Purified Protein Derivative Induced Cytotoxicity in Carcinomatous and Tuberculous Pleurisy

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We have found that natural killer (NK) cells were very active in pleural effusions containing *Mycobacterium tuberculosis*. Cytotoxicity against K562 and Raji was augmented when the mononuclear cells were cocultured for 18 hr with purified protein derivative (PPD) derived from *M. tuberculosis* culture supernatants. In pleural effusions of cancer patients, PPD-activated mononuclear cells were less cytotoxic than their counterparts in peripheral blood. However, in the same patients, interferon and interleukin-2 production was greater in pleural effusions than in peripheral blood. On the other hand, in tuberculosis patients there was no significant difference in cytotoxicity between peripheral blood and pleural effusion mononuclear cells, but the production of interferon and interleukin-2 was higher in pleural effusions than in peripheral blood. Neither group of patients consistently demonstrated a correlation between production of interferon or interleukin-2 in peripheral blood and cytotoxicity. Both PPD-induced cytotoxicity and the production of interferon and interleukin-2 were lower in mononuclear cells of carcinomatous than tuberculous pleural effusion. These results indicate that peripheral blood and pleural effusion mononuclear cells differ in cytotoxicity as well as in interferon and interleukin-2 production. Further, these activities also differ in tuberculous and carcinomatous pleural effusions.

Key Words: Carcinomatous pleurisy, Tuberculous pleurisy, Purified protein derivative, Cytotoxicity

Natural killer (NK) cells belong to a population of lymphoid cells whose functions include the destruction of certain susceptible tumor cells and cells infected with viruses or parasites and the maintenance of immune homeostasis¹⁾. NK cell activity has been studied in several diseases²⁻⁶⁾. We examined the phenotypes and functions of NK cells in patients with carcinomatous and tuberculous pleural effusions from which *Mycobacterium tuberculosis* was cultured, suggesting that *M. tuberculosis* possesses a factor that may affect NK cell activity. In the study reported here, we obtained samples of pleural effusions and peripheral blood from patients with cancer and tuberculosis. We then cultured mononuclear cells

from these samples with purified protein derivative (PPD), derived from the culture supernatant of *M. tuberculosis* and examined the PPD-induced cytotoxicity against K562 and Raji. In the same context we also studied the production of the soluble factors interleukin-2 (IL-2) and interferon (IFN). We describe and discuss the differences in activity between mononuclear cells in peripheral blood and those in pleural effusions of cancer and tuberculosis patients. We also compare the two disease in terms of these immunologic functions within the pleural effusion.

MATERIALS AND METHODS

Samples of peripheral blood and pleural

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effusions were obtained on the same day from 11 patients with cancer and 10 with tuberculosis. Among the eight males and three females with carcinomatous pleural effusions (age range, 37-86 years), the histologically confirmed diagnoses were lung carcinoma in ten and laryngeal carcinoma in one. All the patients except two were positive in tuberculin reactions. The eight male and two female patients with tuberculous effusions ranged in age from 24-77 years. The diagnosis was confirmed by a positive tuberculin reaction (10 patients), demonstration of *M. tuberculosis* by culturing of pleural effusions (two patients), and positive microscopic findings (eight patients). The study was conducted before any of the patients received therapy. Peripheral blood samples from 11 healthy and tuberculin reaction positive donors ranging in age from 24-76 years were used as approximate age-matched controls. Mononuclear cells from both peripheral blood and pleural effusions were isolated and washed, as described previously⁷⁾. Cell suspensions of these fractions contained less than 1% tumor cells.

PPD-induced mononuclear cell cytotoxicity against K562 and Raji was examined. To determine the optimal time of cytotoxicity, samples from healthy controls (2×10^6 : 1 ml) were cultured for 4 days with or without PPD (12.5 μ g/ml) in RPMI 1640 (GIBCO, USA) supplemented with streptomycin (100 μ g/ml), penicillin (100 U/ml), and 10% inactivated fetal calf serum (GIBCO, USA) (that is C-RPMI 1640) at 37°C in a humid atmosphere containing 5% CO₂. Cytotoxicity was examined daily at a target: effector ratio of 1:10, as we described previously^{3,7)}. PPD-induced cytotoxicity was examined under optimal conditions. The viability of target cells was over 98%. Spontaneous ⁵¹Cr release from target cells was under 10%. Maximum release by target K562 and Raji cytolysis was achieved by the addition of 1 N HCl. The percentage of cytotoxicity for individual assays was calculated by the following formula: % cytotoxicity = (experimental release - spontaneous release) / (maximum release - spontaneous release) $\times$ 100.

