

Imbalance of CD45R and CDw29 helper cell subsets after seven days of culture from penicillin hypersensitive patients

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Specific PBMC proliferation to penicillin represents one of the *in vitro* diagnostic procedures to show antigenic sensitization. Nine patients with suggestive clinical history of penicillin hypersensitivity, significant stimulation index in the lymphocyte transformation test (LTT), and increased CD25 expression were selected. Their phenotypic expression of T-cell subset markers was compared with that of six normal subjects after 7 days of culture. Higher percentages of CD45R and CDw29 positive cells were found in penicillin-hypersensitive patients. This group of patients showed a significantly ($P < 0.01$) higher percentage of CD45R positive cells compared with normal subjects, after 7 days of culture. The percentage of CD45R cells did not differ between patients and normal subjects at day 0, and the progressive decrease of CD45R positive cells in culture of normal subjects was not observable in our patients. These results suggest that the patients with penicillin hypersensitivity have an imbalance of CD45R/CDw29 ratio after 7 days of culture, with a higher expression of CD45R positive cells.

Key words: helper T-cell subsets; lymphocyte transformation test; memory T cells (CDw29); naive T cells (CD45R); penicillin hypersensitivity.

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Allergy reactions to penicillin and their derivatives are a major factor limiting their clinical use. This antibiotic can induce most types of immunological reactions. Delayed hypersensitivity reactions, which include exanthema and contact dermatitis, are the most frequent (10). This type of reaction is mainly T cell-mediated, with a specific penicillin proliferative response often being observed *in vitro* (3, 4, 5). Other immunological reactions, although largely mediated by the B-cell compartment, require a specific T-cell help (6). Again, a specific penicillin proliferative response can also be observed in a number of them.

The physiology of T-cell subsets is beginning to be understood. Recent studies suggest that these subsets of T-helper cells correspond to

different stages of maturation (13). According to this interpretation, memory T cells are found within the CDw29 + subset and naive T cells are part of the CD45R + subset (1, 2). The CD4 + CDw29 + subset proliferates poorly upon stimulation with Con A, but responds well on exposure to soluble antigens, providing a good helper signal for PWM-induced immunoglobulin synthesis. In contrast, the CD4 + CD45R + cell subset, proliferates well in response to Con A stimulation, but poorly to soluble antigen stimulation and provides little help to B cells for PWM-induced Ig synthesis (8, 9). Another observation which distinguishes naive (CD45R +) and memory (CDw29 +) T cells is that naive cells respond well to mitogen and alloantigens, but do not proliferate in re-

Table 1.

Type of clinical reaction	Time interval
exanthema	3 months
exanthema	4 months
exanthema	6 months
exanthema	4 months
exanthema	6 months
urticaria	1 year
urticaria	3 years
urticaria, asthma	1 year

sponse to the recall antigen (15). This subset includes cells with suppressor-inducer function (7, 9). The CD4 + CDw29 + subset proliferates in response to recall antigen (13) and includes cells with helper functions (8). Recent studies demonstrated that CD4 + CD45R + cells, when stimulated with mitogens, produced high levels of IL-2 mRNA, but only traces of IL-4 or IFN- γ mRNA (11, 12). In contrast, CD4 + CD45R- cells produced all three lymphokines but less IL-2 mRNA than naive cells after mitogenic stimuli (11).

In the present study, cells from patients who presented a hypersensitivity reaction to penicillin were studied. Their peripheral blood lymphocytes were cultured for 7 days, analysed for their T cell subset distribution, and compared with a group of normal subjects, in the same conditions of culture. Our results show a specific T cell proliferation in response to penicillin, as well as a higher expression of IL-2 receptors in cells from penicillin-hypersensitive patients. After 7 days in culture, the phenotypic expression of the CD4/CD45R phenotype was significantly increased in cells from penicillin-hypersensitive patients, when compared with those from normal subjects.

