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Tests for Penicillin Allergy in Man

I. CARRIER EFFECT ON RESPONSE TO PENICILLOYL CONJUGATES

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Summary. The value of using benzylpenicilloyl (BPO) conjugates rather than benzylpenicillin (B.Pen.) itself in skin tests and in *in vitro* diagnostic tests for penicillin allergy in man is assessed. The effect of various carriers on the outcome of these tests has also been investigated in order to find the most appropriate. Skin tests with B.Pen. and BPO conjugates (with polylysine, PL, and human serum albumin, HSA) in penicillin allergic patients were positive in 36 per cent and up to 50 per cent respectively. The two carriers used were equally effective. Negative results were obtained in the non-allergic control subjects. For *in vitro* studies two tests were selected on the basis of their well established value, the lymphocyte transformation test (LTT) and histamine release from sensitized leucocytes (HRL). In the HRL test BPO conjugates with PL, HSA, bovine serum albumin (BSA) and bovine gamma globulin (BGG) were also compared with B.Pen. The BPO conjugates were all more effective than B.Pen. and the proportion of patients giving positive results with these conjugates was much higher than with B.Pen. (up to 86 per cent compared with 29 per cent). The rank order of effectiveness of the various carriers as judged from maximal histamine release by various penicilloyl conjugates was PL<BSA<HSA<BGG, BGG being the most effective. In the LTT, where BPO:PL, BPO:HSA, BPO:BGG as well as BPO human gamma globulin have been used, the BPO conjugates were also more effective than B.Pen. but the difference was relatively less marked than in HRL test (positive results being obtained with conjugates in up to 92 per cent of patients as compared with 57 per cent with B.Pen.). The rank order of effectiveness of the carriers in the LTT, as judged by comparing the maximum response obtained with each BPO-protein conjugate with the maximum response obtained with BPO:PL, was PL<HSA<HGG<BGG, BGG being the most effective. However, BPO:HGG was effective in lower concentrations. The HRL and LTT were negative in nine out of ten non-allergic subjects, and in control experiments with the carrier molecules alone.

INTRODUCTION

It is generally believed that in order to induce an allergic response to any simple chemical such as penicillin, the drug itself or, more commonly, its metabolites must first

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combine irreversibly with some large carrier molecule such as a protein (Assem, 1967). It is, therefore, important to know whether the antibodies formed are specific also towards the carrier protein molecule. Levine (1962, 1963) has shown that when rabbits and guinea-pigs are immunized with a benzylpenicilloyl–protein conjugate then the antibodies formed are adapted to the entire benzylpenicilloyl group, the lysine side chain and to some adjoining structures of the immunizing carrier protein. The identification of the protein with which penicillin or its metabolites combines *in vivo* would be extremely useful since it could lead to the preparation of the correct complete antigen for use in *in vitro* diagnostic tests. Levine and Price (1964) have compared penicilloyl polylysine (BPO:PL), penicilloyl human serum albumin (BPO:HSA) and penicilloyl human gamma globulin (BPO:HGG) as elicitors of the weal and flare reaction in patients with a past history of penicillin allergy. They found that BPO:PL was more effective than were BPO:HSA and BPO:HGG. This was probably due to the open structure of polylysine which would allow more contact between benzylpenicilloyl groups and the large anti-benzylpenicilloyl combining sites than would be allowed by the more rigid structural configuration of the heterologous benzylpenicilloyl–protein conjugates.

We have used benzylpenicilloyl conjugates with PL, HSA, HGG, bovine serum albumin (BSA) and bovine gamma globulin (BGG). The effectiveness of BPO:PL and BPO:HSA in eliciting a weal and flare response in penicillin allergic patients has been studied and the response compared with that of benzyl penicillin (B.Pen). In general skin testing in drug allergy, even when hapten–macromolecule conjugates were used, was found to be unreliable, and was therefore of little diagnostic value (Assem and Vickers, 1972). It also allows only a limited range of carriers to be tested since only non-antigenic macromolecules can be used. Furthermore, even when homologous or autologous proteins are used as carrier molecules, these proteins may be denatured during the preparation and storage of conjugates, and they may become antigenic. Therefore, one would hesitate to use proteins such as HGG for testing because of the potential risk of inducing the formation of antibodies against HGG.

Since skin tests have such serious limitations, and since they are only semi-quantitative, we have used in addition two reliable *in vitro* tests for allergy which do not have these limitations, namely the release of histamine from sensitized leucocytes and the lymphocyte transformation (stimulation) test (LTT). These tests have been used to study the relative effectiveness of penicilloyl conjugates with various proteins and with polylysine in eliciting an allergic response.

MATERIALS AND METHODS

Patients

A highly selected group of fourteen penicillin allergic patients was studied. All these patients had clinically well-documented allergy and most of them had previously given positive responses to B.Pen. and/or BPO:PL in the *in vitro* tests (histamine release from sensitized leucocytes and passive sensitization of human lung). The main aim of this work was to assess the relative diagnostic value of various antigen preparations; to compare B.Pen. with penicilloyl conjugates with various carriers. It was thus essential to use patients in whom allergy to penicillin could not be disputed.

In addition to this allergic group ten subjects with no personal or family history of penicillin allergy were tested as controls.

Antigens

Benzylpenicillin as the sodium salt Crystapen was obtained from Glaxo Laboratories, Greenford.

