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Interleukin 2 augmentation of the defective natural killer cell activity in patients with primary Sjögren's syndrome

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SUMMARY

Natural Killer (NK) cell activity against K562 target cells was measured in 21 female patients with primary Sjögren's syndrome (primary SS) and in 20 female normal controls matched for age. The *in vitro* effect of α-interferon (IF) and interleukin 2 (IL-2) on NK cell activity was examined and the percentage of large granular lymphocytes (LGL) in blood was measured.

Median baseline NK cell activity in primary SS was 15·4% versus 24·4% in the controls ($P < 0.05$). Median IF-enhanced NK cell activity in the SS group was 35·5% versus 49·6% in the controls ($P < 0.02$). IL-2-enhanced NK cell activity was 35·5% versus 37·6% in the controls (n.s.) The proportion of LGL did not differ in the two groups. Median LGL/lymphocytes was 4·0% in the primary SS patients versus 4·5% in the controls (n.s.).

We conclude that the defective NK cell activity in patients with primary SS is functional, as the number of LGL is normal. Further the NK cell activity off SS was restored by IL-2.

Keywords Natural Killer cells interleukin 2 Sjögren's syndrome

INTRODUCTION

Natural Killer (NK) cells are functionally identified by their ability to lyse a variety of target cells. NK cell activity is closely associated with and probably identical with a subpopulation of lymphocytes, morphologically characterized as large granular lymphocytes (LGL) (Orthaldo & Herberman, 1984).

In addition to mediate NK cell activity, LGL in response to various stimuli release a variety of cytokines including interferon (IF) (Kasahara *et al.*, 1983) and interleukin 2 (IL-2) (Kasahara *et al.*, 1983). The ability of LGL to mediate NK cell activity and antibody dependent cell cytolysis is conditioned by an intact secretory system (Carpen, Virtanen & Saksela, 1981; Pedersen, Norrild & Krebs 1982). The regulation of NK cells is poorly understood, but the fact that LGL rapidly produce IF and IL-2 following stimulation, and that NK cell activity further is augmented by these cytokines provides a mechanism for positive self regulation.

NK cells seem to be of importance in immune surveillance against cancer and microbial infections, and besides evidence for a role of NK cells in autoimmune diseases is forthcoming (Orthaldo *et al.*, 1984; Pedersen 1985). Significantly reduced NK cell activity has been found in patients with Sjögren's syndrome (SS), (Goto, Tanimoto & Horuichi, 1980; Miyasaka *et al.*, 1983;

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Nagahiro *et al.*, 1982) and systemic lupus erythematosus (SLE) (Strannegård, Hermodsson & Westberg, 1982).

SS is a chronic inflammatory connective tissue disease characterized by dryness of mucosal membranes and histologically by lymphoid cell infiltration of exocrine glands, especially salivary and lacrimal glands (Manthorpe *et al.*, 1981). Primary SS is defined by the presence of keratoconjunctivitis sicca and xerostomia in the absence of other chronic inflammatory connective tissue diseases. Frequently extraglandular manifestations, including hypergammaglobulinemia with various kinds of autoantibodies and development of generalized lymphoproliferation ranging from benign, transient lymph node swelling to malignant non-Hodgkin's lymphomas are seen. The relative risk of developing a lymphoproliferative malignancy has been estimated to be 44 (Manthorpe *et al.*, 1981). Whether the low NK cell activity in SS is related to the high incidence of lymphoid neoplasms is however not known.

The present investigation was undertaken in a large group of patients with well-characterized primary SS in order to examine the quality and quantity of NK cells, given that patients with primary SS possess low NK cell activity (Goto *et al.*, 1980; Miyasaka *et al.*, 1983; Nagahiro *et al.* 1982). The study was designed to answer the following questions:

(a) How does IF and IL-2 influence NK cell activity in patients with SS compared to normal controls?

(b) Does the number of LGL in blood differ between the two groups?

