

## Natural Killer Cell Activity in Rheumatoid Arthritis Measured by a Single Cell Cytotoxicity Assay

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*The natural killer(NK) cell activity of mononuclear cells (MNC) from peripheral blood (PB) and synovial fluid (SF) of 40 rheumatoid arthritis (RA) patients was investigated by employing 51-chromium-(<sup>51</sup>Cr) release microcytotoxicity and single cell cytotoxicity assays against K562 target cells. It has been revealed that SF-MNC from RA patients showed a significantly lower NK activity than PB-MNC from the same patients and this might be due to an impaired target binding capacity of the effector cells and not due to a deficiency of active NK cells.*

**Key Words:** Rheumatoid arthritis, NK cells, single cell cytotoxicity

Rheumatoid arthritis (RA) is a chronic inflammatory systemic disease mainly manifested by polyarticular connective tissue inflammation. Though the etiology and the pathogenesis of the disease are not yet elucidated, autoimmune mechanisms are suspected (Waller 1940; Hamerman 1966).

In patients with RA, increased levels of immune complex (IC) in the peripheral blood (PB) and synovial fluid (SF) (Halla *et al.* 1979; Plotz, 1982; Reeback *et al.* 1985) as well as impaired cell-mediated immunities (Waxman *et al.* 1973; Sakane *et al.* 1982) have been reported. The role of natural killer (NK) cells in the pathogenesis of RA is not clearly defined. There have been controversial data showing normal (Barada *et al.* 1982; Neighbour *et al.* 1982), decreased (Dobloug *et al.* 1982; Silver *et al.* 1982) or rather enhanced (Goto *et al.* 1981; Reinitz *et al.* 1982) NK activities in these patients.

In this paper, we report that lymphocytes isolated from the SF of RA patients exhibited a significantly lower NK activity than those from the PB of the same patients. In order to pursue the underlying mechanisms of such a defect in NK activity, we have further undertaken experiments in which both stan-

dard 51 chromium release cytotoxic assay and single cell assay-in-agarose were used and the events involved in the continuous cytotoxic reaction have been more precisely evaluated. By these methods, we have found that target binding cells were significantly reduced in rheumatoid SF.

### MATERIALS AND METHODS

#### Subjects

Fourty RA patients as defined by the American Rheumatism Association (Ropes, 1959), 19 degenerative arthritis (DA) patients and a control group of 40 healthy adults were studied. All patients were being treated at the Out-Patient Department of Yonsei Medical Center in Seoul. There were 14 males and 26 females in the RA group with a mean age of 38 years (range 18-72 years), 7 males and 12 females in the DA group with a mean age of 59 years (range 50-71) and 25 males and 15 females in the control group with a mean age of 33 years (range 21-56 years).

#### Preparation of Effector Cells

Venous blood and SF were collected from the patients before any treatment using heparinized plastic syringes. The collected samples were diluted 1:1 with Roswell Park Memorial Institute (RPMI) 1640 medium, and mononuclear cells (MNC) were isolated by centrifugation on Ficoll-Hypaque density gradients (1.077 mg/ml density, Sigma, U.S.A.) (Böyum, 1968). Cells were washed twice with RPMI medium, suspended

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in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES (4-2-hydroxyethyl-1-piperazine ethane sulfonic acid) buffer, 2 mM L-glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin (hereafter referred to as complete medium) and incubated overnight in plastic dishes at 37°C in a 5% CO<sub>2</sub> humidified incubator. Non-adherent cells were collected and used as effector cells in standard NK assay as well as in single cell assay-in-agarose.

