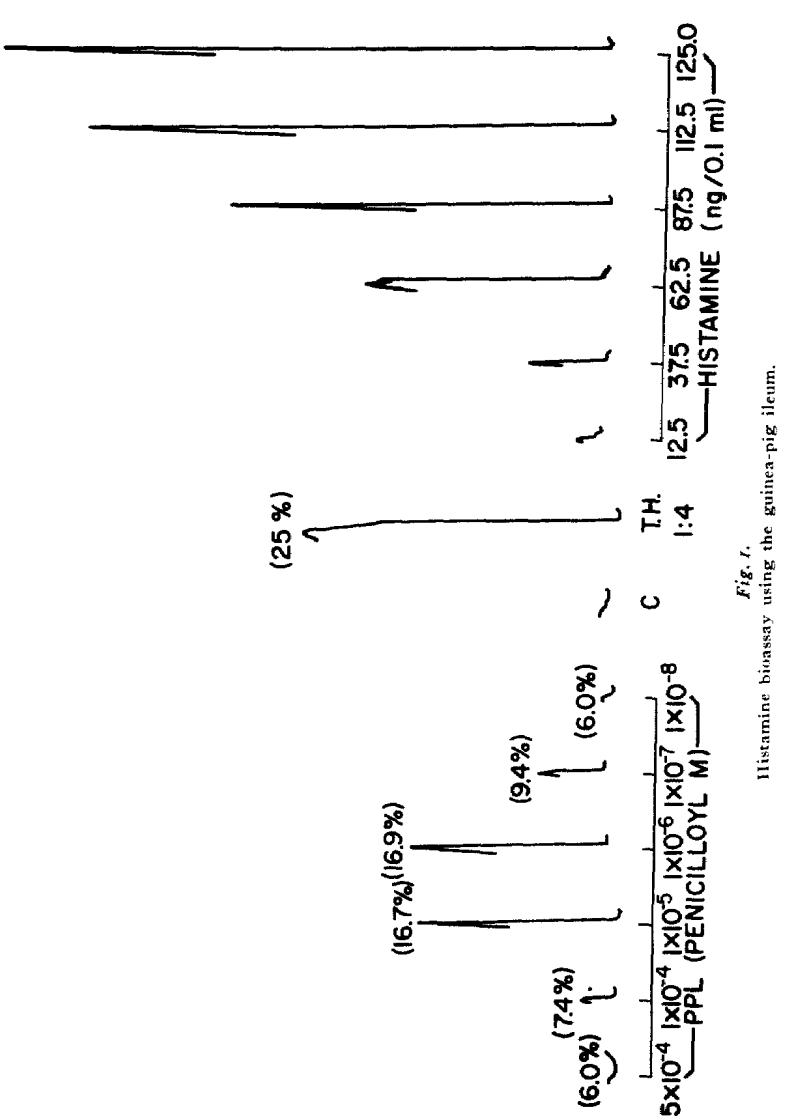

Fig. 1.
Histamine bioassay using the guinea-pig ileum.

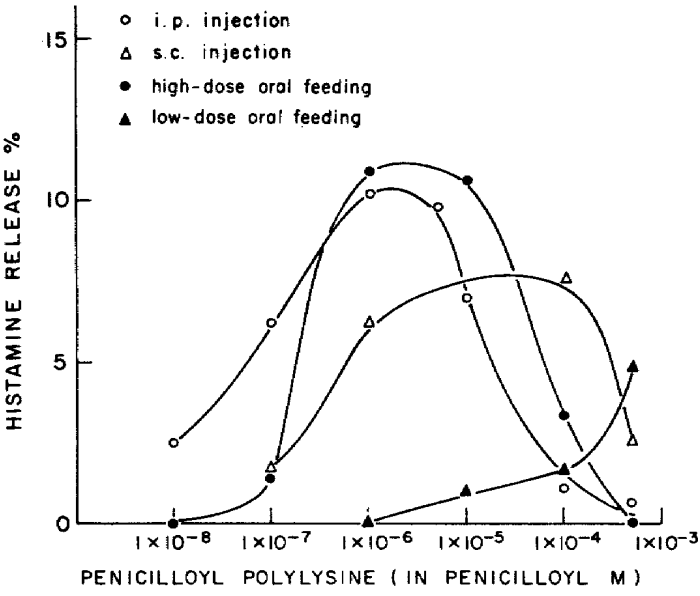

Fig. 2.