MATERIALS AND METHODS

Patients and controls. The patient group consisted of 21 female patients with primary SS. Mean age was 54·3 years (range 36–78). Mean disease duration was 12 years (range 4–13). Seventeen of the patients had extraglandular manifestations, including non-erosive arthritis, myalgia, lymphoma, Raynaud's syndrome, pleuritis sicca, peritonitis sicca, pancreatitis, purpura hypergammaglobulinemica Waldenström and recurrent fever. None of the patients were treated with glucocorticosteroids or any other immunosuppressive drugs. Five patients were treated with bromhexine and one received thiazide. Neither patients nor controls had received any kind of non-steroidal anti-inflammatory drugs 1 week before they were examined for NK cell activity.

Twenty healthy female individuals matched for age served as normal controls. Mean age was 48·7 years (range 28–84).

Definition. SS was diagnosed by objective measurements according to previously established criteria (Manthorpe *et al.*, 1981). Keratoconjunctivitis sicca was defined by the presence of at least two abnormal tests among the following three: (a) Schirmer-I test, (b) break-up time and (c) van Bijsterveld score (semi-quantitative Rose-Bengale test). Xerostomia was defined by the presence of at least two abnormal tests among the following three: (a) lower lip biopsy, (b) unstimulated sialometry and (c) salivary gland scintigraphy.

Bloodsampling. All bloodsamples were collected between 8 and 9 a.m.. Heparine (125 iu/ml) was used as anticoagulants. NK activity was determined immediately after blood sampling.

Determination of NK cell activity. (a) *Effector cells.* Mononuclear cells were isolated by Ficoll Isopaque Gradient centrifugation. The cells were washed three times with Hanks' buffered salt solution and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) to final concentrations of 4×10^6, 2×10^6 and 10^6 cells/ml.

(b) *Target cells.* The K 562 cell line (an erythromyeloid cell line derived from pleural effusion of a patient with chronic myelocytic leukaemia in blast crisis) was maintained in continuous suspension culture in RPMI 1640 supplemented with 10% heat-inactivated FCS. To $2–8 \times 10^6$ target cells in 1 ml were added 0·1 mCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA, USA) specific activity 350–600 mCi/mg. The cells were incubated with the isotope for 1 h at 37°C. The labelled cells were washed through a 4 ml cushion of heat-inactivated FCS. The cell pellet was resuspended in RPMI with 10% heat-inactivated FCS to a final concentration of 5×10^4 cells/ml.

(c) *Cytotoxic assay.* NK cell activity was measured at three effector/target (E/T) cell ratios (80/1, 40/1, 20/1). 400 μl of the labelled target cell suspension was mixed with 400 μl of effector cells and

200 μl of RPMI + 10% heat-inactivated FCS. After incubation for 4 h at 37°C the tubes were centrifuged (1,000 *g*, 10 min), 500 μl of each supernatant were transferred to new tubes, and the radioactivity was counted in a Packard scintillation gamma counter. The experimental release (R) was calculated by the formula:

$$R = \frac{^{51}Cr \text{ activity released in the supernatant}}{^{51}Cr \text{ total activity}} \times 100\%$$

The specific release is the difference between experimental release in the presence of effector cells and experimental release when target cells were incubated with medium only.

Incubation of effector cells with IF. Ten microlitres of 10^5 iu/ml of α-IF (Cantell & Hirronen, 1978) were added to 990 μl of 4×10^6 effector cells/ml. IF was kindly provided by Dr Robert Jordal (The Blood Bank, Copenhagen County Hospital, Gentofte, Denmark). The cells were preincubated with IF for 1 h at 37°C and IF was present during the incubation with target cells as well.