#### Standard NK Assay

NK activity was determined by a 4-hour chromium (<sup>51</sup>Cr) release assay using K562 cells (Lozzio and Lozzio, 1975). Target cells were labeled by incubating 10<sup>6</sup> cells in 0.2 ml of the complete medium with 100 μCi Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (1 mCi/ml NEX-0305, New England Nuclear, Boston, Mass., U.S.A.) at 37°C in a water bath for 1 hour. After 3 washings, 10<sup>4</sup> labeled cells in 100 ul of the medium were incubated in each well of round bottom microtiter plates (Nunc, Denmark) with 5×10<sup>5</sup> effector cells in 100 ul of the medium (50:1 E:T ratio) for 4 hours at 37°C in 5% CO<sub>2</sub>. Three replicate wells were made for each assay. Target cells were also added to replicate wells containing 100 ul of the medium alone to determine spontaneous release and to wells containing 100 ul medium plus detergent (0.25% Triton X-100, Sigma Chemical Co.) to determine maximal release. After the incubation, 50 ul of the supernatant from each well was carefully removed. The radioactivity was measured with a gamma counter. Spontaneous release of <sup>51</sup>Cr ranged from 7 to 14% of the maximal release.

NK cytotoxicity was expressed as:

$$\% \text{ cytotoxicity} = \frac{\text{test release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

#### Single Cell Assay-in-Agarose

Assay was performed by the modified method of Ullberg and Jondal (1981), based on the original descriptions of Grimm and Bonavida (1979). Briefly, an equal number (2×10<sup>6</sup>) of effector cells and unlabeled target cells were mixed in a total volume of 0.2 ml of the complete medium in a 15 cm round bottom tube. Tubes were centrifuged at 500 g for 2 min, incubated at 37°C for 10-20 min and resuspended after 2 to 3 gentle agitations with a Gilson micropipette. The cell mixture was then carefully added to 0.5 ml of 0.5% agarose in RPMI 1640 and poured on-

to a 60 mm petri dish (Costar, 205 Broadway Cambridge, Mass. U.S.A.) that had been precoated with 0.5 ml of 0.5% agarose. After the cell mixture-in-agarose solidified, 5 ml of the complete medium was added, and the plate was incubated for 3h at 37°C in 5% CO<sub>2</sub>. After the incubation, the media was removed, and 2 ml of 0.1% trypan blue was added for 5 min. The plates were then washed three times with cold phosphate-buffer solution (PBS), fixed with 1% formaldehyde and observed under the microscope.

The percentage of target binding cells (%TBC) was determined by counting the number of lymphocytes binding to target cells in 250 lymphocytes counted. The percentage of dead conjugates was determined by counting the number of dead targets in 50 effector-target conjugates. The percentage of spontaneous target cell death was determined by counting the number of dead targets in the absence of effector cells. Corrections were made by applying the following formula to calculate the percentage of dead conjugates: (the % TBC with dead targets) minus (the % spontaneous dead targets) multiplied by (the % TBC with dead targets) (Mathews *et al.* 1983).

The percentage of active NK cells was obtained from the above single cell assay by multiplying (the % TBC with dead targets) with (the % total lymphocytes bound to targets) at the end of the assay.

#### V<sub>max</sub> and Maximal Recycling Capacity (MRC) of NK Cells

The V<sub>max</sub> represents the maximum number of target cells that can be killed by a constant number (10<sup>5</sup>) of effector cells when target cells are present in excess. This was determined by using a minor modification of the method described by Ullberg and Jondal (1981). Briefly, 10<sup>5</sup> effector cells in 0.1ml were incubated in round-bottom microtiter wells with 0.1 ml <sup>51</sup>Cr-labeled K562 cells at five doubling dilutions, from 1×10<sup>5</sup> cells to 0.625×10<sup>4</sup> cells per well. For each E: T ratio, the maximal and spontaneous release was determined. After a 3 hr incubation, 50 ul of supernatant was harvested from each well, and specific <sup>51</sup>Cr-release was calculated from each target cell concentration as described in the standard NK assay. The V<sub>max</sub> was calculated from a linear regression curve obtained by plotting the reciprocals 1/V (y axis) and 1/T (x axis) where V is the number of target cells killed at each target concentration and T is the initial number of target cells. The V<sub>max</sub> is the reciprocal of the intercept of the curve with the y axis and represents the maximum number of target cells that