Histamine release from *in vivo* passively sensitized rat mast cells with the four test sera and challenged with PPL at the molar concentrations indicated.

TABLE 1

Reproducibility of IgE Antibody Detection (i.p. pool) using PPL as Antigen but Different Animals to obtain Sensitized Cells.

PPL in penicilloyl molarity	Histamine release %		
	Exp. 1	Exp. 2	Exp. 3
1×10^{-4}	7.1	7.0	4.2
1×10^{-5}	13.0	–	10.3
1×10^{-6}	16.3	7.2	7.5
1×10^{-7}	10.3	5.7	8.6
1×10^{-8}	8.5	5.3	1.6
0.0	6.0	1.8	2.4
1×10^{-4} (non-sensitized cells)	–	2.0	1.2

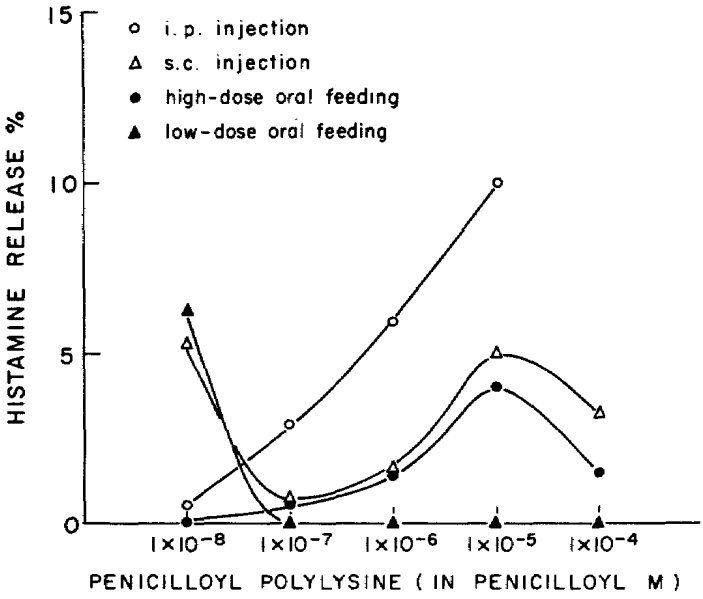

Fig. 3.
Histamine release from rat mast cells in the presence of the four test sera and PPL at the molar concentrations indicated.

test serum (i.p. pool) was used several times for *in vivo* passive sensitization of rat mast cells. There is variation between different experiments as one deals with different animals. The results, however, are reproducible, as on challenge with the optimum concentration of antigen the sensitized cells released histamine anywhere from 2.5 to 4.5 times higher than the controls.

Detection of penicilloyl-specific IgGa antibodies: Experiments were carried out to detect the presence of IgGa antibody in the same sera. All sera tested had IgGa antibodies (Fig. 3), although the optimum penicilloyl concentration varied considerably. Serum from Group I animals (i.p. injection) gave the best results in terms of histamine release. Sera

from animals in the other three groups gave lower values for histamine release, which was antigen concentration dependent.

Detection of 'benzylpenicillin'-specific anaphylactic antibodies: In addition to PPL, PG was also used as antigen for challenge. Table 2 summarizes the results and indicates that PG released histamine in both tests designed to detect IgE and IgGa antibodies. In all cases, histamine release was antigen concentration dependent, and the optimum antigen concentration varied between 1 and 6 mg per ml (1×10^{-3} to 1×10^{-2} M).

TABLE 2

Detection of IgE and IgGa Antibodies using Benzylpenicillin as Antigen.

