

Natural killer cell activity in Sjögren's syndrome and systemic lupus erythematosus: stimulation with interferons and interleukin-2 and correlation with immune complexes

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Abstract

Natural killer (NK) cell activity and its stimulation by interferons (IFNs) and interleukin-2 (IL-2) are diminished in Sjögren's syndrome and systemic lupus erythematosus (SLE). Serum samples of these patients often contain circulating immune complexes, which may influence NK cell activity. Sixteen patients with Sjögren's syndrome (14/16 immune complex positive), 14 with SLE (9/14 immune complex positive), and 11 controls (immune complex negative) were studied. Mononuclear cells collected from a Percoll gradient were preincubated with recombinant IFN- α (rIFN- α) (100 U/ml), rIFN- γ (1000 U/ml), rIL-2 (100 U/ml), or without cytokine. Natural killer cell activity was determined by incubating the mononuclear cells with carboxyfluorescein labelled K562 cells, and the percentage decrease of fluorescence was measured on a FACS Analyzer. In patients with Sjögren's syndrome and SLE NK cell activity and the numbers of cells expressing the NK cell associated antigens CD16 and Leu7 were diminished compared with the controls. Interleukin-2 stimulated NK cell activity significantly in comparison with the non-stimulated value in all studied groups, whereas IFN- γ only stimulated NK cell activity in both patient groups and IFN- α only in patients with Sjögren's syndrome. There was no correlation between NK cell activity, with or without stimulation, and the immune complex concentrations. It is concluded that NK cell activity is decreased in Sjögren's syndrome and SLE and that it may be partially restored by IL-2 and IFN- γ in both diseases, and by IFN- α in Sjögren's syndrome. The decrease of NK cell activity did not correlate with immune complex concentrations; on the other hand, decreased numbers of NK cells (CD16+ or Leu7+) and of cytokine concentrations might be important in the impaired NK cell activity in both diseases.

Natural killer (NK) cell activity is decreased in some autoimmune diseases, including Sjögren's syndrome and systemic lupus erythematosus (SLE).¹⁻³ Normal donor NK cell activity can be inhibited by circulating immune complexes of patients with SLE,⁴ and can be stimulated by interferons (IFNs) and interleukin-2 (IL-2).^{5,6} In Sjögren's syndrome and SLE, however, stimulation by IFNs and IL-2 may be disturbed, though reports are controversial.⁷⁻¹³

In this study NK cell activity was measured flow cytometrically with fluorescein labelled K562 cells

as targets.^{14,15} We investigated the NK cell activity of patients with Sjögren's syndrome or SLE, their numbers of Leu7+ and CD16+ cells, and the influence of IFN- α , IFN- γ , and IL-2 on NK cell activity in both diseases. We also studied the correlation between the presence of circulating immune complexes in the serum and the degree of NK cell activity, with and without stimulation.

Patients and methods

PATIENTS AND CONTROLS

Blood samples were collected from 16 patients with Sjögren's syndrome (one male, 15 female) and 14 with SLE (two male, 12 female), fulfilling respectively the criteria of Fox *et al*¹⁶ and of the American Rheumatism Association.¹⁷ Ten patients received no drugs at the time of the test, six were taking non-steroidal anti-inflammatory drugs, and 18 one or more of D-penicillamine, hydroxychloroquine, methylprednisolone, cyclophosphamide, and azathioprine. Eleven subjects without disease (three male, eight female) served as controls for NK cell activity and the immune complex assays.

MONONUCLEAR CELL ISOLATION

Buffy coat was collected from heparinised blood samples after centrifugation and washed in Hanks's balanced salt solution without Mg⁺⁺, Ca⁺⁺, and phenol red (HBSS-CM, pH 7.4; Gibco Ltd, Paisley, UK). The mononuclear cells were collected from a discontinuous density gradient (Percoll, Pharmacia LKB, Uppsala, Sweden), washed twice in culture medium (RPMI 1640 with 25 mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and L-glutamine (Gibco) containing 50 μ g/ml gentamicin (Gibco) and 30 U/ml nystatin (Gibco)) supplemented with 10% fetal calf serum (Gibco), and concentrated to 12.5×10^6 cells/ml in this medium.