To measure IFN production by mononuclear cells, we cultured samples (2×10^6 : 1ml) with or without PPD for 18 hr in conical tubes (Falcon

2095, USA). After incubation, the supernatants were collected by centrifugation at 300 G for 10 min and were stored at -80°C until assayed. Interferon was assayed by measurement of the cytopathic effect on Sindbis virus following by a dye uptake⁸⁾. The titers were expressed in international units (IU/ml).

IL-2 activity of the supernatants cultured with or without PPD was determined by their ability to support the growth of murine IL-2 dependent CTLL-2 cells that had been washed five times to eliminate IL-2. CTLL-2 cells (1×10^4) in C-RPMI 1640 (0.1 ml) were mixed with 0.1 ml of serial dilutions of the test sample. After 20 hr of incubation, the cultures were pulsed for 4 hours with 3H-thymidine (TdR) (specific activity 6.7 Ci/m mol. NEN Co., USA) and the radioactivity was determined by a liquid scintillation counter (Packard Co. USA). Each assay was compared with a standard recombinant IL-2 supernatant (1U/200 μ l, 1U equivalent 350 NIH units), which was kindly donated by Takeda Chemical Industries Inc., (Japan). For measurement of IL-2 units, the standard and samples were tested at serial twofold dilutions. The results of this assay were evaluated by logarithmic plotting of the counts per minute of 3H-TdR uptake against a twofold dilution of the standard sample. The IL-2 titer in the test samples was calculated by the reciprocal titer of the test samples, which gave 50% of the maximal counts per minute of the standard.

The results (mean standard $\pm$ deviation) were subjected to one-way analysis of variance for calculation of statistical significance.

RESULTS

Kinetics of PPD-induced cytotoxicity.

The kinetics of cytotoxicity against K562 and Raji were examined from day 0 to day 4. As shown in Fig. 1 and 2, maximum cytotoxicity occurred on day 1 (after culture with PPD for 18 hr) in three healthy individuals. On the basis of this result, in subsequent experiments the cytotoxicity against K562 and Raji was investigated after 18 hr of culturing with PPD.

Cytotoxicity against K562.

PPD-induced cytotoxicity of mononuclear cells

Fig. 1. Kinetics of PPD-induced cytotoxicity against K562.

PPD: PPD-induced cytotoxicity
Med: Medium-induced cytotoxicity

Fig. 2. Kinetics of PPD-induced cytotoxicity against Raji.

from peripheral blood and pleural effusions of the patients with cancer and tuberculosis was examined. As shown in Table 1, there was no significant difference in the performance of peripheral blood mononuclear cells between healthy controls and either cancer or tuberculosis patients. Also, there was no difference between cancer and tuberculosis patients. Mononuclear cells from pleural effusions exhibited lower PPD-induced cytotoxicity in cancer patients than in tuberculosis patients ($\alpha=0.05$). In cancer patients PPD-induced cytotoxicity was higher in peripheral blood than in pleural effusion ($\alpha=0.05$), but there was no such difference between in tuberculosis patients.

Cytotoxicity against Raji.

As shown in Table 1, PPD-induced peripheral blood cytotoxicity to Raji was much lower in cancer patients than in either tuberculosis patients or healthy controls ($\alpha=0.05$, $\alpha=0.05$, respectively). There was no significant difference between tuberculosis patients and healthy controls. In pleural effusion, PPD-induced cytotoxicity was lower in cancer patients than in tuberculosis patients ($\alpha=0.01$), but in comparing peripheral blood and pleural effusion, we found no significant intragroup difference in cytotoxicity.

IFN production.

Our finding concerning IFN production are listed in Table 2. Peripheral blood mononuclear cells produced considerably less IFN in the cancer and tuberculosis patients than in the healthy controls ($\alpha=0.05$, $\alpha=0.05$, respectively). However, the values were similar in the two patient groups. Interferon production by pleural effusion mononuclear cells was much lower in cancer patients than in tuberculosis patients ($\alpha=0.05$). In both groups, pleural effusion mononuclear cells produced more IFN than did those in peripheral blood ($\alpha=0.05$, $\alpha=0.01$, respectively).

IL-2 production.

