

Allergen-induced expression of cell surface markers on lymphocytes of *Chi t* I-sensitized patients

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Lymphocyte cultures of persons sensitized to the hemoglobin allergen *Chi t* I show a highly significant response to the allergen measured in the lymphocyte stimulation assay by (³H)-thymidine uptake. In this study, we investigated by flow cytometry the expression of different cell surface markers on lymphocytes after *in vitro* stimulation for 7 d with or without the allergen *Chi t* I. We determined the expression of the low-affinity receptor for IgE (CD23) on lymphocytes of *Chi t* I-sensitized patients and *Chi t* I-exposed as well as nonexposed controls. CD23 expression was significantly higher in patients than in nonexposed controls. Exposed but healthy subjects showed intermediate values. We also determined the expression of activation markers CD25 (IL-2 receptor) and HLA-DR on the lymphocytes of patients and nonexposed controls. HLA-DR expression on non-T cells (CD3[−]) was significantly higher in patients than in controls. HLA-DR on T cells (CD3⁺), and CD25 as well as CD23 expression, could be significantly enhanced after antigen-specific stimulation in patients but not in controls, whereas α/β -T-cell-receptor expression was significantly reduced in patients. Differences between patients and controls were not observed in response to tetanus toxoid (TT) and phytohemagglutinin (PHA). Our results demonstrate antigen-specific influences on the expression of cell surface molecules. These findings may be valuable diagnostic information.

V. Liebers*, M. Raulf, X. Baur

Berufsgenossenschaftliches Forschungsinstitut
für Arbeitsmedizin (BGFA), Institut an der
Ruhr-Universität Bochum, Germany

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Dipl. Biol. Verena Liebers
Berufsgenossenschaftliches Forschungsinstitut
für Arbeitsmedizin (BGFA)
Gilsingstr. 14
4630 Bochum 1
Germany

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Chironomid hemoglobins (*Chi t* I) have been found to be aggressive inhalant allergens of occupational, environmental, and hobby-related relevance (4, 19, 22). For example, persons exposed to the enormous midge swarms in Sudan, Japan, or Wisconsin, as well as people exposed to chironomid larvae, as in fish food production, suffer from rhinitis, conjunctivitis, and bronchial asthma (2, 13, 14, 23).

Chi t I is composed of 12 homologous hemoglobins (components) with known primary structure; the 3-D structure of component III has already been shown by radiographic analysis (26).

As measured in the lymphocyte stimulation assay (14–17), there is in *Chi t* I-allergic patients a significantly higher response on the cellular level than in controls. These data indicate an antigen-specific T-cell activation in allergic patients. Lymphocyte surface markers such as CD25, HLA-DR, and CD23 likewise play a crucial role in initial steps of immune response. The appearance of these mole-

cules at a distinct developmental stage of lymphocytes shows that they may function as receptors for external signals directing cells to the next phase of response.

The objective of the present study was to compare the expression of lymphocyte activation markers CD25, CD23, and HLA-DR in *Chi t* I-allergic patients and nonexposed controls. In addition, the percentages of T and B cells and CD4⁺ and CD8⁺ T cells, as well as of the α/β - and γ/δ -T-cell receptor, were determined. For this purpose, peripheral blood lymphocytes were stimulated with *Chi t* I, PHA, or TT, respectively, to detect possible differences between *Chi t* I-allergic patients and healthy donors.

Material and methods

Study group

Human peripheral blood lymphocytes (PBL) were obtained from 13 *Chi t* I-sensitized patients. Each of them suffered from immediate hypersensitivity reac-

* The study is part of Verena Liebers' PhD thesis.

tions when in contact with fish food containing *Chi t I* (seven reported rhinitis; six, conjunctivitis; six, urticaria; and seven, asthma). Each patient was prick-test positive with *Chi t I* (conc. 0.1 mg/ml). Four subjects were monosensitized, and nine were sensitized to other allergens, too.

Chi t I-specific IgE antibodies were quantitated by the RAST method (radioallergosorbent test); five patients belonged to RAST class 4, three to RAST class 3, and one to RAST class 2. In addition, we investigated lymphocytes of 14 controls not exposed to *Chi t I* and of eight controls exposed but not sensitized to *Chi t I*. Two of the exposed controls and six of the nonexposed controls were nonallergic, whereas the other subjects showed sensitization to one or more common allergens in the prick test. All controls were negative to *Chi t I* in the prick test. All the patients and four exposed controls had daily contact with high amounts of the allergen. Four controls had low exposure to the allergen.

