Comparison between Tuberculous and Carcinomatous Pleurisy

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Tuberculous pleurisy is a good model for resolution of local cellular immunity. It would be expected that tuberculous pleural fluid contains a variety of immunologically important cytokines because of the accumulation of immunocompetent cells in the pleural cavity. We studied interleukin 1 (IL-1), interleukin 2 (IL-2), and interferon gamma (IFN-γ) levels in pleural fluid of 20 patients with tuberculous pleurisy and compared them with those in pleural fluid of 20 patients with malignant pleurisy. We also evaluated adenosine deaminase (ADA) levels in both effusions. Tuberculous pleural fluid had higher levels of IL-1, IL-2, IFN-γ, and ADA than malignant pleural fluid. Although the difference of IL-1 level between tuberculous and malignant pleural fluid was modest, that of IL-2, IFN-γ, and ADA was dominant. These findings suggest that activated T lymphocytes in tuberculous pleural fluid concern the production of lymphokines at the morbid site and they effectively exert local cellular immunity through the action of such lymphokines.

Cytokines, including interleukin 1 (IL-1), interleukin 2 (IL-2), and interferon gamma (IFN-γ) are key mediators of the host response to various infectious, inflammatory and immunologic challenges.

IL-1 has an essential role in T-cell activation. Two distinct polypeptides, IL-1α and IL-1β, mediate IL-1 biologic activities. The primary sources of IL-1 are blood monocytes and tissue macrophages. IL-1β is the major form produced by monocytes and macrophages. The biologic properties of IL-1 and its key role in inflammatory processes suggest its involvement in the pathogenesis of many diseases. Hence, assay for IL-1 is an important tool to study macrophage activation and to investigate the role of IL-1 in various immune and inflammatory processes.

IL-2 plays a crucial role in the mediation of the immune response. It induces and maintains the proliferation of T lymphocytes following mitogen or antigen activation. Moreover, it has been shown to induce production of cytotoxic lymphocytes, natural killer cells, and lymphokine-activated killer cells. IL-2 induces the expression of its own receptor in activated T-effector cells, and receptor binding also leads to proliferation. Then, IL-2 is a key element in clonal expansion of the T-cell population to mediate the immune response.

Although IFN-γ exhibits both antiviral and antineoplastic activities, it is also an important immunoregulator. IFN-γ is produced by T lymphocytes in response to stimulation with specific antigens or nonspecific mitogens, and is capable of modifying the response of other cells to the immune system. It is thus functionally a lymphokine.

These cytokines act synergistically with other cytokines, and the final biologic activity is the result of a network of interactions between these various mediators. The measurement of these cytokines has generally been achieved by a somewhat complicated biologic assay. The availability of specific and sensitive immunoassay for IL-1β, IL-2, and IFN-γ will therefore aid investigators studying these important cytokines.

Adenosine deaminase (ADA) activity is high in tuberculous pleural effusions. Percentages of CD3+ and CD4+ T cells were significantly greater in tuberculous effusion, and levels of ADA activity and percentages of CD4+ T cells in pleural exudates showed a positive correlation. Therefore, ADA could be a new marker of cell-mediated immune activity. We measured IL-1β, IL-2, IFN-γ, and ADA in pleural fluids of patients with tuberculous and carcinomatous pleurisy using a simplified radioimmunoassay (RIA) method or biochemical autoanalyzer and compared with bioassay method in case of IL-2 and IFN-γ.

METHODS

Pleural Fluids

Pleural fluids obtained from 20 patients with tuberculous pleurisy and 20 patients with carcinomatous pleurisy were frozen at −20°C until assayed. Diagnosis was made by positive pleural biopsy or bacteriologic or cytologic study of the pleural fluid. There were 17

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male and three female subjects among the patients with tuberculous pleural effusions and nine male and 11 female subjects with carcinomatous effusions. Patients with tuberculous effusions ranged from 17 to 86 years of age, and those with carcinomatous effusions ranged from 42 to 89 years of age. Cell numbers in seven tuberculous and five carcinomatous pleural effusions were counted, and they were 2,250 ± 720/cu mm and 1,650 ± 520/cu mm (mean ± SEM), respectively. There was no significant difference between them.

**IL-1β Assay**

One hundred microliters of unlabeled standard IL-1β or the pleural fluid sample was incubated with a rabbit anti-IL-1β antiserum (100 µl) during 20 hours at 4°C. The 125I-IL-1β (100 µl) was then added, the incubation being thereafter continued for 4 hours at room temperature. Anti-rabbit γ-globulin antiserum mixed with polyethylene-glycol (1 ml) was then added to all tubes. After incubation for 20 minutes at room temperature, all the complex was precipitated. The tubes were then centrifuged at 1,500 g for 15 minutes, the supernatants were discarded, and radioactivities of the precipitates were determined. A standard curve was constructed and the IL-1β concentrations of the pleural fluid were determined by dose interpolation from this curve. The assay was done using interleukin-1β (125I) RIA kit (Medgenix, Fleurus, Belgium).

**IL-2 Assay**

One hundred microliters of assay standard or pleural fluid sample was pipetted into tubes and incubated with an anti-IL-2 antiserum (100 µl) for 1 hour at room temperature. Then, 100 µl of 125I-labeled IL-2 was added and incubated for 3 hours at room temperature. The second antibody reagent (250 µl) was mixed and shaken gently to ensure a homogeneous suspension for 10 minutes at room temperature. The antibody bound fraction was separated by centrifugation for 10 minutes at 1,500 g. The supernatant was discarded and the radioactivity present in each tube was counted in a gamma scintillation counter. The concentration of unlabeled IL-2 in the sample was determined by interpolation from a standard curve. We used IL-2 125I assay system (Amersham, Buckinghamshire, UK).