Table 2 gives the data concerning IL-2 production. There were no significant differences in peripheral blood IL-2 production among controls, cancer patients, and tuberculosis patients. Much more IL-2 was produced in

Table 1. PPD-induced cytotoxicity against K562 and Raji in cancer and tuberculosis patients with pleural effusion

	Case	K562				Raji			
		Peripheral blood		Pleural effusion		Peripheral blood		Pleural effusion	
		Med/PPD	$\Delta\%$	Med/PPD	$\Delta\%$	Med/PPD	$\Delta\%$	Med/PPD	$\Delta\%$
Carcinomatous pleurisy	1	26.0/55.8	29.8	2.8/ 8.1	5.3	2.7/ 4.0	1.3	0.3/ 1.9	1.6
	2	3.7/22.3	18.6	2.1/ 8.1	6.0	0 / 0	0	0 / 0	0
	3	64.8/79.7	14.9	12.7/18.6	5.9	19.6/28.2	8.6	4.9/ 5.4	0.5
	4	7.6/49.1	41.5	17.9/31.7	13.8	1.0/10.7	9.7	2.5/ 9.9	7.4
	5	4.8/23.3	18.5	2.7/ 9.3	6.6	0 / 0.7	0.7	0.2/ 0.3	0.1
	6	0.1/20.7	20.6	1.8/ 2.9	1.1	0.4/ 2.3	1.9	0 / 1.5	1.5
	7	23.0/55.8	32.8	2.7/16.9	14.2	16.4/28.8	12.4	0 / 4.1	4.1
	8	12.2/74.3	62.1	2.3/39.1	36.8	1.3/ 6.9	5.6	0.1/ 0.5	0.4
	9	14.7/41.0	26.3	25.0/53.0	28.0	1.6/ 8.9	7.3	2.2/14.3	12.1
	10	20.6/71.4	50.8	1.6/30.1	28.5	0.2/ 5.4	5.2	1.7/ 8.3	6.6
	11	8.0/21.0	13.0	3.0/11.4	8.4	1.1/ 1.7	0.6	0 / 2.6	2.6
	Mean $\pm$ SD		30.0 $\pm$ 15.8		14.1 $\pm$ 11.8		4.8 $\pm$ 4.3		3.4 $\pm$ 3.9
Tuberculous pleurisy	1*	7.2/55.9	48.7	6.6/38.1	31.5	0.8/24.9	24.1	6.6/21.3	14.7
	2	10.5/34.7	24.2	59.4/84.6	25.2	0 / 8.4	8.4	36.4/81.2	44.8
	3	2.8/ 9.5	6.7	1.6/15.5	13.9	0 / 0	0	0 / 0	0
	4	46.0/67.3	21.3	17.0/77.0	60.0	2.7/ 9.3	6.6	3.0/30.3	27.3
	5	41.9/78.4	36.5	5.8/34.3	28.5	2.1/38.4	36.3	2.6/18.3	15.7
	6	15.8/67.7	51.9	19.4/74.4	55.0	0.8/17.2	16.4	2.9/28.1	25.2
	7	11.1/44.0	32.9	0 / 8.2	8.2	0.2/12.0	11.8	1.6/ 4.2	2.6
	8	25.8/47.0	21.2	22.4/50.5	28.1	1.8/27.9	26.1	2.1/28.4	26.3
	9	19.6/52.9	33.3	1.6/34.1	32.5	1.2/ 6.6	5.4	1.9/ 5.5	3.6
	10*	18.8/39.9	21.1	1.4/13.3	11.9	5.5/11.4	5.9	1.2/10.8	9.6
	Mean $\pm$ SD		29.8 $\pm$ 13.7		29.5 $\pm$ 17.1		14.1 $\pm$ 11.4		17.0 $\pm$ 14.0
Healthy controls (N=11)	Mean $\pm$ SD		32.1 $\pm$ 9.9		N. D.		12.6 $\pm$ 11.2		N. D.

PPD-induced cytotoxicity was measured in a ^{51}Cr release assay described in "Materials and Methods".

$\Delta\%$: PPD-induced cytotoxicity(%) – medium-induced cytotoxicity(%)

*: *M. tuberculosis* positive in culture

tuberculous than in carcinomatous pleural effusion ($\alpha=0.05$). Also IL-2 production was greater in pleural effusions than in peripheral blood in both patient groups ($\alpha=0.05$, $\alpha=0.05$, respectively).