Sera from treated animals	Type of antibody detected	Histamine release % Benzylpenicillin concentration in mg/ml				
		0.0	6.0	3.0	1.0	0.5
I	IgE	1.3	5.5	8.5	4.6	3.8
II	IgE	1.6	5.0	4.6	5.5	1.6
III	IgE	6.0	6.0	12.2	11.2	6.0
IV	IgE	0.0	1.6	6.6	6.4	0.9
I	IgGa	1.2	3.7	4.8	4.2	1.3
II	IgGa	4.1	6.8	9.0	7.2	4.7
III	IgGa	1.7	7.7	3.3	2.4	2.9
IV	IgGa	0.0	0.0	5.2	0.0	0.0

Effect of NRS on histamine release: In most of the experiments reported in this communication, NRS was present in the test designed to detect IgE antibody, as this was found generally to lower the spontaneous histamine release. A number of experiments were performed in the presence and absence of NRS in order to study if this had any effect on histamine release upon antigen challenge. Slightly higher values were obtained in the presence of NRS (Table 3), but NRS was not a requirement for histamine release.

Isolation of high molecular weight contaminants in PG preparation: To determine if high molecular weight contami-

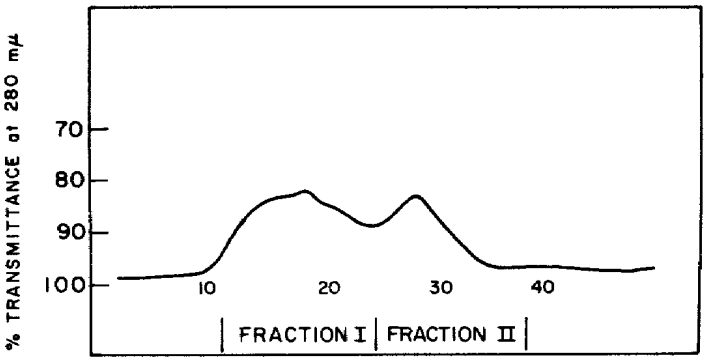

Fig. 4.
Fractionation of PG retentate on Sephadex G-25 column.

TABLE 3
Effect of NRS on Histamine Release from Sensitized Cells on Challenge with PPL.

PPL in penicilloyl molarity	Histamine release in ng/0.1 ml	
	Buffer	NRS
1.2×10^{-4}	161.0	118.0
1.2×10^{-5}	156.0	291.0
1.2×10^{-6}	193.5	296.0
1.2×10^{-7}	257.0	237.0
1.2×10^{-8}	141.0	221.0
0.0	88.5	76.0
1.2×10^{-4} (non-sensitized cells)	30.0	41.5

nants of PG were involved in antigen-antibody reactions, the retentate obtained after dialysis of PG was fractionated on a Sephadex G-25 column. Two fractions were obtained from this column (Fig. 4). Both fractions after concentration were found to contain penicilloyl determinants (1.3×10^{-5} M in Fr. I and 5.6×10^{-6} M in Fr. II). These fractions were used as antigens for challenge in the system measuring IgE antibody with serum from Group I animals, and were found un-

able to release histamine from rat mast cells. They were tested at various penicilloyl concentrations down to 1×10^{-8} M.

DISCUSSION

The results reported in this publication indicate that anaphylactic antibodies were produced in rats that were either immunized with the free drug or fed benzylpenicillin orally. Experiments measuring IgE antibodies to penicilloyl determinant showed that this antibody was induced in all groups of animals used in this study. Biological systems like the one used in this study cannot measure the quantity of antibody present in the sera, but from these results it appears that i.p. immunization with PG and *B. pertussis* vaccine, as well as oral feeding at 380 μg per day for 30 days, were more efficient in inducing IgE antibody production than s.c. immunization of PG with alum or oral feeding at 36.5 μg PG per day for 30 days. Histamine release upon challenge with the antigen (PPL) *in vitro* was not very high, but significantly higher than control values, and reproducible. Considering that rats were exposed to the free drug and that pooled sera were used for testing, one could hardly expect to obtain sera with high IgE antibody content. The interesting point, however, is that this type of antibody was produced in experimental animals not only after immunization but also after oral feeding.

The histamine release from *in vivo* passively sensitized cells on challenge with PPL was antigen concentration dependent (Fig. 2). Optimum penicilloyl concentrations varied with each serum tested. One might be tempted to draw the conclusion that the optimum antigen concentration for each serum tested is related to its antibody content. The results, however, might have been influenced not only by the antibody content but also by the affinity of the antibody for rat mast cell receptors. The fact that different animals were used for each serum tested has introduced another variable that might have affected the results.

