

Investigation of the response to some haptenic determinants in penicillin allergy by skin and *in vitro* allergy tests

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Summary

The role of three penicillin metabolites in human allergic reaction to penicillin has been assessed using sensitive *in vitro* tests in addition to skin testing. Conjugates of the benzylpenicilloyl (BPO), benzylpenicillenate (BPE) and penicillamine (PA) groups with human serum albumin (HSA) and bovine gamma globulin (BGG) have been made. In the passive haemagglutination test antibodies specific for all three determinants were found but there was no difference between allergic and non-allergic groups. However, the ratio IgG/IgM, specific for each determinant, was higher in normals than in allergics. In skin tests, histamine release from leucocytes and the lymphocyte transformation test, the BPO group was confirmed as the major determinant, but in some individuals the BPE or PA groups are more important.

Introduction

In a previous paper (Vickers & Assem, 1974) we presented evidence suggesting that gamma globulins were the most effective carriers for use in *in vitro* diagnostic tests. The identification of the haptenic determinants responsible for allergy to penicillin in man, coupled to the knowledge of carrier requirements, could lead to the preparation of adequate antigens for use as clinical reagents for diagnosis.

Levine (1960) studied allergic cross reactivity among penicillin and its degradation products using allergic contact dermatitis in the guinea-pig as the test system. The penicilloyl group was shown to be the major determinant but the penicillenate and penicillamine groups were also important.

In the rabbit quantitative precipitation (Levine & Ovary, 1961), passive cutaneous anaphylaxis (Levine, 1964) and passive haemagglutination (de Weck, 1962a) have been used as test systems. The penicilloyl group was found to be the major determinant in all systems. de Weck (1962a), using passive haemagglutination, also found penicillenate specific antibodies in serum from rabbits immunized with penicillin.

The penicilloyl group was recognized as the main antigenic determinant in human penicillin allergy independently by Levine & Ovary (1961), Parker *et al.* (1962) and de Weck (1962b). Using passive haemagglutination (Thiel, Mitchell & Parker, 1964)

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specificity was also shown for the penicillenate, penamaldate and penicilloate groups. By direct and passive skin tests (Levine, 1964; Parker *et al.*, 1962) specificity was found for the penicillenate and penicillamine groups. Reactions to penicillin not specific for either penicilloyl, penicillenate, penicillamine or penamaldate groups have been reported (Siegel & Levine, 1964).

Most previous work on the relative importance of the different determinants in human penicillin allergy has been done using skin reactions or haemagglutination techniques. Skin reactions, however, are not as sensitive or reliable as some *in vitro* tests, e.g. histamine release from sensitized leucocytes (HRL) and the lymphocyte transformation test (LTT). The haemagglutination test is of limited value since it detects mainly non-sensitizing antibodies.

A study of all the penicillin metabolites potentially capable of inducing an allergic reaction in man would require almost unlimited time. We have looked at the three metabolites which have most often been indicated as having an important role as determinants in penicillin allergic reactions in man viz. the penicilloyl, penicillenate and penicillamine groups.

Skin tests have been carried out using conjugates with HSA, and the HRL and LTT have been studied using conjugates with BGG. Passive haemagglutination specific for each group has also been carried out and the red cell linked antigen-antibody reaction of Coombs (Coombs *et al.*, 1965) used to find the classes of antibody involved in this reaction.

Materials and methods

Patients. All the penicillin allergic patients submitted to skin tests and *in vitro* testing had clinically well-documented allergy. Most of them had previously given responses to benzylpenicillin and/or penicilloyl conjugates in *in vitro* tests. It was necessary to limit this study to patients whose diagnoses were beyond doubt in order to obtain a true picture of the relative importance of the different determinants. In addition to this group of allergics, ten subjects with no personal or family history of penicillin allergy were used as controls.

Larger numbers of non-penicillin allergics and patients who had given an allergic reaction to penicillin were also tested in the haemagglutination studies.

Antigens. Sodium benzylpenicillin 'Crystapen' was obtained from Glaxo Laboratories, Greenford.

Benzylpenicilloyl HSA (BPO-HSA) and benzylpenicilloyl BGG (BPO-BGG) were prepared by direct reaction of excess benzylpenicillin (B. Pen.) with the protein at high pH (Parker & Thiel, 1963). 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 benzylpenicilloyl group was estimated using the penamaldate assay (Levine, 1962). The preparations were BPO₁₄HSA; BPO₁₂BGG.

Benzylpenicillenate protein conjugates (BPE.HSA and BPE.BGG) were prepared by the method described by de Weck & Eisen (1960). In order to prepare a protein conjugate specific for BPE the protein amino groups must be blocked to prevent the formation of penicilloyl-amine conjugates. This is achieved by using N-acetyl homocysteine thiolactone to acylate the protein. The thiolactone rearranges to homocysteine, and the protein becomes enriched with SH groups. Any penicilloyl groups which do form due to failure in obtaining quantitative substitution of the protein

amino groups can be oxidized to penamaldate using hydrogen peroxide (Parker *et al.*, 1962). It is thus possible to obtain a penicillenate protein conjugate essentially free of penicilloyl residues. After purification the BPE conjugates were estimated from their absorbance at 322 nm (Methods in *Immunology and Immunochemistry*, Vol. 1, 1967). The preparations were BPE₉HSA; BPE₁₁BGG.