MATERIAL AND METHODS

Patients

The selected population consisted of nine patients with histories of penicillin hypersensitivity and significant stimulation index (SI > 2) for penicillin G in a first lymphocyte transformation test. All patients presented skin symptoms (6

maculopapular exanthemas, 3 urticaria) (Table 1) and were cultured *in vitro* with a delay of 1 month to 3 years. Five of six patients tested in skin prick tests were positive for one of the penicillin determinants (PPL or MDM), two were penicillin G RAST-IgE positive and two were atopics, as defined by skin test positivity for eight common major allergens. Patients were free of inflammatory disease or immunosuppressive drugs for the 3 months preceding the *in vitro* culture. The normal control population consisted of six subjects who had received penicillin without an adverse reaction and in whom lymphocyte transformation test to penicillin was negative.

Cell preparation

Peripheral blood mononuclear cells (PBMC) from normal subjects and patients with penicillin allergy were separated from heparinized venous blood by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradients (15 minutes at 500 $\times g$). Leukocytes at the serum Ficoll interface were removed and washed twice in medium and resuspended in the culture medium (RPMI 1640, Gibco Ltd., Scotland) supplemented with 2 mmol/l of L-glutamine, 100 μ g/ml streptomycin and 20% autologous serum.

Stimulation with antigens

Peripheral blood mononuclear cells 10^6 in 1 ml of culture medium were distributed in the plates (Linbro, Flow No. 76.063.04) and supplemented with a selected dilution of antigen added into the culture medium. The optimal concentration of penicillin G was stabilized from several dose-response analyses from different patients. The final concentrations applied to all cultures were for penicillin G, 1000 U/ml, for PPD, 10 μ g/ml used as positive control. The cultures were incubated at 37°C in 95% air, 5% CO₂ humid atmosphere for 7 days.

After this period, the proliferative response to antigens was measured by ³H-thymidine incor-

poration. 2×10^5 cells were placed in microtiter plates (Falcon, Labware, No. 3070) and 20 μ l of methyl- ^{3} H thymidine (20 μ g Ci/ml, specific activity of 5 Ci/mmol) was added 8 h before harvesting. The cultures were harvested with a semiautomatic multifold cell harvester (Skatron, Bergen, Norway) and the radioactivity was counted in a liquid scintillation counter. The results of ^{3} H-thymidine incorporation into DNA are expressed as mean counts per minute, (cpm + SD) and stimulation index (SI) calculated according to the formula:

$$SI = \frac{\text{cpm (+ antigen)}}{\text{cpm (-antigen)}}$$

The test was considered positive when the SI is > 2 .

Monoclonal antibodies

The following monoclonal antibodies were used. OKT4 (CD4) (Ortho Diagnostic Systems Inc., NJ), anti IL2-R (CD25) (Becton Dickinson, Labo Systeme, Heidelberg), anti 4B4 (CDw29), anti 2H4 (CD45R) (Coulter Immunology, Zürich, Switzerland).

Analysis of lymphocyte populations with single and two-color fluorescence flow cytometry

After 7 days of culture the cells are harvested, washed twice in phosphate buffered saline (PBS)

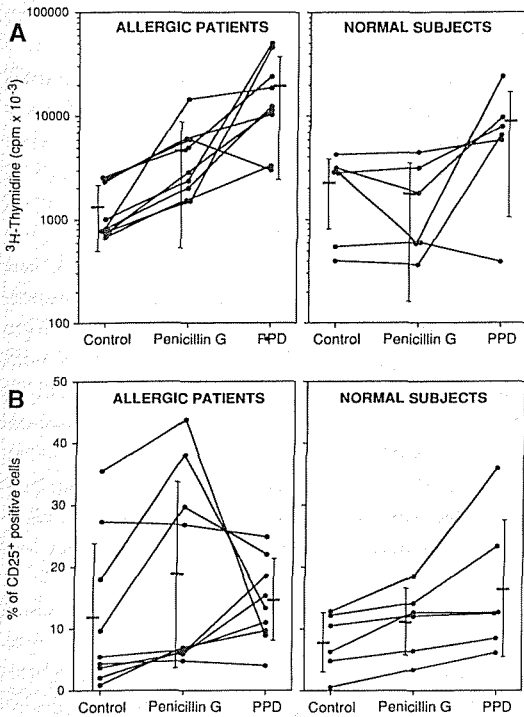


Fig. 1A. PBMC proliferation to penicillin G (1000 U/ml) and PPD (10 μ g/ml) after 7 days in culture from 9 penicillin-allergic patients and 6 normal subjects. The results are expressed in cpm.