Benzylpenicilloyl–polylysine, BPO:PL, was obtained from Sigma Chemical Company, London. The concentration of BPO:PL is expressed in terms of the molarity of the penicilloyl group but no information is freely available as to the number of lysine residues in the polylysine chain or the number of penicilloyl groups per polylysine chain. The manufacturers recommend reference to papers by Parker (1963), Brown, Price and Moore (1964) and Rytel, Klion, Arlander and Miller (1963) for further information about their product. Assuming that BPO–polylysine prepared by Sigma was similar to the preparation in the latter reports, the average number of lysine residues is probably twenty and the average degree of substitution by penicilloyl is between twelve and fifteen groups per molecule, i.e. BPO_{12-15} L_{20}.

The other conjugates, i.e. BPO:HSA, BPO:BSA, BPO:HGG and BPO:BGG were prepared by direct reaction of B.Pen. with the protein at high pH (Parker and Thiel, 1963). Human gamma globulin (HGG) was purified by workers at the Lister Institute and was thought to be largely IgG.

100 mg of protein was dissolved in a minimum of distilled water and 1 ml 1M carbonate buffer, pH 10·4, plus 1·2 grams B.Pen. were added with mixing. The pH was then adjusted to 9·6 with 5 N sodium hydroxide. At 16 and 24 hours after this initial reaction additional 600 mg quantities of B.Pen. were added, the pH being adjusted to 11·0 with 5 N sodium hydroxide after each addition. After the final addition the solution was left for at least 6 hours before purification.

The conjugates were purified by dialysis against 0·01 M phosphate buffer, pH 8·0, and then against distilled water. The preparations were then freeze-dried before estimation. The penicilloyl group was estimated using the penamaldate assay (Levine, 1962). The assay is based on measurement of the difference in absorbance at 285 nm of the penicilloyl–protein conjugate before and after the addition of *p-o*-hydroxymercuri-benzoate, POHMB. The POHMB complexes with the penicilloyl group and the penamaldate group is formed. Differences in absorbance were determined for several known concentrations of the penicilloyl group and a standard curve was plotted. The penicilloyl concentration in the test samples could then be determined from this curve.

The number of penicilloyl groups per molecule of protein was then calculated as: benzylpenicilloyl human serum albumin BPO_{14} HSA; benzylpenicilloyl human gamma globulin BPO_9 HGG; benzylpenicilloyl bovine serum albumin BPO_{11} BSA; benzylpenicilloyl bovine gamma globulin BPO_{12} BGG.

Skin tests

Skin tests were carried out by intradermal injection of 0·02 ml of antigen solution. B.Pen. was given in concentrations of 1·6, 16 and 160×10^{-5} M; BPO:PL and BPO:HSA in concentrations of 1, 5 and 25×10^{-5} M with respect to the penicilloyl group. The diameters of the weal and flare reactions were measured after 15 minutes and patients were asked to report any reactions which might develop later. Skin responses occurring after the first 2 hours were classified as late reactions. These reactions were not necessarily typical delayed-type hypersensitivity reactions. The reactions were graded as follows: (diameter of weal (W) and flare (F) in mm) F<10, W<5 = − ; F10–20, W5–10 = + ; F20–30, W10–15 = ++; F30–40, W15–20 = +++; F>40, W>20 = ++++.

The release of histamine from sensitized leucocytes

This test is an *in vitro* correlate of immediate type allergy, and measures histamine released as a consequence of reaction between allergen and reaginic antibody fixed to basophil leucocytes.

The procedure used to isolate the leucocytes was that described by Assem and McAllen (1970), which is a modification of the technique used by Lichtenstein and Osler (1964). Leucocytes were isolated from heparinized venous blood and challenged with antigen or with Tyrode (as a control). B.Pen. was used in concentrations of 1·6, 16 and 160×10^{-5} M and the BPO–protein conjugates were used in concentrations of 1, 5 and 25×10^{-5} M with respect to the penicilloyl group. The histamine released and the residual cell histamine were measured by bioassay on guinea-pig ileum (Assem and Schild, 1968), and the released histamine calculated as a percentage of the total histamine (released + residual). A positive result was taken as a percentage histamine release from the antigen challenged leucocytes, which was at least twice that from the Tyrode-challenged leucocytes.

The lymphocyte transformation (stimulation) test

The LTT may give positive results in cases of immediate-type or delayed-type allergy. It is valuable in the diagnosis of drug allergy since the allergic reaction of many patients is mediated by a variety of immunological mechanisms (Assem and Vickers, 1972). Furthermore, positive results are frequently obtained with the drug itself, i.e. without the need for preparation of drug–macromolecule conjugates.

The technique used to separate lymphocytes from peripheral blood was that described by Coulson and Chalmers (1967) and DNA synthesis was measured as described by Chalmers, Cooper, Coulson, Inman and Topping (1967). The lymphocytes were suspended in tissue culture medium 199 (Wellcome Laboratories) containing 10 per cent autologous serum and divided into 2·7 ml aliquots. Antigen or medium (0·3 ml) was added, and then the cells incubated for 4 days at 37°. B.Pen. was used in final concentrations of 1·6 and 16×10^{-5} M and the BPO–conjugates were used in final concentrations of 1, 5 and 25×10^{-6} M with respect to the penicilloyl group. After the incubation the incorporation of [^{3}H]thymidine, over a 1-hour period, was measured.

