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Determination of IgE antibodies to the benzyl penicilloyl determinant

A comparison between poly-L-lysine and human serum albumin as carriers

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The influence of two different carriers, poly-L-lysine (PLL) and human serum albumin (HSA) in the binding of specific IgE antibodies to the benzyl penicilloyl hapten (BPO) was determined in a solid-phase assay. Serum samples from patients with a history of immediate reaction to penicillin and which had shown the presence of IgE antibodies to BPO were used. Benzyl penicilloyl derivatized cellulose discs were prepared using PLL of different molecular weight and HSA as carriers. These were treated with different molar ratios of benzyl penicillin. These reagents were compared for uptake of BPO-specific IgE using a pool of sera in a radioallergosorbent test (RAST) type assay. Two PLL systems and two HSA systems were finally compared using 26 individual sera. RAST values were compared by Kruskal-Wallis and Wilcoxon tests. The relationships between the four different assays were evaluated by determining Pearson correlation coefficients and the concordance by determining intraclass correlation coefficients (ICC). Analysis of means by the Wilcoxon test revealed significant differences ($P < 0.01$) only when the different carrier assays were compared. The correlation coefficients between all the assays were significant ($P < 0.0001$), but the ICC was low when the different carrier assays were compared. These results indicate that the nature of the carriers studied (PLL and HSA) influences the capacity for binding IgE antibodies in the RAST procedure. The differences observed indicate that one conjugate cannot be substituted for the other in the determination of IgE antibodies to BPO and that BPO-PLL is preferable.

Key words: IgE antibody; Radioallergosorbent test; Benzyl penicilloyl hapten-poly-L-lysine conjugate; Benzyl penicilloyl hapten-human serum albumin conjugate

Introduction

The major antigenic and allergenic determinant of penicillins has been identified as the

product resulting from the conjugation of benzyl penicillin to HSA carrier molecules (Levine and Ovary, 1961; De Weck, 1962). This chemical structure derives from the opening of the betalactam molecule by the amino group of different carriers. This has been named the major determinant.

Other metabolic products derived from benzyl

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penicillin have been shown to be involved in allergic reactions (Dewdney, 1977; Parker, 1980; Weiss and Adkinson, 1988). Based on the conjugation of BP with either HSA or PLL different methods have been used for in vivo detection of IgE antibodies to penicillins (Basomba et al., 1979; Edwards et al., 1982). Other authors have used as the carrier different proteins (De Haan et al., 1979), polymers (Ohno and Stahmann, 1972) or red cells (De Weck, 1962). Recently systems using penicillins conjugated directly to epoxy activated resin have been reported (Harle and Baldo, 1990). In addition some studies have suggested that penicillin side chain-specific antibodies are also present in some patients' sera (Batchelor et al., 1966; Petersen and Graham, 1974; Blanca et al., 1990, 1991). However, when the same hapten is conjugated to a carrier the nature of the carrier can also influence the capacity of the conjugate to bind to IgE antibodies (Lee et al., 1985; Vega et al., 1989). In this study we compared a variety of BPO-PLL and BPO-HSA conjugates for their ability to bind to BPO-specific IgE antibodies in sera from patients allergic to benzyl penicillin. Reagent preparation and assay conditions were optimized in order to determine whether the differences observed were related to the carrier used.

Materials and methods

BPO-PLL derivatized discs

Poly-L-lysine (1 and 10 mg/ml) of molecular weights 3, 9, 22, 50, 100 and 400 kDa were coupled to cyanogen bromide activated cellulose discs as described previously (Edwards, 1982). These discs were then treated with benzyl penicillin at 1, 10, 25 and 50 mM as described by Edwards et al. (1982).

BPO-HSA derivatized discs

HSA (100 mg/ml) was treated with benzyl penicillin at 200 mg/ml in order to optimize the BPO content of the conjugates as measured by the penalmadate method (Levine, 1962). The optimal BPO-HSA conjugate (1, 5, 10, 50 and 100 mg/ml) was then coupled to cyanogen bromide-activated cellulose discs as above.

Human serum albumin (HSA)

HSA (50 mg/ml) was coupled to cyanogen bromide-activated cellulose discs as described above. These discs were then treated with benzyl penicillin at 200 mg/ml as above.

Positive sera

26 sera were obtained from patients with a history of an immediate reaction to penicillin and were all shown to contain detectable BPO-specific IgE antibody. Positive pools of high and low mean RAST value to BPO were prepared from some of these sera.

