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T lymphocytes expressing CCR3 are increased in allergic rhinitis compared with non-allergic controls and following allergen immunotherapy

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Abstract

Background—In T cell-associated allergic inflammation, homing of T-helper 2 (Th2) effector cells to mucosal sites may be influenced by chemokine receptor expression. Previous studies have identified CCR3 and CCR4 as putative markers of Th2 cells and CCR5 and CXCR3 as markers of Th1 cells. The aim of this study was to assess differential chemokine receptor expression from symptomatic atopic grass pollen-sensitive subjects, compared with patients on high-dose allergen injection immunotherapy (IT) and healthy controls.

Methods—We examined chemokine receptor expression (CCR1–7 and CXCR1–4) by flow cytometry of peripheral blood CD4⁺ and CD8⁺ T cells. We also depleted peripheral blood mononuclear cell (PBMC) populations of CCR3⁺ CD4⁺ cells by magnetic bead separation and cells were stimulated with grass pollen allergen for 6 days. Cytokine production was measured by enzyme-linked immunosorbent assay.

Results—On freshly isolated PBMC, atopic individuals exhibited increased numbers of CCR3⁺ CD4⁺ cells compared with normal controls ($P < 0.01$). CCR3 expression in IT patients was reduced compared with matched atopic rhinitic controls ($P < 0.05$) and comparable with that observed in normal subjects. Depletion of CCR3⁺ CD4⁺ cells from allergen-stimulated PBMC cultures resulted in decreased interleukin (IL)-5 production compared with whole CD4⁺ populations ($P < 0.05$). Freshly isolated CCR3⁺ CD4⁺ cells have significantly higher intracellular IL-4 and lower IFN- γ levels than CCR3[−] CD4⁺ cells. CD4⁺ T cells cultured from both peripheral cells and nasal biopsies demonstrated increased expression of CCR3 in the presence of IL-4 ($P < 0.05$).

Conclusion—CCR3⁺ CD4⁺ T cells are increased in allergic rhinitis, are reduced by allergen IT, have a Th2 phenotype and contribute to allergen-specific responses. Strategies against CCR3⁺ T cells may be effective in human allergic diseases.

Keywords

allergens; allergic disease; CCR3; Chemokine receptors; chemokines; Immunotherapy; T cells; T-helper 2

Chemokines, acting upon their cognate seven-transmembrane receptors, are central regulators of lymphocyte trafficking and recruitment. The two principal chemokine subfamilies are the CC chemokines, acting on CC chemokine receptors (CCRs), and the CXC chemokines, acting on CXC chemokine receptors (CXCRs). The number of chemokines and receptors gives rise to a complex system that can subtly regulate lymphocyte trafficking (1, 2). Many studies have identified potential roles for individual receptors in the regulation of lymphocyte recruitment, and of interest have been those studies which ascribed a T-helper (Th)1 vs Th2 bias to specific receptors. In particular, expression of CXCR3 and CCR5 has been linked to Th1-type T cells (3–5) and CCR3, CCR4 and CCR8 to Th2-type T cells (3, 5–8).

Inflammatory responses in allergic disease are mediated by eosinophils, basophils, lymphocytes and mast cells (9). Activated CD4⁺ cells involved in allergic inflammation are polarized towards a Th2 phenotype and make cytokines such as interleukin (IL)-4 and IL-5 (10). Interleukin-4 promotes B cell switching in favour of immunoglobulin (Ig)E and IL-5 promotes the terminal differentiation and activation of eosinophils. The mechanisms of T cell recruitment to sites of allergic inflammation are not fully understood but elevated levels of the CCR3 ligand CCL11 (eotaxin) in human asthma suggest that CCR3 may be involved in this process (11). Moreover, because eosinophils (12), basophils (13) and polarized Th2 lymphocytes (7) express CCR3, increased CCL11 at sites of allergic inflammation may be responsible for the local infiltration of all these cell types. CCR3 deficient mice do not recruit eosinophils to the lung after allergen challenge in a model of allergic asthma (14).