MONONUCLEAR CELL PREINCUBATION

The cell suspensions were incubated overnight at 37°C in 5% CO₂ in air at 100% humidity with recombinant IFN- α (rIFN- α) (100 U/ml; Produits Roche), rIFN- γ (1000 U/ml; Boehringer, Mannheim, FRG; Janssen Biochimica, Beerse, Belgium), rIL-2 (100 U/ml; Janssen Biochimica), or without cytokine (non-stimulated value). The concentrations of the cytokines were chosen after preliminary experiments indicating that at 100 U/ml IFN- α or IL-2 and 1000 U/ml IFN- γ stimulation reached a maximal level.

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TARGET CELL LABELLING

The K562 cell line¹⁸ was maintained in culture medium supplemented with 0.75% Nutridoma-HU (100 times; Boehringer), 2.5% fetal calf serum (Gibco), and 1 mM sodium pyruvate (Gibco) at 37°C in 5% CO₂ in air at 100% humidity. After one to three days of culture cells were taken for the NK assay, and fresh medium was added. The targets were labelled as described earlier.¹⁴ Briefly, they were washed twice in HBSS-CM, labelled with 150 µg/ml 5(6)-carboxyfluorescein diacetate (Sigma, St Louis, USA) for 15 minutes at 37°C, again washed twice in HBSS-CM, and concentrated to 0.5 × 10⁶ cells/ml in Hanks's balanced salt solution with Mg⁺⁺ and Ca⁺⁺, without phenol red (HBSS+CM, pH 7.4; Gibco). The percentage of labelled cells was measured on an FACS Analyzer (Becton Dickinson, Sunnyvale, USA).

NATURAL KILLER CELL ASSAY

After preincubation the cells were washed in HBSS+CM and resuspended to 12.5 × 10⁶ cells/ml in HBSS+CM containing 10% fetal calf serum. Mononuclear cell suspension (100 µl), 100 µl of target cell suspension, and a mixture of 100 µl of both suspensions (test, ratio 25:1) were incubated for three hours at 37°C. The separately incubated suspensions were mixed just before measuring and used as a blank. After dilution of test and blank in 1 ml HBSS+CM, NK cell activity was determined flow cytometrically on an FACS Analyzer and expressed as the percentage decrease of fluorescent cells in the test suspension as compared with the blank.¹⁵

IMMUNE COMPLEX PRECIPITATION AND DETERMINATION

Immune complexes were precipitated with polyethyleneglycol 6000 (Merck, Darmstadt, FRG).¹⁹ Immune complexes containing IgG were determined by a radioimmunoassay using radioactively labelled protein A (Amersham, UK).¹⁹ IgA, IgM, and IgE immune complex assays were carried out by an enzyme linked immunosorbent assay (ELISA).²⁰ Briefly, microtitre plates (Costar, Cambridge, USA) were coated with goat anti-human IgA (Tago, Burlingame, USA) or rabbit antihuman IgM or IgE (Dako, Glostrup, Denmark). Polyethyleneglycol precipitates diluted 1/500 (IgA and IgM) or 1/1 (IgE) were added to the wells and incubated overnight at 4°C. IgA and IgM were detected with peroxidase conjugated F(ab')₂ goat antihuman IgA and IgM (Tago) respectively, IgE with biotinylated mouse monoclonal antihuman IgE (Diagnostics Pasteur, Marnes, France) and peroxidase labelled streptavidin (Amersham). IgA and IgM immune complex assays were calibrated with N-standard standard serum (Behringwerke AG, Marburg, FRG), immune complexes containing IgE with an IgE reference preparation (Behringwerke AG).

DETERMINATION OF THE NUMBERS OF CD16+ AND Leu7+ CELLS

Mononuclear cells were collected on a Ficoll-Paque (Pharmacia) gradient and concentrated to 10⁷ cells/ml in phosphate buffered saline containing 1% bovine serum albumin. Cell suspension (100 µl) was incubated with 20 µl of anti-Leu11b (CD16) or anti-Leu7 (Becton Dickinson) for 30

minutes at room temperature. After washing, the cells were incubated with 50 µl of goat F(ab)₂ antimouse IgM-fluorescein isothiocyanate (1/20, Tago) for 30 minutes at room temperature, washed, and resuspended in 1 ml phosphate buffered saline-bovine serum albumin. Cells were measured on an FACS Analyzer and expressed as the percentage of positive lymphocytes.

