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Role of Pencillin Binding Proteins in Pencillin Allergy

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Abstract

All β-lactam sensitive bacteria contain enzymatic penicillin binding proteins (PBPs), which are membrane-bound enzymes and targets of β-lactam antibiotics. In this work evaluation of the significance of PBPs in immune response to benzylpenicillin was presented. 35 patients with allergic reactions to penicillin and 17 subjects without penicillin allergy, but exposed, were studied. Proliferative T-cell responses to benzylpenicillin, penicillin and PBPs conjugates (Pc-PBPs) from *E. coli*, *K. oxytoca*, *S. aureus*, *S. epidermidis* or serum protein (Pc-S) were measured. Although each allergic individual responds to Pc-PBPs in several different ways, specific proliferation of T lymphocytes with Pc-PBPs from bacterial membranes was significant higher than with Pc or Pc-S. This observation gives us a real insight into the causes of the drug allergy: individual allergic reaction and susceptibility to the drug is in strict correlation with bacterial infection. It seems likely that acylation of PBPs could be the trigger for primary immune response to the hapten benzylpenicillin.

Key words: drug allergy, hapten, carrier, penicillin binding proteins, adjuvant

Introduction

All eubacteria contain membrane-bound penicillin binding proteins (PBPs), the enzymes that are required for the biosynthesis of the bacterial cell wall (Ghuysen, 1991; Yousif *et al.*, 1985). PBPs catalyze the final steps of the polymerization (transglycosylation) and cross-linking (transpeptidation) of peptidoglycan, an essential component of the bacterial cell wall (Ghuysen and Dive, 1994). PBPs are membrane-bound enzymes and targets of β-lactam antibiotics (Jamin *et al.*, 1993). These enzymes interact with the β-lactam ring *via* serine residue acylation. Bacteria have between three and ten PBPs, depending on the species. Current understanding of how drug allergy arises is based largely on hapten hypothesis. It is a long-standing doctrine stating that to induce immune response (antibody- or cell-mediated) the drug must be conjugated to serum protein (especially to human serum albumin-HSA) (Wal *et al.*, 1991).

In sensitization period benzylpenicillin is administered usually during bacterial infections. Much of the literature on penicillin allergy is devoted to the identification of penicillin and plasma protein derivatives. Little qualitative information is available on the nature of the conjugates and bacterial proteins, that are the target for β-lactam antibiotic. Limited information is available concerning the specificity of antibodies against penicillin-PBP complexes. Furthermore, no documented studies have been reported on T-cell epitopes formation by the conjugates.

The purpose of the study was to determine the origin of penicillin-protein conjugates, which induce primary immune response, in order to test the hypothesis that it is in strict correlation with bacterial infection.

Experimental

Materials and Methods

Antigen. Benzylpenicillin (potassium salt) 50 mg/l was dissolved in physiological saline and used in lymphocyte transformation test (LTT). In preliminary studies optimal concentration of benzylpenicillin and its conjugates for lymphocyte proliferation was tested. Benzylpenicillin and serum conjugates (Pc-S) were obtained just as described previously (Zdziarski, 2000): benzylpenicillin

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(potassium salt) 50 mg/l was dissolved in the autologous serum, gently shaken and incubated overnight at 37°C for 3 h. In the 3rd h *in vitro* the covalent binding of benzylpenicillin was about 1.6%, which closely resembles the dose 9.6 mg/kg *in vivo* (Kitteringham *et al.*, 1987). The concentration corresponds to the benzylpenicillin content of serum *in vivo* (about 1 million IU per 70 kg).