Negative sera

24 sera from atopic subjects with total IgE values in the range 5–1700 IgE units but with no history of penicillin allergy were employed. Pools of some of the sera were used in selected studies.

RAST assays

The assays were carried out as described by Wide et al. (1969) using either 30 μ l of both serum and human anti-IgE (method a) or 50 μ l of both serum and anti-human IgE (method b). Results obtained with BPO derivatized discs were expressed as percentage of label uptake after subtracting the uptake of the single carrier derivatized discs. A test sample was considered positive when it gave a result greater than the mean plus 2 SD from a group of negative sera in the same assay system.

RAST inhibition

These studies were carried out using BPO- ϵ -amino caproic acid (BPO-EACA), prepared as described by Batchelor et al. (1966) at 100, 10, 1, 0.1, and 0.01 mM in the liquid-phase assay with a sample of pooled positive sera. Assays comparing various BPO-PLL and BPO-HSA discs were carried out on the same occasion.

Tests of statistical significance

Total IgE was ascertained in the sera in order to investigate whether there was any relationship between the IgE level and the RAST value for any of the four methods used. A comparison of the variance of the four methods was made using the Kruskal-Wallis test and a comparison of the

means of the values between the BPO-HSA and the BPO-PLL methods was made using the Wilcoxon test (Siegel, 1956). The relationships between the assays were evaluated from Pearson correlation coefficients (r) and concordance was calculated from the analysis of variance (ANOVA) of two factors (serum RAST values and methods) by determining the intraclass correlation coefficient (ICC) (Fleiss, 1975).

Results

Optimization of BPO-PLL reagent discs for uptake of specific IgE antibody

As shown in Fig. 1, 10 mg/ml PLL conjugate 9 kDa coupled to discs gave a higher IgE uptake than did conjugate used at 1 mg/ml and the optimal concentration of benzyl penicillin was 25 mM. The negative control pool gave values of label uptake below 1% of total counts added. Similar results were obtained with the positive control pool and the other molecular weight PLLs used (3, 22, 50, 100 and 400 kDa). Thus there was no increase in the IgE antibody binding capacity

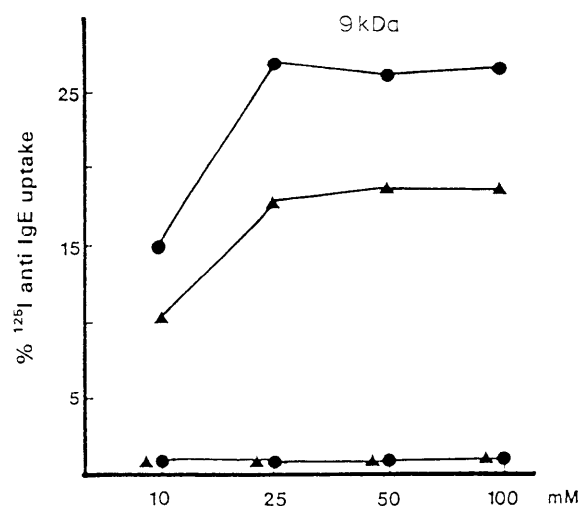


Fig. 1. Percentage ¹²⁵I uptake versus concentration of benzyl penicillin used to prepare BPO-PLL discs after initially coupling 1 (▲) and 10 (●) mg/ml 9 kDa PLL to cyanogen bromide activated cellulose discs. A positive serum pool (upper lines) and negative serum pool (superimposed lower lines) were studied.

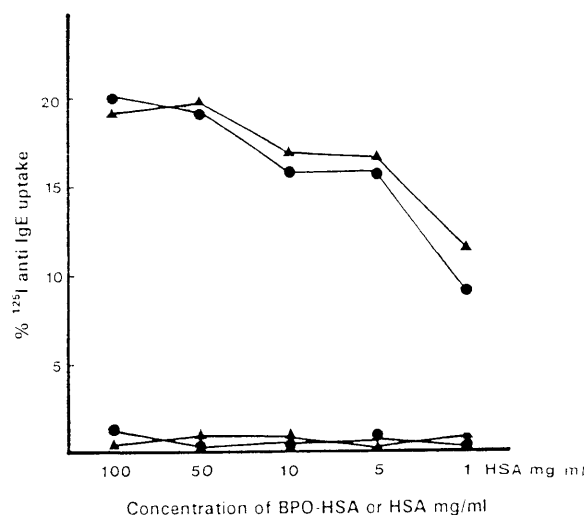


Fig. 2. Percentage uptake of ¹²⁵I anti IgE versus amount of BPO-HSA (▲) using 100 mg/ml HSA and 200 mg/ml of benzyl penicillin (see methods section) or HSA, 100 mg (●) followed by treatment of the discs with 200 mg of benzyl penicillin. A positive serum pool (upper lines) and negative serum pool (lower lines) were studied.

on increasing the molecular weight of the PLLs, although in the negative control pool and individual negative sera there was an increase of the non-specific binding. All subsequent studies employed discs prepared using 9 kDa PLL coupled at 10 mg/ml followed by treatment with 25 mM benzyl penicillin.