Specific immunotherapy (IT) is the only antigen-specific immunomodulatory treatment for IgE-mediated allergic disease (15). Cellular changes that accompany successful grass pollen IT include a reduction in numbers of mast cells, eosinophils and basophils (16). In addition, the balance of cytokine production is skewed towards a Th1 response in IT-treated subjects (17). It is possible that this change in phenotype is due to decreased recruitment of Th2-associated cells to sites of allergic inflammation.

We aimed to identify potential chemokine receptors that may be involved in the trafficking of T lymphocytes in allergic disease. We compared chemokine receptor expression on freshly peripheral blood mononuclear cells (PBMC) isolated from donors with grass pollen allergy with subjects treated with high-dose grass pollen IT and normal healthy subjects. We demonstrate that CCR3 expression is significantly higher on CD4⁺ lymphocytes derived from atopic rhinitic donors compared with both normal controls and those successfully treated for seasonal allergic rhinitis with allergen injection IT. CCR3 expression on T cells is increased under pro-allergic conditions and CCR3⁺ CD4⁺ cells are biased towards preferential Th2 responses.

Materials and methods

Subjects

The studies were approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals NHS Trust and performed with written consent. A total of 32 subjects were recruited (Table 1) who donated either blood (normals, symptomatic atopics and IT-treated subjects; $n = 9$) or a nasal biopsy and blood (normals and symptomatic atopics, $n = 7$). Atopy was defined by one or more positive skin prick tests (SPT) at least 3 mm greater than the negative diluent control to six common aeroallergens [house dust mite (HDM), grass pollen, dog, cat, mixed tree pollen and *Aspergillus fumigatus*; Soluprick, ALK Abelló, Horsholm, Denmark]. Samples of blood and nasal tissue were taken from atopic rhinitic donors during the allergen exposure season, and these donors were all symptomatic at the time of venesection or nasal biopsy. Immunotherapy subjects had received high-dose grass

pollen IT for at least 2 years and SPT, radioallergosorbent test (RAST) and total IgE measurements were taken preimmunotherapy. Immunotherapy and atopic subjects were assessed for a change in hayfever symptoms compared to pretreatment (or previous pollen seasons) using an overall assessment score where +3 was classified as 'a lot better', +2 'better', +1 'a little better', 0 'no change', -1 'a little worse', -2 'worse', -3 'a lot worse'. Normal, nonatopic subjects were all nonsymptomatic, had negative SPTs to the same allergen panel and RAST tests to grass pollen were <0.34 IU/ml.

Reagents

General laboratory reagents were from Sigma, Poole, UK. We used the following anti-CKR Abs, all of which were from Millennium Pharmaceuticals (Cambridge, MA, USA; with grateful thanks to Shixin Qin) except where indicated: anti-CCR1 mAb 2D4 (IgG1 isotype), anti-CCR2 mAb 1D9 (IgG2a), anti-CCR3 mAb 7B11 (IgG2a), anti-CCR4 mAb 1G1 (IgG1), anti-CCR5 mAb 2D7 (IgG1), anti-CCR6 mAb 11A9 (IgG1), anti-CCR7 mAb 3G4 (IgM), anti-CXCR1 mAb 5A12 (IgG2b), anti-CXCR2 mAb 6C6 (IgG1), anti-CXCR3 mAb 1C6 (IgG1) and anti-CXCR4 mAb (IgG2a) was purchased from R&D systems (Abingdon, UK). Control antibodies (mouse IgG1 clone MOPC-21 and IgG2a clone UPC-10) were purchased from Sigma. CCL11 was purchased from PeproTech (London, UK).

Isolation and culture of T cells

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Histopaque 1077. Aliquots of cells were stained immediately for expression of chemokine receptors, or cultured as described (18) in the presence of IL-2 (10 ng/ml) and IL-2 plus IL-4 (20 ng/ml) for 5 days. Cells were then stimulated with phytohaemagglutinin (PHA) (1 µg/ml) and 1×10^6 cells/ml of irradiated PBMC (3000 rads) for a further 7 days. Fresh medium and cytokine was added every 2–3 days. Nasal biopsies, taken under local anaesthesia as described (18), were immediately halved and placed in separate wells of a 24-well culture plate containing 2 ml of complete medium and supplemented with either 10 ng/ml IL-2 alone, or 10 ng/ml IL-2 plus 20 ng/ml IL-4. Following incubation for 5 days, biopsy tissue was removed from culture wells and the remaining lymphocytes restimulated with 1×10^6 cells/ml of irradiated PBMC and 1 µg/ml PHA. Cultures continued to be supplemented with IL-2 or IL-2 plus IL-4, as previously. T cells were expanded for a further 7 days, with fresh complete medium and cytokine added every 2–3 days.

