Recovery from Pneumocystis carinii pneumonia in dexamethasone-treated Wistar rats

A. Sukura*, Y.T. Konttinen**, R. Sepper***, L-A. Lindberg*


ABSTRACT: Our aim was to study histopathological changes in lung tissue at the light microscopic and ultrastructural level during recovery from immunosuppression and Pneumocystis carinii pneumonia.

Male Wistar rats were immunosuppressed by per oral dexamethasone for 12 weeks to induce P. carinii pneumonia, after which dexamethasone was stopped. Recovery was monitored 1, 2 and 4 weeks after cessation of the immunosuppression.

In immunosuppressed animals, CD4+ and CD8+ lymphocytes were both decreased in situ. CD8+ lymphocytes increased above control level at week one. Like CD8+ cells, the ED1+ macrophages increased rapidly in situ. This was accompanied by a progressively increasing migration (more transient for lymphocytes) of macrophages into bronchoalveolar fluid, associated with morphological signs of activation and phagocytosis and proliferation of type II pneumocytes. Bronchoalveolar lavage (BAL) fluid tumour necrosis factor-α (TNF-α) increased from subnormal levels to a 4 week peak, with an inverse correlation between TNF-α and cyst count (r=-0.626).

Our observations suggest a sequence of changes characterized by an increase in CD4+ cells, accompanied by a more rapid and prolonged recruitment/activation of CD8+ cells, macrophages and type II pneumocytes.


Most children have had an asymptomatic primary Pneumocystis carinii infection before their fourth year [1, 2], and pneumonia caused by reactivation of latent P. carinii (PCP) or infection de novo is the leading cause of serious lung disease in acquired immunodeficiency syndrome (AIDS) [3, 4]. Most patients with PCP have CD4+ cells at or below 0.2×10^6 cells/l, suggesting that inducer/helper T-lymphocytes play a critical role in host defence [3, 5]. This finding has been supported by experimental studies using corticosteroid or anti-CD4 antibody-induced immunosuppression and nude or scid mice [6–9]. On this basis, as well as on the basis of in vitro studies, it has become evident that besides CD4+ and B-lymphocytes other cells, in particular lymphocytes of the CD8+ phenotype [10, 11], alveolar macrophages and type II alveolar epithelial cells [12–14], are involved in the resistance to P. carinii and/or in development of pneumonia. In fact, in contrast to antimicrobial effects, some of the commonly used therapeutic regimens, including corticosteroids and pentamidine inhalation, reduce disease severity by inhibiting the deleterious host response [15–17].

Evaluation of the complex interactions between P. carinii and host factors contributing to healing and/or tissue damage in lung tissue and bronchoalveolar fluid is hampered by a lack of appropriate human material. It is difficult to define the exact time of disease onset, to induce recovery and to follow the temporal profile of changes developing during recovery over time. Definition of the local tissue conditions at the time of clinically evident PCP, and changes during recovery in host response in relation to clearance of P. carinii, could contribute to a better understanding of the processes involved both in tissue damage and recovery from P. carinii infection.

Therefore, dexamethasone-immunosuppressed Wistar rats were studied during recovery from P. carinii, with emphasis on histopathological changes in lung tissue and bronchoalveolar lavage (BAL) fluid at light microscopic and ultrastructural level focusing on P. carinii cysts, the main lymphocyte subsets, monocyte/macrophages, and pneumocytes.

Materials and methods

Animals and experimental design

Ten week old male Wistar rats, mean weight 312 g, were obtained from the National Laboratory Animal Centre (Kuopio, Finland). The animals were divided into
four immunosuppressed groups, seven animals in each, and a control group consisting of six animals. As described in a previous study [18], immunosuppression was achieved by adding dexamethasone (1 mg·l⁻¹, dexamethasone phosphate D-1159, Sigma, St. Louis, MO, USA) to the drinking water, and tetracycline (500 mg·l⁻¹, terramycin 5.5%, Pfizer, Brussels, Belgium) was also added to the drinking water to prevent secondary bacterial infections. The control group received no medication, and all the rats consumed a normal commercial diet. To maximize the infection pressure, the immunosuppression groups were placed in an animal room with a rat colony on continuous immunosuppression and infected with *P. carinii*. The control group was placed in a separate, clean animal room. The animals were weighed once a week, and the drinking water with medications was changed twice a week.

