

In vitro lymphocyte proliferation in the diagnosis of allergy to phenoxymethylpenicillin

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Background The aim of this study was to investigate *in vitro* lymphocyte proliferation in the diagnosis of allergy to phenoxymethylpenicillin (PcV), comparing chemically reactive PcV, added to cell cultures in unconjugated form, to a PcV-PLL (poly-L-lysine) conjugate as antigens. Side-chain specificity of lymphoproliferative responses was investigated with reactive benzylpenicillin (PcG) and bacampicillin.

Methods Seventeen patients with a history of hypersensitivity reactions in connection with PcV treatment were studied by means of the lymphocyte transformation test (LTT), the radioallergosorbent test (RAST), skin tests (prick and intracutaneous), and oral challenge with PcV. LTT was also performed in 20 control subjects exposed to PcV therapeutically, and in eight subjects with occupational exposure to this penicillin.

Results Nine patients had a positive *in vivo* test to PcV (five by oral challenge, three by intracutaneous test, and one by both tests), and six were challenge-negative. When reactive PcV was used as antigen in LTT, positive LTT responses were observed in five of the nine patients with a positive *in vivo* test, and two of them were also side-chain specific. Positive LTT responses with reactive PcV also correlated with a positive RAST in five of seven subjects. None of the six patients with a negative challenge test, and only one of the 28 controls showed a positive LTT result with reactive PcV. Thus, the specificity of LTT with reactive PcV was 96%. In contrast, when PLL-conjugated PcV served as antigen, four challenge-negative subjects and 11 controls were LTT-positive.

Conclusions The results of this study indicate that LTT with chemically reactive PcV could be useful as an *in vitro* complement in the diagnosis of PcV allergy and as a tool to reveal the side-chain specificity of peripheral blood lymphocytes. A positive LTT to PLL-conjugated PcV may be an indicator of immunization, but not necessarily allergy, to the penicilloyl structure.

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Key words: antibiotics; benzylpenicillin; challenge test; drug hypersensitivity; human lymphocytes; lymphocyte transformation test; phenoxymethylpenicillin; polylysine; RAST.

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Although the true incidence is difficult to assess, penicillins may induce hypersensitivity reactions in up to 10% of treated patients (1). Generally, *in vitro* techniques for the diagnosis of allergy are

justified, since *in vivo* tests are time-consuming for the patient, sometimes are limited by subjective interpretation of their results (2), and could induce adverse reactions (1) followed by the potentiation of antigen-specific cells and antibodies. Approximately 95% of administered penicillin binds to proteins and creates penicilloyl, formed by the β -lactam ring of the penicillin molecule and the ϵ -amino group of lysine residues on proteins. This

Abbreviations and acronyms. LTT: lymphocyte transformation test; PcG: benzylpenicillin; PcV: phenoxymethylpenicillin; PLL: poly-L-lysine; PPD: purified protein derivative (tuberculin); RAST: radioallergosorbent test.

Table 1. Patients included in study

Subject	Sex	Age (years)	Clinical manifestations upon PcV regimen/onset	Time since last exposure
HP2	M	41	U, Q/immediate	2 years
HP3	F	44	U/late	10 months
HP7	F	45	U/late	3 years
HP9	F	51	U, Q/immediate	3 years
HP10	F	50	E/late	3 years
HP12	F	69	D/immediate	6 years
			A/immediate ^a	5 years
HP13	F	22	U, Q/immediate	10 years
			A/immediate	2 years
HP14	M	32	U, Q/late	2 years
HP15	M	60	U, D, Ang/immediate	12 years
HP17	F	38	U/late	15 years
HP18	F	74	U/late	10 years
HP21	F	31	U, Rh/late	1 year
HP24	F	39	U/late	1 year
HP36	F	43	U/late	5 months
HP44	F	63	P/immediate	3 months
HP50	F	24	P/immediate	3 months
HP53	F	27	G, H/immediate	5 months

A: anaphylaxis; Ang: angioedema; D: dyspnea; E: exanthema; G: gastrointestinal reaction; H: hypotension; P: pruritus; Q: Quincke's edema; Rh: rhinitis; U: urticaria.
^aafter PcG regimen.

