

Down-regulation of natural killer cell activity by autologous polymorphonuclear leucocytes

Role of indomethacin

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It has been recently reported that neutrophils are involved in the regulation of NK cell activity. However, the mechanism of such regulation is unclear. The present study was designed to investigate the mechanisms involved in the regulation of NK cytotoxicity by human neutrophils. The role of indomethacin, an anti-inflammatory drug, in this interaction was studied. NK cells were purified from peripheral blood obtained from normal individuals. NK cell cytotoxicity was tested on K 562 cell line by Cr release assay. Autologous neutrophils obtained from peripheral blood were stimulated by opsonized zymosan either in the presence or absence of indomethacin. The role of neutrophil supernatant containing oxygen radicals and prostaglandins on NK cytotoxicity was examined. It was shown that supernatants from stimulated neutrophils significantly inhibited ($P < 0.05$) the autologous NK cell cytotoxicity. The presence of indomethacin in the *in vitro* reaction mixture, or given orally to donors, partially or completely abolished the inhibitory effect of neutrophil supernatant. Indomethacin inhibited prostaglandin E2 release, and luminol-enhanced, myeloperoxidase-mediated chemiluminescence of activated PMN. Diafiltration of neutrophil supernatant showed that the inhibitory activity was present in the fraction containing molecules lower than 5,000 daltons. In conclusion, our findings indicate that down-regulation of NK cytotoxicity is mediated by prostaglandins produced by stimulated neutrophils and possibly by oxygen radicals.

Key words: indomethacin; natural killer cells; neutrophils; prostaglandins.

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Evidence is accumulating that NK cells play an important role in immune surveillance against cancer and certain infections (9, 16). Because of their spontaneous levels of reactivity and/or their ability to be rapidly activated, NK cells together with macrophages and polymorphonuclear (PMN) cells seem well suited to play an important role in the first line of defense.

A gradual disappearance of NK cell activity with growth and development of tumors, has recently been reported. PMN are prominent in early stages of tumor growth (21), and NK cells

have been shown to accumulate in small primary as well as in transplanted tumors (7). It is therefore possible that products released from activated PMN compromise the functional activity of nearby lymphocytes. Recently it has been reported that PMN seem to either stimulate the NK cell cytotoxicity (23) or down-regulate their activity (6, 22). The present study was undertaken in order to examine the influence of PMN on NK cell activity. The role of indomethacin in this interaction was investigated.

MATERIAL AND METHODS

Determination of NK cell activity

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 1×10^6 cells/ml. In some experiments 1.5 ml of 4×10^6 cells/ml were incubated at 37°C on 60 mm plastic Petri dishes (Teknunc A/S, Roskilde, Denmark) for 1 h . The non-adherent cells were centrifuged (1000 g , 10 min) and resuspended in RPMI with 10% FCS to a final concentration of 4×10^6 cells/ml. This suspension contained 0.2% monocytes measured by peroxidase staining.

Target cells. The K 562 cell line, an erythromyeloid cell line derived from pleural effusion of a patient with chronic myelocytic leukemia in blast crisis, was maintained in continuous suspension culture in RPMI 1640 supplemented with 10% heat-inactivated FCS. To $2\text{--}8 \times 10^6$ target cells in 1 ml was added 0.1 mCi of $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear, Boston, Mass. U.S.A.) Specific activity $350\text{--}600 \text{ mCi/mg}$. 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.

Cytotoxic assay. NK cell activity was measured at three different effector/target (E/T) cell ratios (80/1, 40/1, 20/1). $400 \mu\text{l}$ of the labelled target cell suspension was mixed with $400 \mu\text{l}$ of effector cells and $200 \mu\text{l}$ of RPMI + 10% heat-inactivated FCS. After incubation for 4 h at 37°C the tubes were centrifuged (1000 g , 10 min), $500 \mu\text{l}$ of each supernatant was transferred to new tubes, and the radioactivity was counted in a Packard scintillation gamma counter. The experimental release was calculated by the formula:

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

The specific release (the NK cell activity) 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 indomethacin.

Indomethacin was preincubated with 4×10^6 effector cells/ml for 1 hour at 37°C at a final concentration of $1 \mu\text{g/ml}$. Indomethacin was present during the incubation with target cells as well.