Incubation of effector cells with IL-2. Purified IL-2 was purchased from Boehringer (Mannheim, FRG). This IL-2 was obtained from culture supernatants of pooled human T lymphocytes stimulated with PHA and purified by ammonium sulphate fractionation and DEAE column chromatography (Mier & Gallo, 1980). IL-2 was stored in small aliquots at −20°C until use. IL-2 was preincubated with 4×10^6 effector cells/ml for 1 h at 37°C at a final concentration of 10%. IL-2 was present during the incubation with target cells at a concentration of 10% as well. The IL-2 preparation used in our experiments contained 300 iu/ml, measured as described below.

Measurement of IL-2 activity. IL-2 activity was measured by a slight modification of the method as described by Gillis *et al.* (1978). CTLL IL-2 dependent cells were grown at a concentration of 2×10^3 cells/200 μl of RPMI culture medium and in serial two-fold dilutions of test materials all supplemented with 10% FCS. The cultures were incubated in triplicate for 18 h at 37°C, and pulsed with ^{3}H-thymidine for the last 3 h, before measurement of thymidine incoporation. A laboratory standard was always tested in parallel. This standard had previously been compared with an international reference preparation of Jurkat-derived IL-2 containing 50 iu/ml (specific activity $13{\cdot}1 \times 10^6$ iu/mg, BRMP, Frederick, MD, USA).

Enumeration of large granular lymphocytes (LGL). Samples of 250 μl mononuclear cells at a concentration of 7×10^5 cells/ml were cytocentrifuged in an automatic cytospinner (Shandon). The cells were air dried, fixed in methanol 5 min, and stained with May-Grünwald stain, 5 min, and Giesma, 20 min. The cells were examined by oil immersion microscopy (× 1,000). LGL were identified as medium to large-sized lymphocytes with a relatively high ratio of cytoplasm to nucleus, azurophilic cytoplasmic granules and often an indented nucleus. Monocytes were distinguished from LGL by their morphological appearance.

Statistical evaluations. The percentage of ^{51}Cr release was calculated for samples made in triplicate (s.d. = 0·1 − 3·0%). The within-assay determinations gave a coefficient of variation of 2·9%. The day to day determinations on samples from the same donors gave overall coefficient of variation of 10%. The mean percentage of LGL was blindly determined by two independent observers counting at least 200 lymphocytes. The coefficient of variation was 5%. The significance of the observed differences were calculated by using the Mann–Whitney test.

RESULTS

NK cell activity against K 562 target cells was examined at three effector/target cell ratios: 80/1, 40/1 and 20/1. The results obtained at the three E/T ratios did not differ qualitatively. Only the NK cell activity at E/T cell ratio 80/1 is given below.

As is evident from Fig. 1, baseline NK cell activity of the patients was significantly lower than baseline NK cell activity of the controls ($P < 0{\cdot}05$). Median NK cell activity in patients was 15·4% (range 5·8–50·4) versus 24·4% (range 6·2–47·4) in the controls.

In Fig. 2 baseline and interferon enhanced NK cell activity is shown. After exposure to IF median NK cell activity in the patients was 35·5% (range 17·5–55·0) versus 49·6% (range 22·4–65·4)

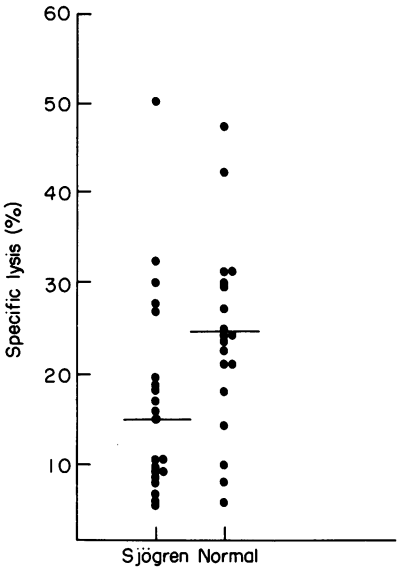

Fig. 1. NK cell activity against K562 target cells was measured in 21 patients with primary Sjögren's syndrome and in 20 controls.