structure is known as the "major antigenic determinant", since, historically, most penicillin-specific antibodies found in man and experimental animals were directed against this immunogen (3–5). One antigenic epitope of the penicilloyl group is the acyl side-chain (6). Its chemical structure differs between penicillin types, and a penicillin-allergic patient may be allergic to one type but tolerate another (7). Antibody-mediated responses to different antigenic epitopes of the penicillin molecule have been more thoroughly investigated (7–13) than cellular responses (14–17), and IgE antibodies against penicilloyl conjugates may show side-chain specificity (7). RAST is a widely used *in vitro* tool for the detection of penicilloyl-specific IgE, but the fairly short half-life of IgE in serum might be a limitation, and cell-mediated allergy cannot be traced. Cellular recognition of the side-chain *in vitro* probably requires the use of reactive penicillin as antigen and has earlier been described in benzylpenicillin (PcG)-allergic individuals (17). In contrast, when the side-chain resides on PcG-poly-L-lysine (PLL) conjugates, a side-chain-specific lymphoproliferative response cannot be induced (16). Studies on occupationally penicillin-sensitized individuals have shown that the lymphocyte transformation test (LTT) can be used in the diagnosis of both IgE- (type I) and cell- (type IV) mediated allergy (14, 15). Phenoxymethylpenicillin (PcV) is the most commonly used penicillin type in Sweden, and 16.5×10^6 doses were prescribed in 1996, as compared to 2.8×10^6 doses of amoxicillin. In this investigation, we have tested the

applicability of LTT, comparing reactive PcV to a PcV-PLL conjugate as antigen, in the diagnosis of allergy to PcV in patients with adverse reactions after therapeutic treatment with this penicillin.

Material and methods

Patients

Seventeen patients (14 women and three men, mean age 44.3 years) with a history of hypersensitivity reactions to penicillin treatment were included in the study (Table 1). Sixteen had experienced adverse reactions in connection with PcV treatment, and one patient with both PcV and PcG.

All patients were referred from the allergology clinic at Huddinge Hospital, Huddinge, Sweden.

Controls

Twenty subjects (17 women and three men, mean age 39.5 years) exposed to PcV without reactions of hypersensitivity and eight healthy penicillin workers (two women and six men, mean age 42.2 years) occupationally exposed to PcV were also included. These subjects were tested with LTT only.

The study was approved by the Ethical Committee for Human Research at Huddinge Hospital, Huddinge, Sweden.

RAST

IgE antibodies specific for penicilloyl G and penicilloyl V were measured in serum using Phadebas RAST[®] (Pharmacia Diagnostics AB, Sweden), performed according to the instructions of the manufacturer.

LTT

The LTT was performed as described earlier (14). Briefly, 50–70 ml of venous blood was collected in plain vacutainer tubes (Venoject, Terumo, Belgium), transferred to a sterile E flask with glass beads, and defibrinated by shaking. To obtain mononuclear cells, 30% of the blood volume was immediately separated on Ficoll[®] (Pharmacia, Sweden), while the remaining 70% was depleted from monocytes by iron treatment before separation. Mononuclear cells were then washed once, pooled, and diluted to 0.5×10^6 /ml in RPMI 1640 with 10% AB+ serum, 10 mM Hepes, gentamicin (Schering, Germany) 4 mg/l, and 4 mM L-glutamine. Falcon[®] tubes (Falcon, New Jersey, USA) of 10 ml were pretreated with 50–200 µl of test substance before 2 ml cell suspension was added to

Table 2. Patients with positive intracutaneous test

Patient code	Test substance						
	PcG	PcV	PcV-PLL	Bacampicillin	Cloxacillin	Ampicillin	MDM
HP3	-	+	-	-	-	-	-
HP10 ^b	+	+	+	+	+	+	+
HP12	+	+	+	+	+	+	+
HP13	-	-	+	-	-	-	-

^aDelayed onset.^bPositive to MDM in skin prick test.