Medium

RPMI 1640 (Gibco, Eggenstein, Germany) was supplemented with 10% human AB serum inactivated by heat (German Red Cross, Munich, Germany), 1% penicillin-streptomycin (Gibco), and 1% L-glutamine (Gibco); it was referred to as complete medium.

Lymphocyte preparation and cell culture

Lymphocytes were separated on a Ficoll-Hypaque density gradient (Pharmacia, Freiburg, Germany), washed twice with Hanks' solution (Gibco), and resuspended in complete RPMI 1640 at a final concentration of 1×10^7 cells/ml for freezing and 1×10^6 cells/ml for incubation with the stimulator. Cells were stored at -196°C until use. For lymphocyte stimulation, *Chi t I* (5 $\mu\text{g/ml}$, purified as described previously (3)), TT (5 $\mu\text{g/ml}$ kindly provided by Prof. Dr Rieber), and phytohemagglutinin (PHA) (30 g/ml, Sigma, Germany) were added to the cells in 24-well, flat-bottom microtiter plates (Falcon, Becton-Dickinson) in 1000-ml volumes and incubated for 7 d at 37°C and 5% CO_2 .

Antibodies for cell surface markers

FITC-(fluorescein-) or PE-(phycoerythrin-) conjugated monoclonal antibodies were purchased from Becton-Dickinson (Mountain View, CA, USA). The following antibodies were used: anti-CD3 = T cells (T-cell-receptor complex); anti-CD19 = B cells; anti-CD4 = T-helper/inducer cells, monocytes; CD8 = T-cytotoxic/suppressor cells, natural killer cell subset; anti-HLA-DR = B cells, monocytes, macrophages,

activated T cells; anti-CD23 = Fc_γR II-expressing cells, B cells, and activated B cells; anti-CD25 anti-interleukin-2 receptor on activated T cells, subset of CD4+ T cells, subset of B cells; anti-CD14/anti-CD45 (leukogate) = monocytes; anti-TCR1 = α/β -T-cell-receptor-bearing cells, lymphocytes; anti-TCR2 = γ/δ -T-cell-receptor-bearing cells, lymphocytes.

Cytofluorographic analysis

For detection of cell surface markers on cultured cells, lymphocytes were stained by FITC- or PE-monoclonal antibodies (mAb). A volume of 100 μl cell suspension (1×10^5 cells) was incubated with 18 μl conjugated mAb for 30 min at 4°C in darkness. Cells were washed and then fixed in 1% paraformaldehyde and stored at 4°C in the dark until analysis. Analysis was performed between days 1 and 4 after fixation. Cytofluorometric analysis was performed with a FACS-Star Plus (Becton-Dickinson). Lymphocytes were distinguished from monocytes by characteristic forward light scatter (FSC) and right light scatter (SSC) profiles. The characteristic scatter conditions were controlled by staining with a mixture of FITC-conjugated anti-CD45 and PE-conjugated anti-CD14 (leukogate). Gated lymphocytes were FITC-anti-CD45 positive and negative by PE-anti-CD14 fluorescence. The gating for forward and side scatter was set around the lymphocyte population defined by the leukogate-antibody, and 10000 particles were analyzed for fluorescence. Unstained cell lymphocytes served as a control to assess cellular autofluorescence. For two-color immunofluorescence studies, the proportions of lymphocytes with positive staining for each antibody were calculated by setting four quadrants. On the basis of control samples, we divided the dot plots for unstained cells (lower left) and single-stained cells, FITC-stained cells (lower right), PE-stained cells (upper left), and double-stained cells (upper right).

Statistical analysis

Data were analyzed by Student's two-tailed t-test (comparison between patients and controls) and by paired t-test (comparison between RPMI and *Chi t I*). A *P* value of 0.05 was taken as the limit of significance.

Results

Characterization of the collective

Fig. 1 shows the total and specific serum IgE levels of the subjects under investigation. Total IgE levels of patients ranged from 2.7 to 669 (mean 206) U/ml.