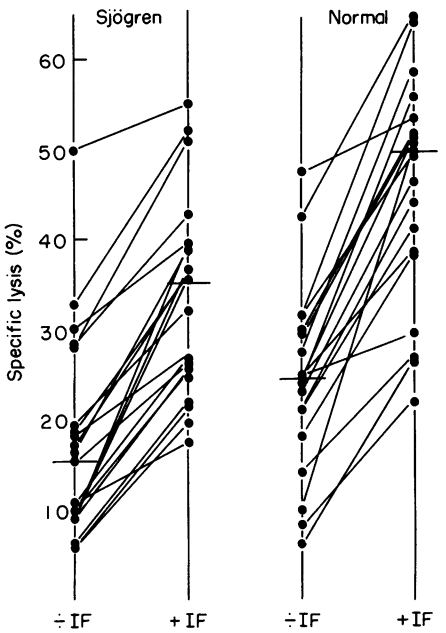

Fig. 2. Baseline and IF-enhanced NK cell activities for patients with primary Sjögren's syndrome ($n=20$) and controls ($n=19$) are shown.

in the controls ($P<0{\cdot}02$). IF-enhanced NK cell activity was significantly higher than baseline NK cell activity in both patients ($P<0{\cdot}01$) and controls ($P<0{\cdot}01$)

IL-2 enhanced the NK cell activity in both patients ($P<0{\cdot}01$) and controls ($P<0{\cdot}01$), Fig. 3. There was no significant difference between NK cell activity after exposure to IL-2 in the two groups. Median NK cell activity after exposure to IL-2 in patients was 35·2% (range 18·8–64·2) versus 37·6% (range 20·7–63·3) in the controls (ns).

Thus baseline and IF-enhanced NK cell activity of primary SS patients was significantly lower

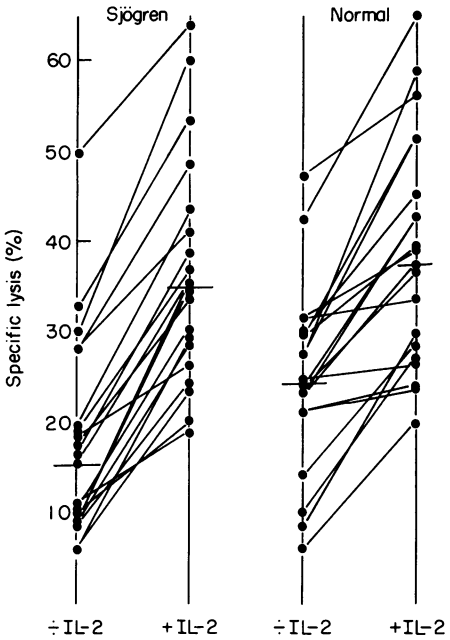

Fig. 3. Baseline and IL-2-enhanced NK cell activities in patients with primary Sjögren's syndrome ($n = 19$) and controls ($n = 20$) are shown.

Table 1. Large granular lymphocytes (LGL) in primary Sjögren's syndrome and controls.

	n	LGL/lymphocytes (%) median (range)
Primary Sjögren's syndrome	21	4·0 (0·5–15·0)
Normal controls	20	4·5 (1·5–8·5)

than in the controls, while there was no significant difference between the IL-2-enhanced NK cell activity of the two groups. IL-2 thus restored the defective NK cell activity of patients with primary SS.

The percentages of LGL among mononuclear cells are shown in Table 1. There was no significant difference between LGL in the two groups. We did not find a significant correlation between the percentage of LGL and NK cell activity.

Neither the presence of extraglandular manifestations, autoantibodies or hypergammaglobulinemia correlated to the NK cell activity or the proportion of LGL. Treatment with bromhexin did not influence NK cell activity. Malignant lymphoma was diagnosed in one of the patients a few weeks after she was tested for NK cell activity. The NK cell activity of this patient was $6{\cdot}2 \pm 0{\cdot}3\%$.