Fig. 1B. CD25 phenotypic expression on PBMC from 9 penicillin-allergic patients, and 6 normal subjects, after 7 days of *in vitro* culture. Control group cultured without antigen versus group cultured with penicillin G ($P < 0.03$). Members of both groups were penicillin allergic. The results are expressed in percentage of positive cells.

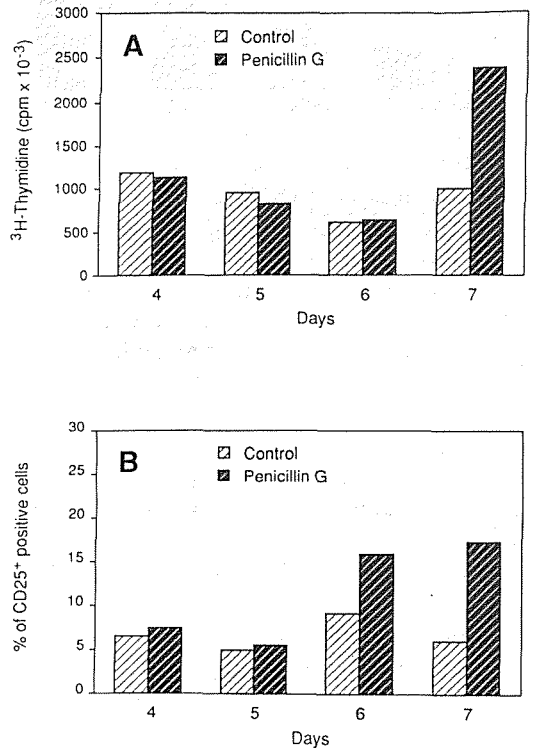


Fig. 2A. Specific penicillin induced proliferation of PBMC *in vitro* at days 4, 5, 6, 7 of culture, from a patient with penicillin hypersensitivity. The results are expressed in cpm. Fig. 2B. CD25 phenotypic expression on PBMC, at days 4, 5, 6, 7 of culture from one patient with penicillin hypersensitivity. The results are expressed in percentage of positive cells.

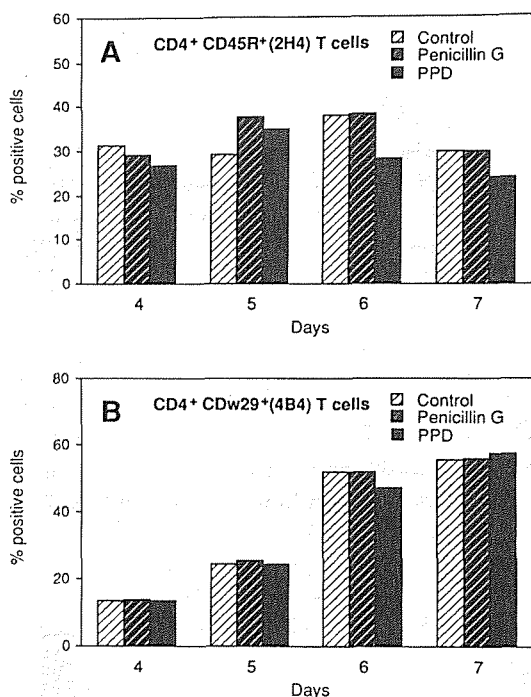


Fig. 3A. CD45R (2H4) phenotypic expression of PBMC at days 4, 5, 6, 7 of culture from a penicillin-hypersensitive patient. The results are expressed in percentage of positive cells.