Clinical strains of *Staphylococcus aureus*, *S. epidermidis, Klebsiella oxytoca* and *Escherichia coli* were used in the study. All strains derived from patients with sepsis or bacterial endocarditis treated with benzylpenicillin plus gentamicin and complicated by penicillin allergy. Bacterial lysate was prepared according to standard methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically (NCCLS, 1997). The samples with minimal bactericidal concentration (MBC) contained bacterial lysate from 10^6 bacterial cells and benzylpenicillin in MBC (in minimal bactericidal concentration). Broken cells in these samples were centrifuged (60 000 rpm for 3 h) for membrane collection. After washing with phosphate buffer, the membranes were stored at –20°C and then used as Pc-PBPs source for T cell stimulation (Hackenbeck *et al.*, 1986). Knowing that each bacterial strain contains a specific set of PBPs, T-cells of penicillin-allergic patients were stimulated with species-specific conjugates and then compared with Pc-S and benzylpenicillin alone.

Test. Lymphocyte proliferation studies were performed as conventional tritiated thymidine incorporation assay. Heparinized blood was collected from the patients. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Paque (1.077 g/cm^3; Pharmacia Uppsala, Sweden) density centrifugation, washed twice and cultured in RPMI 1640 (Flow) enriched with 2 mM L-glutamine (Serva) and 5% autologous serum without addition of various growth enhancers (Maria *et al.*, 1994; Cederbrant *et al.*, 2000). Each well contained 100 µl of the medium.

PBMCs (1×10^5) were cultured for 5 days in culture microplates (Falcon) in triplicate at 37°C and 5% CO2 (Assab) in the presence of benzylpenicillin, its derivatives (*i.e.* Pc-S, Pc-PBPs) or with medium alone. To measure proliferation, 1 µCi tritiated thymidine (^{3}HTdR; IPUR) was added in a volume 10 µl to the triplicate cultures for 12h. Then, the cultures were harvested onto glassfiber filters with a semi-automatic cell harvester (Scatron) and ^{3}HTdR incorporation was measured in beta counter Rack Beta 2017 (LKB). The results are expressed as count per minute (cpm).

Statistical comparison of T cell responses in allergic and healthy donors was made using the analysis of variance Student's t-test for unpaired data with significance level $\alpha = 0.05$.

Results

In the procedure applied benzylpenicillin was bound by PBPs with high affinity, because all strains used in the study were benzylpenicillin sensitive (MBC < 1 µg/ml) (Mouz *et al.*, 1998). The results of thymidine incorporation measurements under the effect of various penicillin derivatives are presented in Table I. A significant difference in cpm with Pc and each Pc-PBPs was observed between allergic and healthy subjects ($p < 0.05$ and 0.00001 respectively), but no statistically significant difference was seen in Pc-S-induced lymphocyte proliferation.

Interestingly, lymphocytes from allergic subjects proliferate in a heterogeneous manner, but it tends to be the rule that the response to several bacterial conjugates was much higher than to benzylpenicillin or serum complexes. Arithmetical means from measurements demonstrate relatively well that bacterial derivates (Pc-PBPs) were the strongest stimulators, although this does not reflect the fact that each patient showed its individual profile (Fig. 1). Surprisingly, some species-specific sets of Pc-PBPs from bacterial membranes were slightly or not at all recognized by T-cells from allergic subjects, whereas the others induced high proliferative response of lymphocytes (Fig. 2). Each allergic person responded to bacterial derivatives in his/her respective way, therefore standard deviation (SD) for each Pc-PBPs was much higher than for Pc or Pc-S, but similar to that in healthy donors (Table I). Despite significant differentiation, it appeared to be the rule that in every patient proliferation under the influence of the most active antigens was by an order of magnitude higher than under the influence of the remaining ones, often demonstrating inhibitory properties ($p < 0.001$). Such a tendency was not observed in the group of subjects responding well

Table I
T-cell proliferation to different benzylpenicillin derivatives in studied subjects

		Medium alone	Pc**	Pc-S***	Pc-PBPs*			
					S. aureus	*S. epidermidis*	*E. coli*	*K. oxytoca*
Allergic N = 35	mean	428.51	898.89	1213.07	1721.30	3195.14	2972.89	3071.15
	SD	271.97	1224.53	2782.25	3652.01	4910.65	4713.80	4864.16
Healthy N = 17	mean	493.98	520.66	583.26	579.16	556.97	704.23	564.82
	SD	715.01	670.84	531.83	629.91	438.26	614.69	353.09

Results are expressed as the mean and the standard deviation of data from experiments for each donor (triplicate cultures for 17 non-allergic and 35 allergic donors).
allergic vs healthy *$p < 0.0000001$ **$p < 0.05$ ***Not significant by Student's t test.

