

In Vitro Study of Nonspecific Cellular Immunity in Rheumatoid Arthritis

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Nonspecific immune parameters such as natural killer (NK) activity, antibody-dependent cellular cytotoxicity (ADCC), production of leukocyte migration-inhibitory factor (LIF) and levels of immune complex (IC) were assessed in 47 patients with rheumatoid arthritis (RA), 20 with degenerative arthritis (DA) and 40 healthy controls. Peripheral blood (PB) as well as synovial fluid (SF) were collected from both RA and DA patients before treatment. Mononuclear cell suspensions and sera were prepared and submitted for the in vitro tests; 4-hr chromium-release assays using human K562 and mouse L1210 cells as targets for NK and ADCC assays respectively, 2-step agarose assay for LIF and platelet aggregation test for IC. Results revealed that 1) LIF activity of PB lymphocytes (PBL) from both RA and DA patients showed a significant ($P<0.05$) decrease as compared with that from healthy controls. 2) PB-NK activity from RA patients showed an insignificant decrease as compared with that from DA or healthy controls. However, mononuclear cells isolated from SF (SFL) of RA patients exhibited significantly ($P<0.02$) lower NK activity than PBL from the same patients. 3) In ADCC assays with PBL, no significant differences were observed among the 3 groups. 4) Higher titers of IC were detected in both PB and SF from RA patients than DA, and a negative correlation was found between serum IC levels and PB-NK activity. These data are discussed in light of previous reports, and a hypothesis regarding a decreased nonspecific cell-mediated immunity in conjunction with an increased humoral immune response, particularly in local sites, is proposed as one of the mechanisms underlying the pathogenesis of RA.

Key Words: Rheumatoid arthritis, NK, ADCC, LIF, Immune complex

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 unknown, autoimmune mechanisms are currently suspected to be responsible (Waalder 1940; Hamerman 1966). Studies on the humoral immunity in patients with RA revealed increased levels of serum gammaglobulin (Kunkel *et al.* 1961) and immune complex (IC) (Reeback *et al.* 1985) as well as IC in synovial fluid (SF) (Winchester *et al.* 1970; Halla *et al.* 1979; Plotz 1982). These findings suggested that enhanced humoral immune responses are involved in the

pathogenesis of the disease.

On the other hand, cell-mediated immune responses such as peripheral suppressor T-cell activity and delayed skin hypersensitivity to recall antigens were depressed in these patients (Sakane *et al.* 1982; Waxman *et al.* 1973). *In vitro* production of interleukin-2 (IL-2) by mononuclear cells from both peripheral blood (PB) and SF following stimulation with phytohemagglutinin (PHA), was also decreased in RA patients, suggesting an impairment of T-lymphocyte function (Combe *et al.* 1985).

More recently, similar decreased cytotoxic activities of natural killer (NK) cells were reported in patients with systemic lupus erythematosus or multiple sclerosis (Neighbour *et al.* 1982) as well as those with RA (Dobloug *et al.* 1982). Yet the underlying mechanisms are not clearly elucidated, it is supposed that the agglutinated IgG or IC could partly interfere with the cytotoxic effect of NK cells (Goto *et al.* 1980).

As to the antibody-dependent cellular cytotoxicity (ADCC), such decreased activity was not observ-

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ed in RA patients (Barada *et al.* 1982). Though the *in vitro* role of NK and ADCC has not yet been fully determined, it is highly suggested that they play an important role in immune surveillance against tumors and microbial infections (Herberman & Holden 1978).

Leukocyte migration inhibitory factor (LIF), a lymphokine that is produced by lymphocytes stimulated with antigens or polyclonal mitogens, has the ability to suppress the migration of granulocytes, and *in vitro* LIF assays can be employed as an immunological procedure to measure T cell function (Astor *et al.* 1973; Rocklin 1974).

In this paper, we have measured different immune parameters in patients with RA and found that while PBL showed a significantly decreased LIF production with normal NK and ADCC activity, SFL of the same patients showed as significantly decreased NK activity with concomitantly increased levels of IC.

MATERIALS AND METHODS

Subjects

The experimental group consisted of 47 classic or definite RA patients as defined by the American Rheumatism Association (Ropes 1959), 24 degenerative arthritis (DA) patients and a control group of 40 healthy adults. All patients were being treated at the Out-Patient Department of Severance Hospital in Seoul. There were 16 males and 31 females in the RA group with a mean age of 38 years (range 19-66 years), 8 males and 16 females in the DA group with a mean age of 59 years (range 46-74) and 25 males and 15 females in the control group with a mean age of 33 years (range 21-56 years).

Preparation of PBL

Prior to treatment venous blood was collected from patients and the control group using heparinized plastic syringes. The blood was diluted 1:1 with Roswell Park Memorial Institute (RPMI) 1640 medium, and PBL were isolated by differential 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 with RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (hereafter referred to as the complete medium) and used for NK and ADCC assays.

