

In vitro reactivity of penicilloyl and penicillanyl albumin and polylysine conjugates with IgE-antibody

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Abstract

Penicilloylated (BPO) and penicillanylated (BPA) poly-L-lysine (PLL) and human serum albumin (HSA) were prepared and characterized by penamaldite assay and proton NMR spectroscopy. The conjugates were coupled to nitrocellulose (NC) discs and cyanogen bromide activated paper discs and their in vitro reactivities with serum IgE antibodies were examined. Results showed that on paper discs, 55.3 and 83% of the sera reacted with PLL conjugates of BPO and BPA, respectively, while 41.5 and 58.1% reacted with HSA conjugates. On NC discs, HSA conjugates gave better results, 75.6 and 70.7%, respectively for BPO and BPA, compared with 38.6 and 50%, respectively for the PLL conjugates. Overall, the BPA–PLL conjugate on paper discs proved to be the most reactive preparation. Addition of the BPO–PLL paper disc preparation detected more positive sera (85.1%) and we believe that the combined use of these two specificities offers the best test for the detection of penicillin-reactive IgE antibodies. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

A number of different penicillin antigens have been described (Dewdney, 1977; Johnson et al., 1949; De Weck, 1983) but, for most of them, little detailed information is available on their allergenicity in man and studies comparing activities and potencies in vivo and in vitro have not been undertaken. The penicillanyl antigen, for example, first suggested over 30 years ago by Feinberg (1970), was

for many years ignored since its formation under physiological conditions seemed unlikely, but findings by Edwards et al. (1982) and Kraft et al. (1981) showed that recognition of this determinant by allergic subjects may be more common than previously assumed.

Although the penicilloyl antigen is the most studied and best defined penicillin determinant, its description as the 'major' determinant refers to its quantitative predominance under physiological conditions rather than its clinical or immunological importance. Since much less is known about all the other defined and putative penicillin antigens, more studies are needed to determine and compare the

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diagnostic relevance of both the major and minor determinants.

In our own routine diagnostic screens on over 1200 sera from subjects with suspected penicillin allergy (Baldo and Pham, unpublished), positive IgE reactions to the penicillanyl group were detected more frequently than the penicilloyl group (204 compared to 108 subjects) suggesting that this antigen may be a useful specificity to employ as a diagnostic agent. As a first step to assess this, a comparison of *in vitro* IgE antibody reactivities of the penicillanyl and penicilloyl antigens was undertaken and, at the same time, the currently accepted carrier for penicilloyl groups, polylysine, was compared to human serum albumin in studies designed to determine which carrier produced the most reactive drug conjugates.

2. Materials and methods

2.1. Sera

Sera from subjects suspected on the basis of their case history as being allergic to penicillins were received from doctors requesting tests for IgE antibodies to penicillins. Forty-seven sera, which were positive in at least one of our different serum IgE tests, were included in this study.

Thirteen sera from adults allergic to house dust mites and/or pollens, 14 normal sera and cord sera were obtained as previously described (Harle and Baldo, 1990; Pham and Baldo, 1996).

2.2. Drugs and chemicals

Benzylpenicillin sodium, poly-L-lysine hydrobromide (PLL) (DP(vis)=50, MW(vis)=10200), human serum albumin (HSA), penicilloyl (BPO) ϵ -aminocaproate sodium, *N,N'*-dicyclohexycarbodiimide and *N*-hydroxysuccinimide were obtained from Sigma Chemical Co. (St. Louis, MO). ^{125} I-anti-human IgE was from Bioclone (NSW, Australia) and cyanogen bromide (CNBr) from Riedel-de Haën AG (Germany). Centriprep was a product of Amicon Inc. (Beverly, MA).

2.3. Preparation of BPO-conjugates

Penicilloylation of HSA and PLL was carried out following the method of Bondaruk et al. (1995) (Fig. 1). HSA or PLL (100 mg) dissolved in 0.5 M K_2CO_3 was reacted with different amounts of benzylpenicillin sodium salt (100–250 mg) for 2 h at room temperature. The HSA and PLL conjugates were purified using a Sephadex G-25 column and dialysis, respectively and then lyophilized.