Supernatant

PMN were preincubated with either indomethacin at a final concentration of $1 \mu\text{g/ml}$ or RPMI 1640 for 60 min at 37°C in a rotor with 20 rpm . The cells were washed and resuspended in RPMI. They were then stimulated with opsonized zymosan (10 mg/ml) for 30 min at 37°C . After the incubation period the mixture was centrifuged at 800 g , and the supernatant was used in NK cytotoxicity assays.

Dia- and ultrafiltration

An Amicon ultrafiltrator cell device (model 8050, Amicon Corp., Danvers, Ma., USA) was used for filtration of the PMN supernatant. The ultrafiltration step was done using a 5.000 MW cut-off filter (YM 5, Amicon). Half the supernatant volume was collected as diafiltrate. The residue was ultrafiltered with RPMI 1640 and resuspended to the volume of the original sample. Filtration was performed with N_2 at 75 psi , 22°C .

Incubation of effector cells with PMN supernatants

4×10^6 effector cells/ml were incubated with PMN supernatants for 1 h at 37°C at a final concentration of 20%. The PMN supernatants

were present during the incubation with target cells at a concentration of 20 % as well, but in one series of experiments the effector cells were washed 3 times in RPMI with 10 % FCS before incubation with target cells. In other experiments the final concentrations of PMN supernatants were 20 %, 10 %, 1 % and 0.1 %, and supernatants from unstimulated PMN and the buffer were incubated with effector cells in parallel as controls.

Prostaglandin E2 assay

PGE2 measurement in the PMN supernatant was made as previously described (5). Proteins in the PMN supernatant were precipitated by 80 % ethanol, and the supernatant collected. Ethanol was evaporated away and the assay buffer was added. A commercial PGE2 radioimmunoassay kit (NEK-020, Du Pont de Nemours, Germany) containing ^{125}I -PGE2 was used for determination of the amount of PGE2 in the supernatant.

Superoxide production

PMN superoxide production was measured by a cytochrome c reduction assay as previously described (11). 0.25 ml PMN suspension (2×10^6 cells/ml) was preincubated with either indomethacin (1 $\mu\text{g}/\text{ml}$) or RPMI 1640 for 60 min at 37°C in a rotor with 20 rpm. At the end of the preincubation period the cells were stimulated with opsonized zymosan (10 mg/ml), and reduction of cytochrome c was determined by measuring absorbance at 550 nm in a spectrophotometer (Shimadzu. Double-beam, UV-190, Kyoto, Japan). Results were converted to nanomoles of cytochrome c reduced by using the extinction coefficient $E_{550} = 2.1 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$.

Chemiluminescence

A previously described luminol-enhanced PMN chemiluminescence assay was used (12). 1.1 ml PMN suspension (1×10^5 cells/ml) was preincubated with 1.1 ml of 10 $\mu\text{g}/\text{ml}$ or 1 $\mu\text{g}/\text{ml}$ indomethacin, or RPMI 1640 for 60

min at 37°C in a rotor at 20 rpm. Each vial contained 5×10^4 preincubated PMN, 4 mg of opsonized zymosan, 50 μl of luminol solution, and 4.1 ml of Krebs-Ringer solution containing 5 mM glucose. The final concentration of luminol in the assay mixture was $5 \times 10^{-8}\text{M}$. Reagents and vials were dark adapted before use, and the experiments were performed under red light. Sequential 0.5 min counts were taken on each vial over a period of 60 min in a scintillation counter.

Treatment with indomethacin

Two healthy males received indomethacin 3×50 mg per day for 7 days. Baseline NK cell activity, indomethacin boosting of NK cell activity and incubation of effector cells with PMN supernatants before testing for NK cell activity were made before, on day 7 (1 h after intake of the last indomethacin dose), and 1 week after indomethacin treatment.

Statistical analysis

The percentage of ^{51}Cr release was calculated for samples made in triplicate ($\text{SD} < 3.0\%$). The significance of the observed differences was calculated by using the Mann-Whitney test.