This test was considered positive when the counts per minute (cpm) of the antigen challenged samples were significantly higher than the cpm of the control samples. Four samples of each type were prepared, and the significance of the difference between various treatments calculated from Student's *t*-test.

RESULTS

SKIN TESTS

Positive skin reactions (immediate and/or late) were obtained only in eight of the fourteen patients (Table 1) even when benzylpenicillin as well as the penicilloyl conjugates were used. The result tabulated is the maximum response obtained for each patient. Several doses of each antigen were tested and generally a maximum response could be obtained with 16×10^{-5} M B.Pen., 5×10^{-5} M BPO:PL and 5×10^{-5} M BPO:HSA. In one patient positive reactions were obtained to B.Pen. in the absence of a response to either BPO:PL or BPO:HSA, and in three patients positive reactions to one or both of these conjugates were found in the absence of a B.Pen. response. More positive results were obtained with BPO:HSA (7/14) than with either B.Pen. (5/14) or BPO:PL (5/14) (Table 1) but the

TABLE 1

SKIN TESTS TO BENZYLPENICILLIN, BENZYLPENICILLOYL POLYLYSINE AND BENZYLPENICILLOYL HSA IN PENICILLIN ALLERGIC PATIENTS

Patient	B. Pen.		BPO:PL		BPO:HSA.	
	Immediate	Late	Immediate	Late	Immediate	Late
S.A.	−	−	−	−	−	−
J.C.	−	+++	−	−	−	−
R.C.	−	−	−	++++	−	+++
C.C.	−	−	−	−	−	−
G.I.	−	−	−	−	−	−
F.M.	−	−	++	++	+	+
M.R.	−	+	−	++	−	+
J.S.	−	−	−	−	−	−
S.S.	++++	−	−	−	++++	−
N.S.	+	−	−	−	++	−
C.T.	−	−	−	−	−	−
E.W.	−	−	+++	++++	++++	−
A.W.	−	−	−	−	−	−
D.S.	++++	−	++++	−	++++	−
Number +ve	3/14	2/14	3/14	4/14	5/14	3/14
Percentage +ve	22	14	22	29	36	22

The results are expressed as follows: diameter of weal (W) and flare (F) in mm. F = <10 W = <5 = 1; F 10–20 W 5–10 = +; F 20–30 W 10–15 = ++; F 30–40 W 15–20 = +++; F>40 W>20 = ++++.

difference is not significant considering the small number involved. No positive responses were obtained in the ten controls tested.

In some patients with severe anaphylactic reactions skin testing can elicit very strong reactions with small concentrations of allergen. One patient (S.S.) who experienced an anaphylactic reaction to penicillin 20 years previously, and has since received no further penicillin therapy, gave a positive response to as little as $0{\cdot}6 \times 10^{-8}$ M BPO:HSA (Fig. 1).

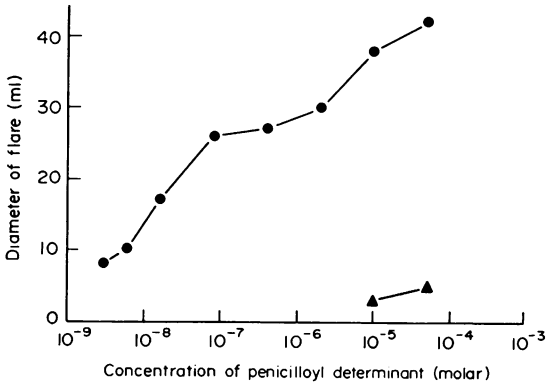

FIG. 1. Weal and flare reaction to intradermal injection of different concentrations of BPO:HSA and BPO:PL in a penicillin allergic patient. (●) Penicilloyl–HSA. (▲) Penicilloyl–polylysine.

Skin responses occurring after the first 2 hours were classified as late reactions. Five patients showed this type of response but only one (RC) seemed to develop a classical 'delayed-type hypersensitivity' reaction which reached its peak after 24 hours. In the other four patients the reaction reached its peak within 2–12 hours.

B.Pen. gave a lower number of positive results than did any of the BPO–protein conjugates tested (Table 2). Only 29 per cent of the penicillin allergic patients gave positive results when tested with B.Pen. compared with 71 per cent with BPO:PL, 79 per cent with BPO:HSA, 86 per cent with BPO:BGG and 70 per cent with BPO:BSA. All the patients who responded to B.Pen. also responded to the BPO protein conjugates. Dose response curves were obtained for each antigen in all patients tested (three examples in

TABLE 2

LEUCOCYTE CHALLENGE TEST IN PENICILLIN ALLERGIC PATIENTS WITH PENICILLIN AND PENICILLOYL–PROTEIN CONJUGATES

Patient	Percentage histamine release *					
	Control	P.Pen	BPO:PL	BPO:HSA	BPO:BSA	BPO:BGG
S.A.	5·2	4·7	8·8	8·0	ND	9·1
J.C.	2·5	1·0	20·8	7·5	ND	20·0
R.C.	1·8	3·4	11·7	13·1	9·0	16·2
C.C.	3·0	2·4	8·2	10·3	7·9	12·2
G.I.	0·7	2·4	5·9	3·0	ND	4·4
F.M.	1·3	1·6	3·8	5·4	3·4	6·8
M.R.	2·3	10·1	15·1	11·2	13·0	13·6
J.S.	2·8	3·9	3·6	8·9	6·7	17·2
S.S.	6·9	23·5	14·3	14·7	7·6	16·7
N.S.	4·4	4·4	4·6	5·0	5·8	6·8
C.T.	0·5	2·1	8·6	4·5	ND	2·2
E.W.	3·5	0·4	10·0	9·0	10·0	8·3
A.W.	0·4	0·5	12·0	3·9	2·3	9·5
D.S.	16·8	21·9	8·8	6·7	5·6	37·0
Number +ve		4/14	10/14	11/14	7/10	12/14
Percentage +ve		29	71	79	70	86

The histamine released is expressed as a percentage of the total histamine (histamine released in supernatant plus residual cell histamine). The test is considered positive if the histamine release in allergen challenged aliquots is twice the histamine release in tyrode challenged aliquots.