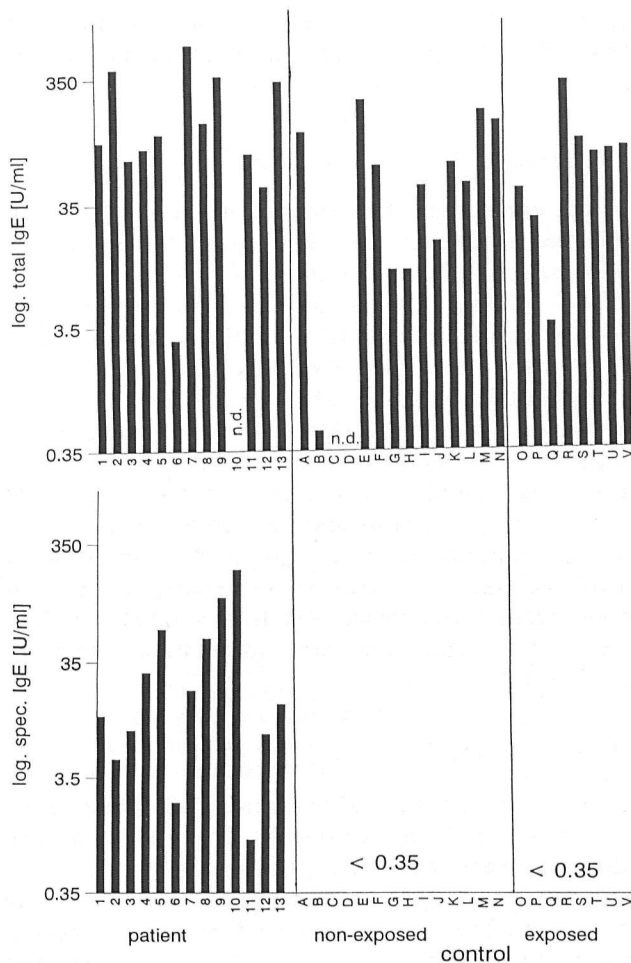


Fig. 1. Characterization of subjects under investigation by total and specific IgE in serum. Significant differences regarding total IgE in patients and controls were not detectable. Patients were characterized by specific IgE levels ≥ 1 unit/ml.

Values between 0.5 and 320 U/ml were measured for exposed (mean 96.8 U/ml) and nonexposed (mean: 82.3) controls. Specific IgE for *Chi t I* was measurable only in sera of patients.

Time-dependence of cell surface marker expression

The first series of preliminary experiments was undertaken to study the expression of cell surface markers CD23, CD25, and HLA-DR on T cells (HLA-DR + CD3 +) in *Chi t I*-allergic patients and nonexposed controls in the absence of stimuli (incubation time day 0). Significant differences in the expression of the tested markers between the six patients and the 13 nonexposed controls were not observed. Because of great individual variability, the standard deviation in both groups was very high (Fig. 2).

To characterize differences between allergic and nonallergic persons, we examined the expression of cell surface markers in response to an antigen-

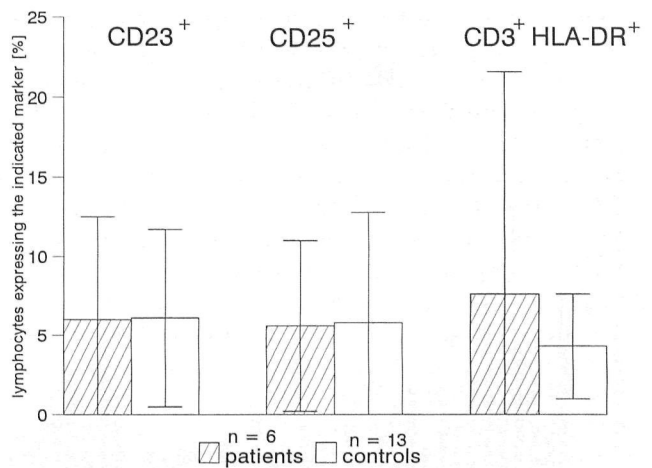


Fig. 2. Expression of cell surface markers CD23, CD25, and CD3 HLA-DR was studied on lymphocytes of six patients and 13 nonexposed controls directly after blood drawing (without incubation). Since individual variability was very high, significant differences were not detectable.

specific stimulus by incubation for different days. To determine the time-dependent response of the cells to the allergen *Chi t I*, we determined the expression of CD25 and HLA-DR + CD3 + in two cases at days 0, 3, 5, and 7 of culture. These preliminary experiments showed that *Chi t I* enhanced the expression of CD25 and HLA-DR + CD3 + at day 7 (data not shown). Because of these results and literature data (7, 11), the following stimulation experiments were always performed over 7 d.