Penicillamine protein conjugates (PA.HSA and PA.BGG) were prepared by the method described by Eisen, Belman & Carsten (1953). The procedure involves the reduction of the protein by penicillamine in urea solution. An oxidizing agent is then added, and the sulphhydryl groups of the reduced protein combine with penicillamine, forming a mixed disulphide bond. After purification penicillamine was estimated using the method described by Pal (1959). The preparations were PA₁₀HSA; PA₉BGG.

The carrier proteins used for the preparation of conjugates of various haptens had been used in control studies in the leucocyte and lymphocyte tests to be described subsequently. Tests with these proteins in five non-allergic subjects, and in five penicillin-allergic patients were completely negative, i.e. no falsely-positive results were obtained with the carrier proteins (Vickers & Assem, 1974).

Skin tests

Skin testing was performed by intradermal injection of 0.02 ml of antigen solution. BPO.HSA, BPE.HSA and PA.HSA were given in concentrations of 1, 5 and 25×10^{-5} M with respect to the haptenic group. B.Pen. was given in concentrations of 1.6, 16 and 160×10^{-5} M. The diameters of the weal and flare reactions were measured after 15 min and the patients reported any reaction which developed later. Skin responses occurring after the first 2 hr were termed late reactions, but were not necessarily delayed-type hypersensitivity reactions. The skin reaction was considered positive when the diameter of the weal was greater than 5 mm and the diameter of the flare was greater than 10 mm.

The release of histamine from sensitized leucocytes

This test measures histamine released as a consequence of reaction between allergen and reaginic antibody fixed to basophil leucocytes.

Leucocytes were isolated as described by Assem & McAllen (1970). Leucocytes were isolated from heparinized blood and challenged with antigen or with Tyrode solution control. BPO.BGG, BPE.BGG and PA.BGG were used in concentrations of 1, 5 and 25×10^{-5} M with respect to the haptenic group. B.Pen. was used in concentrations of 1.6, 16 and 160×10^{-5} M. The histamine released and the residual cell histamine were measured by bioassay on guinea-pig ileum (Assem & Schild, 1968), and the released histamine calculated as a percentage of the total histamine (released + residual). The test was considered positive when the percentage histamine released from antigen-challenged samples was twice that from Tyrode challenged samples.

The lymphocyte transformation test

This test can give positive results in cases of immediate and delayed-type allergy. Lymphocytes were separated from peripheral blood, as described by Coulson & Chalmers (1967), and DNA synthesis was measured as described by Chalmers *et al.* (1967). The lymphocytes were suspended in tissue culture medium 199 without antibiotics (Wellcome Laboratories), containing 10% autologous serum, and divided into 2.7 ml aliquots. Antigen or medium (0.3 ml) was added, and the cells incubated

for four days at 37°C. BPO.BGG, BPE.BGG and PA.BGG were used in a final concentration of 5×10^{-5} M, which had previously been shown to give the maximum response for each determinant. B.Pen. was used in a final concentration of 1.6 and 16×10^{-5} M. After incubation the incorporation of (^{3} H) thymidine, over a 1-hour period, was measured. This test was considered positive when the counts per minute (ct/min) of the antigen challenged samples were significantly higher than the ct/min of the control samples. Four or five replicates of each treatment were prepared, and the significance of the difference between treatments calculated from Student's *t* test.

Haemagglutination techniques

Preparation of sheep red blood cells (SRBC) coated with antigen. The method used was an adaptation of that described by Fulthorpe *et al.* (1961). Two changes were made to their method in order to reduce haemolysis of red cells to a minimum; the cells were formalinized before tanning and coating with antigen, and a preservative, thiomersalate, was added to the borate-succinate buffer, which helped to keep a constant pH.

The following preparations were made: SRBC coated with (i) HSA; (ii) BPO.HSA; (iii) BPE.HSA; and (iv) PA.HSA.

Passive haemagglutination procedure

This was used to detect antibodies in the serum which reacted with the BPO, BPE and PA groups. The serum to be tested was diluted 1 in 5 with control cells (i.e. those coated with HSA) and then left overnight at 4°C. The cells were then spun down and the serum removed. This procedure removes any antibodies in the serum which react non-specifically with the SRBC. Haemagglutination tests were carried out using a Takatsy microtitrator, dropping pipette and plexiglass blocks with eight rows of twelve small wells. All dilutions were made using 0.6% w/v dextran (mol. wt 110,000) in 0.9% saline. This was found to give maximum sensitivity, and it was possible to detect 3.5×10^{-7} µg/ml antibenzylpenicilloyl antibody in rabbits. Two-fold dilutions of serum were made and an equal volume of coated SRBCs added to each well. For each serum a control was made, using the cells coated with HSA. The contents of the wells were mixed by gentle rotation and left at room temperature for about 3 hr to allow the agglutination pattern to develop. The titre of the serum was taken as the reciprocal of the last dilution, which gave a clearly defined agglutination of the cells. A titre of twenty was considered positive.

