

## Natural killer (NK) cell activity of peripheral blood, synovial fluid, and synovial tissue lymphocytes from patients with rheumatoid arthritis and juvenile rheumatoid arthritis

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**SUMMARY** Natural killer (NK) cell activity was investigated in peripheral blood, synovial fluid, and synovial tissue lymphocytes from patients with rheumatoid arthritis (RA) and juvenile rheumatoid arthritis (JRA). Unfractionated lymphocytes, T lymphocytes, and non-T lymphocytes from the 3 compartments of JRA patients had reduced activity compared with that of normal peripheral blood lymphocytes (with *p* values usually between 0.05 and 0.1). Unfractionated synovial tissue lymphocytes of RA patients also showed reduced cytotoxicity ( $0.05 < p < 0.1$ ), whereas peripheral blood lymphocytes exerted normal NK cell activity. The NK activity was exerted by cells both with and without Fc $\gamma$  receptors. The highest cytotoxicity was observed in Fc $\gamma$  receptor-positive cells, both in peripheral blood and synovial fluid, since more than 70% reduction in NK activity was found after depletion of Fc $\gamma$  receptor-positive cells. No evidence of lymphocytotoxic antibodies or other factors with influence on NK cells was observed in the patients' sera.

NK cells are important for the surveillance of cancer cell growth and possibly also for protection against viral infections.<sup>1-3</sup> Little is known, however, about other possible functions of the NK cells, for instance, their contribution to tissue damage in autoimmune diseases. An immunodeficiency disorder involving a selective defect in NK cells has been described,<sup>4</sup> but whether there exist disease states with NK cell abnormalities restricted to certain lymphocyte populations or subpopulations is not known.

In the mouse the NK cells bear a unique surface antigen, the Ly5 antigen.<sup>5</sup> In man these cells seem to be heterogeneous with respect to surface markers, and the activity is exerted by both T and non-T lymphocytes, with and without Fc $\gamma$  receptors.<sup>6,7</sup>

In addition to genetic predispositions and autoimmune mechanisms the possibility also exists that viral infections may play a role in the pathogenesis of rheumatoid arthritis (RA) and juvenile rheumatoid arthritis (JRA).<sup>8,9</sup> Such viral infections could be due to a defective antiviral immunity, reflected in

derangements in NK cell activity and in interferon levels.<sup>1,3,5</sup>

In this study we have investigated the NK cell activity of peripheral blood, synovial fluid, and synovial tissue lymphocytes from RA and JRA patients. The relation between NK cells and Fc $\gamma$  receptor-bearing cells was studied, and we have also looked for a possible association between NK cell activity and interferon titres.

### Materials and methods

**Patients.** Eight adult patients with classical or definite RA according to the ARA criteria<sup>10</sup> and 13 patients with JRA according to the criteria of Brewer *et al.*<sup>11</sup> were studied. The age distribution in the RA group ranged from 37 to 67 years (mean 47 years) and in the JRA group from 9 to 17 years (mean 12 years). Seven (88%) of the RA patients and 2 (15%) of the JRA patients were seropositive and 3 (38%) of the adult patients and 3 (23%) of the children were positive for antinuclear antibodies (ANA positive).

Of the JRA patients, 2 (15%) had the systemic onset type of the disease, 8 (62%) had the pauci-articular type, and 3 (23%) had polyarticular type.<sup>11</sup>

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Two (25%) of the RA patients and one (8%) of the JRA patients were receiving long-term steroid treatment. Two JRA patients were on cytostatic drugs (chlorambucil and azathioprine), whereas 3 JRA patients were not on drug treatment. All the other patients were treated with various anti-inflammatory drugs in conventional doses.

**Effector cells.** Peripheral blood mononuclear cells (PB-MNC) were isolated from heparinised blood by Isopaque-Ficoll gradient centrifugation.<sup>12</sup> The patient's blood was taken at the time of synovial fluid aspiration or on the first day after synovectomy. Synovial fluids were drawn aseptically from knee joints or wrists into heparinised tubes, and the synovial fluid lymphocytes were isolated as described elsewhere.<sup>13</sup> Only synovial fluid specimens containing >95% MNC after Isopaque-Ficoll centrifugation were used. Synovial tissues were obtained by synovectomy in temporary ischaemia under general or local anaesthesia from 8 RA patients and 3 JRA patients. The synovial tissue MNC were eluted according to Abrahamsen *et al.*<sup>14</sup> Cells with an appearance similar to PB-MNC constituted approximately 70% of all the eluted cells, and the viability always exceeded 80% as judged by trypan blue exclusion.