Flow cytometry

Cells were washed with staining buffer (PBS + 1% BSA + 0.1% azide), resuspended at 0.5×10^6 cells/ml and incubated with anti-CKR or control antibodies for 30 min (all at 10 µg/ml, except anti-CCR3 mAb 7B11, 3 µg/ml). Staining was detected using goat anti-mouse RPE-conjugated F(ab')₂ antibodies (DakoCytomation, Ely, UK), except for CCR7 which was detected using FITC-conjugated goat anti-mouse IgM (Sigma). Un-utilized secondary antibody binding sites were then neutralized by a blocking step with mouse IgG, prior to labelling with anti-CD8-FITC and anti-CD4-APC (BD Pharmingen, Cowley, UK), except for CCR7 where anti-CD8-PE (BD Pharmingen) was used to complement the FITC secondary antibody used for CKR staining. Cells were finally resuspended in PBS containing To-Pro 3 (Molecular Probes, Eugene, OR, USA), a dye that excludes dead cells similarly to staining with propidium iodide (19). Samples were analysed using a dual laser FACSCalibur flow cytometer with appropriate compensation settings (Becton Dickinson, Mountainview, CA, USA).

Cellular depletion experiments

CD4⁺ T cells were isolated from the PBMC by negative immunomagnetic selection using MACS CD4⁺ T-cell isolation kit II (Miltenyi Biotech, Bisley, UK). Purity of CD4⁺ T cells was assessed by flow cytometry and ranged from 92% to 96%. CD4⁺ cells were stained with 7B11 as described earlier. Rat anti-mouse IgG2a/b microbeads (Miltenyi Biotech) were used to capture CCR3⁺ cells and labelled cells were passed over a second column. The negative cell fraction, representing a population of CD4⁺ cells depleted of CCR3⁺, were stimulated with either 20 µg/ml of grass pollen allergen (*Phleum pratense*, a kind gift from ALK Abellø) or 10 µg/ml of purified protein derivative (PPD) (Evans Vaccines Ltd, Liverpool, UK) for 6 days. Irradiated (3000 rads) CD4⁺ cells were used as antigen-presenting cells. CD4⁺ cells were stimulated in parallel under identical conditions. CD4⁺ cells and cell populations depleted of CCR3⁺ cells were assessed for their expression of CCR3 and an average of 60 ± 12% of CCR3⁺ cells were depleted from the CD4⁺ population.

Cytokine enzyme-linked immunosorbent assays

Supernatants from allergen-stimulated PBMC cultures were assayed for the presence of IL-5 and IFN-γ by enzyme-linked immunosorbent assay. Assays utilized BD Pharmingen matched antibody pairs and human recombinant cytokines as standards (PeproTech EC). The limits of assay detection were 4–10 pg/ml.

Intracellular cytokine analysis

Freshly isolated PBMC populations were activated with phorbol 12-myristate 13-acetate (25 ng/ml) and ionomycin (1 µg/ml) for 5 h. Brefeldin A (10 µg/ml) was added for the final 4 h of stimulation. Cells were subsequently stained extracellularly for CCR3 and CD4 and fixed using CellFix (Becton Dickinson). Cells were permeabilized using saponin (0.1% saponin, 1% FCS in staining buffer) and stained for intracellular IFN-γ and IL-4 (Becton Dickinson) according to manufacturer's instructions. Samples were analysed by flow cytometry as above.

Statistical analysis

Data was analysed using Graphpad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). Statistical comparisons used the Student's *t*-test where *P* < 0.05 was considered significant.