The red-cell-linked antigen-antiglobulin reaction

This was used to detect the classes of antibody involved in the passive haemagglutination reaction. The procedure used was that described by Steele & Coombs (1964) and Coombs *et al.* (1965). Serum to be tested was diluted 1 in 5 with HSA coated SRBCs, and left overnight at 4°C. The cells were then spun down and the serum removed. Twofold dilutions of the serum were made in 0.6% w/v dextran (mol. wt 110,000) in 0.9% saline, and equal volumes of SRBCs coated with BPO.HSA, BPE.HSA or PA.HSA were added to the serum. After incubation for 3 hr at room temperature the cells were washed twice with 0.6% w/v dextran solution to remove any uncombined serum protein. 0.025 ml aliquots of treated cell suspension were placed in agglutination wells containing 0.025 ml aliquots of diluted antisera to human IgA, IgD, IgE, IgG and IgM. Antihuman IgM was used in a dilution of 1:400 and the other

antisera in dilutions of 1:10,000. The contents of the well were mixed by gentle rotation, and the agglutination pattern read after incubation for 3 hr at room temperature. The titre was taken as the reciprocal of last dilution which gave a clearly visible agglutination. A titre of twenty was considered positive.

Results

Passive haemagglutination

Specificity was clearly shown for the three determinants tested, BPO, BPE and PA. Fig. 1 shows the distribution of titres found for each determinant, and indicates that

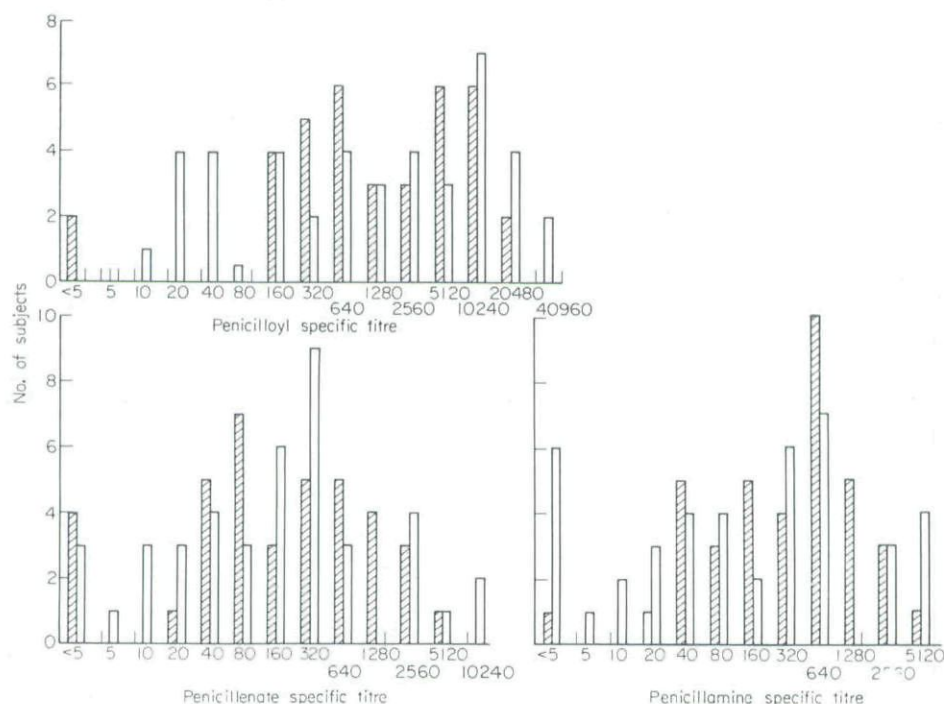


Fig. 1. Frequency of distribution of haemagglutination titres specific for the benzylpenicilloyl benzylpenicillenate and penicillamine determinants in penicillin allergic and in non-allergic subjects. Hatched, normal; blank, allergic.

there is no difference between allergic and non-allergic subjects. Taking a titre of twenty as positive, forty-one/forty-two allergics and thirty-six/thirty-eight non-allergics showed specificity for BPO, thirty-five/forty-two allergics and thirty-four/thirty-eight non-allergics showed specificity for BPE and thirty-three/forty-two allergics, thirty-seven/thirty-eight non-allergics showed specificity for PA. The BPO determinant produced the highest titres in over half the cases (twenty-four/forty-two allergics, twenty-eight/thirty-eight non-allergics). One normal and four allergics showed specificity only for BPO; a further one normal and two allergics showed no specificity for BPE, and a further five allergics showed no specificity for PA. Ten/forty-two allergics and seven/thirty-eight normals gave their highest titres with PA and three subjects, two normal and one allergic, had PA specificity in the absence of BPO

specificity. Eight/forty-one allergics and three/thirty-eight normals gave their highest titre with BPE but in no case was BPE specificity found in the absence of specificity for BPO.

The red cell linked antigen antiglobulin reaction (RCLAAR)

No IgA or IgD antibodies were found. The number of subjects giving positive haemagglutination titres specific for the IgE, IgG and IgM classes is shown in Table 1.