T lymphocytes were isolated as previously described.<sup>15</sup>

**Target cells.** The myeloblastoid cell line K-562 was maintained in continuous suspension culture in complete medium.  $1 \times 10^6$  cells suspended in 0.3 ml fetal calf serum (FCS) were labelled with 300  $\mu$ Ci  $^{51}\text{Cr}$  (Amersham Radiochemicals, England) for 2 h to achieve approximately 2 cpm/cell. The cells were then washed 3 times in phosphate buffered saline (PBS) supplemented with 5% FCS, and resuspended to a concentration of  $2 \times 10^4$  cells/ml in Roswell Park Memorial Institute solution (RPMI) 1640 + 20% FCS.

**Cytotoxicity assay.** 0.1 ml of effector cells ( $2 \times 10^6$  cells/ml) (unfractionated, T or non-T lymphocytes) were mixed with 0.1 ml of target cells ( $2 \times 10^4$  cells/ml) in V-bottom microtitre plates (Costar, Cambridge, Mass), centrifuged at 50 g and 20°C for 5 min, and incubated at 37°C in 5%  $\text{CO}_2$  and 100% humidity. After incubation for 4 h the supernatants were collected by a microplate technique<sup>16</sup> and the radioactivity was counted in a gamma counter. The cytotoxicity index (CI) was calculated according to the formula

$$\text{CI} (\%) = \frac{C_{\text{exp}} - C_{\text{background}}}{C_{\text{total}} - C_{\text{background}}} \times 100$$

where  $C_{\text{exp}}$  and  $C_{\text{background}}$  denote counts released

into the supernatants in the presence or absence of effector cells respectively, and  $C_{\text{total}}$  denotes counts in 0.1 ml of  $^{51}\text{Cr}$  labelled target cells.

**Depletion of Fc $\gamma$  receptor bearing cells and blocking of Fc $\gamma$  receptors.** Detection and removal of Fc $\gamma$  receptor-bearing cells was performed with human O,Rh+ erythrocytes sensitised with anti-Rh Ripley antibodies.<sup>15-17</sup> Furthermore, heat-aggregated (63°C, 15 min) pooled human IgG was added directly to the wells containing effector and target cells at a final concentration of 0.05, 0.5 and 5 mg/ml, and the NK activity was observed.

**Treatment of normal lymphocytes with sera from JRA patients.** Sera from 4 JRA patients with the lowest peripheral blood NK cell activity were tested for the occurrence of lymphocytotoxic antibodies (anti-NK-cell antibodies).  $2 \times 10^6$  normal peripheral blood lymphocytes were resuspended in 0.1 ml of heat-inactivated JRA patient serum, and 0.3 ml of human AB serum was added as complement source.<sup>18</sup> The mixtures were incubated at 15°C for 30 min. The treated cells were then washed 3 times in complete medium, and afterwards adjusted to  $2 \times 10^6$  viable cells/ml, and used as effector cells in the NK cytotoxicity assay.

**Determination of interferon in patient sera.** Interferon (IFN) titres were assayed by an infectivity inhibition microtest in human embryonal fibroblast cells with vesicular stomatitis virus as challenge virus according to methods described elsewhere.<sup>19</sup>

**Statistics.** The results are given as mean values  $\pm$  standard error of the mean (SEM). The significance of the observed differences were calculated by using the Mann-Whitney U test. Probability values less than 0.05 2-sided were considered significant, whereas  $0.05 < p < 0.1$  was considered as being of low significance.

## Results

**NK cell activity in peripheral blood, synovial fluid, and synovial tissue lymphocytes from patients with RA and JRA.** The mean cytotoxic indices of peripheral blood, synovial fluid, and synovial tissue lymphocytes of JRA patients are given in table 1. Reduced NK activity was observed in T lymphocytes from both peripheral blood and synovial fluid as well as in non-T lymphocytes from peripheral blood. Non-T lymphocytes from synovial fluid of JRA patients, however, showed an increased activity compared with that of the corresponding fraction in JRA peripheral blood ( $26.2 \pm 5.3\%$  compared with  $10.6 \pm 3.0\%$  ( $p < 0.05$ )) and also compared with normal peripheral blood non-T lymphocytes.