Expression of CD23

We studied the expression of CD23 in 13 patients, 11 non-*Chi t I*-exposed controls, and eight *Chi t I*-exposed, nonsensitized subjects. Fig. 3 shows the individual values of CD23 expression on lymphocytes incubated with or without the allergen *Chi t I*. Cells of 11 of 13 patients showed increased CD23 values after antigen-specific stimulation. As compared with nonexposed controls, the level of CD23 was significantly higher in stimulated ($P < 0.01$) as well as in nonstimulated cultures of patients ($P < 0.05$). Exposed controls showed intermediate values and no significant enhancement of CD23 expression after antigen-specific stimulation. A correlation between the low-affinity receptor for IgE and total or specific serum-IgE could not be found; for example, patient no. 6 with the highest CD23 expression (with and without allergen-stimulation) showed the lowest amount of total and specific IgE.

Expression of activation markers

Nine patients and six nonexposed controls were examined for HLA-DR and CD25. As shown in Fig. 4,

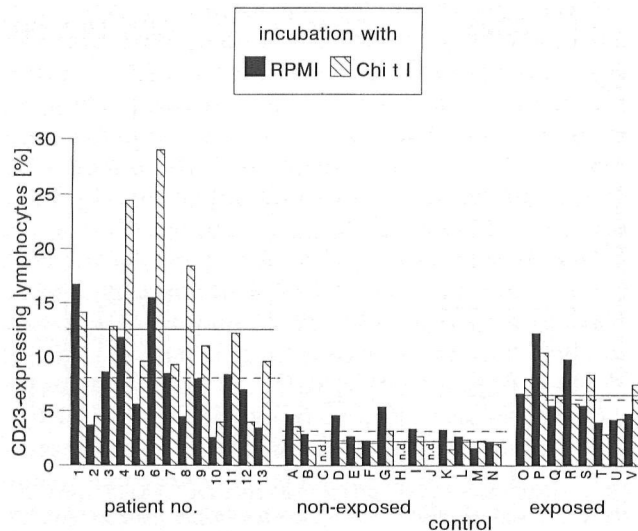


Fig. 3. CD23 expression in group of 13 *Chi t I*-allergic patients, 11 non-*Chi t I*-exposed controls, and 8 *Chi t I*-exposed controls. Expression of CD23 was evaluated on lymphocytes after incubation in RPMI or stimulation with *Chi t I* for 7 d. Mean value of CD23 expression in patients was significantly higher than in non-exposed controls ($P < 0.05$). Correlation of CD23 expression and specific or total IgE was not detectable. ---- mean value after incubation in RPMI; — mean value after stimulation with *Chi t I*.

a significant increase of CD25 ($P < 0.01$) and CD3 HLA-DR ($P < 0.01$) after antigen-specific stimulation of patients' lymphocytes was measurable. There was no influence of antigen-stimulation in the control group. As compared with controls, HLA-DR on non-T cells (CD3⁻; e.g., B cells) showed signifi-

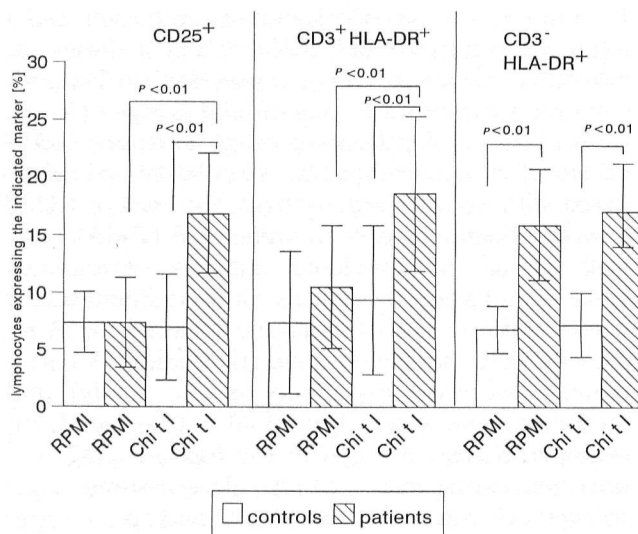


Fig. 4. Expression of cell surface marker HLA-DR on T cells (CD3⁺) and non-T cells (CD3⁻) and of CD25 (IL-2 receptor). Mean values $\pm$ SD of nine patients (patients 1–9) and six non-allergen-exposed controls (controls A–F) after incubation of lymphocytes in RPMI or stimulation with *Chi t I* for 7 d are shown.

cantly enhanced values without stimulation ($P < 0.01$). Antigen-specific stimulation had no effect.