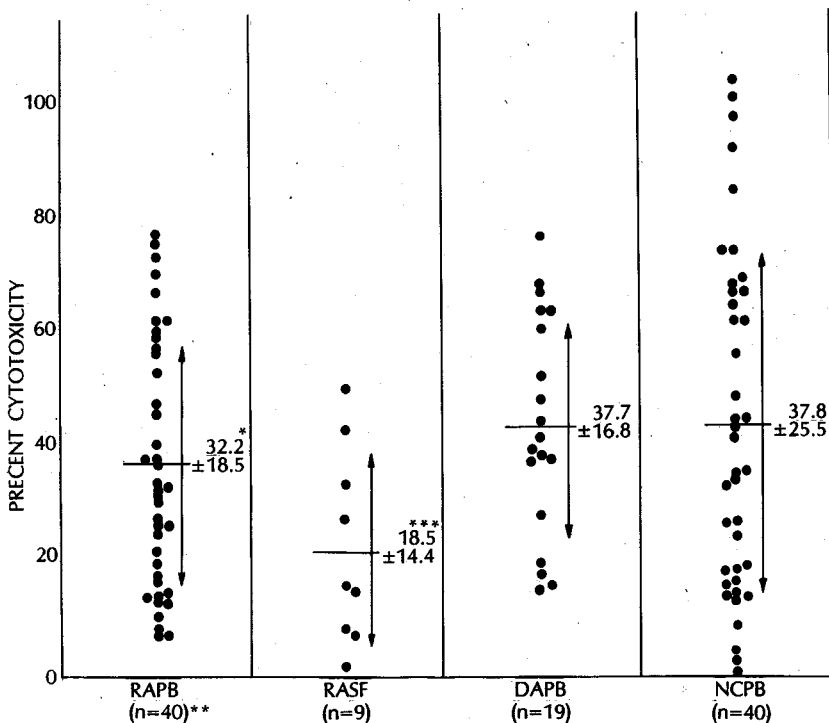
can be killed by  $10^5$  effector cells (Table 1). Experimental data thus obtained can be closely approximated by determining the percent  $^{51}\text{Cr}$ -release at a 50:1 E:T

ratio and then employing the formula:  $V_{\max} = 3.48 \times 10^2 \times (\% ^{51}\text{Cr}\text{-release at 50:1 ratio}) - 1.2 \times 10^3$ . This formula was devised in order that this parameter could

**Table 1. A representative experiment showing data used for calculation of  $V_{\max}$**

Dilution number	Number of effector cells	Number of target cells (T)	Percentage killed target cells	Number of killed target cells (V)	1/T = X	1/V = Y
1	$1 \times 10^5$	$1 \times 10^5$	10.58	$1.05 \times 10^4$	$1 \times 10^{-5}$	$9.45 \times 10^{-5}$
2	$1 \times 10^5$	$5 \times 10^4$	15.61	$7.80 \times 10^3$	$2 \times 10^{-5}$	$1.28 \times 10^{-4}$
3	$1 \times 10^5$	$2.5 \times 10^4$	31.31	$7.83 \times 10^3$	$4 \times 10^{-5}$	$1.28 \times 10^{-4}$
4	$1 \times 10^5$	$1.25 \times 10^4$	47.08	$4.64 \times 10^3$	$8 \times 10^{-5}$	$2.15 \times 10^{-4}$
5	$1 \times 10^5$	$0.625 \times 10^4$	49.46	$3.09 \times 10^3$	$1.6 \times 10^{-4}$	$3.24 \times 10^{-4}$

Regression analysis results:  $Y = a + bX$ ;  $r^2 = 0.984$ ;  $a = 8.41 \times 10^{-5}$ ,  $b = 1.52$ ;  $V_{\max} = 1/a = 1.19 \times 10^4$ ; Data are given from a  $^{51}\text{Cr}$ -release assay, as described in Materials and Methods



**Fig. 1.** NK cytotoxicity measured by 4h- $^{51}\text{Cr}$  release assay against K562 target cells (50:1 E:T ratio)

RAPB-Rheumatoid arthritis PB-MNC

RASF-Rheumatoid arthritis SF-MNC

DAPB-Degenerative arthritis PB-MNC

NCPB-Normal control PB-MNC

\* mean  $\pm$  S.D.

\*\* Numbers in parenthesis indicate the number of subjects examined

\*\*\*  $P < 0.05$  vs RAPB, DAPB & NCPB

be approximated from the  $^{51}\text{Cr}$ -release data.