LIF production and assay

PBL were suspended at concentration of 1×10^6 /ml

in RPMI medium containing 15% heat-inactivated fetal calf serum, 1.6 mM L-glutamine and antibiotics. One hundred microliters of cells were pipetted into round bottom wells of microtiter plates (Costar, U.S.A.) and incubated in the presence of 13 µg/ml of PHA (M-form, Gibco, U.S.A.) for 3 days at 37°C in a humidified 5% CO₂ atmosphere. Cell-free LIF preparations were separated by centrifugation and stored at -20°C until assayed. Control samples consisted of unstimulated lymphocyte-culture supernates similarly prepared (Du Bois *et al.* 1974).

LIF was assayed by the indirect two-stage agarose technique (Clausen 1973). Neutrophils (PMN) were isolated from fresh normal PB by sequential application of Ficoll-Hypaque centrifugation, 5% dextran sedimentation and hypo-osmolar lysis of remaining erythrocytes (Weisbart *et al.* 1982). Pooled 4×10^6 PMN from three people were suspended in 30 µl of LIF preparations or control samples and left for 30 min at room temperature with constant agitation. Ten µl of PMN suspension were pipetted into each well of a 1% agarose plate previously prepared as follows:

0.6g agarose was dissolved in 30 ml distilled water by heating to 100°C and put in a 50°C water bath. In a separate flask, 30 ml of the solution was prepared: 5.4 ml RPMI (10× concentrated), 6.0 ml heat-inactivated horse serum, 1.0 ml penicillin (10,000 IU/ml) and streptomycin (10,000 µg/ml), 0.6 ml Na₂HCO₃ (10%) and 17.0 ml distilled water. The flask was put in a 50°C water bath and the added to the agarose solution. The agarose-medium mixture (4.5 ml) was pipetted into each plastic tissue culture plate (60×15 mm, Nunc, Denmark) and left at room temperature to solidify. In each agarose plate, wells (3 mm diameter), arranged in a circle with equal distances, were made by cutting the hardened agarose out and 10 µl of the PMN suspension were put into each well in triplicate.

After an 18 hour incubation at 37°C, 2% CO₂, the agarose plate was fixed with 2% glutaraldehyde for 2 hours, washed and stained with 2% Giemsa solutions. The migration area was measured by Yonsei Anatomy Program 1, an automatic cell measurement system, under microscope (16×). LIF content was expressed as a migration index

$$\text{Migration index} = \frac{\text{mean migration area for test sample}}{\text{mean migration area for control sample}} \times 100$$

NK cytotoxicity

PBL were suspended in complete medium contain-

ing 10 mM HEPES (4-2-hydroxyethyl-1-piperazine ethane sulfonic acid) buffer and incubated in plastic dishes at 37°C in a 5% CO₂ humidified incubator overnight. Non-adherent cells were collected and used as effector cells.

NK activity was determined by a 4-hour chromium (⁵¹Cr)-release assay using K562 cells derived from human myeloid leukemia as targets (Lozzio & Lozzio, 1975). Target cells were labeled by incubating 10⁶ cells in 0.2 ml of the complete medium with 100 µCi Na₂⁵¹CrO₂ (1 mCi/ml NEZ-0305, New England Nuclear, Boston, Mass., U.S.A.) at 37°C in a water bath for 1 hour. After 3 washings, 10⁴ labeled cells in 100 µl were incubated in each well of a round-bottomed microtiter plate with 5×10⁵ effector cells in 100 µl (1:50) for 4 hours at 37°C in a 5% CO₂ incubator. After incubation, 100 µl of the supernatants from each wells were removed. The radioactivity was measured with a gamma counter. Three replicate wells were made for each assay. Target cells were also added to replicate wells containing 100 µl of the medium alone to determine spontaneous release and to wells containing 100 µl medium plus detergent (0.25% Triton X-100, Sigma, U.S.A.) to determine maximal release. Spontaneous release of ⁵¹Cr ranged from 7 to 14% of the maximal release. NK activity was expressed as:

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

ADCC assay

Target cells used in this assay were murine L1210 cells (Moore *et al.* 1966) known to be resistant to human NK cells. Immune serum was raised in NZW (New Zealand white) rabbits by giving them weekly injection of 3-5×10⁷ L1210 cells for 6 weeks and them stored at -20°C until use. The highest dilution of this serum inducing a maximum specific lysis was 1:800, therefore, the final dilution of 1:400 was used throughout the experiments for ADCC assay. The same 4-hour ⁵¹Cr-release assay that used for NK activity was used by adding 50 µl of diluted immune serum to each well containing 10⁴ targets and 5×10⁵ effector cells (1:50). ADCC activity was calculated as follows:

$$\text{ADCC activity} = \% \text{ cytotoxicity with immune sera} - \% \text{ cytotoxicity without immune sera}$$

Detection of IC

IC in PB or SF was detected by platelet aggregation test (PAT) (Myllyla *et al.* 1971). The platelets were collected from fresh human PB by differential centrifugation, washed twice with saline and suspended at a concentration of 200,000/mm³ in a basal salt solution containing 0.025% glucose (pH=7.5). Test samples were diluted in a round bottom microtitration plate and 50 µl of platelets were added to each well. After an 18 hour incubation at 4°C, the platelet aggregation was examined macroscopically.