After 12 weeks of immunosuppression, one group was sacrificed, and in the recovering groups the immunosuppression with dexamethasone was stopped; 4 days later the prophylactic tetracycline was also stopped. The recovering groups were sacrificed 1, 2 and 4 weeks after cessation of the immunosuppression. The control group underwent euthanasia at the same time as the 2 week recovery group.

The study was approved by the College of Veterinary Medicine committee on animal experimentation.

**Sampling**

Under intraperitoneal sodium pentobarbital anaesthesia (30 mg i.p.; Mebunat®, Orion, Helsinki, Finland) the thorax was opened, and a blood sample was taken from the right ventricle. Thereafter, all vessels caudal from the liver were cut, and the animal was bled to death.

The lungs with the attached trachea were removed from the thorax in toto and weighed, and their relative weights ((lung weight - tracheal weight)/total body weight) were calculated. The left lobe was sacrificed and used for BAL sampling. A blunt 23-gauge needle was placed gently in the bronchus via the trachea and fixed in place. Lavage was performed with five 300 µl aliquots of 37°C physiological saline. The initial wash was disregarded and the four subsequent washes composed the BAL sample.

The right caudal lobe was processed to cryosamples, which were frozen in liquid nitrogen and stored at -70°C, and to routine histology samples, which were fixed in 4% formaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Samples for transmission electron microscopy were taken from the right medial lobe, which was cut into 1 mm³ pieces and fixed for 2 h in 2.5% glutaraldehyde in 0.1 M Sörensen phosphate buffer, pH 7.3, before embedding in Epon.

Imprint samples were taken from the right medial lobe by pressing the freshly cut surface to an objective slide. The slides were then air dried and fixed in (4°C) acetone. The rest of the lung was stored at -20°C for quantitation of the cysts, as described under "Cyst quantitation" below.

**Blood samples.** Total leucocyte count was calculated with an automatic Coulter® T-850 (Coulter Electronics Ltd, Luton, UK) analyser. Differential count was performed from May-Grünwald Giemsa stained blood smears with an oil-immersion objective (×1,000).

**Bronchoalveolar lavage samples.** Living cells, dead cells and erythrocytes were counted in BAL fluid using trypan blue stained samples and Bürker's chambers. Samples were centrifuged for 5 min at 160 g, the pellets were resuspended in 0.9% NaCl for preparation of cytocentrifuge (Cytopsin 3 Shandon, Shandon Scientific Ltd, Cheshire, UK) specimens, which were air dried and fixed in (4°C) acetone. Differential countings were performed with May-Grünwald Giemsa stained slides.

**Cyst quantitation.** The lung sample was weighed (average weight 0.37 g) and digested in 10 ml of 0.2% collagenase in 0.9% NaCl at 40°C under continuous stirring. After centrifugation for 5 min at 650 g and three washes, the pellet was resuspended in 0.9% NaCl, and an aliquot was processed to a cytocentrifuge specimen [19], which was stained with toluidine blue O, before counting using an ocular counting square. The final concentration of cysts·g⁻¹ of lung tissue was calculated. The detection level of the method used is approximately >10⁴ cysts·g⁻¹ lung tissue.

**Indirect immunoperoxidase staining**

Mouse anti-rat monoclonal antibodies against CD2, CD4 (W3/25) and CD8 were derived from clones OX-34, OX-35 and OX-8, respectively. Macrophages were identified using monoclonal antibodies derived from clone ED1. All antibodies were purchased from Serotec Ltd (Blackthorn, Bicester, UK). Cryostat sections were air dried, fixed in (4°C) acetone for 5 min and treated with chloroform for 30 min.