Results

Chemokine receptor expression on freshly isolated T cells from subjects with symptomatic allergic rhinitis, patients with allergic rhinitis receiving grass pollen immunotherapy and normal controls

Chemokine receptor expression was highly variable on CD4⁺ T cells with some receptors expressed on a very small proportion of cells (e.g. CCR1) whereas others were expressed on almost all cells (e.g. CXCR4) (Fig. 1). Expression patterns of most chemokine receptors were similar on T cells from normal and atopic rhinitic subjects. However, expression of CCR3 and CXCR1 was significantly higher on cells derived from atopic donors compared with normal subjects (*P* < 0.01). Extensive analysis of CXCR1 expression is published elsewhere (20). Examination of CD8⁺ lymphocytes did not reveal any differences in chemokine receptor expression between normal and atopic donors (data not shown).

Detailed examination of CCR3 expression on T cells revealed that more CD4⁺ cells express this receptor compared with CD8⁺ cells (Fig. 1B). In addition, significant differences between normal and atopic donors were only apparent on CD4⁺ cells.

We next examined the expression of CCR3 on freshly isolated PBMC derived from subjects receiving grass pollen IT. These subjects reported significant improvements in symptom scores compared with matched untreated atopic controls ($P < 0.05$) (Fig. 2A). Data show that significantly fewer CD4⁺ T cells derived from IT donors expressed CCR3 compared with atopic controls ($P < 0.05$) (Fig. 2B).

Depletion of CCR3⁺ cells from allergen-stimulated PBMC cultures decreases IL-5 production

To assess the contribution of CCR3⁺ CD4⁺ T cells to cytokine production in allergen-stimulated cells cultures, we depleted CCR3⁺ cells from CD4⁺ cells derived from six grass-pollen sensitive donors. On average, 60% of CCR3⁺ cells were removed from the CD4⁺ population as assessed by flow cytometry (Fig. 3). Stimulation of CCR3-depleted cells with grass pollen allergen induced significantly less IL-5 production compared with the whole CD4⁺ population (whole CD4⁺ cells = 549 ± 317 pg/ml (mean $\pm$ SE); CCR3-depleted cells = 319 ± 147 pg/ml; $P = 0.03$). In contrast, IFN- γ production was increased in cultures depleted of CCR3⁺ cells although this did not reach statistical significance (whole CD4⁺ cells = 224 ± 128 pg/ml; CCR3-depleted cells = 342 ± 191 pg/ml; $P = 0.06$). Whole CD4⁺ and CD4⁺ CCR3⁻ populations produced similar amounts of IL-5 and IFN- γ after stimulation with an irrelevant antigen, PPD.

Intracellular cytokine expression by CCR3⁺ T cells

We next investigated the intracellular cytokine profile of CCR3⁺ CD4⁺ cells and compared cytokine expression with CCR3⁻ CD4⁺ cells from freshly isolated PBMC obtained from atopic donors (Fig. 4). Results show that CCR3⁺ cells produce significantly more IL-4 ($P < 0.05$) and significantly less IFN- γ ($P < 0.001$) compared with CCR3⁻ cells.

CCR3 expression is upregulated under Th2 conditions

In order to investigate the effect of a Th2 environment on CCR3 expression we generated short-term T-cell lines grown in IL-4. Cells were grown from both PBMC populations and nasal biopsies derived from normal and atopic donors. Results shown in Fig. 5 reveal that culture of blood-derived cells in the presence of IL-4 and IL-2 could significantly increase CCR3 expression from both atopic and normal donors compared with cells cultured in IL-2 alone ($P < 0.001$ and $P < 0.01$ respectively). These significant differences in CCR3 expression were also evident on nasal biopsy-derived cells obtained from atopic and normal donors ($P = 0.02$ and $P < 0.01$ respectively). Differences in CCR3 expression between normal and atopic cells were no longer observed in cell lines.

Discussion

We observed that CCR3 was expressed on a distinct subset of human CD4⁺ T cells, and that in freshly isolated blood these receptors were represented at higher numbers in symptomatic atopic rhinitis donors. Depletion of CCR3⁺ cells in allergen stimulated cultures resulted in reduced IL-5 production suggesting allergen-specific CCR3⁺ cells contribute to pro-allergic Th2 cytokine production. *In vitro* characterization revealed that this chemokine receptor is upregulated on both peripheral blood and nasal-derived CD4⁺ T cells under Th2-skewing conditions. Intracellular cytokine analysis shows that CCR3⁺ CD4⁺ cells produce significantly more IL-4 and less IFN- γ compared with CCR3⁻ CD4⁺ cells.