Optimization of BPO-HSA reagent discs

The optimal BPO content of BPO-HSA conjugates was obtained by treating 100 mg/ml of HSA with 200 mg/ml benzyl penicillin. With this ratio, the number of BPO haptens in the carrier was 22. As shown in Fig. 2, coupling of 100 mg/ml BPO-HSA to cyanogen bromide-activated cellulose discs, followed by treatment with 200 mg/ml benzyl penicillin, gave a similar optimal uptake of label using both positive and negative serum pools. Subsequent studies used discs prepared by coupling HSA at 50 mg/ml followed by treatment with 100 mg/ml BP.

Comparison by RAST inhibition of BPO-PLL and BPO-HSA RAST discs

The results of these studies are shown in Fig. 3. In all cases BPO-EACA fully inhibited anti-

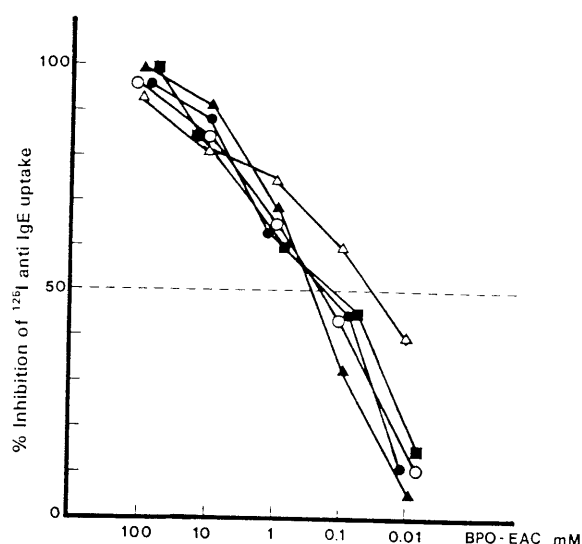


Fig. 3. RAST inhibition studies. Molecular weight of the PLLs of the conjugates were: 3 kDa (▲), 9 kDa (●), 50 kDa (■), 100 kDa (○). BPO-HSA (△). Monomers of BPO-EACA were used for inhibition in the fluid phase.

body binding, confirming the specificity of the assay system. Although all BPO-PLL discs gave similar inhibition curves, BPO-HSA discs required ten-fold less BPO-EACA for 50% inhibition. This suggests that all the BPO-PLL discs had a higher hapten density of BPO groups available for antibody binding and this would account for the generally higher antibody uptake with test sera (see later).

RAST inhibition studies using individual sera (2, 6 and 9 of Table I) are presented in Fig. 4 comparing the BPO-PLL of 9 kDa and the BPO-HSA discs. With all three sera studied higher molar concentrations of BPO-EACA were required for inhibition when BPO-PLL discs were used. These differences were of the order of 100 mM for sera 2 and 6 and 10 mM for sera 9.

Total IgE levels

These are shown in Table I. There was no significant correlation between the level of IgE and the RAST value determined by any of the methods used. The *r* value was 0.031 for BPO-PLL_a, 0.005 for BPO-PLL_b, 0.027 for BPO-HSA_a and 0.09 for BPO-HSA_b.

Comparison of RAST values of individual sera

The mean and SD values for the negative control group were 0.98 and 0.68 for the BPO-PLL_a, 0.92 and 0.20 for the BPO-PLL_b, 0.25 and 0.36 for the BPO-HSA_a and 0.35 and 0.20 for BPO-HSA_b.

RAST values 2 SD above the mean of the negative sera were considered to be positive. The results for the sera studied are presented in Table I. In most cases method 'a' gave slightly higher results than method 'b' for a given carrier system, but comparisons between PLL and HSA discs within a given method showed more striking differences. Two sera in particular, 10 and 16, were negative using HSA methods. Additionally sera

TABLE I

SPECIFIC IGE ANTIBODIES DETERMINED IN 26 SERA TO BPO-PLL AND BPO-HSA CONJUGATES

BPO-PLL_a and BPO-HSA_a: 30 µl of sera and anti-IgE were used. BPO-PLL_b and BPO-HSA_b: 50 µl of sera and anti-IgE were used. Mean value and SD for each determination was: BPO-PLL_a 16 and 14.5, BPO-PLL_b 13.8 and 13.7, BPO-HSA_a 8.3 and 9.3 and BPO-HSA_b 6.4 and 7.7.

