Phenotypic and Functional Study of Natural Killer Cells in Tuberculous Pleurisy Patients

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The phenotypic and functional characteristics of the cells that mediate "natural killer" (NK) cytotoxicity were examined by using anti-Leu 7 monoclonal antibody specific for NK cell in peripheral blood mononuclear cells (PMC) and pleural effusion mononuclear cells (PEMNC) from the tuberculous pleurisy patients. The proportion of Leu 7+ cells in PEMNC was observed significantly lower than in PMC, whether the patients were treated or not. Before treatment NK cell activity, precisely cytotoxicity against K 562, in PEMNC was apparently higher than in PMC. There was little relationship between proportion of leu 7+ cells and NK cell activity. Further, the mechanism of high NK cell activity in PEMNC was analysed by coculture with a healthy control's PMC and a tuberculous pleural effusion. The NK cell activity of Leu 7+ cells was augmented by low molecular soluble factor(s).

Key Words: NK cell, Tuberculous pleurisy, Monoclonal antibody, Local immunity

Lymphoid cells that lyse certain tumor cell lines in vitro without prior deliberate immunization have been collectively designated natural killer (NK) cells¹⁾. Effectors of such spontaneous or natural killer cells have been described in several species. There have been suggestions that this cell type plays important roles in host defence against nascent tumors and certain microbial pathogens. An immunodeficiency disorder²⁾ as well as rheumatoid arthritis and juvenile rheumatoid arthritis³⁾ involving a selective defect in NK cells have been described. Further, a number studies of NK cells in malignant effusions⁴⁾ and of soluble factor in pleural effusion⁵⁾ have been reported. It is well known that many lymphocytes are observed in tuberculous pleural effusion. Different distribution and the responses of T cells^{6,7)} in tuberculous pleural effusion and peripheral blood have been reported. Little is known, however, about the pathophysiological importance and different functions of NK cells between peripheral blood and

pleural effusion in tuberculous pleurisy patients.

In the present study, the proportion of NK cells in peripheral blood mononuclear cells (PMC) and pleural effusion mononuclear cells (PEMNC) of tuberculous pleurisy patients (pts) were examined by using anti-Leu 7 monoclonal antibody (mAb) specific for NK cells. In addition, NK cell activity of PMC and PEMNC was measured by K 562 target cell cytotoxicity. The proportion of Leu 7+ cells in peripheral blood was much larger than in pleural effusion, while NK cell activity in peripheral blood was much lower than in pleural effusion. In other words, there is little relationship between the phenotype of NK cells and NK cell activity.

To elucidate further the phenomenon of high NK cell activity of PEMNC before treatment, we examined the augmenting activity of tuberculous pleural effusion for lytic activity against K 562 as well as surface antigens of the cells which were augmented in natural cytotoxic assay. It has

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been demonstrated that soluble factor(s) which have augmenting activity for NK cell activity exist in pleural effusion by the method of gel filtration. Neither Interleukin-2 (IL-2) nor Interferon^{8,9)} in NK cell augmenting factors could be detected in this experiment. Furthermore, Leu 7+ cells had high lytic potential activity against K 562 by incubation with the tuberculous pleural effusion.

MATERIALS AND METHODS

Preparation of Peripheral Blood Mononuclear Cells and Pleural Effusion Mononuclear Cells.

The series consisted of 12 pulmonary tuberculosis patients with pleural effusion (34–83 yr, 59.0±15.3: 9 male, 3 female) hospitalized at Shinshu University Hospital. The final diagnosis was made by positive tuberculin reaction, the presence of acid-fast bacilli in repeated sputum samples and by demonstration of *mycobacterium tuberculosis* in pleural effusion by culture. Positive tuberculin reaction was observed in all 12 pts. Two pts had acid-fast bacilli in their sputum. Only four pts, however, were positive on culture from effusion. Four of the pts had already taken anti-tuberculous drugs (INH, Streptomycin, Rifampicin) for more than one month and/or glucocorticoids (30 mg/day) for two weeks. Twenty-six healthy donors (22–74 yr, 47.0±20.6:15 male, 11 female) served as controls in peripheral blood. Peripheral blood and pleural effusion samples were collected on the same day. Peripheral blood mononuclear cells and pleural effusion mononuclear cells were obtained by Lymphoprep (Nyegaard Co., Oslo, Norway) density centrifugation as described before⁶⁾. After the separation, PMC and PEMNC were washed with RPMI 1640 (GIBCO, Grand Island, N.Y.) and then suspended in RPMI 1640 supplemented with 10% inactivated fetal bovine serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml), that is, complete RPMI 1640 (C-RPMI 1640).