* Maximum histamine release over concentration range used.

ND = not done.

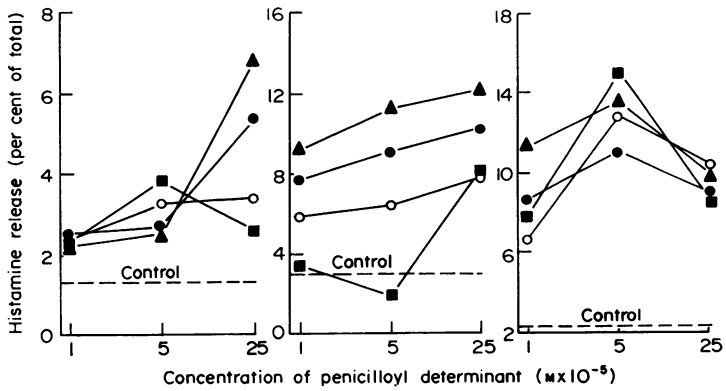

FIG. 2. Three typical dose response curves obtained on challenge of the leucocytes of penicillin allergic patients with BPO:PL, BPO:HSA, BPO:BSA and BPO:BGG. (■) Penicilloyl–polylysine. (○) Penicilloyl–BSA. (●) Penicilloyl–HSA. (▲) Penicilloyl–BGG.

Fig. 2) and generally the maximum response was obtained at $1{\cdot}6 \times 10^{-5}$ M B.Pen. and 5×10^{-5} M with respect to the penicilloyl group for the BPO–protein conjugates. The maximum response given is tabulated (Table 2). Antigen concentrations higher than those needed to produce the maximum response produced inhibition of histamine release.

The BPO:HGG conjugate was not studied in the leucocyte challenge test. This was due in part to the relatively high concentrations of antigen (conjugates in this case) required for the leucocyte test, and partly because only a small amount of purified HGG was available.

CONTROL TESTS IN NON-ALLERGIC SUBJECTS

The BPO–protein conjugates were tested for their ability to release histamine from the leucocytes of ten non-allergic controls (Table 3). Only one patient (YH) gave positive results. This patient had a history of allergy to animal fur and pollen, and it is possible that she had unrecognized penicillin allergy.

TABLE 3

LEUCOCYTE CHALLENGE TEST IN CONTROL SUBJECTS WITH PENICILLOYL–PROTEIN CONJUGATES

Patient	Percentage histamine release *				
	Control	BPO:PL	BPO:HSA	BPO:BGG	BPO:BSA
E.A.	4·0	4·1	4·5	4·4	ND
M.V.	1·9	0·3	0·8	0·7	ND
K.Y.	3·2	4·1	2·9	2·7	2·5
G.A.	4·4	4·1	5·3	4·0	4·4
J.J.	3·2	3·6	3·7	3·7	3·2
J.C.	2·3	2·0	2·9	4·0	2·7
J.W.	0·8	0·9	0·5	1·1	1·1
Y.H.	1·7	2·6	2·5	3·3	4·9
C.S.	3·1	4·2	4·0	4·3	4·3
D.T.	2·1	2·8	2·9	2·9	2·6
Number +ve		0/10	0/10	1/10	1/8
Percentage +ve		0	0	10	12·5

The histamine released is expressed as a percentage of the total histamine (released plus residual cell histamine). The test is considered positive if the histamine release in allergen challenged aliquots is twice the histamine release in tyrode challenged aliquots.

* Maximum histamine release over concentration range $1\text{–}25 \times 10^{-5}$ M (BPO).
ND = not done.

CONTROL TESTS WITH CARRIER PROTEINS

In five allergic patients a comparison was made between the responses elicited by the penicilloyl conjugate and its carrier protein alone treated as in the preparation of conjugates (Table 4). In this group of experiments the concentration of the conjugate was 5×10^{-5} M with respect to the penicilloyl group and the concentration of protein was equivalent to the amount of protein present in this concentration of conjugate. In every case the protein alone failed to release significantly more histamine than control samples. In two subjects the response to BPO:HSA was not positive (according to the criterion which we have adopted, i.e. the test is positive only if the percentage histamine release is at least twice that in non-allergen challenged samples), but HSA gave an even lower

response, suggesting that even the apparently negative tests with BPO:HSA may not be truly negative if the control samples routinely included tests with the carrier protein.