2.4. Preparation of benzylpenicillanyl (BPA) conjugates

2.4.1. *N*-Hydroxy succinimide ester of benzylpenicillin

The procedure followed was based on the method of Edwards et al. (1982). Benzylpenicillin (the free acid) was treated with *N*-hydroxysuccinimide (62 mg) and *N,N'*-dicyclohexycarbodiimide (110 mg) at room temperature. Two hours later the mixture was centrifuged and the supernatant evaporated to a foamy solid which, after trituration in 40–70°C petroleum ether, yielded 150 mg of light yellow powder. Thin-layer chromatography (silica; 2:1 ethyl acetate: 40–70°C petroleum ether) with iodine as indicator showed the presence of one major component ($R_f=0.49$).

2.4.2. BPA–HSA and BPA–PLL

N-hydroxysuccinimide ester of benzylpenicillin (100 mg for HSA, 200 mg for PLL) dissolved in ethanol (50 mg/ml) was added to HSA or PLL (100 mg) solution in 0.1 M sodium phosphate buffer pH 7.0 in 10 aliquots during a 2-h period at room temperature. After a further 1.5 h the mixture was centrifuged to remove the precipitate and the product was purified using a Centriprep concentrator (Fig. 1). In order to get a higher degree of substitution, treatment of the resulting conjugate with the *N*-hydroxysuccinimide ester of benzylpenicillin was repeated.

2.5. Characterization of the conjugates

2.5.1. Penamaldate assay of the BPO groups

The degree of substitution of BPO conjugates was

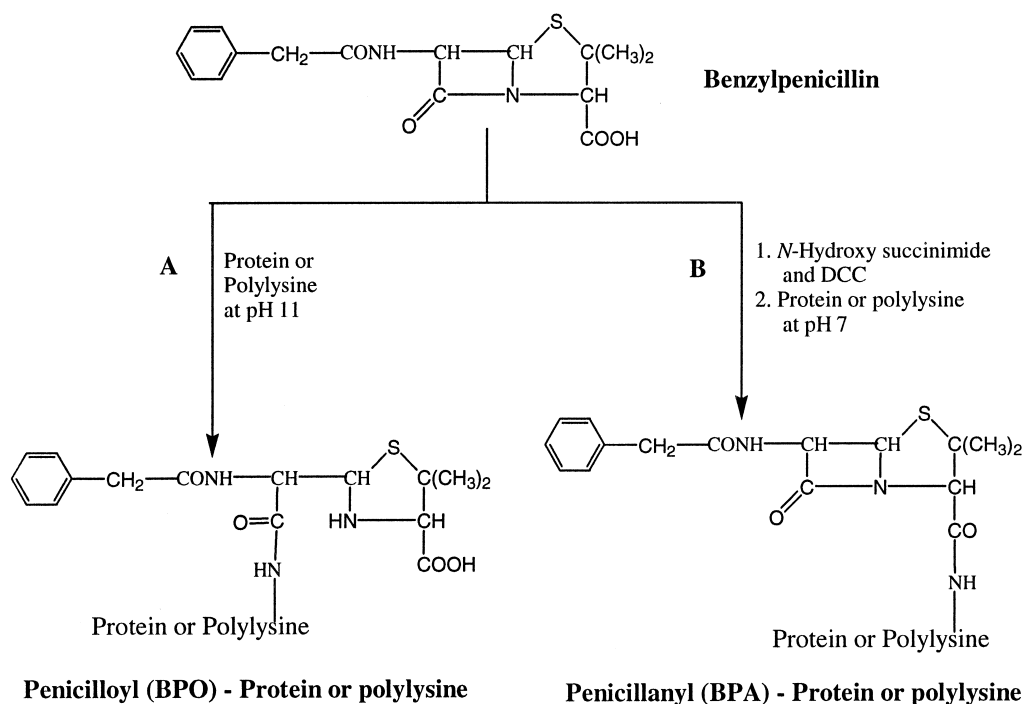


Fig. 1. Preparation and structure of (A) penicilloyl (BPO) and (B) penicillanyl (BPA) conjugates.

determined according to the method of Rolli and Schneider (1984).

2.5.2. NMR

^1H NMR spectra were acquired at 30°C on a Varian XL-400 spectrometer. Samples (3–5 mg) were dissolved in D_2O and the apparent pH was measured directly without further correction. Pre-irradiation of the HDO signal was used to suppress that peak. Chemical shifts are reported downfield from sodium 3-(trimethylsilyl) propionate (TPS), using the HDO peak at 4.725 ppm as an internal reference. The location of the HDO peak was determined in a separate experiment using TPS in D_2O at the same temperature and pH.