Antibodies.

Monoclonal antibodies (anti-Leu 7 and anti-Leu 1) for human NK cell antigens and human T cells, respectively, were purchased from Becton Dickinson (Sunnyvale, C.A.)^{10,11)}.

Immunofluorescence Assay.

1×10⁶ (1 ml) PMC or PEMNC from tuberculous pleurisy patients and PMC from healthy controls reactive with anti-Leu 7 mAb were enumerated by indirect immunofluorescence assay. Anti-Leu 7 mAb was used at a concentration of 5 µg/ml and reacted with the cells for 30 min on ice. The samples were centrifuged at 400 G for 10 min and washed with phosphate buffered saline (PBS, 0.005 M, pH 7.4) once. In the second step the cells were reacted with FITC-goat anti-mouse immunoglobulins (Tago Inc, Burlingame, C.A.). After an additional 30 min incubation on ice, the cells were washed twice with PBS. After this, Leu 7+ cells were immediately enumerated with fluorescence microscopy. Two hundred cells were counted: only cells with lymphocyte morphology and a cap-like or circular rim staining were counted as Leu 7+ cells. One tenth percent of NaN₃ was present throughout the treatment and all procedures were carried out at 4°C.

Cytotoxic Assay.

Target K 562 cells were labeled with 100–150 µCi sodium 51Cr chromate (Nippon Atomic Power Institute; Tokyo) at 37°C, 5% CO₂ in a humid atmosphere for 60–90 min and washed three times with C-RPMI 1640. The concentration of PMC or PEMNC from tuberculous pleurisy patients and PMC from healthy controls was adjusted to give the required effector (1×10⁵) to target (1×10⁴) ratio (10:1), and the cells were dispensed in 200 µl aliquots. After 4 hr incubation in an atmosphere of 5% CO₂ in air the assay was terminated by centrifugation at 100 G, and 100 µl of the supernatant was counted for radioactivity. All the experiments were done in triplicate. The percentage of cytotoxicity was calculated according to the following formula:

$$\% \text{ cytotoxicity} = (\text{Experiment 51Cr release} - \text{spontaneous 51Cr release} / \text{Maximum 51Cr release} - \text{spontaneous 51Cr release}) \times 100.$$

The variations among triplicates were less than 8% and the percentage of spontaneous release without effector cells was below 8%.

Effect of Tuberculous Pleural Effusion on NK Cell Activity.

Peripheral blood mononuclear cells from the

patients with positive demonstration of *Mycobacterium tuberculosis* in pleural effusion as well as high NK cell activity of PEMNC were cocultured with autologous or allogeneic tuberculous pleural effusion supplemented with C-RPMI 1640 (final dilution 1:2) or C-RPMI 1640 alone at 37°C, 5% CO₂ in a humid atmosphere for 6 days. NK cell activity was examined every day. Further, for the purpose of analysing NK cell augmenting factor(s), PMC from a healthy control were cocultured with each fraction (final dilution 1:2) obtained from gel-filtration or IL-2 (ENI Co., USA). NK cell activity was assayed on day 3.

IL-2 and Interferon Immunobiologic Assay.

IL-2 activity was determined by IL-2 concentration-dependent stimulation of CTLL-2 proliferation, a cloned murine cytotoxic T lymphocyte line. CTLL-2 (4×10^4 cells/ml) proliferation was monitored by ³H-TdR incorporation (0.5 μ Ci/well, 6 Ci/mmol specific activity; New England Nuclear, Boston, MA) during the 8 hr of a 36 hr culture in the presence of the IL-2 or tuberculous pleural effusion from 12 tuberculous pleurisy patients. Further, interferon levels in tuberculous pleural effusion were measured by a plaque inhibition assay employing human neonate skin fibroblast monolayers and Sindbis virus. The levels are the reciprocals of the dilutions producing a 50% reduction in cytopathic effect.

Complement-mediated Cytolysis.