STATISTICS

Results were analysed by the Mann-Whitney U test, Wilcoxon matched pairs signed ranks test, and by calculation of the Spearman rank correlation coefficient where appropriate; a p value <0.05 was considered significant. Stimulation was considered significant if the percentage NK cell activity was increased by 17% as compared with the non-stimulated percentage. This cut off value was calculated after normalising the curve by an arcsine transformation.²¹ Total immune complex score was defined as the sum of the immune complex values of the different isotypes divided by their respective cut off values. As this score estimates the total concentration of immune complexes, taking into account a weighted value of each isotype, it was used to determine the relation between NK cell activity and immune complexes.

Results

In patients with Sjögren's syndrome or SLE NK cell activity was less than in the controls (mean values (SD): 43 (20)%, 39 (23)%, and 78 (19)% respectively; Sjögren's syndrome *v* controls *p*=0.0009, SLE *v* controls *p*=0.0004; Mann-Whitney U test).

The percentage of Leu7+ cells was only diminished in SLE (SLE *v* controls *p*=0.004; Mann-Whitney U test); the percentages of CD16+ cells were normal in both diseases. The absolute numbers of lymphocytes, CD16+, and Leu7+ cells were significantly lower in patients with Sjögren's syndrome and in those with SLE than in the controls (Sjögren's syndrome *v* controls: CD16+ *p*=0.006, Leu7+ *p*=0.02; SLE *v* controls: CD16+ *p*=0.02, Leu7+ *p*=0.03); the number of lymphocytes in patients with Sjögren's syndrome was lower than in those with SLE (*p*=0.02; Mann-Whitney U test) (fig 1).

Natural killer cell activity was stimulated significantly with IL-2 in 3/11 controls, 9/15 patients with Sjögren's syndrome, and 7/14 patients with SLE (controls *p*=0.02, Sjögren's syndrome *p*=0.002, SLE *p*=0.001; Wilcoxon test). Interferon gamma stimulated NK cell activity significantly in 0/11 controls, 5/14 patients with Sjögren's syndrome, and 2/14 patients with SLE (Sjögren's syndrome *p*=0.02, SLE *p*=0.01; Wilcoxon test). Interferon alfa stimulated NK cell activity in 0/11 controls, 3/15 patients with Sjögren's syndrome (*p*=0.02; Wilcoxon test), and 0/14 patients with SLE. Interleukin-2 stimulated NK cell activity more than both IFNs (fig 2), but it did not reach the non-stimulated control group values.

Fourteen of 16 serum samples from patients with Sjögren's syndrome and 9/14 from those with SLE contained immune complexes, mostly complexes containing IgG and IgM; the control sera contained no immune complexes (fig 3). The immune complex concentrations of the different isotypes and the total immune complex score did not correlate with NK cell activity, with or without stimulation (Spearman rank test).

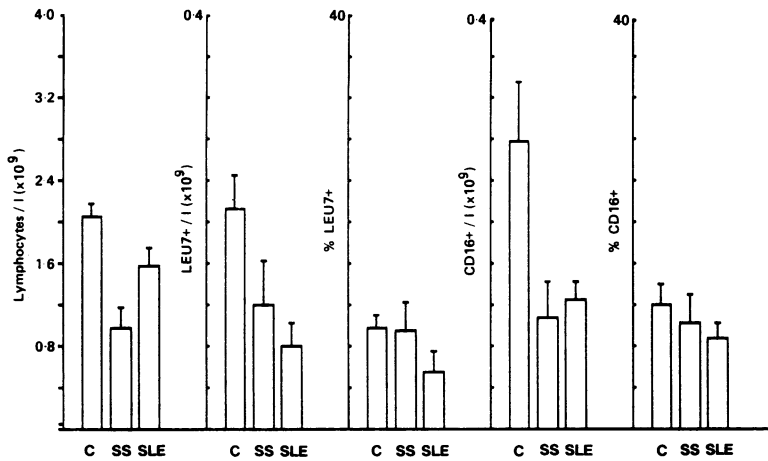


Figure 1 Absolute numbers of lymphocytes/l and absolute numbers/l and percentages of Leu7+ and CD16+ cells in controls (C, n=26), patients with Sjögren's syndrome (SS, n=12), and patients with systemic lupus erythematosus (SLE, n=12). Mean (SE).