TABLE 4

LEUCOCYTE CHALLENGE TEST IN PENICILLIN ALLERGIC PATIENTS TO COMPARE THE RESPONSE TO THE CARRIER PROTEIN ALONE WITH THE RESPONSE TO THE APPROPRIATE BPO–PROTEIN CONJUGATE

Patient	Percentage histamine release				
	Control	BPO:BGG	BGG	BPO:HSA	HSA
(1)	1·2	6·9	2·2	5·7	1·6
(2)	1·1	4·2	1·6	4·3	1·7
(3)	2·5	5·9	1·2	4·8	2·5
(4)	3·6	12·0	3·8	8·1	4·2
(5)	4·7	12·1	4·6	6·2	3·1

BPO:BGG and BPO:HSA are used at a final concentration of 5×10^{-5} M with respect to the BPO hapten; BGG and HSA are used in the concentrations in which they are present in the conjugates. The histamine released is expressed as a percentage of the total histamine (released plus residual cell histamine). The test is considered positive if the histamine release in allergen challenged aliquots is twice the histamine release in tyrode challenged aliquots.

RANKING OF THE RELATIVE VALUE OF VARIOUS CARRIERS

To assess the effects elicited by the different conjugates it was necessary to rank the responses. This was done using two parameters: the maximum histamine release obtained with BPO:HSA, BPO:BGG and BPO:BSA was expressed as a percentage of the maximum histamine release obtained with BPO:PL; if two conjugates gave the same percentage then the dose at which the maximum response was obtained was also considered. The ranking of the various carriers for ten patients is shown in Table 5. The ranking was

TABLE 5

RANKING OF THE CARRIER PROTEINS FROM THE RESULTS OF THE LEUCOCYTE CHALLENGE TEST IN PENICILLIN ALLERGIC PATIENTS

Patient	Carrier protein			
	PL	BSA	HSA	BGG
R.C.	1	3	2	4
C.C.	1	2	3	4
F.M.	1	2	3	4
M.R.	1	2	3	4
J.S.	1	2	3	4
S.S.	2	1	3	4
N.S.	1	3	2	4
E.W.	4	3	2	1
A.W.	3	2	1	4
D.S.	2	1	3	4
Total of ranks	17	21	25	37
Order of ranks	1	2	3	4

Rank 1 = least effective carrier; rank 4 = most effective carrier.

TABLE 6

LEUCOCYTE CHALLENGE TEST IN PENICILLIN ALLERGIC PATIENTS WITH BPO CONJUGATES WITH BSA, HSA, AND BGG

Patient	Carrier protein		
	BSA	HSA	BGG
S.A.	ND	107	144
J.C.	ND	163	641
R.C.	322	317	644
C.C.	356	506	622
G.I.	ND	169	494
F.M.	131	208	262
M.R.	120	111	144
J.S.	189	260	506
S.S.	100	135	153
N.S.	126	109	148
C.T.	ND	188	98
E.W.	100	90	83
A.W.	45	35	226
D.S.	75	105	358
Total	1564	2504	4523
Average Carrier	156	179	323
cf. PL. by *t*-test	$0{\cdot}05 < P < 0{\cdot}1$	$0{\cdot}001 < P < 0{\cdot}01$	$P < 0{\cdot}001$

The results are expressed as the maximum histamine released by antigen as a percentage of the maximum histamine released by BPO: PL (= 100). The carrier proteins are then compared to PL, and an analysis is made of the statistical significance of the increase in effectiveness of BPO conjugates with BSA, HSA, and BGG as compared to the effectiveness of the BPO: PL conjugate. Student's *t*-test is used to obtain *P* values.

analysed using the coefficient of concordance ratio and Snedecor's distribution. The rank order PL, BSA, HSA, BGG was found to be significant at the 5 per cent level. Further analysis of all the results (Table 6) confirmed that the most effective carrier of those tested was BGG. The general rank order was PL, BSA, HSA, BGG; PL being the least effective carrier. The responses to BPO conjugates with BGG and HSA were significantly higher than the response to BPO:PL. Also BPO:BGG gave a significantly higher response than either BPO:HSA ($0{\cdot}02 < P < 0{\cdot}05$) or BPO:BSA ($0{\cdot}01 < P < 0{\cdot}02$). The response to BPO:HSA was higher than that to BPO:BSA but the difference was not statistically significant ($0{\cdot}6 < P < 0{\cdot}7$).

LYMPHOCYTE TRANSFORMATION TEST

In the LTT the difference between B.Pen. and the BPO–protein conjugates was less marked (Table 7). The BPO:HGG conjugate, however, gave positive results in 92 per cent of patients compared with 57 per cent for B.Pen. and 79 per cent for the other conjugates. All the patients who responded to B.Pen. also responded to the BPO–protein conjugates although one patient (E.W.) gave a greater response to B.Pen.

Dose–response curves were obtained for each antigen in all patients tested. Allergen dose–response curves for all carriers could not be carried out in a single experiment.

Thus three experiments were done for each patient, comparing control samples (no allergen) and aliquots challenged with BPO:PL and one other carrier per experiment. Three examples from different patients are shown in Fig. 3. The maximum response for BPO:HGG was obtained at 1×10^{-6} M (with respect to the penicilloyl group) while for BPO:PL, BPO:HSA and BPO:BGG the maximum response was obtained at 5×10^{-6} M.