Complement-mediated cytolysis was done as described before¹²). Briefly, the suspension of the PMC (2×10^6 /ml) was treated with anti-Leu 7 mAb or anti-Leu 1 mAb on ice for 30 min. After the treatment with these antibodies, the cells were washed once and then treated with 100 μ l of rabbit anti-mouse immunoglobulins (1:500 dilution) on ice for 30 min. After two washings with RPMI 1640, well-selected rabbit complement (1:4 dilution) was added to each group and the cells were incubated at 37°C for 1 hr. After two more washings the cells were suspended in C-RPMI 1640 supplemented with allogeneic tuberculous pleural effusion and cultured at 37°C, 5% CO₂ in a humid atmosphere. NK cell activity was measured on day 3 as described above. One-tenth

percent of NaN₃ was added during the treatment.

Gel Filtration of Tuberculous pleural effusion.

The sample (2 ml) that showed high NK cell activity of PEMNC as well as positive demonstration of *Mycobacterium tuberculosis* in pleural effusion was applied to a 1.5×90 cm Sephadex G 200 (Pharmacia Fine Chemicals, Sweden) equilibrated PBS (pH 7.4). Elution was performed at a flow rate of 8 ml/hr with the same buffer. Two milliliter fractions were collected and the protein content (optimal density, 280 nm) and NK cell augmenting biologic activity were determined. The column was calibrated with albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000) and ribonuclease A (13,700) (Pharmacia Fine Chemicals, Sweden).

Statistical Analysis.

The results are given as mean values $\pm$ standard deviation. The significance of the observed differences were calculated by Student's t-test.

RESULTS

Immunofluorescence Assay.

As shown in Fig. 1, the percentage of Leu 7+ cells was $22.5 \pm 8.6\%$ in peripheral blood of healthy controls, $27.6 \pm 12.3\%$ in peripheral blood of tuberculous pleurisy patients ($p < 0.25$ compared with that in normal controls) and $7.7 \pm 3.4\%$ in tuberculous pleural effusion. These data show that the distribution of Leu 7+ cells is different between peripheral blood and pleural effusion. Further, whether the patients were treated or not, the Leu 7+ cell population in pleural effusion was much smaller than in peripheral blood.

Natural Cytotoxic Assay.

NK cell activity was estimated by cytolysis of ⁵¹Cr labeled K 562. As shown in Fig. 2, NK cell activity of PMC from healthy controls was $24.3 \pm 15.3\%$. NK cell activity of PEMNC and PMC from tuberculous pleurisy patients was $30.8 \pm 21.7\%$ and $21.2 \pm 13.2\%$, respectively. There was no difference of NK cell activity of PMC between tuberculous pleurisy patients and healthy controls. Further, Leu 7+ cells in pleural effusion exhibited a very small population, while NK cell

Fig. 1. Proportion of Leu 7+ cells. Leu 7+ cells were enumerated by indirect immunofluorescence assay. ○—○ treated patients PBL: peripheral blood ●—● non-treated patients PL: pleural effusion *: *Mycobacterium tuberculosis* positive patients

Fig. 2. NK cell activity of tuberculous pleurisy patients and healthy controls. NK cell activity was measured ⁵¹Cr release assay at E:T ratio (10:1). PBL, PL: See footnote in Fig. 1 Symbols as in Fig. 1

activity of PEMNC was observed to be high. These data show that there is little relationship between the proportion of Leu 7+ cells and NK cell activity. Further, NK cell activity of PEMNC from the 4 treated pts was observed to be low, compared to that of PMC from the same patients.

The Effect of Tuberculous Pleural Effusion on NK Cell Activity.

High NK cell activity of PEMNC could be observed before treatment in most of the patients. In the next experiments, the effect of tuberculous pleural effusion on NK cell activity was examined. As shown in Fig. 3, NK cell activity of a tuberculous pleurisy patient's PMC increased apparently from day 2 to 5 by coculture with either autologous or allogeneic tuberculous pleural effusion whose PEMNC were shown to be high. The results were reproduced in three separate experiments. These data indicate that soluble factor(s) that augment NK cell activity exist in