Sera	BPO-PLL _a	BPO-PLL _b	BPO-HSA _a	BPO-HSA _b	IgE
1	10.38	8.07	4.1	3.1	38
2	47.98	41.65	21.81	18.02	90
3	27.5	22.38	17.16	10.99	65
4	44.6	48.36	20.05	18.66	40
5	14.74	11.79	10.13	8.16	1000
6	44.77	40.7	21.13	12.94	260
7	29.1	24.1	1.8	1.19	210
8	6.91	7	5.48	4.37	120
9	16.94	11.67	13.77	9.68	80
10	15.8	16.05	0.76	0.60	40
11	9.9	8.5	6.4	5.6	60
12	10.25	9.48	3.59	2.22	100
13	5.87	4.79	4.09	3.27	16
14	9.5	3.49	4	3.35	35
15	10.09	6.21	3.6	2.68	420
16	5.85	4.92	0.03	0.25	120
17	9.82	8.96	1.18	1.18	68
18	49.63	42.8	41.3	35.67	66
19	4.25	3.97	1.05	1.42	89
20	2.79	3.79	4	2.31	100
21	6.16	5.05	4.6	2.7	35
22	6.44	4.69	3.8	2.78	20
23	4.87	2.9	3.85	2.63	120
24	5.95	5.21	1.6	1.16	210
25	16.32	12.54	15.28	11	160
26	2.4	1.6	1.9	1.58	25

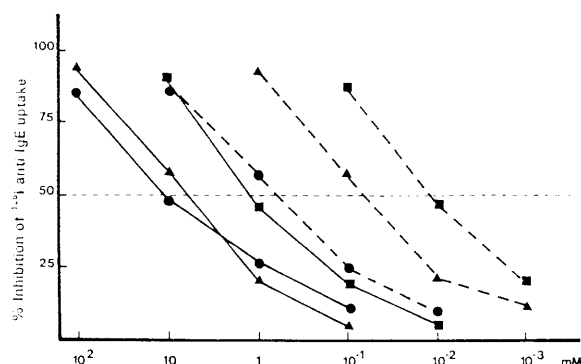


Fig. 4. RAST inhibition studies. Serum 2 ($\blacktriangle$), serum 6 ($\blacksquare$) and serum 9 ($\bullet$) were inhibited using solid-phase BPO-HSA RAST discs (dashed line) and BPO-PLL RAST discs (continuous line). In the fluid phase monomers of BPO-EACA were used.

12 and 17 were weakly positive in the HSA systems but clearly measurable with both PLL methods. The reasons for these differences are not clear but might be explained by the fact that the PLL solid phase is more efficient at binding low affinity antibodies such as may be present in these sera. When the different assays were compared (see Table I) there were significant differences between the BPO-PLL and BPO-HSA methods ($P < 0.001$), but when the results obtained by methods utilizing the same carrier were compared the differences were not significant. The Pearson correlation coefficients are presented in Table II. In all cases the r values were

TABLE II
PEARSON CORRELATION COEFFICIENTS AND ICC VALUES BETWEEN THE FOUR DIFFERENT ASSAYS
The numbers in brackets are the ICC values.

	R value (ICC value)			
	BPO-PLL _a	BPO-PLL _b	BPO-HSA _a	BPO-HSA _b
BPO-PLL _a	1 (1)	0.98 (0.75)	0.84 (0.12)	0.83 (0.13)
BPO-PLL _b	0.98 (0.75)	1 (1)	0.81 (0.11)	0.81 (0.10)
BPO-HSA _a	0.84 (0.12)	0.81 (0.12)	1 (1)	0.98 (0.61)
BPO-HSA _b	0.83 (0.13)	0.81 (0.10)	0.98 (0.61)	1 (1)

significant ($P < 0.0001$). However, the ICC values were low when the different methods were compared (see Table II).