TABLE 7

LYMPHOCYTE TRANSFORMATION TEST IN PENICILLIN ALLERGIC PATIENTS

Patient	Incorporation of [^{3}H]thymidine by lymphocytes (cpm)									
	B.Pen.		BPO:PL		BPO:HSA		BPO:BGG		BPO:HGG	
	Control	AG	Control	AG	Control	AG	Control	AG	Control	AG
S.A.	197	589*	408	841*	267	271	408	1142*	407	802*
J.C.	728	1606*	458	1208*	458	1200*	1008	2377*	ND	—
R.C.	183	186	513	1377*	276	1323*	513	2345*	284	991*
C.C.	283	339	490	1030*	490	661*	392	642	1545	1323
G.I.	473	872*	720	1233*	268	1652*	720	1093	973	1448*
F.M.	536	1200*	1476	1673	406	675*	1476	2522*	536	972*
M.R.	443	298	582	1069*	582	676	257	834*	469	1408*
J.S.	729	1252*	205	913	205	1253*	265	454*	415	767*
S.S.	712	1773*	408	1367*	292	308	408	1359*	385	1344*
N.S.	424	547	1083	1610*	1083	2186*	876	1714	756	2304*
C.T.	610	381	624	1031*	624	1325*	177	367*	861	1414*
E.W.	136	1007*	430	726	491	927*	430	1350*	107	196*
A.W.	247	353	1369	2200*	1396	3059*	1369	4934*	857	3364*
D.S.	654	7464*	346	22613*	346	12560*	492	21060*	ND	—
Number +ve	8/14		11/14		11/14		11/14		11/12	
Percentage +ve	57		79		79		79		92	

The results are expressed as the mean cpm of four samples. For the allergen-challenged samples the maximum thymidine incorporation over the concentration range used is given. The test is considered positive when the incorporation of [^{3}H]thymidine by the antigen challenged aliquots is significantly higher than control incorporation.

* Positive result (significant by Student's *t*-test).

CONTROL TESTS IN NON-ALLERGIC SUBJECTS

The results of the LTT in ten non-allergic subjects are given in Table 8. Only one subject (1/10 patients) responded to all three carrier proteins tested. This subject (E.A.) has worked with penicillin for many years.

CONTROL TESTS WITH CARRIER PROTEINS

A comparison of the carrier alone with the BPO conjugates (Table 9) showed that the carrier protein treated as in the preparation of conjugates was not capable of stimulating lymphocyte transformation by itself.

RANKING OF THE RELATIVE VALUE OF VARIOUS CARRIERS

A quantitative comparison of the responses of the carriers using the LTT was extremely difficult. The LTT is reproducible qualitatively but not quantitatively (i.e. the height and shape of the dose–response curve) and it is impossible to compare all the conjugates

TABLE 8

LYMPHOCYTE TRANSFORMATION TEST IN CONTROL SUBJECTS

Patient	Incorporation of [^{3}H]thymidine by lymphocytes (cpm)			
	Control	BPO:HSA	BPO:BGG	BPO:HGG
E.A.	399	689*	2014*	536*
M.V.	546	683	735	548
K.Y.	336	418	576	282
G.A.	1394	1273	1034	1334
J.J.	1412	1612	1798	1335
J.C.	1316	1574	781	751
J.W.	899	702	823	739
Y.H.	1053	656	1099	803
C.J.	3317	4305	4550	1792
D.T.	801	777	653	679
Number +ve		1/10	1/10	1/10
Percentage +ve		10	10	10

The results are expressed as the mean cpm of four samples. The test is considered positive when the incorporation of [^{3}H]thymidine by the antigen challenged aliquots is significantly higher than control incorporation.

* Positive response (significant by Student's *t*-test).

TABLE 9

LYMPHOCYTE TRANSFORMATION TEST IN PENICILLIN ALLERGIC SUBJECTS TO COMPARE THE EFFECT OF THE CARRIER PROTEIN ALONE WITH THE APPROPRIATE BPO–PROTEIN CONJUGATE RESPONSE

Patient	Incorporation of [^{3}H]thymidine by lymphocytes (cpm)						
	Control	BPO:BGG	BGG	BPO:HGG	HGG	BPO:HSA	HSA
(1)	363	304	333	704*	338	753*	421
(2)	367	770*	388	944*	798	618*	533
(3)	749	1041*	623	1052*	613	646	530
(4)	201	319*	183	1151*	93	596*	140
(5)	362	491*	309	583*	218	612*	396

BPO:BGG and BPO:HSA are used at a final concentration of 5×10^{-6} M (BPO); BPO:HGG is used at a concentration of 1×10^{-6} M (BPO); BGG, HSA and HGG are used in the concentrations in which they are present in the conjugates.

The results are expressed as the mean cpm of four samples. The test is considered positive when the incorporation of [^{3}H]thymidine by the antigen challenged aliquots is significantly higher than control incorporation.