Fig. 3. The kinetic effect of NK cell activity of a tuberculous pleurisy patient's PMC by coculture with tuberculous pleural effusion or C-RPMI 1640 alone at 37°C, 5% CO₂ for 6 days. NK cell activity was examined every day. ○—○ PBL, PL: HLA Aw24, Bw52, Cw1, Cw3, DR2. ●—● PBL: HLA Aw24, Bw52, Cw1, Cw3, DR2. PL: HLA Aw24, Bw44, Bw35, Cw3, DR4. ▲—▲ PBL: HLA Aw24, Bw52, Cw1, Cw3, DR2. C-RPMI 1640.

tuberculous pleural effusion. In the next experiments, IL-2 bioactivity in tuberculous pleural effusion was examined. The responses of CTLL-2 could be observed significantly on adding IL-2 (Exp. 1 IL-2 (64 U) 10,930 Δcpm, Exp. 2 IL-2 (64 U) 9,730 Δcpm, Δcpm: experiment responses-medium alone responses), while the responses could not be observed on adding tuberculous pleural effusion (Exp. 1 PL. 1 820 Δcpm, PL. 2 200 Δcpm; Exp. 2 PL. 3 350 Δcpm, PL. 4 0< Δcpm). The same tendency was obtained in experiments on other pleural effusion. These data suggest that IL-2 did not exist in tuberculous pleural effusion. Further, we checked for the presence of interferon in tuberculous pleural effusion by the inhibition of cytopathogenicity of sindbis virus. None was detected.

Analysis of Soluble Factor in Tuberculous Pleural Effusion.

Analysis of soluble factor(s) in pleural effusion was examined by the method of gel filtration. The eluted fraction was collected and divided into 5 fractions (Fr. 1 Fraction number 24—28, Fr. 2 Fraction number 29—35, Fr. 3 Fraction number

36–47, Fr. 4 Fraction number 56–60, Fr. 5 Fraction number 61–75). In this experiment, Fr. 1, 2 and 3 mainly included Ig M, Ig G and albumin, respectively. Lower molecular substance(s) smaller than ribonuclease A (13,700) was included in Fr. 4 and 5. A healthy control's PMC were cocultured with each group and then NK augmenting activity was examined on day 3. NK cell activity of PMC (Control 25.1%) could be augmented by coculture with either Fr. 4 (63.5%) or Fr. 5 (63.0%), but not with either Fr. 1 (32.9%), Fr. 2 (33.1%) or Fr. 3 (28.2%). NK cell activity augmenting factor(s) were thus included in Fr. 4 and 5.

Characterization of the Cells Augmented by Soluble Factor.

The population of the cells whose NK cell activity was augmented by pleural effusion was characterized by anti-Leu 7 and anti-Leu 1 mAbs. As shown in Table 1, NK cell activity of the healthy controls' PMC incubated with the tuberculous pleural effusion was augmented. This augmented NK cell activity of allogeneic PMC, however, was apparently reduced by treatment with anti-Leu 7 mAb plus C, but not affected by treatment with anti-Leu 1 mAb plus C. These data thus indicate that Leu 7+ cells mediated NK cell activity by the effects of tuberculous pleural effusion.

DISCUSSION

In the experiments reported in this paper, Leu 7+ cells were enumerated in a group of 12 tuberculous pleurisy patients ranging in age from 34–83 yr and 26 healthy controls who ranged in age from 22–74 yr. No difference in Leu 7+ cell population between tuberculous pleurisy patients and healthy controls was found in PMC. It has been reported that, in younger people, Leu 7+ cell population is much smaller than in older people¹³⁾. Even with the wide age-range of the present patients, however, the proportion of Leu 7+ cells in PMC of patients did not differ from that of healthy controls.

It has been reported that NK cell activity, manifested as cytolysis of appropriate targets in vitro, was decreased shortly after the administration of corticosteroids¹⁴⁾ and by chemical substances

Table 1. Analysis of phenotypic expression of the cells by coculture with tuberculous pleural effusion

	Treatment	T/E (1:10)
Exp. 1	Complement (C)	40.3
	Anti-Leu 7 + C	14.3
	Anti-Leu 1 + C	50.1
Exp. 2	Complement (C)	70.0
	Anti-Leu 7 + C	24.2
	Anti-Leu 1 + C	68.9

a) The cells were treated with complement (C) alone, anti-Leu 7 mAb plus C, or anti-Leu 1 mAb plus C. After each treatment, these cells were cocultured with tuberculous pleural effusion. NK cell activity was assayed on day 3.

Exp. 1 PBL:HLA; A2, A26, Bw35, Bw44, Cw3, DR4, DRw8.