RESULTS

When mononuclear cells from 15 healthy donors were incubated with the stimulated PMN supernatants from the same donors the NK cell activity was significantly inhibited ($P < 0.05$). 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. Median baseline NK cell activity was 41.5 % (range 15.2 to 57.0), while median NK cell activity after incubation of the cells with PMN supernatant was 27.2 % (range 8.2 to 50.7), as presented in Fig. 1. The results show that the PMN supernatant from two of the donors did not inhibit the NK cell activity. One of these donors and another donor with strong inhibitory activity of PMN

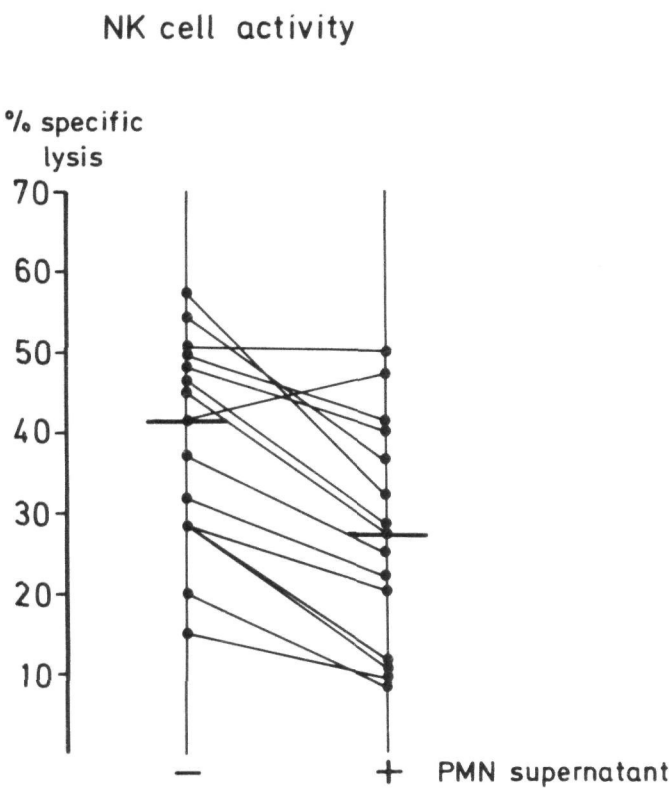


Fig. 1. Median NK cell activity of monuclear cells from 15 donors incubated with or without PMN supernatants. E/T ratio is 80/1.

supernatant were examined repeatedly. It was found that the PMN supernatant of the latter donor constantly inhibited the NK cell activity, while that of the former did not.

PMN supernatant inhibited the NK cell activity

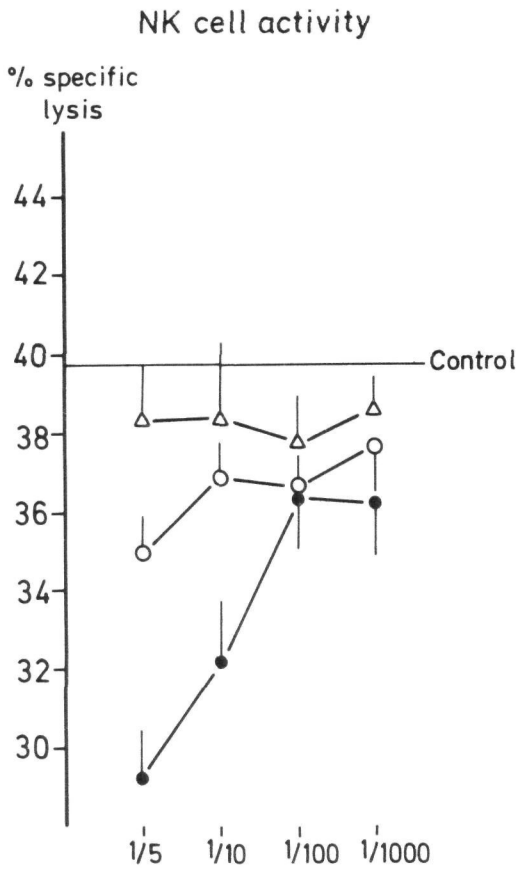


Fig. 2. NK cell activity of mononuclear cells incubated with different concentrations of activated PMN supernatant (●), unstimulated PMN supernatant (○) or buffer (Δ). E/T ratio is 80/1.

in a dose-dependent manner. Supernatant from non-stimulated PMN inhibited the NK cell activity, but to a lesser degree than stimulated

Table 1
The effects of indomethacin and PMN supernatant on NK cell activity

		NK activity		
		% Cytotoxicity	% Augmentation	% Inhibition
I	Controls	34.6 ± 13.1		
II	Indomethacin	42.5 ± 12.0	22.8	
III	PMN supernatant	25.5 ± 12.5		26.3
IV	PMN supernatant + wash*	33.2 ± 11.4		4.0
V	PMN + indomethacin supernatant**	29.5 ± 11.8		14.7
I	versus	II : $P < 0.01$		
I	versus	III : $P < 0.05$		
I	versus	IV : NS		
I	versus	V : NS		
III	versus	V : NS		
$n = 8$				

*Effector cells were preincubated with PMN supernatant and washed before incubation with target cells.