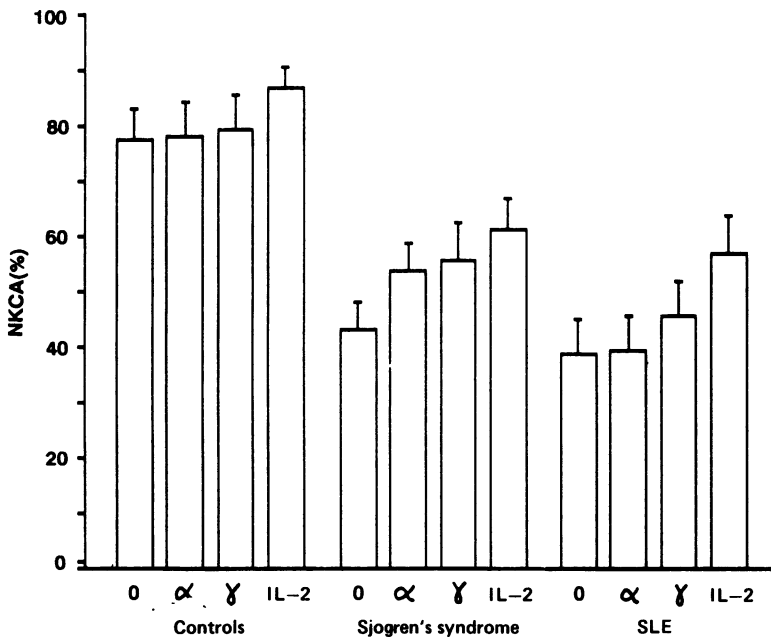
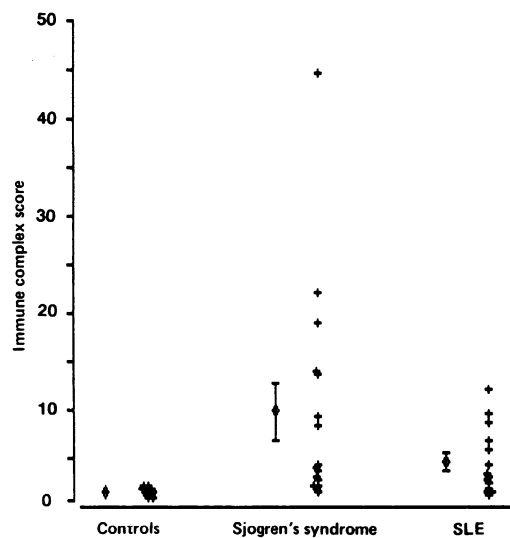


Figure 2 Natural killer cell activity (NKCA; mean (SE)) in controls (n=11), patients with Sjögren's syndrome (n=16), and patients with systemic lupus erythematosus (SLE) (n=14) without stimulation (0) and after stimulation with interferon alpha (α), interferon gamma (γ), and interleukin-2 (IL-2).

Figure 3 Total immune complex score and mean (SE) in controls, patients with Sjögren's syndrome, and patients with systemic lupus erythematosus.



Immune suppressive treatment only suppressed IFN- γ stimulated NK cell activity in patients with SLE ($p=0.03$, Mann-Whitney U test); it had no significant effect on NK cell activity with or without stimulation in patients with Sjögren's syndrome, nor on IL-2 stimulated, IFN- α stimulated, and non-stimulated NK cell activity in SLE. In this study the numbers of lymphocytes, CD16+, and Leu7+ cells were not influenced by immune suppressive drugs.