Table 1. Classes of antibody involved in the haemagglutination reaction specific for benzylpenicilloyl, benzylpenicillenate and penicillamine determinants in penicillin allergic and in non-allergic subjects

Serum haemagglutinating antibody specific for		Number Positive Comparison			Average titre Comparison		
		Normal	Allergic	<i>P</i> values	Normal	Allergic	<i>P</i> values
IgE	Penicilloyl	10/38	11/42	n.s.	11	13	n.s.
	Penicillenate	2/38	7/42	n.s.	6	9	n.s.
	Penicillamine	2/38	4/42	n.s.	6	8	n.s.
IgG	Penicilloyl	36/38	32/42	n.s.	270	79	<0.001
	Penicillenate	31/38	21/42	<0.01	102	47	<0.01
	Penicillamine	33/38	25/42	n.s.	168	31	<0.01
IgM	Penicilloyl	22/38	36/42	<0.01	32	640	<0.001
	Penicillenate	12/38	26/42	<0.01	20	81	<0.01
	Penicillamine	16/38	31/42	<0.01	20	111	<0.01

The IgG level was higher in normals than in allergics, and the IgM level was higher in allergics than in normals (Table 1). If the ratio IgG:IgM is taken a significant difference between allergics and normals is seen. The spread of this ratio for allergics and normals, specific for the three groups tested, is shown in Fig. 2.

Only a small proportion of subjects had IgE haemagglutinating antibodies, and there was no difference between allergic and non-allergic subjects (Table 1). However, among those who showed BPO specificity for IgE and IgG there was a much higher average IgG:IgE ratio for the normal group than for the allergic group, 22:1 as compared with 3:1 ($P = <0.01$). For BPE the normal average IgG:IgE ratio was 10:1, and for allergics 3:1 ($P = <0.05$). For PA the difference was not significant, the normal ratio being 7:1 and the allergic 6:1.

Skin reactions

The results given are the maximum obtained over the stated dose range. No positive skin responses were obtained for any determinant in the ten control subjects. Of the twenty-two penicillin-allergic patients tested, ten responded to BPO, eight to BPE and ten to PA (Table 2). The BPO responses were generally greater than the BPE and the PA responses, and four patients responded to BPO in the absence of a response to BPE and PA. Three patients responded to PA and BPE but not to BPO; another responded only to PA, and yet another only to BPE. Six of the twenty-two patients responded to B.Pen. (Table 2). All of these patients also reacted to at least one determinant, although in two cases (J.C. and D.S.) the highest response was given to B.Pen.

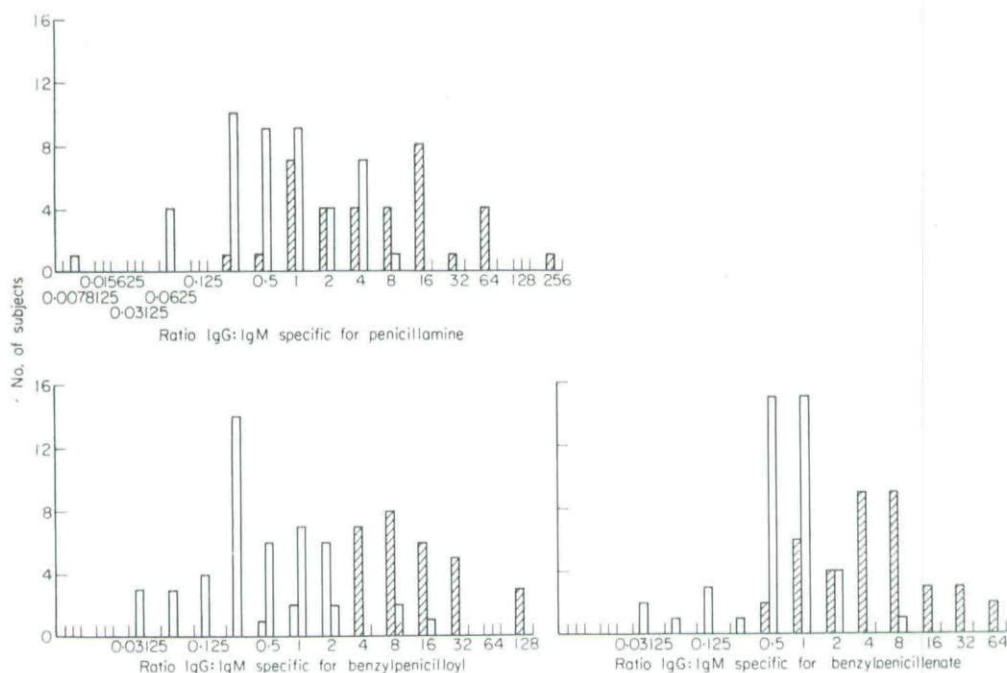


Fig. 2. Frequency of distribution of ratio IgG:IgM haemagglutinating antibody specific for benzylpenicilloyl, benzylpenicillenate and penicillamine groups in penicillin allergic and non-allergic subjects. Hatched, normal; blank, allergic. In all three $P < 0.001$.

Histamine release from leucocytes

The responses given (Tables 3 and 4) are the maximum obtained over the concentration range $1, 5, 25 \times 10^{-5}$ M with respect to the haptenic group. The maximum response was usually obtained at 5×10^{-5} M, and some typical dose response curves are shown in Fig. 3.