each tube. Three parallel cultures without antigen served as controls for background proliferation. The following test substances were used: PcV (Astra AB, Sweden) 100–300 µg/ml, PcG (Astra AB, Sweden) 100–300 µg/ml, bacampicillin (Astra AB, Sweden) 20–100 µg/ml, and penicilloyl V PLL (prepared as described below) 28–112 µg/ml (referring to the amount of penicilloyl). Cell cultures treated with test substance were prepared in duplicates or as single cultures. Purified protein derivative (PPD) (Statens Seruminstitut, Denmark) 1 µg/ml was used as a positive control for lymphocyte proliferation. (Most of the adult Swedish population have been immunized with Calmette-Guérin bacillus and exhibit an increased *in vitro* lymphoproliferative response to PPD and a positive Mantoux test.) After 5 days of incubation at 37°C and in 5% CO₂, the cells were pulsed with ³H-labeled thymidine (Amersham, UK). The cell cultures were then harvested on a semiautomatic cell harvester (Flow Laboratories, UK), and the radioactivity measured in a liquid scintillation β-counter (Wallac, Finland). The results of ³H-labeled thymidine incorporation were expressed as a stimulation index (SI) calculated as:

$$SI = \frac{\text{mean cpm in antigen-treated culture}}{\text{mean cpm in background control cultures}}$$

An SI value of ≥ 2 was regarded as a positive response and SI of < 2 as a negative response.

Preparation of PcV-PLL conjugate

An amount of 4.51 g PcV (Astra AB, Södertälje, Sweden) was dissolved in 5.4 ml 10.5 mM PLL-HBr, MW: 5300 g/mol (Miles Yeda Ltd, Israel), diluted in 0.44 M Na₂CO₃. The solution was left for 1.5 h at room temperature under constant agitation and then dialyzed against distilled water for 3–6 days at 4°C. The preparation was lyophilized and analyzed by the penemaldate assay (18) to measure the number of penicillin molecules bound to each carrier molecule (epitope density). Per PLL carrier, 16 PcV molecules were found. No PcV monomers were detected upon HPLC analysis.

Skin tests

The reagents were prepared in single-use ampoules and lyophilized. The test substances

Table 3. Test results with RAST, intracutaneous test, and LTT with PcV/PcV-PLL, and oral challenge

Patient code	RAST penicilloyl-V/G	Intravenous test PcV/PcV-PLL	LTT PcV/PcV-PLL	Challenge PcV
HP3	+/-	+/-	+/-	nd
HP12	+/+	+/+	+/+	nd
HP13	+/+	-/+	+/-	nd
HP7	+/+	-/-	+/+	nd
HP15	+/+	-/-	+/-	nd
HP9 ^a	+/-	-/-	-/-	+
HP10 ^a	+/+	+ /nd	-/+	+
HP36 ^b	-/-	nd	+ /nd	+
HP50 ^c	-/-	nd	+/+	+
HP44 ^d	-/-	nd	-/-	+
HP53 ^e	-/-	nd	-/+	+
HP2, HP14	-/-	-/-	-/-	-
HP17, HP18, HP21, HP24	-/-	-/-	-/+	-

+ : positive result; - : negative result; nd: not done.

Symptoms upon challenge: ^arash, ^burticaria, ^cpruritus and edema, ^dpruritus, ^egastrointestinal.