IgGa antibodies to penicilloyl determinant were also pre-

sent in all sera tested (Fig. 3). The titers, however, of these sera appeared to be low, which is expected particularly for sera from Group I and Group II animals. The immunization schedule was not favourable for the production of this type of antibody. The optimum penicilloyl concentration varied considerably among the four pooled sera tested. Histamine release was low but reproducible.

In addition to PPL, PG was also used as antigen for challenge in both systems detecting IgE and IgGa antibodies. It was found that PG at high concentrations (1.0 to 6.0 mg per ml (1×10^{-3} to 1×10^{-2} M)), was capable of releasing histamine from sensitized or from normal rat mast cells in the presence of these sera (Table 2). These results are somewhat unexpected as PG is a hapten. It has been shown repeatedly, however, that PG releases histamine in our system. Histamine has also been released from sensitized human leucocytes on challenge with PG (13). It is a well-known fact that PG is an extremely active molecule and undergoes a number of transformations. It is conceivable that during incubation PG, or rather degradation products of PG, could react with either serum components or BSA that is present in Tris-ACM buffer, or possibly polymerize to form a polyvalent hapten. There is also the possibility to be considered that PG binds to cells and thus becomes polyvalent. The fact that quite high concentrations of the drug are required to elicit a response could be taken as an indication that the drug undergoes some transformation before being capable of reacting with antibodies in our system.

It has been reported that preparations of PG are contaminated by either proteinaceous material not related to PG or some forms of polymers derived from PG (9). Thus the possibility existed that contaminants in PG could be responsible not only for the antigen-antibody interaction but also for antibody induction in our animals. This, however, was not the case. The retentate of PG after extensive dialysis was chromatographed on Sephadex G-25 column, and two fractions eluted from this column (Fig. 4) gave a positive penamaldate

reaction (5), indicating the presence of penicilloyl groups in these fractions. These two fractions, when tested in our system at penicilloyl concentrations similar to PPL that gave positive histamine release, were found inactive in releasing histamine from sensitized rat mast cells with the serum from Group I animals. We do not know their structure, except that they have penicilloyl groups. The serum used for testing contained antibodies with penicilloyl specificity, but if the penicilloyl groups in these fractions are not exposed but hidden, they do not necessarily react with the antibody. These results are in agreement with De Weck's findings (2) that polycondensation products arising from PG are not immunogenic.

Another interesting point is that rats fed PG produced anaphylactic antibodies. Oral immunization of animals is not a common practice for inducing antibody production. Rothberg et al. (11), however, demonstrated that the qualities of antibodies present in the sera from orally immunized rabbits were similar to the antibodies from parenterally immunized rabbits using BSA as antigen. Furthermore, it is a well-known fact that oral administration of PG does sensitize people to this drug (4). In the present study, as well as in another study (10) in which rats were kept on low dose, long oral exposure to PG, anaphylactic antibodies have been produced to drug determinants. It is obvious that more work has to be done in this area to understand the mechanism of oral immunization, considering that food allergies and drug hypersensitivity reactions occur in human as a result of oral immunization.

This study has indicated that this approach to studying anaphylactic antibody production to drug determinants using experimental animals was successful. In view of the low histamine release obtained in this study, future studies may require the development of a more sensitive technique for the detection of this type of antibody.

SUMMARY

Studies were undertaken to determine if animals exposed to benzylpenicillin could produce anaphylactic antibodies to the drug moiety. For this purpose, Albino Wistar rats were divided into four groups. Two groups of animals were immunized with benzylpenicillin using either the intraperitoneal or the subcutaneous route. The remaining two groups were used for feeding experiments. The animals were bled upon termination of the experiments; their sera were collected and pooled per group. The pooled sera were tested for the presence of anaphylactic antibodies. Histamine release from rat mast cells under the appropriate conditions was used as an indicator of antibody production. Penicilloylpolylysine and benzylpenicillin were both employed to determine antibody specificity. It was found that sera from all groups of animals contained anaphylactic antibodies with specificity towards the penicilloyl group and 'benzylpenicillin'.

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