Discussion

In this study we compared the influence of IFNs, IL-2, and immune complexes on NK cell activity in Sjögren's syndrome and SLE. Interferon gamma and IL-2 stimulated NK cell activity in both diseases, whereas IFN- α stimulated NK cell activity only in Sjögren's syndrome.

Recombinant cytokines were used to avoid effects due to other possible modulating factors which might have been present in other biological preparations.

We chose to determine NK cell activity flow cytometrically with fluorescein labelled K562 cells as targets.^{14 15} This test has several advantages over the conventional chromium release assay: its costs, duration, and percentage of spontaneous release are lower, it allows direct target cell measurement, and the use of radioactivity is avoided.

Depression of NK cell activity in SLE^{2 3} may be due to several factors, including a decreased number of active NK cells¹³ and an abnormal NK cell function caused by intrinsic cell defects or serum factors.²² Diminished NK cell activity with a normal or reduced number of cells,^{1 13 23} as well as normal NK cell activity with a reduced number of NK cells^{8 24} have been reported in Sjögren's syndrome. We showed an almost equal decrease of NK cell activity and absolute numbers of CD16+ and Leu7+ cells in Sjögren's syndrome and SLE as compared with controls. The percentage of Leu7+ cells was decreased in SLE, however, indicating a selective deficiency of these cells in SLE, whereas the diminished Leu7+ cells in Sjögren's syndrome, and CD16+ cells in both diseases might be explained by the decreased total number of lymphocytes.

It has been shown that in normal subjects NK cell activity can be stimulated by IFN- α , IFN- γ , and IL-2.^{5 6} We found that NK cell activity in controls was stimulated only by IL-2, probably owing to the fact that basal NK cell activity is high already and a significant increase cannot be measured as it would exceed 100%. In SLE and Sjögren's syndrome NK cell activity was significantly stimulated with IL-2 and with IFN- γ . Interferon alpha weakly increased NK cell activity in Sjögren's syndrome, but no response to this cytokine could be seen in SLE. These results suggest that deficiency of IFN- γ and IL-2 in SLE and Sjögren's syndrome and of IFN- α in Sjögren's syndrome might contribute to the diminished NK cell activity in these autoimmune diseases. Some reported experimental data favour this hypothesis,^{8 22 23 25-28} others do not.²⁹⁻³¹ The differences between NK cell responses to IFN- α in Sjögren's syndrome and SLE might be explained by increased concentrations of IFN- α in SLE³¹ possibly causing priming, down regulation of the receptor, NK cytotoxic factor depletion, or activation of suppressor cells.³²

In our experiments none of the cytokines

studied could restore NK cell activity to normal, suggesting that factors other than deficiency might also be important: a decreased number of active NK cells,^{13 22 24 31 33} impaired induction of high affinity IL-2 receptors,³⁴ an abnormality in post-receptor signalling,³⁵ or the presence of antibodies to lymphocytes.^{1 36}

Other inhibitory factors of NK cell activity might be circulating immune complexes,⁴ which are indeed often found in the serum of patients with Sjögren's syndrome or SLE. In our experiments, however, we could not show a significant correlation between the levels of immune complexes and the decrease of NK cell activity or the impairment of stimulation by IFN and IL-2.

Yet, we have to consider that 4/16 patients with Sjögren's syndrome and 8/14 with SLE received immune suppressive drugs at the time of the test. This, however, did not influence their total number of lymphocytes, CD16+ cells, Leu7+ cells, NK cell activity nor its stimulation with IL-2 or IFN- α . Only in patients with SLE treated with corticosteroids or cyclophosphamide was NK cell activity stimulated by IFN- γ diminished as compared with the other patients with SLE.

In conclusion, we found diminished NK cell activity in Sjögren's syndrome and SLE, which could be stimulated with IFN- γ and IL-2 in both diseases and with IFN- α in Sjögren's syndrome, but which could not completely be restored. This suggests that diminished production of these cytokines might contribute to the decreased NK cell activity, but other causes should also be considered, one of which might be the decreased number of effector cells. The presence of immune complexes does not seem to inhibit NK cell activity in these diseases.

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