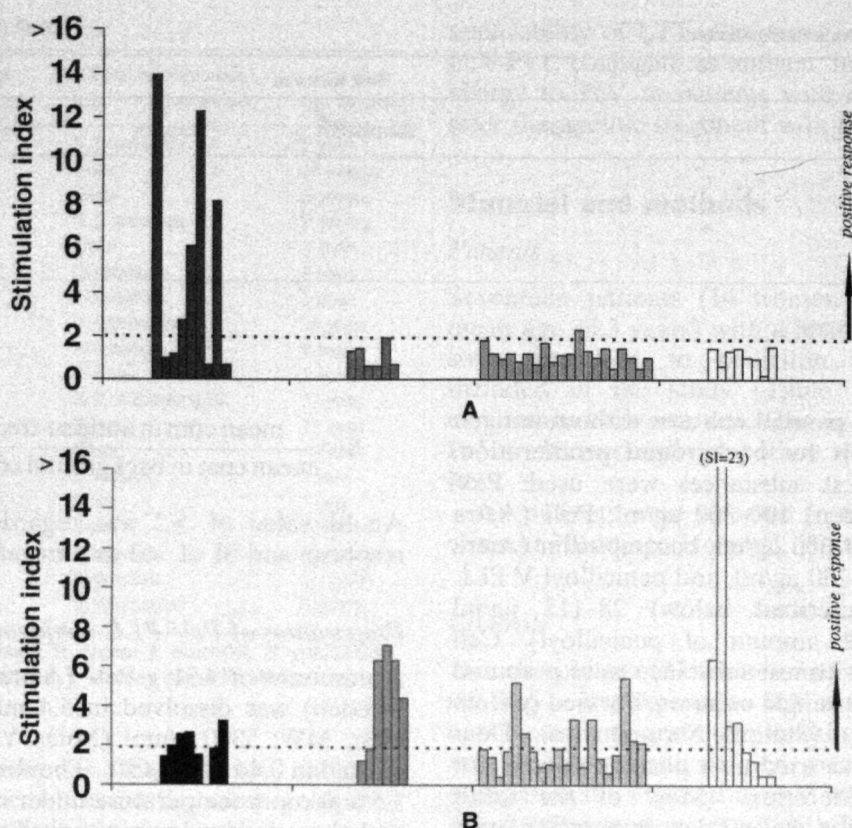


Fig. 1. Maximal LTT results (SI $\pm$ SD) with reactive PcV (A) and PLL-conjugated PcV (B). Black: patients with positive *in vivo* test (mean background proliferation [cpm $\pm$ SD] = 1667 $\pm$ 1381, mean of PPD responses [SI $\pm$ SD] = 104 $\pm$ 72); gray: patients with negative provocation test (1186 $\pm$ 1301, 77 $\pm$ 47); light gray: therapeutically exposed controls (1326 $\pm$ 1260, 155 $\pm$ 227); white: occupationally exposed controls (1692 $\pm$ 1192, 60 $\pm$ 34). SI $\geq$ 2: positive response.

were diluted as described by Juhlin et al. (19), except for bacampicillin, which was diluted in sodium chloride. To avoid aggregation of PcG (20), in particular, the contents were reconstituted immediately before use. The following 1:1 (w/v) solutions were used: PcG (10 mM), PcV (10 mM), PcV-PLL (10 μ M), MDM (10 mM benzylpenicilloate plus 10 mM benzylpenicilloate), bacampicillin (10 mM), cloxacillin (10 mM), and ampicillin (10 mM). All test reagents were provided by Astra AB, Sweden. Skin prick tests were performed with 1:1 concentrations and, if negative, an intracutaneous test was performed with 1:100 followed by 1:1 if negative.

Oral challenge test with PcV

As a starting dose, 125 mg of PcV was used. Blood pressure, pulse, and PEF (peak expiratory flow) were checked after 15 min and every 30 min thereafter. If no reaction was recorded, 1 g of PcV was given 2 h after the test dose, and followed by a 5-day regimen.

Results

Nine of the 17 patients had a positive *in vivo* test to PcV presented as a positive skin intracutaneous test ($n=3$) (Table 2), or a positive challenge test ($n=5$), and one patient (HP10) was positive in both tests. Two of the patients with a positive skin test were also RAST-positive. These subjects were not challenged due to a history of severe adverse reactions. The third patient with a positive skin test did not agree to a challenge test. All skin prick tests were negative except in one patient (HP10) who was positive to MDM. Six patients had negative challenge tests. Two skin-test-negative patients (HP7 and HP15) did not agree to a challenge test and thus could not be classified.

An overview of test results from RAST, skin test and LTT with PcV/PcV-PLL, and oral challenge with PcV is given in Table 3.