For the first time, this study reports lower numbers of CCR3-expressing T cells in subjects with allergic rhinitis treated with allergen IT compared with untreated patients and comparable with levels in nonallergic controls. It would be of interest also to examine CCR3 expression before and after grass pollen IT using a larger cohort of subjects in a longitudinal

study. None the less our data show a clearly reduced population of CCR3⁺ cells that paralleled clinical improvement compared with untreated controls.

Of the chemokine receptors studied we only identified alterations in expression of CCR3 and CXCR1. CCR8 was not examined in detail in view of technical difficulties encountered with the limited monoclonal antibodies available. Thus, CCR8, and other recently identified chemokine receptors associated with potential Th2 responses, remain to be formally examined in the context of human allergic disease.

Our findings are consistent with previous *in vitro* observations which identified a strong association of the receptor CCR3 with Th2-type differentiated cells (5, 7) and evidence from murine models of allergic inflammation (21). Association of CCR3 and human allergic disease has, however, been somewhat controversial. In atopic dermatitis, expression of CCR3 as determined by immunohistochemistry in lesional skin was shown to be elevated compared with normal controls (22). Serial sections stained for CD3 suggested that these cells were T lymphocytes. Moreover, nonlesional skin biopsies from patients with atopic dermatitis revealed elevated CCR3 expression at both mRNA and protein level. CCR3⁺ T cells have also been identified in nasal polyps compared with control nasal mucosal biopsies (23). Wang et al. (24) recently demonstrated that PBMC from subjects allergic to HDM had significantly increased numbers of CCR3⁺ CD4⁺ cells compared with nonatopic normal controls. Other groups, however, have not observed such associations between CCR3 and atopic disease. In one study, CCR3 expression on CD4⁺ CD45RO⁺ cells obtained from PBMC of grass pollen-allergic subjects was low compared to controls and there were no differences in CCR3 before/during the grass pollen season (25). Because of the low expression of CCR3 on CD4⁺ T cells small variations of antibody binding may have implications in identifying this population. Whilst other studies used a directly conjugated antibody, we utilized a primary anti-CCR3 antibody, followed by a labelled secondary antibody which may act to enhance the signal from the primary antibody. Another study, using the same antibody as utilized in this study, examined chemokine receptor expression on human lung T cells from four normal and asthmatic donors (26) and found no difference in CCR3 expression, although no numerical data was presented. It is possible that CCR3 expression is downregulated after homing to peripheral organs, or possibly CCR3 expression is not associated with asthma *per se*, in the absence of allergy. A recent study by Morgan et al. (27) again showed no difference in CCR3 expression on CD3⁺ cells between asthmatics (of which 64% were atopic) and controls although did associate CCR3 expression with IL-4 producing T cells.

In the present study, increases in CCR3⁺ T cells were observed following culture in the presence of IL-4 in both blood- and nasal-derived lines. Stimulation of CCR3-depleted CD4⁺ cells with grass pollen allergen resulted in decreased production of IL-5 and concomitant increases in IFN- γ compared with control cultures. This deviation away from the allergic Th2 state was associated with a loss of allergen-specific T cells, as no change in cytokine production was observed after stimulation with an irrelevant antigen. Using antibody-conjugated magnetic beads we were able to deplete approximately 60% of CCR3⁺ CD4⁺ T cells from PBMC. We speculate that this incomplete depletion reflects the low level of surface CCR3 expression on T cells when compared with other phenotypic markers more routinely used for cell separation. Nevertheless, we observed a decrease of 30% in IL-5 production suggesting that allergen-specific CCR3⁺ cells contribute a significant fraction of the Th2-associated cytokine production. These findings do not exclude the possibility that CCR3⁺ cells co-express other Th2-associated chemokine receptors such as CCR4 or CXCR1 (20, 26) that may also contribute to allergic responses.

In summary, we have confirmed that CCR3 expression is closely associated with Th2 cytokine production and have shown, for the first time, that CCR3-expressing CD4⁺ T lymphocytes are increased in allergic rhinitis and are inhibited following successful allergen IT. Small molecule antagonists of CCR3 have been developed and may provide a mechanism by which CCR3-mediated functions are inhibited (28). Moreover, blockade of this receptor could potentially prevent the migration of not only Th2 lymphocytes but also eosinophils, basophils and mast cells all of which are implicated in allergic disease. Our data support that targeting the CCR3 receptor may be a useful therapeutic strategy in allergic disease.