PL:HLA; Aw24, Bw52, Cw1, Cw3, DR2.

Exp. 2 PBL:HLA; Aw24, Bw44, Bw35, Cw3, DR9.

PL:HLA; Aw24, Bw52, Cw1, Cw3, DR2.

(estrogen¹⁵⁾, prostaglandin¹⁶⁾ and adenosin¹⁷⁾) in the in vitro assay culture system. It may be that another cause of reduced NK cell activity of PEMNC by treatment is the effect of anti-tuberculous drugs. Further, it seems that NK cell activity of PEMNC in non-treated patients can be divided into 2 groups with one group showing high NK cell activity of PEMNC and the other showing low activity. We can not explain the reason for the variety of activity as regards age, sex, physical examination and stage of the disease. Luft et al¹⁸⁾ and Hauser et al¹⁹⁾ reported that NK cell activity in peritoneal cells in mice was enhanced by injection of *Corinebacterium* or *Toxoplasma gondii*. It seems therefore that NK cell activity correlates to some degree with immunity to infection and the stage of infection.

In these experiments, there was little relationship between the proportion of Leu 7+ cells and NK cell activity. Recently, another mAb, anti-Leu 11 has been produced against human granular lymphocytes²⁰⁾. We have examined PMC and PEMNC from tuberculous pleurisy patients using anti-Leu 11b mAb. Leu 11b+ cell population in tuberculous pleural effusion was much smaller than in peripheral blood, as in the same as Leu

7+ cells (data not shown). These data can not fully explain the point of the phenotypic marker of the cells (Leu 7 and Leu 11) for high NK cell activity in pleural effusion. Unfortunately, we can not determine what cell phenotype is responsible for the cytolytic activity by using available monoclonal antibody.

Another reason for high NK cell activity in PEMNC is thought to be the augmenting soluble factor(s) in pleural effusion. The existence of augmenting factor(s) was observed in tuberculous pleural effusion. In other diseases including SLE (N=2), malignant lymphoma (N=2), lung cancer (N=5), *paragonimus miyazakii* (N=1) we could not observe any augmenting soluble factor in pleural effusion. It has been reported that NK cell activity was augmented by IL-2 or interferon^{8,9)}. However, we could detect neither IL-2 nor interferon (α , β , γ). Further, data from Sephadex G-200 gel filtration showed that NK cell augmenting factor(s) existed in the lower molecular fraction. We could detect neither IL-2 nor Interferon (α , β , γ) in the lower molecular band by the method of sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) (data not shown).

HLA typing was analysed in the coculture experiments of peripheral blood and pleural effusion. NK augmenting effects could be clearly observed in the following combinations:

1. PBL:HLA A2, A26, Bw35, Bw44, Cw3, DR4, DRw8. PL:HLA Aw24, Bw52, Cw1, Cw3, DR2.
2. PBL:HLA Aw24, Bw35, Bw44, Cw3, DR9. PL:HLA Aw24, Bw52, Cw1, Cw3, DR2. 3. PBL:HLA Aw24, Bw52, Cw1, Cw3, DR2. PL:HLA Aw24, Bw35, Bw44, Cw3, DR4.

It, however, has not been clear whether some HLA typing is related with the effects of NK augmenting factor, and further investigation is necessary to establish the relationship between HLA-typing and the augmenting factor. The present study demonstrated that low molecular soluble factor(s) exist in tuberculous pleural effusion.

The surface antigen of NK cell activity elicited by coculture with the tuberculous pleural effusion at the induction phase (day 1) was examined. As shown in Table 1, it was necessary to have Leu 7+ cells to elicit NK cell activity, but not Leu 1+ cells. Further, at the effector phase (day 3), NK

cell activity of the PMC cocultured with tuberculous pleural effusion was examined. The NK cell activity was eliminated by treatment with anti-Leu 7 mAb + Complement (C) (C alone 29.0%, anti-Leu 7 mAb plus C 4.8%, T/E 1:10). Thus, it was clearly shown that NK cell activity of Leu 7+ cells was augmented by the soluble factor(s) in some of the tuberculous pleural effusion. The proportion of Leu 7+ cells, however, did not increase by coculture with the effusion (data not shown). Experiments in progress are chemically analysing the factor in tuberculous pleural effusion.

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