Patients with positive in vivo tests to PcV (n=9)

Three of these patients were RAST-positive to both penicilloyl V and penicilloyl G. Two patients

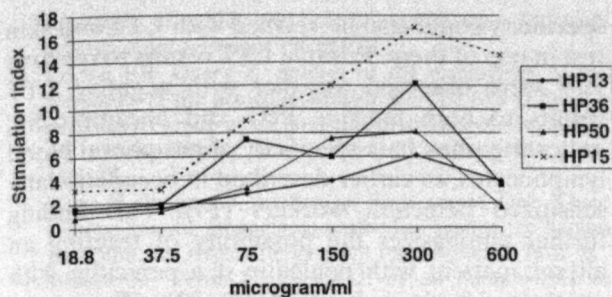


Fig. 2. Dose-response relationship of lymphoproliferative responses (SI) in patients exhibiting positive LTT result with reactive PcV or PcG (dotted line). SI $\geq$ 2: positive response.

were RAST-positive to penicilloyl V only, and the remaining four were RAST-negative. Five patients exhibited a positive LTT result to reactive PcV (Fig. 1A) and two were LTT-positive to PcV-PLL (Fig. 1B). Two were not tested with PcV-PLL.

Patients with a negative challenge test (n=6)

None of these patients were RAST-positive, and none of them were LTT-positive with reactive PcV (Fig. 1A). Four were LTT-positive to PcV-PLL (Fig. 1B).

Therapeutically PcV-exposed controls (n=20)

One of these subjects was LTT-positive to reactive PcV, and the remaining 19 were LTT-negative (Fig. 1A). Seven exhibited a positive LTT to PcV-PLL (Fig. 1B).

Occupationally PcV-exposed controls (n=8)

None of these subjects were LTT-positive to reactive PcV (Fig. 1A). Four exhibited a positive LTT to PcV-PLL (Fig. 1B).

Characteristics of in vitro lymphoproliferative responses

There was a correlation between RAST and LTT with reactive PcV. Five of the seven RAST-positive patients also had a positive LTT to reactive PcV. Eight of the 10 RAST-negative patients were LTT-negative with reactive PcV, but six were positive to PcV-PLL (Table 3). That is, patients with a positive LTT to reactive PcV did not always show a positive LTT with PcV-PLL. Out of six patients with a positive LTT result to reactive PcV, three were also LTT-positive to PcV-PLL. The specificity of LTT with reactive PcV was 96%, since one of the 28 controls was LTT-positive. Reactive PcG and PcV were generally used at 300 and 100 µg/ml, but four patients were retested with additional test concen-

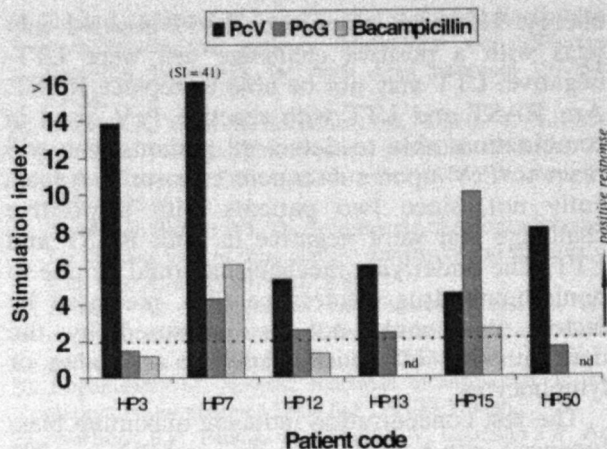


Fig. 3. Side-chain specificity of maximal lymphoproliferative responses (SI $\pm$ SD) to reactive penicillins. SI $\geq$ 2: positive response.

trations. The lymphoproliferative responses showed a dose-response relationship, with an optimum at 300 µg/ml, as illustrated in Fig. 2.

Side-chain specificity of lymphoproliferative responses comparing reactive PcV, PcG, and bacampicillin as antigens is illustrated in Fig. 3. Two patients were positive to reactive PcV only.

Discussion

When reactive PcV was used as antigen in LTT, five out of nine patients with a positive *in vivo* test to this penicillin were positive. In contrast, challenge-negative patients were consistently LTT-negative. Only one of the 28 PcV-exposed control subjects was LTT-positive, indicating a specificity of 96% with this antigen, compared to 85% when LTT is used with drugs in general (21). For investigation of the sensitivity of LTT, as recently described with drugs including penicillins (21), a larger group of patients with verified PcV-allergy needs to be tested. PcV-PLL was found to be less suitable to distinguish patients from healthy controls, since 11 control subjects were LTT-positive when this conjugate was used as antigen.