After the sections has been washed in 10 mM PBS, endogenous peroxidase was inactivated with 0.3% H₂O₂ in 10 mM PBS containing 0.1% NaN₃, to reduce tissue-breaking bubble formation. After two washes in 0.1% bovine serum albumin (BSA) in 10 mM PBS (referred to as PBS-BSA), 5 min each, sections were treated in a humidity chamber with: 1) primary antibodies (1:50–1:100 in PBS-BSA) for 60 min; 2) normal donkey serum (1:20 in PBS-BSA), Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min; 3) peroxidase conjugated donkey anti-mouse immunoglobulin (1:50 in PBS-BSA, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 60 min; 4) 50 mM Tris, pH 7.6, for 5 min; and 5) 0.01% H₂O₂ and 0.05% diaminobenzidine (DAB) in 50 mM Tris. The developing time, 2–10 min, was controlled under light microscopy. All incubations were carried out at 20°C, and slides were washed in PBS three times, for 5 min each, between each step. Counterstaining was performed with Mayer’s haematoxylin before dehydration and mounting.

Specificity of the staining was confirmed: 1) by omission of the primary antibody from the staining sequence;
and 2) by use of normal mouse serum (1:100 in PBS-BSA) instead of the primary antibody. Rat spleen cryosections were used as positive sample controls [20].

Quantitation of immunoreactive cells

Inflammatory cell subclasses were calculated from the stained slides (6 μm tissue sections) using an ocular counting square and an oil-immersion objective (×1,000 magnification). The starting point of the counting was chosen randomly, and counting was continued using adjacent fields, with the exclusion of the bronchial associated lymphatic tissue (BALT). The investigator counting the cells did not know the source of the sample.

Post-embedding immunoelectron microscopy

For post-embedding immunoelectron microscopy, Epon-embedded ultrathin sections were incubated with P. carinii cyst stage specific monoclonal antibody, as described in detail elsewhere [21].

Radioimmunoassay

Tumour necrosis factor-α (TNF-α) was measured with a competitive double-antibody radioimmunoassay as described in detail previously [22]. The detection level of the assay was 5 ng·l⁻¹.

Statistics

Data are expressed as mean±SEM. The significance of the differences between mean values was tested using one-way analysis of variance (ANOVA) or, when appropriate, Kruskal-Wallis one-way ANOVA. Pairwise comparisons were performed with Bonferroni test or Mann-Whitney U-test. The relationship between variables was studied using Spearman’s rank correlation. Animal weights were subjected to repeated measurements ANOVA. Analyses were performed using Statistix 4.0 statistical software package [23], except repeated analysis of variance, which was carried out using the general linear model procedure in SAS [24]. A difference was considered significant when p was less than 0.05.

Results

General effects of immunosuppression and PCP

All animals survived throughout the experiment. The animals in the control group gained weight (49±2.1% by the time immunosuppression was finished in the other study groups), whereas the animals on immunosuppression lost weight during the dexamethasone treatment (20±2.5%), but started to gain weight after the treatment was stopped; the 4 week recovery group achieving

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Differential cell count</th>
<th>Relative weight</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Neutrophils</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>IS</td>
<td>7</td>
<td>3.06±0.61</td>
<td>2.70±0.60</td>
<td>0.30±0.02</td>
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<tr>
<td>1 post-IS</td>
<td>7</td>
<td>1.40±0.19*</td>
<td>0.60±0.12</td>
<td>0.77±0.09</td>
</tr>
<tr>
<td>2 post-IS</td>
<td>7</td>
<td>3.13±0.78</td>
<td>2.06±0.73*</td>
<td>1.00±0.11*</td>
</tr>
<tr>
<td>4 post-IS</td>
<td>7</td>
<td>2.94±0.19</td>
<td>1.24±0.13*</td>
<td>1.63±0.17*</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>3.22±0.34</td>
<td>0.57±0.14</td>
<td>2.57±0.23</td>
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</table>

Data are presented as mean±SEM. IS: 12 weeks immunosuppression; PCP: weeks after immunosuppression; Control: control group underwent euthanasia at the same time as the 2 week post-IS group. *: p<0.05 versus control, Mann-Whitney U-test.