2.6. Preparation of conjugate discs

The pure nitrocellulose (NC) membrane, pore $0.45\ \mu\text{m}$, and 542 filter paper were from Bio-Rad Laboratories (Hercules, CA) and Whatman International Ltd (UK), respectively.

2.6.1. NC discs

Drug conjugates were adsorbed to NC discs according to the method of Pham and Baldo (1996).

2.6.2. BPO-carrier paper disc conjugate

CNBr activated paper discs (Ceska et al., 1972) were soaked with agitation in a solution of BPO-carrier conjugates in freshly made $0.1\ \text{M}\ \text{NaHCO}_3$ at a concentration of $20\ \mu\text{g}$ of conjugate per disc at 4°C for 20 h. The discs were then washed three times with $0.1\ \text{M}\ \text{NaHCO}_3$ and blocked with $0.1\ \text{M}$ ethanolamine in $0.1\ \text{M}\ \text{NaHCO}_3$ (pH 9.0) at room temperature for 3 h. After three washes with both $0.1\ \text{M}$ acetate buffer (pH 4.0) and phosphate buffered saline (PBS), the discs were stored in PBS at 4°C .

2.6.3. BPA-carrier paper disc conjugate

PLL or HSA were coupled to CNBr-activated paper discs as above before treatment at room temperature for 2 h with benzylpenicillin succinimide ester suspended in $0.1\ \text{M}$ phosphate buffer (pH 7.0) at a concentration of $250\ \mu\text{g}$ of the ester per

disc. The discs were washed six times with PBS and stored in PBS at 4°C.

2.7. Detection of drug-reactive IgE antibodies by radioimmunoassay

Antibodies were detected as previously described (Harle and Baldo, 1990). The percentage radioactive uptake of ^{125}I -anti-human IgE, i.e. the per cent of counts added was calculated and the presence of drug-specific IgE antibodies was determined by deducting the corresponding carrier background from the results of drug-carrier conjugate discs. Mean percentage uptakes obtained when the 27 control sera were used with BPO-PLL paper, BPA-PLL paper, BPO-HSA paper, BPA-HSA paper, BPO-PLL nitrocellulose (NC), BPA-PLL NC, BPO-HSA NC and BPA-HSA NC discs were 0.02 ± 0.13 , 0.07 ± 0.14 , 0 ± 0.15 , 0.02 ± 0.11 , 0.02 ± 0.04 , 0 ± 0.06 , 0.11 ± 0.14 and 0.03 ± 0.06 , respectively. To compare results in test groups, a percentage of ^{125}I uptake $\geq$ mean ± 3 S.D. of the corresponding control group for each disc was taken as the cut-off value to distinguish the positive sera.

2.8. Inhibition test

For inhibition studies, serum (20 μl), appro-

priately diluted, was incubated for 2 h at room temperature with 20 μl of a solution of inhibitor in PBS pH 7.2 before the addition of a penicillin-carrier conjugate-disc. The assay was then continued as described above.

2.9. Statistical analysis

Comparison of differences between test groups was made by chi-square analysis.

3. Results

3.1. Characterization of the conjugates

BPO-conjugates containing from 11 to 67% BPO groups for BPO-PLL and 24–59% for BPO-HSA were prepared and characterized by the penamaldate assay (Rolli and Schneider, 1984). The penamaldate assay is a well established method to characterize BPO conjugates, whereas there is, as yet, no validated method to characterize BPA conjugates.

The ^1H NMR spectra of the BPO-PLL conjugates were similar to those reported previously by Caneva et al. (1993) with broad peaks for the H_α , H_β , H_γ and H_δ protons of polylysine at 4.30, 1.67–1.77, 1.40 and 1.67 ppm, respectively (Fig. 2A). At pH