Expression of the T-cell receptor

After antigen-specific stimulation, the α/β -T-cell-receptor expression was significantly lower on lymphocytes of patients than those of controls ($P < 0.05$) as well as on nonstimulated cells ($P < 0.01$; Fig. 5). Similar results were found for the CD3 molecule, which is part of the T-cell-receptor complex. A significant decrease of CD3 expression was measurable in patients ($P < 0.01$; mean value without stimulation $64.6 \pm 14.3\%$, mean value after antigen-specific stimulation $57.6 \pm 11.8\%$), but not in controls ($69.2 \pm 16.7\%$ without and $68 \pm 16.5\%$ with stimulation). A concomitant (nonsignificant) increase of γ/δ -T-cell-receptor-expressing lymphocytes in six of nine patients was measurable (mean value in RPMI $6.9 \pm 6.5\%$; mean value after stimulation with *Chi t I*, $8.2 \pm 5.9\%$).

Other cell surface markers

B cells were determined by anti-CD19. Their range for patients and controls was 5–17%. CD4⁺ T cells showed values of $38 \pm 16\%$ (patients) and $42 \pm 13\%$ (controls) in RPMI, and values of $31 \pm 11\%$ (patients) and $22 \pm 18\%$ (controls) after antigen-specific stimulation. For CD8⁺ T cells, we determined $29 \pm 10\%$ (patients) and $29 \pm 7\%$ (controls) in RPMI, and $30 \pm 7\%$ (patients) and $27 \pm 9\%$ (controls) after stimulation with *Chi t I*. This means that

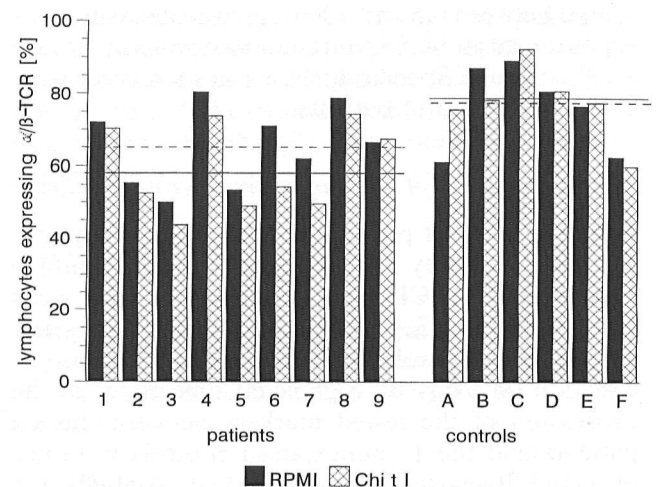


Fig. 5. Expression of α/β -T-cell receptor on lymphocytes of *Chi t I*-sensitized patients and nonexposed controls with (*Chi t I*) or without (RPMI) antigen-specific stimulation. After stimulation, α/β -T-cell-receptor expression was significantly lower in patients than in controls ($P < 0.05$; t-test) or on nonstimulated lymphocytes ($P < 0.01$; paired t-test).