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Statistical analysis

Independent t-test was used to compare the two patient groups.

RESULTS

LIF activity

LIF activity of PBL was measured in 32 RA patients, 19 DA patients and 19 healthy controls.

As shown in Table 1 the leukocyte migration indexes of both RA and DA groups were significantly higher than normal controls. Thus, RA and DA patients showed a significant decrease in LIF activity as compared to that of healthy controls. No difference could be detected, however, between RA and DA patients.

Table 1. LIF activity of PBL

Group	Number of patients	Migration index (mean±S.E.)
Rheumatoid arthritis	32	77.6± 3.8*
Degenerative arthritis	19	76.1± 4.6*
Normal control	19	53.8±14.7

* P<0.05 as compared with Normal control

NK activity

NK activity of PBL was measured in 47 RA patients, 24 DA patients and 40 healthy controls, and the mean percent of cytotoxicity is shown in Table 2. No difference was observed among the 3 groups. However, mononuclear cells isolated from the synovial fluid of 8 RA patients exhibited a highly significant decrease in NK activity as compared with that of PBL of the same patients (Table 3).

Table 2. NK activity of PBL

Group	Number of patients	% cytotoxicity (mean±S.E.)
Rheumatoid arthritis	47	33.1±2.6 ^(N.S.)
Degenerative arthritis	24	36.7±3.7
Normal control	40	37.8±4.0

N.S.: not significant as compared with Degenerative arthritis or Normal control groups.

Table 3. Comparison of NK activity between PBL and SF in 8 RA patients

Source of lymphocytes	% cytotoxicity (mean±S.E.)
Peripheral blood	42.9±8.5*
Synovial fluid	20.9±4.9

* P<0.05

ADCC activity

The results obtained from ADCC assays with PBL from 35 RA patients, 13 DA patients and 16 normal controls are shown in Table 4. No significant differences were observed among these groups.

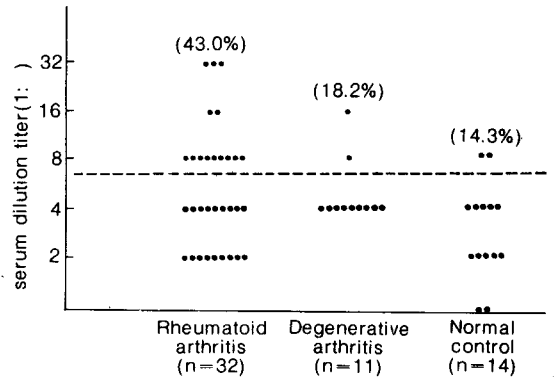
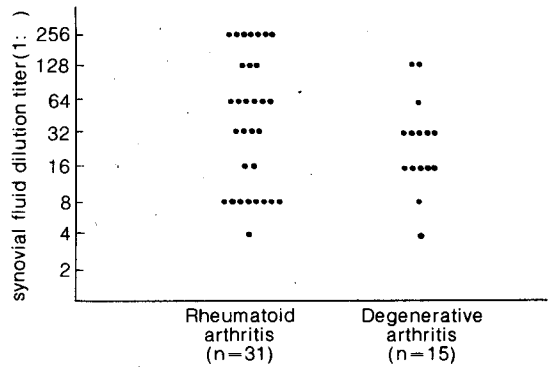
Table 4. ADCC activity of PBL

Group	Number of patients	ADCC activity (%) (mean±S.E.)
Rheumatoid arthritis	35	63.1±2.7 ^(N.S.)
Degenerative arthritis	13	62.2±3.5 ^(N.S.)
Normal control	16	56.6±4.0

N.S.: not significant as compared with Normal control.

IC levels in PB and SF.

The levels of IC were measured by the platelet aggregation method with sera from 32 RA patients, 11 DA patients and 14 normal controls. As shown in Figure 1, the number of positive sera detected in dilutions greater than 1:8 was higher (43.8%) in RA pa-


Fig. 1. IC in sera of RA patients.

Fig. 2. IC in SF of RA patients.
Table 5. Correlation of PB-NK activity and serum IC level in 28 RA patients

NK activity (% cytotoxicity)	Number of patients	% of positive* IC in sera
0-20	9	56.6%
21-40	12	41.7%
Over 41	7	28.6%

* positive in sera diluted 1:8 or above

tients than DA (18.2%) or normal controls (14.3%), but the difference was statistically at the limit of significance.