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Abbreviations

CCR	CC-chemokine receptor
CXCR	CXC-chemokine receptor
PBMC	peripheral blood mononuclear cells
IL	interleukin
IT	immunotherapy
RAST	radioallergosorbent test
PHA	phytohaemagglutinin
PPD	purified protein derivative

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Figure 1.

Chemokine receptor expression on peripheral blood mononuclear cells (PBMC) from atopic and normal donors. (A) Freshly isolated PBMC from normal and atopic donors ($n = 9$) were stained for chemokine receptor (CKR) expression and the percentage expression of each CKR on CD4⁺ T cell is shown. (B) CCR3 expression on CD8 and CD4 cells.

Figure 2.

(A) Overall assessment score of atopic and immunotherapy (IT) subjects. Subjects were asked if their hayfever symptoms were worse (negative score) or better (positive score) compared with 2 years previously (before the start of immunotherapy treatment). (B) CCR3 expression on CD4⁺ cells from freshly isolated peripheral blood mononuclear cells derived from normal, atopic and IT subjects.

Figure 3.

Depletion of CCR3⁺ cells from allergen-stimulated cultures. CD4⁺ cells were isolated from peripheral blood mononuclear cells (PBMC) derived from atopic donors ($n = 6$) by negative immunomagnetic separation. A CD4⁺ cell population depleted of CCR3⁺ cells was also obtained by negative selection. The numbers of CCR3⁺ cells in the CD4⁺ and in the CCR3-depleted fraction were assessed by flow cytometry. CD4⁺ cells and CCR3-depleted CD4⁺ cells were cultured with grass pollen allergen for 6 days in the presence of irradiated CD4⁺ cells acting as antigen-presenting cells. Cytokine production was measured by enzyme-linked immunosorbent assay. The graph represents the relative change in CCR3 expression or cytokine production comparing the CD4⁺ fraction with the CD4⁺ CCR3-depleted fraction. Subjects who did not produce detectable levels of cytokine in either whole or depleted PBMC were excluded from calculations of relative change.

Figure 4.

Intracellular cytokine staining of CCR3⁺ CD4⁺ cells. Peripheral blood mononuclear cells derived from atopic donors ($n = 10$) were activated for 5 h, fixed and subsequently stained for intracellular IFN γ and IL-4. The results show the percentage of cells staining for IL-4 and/or IFN- γ .

Figure 5.

Expression of CCR3 on CD4⁺ cells cultured under Th2-skewing conditions. Peripheral blood mononuclear cells ($n = 6$) or cells derived from nasal biopsies ($n = 7$) were cultured with IL-2 alone or in the presence of IL-2 and IL-4 for 7–10 days. CCR3 expression on CD4⁺ cells was measured by flow cytometry. *Significant change compared with cells cultured in IL-2 alone.

Table 1

Characterisation of participants with allergic rhinitis and normal controls. Venesection was carried out during the UK pollen season and all allergic donors had symptoms of rhinitis at the time of nasal biopsy. Data are shown as median (upper quartile, lower quartile)

Subjects – blood	Allergic	Normal	Immunotherapy
No. of patients	9	9	9
Sex (M/F)	5F/4M	4M/5F	4F/5M
Age (years)	30 (21,49)	34 (26,46)	38 (33,39)
Skin prick test (mm)	6 (3,8)	<0.34 (<0.34, <0.34)	8 (8,10)
Radioallergosorbent tests (IU/ml)	35 (1.5–101)	<0.34 (<0.34, <0.34)	58 (41,100)
Total IgE	234 (90,526)	15 (10,91)	178 (76,568)
Subjects – nasal biopsy		Allergic	Normal
No. of patients		7	7
Sex (M/F)		2M/5F	2M/5F
Age (years)		29 (22–35)	34 (25–46)
Skin prick test (mm)		7 (5–12)	<0.34 (<0.34, <0.34)
Radioallergosorbent tests (IU/ml)		55 (14,99)	<0.34 (<0.34, <0.34)