Discussion

For the determination of antibodies of different isotypes to penicillins the nature of both the carrier (Vega et al., 1989) and the hapten (Blanca et al., 1991) may influence the capacity for binding IgE antibodies. There is evidence using rabbit polyclonal (Atsumi et al., 1967) and mouse monoclonal antibodies (De Haan, 1985; Fukushima et al., 1987) that different parts of the benzyl penicillin molecule may form different epitopes. This has also been shown with IgE antibodies from patients allergic to penicillins (Van Dellen, 1971; Blanca et al., 1988; Blanca et al., 1991). With regard to the nature of the carrier, different structures have been used for the estimation of both IgE and IgG antibodies such as proteins (Wide et al., 1971; De Haan, 1979), polylysines (Edwards et al., 1982; Blanca et al., 1990), bacteriophages (Haimovich et al., 1967) viral particles (Mamas et al., 1979), polyacrylamide (Ohno et al., 1972) and red cells (De Weck, 1964; Thiel et al., 1964). The nature of the carrier and the density of the hapten in the conjugate may influence the antibody binding capacity (Lee et al., 1985). In this study the two most commonly used carriers for determining IgE antibodies to the BPO determinant were compared in the same radioimmunoassay in order to determine whether there were differences in their capacity to bind IgE antibodies.

Preliminary results from our group indicated that differences did exist using these two different carriers (Vega et al., 1989). The PLLs may have the theoretical advantage of producing conjugates with a high density of hapten since more amino groups per molecule are available for conjugation than in HSA. Moreover, in the HSA molecule not all the BPO groups are exposed to the antibody (Lafaye et al., 1988; Yvon et al., 1990). In our study there were only slight differences between different molecular weight PLLs, however, when BPO-PLL and BPO-HSA reagents were compared by RAST inhibition using a posi-

tive serum pool and BPO-EACA as an inhibitor the results did suggest a higher hapten density on the PLL discs. When the two carriers were compared using 26 individual sera from penicillin allergic patients the two BPO-PLL systems gave higher values and four sera were either very weakly positive or were negative to BPO-HSA discs.

The data indicate that in the case of the methods using the same carrier there were no differences in the results obtained. However, the values of the means were higher when the BPO-PLL methods (a and b) were compared to the BPO-HSA methods. When the methods using different carriers were compared there was a good correlation in every case, whereas the ICC values were low when different methods were compared. Whether these differences are related with the backbone of the carrier and/or to the specificity and affinity of the antibodies is not known at present and further studies will be required to resolve these points.