**PMN were preincubated with indomethacin, washed and stimulated with zymosan.

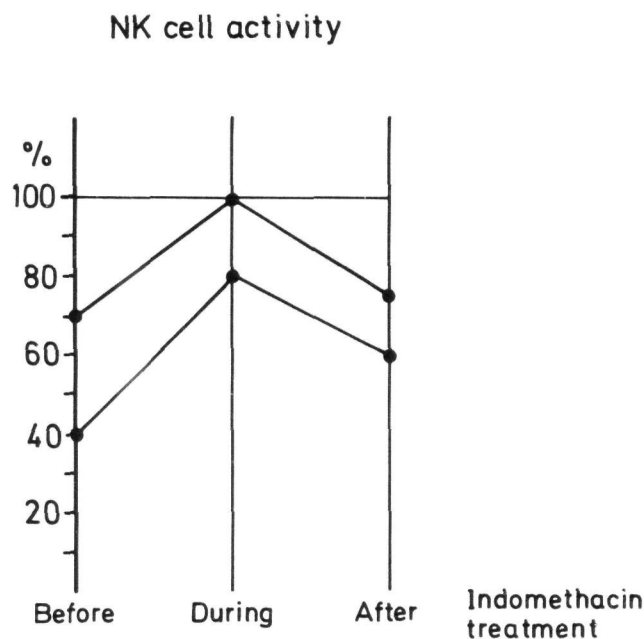


Fig. 3. NK cell activity after exposure to PMN supernatant in percentage of baseline NK cell activity is shown for two donors before, during and after treatment with indomethacin. E/T ratio is 80/1.

PMN (Fig. 2). The results shown were obtained by the use of mononuclear cells separated on Ficoll Isopaque. Experiments with monocyte-depleted effector cells were made in parallel and gave identical results. PMN supernatants were fractionated into a low molecular fraction < 5,000 D and a fraction > 5,000 D. The effect of each fraction was tested on NK cell activity and it was found that only the low molecular weight fraction inhibited NK cell cytotoxicity.

In vitro effect of indomethacin

Incubation of mononuclear cells with indomethacin at pharmacological concentrations (1 µg/ml) augmented the NK cell activity. When PMN leucocytes were stimulated with zymosan in the presence of indomethacin followed by wash before incubating the supernatants with effector cells, the inhibitory effect of the PMN supernatant was partly abolished, but this was, however, not significant (Table 1). Inhibition of the NK cell activity was reversible, as washing of the NK cell activity after incubation with supernatant from stimulated PMN abolished the inhibitory effect of the supernatant (Table 1).

In vivo effect of indomethacin

Baseline NK cell activity and NK cell activity after exposure to PMN supernatants were measured in two donors before, during and after treatment with indomethacin. As shown in Fig. 3 the inhibitory effect of the PMN supernatants was abolished during treatment with indomethacin. Drug treatment did not influence NK cell activity or *in vitro* boosting of NK cells with indomethacin.

In vitro effect of indomethacin on PMN prostaglandin E2 production

The amount of PGE2 in the supernatant of PMN activated by opsonized zymosan in the absence of indomethacin was 0.6 ± 0.1 ng/ml, whereas in the presence of 1 µg/ml indomethacin it was reduced to 0.1 ± 0.02 ng, corresponding to a 6-fold decrease (1 ng/ml PGE2 = 3 × 10⁻⁹M).

In vitro effect of indomethacin on PMN oxidative metabolism

The results are shown in Table 2. Indomethacin at concentrations up to 10 µg/ml did not show any influence on PMN superoxide generation. On the other hand, indomethacin inhibited the luminol-enhanced, myeloperoxidase-mediated, H₂O₂-dependent PMN chemiluminescence response by 35 % in one donor and by 59 % in the other.