DISCUSSION

It has been reported that biological response modifiers augment NK cell activity in mice⁹⁻¹¹⁾ and in humans¹²⁾. We found that NK cells were highly active in tuberculous pleural effusions in which *M. tuberculosis* was confirmed by culture. Using PPD as a modifier in healthy controls, we observed that cytotoxicity and production of IFN and IL-2 in tuberculin positive group were a little higher than those in tuberculin negative group. In this experiment, only two patients with carcinomatous pleural effusion were negative tuberculin

reaction. We attempted to elucidate the difference of local immunity in carcinomatous and tuberculous pleural effusions. As shown in Figs. 1 and 2 and in Table 1, PPD enhancement of cytotoxicity was observed in healthy controls and also in cancer and tuberculosis patients. In the kinetic study, maximum cytotoxicity was observed on day 1, after which PPD augmentation gradually decreased. In addition we did not detect any augmentation of cytotoxicity in medium not treated with PPD. We found no differences in peripheral blood cytotoxicity to K562 among cancer patients, tuberculosis patients, and healthy controls. However, against Raji it was much lower in cancer patients than in tuberculosis patients and healthy controls. Thus, there were clear difference in cytotoxicity to K562 and Raji. Further, the

Table 2. Interferon and interleukin-2 production in cancer and tuberculosis patients with pleural effusion

		Peripheral blood				Pleural effusion			
		Med		PPD		Med		PPD	
	Case	IFN	IL-2	IFN	IL-2	IFN	IL-2	IFN	IL-2
Carcinomatous pleurisy	1	<6	<1	76	<1	<6	<1	2,340	17
	2	<6	<1	155	2	<6	<1	16	12
	3	<6	<1	161	<1	<6	<1	1,060	16
	4	<6	<1	17	<1	<6	<1	2,430	6
	5	<6	<1	599	<1	<6	<1	120	1
	6	<6	<1	378	4	<6	<1	460	20
	7	<6	<1	28	<1	<6	<1	3,140	44
	8	<6	<1	60	<1	14	<1	2,540	1
	9	<6	<1	650	8	<6	<1	482	11
	10	<6	<1	444	<1	<6	<1	486	8
	11	<6	<1	38	<1	<6	<1	469	1
Mean ± SD				237 ± 238				1,231 ± 1,143	12 ± 12
Tuberculous pleurisy	1*	<6	<1	799	7	16	<1	6,330	49
	2	<6	<1	99	6	<6	<1	10,000	173
	3	<6	<1	6	<1	<6	<1	264	7
	4	<6	<1	161	<1	<6	<1	5,730	360
	5	<6	<1	452	1	<6	<1	1,320	24
	6	<6	<1	20	<1	<6	<1	5,730	500
	7	<6	<1	706	<1	<6	<1	2,120	13
	8	<6	<1	549	14	<6	<1	1,930	74
	9	<6	<1	114	<1	<6	<1	1,470	50
	10*	<6	<1	108	2	<6	<1	889	20
Mean ± SD				301 ± 297				3,578 ± 3,174	127 ± 170
Healthy controls (N=11)	Mean ± SD			952 ± 905	7 ± 14			N. D.	N. D.

Interferon and IL-2 production were measured as described in "Materials and Methods".

IFN: interferon (IU/ml)

IL-2: interleukin-2 (1×10^{-3} U/ml)

*: *M. tuberculosis* positive in culture

cytotoxicity of carcinomatous pleural effusion to both K562 and Raji was lower than that of tuberculous effusion. The same tendency of the cytotoxicity was observed at a target: effector ratio of 1:30 (data not shown). It is well known that K562, the cell line derived from a patient with chronic myeloid leukemia in blast crisis, is more susceptible, while Raji, derived from a patient with Burkitt's lymphoma, is resistant to natural killer cell activity. This may explain the differences in cytotoxicity to K562 and Raji among the three groups compared.

By using Raji as a target we showed clearly that mononuclear cells in carcinomatous pleural effusion have very weak cytotoxic activity. However, we cannot fully explain this finding from the

standpoint of susceptibility¹³⁾. We also investigated the production of soluble factors that affect PPD-enhanced cytotoxicity. It has been reported that the cytotoxicity of NK cells can be strongly augmented by either IFN¹⁾ or IL-2¹⁴⁾. We found that IFN production in peripheral blood was much higher in healthy controls than in either group of patients. We did not discover the reason for this, but it may be related to the fact that mononuclear cells are abundant in pleural effusion because of migration from peripheral blood to pleural space. Also, for both carcinomatous and tuberculous pleural effusions, we did not always observe consistent linear correlations between cytotoxicity and IFN or IL-2 production (numbering of patients is same in Table 1 and in Table 2). The