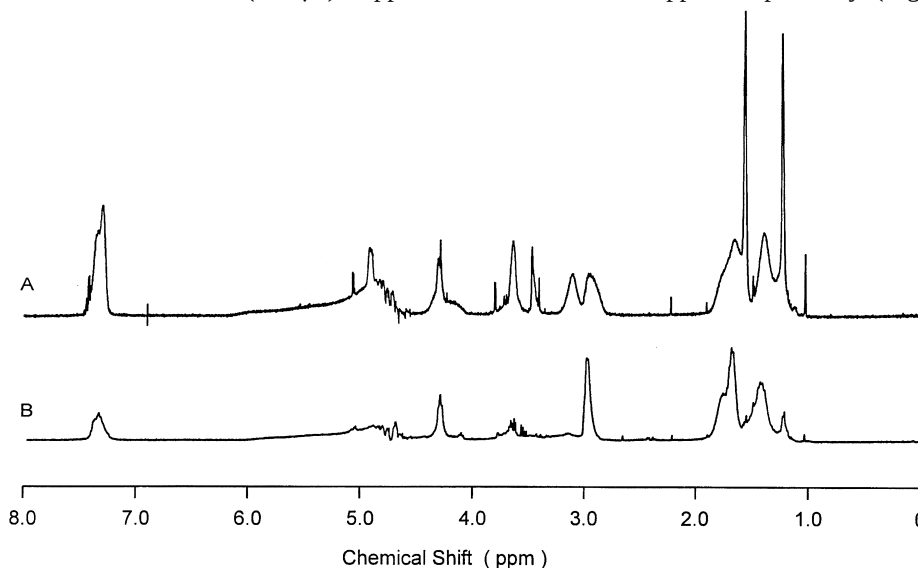


Fig. 2. ^1H NMR spectra of (A) BPO-PLL with 43% (48% in the penamaldate assay) of substitution and (B) BPA-PLL with 8% of substitution calculated by the relative areas of the H_δ peak of substituted and unsubstituted lysines.

5.65, the H_ϵ resonances occurred at 2.95 (unsubstituted) and 3.11 ppm (substituted). The peak produced by unsubstituted residues moved upfield to 2.58 ppm when the pH was increased to 11.8, while the position of the H_ϵ peak for substituted residues remained unchanged. The relative areas of these peaks provide a simple and direct measurement of the extent of derivatization of the lysine residues. The percentage substitution of BPO groups in the PLL carrier was 43%. Assaying penamaldate in the same sample gave a value of 48%.

The BPO resonance positions were also similar to those reported previously (Caneva et al., 1993) with methyl resonances at 1.24 and 1.57, H_2 at 3.47, benzylic CH_2 at 3.64 and aromatic hydrogens near 7.3 ppm (Fig. 2A). The H_6 proton was observed as a doublet at 4.91 ppm with a 7–8 Hz coupling constant as expected for the quasi-free rotation around the C_5 – C_6 bond resulting from opening of the β -lactam ring. The degree of substitution can also be estimated from the ratio of the sum of the areas of the lysine H_ϵ peaks to the area of the benzylic proton peak or from the ratio of the lysine peak areas to that of the aromatic proton peak. Estimates obtained in this way were within a few percent of those obtained from the ratio of the substituted and unsubstituted lysine H_ϵ peaks.

The level of substitution of the BPA conjugates was low (8%) and the aromatic and benzyl protons gave the only clear signals in the ^1H NMR spectrum from the penicillanyl part of the molecule (Fig. 2B). There was a small doublet at 5.07 ppm with a coupling constant of 4.5 Hz. The different chemical shift and the small coupling constant strongly suggest that the β -lactam ring is present.

3.2. Effect of degree of substitution on reactivity of conjugates

In preliminary studies, the BPO- and BPA-PLL conjugates on CNBr-activated paper discs showed the highest reactivities. Since the degree of substitution of PLL by BPA is not measurable when the BPA is coupled to PLL paper discs, BPO-PLL paper discs were used to conduct the study. The results (Fig. 3) showed that some sera were reactive to changes in the degree of substitution (Nos. 45, 44 and 20) whereas others gave similar uptakes (Nos.

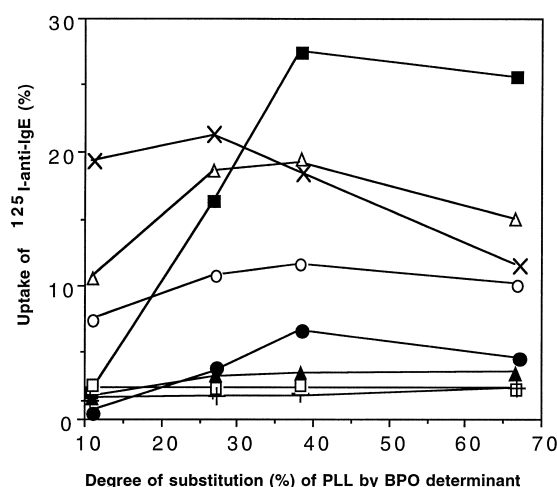


Fig. 3. The effect of degree of substitution of PLL by BPO groups on the IgE reactivities of the conjugates linked to paper discs with sera 11 (1:3) (○), 20 (1:3) (●), 10 (1:3) (+), 18 (×), 38 (□), 45 (■), 44 (△), and 44 (1:3) (▲).