Fig. 3B. CDw29 (4B4) phenotypic expression of PBMC at days 4, 5, 6, 7 of culture from a penicillin-hypersensitive patient. The results are expressed in percentage of positive cells.

and supplemented with 5% goat serum. The antibody (5 μ l for 2×10^5 cells) was added to the

cells and incubated for 30 min at 4°C. The cells were washed twice with PBS 5% goat serum, and 50 μ l of conjugated goat antimouse immunoglobulin F(ab₂) fragment (GAM-FITC) was added and incubated for 30 min in the dark at 4°C. Cells were washed twice and analysed with cytofluorograph (FACS EPICS IV, Coulter Electronics).

Two-color fluorescence was performed using anti-T4 FITC and anti-4B4 phycoerythrin (PE); anti-T4 FITC and anti-2H4 phycoerythrin (PE). In brief, the cells are washed twice and the antibody (10 μ l for 2×10^5) added and incubated 10 min at room temperature. Cells were washed twice and analysed.

Statistical analysis

Student t test and analysis of variance were used to compare the results from the different groups.

RESULTS

When cultured *in vitro* with penicillin G, the PBMC of nine patients with penicillin hypersensitivity, showed an increased incorporation of ³H-thymidine on day 7 when compared with those of six normal subjects ($P < 0.05$) (Fig. 1A). Cells in culture showed a maximal proliferation at day 7 (Fig. 2A), as well as an increased expression of CD25+ cells (Fig. 2B). In addition, among the cultures of penicillin-hypersensitive patients, the percentage of

Table 2.

PBMC phenotypic expression in penicillin-hypersensitive patients and normal subjects after 7 days of culture

Patients	Number	CD4 +	CD4 + CD45R +	CD4 + CDw29 +
A. Penicillin-allergic				
Control	9	53 $\pm$ 13	19 $\pm$ 10*	37 $\pm$ 14****
Penicillin	9	57 $\pm$ 9	18 $\pm$ 9**	33 $\pm$ 13
PPD	9	48 $\pm$ 10	15 $\pm$ 8***	33 $\pm$ 11
B. Normal subjects				
Control	6	42 $\pm$ 14	6 $\pm$ 3*	22 $\pm$ 12****
Penicillin	6	51 $\pm$ 15	6 $\pm$ 3**	22 $\pm$ 15
PPD	6	51 $\pm$ 5	7 $\pm$ 4***	23 $\pm$ 14

* $P < 0.01$, ** $P < 0.01$, *** $P < 0.03$, **** $P < 0.07$.

CD25 + cells was significantly higher ($P < 0.03$) in those cultures supplemented with penicillin G, than in those without the antigen (Fig. 1B). This percentage was not significantly increased in culture of normal subjects.

We observed the phenotypic expression of PBMC from one patient with penicillin hypersensitivity at different times during culture, and found a progressive increase in the percentage of CDw29 + cells up to day 7 (Fig. 3B). When we studied the CD45R + cells of the same patient, we found the persistence of the same percentage of positive cells until day 7 (Fig. 3A). The specific T cell subset markers were analysed in PBMC from penicillin-hypersensitive patients and compared with those from normal subjects. We found a modification in the percentage of CD4 + CDw29 + and CD4 + CD45R + cells in penicillin-hypersensitive patients. Compared with normal subjects after 7 days of culture, PBMC from penicillin-hypersensitive patients exhibited a significantly higher ($P < 0.01$) percentage of cells expressing the CD4 + CD45R + markers (Table 2) in the presence of either relevant or irrelevant antigen, or medium alone. When we analysed the same group (patients and normal subjects) at day 0, we found that the percentage of CD45R + cells from normal subjects was higher at day 0 than at day 7 (20 versus 6 respectively), but without changes in cultures of penicillin-hypersensitive patients (18 versus 19) (Table 3). This percentage was in the same order of magnitude, at day 0, in penicillin-hypersensitive patients and normal subjects (18 versus 20, respectively).