The MRC is an estimation of the average number of target cells that an active NK cell can kill during the incubation period when target cells are present in complete saturation. This was calculated by the data obtained from the  $^{51}\text{Cr}$ -release assay used to determine  $V_{\text{max}}$  and the simultaneous single cell assay as follows:  $V_{\text{max}}$  divided by the total number of active NK cells in  $V_{\text{max}}$  assay.

### Statistical Calculations

The data were analyzed by the Student's t-test.

## RESULTS

### NK Activity of RA and DA Patients and Normal Healthy Controls

Nonadherent MNC from the PB (PB-MNC) of 40 RA and 19 DA patients and 40 normal healthy controls as well as those from the SF (SF-MNC) of 9 RA patients were submitted to standard NK assays using K562 cells as targets at an E:T ratio of 50:1.

As shown in Figure 1, the mean percent cytotoxicities of PB-MNC were not significantly different among these groups:  $32.2 \pm 18.5\%$  for RA patients,  $37.7 \pm 16.8\%$  for DA patients and  $37.8 \pm 25.5\%$  for normal controls. However, SF-MNC of 9 RA patients showed significantly lower NK activity,  $18.5 \pm 14.4\%$ , than those of PB-MNC from all of the subjects.

### Single Cell Assay-in Agarose

In order to enumerate TBC and to determine the

fraction of dead conjugates and active NK cell, single cell assays-in-agarose were performed in parallel with a portion of each MNC used for the standard NK assays.

As shown in Table 2, the percentages of TBC of SF-MNC from RA patients was significantly decreased as compared with those of PB-MNC either from RA and DA patients or from normal healthy controls:  $5.0 \pm 1.7\%$  for SF-RA patients,  $7.0 \pm 2.2\%$  for PB-RA patients,  $6.8 \pm 2.6\%$  for PB-DA patients and  $7.7 \pm 2.5\%$  for PB-normal controls. The percentages of dead conjugates and active NK cells were similar in both PB and SF-MNC.

To estimate the MRC of effector cells, the single cell assay-in-agarose was combined with the standard  $^{51}\text{Cr}$ -release assay as described in Materials and Methods. The  $V_{\text{max}}$  which represents the maximum killing potential of effector cells was calculated from the data obtained from a representative experiment as shown in Table 1.

A significantly reduced value of  $V_{\text{max}}$  was obtained with SF-MNC,  $(5.2 \pm 5.0) \times 10^3$ , as compared to those of PB-MNC,  $(10.0 \pm 6.4 \text{ to } 12.0 \pm 8.9) \times 10^3$ .

Similarly, the MRC of the former was lower ( $2.9 \pm 2.8$ ) than those of the latter ( $3.5 \pm 1.9 \text{ to } 5.1 \pm 2.1$ ), but the differences were at the limit of significance.

## DISCUSSION

Accumulating evidence suggests that NK cells play an important role in immune surveillance against tumors and microbial infections as well as in the regulation of hematopoietic stem cells (Herberman

Table 2. NK cytotoxicity,  $V_{\text{max}}$ , TBC, fraction of dead conjugates and estimates of active NK & MRC

Source of effectors	Number of subjects	NK cytotoxicity* (%)	$V_{\text{max}} (\times 10^3)$	TBC (%)	Fraction of dead conjugates (%)	Active NK cells (%)	Estimated MRC
RAPB	40	$32.2 \pm 18.5$	$10.0 \pm 6.4$	$7.0 \pm 2.2$	$9.8 \pm 4.5$	$0.7 \pm 0.4$	$3.5 \pm 1.9$
RASF	9	$18.5 \pm 14.4^{**}$	$5.2 \pm 5.0^{***}$	$5.0 \pm 5.0^{****}$	$8.9 \pm 3.6$	$0.5 \pm 0.3$	$2.9 \pm 2.8$
DAPB	19	$37.7 \pm 16.8$	$11.9 \pm 5.9$	$6.8 \pm 2.6$	$8.2 \pm 3.4$	$0.6 \pm 0.3$	$5.1 \pm 2.1$
NCPB	40	$37.8 \pm 25.5$	$12.0 \pm 8.9$	$7.7 \pm 2.5$	$8.1 \pm 4.4$	$0.6 \pm 0.4$	$4.0 \pm 3.0$

RAPB – Rheumatoid Arthritis PB-MNC

RASF – Rheumatoid Arthritis SF-MNC

DAPB – Degenerative Arthritis PB-MNC

NCPB – Normal Control PB-MNC

\*: Mean  $\pm$  S.D.