The cytotoxic indices of peripheral blood and synovial tissue lymphocytes of the RA patients are

Table 1 NK cell activity (CI in percent) of peripheral blood, synovial fluid, and synovial tissue lymphocytes from patients with JRA

Lymphocytes	JRA patients	Controls
<i>Peripheral blood</i>		
Unfractionated	24.3±3.2† n=13*	31.8±4.0 n=10
T	5.2±0.8§ n=9	10.4±1.9 n=8
Non-T	10.6±3.0§ n=9	18.1±2.3 n=8
<i>Synovial fluid</i>		
Unfractionated	21.8±3.6‡ n=10	—
T	6.4±1.5‡ n=7	—
Non-T	26.2±5.3† n=6	—
<i>Synovial tissue</i>		
Unfractionated	16.2±6.7‡ n=3	—
T	20.0 n=1	—
Non-T	26.4 n=1	—

\*Number of experiments. Levels of significance: †p>0.1. ‡0.05<p<0.1, §p<0.05 when compared with the corresponding control cell fractions. Normal peripheral blood lymphocytes were used as controls. CI=cytotoxicity index.

given in Table 2. Similar NK cell activity was seen in T and in non-T lymphocytes from peripheral blood. In one of the RA patients a dissociation in NK cell activity between peripheral blood and synovial tissue non-T lymphocytes was seen (7.3% versus 15.9%). Both unfractionated T and non-T lymphocytes from the synovial tissue of this RA patient exerted much lower activities than the corresponding fractions of the JRA patients (12.4%, 7.9%, and 15.9% versus 29.5%, 20.0%, and 26.4% respectively).

**NK cell activity related to sex, treatment, and clinical parameters.** Of the 13 JRA patients the 10 girls had a mean peripheral blood NK activity of 20.5±3.1% compared with a mean of 37.1±3.8% of the 3 boys (p<0.05). No correlation was found between peripheral blood NK cell activity and disease activity, the use of antirheumatic drugs, immunoglobulin levels, or the occurrence of autoantibodies in the RA

Table 2 NK cell activity (CI in percent) of peripheral blood and synovial tissue lymphocytes from patients with RA

Lymphocytes	RA patients	Controls
<i>Peripheral blood</i>		
Unfractionated	32.6±5.3† n=8*	35.0±5.5 n=8
T	10.5±2.3 n=6	8.1±1.9 n=6
Non-T	14.1±2.1 n=5	18.1±4.7 n=5
<i>Synovial tissue</i>		
Unfractionated	23.8±5.6‡ n=7	—
T	7.9 n=1	—
Non-T	15.9 n=1	—

For symbols see Table 1.

and JRA patient sera. Neither was there any association between NK cell activity and the lymphocyte count in the synovial fluid.

**The role of Fcγ receptor bearing cells from peripheral blood and synovial fluid as effector cells in the NK cytotoxicity assay.** The percentages of Tγ cells in peripheral blood, synovial fluid, and synovial tissue lymphocytes of the RA and JRA patients are given in Table 3. In 4 experiments Fcγ receptor-bearing cells were removed by erythrocyte-antibody rosette-forming cells (EA-RFC) depletion from peripheral blood and synovial fluid T lymphocytes and from peripheral blood non-T lymphocytes. After depletion of Tγ cells normal peripheral blood T cell cytotoxicity was reduced by 69.3±6.4% (Table 4). JRA synovial fluid T cell cytotoxicity was reduced by 26.5±10.2%. The cytotoxicity of normal peripheral blood non-T lymphocytes was reduced by 73.4±5.4% following depletion of EA-RFC.

To elucidate whether NK cell activity is dependent on intact (unblocked) Fcγ receptors on the effector cells or not aggregated IgG was added to the assay in varying concentrations. Inhibition was observed of all cell fractions tested from both peripheral blood and synovial fluid, and a significant inhibition was observed with IgG concentrations between 0.05 and 0.5 mg/ml (data not shown).

Table 3 Percentage Tγ cells in peripheral blood, synovial fluid, and synovial tissue lymphocytes of patients with RA and JRA

Lymphocytes from	RA patients	JRA patients	Controls
Peripheral blood	13.1±2.0 n=8*	8.4±1.2 n=13	13.6±1.3 n=30
Synovial fluid	NT	11.6±1.5 n=7	NT
Synovial tissue	15.5±3.9 n=4	8.0±7.1 n=2	NT

\*Number of experiments. NT=not tested owing to lack of material.

Table 4 NK cell activity (CI in percent) in normal peripheral blood and JRA synovial fluid lymphocytes before and after depletion of Fcγ receptor bearing cells (EA-RFC)

Cells tested	CI	CI after depletion of EA-RFC
<i>Normal peripheral blood lymphocytes</i>		
Unfractionated	47.1±6.8	11.4±1.0 (76.2±4.5)*
T	17.7±3.4	5.0±0.7 (69.3±6.4)
Non-T	20.7±4.1	5.9±0.2 (73.4±5.4)
<i>JRA synovial fluid lymphocytes</i>		
Unfractionated	23.8±8.7	6.7±1.6 (71.8±5.1)
T	8.7±0.8	6.4±1.2 (26.5±10.2)

\*Per cent reduction in NK cell activity.