In comparison of LTT and RAST as diagnostic tools, antigen-specific lymphocytes have a longer half-life than antibodies. Another advantage of LTT is the possibility of detecting both cell- (14, 22) and antibody-mediated reactions (14, 15), since T cells are the key conductors of both these responses. As shown in the present study, most RAST-positive patients (five out of seven) also exhibited a positive LTT to reactive PcV. Furthermore, two patients with a positive challenge test to PcV were RAST-negative but LTT-positive, a fact which suggests that LTT could be useful as a complementary test for the diagnosis of penicillin

allergy. However, since two RAST-positive subjects with a positive challenge test were LTT-negative, LTT may not be able to replace RAST. Are RAST and LTT with reactive PcV, used in combination, able to detect all patients who will react to PcV upon subsequent exposure? Apparently not, since two patients with a positive challenge test were negative in both RAST and LTT. The underlying mechanisms could be due to nonimmune drug intolerance (23), mediated by factors that could not be measured by the determination of penicillin-specific antibodies or lymphocytes.

The test concentration inducing maximum blastogenesis with both reactive PcG and PcV was 300 µg/ml, the same as that found by Brander et al. (17). PcG-allergic individuals are known to mount lymphoproliferative responses to reactive PcG *in vitro* (16). PcG-specific T-cell clones are HLA I and HLA II restricted, although antigenic processing is not necessary, since fixed antigen-presenting cells can still present PcG (17). PcV has recently been shown capable of generating antigenic determinants on the surface of mouse spleen cells (24). Thus, penicillin probably activates specific T cells of allergic subjects either by binding directly to MHC molecules or to peptides present in the antigen-presenting groove of these structures, as observed with nickel (25) and other haptens (26–30). On the contrary, when the penicillin molecule is rendered nonreactive by conjugation to carrier molecules, such as PLL, such lymphocyte proliferation is no longer conceivable (16). This agrees with our results since only three out of six patients with a positive LTT to reactive PcV were LTT-positive with the PcV-PLL conjugate.

Unexpectedly, positive LTT results with PLL-conjugated penicillins were found in four out of six (67%) patients with a negative challenge test, in seven out of 20 (35%) therapeutically exposed controls, and in four out of eight (50%) occupationally exposed controls. We propose that a lymphoproliferative response to penicilloyl conjugates reflects an immunogenic, but not an allergenic, response to this determinant. An immunogenic property of penicilloyl has earlier been suggested (6) and penicilloyl-specific antibodies have been found in healthy individuals (10). Thus, our LTT findings could reflect the cellular equivalent of these penicilloyl-specific antibodies.

The role of the penicillin side-chain has attracted much interest during the last 10 years. Recently, the presence of PcV-selective IgE, without cross-reactivity to PcG, was described (31). Two patients in our study were RAST-positive to penicilloyl V but negative to penicilloyl G, and this side-chain

specificity could also be verified with LTT and skin test in one of them. Positive LTT results to reactive PcV were observed together with negative LTT results to both reactive PcG and bacampicillin, indicating side-chain specificity of peripheral blood lymphocytes, as earlier described in occupationally sensitized penicillin workers (14). This finding further emphasizes the possibility of treating an allergic patient with penicillin if a penicillin with another side-chain is used (7, 32). The cross-reactivity between PcG and the aminopenicillin amoxicillin is low, and amoxicillin-allergic individuals can tolerate treatment with PcG (7). Nevertheless, individual differences seem obvious, and some patients reacted to several penicillin types as well. Two patients had positive skin tests to all penicillin types tested, and four patients showed positive LTT responses to more than one penicillin. These and other results (33) indicate that penicillin sensitivity is individual and also may involve the nuclear portion of the penicillin molecule. Thus, diagnosis of penicillin allergy should be performed on a case-by-case basis.

In conclusion, LTT with reactive PcV could be useful as a complementary test in the diagnosis of allergy to this penicillin, especially in patients with a negative RAST, and also for mapping the side-chain specificity of *in vitro* lymphoproliferative responses. Using this method for prediction, renewed penicillin treatment might be possible, provided that a penicillin with another side-chain is used. The PcV-PLL conjugate, on the other hand, could not be used to distinguish between healthy and truly allergic individuals. A positive LTT response to this conjugate may be an indicator of immunization, but not allergy, to the penicilloyl structure.

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