P. carinii cysts and lung weight during recovery

None of the animals in the control group had any detectable P. carinii cysts in the toluidine blue O-stained imprint slides. All animals on immunosuppression, with one exception in the 4 week post-immunosuppression group, had cysts in imprint samples, and even the one without cysts in the imprint sample had cysts in the digested lung sample, representing the lowest recorded number of cysts·g⁻¹ of lung tissue (4.15×10⁶ cysts·g⁻¹). The cyst concentration declined in post-immunosuppression groups during the recovery period (p<0.05), although an average of 9.5×10⁶ cysts·g⁻¹ were still present at week four (table 2).

The relative lung weight (lung weight/body weight) was high in PCP groups compared to controls (p<0.001; pooled data).

Radioimmunoassay

Tumour necrosis factor-α (TNF-α) was measured with a competitive double-antibody radioimmunoassay as described in detail previously [22]. The detection level of the assay was 5 ng·l⁻¹.

Statistics

Data are expressed as mean±SEM. The significance of the differences between mean values was tested using one-way analysis of variance (ANOVA) or, when appropriate, Kruskal-Wallis one-way ANOVA. Pairwise comparisons were performed with Bonferroni test or Mann-Whitney U-test. The relationship between variables was studied using Spearman’s rank correlation. Animal weights were subjected to repeated measurements ANOVA. Analyses were performed using Statistix 4.0 statistical software package [23], except repeated analysis of variance, which was carried out using the general linear model procedure in SAS [24]. A difference was considered significant when p was less than 0.05.

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<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cyst count</th>
<th>Relative weight</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS</td>
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<td>2.1±0.35</td>
<td>0.56±0.043*</td>
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<td>1 post-IS</td>
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<td>1.3±0.24</td>
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<td>4.9±2.7</td>
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<tr>
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<td>7</td>
<td>0.75±0.25*</td>
<td>0.62±0.064*</td>
<td>20.3±6.2</td>
</tr>
<tr>
<td>4 post-IS</td>
<td>7</td>
<td>0.95±0.20*</td>
<td>0.61±0.062*</td>
<td>28.0±4.2*</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0±0</td>
<td>0.33±0.012</td>
<td>23.7±8.9</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM. IS: 12 weeks immunosuppression; post-IS: weeks after immunosuppression; Control: control group underwent euthanasia at the same time as the 2 week post-IS group. *: statistically significant difference (p<0.05); cyst counts and TNF-α compared to immunosuppression group and relative weights to control group.
Histopathology

Perivascular lymphocyte infiltrates in lung tissue and accumulation of macrophages in alveolar spaces, which were partly filled with a foam-like material, were the main histopathological changes in the immunosuppressed groups. Typically, alveolar septa were thick, and large areas of lung sections were atelectatic in the immunosuppressed groups. Alveolar epithelization by type II pneumocytes was evident, particularly in the recovering groups.

ED1 positive monocyte/macrophages (fig. 1) were numerous in all recovering groups compared to the control group (p<0.05), which did not differ from the immunosuppressed group in this respect (fig. 2).

The number of CD4+ lymphocytes was low in the immunosuppression group and at week one after immunosuppression compared to other groups (p<0.05). In contrast, the number of CD8+ cells, which was also low in the immunosuppression group (p<0.05), increased by week one post-immunosuppression and then decreased gradually to the control level (fig. 2). CD8+ cells were scattered throughout the interstitium (fig. 1), whilst CD4+ cells were found mainly in the peribronchial and perivascular lymphocyte infiltrates (not shown).

Bronchoalveolar lavage fluid

Mean recovery of the bronchoalveolar washings was 68±4.8%, with no significant differences among the means in pairwise comparisons (data not shown). The number of dead cells was high in the 4 week post-immunosuppression group (p<0.05). The number of macrophages increased gradually in post-immunosuppression groups (p<0.05) (fig. 3), whereas the lymphocytes peaked at week one after immunosuppression (p<0.01) (fig. 3). In the immunosuppression group, BAL fluid macrophages were large in size, with light staining and highly vacuolated cytoplasm.