Table 2

Effect of indomethacin on stimulated neutrophil oxidative metabolism. Results are mean ± SD of data from a single experiment performed in duplicate

Donor	Drug conc (µg/ml)	Chemiluminescence (mean 0.5 min cpm 10 ⁻³ ± SD)	nmol of cytochrome c reduced/1 × 10 ⁶ PMN ± SD
1	10	15 ± 3.5	5.95 ± 0.07
1	1	14 ± 1.0	5.90 ± 0.00
1	0	23 ± 3.5	5.85 ± 0.21
2	10	7 ± 3.5	5.95 ± 0.07
2	1	3 ± 4.2	6.00 ± 0.00
2	0	17 ± 4.9	5.75 ± 0.35

DISCUSSION

The regulation of NK cell activity has attracted considerable attention, and it has been demonstrated that a number of agents influence the levels of activity of NK cells. Interferon (14) and interleukin 2 (15) augment the cytolytic function of NK cells, while azathioprine (20), glucocorticosteroids (19) and prostaglandins (1, 4) depress NK cell activity. However, very little is known about the modulation of NK cell activity by other cells.

Our results show that the NK cell activity was inhibited by autologous PMN supernatant. Unstimulated PMN inhibited the NK cell activity but to a lesser degree than stimulated. Washing of the effector cells before incubation with target cells abolished the inhibitory effects.

Stimulated PMN leucocytes produce prostaglandins and free oxygen radicals (13). In this investigation indomethacin was used as a tool to determine which component of PMN products is involved in the inhibition of NK cell activity. *In vitro* incubation of effector cells with indomethacin and other drugs which inhibit prostaglandin production enhances NK cell activity (3), while *in vivo* treatment of normal donors with indomethacin does not influence the NK cell function (18).

Our finding that *in vivo* treatment with indomethacin partly abolished the inhibitory effect of PMN supernatant, suggests that the neutrophil prostaglandins are in part responsible for the depressing effect. This point is supported by the finding that indomethacin inhibited the PGE₂ production by activated PMN. The amount of PGE₂ in the PMN supernatant which inhibited NK cytotoxicity was high, and comparable to concentrations of PGE₂ which are known to inhibit NK cytotoxicity *in vitro* (10). Further, the results of ultrafiltration experiments indicate that the inhibitory activity of PMN supernatant is due to substances smaller than 5,000 daltons, corresponding to prostaglandin molecular weight.

Indomethacin had no effect on PMN superoxide anion generation, while the luminol-enhanced myeloperoxidase-mediated chemiluminescence was reduced by indomethacin. It

has been shown that hypochlorous acid is involved in the generation of luminol-enhanced chemiluminescence response (2). HOCl is formed by a myeloperoxidase-mediated reaction between chloride and hydrogen peroxide which is generated by stimulated PMN (8). Therefore, it is possible that hydrogen peroxide generated by stimulated neutrophils in our system is also involved in the inhibition of NK cell cytotoxicity. This finding is in agreement with those of El-Hag & Clark (6) and Seaman et al. (22).

The clinical importance of the interaction between PMN and NK cells is not known. It is, however, possible that the *in vivo* production of oxygen radicals and prostaglandins by PMN or monocytes, e.g. in tumor tissue, constitutes a mechanism whereby tumor cells are defended against attack by cytotoxic effector cells. Though we recently found that indomethacin treatment of normal donors did not inhibit the NK cell activity, this finding does not exclude the possibility that indomethacin might enhance the NK cell activity of patients with high prostaglandin or oxygen radical production. The results presented here show that the inhibitory effect of stimulated PMN supernatant on autologous NK cells is reduced during *in vivo* treatment with indomethacin.

Since NK cells seem to play an important role in immune surveillance (9, 16) studies of the role of drugs which inhibit prostaglandin production modifying immune response in disorders such as cancer, premalignant disease and severe infections are warranted.

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REFERENCES

1. Brunda, M. J., Perlman, P. & Schneider, M. V.: Inhibition of murine natural killer cell activity by prostaglandins. *J. Immunol.* 124, 2682-2687.
2. De Chatelet, L. R., Long, G. D., Shirley, P. S., et al.: Mechanisms of the luminol-dependent chemiluminescence of human neutrophils. *J. Immunol.* 129, 1589-