The numbers represent mean%±SEM of 4 separate experiments. Due to the low number of non-T lymphocytes in synovial fluid mononuclear cells, EA-RFC depletion from these cell fractions could not be performed. After depletion less than 0.5% rosette forming cells (Tγ cells) was left in the T cell fractions and less than 3.0% in the non-T cells.

NK cell activity of normal peripheral blood lymphocytes treated with JRA patient's sera and complement. Normal peripheral blood lymphocytes were treated with sera from the 4 JRA patients with lowest NK cell activities (mean CI of these 4 patients was  $12.5 \pm 1.8\%$ ). No alteration in NK cell activity or in cell viability was observed after the treatment (data not shown).

*Relation between NK cell activity and serum interferon (IFN) levels.* Total IFN levels were determined in the sera of the 8 RA and of 8 JRA patients. No significant differences were observed between the RA and the JRA patient groups (mean titres of  $12.0 \pm 1.1$  and  $11.3 \pm 0.6$  in the RA and the JRA group respectively) nor between the patient groups and the controls. Significant IFN titres were detected in 3 RA and 4 JRA patients, and further specificity testing indicated that the activity was due to immune ( $\gamma$ ) IFN. No association between high titres of immune ( $\gamma$ ) IFN and high NK cell activity was seen, since high IFN levels were observed in RA and JRA patients with both high and low NK cell activity.

## Discussion

In this study we found reduced NK cell activity in peripheral blood, synovial fluid, and synovial tissue lymphocytes of JRA patients. In contrast only a slightly reduced spontaneous cytotoxicity was seen in the peripheral blood of RA patients, whereas RA synovial tissue lymphocytes also showed reduced cytotoxicity. Thus a local reduction in NK activity compared to peripheral blood lymphocytes from healthy individuals ( $p$  values usually between 0.05 and 0.1) seems to be a constant feature of both RA and JRA patients. However, there was a large individual variation of NK activity in the JRA and RA lymphocyte experiments from the various compartments. The reduced cytotoxicity could be due to blocking of NK cell Fc $\gamma$  receptors by immune complexes.<sup>20, 21</sup> The reduction could also reflect low interferon concentrations, as NK cells are activated by this substance.<sup>5</sup> However, no association between serum interferon titres and peripheral blood NK cell activity was observed either in the patient groups or in individual patients.

It is currently believed that NK cells play an important role in preventing the development of cancer.<sup>2, 3</sup> They may also participate in the defence against viral infections.<sup>1</sup> Thus decreased NK cell activity has been observed in patients with certain malignant and pre-malignant diseases<sup>22</sup> as well as in a few other disease states.<sup>4, 18</sup> There is to our knowledge no increased frequency of malignant diseases among RA and JRA patients, but these patients frequently have antiviral antibodies in their sera and also antiviral antibody-

producing cells in the synovial tissue.<sup>23</sup> Local impairment of cellular immunity to viral antigens has also been demonstrated in these groups of patients.<sup>24, 25</sup> The reduced NK cell activity now observed could permit virus to persist and replicate in the organism, for example, locally in the synovial tissue, thus leading to prolonged stimulation of the immune system. Chronic viral infections might therefore participate in the pathogenesis of these diseases.

The NK cell activity and the percentage T $\gamma$  cells were both reduced in the peripheral blood, synovial fluid, and synovial tissue lymphocytes of the JRA patients, whereas both were normal in the peripheral blood of the RA patients. However, in synovial tissue of RA patients the percentage T $\gamma$  cells was increased, whereas the NK cell activity was reduced. This discrepancy between membrane markers and cell function may in part be explained by a local expansion of Fc $\gamma$  receptor-bearing cells without NK activity, for example, due to local activation. Proliferation of Fc $\gamma$  receptor-bearing cells is also a constant feature after stimulation *in vitro*.<sup>26</sup> The high number of HLA DR positive T lymphocytes,<sup>27</sup> the high spontaneous uptake of <sup>3</sup>H-thymidine,<sup>28</sup> together with the high percentage of plasma cells in synovial tissue<sup>29</sup> also strongly indicate local activation.

Evidence of lymphocytotoxic antibodies with specificity for NK cells was not obtained either in the JRA or in the RA patient sera. Antilymphocytic antibodies have been demonstrated in JRA patients with suppressor cell defects.<sup>29</sup> Neither were other factors with influence on NK cell activity detected in the patient sera.

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