11 and 38). For sera of low uptake (Nos. 10, 44 and 38), the highest degree of substitution (35–65%) gave the best uptake. For the strongest reacting sera, the highest uptakes occurred with conjugates with lower (25–40%) degrees of substitution. The results obtained using BPO-HSA NC discs were similar (data not shown). Since the main purpose of this study was to develop a method to detect as small an amount of IgE antibody as possible, the highest degree of substitution was used in all later experiments.

3.3. The effect of carrier on the reactivities of the conjugates

Using BPO conjugates coupled to CNBr-activated paper discs, the percentages of positive sera detected were 55.3 and 41.5%, respectively ($P < 0.25$) for PLL and HSA carriers. For the equivalent BPA-paper disc preparations, the percentages were 83 and 58.1%, respectively ($P < 0.01$). In the case of NC discs, BPO-HSA and BPA-HSA discs detected 75.6 and 70.7% of positive sera, and these results were higher than the results obtained with BPO-PLL NC discs (38.6%, $P < 0.001$) and BPA-PLL NC discs (50%, $P < 0.1$), respectively (Fig. 4).

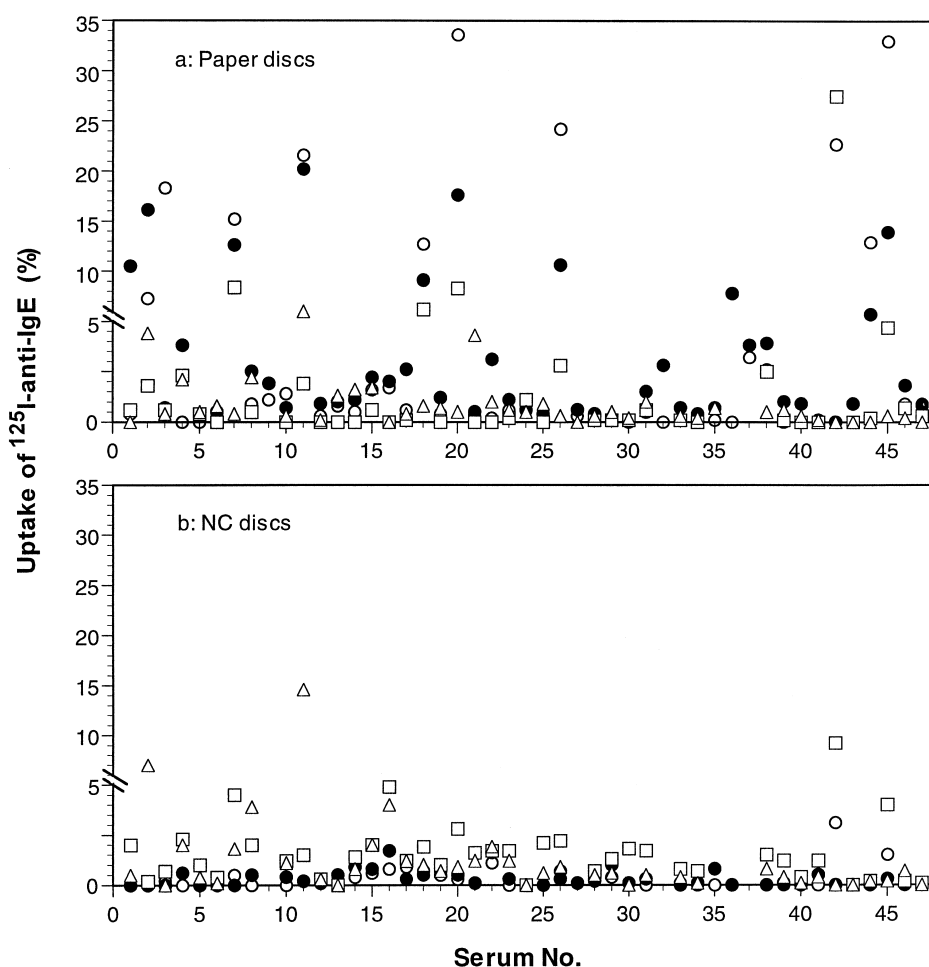


Fig. 4. Specific reactivity (as measured by uptake of ^{125}I -anti-human IgE) of 47 positive sera with CNBr activated paper discs (a) or NC discs (b) that had previously been coupled to BPO-PLL ($\circ$), BPA-PLL ($\bullet$), BPO-HSA ($\square$) or BPA-HSA ($\triangle$). The percentages of positives classified as mean+2 S.D., mean+3 S.D. or mean+5 S.D. of control sera were 68.1, 55.3 or 42.6 for BPO-PLL paper discs; 89.4, 83.0 or 66.0 for BPA-PLL paper discs; 46.3, 41.5 or 26.8 for BPO-HSA paper discs; 72.1, 58.1 or 39.5 for BPA-HSA paper discs; 50.0, 38.6 or 34.1 for BPO-PLL NC discs; 50.0, 50.0 or 31.8 for BPA-PLL NC discs; 80.5, 75.6 or 65.9 for BPO-HSA NC discs; and 75.6, 70.7 or 68.3 for BPA-HSA NC discs, respectively.

3.4. The effect of solid support on the reactivities of the conjugates

For BPO-PLL conjugates, the percentages of positive sera were 55.3 and 38.6%, respectively ($P < 0.25$) when CNBr-activated paper discs and NC discs were used as the support. The corresponding results for BPA-PLL conjugates, viz. 83 and 50%, respec-