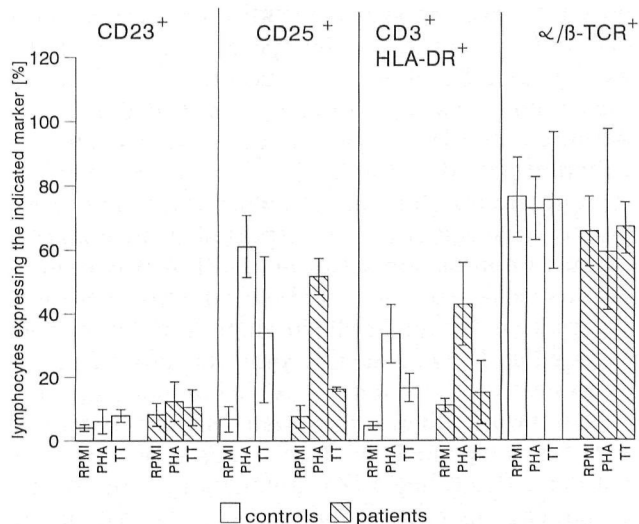


Fig. 6. PHA- and TT-dependent expression of low-affinity receptor for IgE (CD23), IL-2-receptor (CD25), activation marker HLA-DR, and α/β -T-cell receptor on lymphocytes of three *Chi t* I-sensitized patients (patients 4, 8, 9) and three nonexposed controls (controls A, B, C). Cell surface marker expression was determined after incubation with RPMI (medium control), and stimulation with PHA and TT for 7 d. Differences between *Chi t* I-sensitized patients and nonexposed controls in responses to PHA and TT were not detectable.

the relationship of CD4 to CD8 is 0.3–2.3 for *Chi t* I-sensitized subjects as well as for the control group.

Response to TT and PHA

We compared the expression of cell surface markers between patients and controls in response to TT and PHA. Differences between *Chi t* I-sensitized patients and nonsensitized subjects were not detectable. Fig. 6 shows results for CD23, CD25, CD3 + HLA-DR, and the α/β -T-cell receptor. In comparison with the results obtained by RPMI, we measured, after unspecific stimulation with the mitogen PHA, a significantly enhanced expression of HLA-DR ($P < 0.01$) and CD25 ($P < 0.01$) in *Chi t* I-allergic patients as well as in controls. Furthermore, in both groups, stimulation with PHA increased the percentage of CD8-positive cells, resulting in decreased CD4/CD8 ratios.

All subjects had been TT-vaccinated, and after specific stimulation with TT an increase of the activation markers HLA-DR and CD25 (as compared with the RPMI value) could be observed. Therefore, the response to TT as well as to PHA was similar in *Chi t* I-sensitized patients and in controls.

Discussion

The aim of this study was to find out whether an allergen-induced difference in the expression of lym-

phocyte cell surface markers between *Chi t* I-allergic patients and non-allergen-exposed as well as allergen-exposed controls exists.

In the absence of any stimuli and without incubation for several days, a high variability in the expression of each marker was observed. Differences between lymphocytes of patients and those of non-sensitized controls were not detectable. Therefore, we tested the expression of cell surface markers under standardized *in vitro* conditions (antigen-specific stimulation of PBL for 7 d). A significant increase of the activation marker HLA-DR and of CD25 and CD23 could be observed after PBL stimulation with *Chi t* I in patients. It is not known whether this enhancement is a direct effect of *Chi t* I or a secondary effect; for example, a lymphokine-mediated response. Further studies measuring lymphokines in the supernatants of cells will reveal the role of mediators in the expression of cell surface markers.

Our data clearly show that the increase of HLA-DR expression was due to an enhancement of activated T cells (CD3 +). HLA-DR was also significantly higher in patients than in controls after incubation with RPMI. This difference was due to a higher percentage of CD3 – cells expressing HLA-DR; e.g., B cells. Although two of the control persons were atopic, the enhanced percentage of HLA-DR + cells does not seem to be a general characteristic of IgE-mediated type I responses. However, a relationship between HLA-DR + non-T cells and exposure to an allergen is conceivable. High exposure to the respective allergen may lead to enhanced circulation of HLA-DR-expressing cells. All patients had contact with high amounts of the allergen *Chi t* I. Measurements of HLA-DR-expressing lymphocytes in allergen-exposed nonsensitized persons will be necessary to clarify this statement. HLA-DR associations were described for different antigens (18, 27) including *Chi t* I. The association of *Chi t* I allergy with HLA-DR type DRB1*0101 in monosensitized subjects is described. Therefore, the presence of increased levels of HLA-DR-expressing cells may help to promote the presentation of the respective allergen.

CD25, also known as Tac-antigen, is predominantly expressed on activated T cells. This receptor mediates the pleiotropic effects of IL-2. The expression of CD25 can be induced by its ligand as well as after mitogenic cell activation. Regarding the enhanced expression of CD25 after stimulation with *Chi t* I, similar results were found by antigen-specific stimulation of blood mononuclear cells of asthmatic patients allergic to house dust (12). House-dust-stimulated cultures showed a significantly higher percentage of IL-2-receptor-positive cells. CD4 + T-cell activation is also known to be important in the

pathogenesis of acute severe *asthma*. Contrary to controls, a significant increase in the expression of CD25 (IL-2 receptor), HLA-DR, and very late activation antigen (VLA-1) in patients with acute severe asthma was reported (6).