DISCUSSION

The analysis of NK cell activity in patients with autoimmune diseases is difficult as these patients often receive medicaments, e.g. prednisone, azathioprine and cyclophosphamide, known to influence NK cell activity (Berge *et al.*, 1982; Pedersen & Beyer, 1985; Pedersen *et al.*, 1985; 1984a,b). For ethical reasons medication can be withheld only rarely. Patients with primary SS are however seldom treated with these drugs and thus constitute an ideal patient group for the analysis of NK cell activity in relation to autoimmunity.

We studied the NK cell activity of 21 female patients with primary SS and 20 female controls.

Primary SS was diagnosed by reproducible and objective criteria. Baseline NK cell activity was significantly suppressed in the primary SS group.

Goto *et al.* (1980) reported low NK cell activity in seven patients with primary SS and in 18 patients with SS associated with RA or other connective tissue diseases—secondary SS. In eight patients with primary SS and five patients with secondary SS, Nagahiro *et al.* (1982) found normal baseline but defect IF-enhanced NK cell activity. Miyasaka *et al.* (1983) found low NK cell activity in 20 patients with primary SS and in seven patients with secondary SS, while Ichikawa *et al.* (1985) in nine patients with primary SS and 20 patients with secondary SS found normal NK cell activity. The reason why some discrepancy is found between our results and other studies could be due to differences in number of patients with primary SS included. Thus, only Miaysaka *et al.* (1983) studied a sufficiently large number of patients with primary SS, while the other groups in addition included many patients with secondary SS. The discrepancy in results might also be due to differences in diagnostic criteria tests for primary SS and minor differences in the NK cell assays. Further the effect of IL-2 stimulation on NK cell activity has not been investigated before.

In vitro stimulation with IF and IL-2 significantly enhanced the NK cell activity in our patients and controls, but the IF-enhanced NK cell activity was significantly lower in SS. In contrast the IL-2-enhanced NK cell activity did not differ in the two groups. Therefore we conclude that the NK defect of patients with primary SS is functional. In accordance with this concept, the proportion of LGL among blood mononuclear cells in the patient group was normal.

IL-2 which stimulates the proliferation of normal T lymphocytes and the generation of cytotoxic T cells (Farrar *et al.*, 1982; Gillis & Watson, 1978) is produced by antigen- or mitogen-activated T lymphocytes, some T cell lines and T-T hybridoma cell lines (Farrar *et al.*, 1982). In addition it was recently shown that LGL produced IL-2 and on a per cell basis seemed to be even more effective than T lymphocytes in producing lymphokines including IF and IL-2 (Kasahara *et al.*, 1982). LGL also proliferate in response to IL-2 and when LGL were tested for cytotoxic activity in the presence of IL-2, a dose dependent linear increase in the NK cell activity was observed (Domzig, Stadler & Herberman, 1983).

Our results suggest that the defective NK function in patients with primary SS is secondary to an abnormal IL-2 production. Miyasaka *et al.* (1984) described significantly low IL-2 production in 22 patients with SLE and 14 patients with RA, while patients with SS showed a wide range of variation. Their data show however that five of the eight SS patients had lower IL-2 production than any of the 19 controls, while three had very high IL-2 production.

Few studies have focused on the influence of IL-2 on NK cells *in vivo*. In patients with acquired immunodeficiency syndrome (AIDS) a severe defect in cell-mediated immunity is found (Gerstoft *et al.*, 1982). In addition to reversal of the ratio T helper (CD4) to T suppressor (CD8) cells, patients with AIDS have reduced NK cell activity (Gerstoft *et al.*, 1982). Recently it was shown that IL-2 was capable of directly stimulating the NK cell activity of AIDS patients but the absolute level of their NK cell activity after IL-2 incubation was below that detected in healthy heterosexual and healthy homosexual subjects (Reddy, Pinyavay & Grilco, 1984). In patients with advanced malignant solid tumours the NK cell activity was significantly correlated to IL-2 production, but IL-2 boosting of the NK cells was not performed (Rey *et al.*, 1983). IL-2 deficiency occurs also in autoimmune strains of mice (Altman *et al.*, 1981), and it has been demonstrated that mouse NK cells, activated *in vitro* by IL-2, inhibit the growth of established melanoma pulmonary metastases (Mazumder & Rosenberg, 1984). Further more administration of IL-2 to mice augments the NK cell activity (Henney *et al.*, 1981).