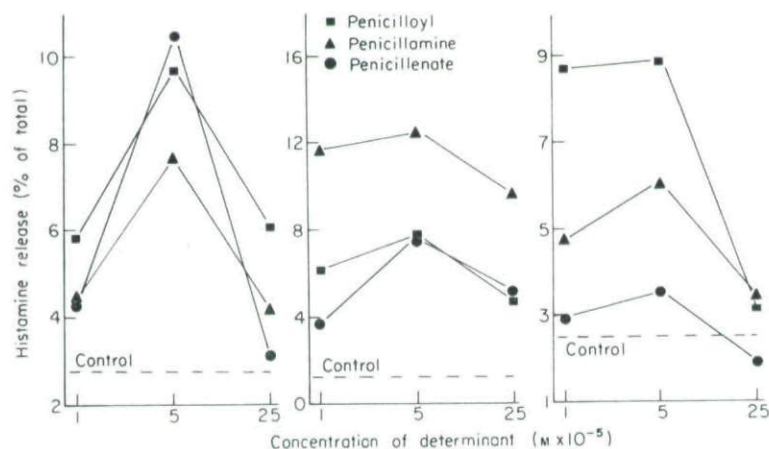


Fig. 3. Three typical dose response curves obtained on challenge of the leucocytes of penicillin allergic patients with benzylpenicilloyl, benzylpenicillenate and penicillamine HSA conjugates.

Table 2. Intradermal skin tests in penicillin allergic patients with benzylpenicilloyl, benzylpenicillenate and penicillamine HSA conjugates, and with benzylpenicillin

Patient	Diameter of flare (mm)							
	Benzylpenicilloyl/ HSA		Penicillamine/HSA		Benzylpenicillenate/ HSA		Benzylpenicillin	
	Immediate	Late	Immediate	Late	Immediate	Late	Immediate	Late
S.A.	6		7		5		6	
J.C.	6		18*		12*		6	32*
R.C.	5	34*	15*	8	6	12*	5	7
C.C.	6		6		5		6	
G.I.	7		9		8		6	
F.M.	18*		10*		32*		8	
M.R.	5	19*	2		3		3	10*
J.S.	6		0		0		0	
S.S.	42*		9		9		50*	
N.S.	21*		10*		8		19*	
C.T.	0		15*		10*		0	
E.W.	40*		11*		10*		4	
A.W.	6		9		8		8	
D.S.	21*		10*		8		64*	
J.W.	0		0		0		0	
A.A.	60*						10*	
T.D.	5		4	10*	5	10*	4	
M.	12*		0		0		0	
K.	21*		31*		41*		1	
C.H.	0		9		11*		0	
G.L.	8		4		9		6	
C.B.	0		11*		0		0	
No. pos.	8/22	2/22	9/22	1/22	6/22	2/22	4/22	2/22
No. pos.		10/22		10/22		8/22		6/22

* Positive test.

No response to PA was found in the ten controls tested; response to BPO occurred in one, and response to BPE was obtained in three (Table 3). However, these responses were very small, and on the borderline of the positive ratio value (twice the control release).

In the penicillin allergic group fourteen/twenty-one patients responded to BPO, twelve/twenty-one to PA and sixteen/twenty-one to BPE (Table 4). BPO elicited the highest response in eight patients, and one patient (J.C.) responded only to BPO. One patient responded to PA in the absence of a BPO reaction, and two patients responded to BPE and not to BPO. Another four patients gave higher responses to PA than to BPO, and a further six gave higher responses to BPE than to BPO. Five of the twenty-one patients also reacted to B.Pen. and in one case (C.T.), the B.Pen. response was the highest.

Lymphocyte transformation test

One of the ten control subjects (EA) gave positive results to all these determinants

Table 3. Leucocyte histamine release test with benzylpenicilloyl, penicillamine and benzylpenicillenate protein conjugates in non-penicillin allergic subjects

Patient	Histamine release (% of total)			
	Control	Benzylpenicilloyl BGG	Penicillamine BGG	Benzylpenicillenate BGG
E.A.	4.0	4.4	3.5	4.3
M.V.	1.9	0.7	ND	ND
K.Y.	3.2	2.7	2.3	2.6
G.A.	4.4	4.0	3.8	5.3
J.J.	3.2	3.7	3.2	6.3
J.C.	2.3	4.0	3.3	2.9
J.W.	0.8	1.1	0.8	1.7*
Y.H.	1.7	3.3*	2.1	3.4*
C.J.	3.1	4.3	3.4	3.8
D.T.	2.1	2.9	2.6	2.4
No. pos.		1/10	0/9	2/9

* Positive response.

Table 4. Leucocyte histamine release test with benzylpenicilloyl, penicillamine and benzylpenicillenate protein conjugates in penicillin allergic subjects and with benzyl-penicillin