\*\* :  $P < 0.05$  vs RAPB       $P < 0.01$  vs DAPB & NCPB

\*\*\*:  $P < 0.05$  vs RAPB       $P < 0.01$  vs DAPB & NCPB

\*\*\*\*:  $P < 0.05$  vs DAPB       $P < 0.01$  vs RAPB & NCPB

1985). The role of NK cells in autoimmune disorders is not clearly defined. Depressed NK activities have been reported in systemic lupus erythematosus and multiple sclerosis (Neighbour et al. 1982). In patients with RA, there have been controversial reports showing normal (Barade et al. 1982; Neighbour et al. 1982), decreased (Dobloug et al. 1982; Silver et al. 1982) or rather enhanced (Goto et al. 1981; Reinitz et al. 1982) peripheral NK activity as compared with that of healthy controls.

In our study, PB-MNC from 40 RA patients showed that their NK activities were no different than those obtained from PB-MNC from 19 DA patients and 40 normal controls. However, it has been revealed that SF-MNC from 9 RA patients exhibited significantly decreased NK activity when compared with PB-MNC. NK activity from all other subjects studied (Figure 1). These results are compatible with those reported by Silver et al. (1982) and Dobloug et al. (1982). The mechanisms for such differences in NK activity between SF-MNC and PB-MNC are uncertain. NK-like effector cells in rheumatoid SF which differ qualitatively from those of PB have been suggested (Dobloug et al. 1982).

NK cells could exert their cytotoxic actions through the following continuous steps: a) recognition of and binding to a target cell, 2) lysis of the target cells and 3) recycling. In order to define the nature of such defect in NK activity in SF, we have performed experiments using a combined standard  $^{51}\text{Cr}$ -release and single cell-in-agarose assays with MNC from both the SF and PB of the patients and healthy controls. By these methods, it has been possible to estimate the TBC, active cytotoxic cells and MRC of effector cells and thus the critical site(s) in the cytotoxic reaction could be precisely localized.

As shown in Table 2, the SF-MNC from RA patients showed significantly decreased TBC without affecting lytic and recycling capacities. Though the underlying mechanisms are unknown, it is probable that various soluble factors, pathologically produced and accumulated in the SF, such as rheumatoid factor, IC and other inflammatory modulators could interfere with the recognition and binding steps of the effector cells. In our previous experiments, high titers of IC were detected in both the PB and SF of RA patients and a negative correlation was found between IC levels and NK activity (Hong et al. 1987).

In contrast, Reinitz et al. (1982) has reported an increased NK activity in SF-MNC in comparison with PB-MNC from RA patients. However in their study, all patients examined were treated with nonsteroidal anti-inflammatory drugs (NSAIDs) and some patients also

with gold salts. These treatments could interfere with the activity of inflammatory modulators such as prostaglandin (PGE<sub>2</sub>) which inhibit NK activity.

There are many reports showing the impairment of cell-mediated immune parameters in RA patients: depressed cutaneous hypersensitivity responses to recall antigens, impaired in-vitro lymphocyte blastogenesis (Emery et al. 1984) and decreased production of interleukin-2 (Combe et al. 1985) and leukocyte migration inhibitory factor by PB-MNC following phytohemagglutinin (PHA) stimulation (Hong et al. 1987).

Taken together, it can be postulated that an impaired cellular immune function including NK activity, particularly in local sites, is a characteristic feature of RA patients. This might be the result not only of an intrinsic functional defect in effector cells themselves but also due to pathologically produced soluble factors which interfere ultimately with effector mechanisms. Further investigations which are designed to dissociate these possibilities will be necessary to elucidate more precisely the pathogenesis of RA.

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