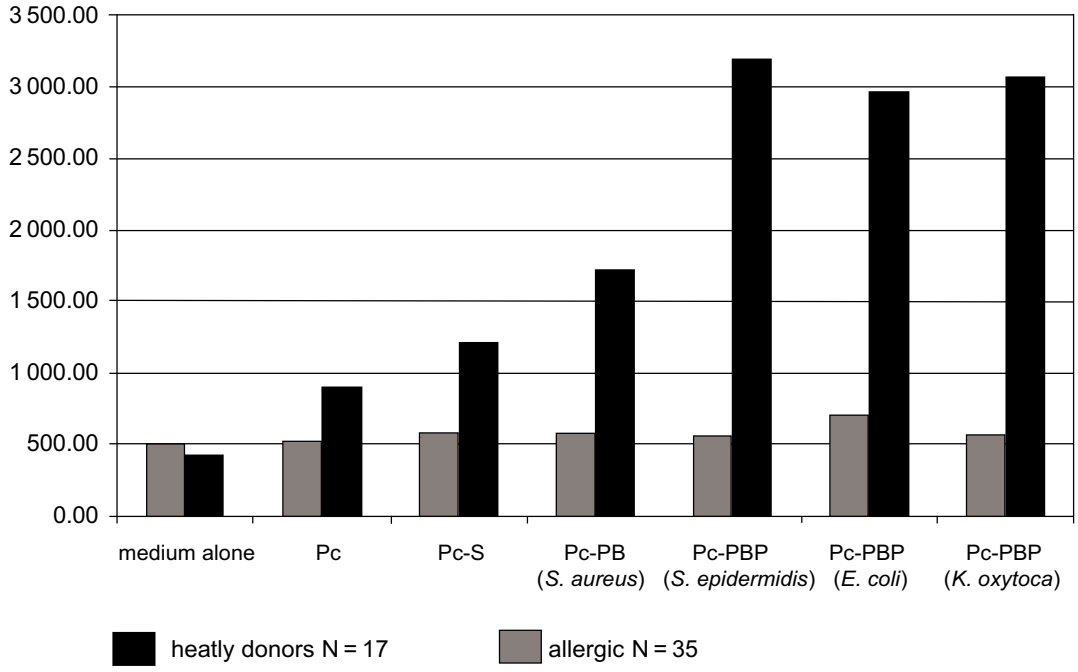

Fig. 1. T-cell proliferation to hapten benzylpenicillin (Pc) and benzylpenicillin derivatives with serum proteins (Pc-S) or species-specific penicillin binding proteins (PBPs) in bacterial lysates.
The proliferative responses are expressed in cpm and given as the mean.