tively were significantly higher ($P < 0.001$). Hence, for both specificities, a higher number of positive reactions was detected with the CNBr-activated paper support. The percentages of positive sera for BPO-HSA on CNBr-activated paper discs and NC discs were 41.5 and 75.6%, respectively ($P < 0.01$) while for BPA-HSA, 58.1 and 70.7% of sera were classified as positive ($P < 0.5$) (Fig. 4). In other

words, for the HSA conjugates, the NC solid phase revealed more positive reactions than the drug-HSA-paper disc support.

3.5. Comparison of the BPO and BPA conjugates

When the reactivities of BPO conjugates were compared with the BPA conjugates on different carriers and different solid supports, the BPA conjugates detected higher percentages of positive sera with 83 vs. 55.3% with PLL conjugates on CNBr-activated paper discs, 58.1 vs. 41.5% (HSA conjugates on CNBr-activated paper discs) and 50 vs. 38.6% (PLL conjugates on NC discs). For the drug-HSA conjugates on NC discs BPO conjugates gave better results (75.6 vs. 70.7%) (Fig. 4). Differences were not significant ($P>0.1$) except for PLL conjugates on CNBr-activated paper discs ($P<0.01$).

3.6. The choice of the most reactive drug-carrier-solid phase complex

Fig. 4 shows that BPA-PLL coupled to CNBr-activated paper discs detected the highest percentage of positive sera. If a serum was clearly positive (for example, $>2\%$) with any other drug-solid phase complex, it was also positive with BPA-PLL or BPO-PLL-paper discs except for only one serum (No. 25). When the BPO- and BPA-PLL-paper discs were compared, in most cases the BPA complex gave higher percentage uptakes of ^{125}I -anti-IgE when the uptake was less than 10%. For sera

showing an uptake $>10\%$, BPO-PLL-paper discs showed the highest uptakes. Thirteen sera were found to be positive only with BPA-PLL-paper discs whereas only one serum was positive only with the BPO-PLL-paper disc complex reinforcing the structural difference between the BPO-PLL and BPA-PLL antigens.

3.7. Preliminary inhibition studies

Three sera were examined (Fig. 5). The patterns of inhibition by penicillin G and benzylpenicilloylated ϵ -aminocaproic acid (BPO-ACA) were different among the sera. For serum 11, the potencies of inhibition of both inhibitors were nearly the same when BPO-PLL-paper discs were used, but the potency decreased significantly for BPO-ACA when BPA-PLL-paper discs were used. For sera 45 and 44, BPO-ACA was always the better inhibitor with both discs.