- 1593, 1982.
3. Droller, M. J., Perlman, P. & Schneider, M. V.: Enhancement of natural and antibody-dependent lymphocyte cytotoxicity by drugs which inhibit prostaglandin production by tumor target cells. *Cell. Immunol.* **39**, 154-164, 1978.
4. Droller, M. J., Schneider, M. U. & Perlman, P.: A possible role of prostaglandins in the inhibition of natural and antibody-dependent cell-mediated cytotoxicity against tumor cells. *Cell. Immunol.* **39**, 165-177, 1987.
5. Du Pont Catalog NO. NEK-020 Instruction manual for the measurement of prostaglandin E2 in plasma and tissue samples.
6. El-Hag, A. & Clark, R. B.: Down-regulation of human natural killer activity against tumors by the neutrophil myeloperoxidase system and hydrogen peroxide. *J. Immunol.* **133**, 3291-3297, 1984.
7. Gerson, J. M.: Systemic and in situ natural killer activity in tumor-bearing mice and patients with cancer. In Herberman, R. B. (ed.): *Natural cell mediated immunity against tumors*, pp. 1047-1062. Academic Press, New York, 1980.
8. Harrison, J. E. & Schultz, J.: Studies on the chlorinating activity of myeloperoxidase. *J. Biol. Chem.* **251**, 1371-1374, 1976.
9. Herberman, R. B.: NK cells and their possible roles in resistance against disease. *Clin. Immunol. Rev.* **1**, 1-65, 1981.
10. Herman, J. & Rabson, A. R.: Prostaglandin E2 depresses natural cytotoxicity by inhibiting interleukin-1 production by large granular lymphocytes. *Clin. Exp. Immunol.* **57**, 380-384, 1984.
11. Kharazmi, A., Döring, G., Høiby, N. & Valerius, N. H.: Interaction of pseudomonas aeruginosa alkaline protease and elastase with human polymorphonuclear leukocytes in vitro. *Infect. Immunol.* **43**, 161-165, 1984.
12. Kharazmi, A., Høiby, N., Döring, G. & Valerius, N. H.: Pseudomonas aeruginosa exoproteases inhibit human neutrophil chemiluminescence. *Infect. Immunol.* **44**, 587-591, 1984.
13. Klebanoff, S. J. & Clark, R. A.: *The neutrophil. Functional and clinical disorder*. North-Holland Publishing Co., Amsterdam, 1978.
14. Ortaldo, J. R., Mantovani, A., Hobbs, D., Rubinstein, M., Pestka, D. & Herberman, R. B.: Effects of several species of human leucocyte interferon cytotoxic activity of NK cells and monocytes. *Int. J. Cancer* **31**, 285-293, 1983.
15. Ortaldo, J. R., Gerard, J. P., Henderson, L. E., Neubauer, R. H. & Rabin, H.: Responsiveness of purified natural killer cells to pure interleukin 2 (IL-2). In: Oppenheim, J. J. & Rabin, H. (eds.): *Lymphokines and cytokines*. Academic Press, New York, 1983.
16. Pedersen, B. K.: Natural killer cells in relation to disease and treatment. A review. *Allergy* **40**, 547-557, 1985.
17. Pedersen, B. K., Oxholm, P., Manthorpe, R. & Andersen, V.: Interleukin 2 augmentation of the defective natural killer cell activity of patients with primary Sjögren's syndrome. *Clin. Exp. Immunol.* **63**, 1-7, 1986.
18. Pedersen, B. K., Oxholm, P. & Klarlund, K.: Characterization of the in vivo and vitro effects of indomethacin on human natural killer cell activity. *Allergy* **41**, 532-536, 1986.
19. Pedersen, B. K., Beyer, J. M., Rasmussen, A., Klarlund, K., Pedersen, B. N. & Helin, P.: Methylprednisolone pulse therapy induced fall in natural killer cell activity in rheumatoid arthritis. *Acta Pathol. Microbiol. Immunol. Scand. [C]* **92**, 319-323, 1984.
20. Pedersen, B. K., Beyer, J. M., Rasmussen, A. et al.: Azathioprine as single drug in the treatment of rheumatoid arthritis induces complete suppression of natural killer cell activity. *Acta Pathol. Microbiol. Scand. [C]* **92**, 221-225, 1984.
21. Russel, S. W., Doe, W. F., Hoskins, R. G. & Cochrane, C. G.: Inflammatory cells in solid murine neoplasms. I. Tumor disaggregation and identification of constituent inflammatory cells. *Int. J. Cancer* **18**, 322-330, 1976.
22. Seaman, W. E. T. D., Gindhardt, M. A., Blackman, L. B., Dalad, N., Talal, N. & Werb, Z.: Suppression of natural killing in vitro by monocytes and polymorphonuclear leukocytes. *J. Clin. Invest.* **69**, 876-888, 1982.
23. Yang, J. & Zucker-Francklin, D.: Modulation of natural killer (NK) cells by autologous neutrophils and monocytes. *Cell. Immunol.* **86**, 171-182, 1984.

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