* Positive response (significant by Student's *t*-test).

in one experiment because of the volume of blood which would be required (5 ml per aliquot, and four replicates of each treatment are required for statistical analysis) and the technical difficulties involved. Thus only two conjugates were tested in any single experiment, BPO:PL being used as reference (e.g. Fig. 3) and then the maximum response obtained with BPO conjugates to other carriers was expressed as a percentage of the maximum response obtained with BPO:PL. Ranking was then based on this value and on the dose at which the maximum response was obtained should two values be equal (Table 10). The ranking order was PL, HSA, HGG, BGG; PL being the least effective carrier. When the ranking order (for twelve patients) was analysed as in the leucocyte test, it was significant at the 5 per cent level. A more detailed analysis of all the results

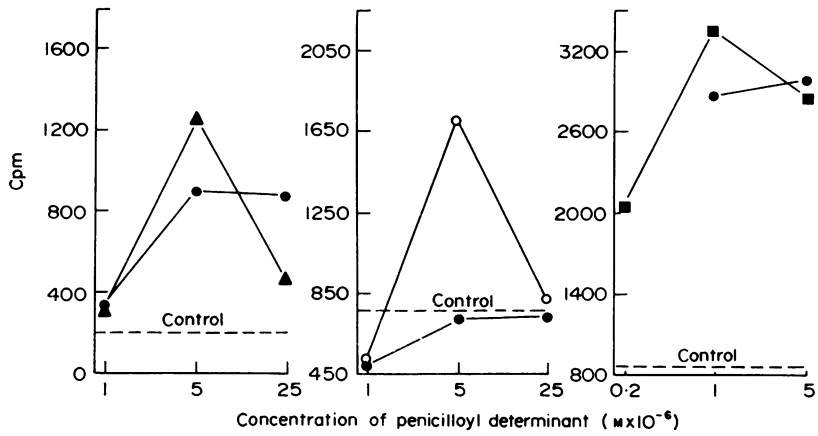

FIG. 3. Comparison of BPO: PL with BPO: HSA, BPO: BGG or BPO: HGG in the lymphocyte transformation test in three penicillin allergic patients. Note that the maximum response to BPO: HGG was obtained at a concentration of 1×10^{-6} M BPO as compared to 5×10^{-6} M BPO for the other BPO protein conjugates. (●) Penicilloyl–polylysine. (▲) Penicilloyl–HSA. (○) Penicilloyl–BGG. (■) Penicilloyl–HGG.

TABLE 10

RANKING OF THE CARRIER PROTEINS FROM THE RESULTS OF THE LYMPHOCYTE TRANSFORMATION TEST IN PENICILLIN ALLERGIC PATIENTS

Patient	Carrier protein			
	PL	HSA	HGG	BGG
S.A.	3	1	2	4
R.C.	2	4	1	3
C.C.	2	1	3	4
G.I.	2	4	3	1
F.M.	3	1	2	4
M.R.	3	1	4	2
J.S.	2	4	3	1
S.S.	3	1	4	2
N.S.	1	2	3	4
C.T.	1	2	3	4
E.W.	1	3	2	4
A.W.	1	2	3	4
Total of ranks	24	26	33	37
Order of ranks	1	2	3	4

Rank 1 = least effective carrier; rank 4 = most effective carrier.

from the penicillin allergic patients (Table 11) confirms the rank order. HGG and BGG were significantly more effective as carriers than PL. BGG was significantly more effective than HSA ($0{\cdot}05 < P < 0{\cdot}01$) but the difference from HGG was not significant ($0{\cdot}3 < P < 0{\cdot}4$).

TABLE 11

LYMPHOCYTE TRANSFORMATION TEST IN PENICILLIN ALLERGIC PATIENTS WITH BPO CONJUGATES WITH HSA, HGG AND BGG

Patient	Carrier protein		
	HSA	HGG	BGG
S.A.	80	87	136
J.C.	99	ND	119
R.C.	274	93	170
C.C.	64	165	266
G.I.	175	124	89
F.M.	86	92	151
M.R.	63	166	89
J.S.	137	102	96
S.S.	84	152	99
N.S.	136	202	235
C.T.	129	130	162
E.W.	172	159	186
A.W.	159	185	224
D.S.	56	ND	297
Total	1714	1657	2309
Average	122	138	165
Carrier cf. PL. by *t*-test	$0{\cdot}1 < P < 0{\cdot}2$	$0{\cdot}001 < P < 0{\cdot}01$	$P < 0{\cdot}001$

The results are expressed as the maximum number of cpm obtained using antigen as a percentage of the maximum number of cpm obtained using PL = 100). Each value is calculated from the mean of four samples. The carrier proteins are compared to PL and an analysis is made of the statistical significance of the increase in effectiveness of BPO conjugates with HSA, HGG, and BGG as compared to the effectiveness of the BPO:PL conjugate. Student's *t*-test is used to obtain *P* values.

DISCUSSION

The skin test results do not correlate well with the clinical diagnosis and the findings in *in vitro* tests. All fourteen patients studied had clinically and experimentally confirmed penicillin allergy, yet in skin tests only 36 per cent responded to B.Pen., 36 per cent to BPO:PL, and 50 per cent to BPO:HSA. These relatively low figures may not represent the general incidence of positive skin tests in penicillin allergy since in a larger study (forty-nine patients) by Assem and Vickers (1972), which included these fourteen patients, positive skin tests were obtained in 55 per cent of patients with clinically established diagnosis of drug allergy.

One patient gave a late positive reaction to B.Pen. in the absence of reaction to BPO:PL or BPO:HSA, and this may indicate reaction to minor determinants. Three patients gave reactions to the conjugates in the absence of reaction to B.Pen. This may be due to the failure of B.Pen. to achieve bridging of antibody molecules. Alternatively, it could represent a high degree of carrier specificity in these patients.