Patient	Histamine release (% of total)				
	Control	Benzylpenicilloyl BGG	Penicillamine BGG	Benzylpenicillenate BGG	Benzylpenicillin
S.A.	0.5	6.2*	19.0*	5.8*	0.5
J.C.	0.6	10.3*	1.0	0.4	0.2
R.C.	1.5	24.3*	3.6*	9.6*	2.9*
C.C.	1.2	7.7*	7.1*	12.5*	1.0
G.I.	2.2	11.8*	9.8*	8.1*	7.5*
F.M.	3.2	10.0*	8.7*	12.3*	3.8
M.R.	0.9	9.2*	4.2*	8.7*	4.0*
J.S.	2.5	8.9*	3.5	6.1*	3.5
S.S.	2.8	9.8*	10.5*	7.7*	9.5*
N.S.	3.1	5.9	7.9*	2.2	3.1
C.T.	4.2	6.6	3.4	8.4*	17.2*
E.W.	2.2	2.0	1.0	7.7*	0.2
A.W.	5.0	14.4*	15.5*	19.5*	6.5
D.S.	49.8	61.2	69.6	72.8	64.7
J.W.	6.3	8.7	5.1	6.9	
A.A.	11.9	28.0*	22.1	27.5*	12.5
M.	2.8	34.1*	18.3*	26.8*	2.5
C.H.	4.5	10.0*	40.0*	17.0*	7.6*
T.D.	2.1	4.5*	3.2	4.6*	2.1
K.	11.5	11.0	7.9	12.2	
J.K.	4.0	7.7	10.6*	10.2*	2.2
No. pos.		14/21	12/21	16/21	5/21

* Positive response.

Table 5. Lymphocyte transformation test in non-penicillin allergic subjects with benzylpenicilloyl, penicillamine and benzylpenicillenate protein conjugates

Counts per minute (mean of four samples) $\pm$ s.d.				
Patient	Control	Benzylpenicilloyl BGG	Penicillamine BGG	Benzylpenicillenate BGG
E.H.	399 $\pm$ 98	2014 $\pm$ 343*	755 $\pm$ 202*	859 $\pm$ 309*
M.V.	546 $\pm$ 218	735 $\pm$ 290	842 $\pm$ 50*	649 $\pm$ 124
K.Y.	336 $\pm$ 101	576 $\pm$ 146	628 $\pm$ 312	327 $\pm$ 154
G.A.	1394 $\pm$ 227	1034 $\pm$ 135	745 $\pm$ 174	1479 $\pm$ 233
J.J.	1412 $\pm$ 518	1798 $\pm$ 493	1144 $\pm$ 130	1147 $\pm$ 224
J.C.	1316 $\pm$ 308	781 $\pm$ 304	1058 $\pm$ 24	1889 $\pm$ 221
J.W.	898 $\pm$ 123	824 $\pm$ 260	581 $\pm$ 121	668 $\pm$ 127
Y.H.	1053 $\pm$ 36	1099 $\pm$ 607	668 $\pm$ 101	759 $\pm$ 237
C.J.	3317 $\pm$ 1480	4550 $\pm$ 1813	2900 $\pm$ 1805	1786 $\pm$ 287
D.T.	801 $\pm$ 164	777 $\pm$ 147	615 $\pm$ 147	815 $\pm$ 297
No. pos.		1/10	2/10	1/10

* Positive response.

Table 6. Lymphocyte transformation test in penicillin allergic subjects with benzylpenicilloyl, penicillamine and benzylpenicillenate protein conjugates, and with benzylpenicillin.

Counts per minute (mean of four samples) $\pm$ s.d.					
Patient	Control	Benzylpenicilloyl BGG	Penicillamine BGG	Benzylpenicillenate BGG	Benzylpenicillin
S.A.	199 $\pm$ 17	824 $\pm$ 148*	388 $\pm$ 129*	732 $\pm$ 336*	589 $\pm$ 105*
J.C.	728 $\pm$ 247	1089 $\pm$ 311*	904 $\pm$ 324	2292 $\pm$ 430*	1606 $\pm$ 736*
R.C.	183 $\pm$ 53	324 $\pm$ 255	374 $\pm$ 222	298 $\pm$ 175	186 $\pm$ 224
C.C.	283 $\pm$ 92	428 $\pm$ 278	291 $\pm$ 84	452 $\pm$ 144	339 $\pm$ 14
G.I.	473 $\pm$ 206	755 $\pm$ 61*	831 $\pm$ 66	986 $\pm$ 124*	872 $\pm$ 168*
F.M.	791 $\pm$ 132	618 $\pm$ 174	863 $\pm$ 18	1945 $\pm$ 386*	1583 $\pm$ 168*
M.R.	773 $\pm$ 518	681 $\pm$ 264	879 $\pm$ 183	1231 $\pm$ 335*	298 $\pm$ 59
J.J.	923 $\pm$ 306	2944 $\pm$ 1486*	873 $\pm$ 34	1001 $\pm$ 130	1434 $\pm$ 134*
S.S.	712 $\pm$ 161	1042 $\pm$ 159	1030 $\pm$ 116*	993 $\pm$ 88*	1773 $\pm$ 296*
N.S.	424 $\pm$ 254	660 $\pm$ 133*	2405 $\pm$ 1021*	680 $\pm$ 202	547 $\pm$ 32
C.T.	486 $\pm$ 79	919 $\pm$ 177*	649 $\pm$ 145	571 $\pm$ 87	304 $\pm$ 25
E.W.	136 $\pm$ 46	173 $\pm$ 34*	172 $\pm$ 66*	108 $\pm$ 38	1007 $\pm$ 130*
A.W.	7271 $\pm$ 108	10571 $\pm$ 600*	9351 $\pm$ 322*	7409 $\pm$ 329	10391 $\pm$ 1584
D.S.	654 $\pm$ 50	10664 $\pm$ 599*	1109 $\pm$ 322*	1271 $\pm$ 329*	7464 $\pm$ 355*
A.A.	657 $\pm$ 50	2144 $\pm$ 1470*	925 $\pm$ 1230*	229 $\pm$ 32	370 $\pm$ 55
T.D.	300 $\pm$ 75	233 $\pm$ 76	140 $\pm$ 55	167 $\pm$ 17	195 $\pm$ 36
K	331 $\pm$ 128	339 $\pm$ 117	327 $\pm$ 163	924 $\pm$ 288*	262 $\pm$ 30
C.L.	412 $\pm$ 71	424 $\pm$ 22	383 $\pm$ 276	379 $\pm$ 104	418 $\pm$ 87
C.B.	244 $\pm$ 84	287 $\pm$ 80	356 $\pm$ 203	291 $\pm$ 72	207 $\pm$ 28
K.A.	183 $\pm$ 74	361 $\pm$ 118	185 $\pm$ 33	352 $\pm$ 119	239 $\pm$ 91
No + ve		11/20	7/20	8/20	8/20