The reason why low NK cell activity is found in disorders such as cancer and autoimmune diseases is not fully understood, but it is possible that the observed defective NK cell activity is in part due to a defect in IL-2 production. Since NK cells seem to play an important role in immune surveillance (Pedersen, 1985; Pedersen & Beyer 1985), consideration of the use of IL-2 as a potential therapeutic agent to modify immune responses in disorders such as cancer, premalignant diseases and autoimmune diseases is warranted.

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REFERENCES

ALTMAN, A., THEOFILOPOULOS, DA.N., WEINER, R., KATZ, D.H. & DIXON, F.J. (1981) Analysis of T cell function in autoimmune murine strains. Defects in production of and responsiveness to interleukin 2. *J. exp. Med.* **154,** 791.

BERGE, R.J.M. T, WALBECK, H.K. & SCALLEKENS RH.A. (1982) Evaluation of the immunosuppressive effects of cyclophosphamide in patients with multiple sclerosis. *Clin. exp. Immunol.* **50,** 495.

CANTELL, K. & HIRRONEN, S. (1978) large-scale production of human leucocyte interferon containing 10 units per ml. *Gen. Virol.* **39,** 541.

CARPEN, O., VIRTANEN, I. & SAKSELA, E. (1981) The cytotoxic activity of human natural killer cells requires an intact secretory apparatus. *Cell. Immunol.* **58,** 97.

DOMZIG, W., STADLER, B.M. & HERBERMAN, R.B. (1983) Interleukin 2 dependence of human natural killer (NK) cell activity. *J. Immunol.* **130,** 1970.

FARRAR, J.J., BENJAMIN, W.R., HILFIKER, M.L., HAWARD, M., FARRAR, W.L. & FULLER-FARRAR, J. (1982) The biochemistry, biology and role of interleukin 2 in the induction of cytotoxic T cell and antibody forming B cell responses. *Immunol. Rev.* **63,** 129.

GERSTOFT, J.A., MALCHOW-MØLLER, A., BYGBJERG, I., DICKMEISS, E. & ENK, C. (1982) Severe acquired immunodeficiency in European homosexual men. *Br. Med. J.* **285,** 17.

GILLIS, S., FERM, M.M., OU, W. & SMITH, K.A. (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027.

GILLIS, S. & WATSON, J. (1978) Long term culture of tumour specific cytotoxic T cells. *Nature*, **268,** 154.

GOTO, M., TANIMOTO, K. & HORUICHI, Y. (1980) Natural killer cell-mediated cytotoxicity in Sjögren's syndrome and rheumatoid arthritis. *Arthritis Rheum.* **24,** 1274.

HENNEY, C.S.K., KURIBAYASHI, K., KERN, D.E. & GILLIS, S. (1981) Interleukin 2 augments natural killer cell activity. *Nature*, **291,** 335.

ICHIKAWA, Y., YOSHIDA, M., TAKAYA, M., UCHIYAMA, M., SHIMIZU, H. & ARIMORI, S. (1985) Circulating natural killer cells in Sjögren's syndrome. *Arthritis Rheum.* **28,** 182.

KASAHARA, T., DJEU, J.Y., DOUGHERTY, S.F. & OPPENHEIM, J.J. (1983) Capacity of human large granular lymphocytes (LGL) to produce multiple lymphokines: Interleukin 2, interferon, and colony stimulating factor. *J. Immunol.* **131,** 2379.