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References

- Atsumi, T., Nishida, K., Kinoshita, Y., Shibata, K. and Horiuchi, Y. (1967) The heterogeneity of combining sites of benzyl penicillin antibodies obtained from single rabbit. *J. Immunol.* 99, 1286.
- Basomba, A., Villalmazo, I.G., Campos, A., Pelaez, A. and Berglund, A. (1979) IgE antibodies against penicillin as determined by Phadebas RAST. *Clin. Allergy* 9, 515.
- Batchelor, F.R., Dewdney, J., Waston, R.D. and Wheeler, A.W. (1966) The immunogenicity of cephalosporin derivatives and their cross-reactions with penicillin. *Immunology* 10, 21.
- Blanca, M., Perez, E. and Garcia, J. (1988) Anaphylaxis to amoxicillin with good tolerance to benzyl penicillin. In vivo and in vitro studies of specific IgE antibodies. *Allergy* 43, 812.
- Blanca, M., Vega, J.M., Garcia, J., Carmona, M.J., Terrados, S., Avila, M.J., Miranda, A. and Juarez, C. (1990) Allergy to amoxicillin with good tolerance to other penicillins. Study of the incidence in subjects allergic to other penicillins. *Clin. Exp. Allergy* 20, 475.
- Blanca, M., Perez, E., Mayorga, C., Sanchez, F., Fernandez, J., Juarez, C. and Suau, R. (1991) Differences in serum IgE antibody activity to benzyl penicillin and amoxicillin measured by RAST in a group of penicillin allergic patients. *Allergy* 46, 632.
- De Haan, P., Boorsma, M.D. and Kalsbeck, G.L. (1979) Penicillin hypersensitivity: determination and classification of anti-penicillin antibodies by the enzyme linked immunosorbent assay. *Allergy* 34, 111.
- De Haan, P., De Jonge, A.J.R., Veerbrugge, T. and Boorsma, M.D. (1985) Three epitope specific monoclonal antibodies against the hapten penicillin. *Int. Arch. Allergy Appl. Immunol.* 76, 42.
- Dewdney, J.M. (1977) Immunology of the antibiotics. In: M. Sela (Ed.), *The Antigens*, Vol. IV. Academy Press, New York, pp. 115-121.
- De Weck, A.L.B. (1962) Studies on penicillin hypersensitivity. I. The specificity of rabbit anti-penicillin antibodies. *Int. Arch. Allergy Appl. Immunol.* 21, 20.
- De Weck, A.L. (1964) Penicillin allergy: Its detection by an improved haemagglutination technique. *Nature* 202, 975.
- Edwards, R.G., Speckman, D.A. and Dewdney, J.M. (1982) Development and use of three radioallergosorbent tests in the diagnosis of penicillin allergy. *Int. Arch. Allergy Appl. Immunol.* 68, 352.
- Fleiss, J.L. (1975) Measuring agreement between two judges on the presence or absence of a trait. *Biometric* 31, 651.
- Fukushima, H., Misaki, R., Takeuchi, M., Ninomi, Y. and Harada, M. (1987) Mouse anti-benzyl penicilloyl IgE monoclonal antibody: preparation, characterization and cross-reactivity. *Clin. Exp. Immunol.* 68, 427.
- Haimovich, J., Sela, M., Dewdney, J.M. and Batchelor, F.R. (1967) Anti penicilloyl antibodies: detection with penicilloylated bacteriophage and isolation with a specific immunosorbent. *Nature* 214, 1369.
- Harle, D.G. and Baldo, B.A. (1990) Identification of penicillin allergenic determinants that bind IgE antibodies in the sera of subjects with penicillin allergy. *Mol. Immunol.* 27, 1063.
- Lafaye, P. and Lapresle, C. (1988) Fixation of penicilloyl groups to albumin and appearance of anti-penicilloyl antibodies in penicillin treated patients. *J. Clin. Invest.* 88, 7.
- Lee, D., Dewdney, J.M. and Edwards, R.G. (1985) The influence of the hapten density on the assay of penicilloylated proteins in fluids. *J. Immunol. Methods* 84, 235.
- Levine, B.B. and Ovary, Z. (1961) Studies on the mechanism of the formation of the penicillin antigen. III. The *N*-(*D*- α -benzyl penicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin G. *J. Exp. Med.* 114, 875.
- Mamas, S. and Dray, F. (1979) Viro immunoassay for detection of anti-penicilloyl antibodies and penicilloyl residues: comparison of results obtained by radio, viro and enzyme immunoassay. *Ann. Immunol.* 130C, 407.
- Ohno, Y. and Stahman, M.A. (1972) A new detection method for penicillin antibodies and hypersensitivity in humans

- using penicillin coupled to polyacrylamide particles. *Immunochemistry* 9, 1087.
- Parker, C.W. (1980) Drug allergy. In: C.W. Parker (Ed.), *Clinical Immunology*, Vol. II. Saunders, Philadelphia, PA, pp. 1219-1260.
- Petersen, B.H. and Graham, J. (1974) Immunologic cross-reactivity of cephalexin and penicillin. *J. Lab. Clin. Med.* 83, 860.
- Siegel, S. (1956) *Non-parametric statistics for the behavioral sciences*. McGraw Hill, New York.
- Thiel, J.A., Mitchel, A. and Parker, C. (1964) The specificity of haemagglutination reactions in human and experimental penicillin hypersensitivity. *J. Allergy* 35, 399.
- Van Dellen, R.G., Walsh, W.E. and Peters, G.A. (1971) Different patterns of wheal and flare reactivity in patients allergic to penicillins. *J. Allergy Clin. Immunol.* 47, 230.
- Vega, J.M., Blanca, M. and Miranda, A. (1989) Comparison of two methods for determining IgE antibodies to benzyl penicilloyl. *J. Allergy Clin. Immunol.* 83, 271.
- Weiss, M.E. and Adkinson, N.F. (1988) Immediate hypersensitivity to penicillin and related antibiotics. *Clin. Allergy* 18, 515.
- Wide, L. (1969) Radioimmunoassays employing immunosorbents. In: E. Diczfalussy (Ed.), *Immunoassays for Gonadotrophins*. *Acta Endocrinol.* 142 (suppl.), 207-213.
- Wide, L. and Juhlin, L. (1971) Diagnosis of penicillin allergy of the immediate type by radioimmunoassay of reagins (IgE) to penicilloyl conjugates. *Clin. Allergy* 1, 171.
- Yvon, M., Anglade, P. and Wal, J.M. (1990) Identification of the binding sites of benzyl penicilloyl, the allergenic metabolite of penicillin, on the serum albumin molecule. *J. Clin. Invest.* 263, 237.