* Positive response.

(Table 5). This subject had not shown any clinical symptoms of allergy although he has worked with penicillin for many years. He was also one of the subjects with very high haemagglutination titres. Another control gave a small response to PA.

In the penicillin allergic group eleven/twenty responded to BPO, seven/twenty to PA and eight/twenty to BPE (Table 6). Three patients responded to BPE in the absence of a BPO response. None responded to PA in the absence of response to BPO.

Discussion

Haemagglutinating antibody

There was no difference between the serum from the penicillin allergic and the non-allergic group with regard to the ability to agglutinate SRBC coated with the three penicillin determinants tested. This finding is in agreement with the early observations of Ley *et al.* (1958), Watson (1962) and Watson, Joubert & Bennett (1959, 1960), but differs from that of various other workers who did find a difference between allergic and non-allergic groups (Epp, 1959; Heggie, 1960; Harris & Vaughan 1961; Schwartz & Vaughan, 1963; Van Arsdel *et al.*, 1963; de Weck, 1964). The discrepancies can probably be explained by examining the patients within the groups. In most reports the allergic subjects were hospitalized and the serum samples tested taken close to the time of their allergic reaction, whereas in the allergic group reported here all patients have a clearly documented clinical history of allergy to penicillin, but the serum sample tested was not necessarily taken close to the time of reaction. Most of our non-allergic controls had had penicillin therapy at some time, but none had recently received large doses of penicillin. It is probable that the differences observed by other groups were due to recent exposure to penicillin in the allergic group and not to a difference between allergic and non-allergic subjects.

The proportion of subjects possessing haemagglutinating antibodies in their serum was found to be much higher than has previously been reported. This is presumably due to the sensitivity of the method used. We could detect as little as 3.5×10^{-7} $\mu\text{g/ml}$ antibenzylpenicilloyl antibody compared with 3.0×10^{-4} $\mu\text{g/ml}$ detected by Levine, Fellner & Levitska (1966).

The preparation of complete antigens and the coating of SRBC with these hapten-protein conjugates makes possible the direct detection of haemagglutinating antibodies specific for the various determinants. The BPO group appears to be the major determinant. Some patients, however, gave higher titres with BPE or PA, and three subjects had PA specificity in the absence of BPO specificity. Specificity for the BPE grouping has been reported (de Weck, 1964; Levine *et al.*, 1966a, b) but no reports of haemagglutinating antibody specific for PA have been published.

The red cell linked antigen antiglobulin reaction

The ratio IgG:IgM was found to be significantly higher in normals than in allergics. This result conflicts with Levine's finding (Levine *et al.*, 1966b; Levine *et al.*, 1966c) that there was little difference between allergic and non-allergic groups with regard to the level of penicilloyl-specific IgM or IgG. In contrast, Wheeler (1971) reported that serum from allergic patients generally had higher levels of penicilloyl-specific antibodies of all classes than had normals.

The finding that normals have more haemagglutinating IgG specific antibody is difficult to interpret unless the concept of blocking antibody is accepted. Then the

high levels of haemagglutinating IgG specific antibody in normals exposed to penicillin could account for these subjects failing to exhibit symptoms of allergy even though they may have some reaginic antibody. It is possible that the normals may either develop blocking IgG antibody in preference to reaginic antibody or that the high levels of IgG may in some way neutralize the effect of any reaginic antibody formed. It is necessary here to postulate that the IgG detected by haemagglutination is not reaginic-type IgG.

Wheeler (1971) found that the ratio of penicilloyl-specific IgG:IgE in the serum of normals was higher than that in allergics, and he suggested that this could indicate a protective function for IgG. In our study the number of subjects with detectable IgE-specific haemagglutinating antibody was comparatively small, but the ratio IgG:IgE, specific for BPO and BPE but not for PA, was significantly higher in normals than in allergics.

Skin sensitizing antibody

As a diagnostic test the skin reaction is disappointing, being positive in a relatively small number of penicillin allergic patients.

The BPO group emerges as the major determinant although as many patients respond to PA, and nearly as many to BPE. As with haemagglutinating antibody some individuals have specificity for PA and BPE, and not for BPO. This finding tends to eliminate the possibility that the response to BPE could be due to cross reaction with BPO-specific antibody. One patient (J.C.) gave a large response to B.Pen. in the absence of a response to BPO, and with comparatively small responses to PA and BPE. In another two patients B.Pen. elicited the largest reaction. These responses may be due to specificity for penamaldate, penicilloate or some other penicillin metabolite.