In addition, the levels of IC measured by the same method, with SF from 31 RA and 15 DA patients, showed significantly higher rates with increasing dilution in RA than DA patients, thus, in 7 patients out of 31 in the RA group (or 22.6%), IC could be detected even in SF diluted 1:256 (Fig. 2).

Correlation of NK activity and IC levels

The results obtained from 28 RA patients revealed that those who had low NK activity (below 20% cytotoxicity) showed high levels of serum IC, and on the contrary, those who had high NK activity (over 41%) showed low levels of the latter (Table 5).

DISCUSSION

Studies on the pathogenesis of RA have been primarily concerned with humoral and cellular immune functions (Paget and Gibofsky 1981). The role of humoral immunity in RA has been suggested by the increased levels of serum gammaglobulin (Kunkel *et al.* 1961) and IC in both PB (Alspauch *et al.* 1983; Hack *et al.* 1984; Reeback *et al.* 1985) and SF (Plotz 1982). Lymphopenia and impaired blastogenesis to mitogens (Lockshin *et al.* 1975; Horwitz and Garrett 1977; Horwitz and Juul-Nielsen 1977; Felder *et al.* 1985), as well as a decreased production of IL-2 in RA patients have been reported (Miyasaka *et al.* 1893; Combe *et al.* 1985).

LIF is a class of lymphokine produced by stimulated T lymphocytes, and its function is to inhibit the migration of polymorphonuclear cells from an inflammatory site (Rocklin 1974). It has been shown that the *in vitro* productivity of LIF by lymphocytes following stimulation with different antigens such as PPD, streptokinase-streptodornase and candida, has correlated perfectly with the *in vivo* delayed hypersensitivity using the same antigens in lymphocyte donors (Astor *et al.* 1973). Thus, LIF assays can be also assumed to be a reliable immunological procedure to determine the competence of T cell-mediated immunity.

We have found in the present study that LIF activity of PBL from RA patients was significantly decreased as compared to that of healthy controls. Whether such decreased activity is specific for RA or not is uncertain from our data, since a similar degree of LIF activity was also observed in DA patients. Alternatively, the decreased activity of the latter might be, to some extent, due to age-related nonspecific impairment of immune functions; mean age of 59 years for DA patients, 38 years for RA patients and 33 years for healthy controls.

It has been reported that peripheral NK cell function was significantly depressed in chronic diseases such as multiple sclerosis and systemic lupus erythematosus, except in patients with RA who showed NK activity no different from that of normal healthy

controls (Neighbour *et al.* 1982). In contrast, an enhanced NK activity in RA patients has also been reported (Goto *et al.* 1980). In our study, PBL from the latter showed a slight but not significant decrease in activity if compared with that of DA patients or healthy controls. In addition, the lymphocytes isolated from the SF of the same patients showed very significantly decreased NK activity. These results are compatible with those reported by Silver *et al.* (1982), Dobloug *et al.* (1982) and Reinitz *et al.* (1982). It has been suggested that SFL differ from PBL in patients with RA (Fox *et al.* 1982) and that activated NK-like effector cells which differ from those of PB have been found in rheumatoid synovial fluid (Silver *et al.* 1982).

Concerning the ADCC activity, no significant differences were observed among the 3 groups as reported by other investigators (Burmester *et al.* 1975; Diaz-Jouanen *et al.* 1976; Feldmann *et al.* 1976; Barada *et al.* 1982). Though the ADCC activity of lymphocytes from the SF of these patients could not be tested in our study because of the poor cell yields, it will be interesting to determine whether the ADCC activity of SFL is also lower than that of PBL in the same patients as observed with their NK activity. If some differential effects are observed between these activities, it would indirectly suggest that the effector cells of both activities are functionally different even though they share common surface markers.

We have found that the IC levels in both PB and SF from RA patients were increased as compared with those of DA patients or normal healthy controls. Seven patients out of 31 showed a positive reaction even in SF diluted 1:256, whereas in DA patients, no positive reactions were observed in SF equally diluted. Moreover, RA patients who had higher serum IC levels showed lower PBL-NK activity and vice versa. This suggests an interference by IC on NK activity probably through the binding of IC to the Fc receptors of NK cells. A similar mechanism for decreased NK activity has been suggested in RA patients (Dobloug *et al.* 1982) as well as in patients with systemic lupus erythematosus (Silverman and Cathcart 1982).

As a whole, a decreased cell-mediated immune function concomitantly with an increased humoral immunity, particularly at local sites as in joints, may represent general immunological features in RA. Further studies on the relationships among these different immune parameters will certainly be necessary to further elucidate the mechanisms implicated in the pathogenesis of this disease.

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