MANTHORPE R., FROST-LARSEN K., ISAGER H. & PRAUSE J.U. (1981) Sjögren's syndrome. A review with emphasis on immunological features. *Allergy*, **36,** 139.

MAZUMDER, A. & ROSENBERG, S.A. (1984) Successful immunotherapy of natural killer resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated *in vitro* by interleukin 2. *J. exp. Med.* **139,** 495.

MIER, J.W. & GALLO, R.C. (1980) Purification and some characteristics of human T-cell growth factor from phytohemagglutinin-stimulated lymphocyte conditioned media. *Proc. natn. Acad. Sci.* **77,** 6134.

MIYASAKA, N., NAKOMA, T., RUSSELL, I.J. & TALAL, N. (1984) Interleukin 2 deficiencies in rheumatoid arthritis and systemic lupus erythematosus. *Clin. exp. Immunopathol.* **31,** 109.

MIYASAKA, N., SEAMAN, W., BAKSHI, A., SAUVEZIE, B., STRAND, V., POPE, R. & TALAL, N. (1983) Natural killing activity in Sjögren's syndrome. An analysis of the defective mechanisms. *Arthritis Rheum.* **26,** 954.

NAGAHIRO, J.K., AHIRA, T., SHOGO, K. & FULMANARO, T. (1982) Studies of the function of natural killer-interferon systems in patients with Sjögren's syndrome. *Clin. Invest.* **69,** 581,

ORTHALDO J.R. & HERBERMAN R.B. (1984) Heterogeneity of natural killer cells. *Ann. Rev. Immunol.* **2,** 359.

PEDERSEN, B.K. (1985) Natural Killer cells in relation to disease and treatment. A review article. *Allergy*, In press.

PEDERSEN, B.K. & BEYER, J.M. (1985) A longitudinal study on the influence of azathioprine on natural killer (NK) cell activity. Submitted.

PEDERSEN, B.K., BEYER, J.M., KLARLUND, K. & CLEMMENSEN, I.H. (1985) Baseline and interferon enhanced natural killer cell activity in rheumatoid arthritis. *Acta Path. Microbiol. Immunol. Scand. sect. C*, **93,** 79.

PEDERSEN, B.K., BEYER, J.M., RASMUSSEN, A., KLARLUND, K., PEDERSEN, B.N. & HELIN P. (1984a). Methylprednisolone pulse therapy induced fall in natural killer cell activity in rheumatoid arthritis. *Acta Path. Microbiol. Immunol. Scand. sect. C*, **92,** 319.

PEDERSEN, B.K., BEYER, J.M., RASMUSSEN, A., KLARLUND, K., HØRSLEV-PETERSEN, K., PEDERSEN, B.N. & HELIN, P. (1984b) Azathioprine as single drug in the treatment of rheumatoid arthritis induced complete suppression of natural killer cell activity. *Acta Path. Microbiol. Immunol. Scand. sect. C*, **92,** 221.

PEDERSEN, B.K., NORRID, B. & KREBS, H.J. (1982) The effect of monensin on the antibody dependent cell-mediated killing. *Cell. Immunol.* **72,** 208.

REDDY, M.M., PINYAVAY, N. & GRILCO, M.H. (1984) Interleukin 2 augmentation of natural killer activity in homosexual men with acquired immune deficiency syndrome. *Infect. Immun.* **44,** 339.

REY, A., KLEIN, B., ZAGURY, D., THIERRY, C. & SERROW, B. (1983) Diminished interleukin 2 activity production in cancer patients bearing solid tumors and its relationship with natural killer cells. *Immunology letters*, **6,** 175.

STRANNEGÅRD O., HERMODSSON S. & WESTBERG G. (1982) Interferon and natural killer cells in systemic lupus erythematosus. *Clin. exp. Immunol.* **50,** 246.