It has been suggested (Voss, Redmond & Levine, 1966; Levine *et al.*, 1966c) that BPO skin sensitizing antibody does not usually mediate immediate-type allergic reactions to penicillin, but that this type of reaction is associated with skin-sensitizing antibody of minor determinant specificity. In this study the BPO group produces as many, and often larger reactions than the BPE and PA groups. Other workers have used a BPO.PL to produce a BPO-specific response, whereas we have used BPO.HSA, which has been shown to produce a higher percentage of positive results in skin testing (Vickers & Assem, 1974). In addition, because of the relatively small numbers of patients involved, a difference in patient population may account for these discrepancies.

There was no apparent correlation between the skin test results and the haemagglutination titres. It has been suggested (Voss *et al.*, 1966) that a positive BPO skin response is associated with a low level of IgG BPO-specific haemagglutinating antibody. Insofar as the non-allergic group have high IgG levels and no skin response, this is true. However, there was no correlation between the IgG levels in allergics and their skin response. This lack of correlation was found for all three determinants tested.

Histamine release from sensitized leucocytes

It was thought that both immediate skin reactions and histamine release from leucocytes measure tissue-bound allergen-specific IgE antibody (Lichtenstein & Levy, 1969; Ishizaka *et al.*, 1969; Assem & McAllen, 1970). Perelmutter & Eisen (1970) found a correlation between skin tests and leucocyte histamine release in penicillin allergy. Our results do not confirm this correlation.

Far more patients gave positive leucocyte histamine release (BPO fourteen/twenty-one, BPE sixteen/twenty-one, PA twelve/twenty-one, B.Pen. five/twenty-one) than gave positive immediate skin reactions (BPO eight/twenty-two, BPE six/twenty-two, PA nine/twenty-two, B.Pen. four/twenty-two). Further, there was no correlation in individual patients between the size of skin reaction and the percentage histamine released.

It is possible that one subclass of IgE is bound to mast cells in the skin, and another IgE subclass to circulating basophils. Alternatively the release of histamine from sensitized leucocytes by penicillin protein conjugates may be partly mediated by another class of reaginic antibody. Assem & McAllen (1970) showed that the IgG, IgA and IgM classes of antibody could also be involved in histamine release from leucocytes. Earlier Assem & Schild (1968) reported that serum from penicillin allergic patients sensitized monkey lung more readily than human lung, and Assem (1972) then found that monkey lung could be sensitized by heat-stable human antibody. We have also found (unpublished observations) that when IgE antibody has been absorbed from the serum of penicillin allergic patients, this serum is still able to sensitize monkey lung passively. Histamine release from the passively sensitized monkey lung could then be obtained with anti-IgG but not with anti-IgE sera. In some preliminary experiments on leucocytes from five patients allergic only to penicillin, using the anti-IgE reversed leucocyte test as a probe to detect cell-bound IgE, it was clear that IgE could not fully account for the histamine released with penicillin metabolites.

It would appear, therefore, that IgG rather than IgE may be the main antibody involved in penicillin-induced histamine release from human leucocytes. Stanworth & Smith (1972) found that local injection of human myeloma IgG₄, in contrast with myeloma proteins of the other three IgG subclasses, is capable of inhibiting any subsequent skin sensitization of baboons by human reaginic antibody. It is possible that IgG₄ could prove to be important in human reaginic reactions.

Only three patients failed to respond to at least one of the three determinants tested. One of these patients (O.S.) repeatedly had a high spontaneous histamine release (of the order of 50%), and although the histamine release produced by all three determinants was very high (BPO 61%, BPE 73%, PA 70%), it was not twice the control release, which is the criterion used to assess a positive response.

The BPO group again emerges as the major determinant but in some individuals greater reaction is elicited by BPE or PA. No patient responded to B.Pen. in the absence of a reaction to one of the determinants, but one patient gave her greatest response to B.Pen. and this may reflect a reaction to determinants other than those tested. It has been suggested from skin tests (Voss *et al.*, 1966; Levine *et al.*, 1966c) that determinants other than BPO are more important in eliciting immediate-type reactions. Our results suggest that PA and BPE are as important but not more important than BPO.

Lymphocyte transformation test

Specificity is shown for the three determinants tested, BPO, BPE and PA. The BPO group is clearly indicated as the major determinant, but in some individuals greater responses were obtained with BPE and PA groups.

The type of reaction initially experienced by the patient does not appear to influence the extent of transformation. Positive LTT can be obtained in patients who

have given typical anaphylactic immediate-type reactions, in those with typical delayed-type reactions, and in patients whose reactions are not easily classified.

The low number of positive results in the LTT is unexpected. In comparison with the skin and leucocyte tests the relative proportion of positive results obtained with BPE and PA is low. This may indicate that these determinants are more important in immediate than in delayed-type reactions.

In conclusion, we have shown by reliable *in vitro* test systems that at least three determinants, the BPO, BPE and PA groups, are involved in the human allergic response to penicillin. It should be possible to detect most cases of penicillin allergy using the three determinants tested here. However, more work needs to be done to examine the role of other penicillin metabolites.

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