Unlike CD25, the CD23 antigen (Fc ϵ -receptor II) functions in B-cell growth and differentiation (29) and in IgE-mediated immunity (5). This molecule was found not to be restricted to B cells. It could also be induced on monocytes, platelets, Langerhans' cells, and natural killer cells (10). According to Prinz et al. (23), the Fc ϵ R II for IgE is expressed on a subpopulation of activated human T cells. This expression is selectively induced by the stimulation of T cells of allergic patients with the respective allergen.

Our data show that patients have a significantly higher level of Fc ϵ R II (CD23) after stimulation with *Chi t I* ($P < 0.01$) than do controls. Furthermore, the expression of CD23 on lymphocytes of *Chi t I*-sensitized patients significantly increased after stimulation with *Chi t I*, as compared with incubation with RPMI ($P < 0.05$). This increase was measurable neither in exposed nor in nonexposed controls. Since we used an antibody recognizing all Fc ϵ R II-bearing cells, we were not able to determine whether the elevated CD23 level is due mainly to T or B cells.

Our results with *Chi t I* show that CD23 in patients is significantly upregulated in the presence of the allergen *Chi t I*, suggesting a connection of CD23 and allergic IgE-mediated diseases. The role of CD23 expression was described for different atopic diseases (5, 24, 25). Although the role of CD23 in IgE regulation was reported (20), there are data documenting biologic activities of CD23 that are unrelated to IgE as well as, for instance, T-cell growth and antigen presentation (9, 21).

We also studied the expression of T-cell receptors in patients and controls. The number of α/β -chains expressing lymphocytes was significantly lower ($P < 0.05$) in patients than in controls after incubation with *Chi t I*, and in *Chi t I*-stimulated than in nonstimulated cells ($P < 0.01$). After incubation in RPMI, there was no significant difference between patients and controls in the number of α/β -chain-expressing lymphocytes. The decrease of α/β -T-cell-receptor-expressing lymphocytes in response to the allergen was accompanied by a slight, nonsignificant increase of γ/δ -T-cell-receptor-positive cells. An increase of γ/δ -T cells has been observed in some immunologic disorders (1, 28).

The downregulation of the α/β -T-cell receptor can be explained by signal transduction in activated T cells, probably by internalization. Whether a concomitant increase in the number of T-cell-receptor α - and β -chain mRNA transcripts occurs remains to

be determined (8). We also studied the expression of cell surface markers in response to mitogen PHA and antigen TT after stimulation for 7 d. Although the optimal reaction is not expected at day 7 (the optimum for PHA is day 3), we chose the same incubation period as for *Chi t I*. Our interest was not to compare absolute and maximal values but to examine the specificity of the reported surface marker expression in response to *Chi t I*. PHA was used as an unspecific stimulus to show whether there is a difference in the unspecific immune response of non-exposed and *Chi t I*-allergic subjects, and TT serves as control for the antigen-specific immune response. All examined subjects had been TT-vaccinated and therefore responded with an increase of activation markers. Regarding PHA stimulation, lymphocytes of patients as well as of controls showed an increased expression of HLA-DR and CD25. These results correspond to the investigations of Kus (12). In his studies, the mean percentages of IL-2-receptor-positive cells in PHA-stimulated cultures of asthmatic patients and of the control group were not different either. The reactivity of lymphocytes in cell surface marker expression was similar in patients and controls. This indicates that the described differences in cell surface marker expression are specific to the antigen *Chi t I*.

In conclusion, our results show that lymphocytes of *Chi t I*-allergic patients may be activated by an antigen-specific stimulus. Differences between allergic and nonsensitized donors were mainly measurable in response to the specific antigen *Chi t I*. CD25, CD23, and HLA-DR were significantly enhanced on lymphocytes in the presence of *Chi t I*. In addition, a higher percentage of lymphocytes expressing HLA-DR and of CD23+ lymphocytes in *Chi t I*-allergic persons was detectable after incubation with RPMI than in nonexposed controls. Antigen-specific activation occurred also with TT in TT-vaccinated persons. The reactions to TT and the mitogen PHA were independent of *Chi t I* allergy. The observation of antigen-specific expression of lymphocyte cell surface markers in allergen-sensitized subjects may be a valuable diagnostic indicator.

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