The percentage of CD4 + CDw29 + T cells from penicillin-hypersensitive patients after 7 days of culture with penicillin, was not significantly different from those of normal subjects (Table 2). Similarly, no significant difference could be observed ($P < 0.07$) when the control

conditions of penicillin-hypersensitive patients were compared with those of normal subjects. The percentage of CDw29 + cells increased from day 0 to day 7 in penicillin-hypersensitive patients (28 versus 37), whereas it decreased in normal subjects (28 versus 22). Although this observation was of interest it was not significant given the number of patients studied.

DISCUSSION

As reported (3, 4, 5), we were able to show in this study a specific *in vitro* lymphoproliferation for penicillin-hypersensitive patients. There was a concordant relationship between thymidine incorporation and expression of CD25 after 7 days of culture. The phenotypic expression of the IL-2 receptor (CD25) was higher in cultures with penicillin from penicillin-hypersensitive patients than in those from normal subjects, indicating that cells were specifically activated by penicillin antigen. The maximal expression of CD25 positive cells occurred late, when compared with a common antigen such as PPD, for which the CD25 expression peaked at day 4 (data not shown). Our understanding is that the amount of precursors is most likely much lower for penicillin than for PPD, explaining the delayed maximal activation.

The proliferating T cells have been shown to express the CDw29 phenotype (12). This phenomenon was observed on the T cells from normal subjects and penicillin-hypersensitive patients, and most likely represented the expansion in culture of memory T cells. It has been demonstrated recently that T cells in culture increase their CDw29 expression and lose the CD45R phenotype (1, 15). More convincingly, when T-cell clones with CD45R phenotype were cultured for 14 days the cells lost the expression of the CD45R marker (11). In our

Table 3.

PBMC phenotypic expression in penicillin-hypersensitive patients and normal subjects at day 0

Patients	Number	CD4 +	CD4 + CD45R +	CD4 + CDw29 +
A. Penicillin-allergic	5	49±9	18±11	28±14
B. Normal subjects	5	55±6	20±4	28±7

study, we found that the cells from normal subjects lose the CD45R phenotype after 7 days of culture, whereas cells from penicillin-hypersensitive patients maintained a higher expression of CD45R in the same conditions of culture. This phenomenon could correspond to a modification of the capacity of these cells to proliferate in culture, given that it appeared to be antigen-independent. Indeed, the cells maintained in culture medium as well as those cultured with PPD displayed a similar percentage of the CD4 + CD45R + phenotype, which was significantly different from those observed in normal subjects. We do not know at the moment, whether a soluble factor present in supernatants at day 7 in penicillin-hypersensitive patients could modulate the expression of CD45R positive cells of normal subjects. Alternatively, the difference between the two groups could be explained by the selection of our patients on the basis of a response of cells in culture to penicillin. However, these patients did not respond specifically to other antigen and their responses to phytohemagglutinin (PHA) were in the normal range (data not shown).

Since the persistence of the CD45R expression on cells in culture seemed to be related to the capacity of the cells to proliferate *in vitro*, we analysed a possible relationship with the time interval elapsed from the onset of the clinical symptoms. Indeed, a regulatory factor at the level of T cell not found in normal subjects could still be active in the penicillin-hypersensitive patients. However, no correlation between the decrease in CD45R expression at day 7 of culture and the time elapsed from clinical symptoms could be demonstrated (data not shown).

The phenotypic expression of CDw29 of PBMC after 7 days in cultures of penicillin-hypersensitive patients was not significantly different from those of normal subjects. We found at day 7 a non-significant increase of the expression of the CDw29 phenotype in penicillin-hypersensitive patients, regardless of the presence of stimulatory agents (control culture, irrelevant or relevant antigen), when compared with that in normal subjects. When analysed at day 0, a similar percentage of positive cells was found in both groups.

In the patients who responded *in vitro* to penicillin antigen we have found a modification of the expression of the T cell subset markers (CDw29 and CD45R) that could account for an increased cell reactivity.

Along the same line an imbalance of CD45R + and CDw29 + T helper cell subsets in patients with atopic dermatitis has been described (16). Similarly to our results this study suggests that CD45R + cells are incapable of differentiation into CDw29 + helper T cells in atopic dermatitis.

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