same tendency was evident in the peripheral blood of cancer and tuberculosis patients. Further, even the difference of IL-2 production in pleural effusion between cancer and tuberculosis patients was observed, the level of IL-2 production was very low. It seems that IFN is more essential than IL-2 for PPD-induced cytotoxicity. Bloom et al.¹⁵⁾ reported that, in healthy controls, NK cell activity in target binding cells was augmented by culturing with monocytes. Also, Uchida et al.¹⁶⁾ reported that presence of monocyte/macrophage and nylon-wool non adherent lymphocytes in carcinomatous pleural effusions reduced NK cell activity. Such findings may reflect factors that would explain the nonlinear relationship between PPD-induced cytotoxicity and the production of soluble mediators. Further, we observed that there was little difference of cytotoxicity between carcinomatous and tuberculous pleural effusion mononuclear cells when the pleural effusion mononuclear cells were cultured with either IFN or IL-2. It seems that low PPD-induced cytotoxicity of carcinomatous pleural effusion mononuclear cells is due to small amount of IFN and IL-2 production. However, we cannot explain low cytotoxicity of carcinomatous pleural effusion mononuclear cells by IFN and IL-2 production. Another factor may involve in PPD-induced cytotoxicity.

On the other hand, we have already reported that the effector cells derived from peripheral blood and pleural effusions were Leu 11+ cells in PPD-induced cytotoxicity. It is thought that Leu 11+ cells are activated through IFN and/or IL-2.

The results of this investigation showed impaired cytotoxic activity, particularly in carcinomatous pleural effusions. Further, immunological differences were clearly demonstrated between cancer and tuberculosis patients as well as peripheral blood and pleural effusion.

REFERENCES

- 1) Herberman RB, Hoden HH: Natural cell mediated immunity. *Adv Cancer Res* **27**: 305, 1978.
- 2) Dobloug JH, Forre O, Kvien TK, et al: Natural killer (NK) cell activity of peripheral blood, synovial fluid, and synovial tissue lymphocytes from patients with rheumatoid arthritis and juvenile rheumatoid arthritis. *Ann Rheum Dis* **41**: 490, 1982.
- 3) Okubo Y, Nakata M, Kuroiwa Y, et al: Phenotypic and functional study of natural killer cells in tuberculous pleurisy patients. *Jpn J Med* **25**: 250, 1986.
- 4) Agostini C, Trentin L, Zambello R, et al: Phenotypical and functional analysis of natural killer cells in sarcoidosis. *Clin Immunol Immunopathol* **37**: 262, 1985.
- 5) Baley JE, Schacter BZ: Mechanisms of diminished natural killer cell activity in pregnant women and neonates. *J Immunol* **134**: 3042, 1985.
- 6) Semenzato G, Agostini C, Zambello R, et al: Lung T cells in hypersensitivity pneumonitis: phenotypic and functional analyses. *J Immunol* **137**: 1164, 1986.
- 7) Okubo Y, Nakata M, Kuroiwa Y, et al: NK cells in carcinomatous and tuberculous pleurisy. Phenotypic and functional analyses of NK cells in peripheral blood and pleural effusions. *Chest* **92**: 500, 1987.
- 8) Kohase M, Kohno S, Yamazaki S, et al: The clinical potential of interferons: Potency standardization of human interferon preparation for clinical trials. p299, University of Tokyo Press, 1980.
- 9) Wolfe SA, Tracey DE, Henney CS: Induction of "natural killer" cells by BCG. *Nature* **262**: 584, 1976.
- 10) Wiltout RH, Mathieson BJ, Talmadge JE, et al: Augmentation of organ-associated natural killer activity by biological response modifiers. Isolation and characterization of large granular lymphocytes from the liver. *J Exp Med* **160**: 1431, 1984.
- 11) Kalland T, Alm G, Stalhandske T: Augmentation of mouse natural killer cell activity by 2616, a new immunomodulator. *J Immunol* **134**: 3956, 1985.
- 12) Salata RA, Kleinhenz ME, Schacter BZ, et al: Augmentation of natural killer cell activity by lipopolysaccharide through separable effects on the binding of non adherent lymphocytes to tumor target and tumor killing. *Cancer Res* **44**: 1044, 1984.
- 13) Storkus WJ, Howell DN, Salter RD, et al: NK susceptibility varies inversely with target cell class 1 HLA antigen expression. *J Immunol* **138**: 1657, 1987.
- 14) Henney CS, Kuribayashi K, Kern DE, et al: Interleukin-2 augments natural killer cell activity. *Nature* **291**: 335, 1981.
- 15) Bloom ET, Babbitt JT, Kawakami K: Monocyte-mediated augmentation of human natural killer cell activity: conditions, monocyte and effector cell characteristics. *J Immunol* **137**: 172, 1986.
- 16) Uchida A, Micksche M: Suppressor cells for natural killer activity in carcinomatous pleural effusions of cancer patients. *Cancer Immunol Immunother* **11**: 255, 1981.