In this study only PL and HSA conjugates were used. BPO:HGG, which proved most effective in *in vitro* tests was not used because of the possible danger of inducing the formation of antibodies against HGG (HGG is almost certainly denatured during the prepara-

tion of conjugates). A greater number of positive responses were obtained with BPO:HSA than with BPO:PL but the difference is not significant. Parker (1963), using similar preparations to ours, has reported that BPO:PL, BPO:HSA and BPO–HGG are equally effective in eliciting weal and flare responses. A report by Levine (1964), however, showed that BPO:PL is a more effective elicitor of the allergic skin reaction than BPO:HSA, which in turn is generally more effective than BPO:HGG. The BPO:PL preparation used by Levine is more closely similar to his BPO–protein conjugates as far as molecular size is concerned than the preparations used in this study. However, the degree of BPO substitution in BPO:PL is far greater than in the BPO–protein conjugates. It is probable that had Levine used a BPO:PL preparation with approximately the same number of BPO groups as in the other conjugates, the steric advantages of his PL preparation would not be so apparent.

In general, from the results presented here, skin testing seems to be of limited diagnostic value in penicillin allergy, even when penicillin conjugates rather than penicillin itself are used. Evidence from *in vitro* tests suggests that a more reliable result will always be achieved from these tests than from skin tests. A positive skin test will confirm allergy but a negative test will not exclude it. The study of effectiveness of different carriers in skin testing is restricted by the number of carriers which can ethically be tested. Furthermore, *in vitro* tests provide better quantitative comparison between the various conjugates than does the skin test.

In the *in vitro* tests BPO conjugates were generally much better than B.Pen. in eliciting responses. The conjugates were relatively more useful in the leucocyte challenge test than in the LTT. In the leucocyte test B.Pen. would fail to achieve bridging of reaginic antibodies; thus the release of pharmacological mediators may not occur, whereas in the LTT the relatively more favourable results with B.Pen. may be explained by the formation of conjugates in the lymphocyte culture medium. Weck (1971) has suggested that a more likely explanation may be the conjugation of B.Pen. to the membrane of a non-sensitized lymphocyte and the presentation of BPO groups to sensitized lymphocytes by this BPO–carrier cell conjugate. We have obtained fewer positive results in the LTT than Weck, and it is felt that even though BPO–carrier cell conjugates may form in lymphocyte cultures, such conjugates may not present BPO groups to sensitized lymphocytes in the most appropriate way.

In both the leucocyte test and the LTT the BPO:PL conjugate was less effective than the other BPO–protein conjugates. In the LTT the number of positive reactions elicited by BPO:PL was not less than for BPO:HSA or BPO:BGG but the response to BPO:PL was much lower. This difference cannot be explained by any non-specific action of the proteins. Control subjects did not respond to the protein conjugates in *in vitro* tests. Further, in the LTT there was no stimulation by HGG, BGG or HSA alone, and in the leucocyte test no histamine release by BGG or HSA. Limited studies carried out on HGG suggest that HGG also will not release histamine from leucocytes non-specifically. The more likely explanation for the differences observed between BPO:PL and the BPO-protein conjugates would be a specific requirement for areas of the protein beyond the lysine side chain, probably for definite amino acids. Further, if there is specificity for a particular tertiary protein structure (Levine, 1963) then the physical characteristics of PL would be a disadvantage.

The experiments were done on an equimolar basis with respect to the BPO group so that a respectively small PL molecule with BPO groups separated by short distances is

being compared with large protein molecules with BPO groups separated by much larger distances. It is possible that a minimum distance is required between two antigenic determinants in order to produce a maximal response. For example, such an arrangement in the leucocyte test may produce efficient bridging of IgE antibody molecules bound to the surface of basophils. The BPO:PL, on the other hand, may fail to achieve efficient bridging because the BPO groups occur at too frequent intervals.

If it is assumed that the natural antigen is the most perfect one, and this may not necessarily be true, then the differences in effectiveness of the carrier proteins tested presumably represent the degree to which these proteins differ from the natural carrier protein. The differences in effectiveness obtained cannot be explained by differences in the extent of substitution by BPO since the preparations we used were BPO_{14}HSA, BPO_{11} BSA, BPO_9HGG and BPO_{12}BGG, and Levine (1964) has shown that there is little difference in the effectiveness of BPO_{10}HGG as compared with BPO_{22}HGG.

The use of the BPO:HGG conjugate in this investigation was limited to the LTT because of the requirement in the leucocyte challenge test of relatively high concentrations of BPO conjugates, and hence, larger amounts of HGG than were available. The LTT did not show a significant difference in the maximal responses produced by BPO:BGG and BPO:HGG. However, BPO:HGG was effective in more cases than BPO:BGG and at a lower concentration suggesting, as expected, that HGG resembles the natural carrier more closely than BGG. Differences in arrangement of amino acids between the BPO groups and different tertiary configurations of the molecules may be important factors.

Generally, the results from *in vitro* tests show them to be more reliable than skin tests, and to correlate well with the patient's history. The choice of antigen for use in these tests and the concentration at which the antigen is used is critical. If B.Pen. is used in the leucocyte test then the test is only as effective as skin testing. However, when a BPO-protein conjugate is used, up to 86 per cent positive results are obtained. In the LTT the conjugates are of relatively little advantage but with BPO:HGG 92 per cent positive results were obtained compared with 57 per cent for B. Pen.

The BPO:HGG preparation would appear to be the best conjugate for diagnostic testing *in vitro*. However, since BPO:BGG produces positive results in the majority of allergic patients, and since BGG is readily available commercially, BPO:BGG should provide an adequate alternative.

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