TNF-α content was low in the immunosuppression and the 1 week post-immunosuppression groups, but reached the normal level by week two, with the highest value seen at week four (p<0.05) (table 2). TNF-α concentration and the cyst count (per gram of lung tissue) were in a negative correlation to each other (r=-0.626; p<0.01).

Ultramorphology

Alveolar macrophages in post-immunosuppression groups often contained phagocytosed material identified
RECOVERY FROM \textit{P. carinii} PNEUMONIA IN RATS

Fig. 4. – Alveolar macrophages in male Wistar rats recovering from \textit{P. carinii} pneumonia (PCP) after cessation of 12 weeks of immunosuppression with dexamethasone. Alveolar macrophages which have phagocytosed: A) a trophozoite-like particle (arrow) or: B) a cyst-like particles (arrow). The alveolar macrophages show morphological signs of activation, including large size and ragged appearance. Transmission electron micrograph. (Internal scale bar=1.5 µm in panel (A); and 0.89 µm in panel (B)).

Fig. 5. – Postembedding immunoelectron micrograph of \textit{P. carinii} cyst with intracystic bodies. A) Indirect staining with primary monoclonal antibody directed against a marker antigen of the electronlucent layer of the cyst pellicle (arrow, 15 nm gold particles). (Internal scale bar=0.52 µm). B) An alveolar macrophage has phagocytosed a \textit{P. carinii} cyst, visible in a phago(lyso)some. Some label (arrows) also visible outside the

Fig. 6. – One week recovery group. Activation both of T-cell and B-cell lineage was evident. In transmission electron microscopy, lymphoblasts characterized by large size (>10 µm in diameter): A) T-lymphocytes by free ribosomes. (Internal scale bar=1.9 µm). B) Plasmablasts and plasma cell by a typical nuclear chromatin pattern and rough endoplasmic reticulum. (Internal scale bar=2.25 µm).
as trophozoites or cysts (fig. 4). Furthermore, postembedding immunoelectron microscopy suggested intracellular degradation and transport of the electronlucent layer cyst-pellicle antigen (fig. 5). This macrophage activation was accompanied by T- and B-lymphocyte activation, as evidenced by the appearance of T-lymphoblasts and plasmablasts/plasma cells, respectively (fig. 6). In addition, resident type II pneumocyte involvement was suggested by their increased numbers and changed morphological characteristics, in particular by their ragged appearance (fig. 7).

**Discussion**

*P. carinii* pneumonia is today the most frequent cause of death from infectious disease in the United States [25]. This astounding increase has resulted from the AIDS epidemic, in which about 85% of patients acquire PCP [26], those with low CD4+ cells being at high risk [5, 27]. However, CD4+ cells by themselves display little direct antifungal or proinflammatory potential. Instead, by being specific immunocompetent cells, they can be specifically activated by processed antigenic endotopes, which activation leads to production of CD4+ cytokines, such as interleukin-2 (IL-2), and interferon-gamma (IFN-γ) produced by murine Th1-type CD4+ cells. In fact, recent studies suggest that aerosol administration of IFN-γ reduces the intensity of PCP by augmentation of host alveolar macrophage response, despite persistent CD4+ cell depletion [28]. *In vitro* IFN-γ activates alveolar macrophages to produce TNF-α, whereas type II pneumocytes produce TNF-α whether IFN-γ is present or not [29]. TNF-α seems to be cytotoxic to *P. carinii* [14], which mechanism may involve reactive oxygen species and nitric oxide [30].

Numerous clinical studies have revealed various factors associated with increased risk for PCP [10, 11]. Similarly, specific anti-CD4 [7] treatment and immunoneutralization and/or supplementation studies with various cytokines [31] have suggested which factors might be involved in disease susceptibility on the one hand and which, on the other hand, may contribute to the clinical disease manifestations produced by the host response against *P. carinii*. We hypothesized that a complementary approach, namely documentation of the changes associated with the full-blown disease and sequential changes during recovery from immunosuppression and *P. carinii* pneumonia, might provide new information and a test for the emerging hypothesis on the role of the immunocompetent T-lymphocytes and the more or less nonspecific and potentially tissue-damaging effector systems briefly discussed above.