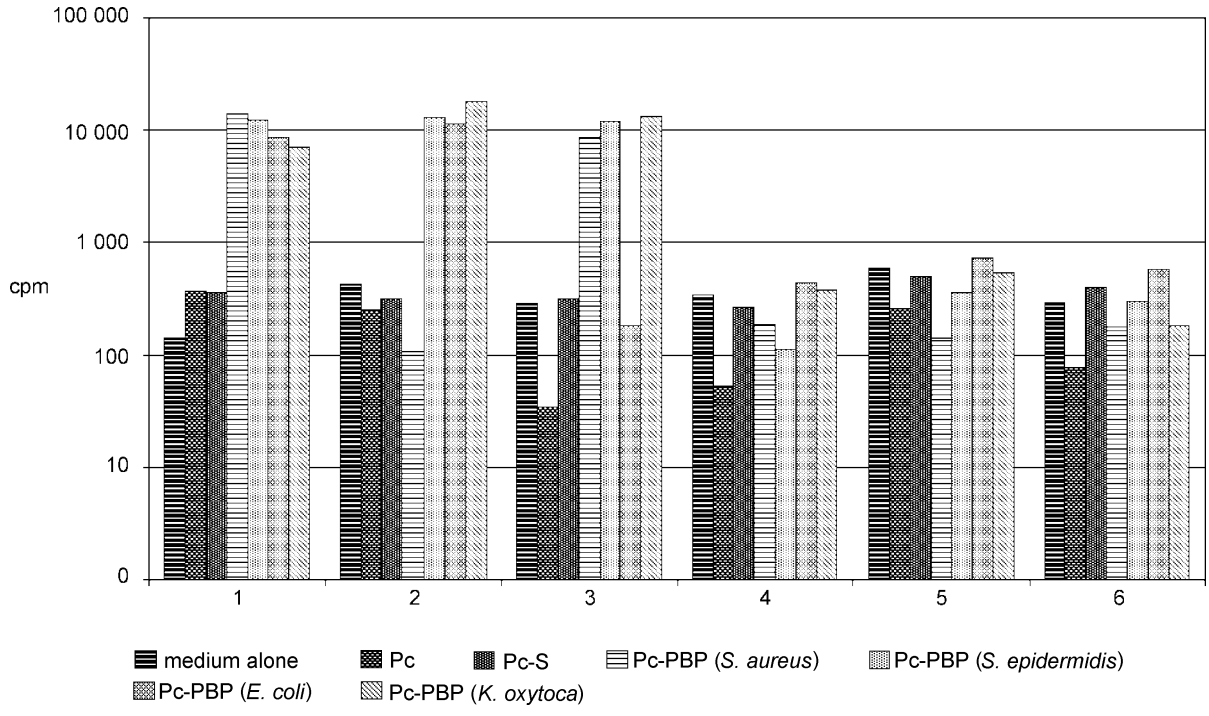

Fig. 2. Lymphocytes proliferative response to the applied antigens in 3 allergic (1–3) and 3 healthy (4–6) patients

to therapy – differences between particular antigens were insignificant and did not reach significance threshold (data not shown).

Twenty seven (79%) allergic patients had positive result of LTT with Pc, 20 (57%) – with Pc-S, 14 (40%) – with bacterial lysates from *S. aureus*, 23 (66%) – *S. epidermidis*, 24 (69%) – *E. coli* and 22 (63%) with *K. oxytoca*, respectively (data not shown).

Discussion

It is a general paradigm that reactivity to hapten benzylpenicillin requires the association of the drug to a putative carrier protein (Warbrick, 1995). Although this phenomenon was not controlled in the study (neither *in vivo* nor *ex vivo* (Maria *et al.*, 1994)), benzylpenicillin shows very high reactivity and can easily bind to serum albumin and globulin in the system (Zdziarski, 2000). Covalent binding of benzylpenicillin to serum proteins *in vitro* is of similar magnitude to that observed *in vivo* (Kitteringham *et al.*, 1987). In spite of the hapten hypothesis no statistically significant difference between healthy and allergic donors was seen in Pc-S-induced lymphocyte proliferation (Table I). Sometimes Pc-S inhibited spontaneous tritiated thymidyne incorporation (cpm for medium alone was higher than cpm for Pc-S) (data not shown).

Penicillin-binding proteins (PBPs) are vitally important targets in the killing of bacteria by β-lactams. PBPs have often been detected by labelling bacterial membrane preparations with ^{3}H-, ^{14}C-, or ^{125}I-labelled benzylpenicillin, separating the labelled proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and exposing the gels to X-ray films (Masson *et al.*, 1983). Benzylpenicillin can easily form a major determinant *via* acylation of serine residue and block catalytic sites of PBPs, then epitope density of benzylpenicillin-PBP conjugates is concentration independent (data not shown). Furthermore, PBPs are a better carrier for benzylpenicillin than serum proteins as a result of: a) structure and high molecular weight (M = 30000–90000); b) phylogenic distinction between PBPs and histocompatibility proteins; c) high affinity of PBPs to benzylpenicillin (dissociation constant $k \approx 10^{-3}/s$); d) a strong, antigen-specific immune response induction by PBPs (Den Blaauwen *et al.*, 1989).