4. Discussion

PLL conjugates of penicillins have been the most widely used penicillin antigens for allergy testing (De Weck, 1983; Parker et al., 1962; Levine, 1964) although Parker et al. (1962) found that BPO-polylysine and BPO-protein conjugates were about equally effective in eliciting wheal and erythema responses in humans. More recently, Blanca et al. (1992) found that BPO-PLL was superior to BPO-

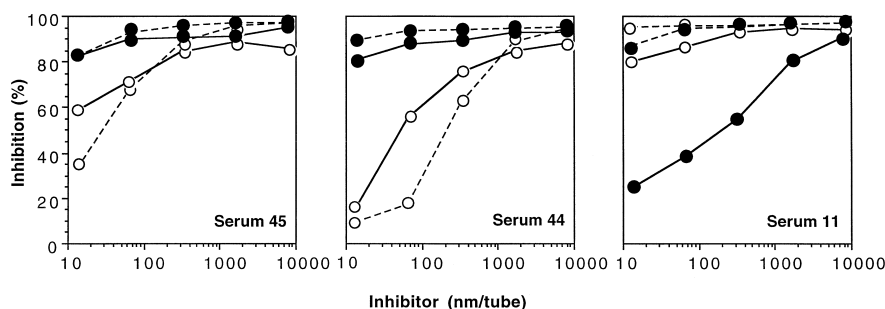


Fig. 5. Inhibition of IgE antibody binding to BPO-PLL (---○---) and BPA-PLL (—●—) linked to paper discs by sodium benzylpenicillin (○) and benzylpenicilloyl ϵ -aminocaproate (●). Sera 45, 44 were used undiluted; serum 11 was diluted (1/2).

HSA in both inhibition and direct IgE antibody binding studies.

Perhaps the single most important result in this study was the finding that overall, more positive reactions were detected with the BPA than with the BPO specificity. This was most marked with conjugates linked to the paper disc solid phase and least apparent with the HSA conjugates on NC where BPO conjugates proved slightly more reactive. While NC proved to be the superior solid phase for the HSA conjugates, uptakes were generally low and certainly inferior to the results obtained with PLL conjugates covalently coupled to paper. This finding is consistent with the results of Blanca et al. (1992). For both the BPO and BPA specificities paper discs proved a superior support to NC perhaps because retention of PLL by NC is poor or at least not as good as it is with HSA.

An interesting finding was the occasional positive reaction obtained with the BPA but not the BPO determinant (for example, sera 1 and 4 with BPA–PLL paper discs) and the reverse viz. recognition of the BPO but not the BPA specificity (e.g. serum 42). This provided extra evidence that the BPO specificity was not contaminating the BPA conjugates and vice versa.

In addition to the need to assess and compare allergenic reactivities of the various putative penicillin antigens (De Weck, 1983) both in vitro and in vivo, different antigenic complexes prepared for study and clinical use need to be characterized and standardized. An important aspect of such definitions is the relationship between the degree of substitution of penicillin derivatives in the conjugate and the biological activity of the conjugate. Although the penamaldate assay is a well established method for the characterization of BPO conjugates (Rolli and Schneider, 1984), the need for sensitive and reliable methods for the characterization of BPA conjugates remains. For BPO–PLL conjugates, the penamaldate assay is routinely used for characterization and our findings lead us to recommend this assay together with NMR spectroscopy for the characterization of BPO conjugates. With the BPA conjugates, the picture is less clear. For the characterization of BPA–PLL, we currently employ proton NMR spectroscopy. It is a useful and convenient method for estimating the degree of substitution of polylysine

conjugates and has the advantage that it measures the number of substituted and unsubstituted lysine residues directly. The one disadvantage is that the need to estimate the position of the baseline can lead to uncertainties of the order of $\pm 4\%$. Other methods including a modified penamaldate assay, employment of radioactive tracer and estimation of free amino groups are currently being investigated in an effort to determine accurately the hapten-carrier structure, the degree of hapten substitution and the correlation between the results of the different analytical methods.

In conclusion, even though the BPO–HSA conjugate on NC discs showed a high frequency of recognition of IgE antibodies, the high recognition frequency obtained with BPA–PLL, the higher uptakes of ^{125}I -anti-IgE obtained with BPO–PLL and BPA–PLL and the use of both the BPO and BPA specificities offers, we believe, the best combination of tests for the detection of penicillin-reactive IgE antibodies.

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