The most widely utilized animal models used in studies of pathomechanisms of PCP are corticosteroid-treated animals and congenitally immunodeficient nude and scid mice (for discussion of these models, see the recent review [13]). Although none of these models is perfect, and although *P. carinii* strains in man and experimental animals differ [32, 33], they do reproduce the histopathological hallmarks of typical PCP, and have provided valuable information about the pathogenesis and the host/*P. carinii* interactions.

In the present study, a 12 week induction period was followed by termination of immunosuppression, after which histopathology and BAL fluid changes were followed over time during recovery. Contrary to expectations, the numbers of CD8+ (and not CD4+) cells increased rapidly *in situ* to values well above normal, and remained elevated throughout the study period. In contrast, lymphocytosis was seen in BAL fluid only at week one. Focal lymphocyte activation was also suggested by the appearance of T-lymphoblasts and plasma cells *in situ*. This suggests that CD8+ cells acting synergistically with CD4+ cells may augment the initial process, possibly by producing IFN-γ, as suggested by HUFFNAGLE et al. [34]. CD4+ cell changes showed a different profile, being low *in situ* in immunosuppressed animals, and gradually returning to normal without exceeding the control values during the 4 week follow-up period.

Interestingly, cells of the mononuclear phagocyte series, mostly alveolar macrophages, also rapidly increased in
numbers in alveoli containing both *P. carinii* trophozoites and cysts. The number of type II pneumocytes in the septa also increased. Furthermore, they showed morphological signs of activation, such as increased size and ruffling. Monocytes/macrophages contained remnants of both trophozoites and cysts. In macrophages, the electronlucent layer antigen of the *P. carinii* pellicle was found in phagolysosomes, but also elsewhere in the cell cytoplasm. This might represent antigen processing, which may play a critical role in the induction of the antigen-specific T-lymphocyte response thought to play a crucial role in the host response to *P. carinii* during natural recovery [7, 9].

The present study demonstrates a clear compartmentalization of the cells participating in the afferent and efferent arm of the local immune response against *P. carinii* in Wistar rat lung. Lymphocytes were usually (except at week one) confined to the lung interstitium, whereas alveolar macrophages, and possibly also monocyte/macrophages recruited from the circulation, seemed to locate in lung alveoli, where many type II pneumocytes were also observed. Phagocytosis and intracellular killing of *P. carinii* by alveolar macrophages and the directly cytotoxic humoral factors secreted by type II pneumocytes, therefore, seem to be responsible for the clearance of *P. carinii* organisms. Our findings support the demonstration by Pesanti [29] of TNF-α release into BAL fluid during recovery. Macrophages showed signs of direct interactions with *P. carinii*, including phagocytosis and antigen-processing coupled with morphological signs of activation. Active T-lymphocyte involvement, perhaps mediated by IFN-γ as suggested by Beck et al. [28], might contribute to this activation. However, it was of interest that under the *in vivo* conditions, which allow growth and replication of *P. carinii* organisms, the cyst numbers decreased relatively gradually, and the main effector cells, pneumocytes, seemed to remain active right to the end of the 4 week follow-up period.

Migration and homing of immunocompetent CD4+ (and CD8+) lymphocytes into the lung interstitium suggest involvement of these cells, an involvement which might, based on the topography and kinetics of the host defence, be mediated indirectly by lymphokines. This would be compatible with the work by Shear et al. [35] suggesting a role for IFN-α, produced by both CD4+ and CD8+ cells, in host defence against *P. carinii*. This conclusion is supported by the present observation of phagocytosis and, possibly also, of the processing and presentation of *P. carinii*-derived peptides to local lymphocytes, which, based on ultramorphological criteria, showed signs of activation in both T- and B-lymphocyte lineage.

**References**