Undoubtedly at least one bacterial adjuvant *i.e.* muramyl dipeptide (MDP) was present in each bacterium used in the study. It is the smallest adjuvant active part of complete Freund's adjuvant and the product of enzymatic lysis of murein (peptydoglycan) by lysosyme. Intraperitoneal injections of penicillin and Freunds adjuvant or bacterial lysate from *S. aureus* 209P lead to anaphylactic shock and death of guinea pigs (Zdziarski, 1996/97). The influence of bacterial adjuvants is also partly PBPs-dependent. Acylation of active site of PBPs by β-lactam antibiotics induces large increases in both free and total endotoxin. β-lactam antibiotics greatly enhanced the release of lipoteichoic acid and peptidoglycan. Further, bacterial products from bacteria, such as peptidoglycan, teichoic acid and toxins, activate mononuclear cells to produce tumor necrosis factor alpha (Prins *et al.*, 1995). This puzzling observation concerns the specificity of the lymphocyte proliferation in our procedure. On the other hand, a similar approach has been used successfully to detect antibody against benzylpenicilloyl moiety in Pc-PBPs (Hackenbeck *et al.*, 1986). In addition, all bacterial membranes used in the study hardly induced lymphocyte proliferation in all healthy donors, but these compounds enhanced immune response when administered with specific antigen (Pc-PBPs) (Table II), insomuch each allergic individual responds to Pc-PBPs in several different ways (Fig. 2). Although bacterial lysate used in the study contains several active compounds (*i.e.* murein, microbial glycolipids, endotoxin), much higher lymphocyte proliferation for several bacterial membranes with Pc-PBPs than with medium alone was found exclusively in allergic donors. In general, acylation of PBPs in sensitive bacteria caused lysis and subsequent enhancement of inflammatory cascade, than led to increase of presentation of antigen and antigen-specific lymphocyte proliferation. The activity of hapten-specific T cells is modulated by the integration of different signals *i.e.* specific *via* T cell receptor complex and costimulatory from bacterial membranes via other receptors (Table II), therefore lymphocytes from allergic subjects proliferate in a heterogeneous manner (Fig. 2).

These findings taken together indicate that penicillin allergy pathogenesis may be associated with bacterial infection. Further support the hypothesis is based on weak lymphocyte stimulation by Pc-S (Fig. 2).

Table II
Cross talk between signal transduction pathways in lymphocyte

SIGNALS costimulatory / Antigen specific	*S. aureus*	*S. epidermidis*	*E. coli*	*K. oxytoca*
+ (allergic)	4.02	7.46	6.94	7.17
– (healthy donors)	1.17	1.13	1.43	1.14

+ – presence, – –absence of antigen-specific signal
Results are expressed as stimulation index *i.e.* cpm of cultures with cell wall /cpm with medium alone

This study reinforces our clinical finding: 100 patients with negative skin test before and with symptoms of penicillin hypersensitivity during antibiotic treatment were diagnosed. We report only 4 cases, where allergy to penicillin was confirmed by the same skin test. The patients received penicillin during serious bacterial infections and experienced allergic symptoms (Zdziarski, 1999). Eastaway and coworkes published similar case report of gas gangrene complicated by penicillin allergy (Eastaway and Soutar, 1994).

Thus, these data for the first time provide insight into the origin of the immunogen responsible for penicillin allergy. Molecular structure of several PBPs and the presence of bacterial adjuvant may largely improve immunogenicity of hapten benzylpenicillin for lymphocytes.

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