In a biologic assay, the amount of IL-2 present in the pleural fluids was quantitated by measuring its activity using cloned, IL-2 dependent cytotoxic mouse T-cell line CTL N (kindly provided by Dr. Claudio Fiocchi, Cleveland Clinic Foundation, Cleveland, Ohio). Briefly, triplicate twofold serial dilutions of the pleural fluid (100 µl) were combined with 104 CTL N cells per well in round bottom microtiter plates and incubated for 18 hours at 37°C in a CO2 incubator. The cultures were then pulsed with 0.5 µCi [3H]-thymidine (sp act, 2 Ci/mmole; New England Nuclear, Boston, Mass) for 6 hours, harvested on glass fiber filters using a semiautomated cell harvester (MASH II, Microbiological Associates, Bethesda, Md), and counted for radioactivity in a liquid scintillation counter. A unit of IL-2 activity was defined as the inverse of the sample dilution yielding a counts per minute response that is 50 percent of the maximum response of a standard IL-2 solution, as determined by probit analysis. A recombinant IL-2 (Shionogi Pharmaceutical Co, Ltd, Osaka, Japan) was used as the standard IL-2.

**IFN-γ Assay**

Two hundred microliters of standard or pleural fluid sample was transferred into the assay tubes, then 50 µl of 125I-anti-IFN-γ monoclonal antibody was added into all assay tubes. The tubes were mixed and incubated for 2 hours at room temperature. The analyte 125I-labeled antibody complex was then immobilized by incubation with 50 µl of a sheep anti-IFN-γ antibody coupled to solid phase. The tubes were agitated for 2 hours at room temperature. Tubes were added with 1 ml buffer and 2 ml sucrose reagent and settled for 15 minutes. The tube contents were aspirated leaving approximately 0.3 ml in the bottom of each tube. Radioactivity in the assay tubes was counted using a gamma scintillation counter. Using the standard curve, IFN-γ concentrations for the samples were interpolated and expressed by units per milliliter. For IFN-γ immunoassay, a specific kit (Boots-Celltech Diagnostic Ltd, Berkshire, UK) was used.

The dye-uptake method using Fugh and Lund (FL) cells and Sindbis virus for a biologic assay has been described in detail elsewhere. In brief, serial dilutions of pleural fluid were combined with 5 x 10⁴ FL cells per well in flat bottom microtiter plates and incubated overnight. Then, medium in each well was discarded and infected with 100 TCID₅₀ of Sindbis virus. We used Sindbis virus instead of vesicular stomatitis virus in the present study. Several wells in each plate served as virus controls, cell controls, and standard interferon. Cells were observed for dye uptake when the virus controls showed complete destruction by the virus. For the optical density (OD) reading, each well was mixed with 100 µl of 0.02 percent neutral red solution for 2 hours, and then washed with phosphate-buffered saline (PBS) solution two times. OD was read at 550 nm after pouring into each well 100 µl of 50 percent ethanol containing 0.01N HCl. The highest dilution of the sample causing 50 percent protection of cells was considered the end-point. A recombinant IFN-γ (Takeda Pharmaceutical Co, Ltd, Osaka, Japan) was used as the standard.

**ADA Activity Assay**

ADA activity was measured by autoanalyzer using a reagent (purchased from Denkaseiken Co, Ltd, Tokyo, Japan). Briefly, ADA catalyses adenosine + H₂O to inosine + NH₃. Glutamic acid dehydrogenase then catalyses NH₃ + α-ketoglutaric acid to L-glutamic acid + H₂O with reduced nicotinamide-adenine dinucleotide phosphate (NADPH). Decrease of NADPH was estimated with the decrease of absorbance at 340 nm. Pleural fluid with hemolysis was omitted.

**Statistical Analysis**

Groups were compared by the Mann-Whitney rank sum test or the regression analysis.

**RESULTS**

IL-1β concentrations in pleural fluid from 20 patients with tuberculous pleurisy and 19 patients with carcinomatous pleurisy are shown in Figure 1. One pleural fluid sample from a patient with carcinomatous pleurisy was such a small amount that we could not measure the IL-1β level in this patient. Although IL-1β levels were significantly higher (p<0.05) in tuberculous pleural fluid (median 0.40 ng/ml, range 0.1 to 0.7 ng/ml) than in carcinomatous pleural fluid (median 0.30 ng/ml, range 0.2 to 0.6 ng/ml), the difference was modest.

IL-2 levels in 20 tuberculous pleural fluids were distributed between less than 0.8 U/ml and 6.7 U/ml and the median was 1.9 U/ml. Those in 20 carcinomatous pleural fluids ranged from less than 0.8 U/ml to 5.9 U/ml, with a median of <0.8 U/ml. There was a significant difference between the two groups (p<0.01). IL-2 levels in five (25 percent) tuberculous pleural fluid samples and 15 (75 percent) carcinomatous pleural fluid samples were less than the measurable limit (<0.8 U/ml) as shown in Figure 2. In a bioassay, pleural fluid samples were toxic for CTL N.
cells and we could not evaluate IL-2 levels.

Pleural fluid IFN-γ levels in 20 patients with tuberculous pleurisy were 73 U/ml in median and 1.5 to 410 U/ml in range, and those in 20 patients with carcinomatous pleurisy were less than 1.0 U/ml in median and less than 1.0 to 570 U/ml in range (Fig 3). There was a significant difference between the two groups (p<0.01). Fifteen (75 percent) samples in carcinomatous pleural fluid were less than 1.0 U/ml, which was the measurable limit, against none in tuberculous pleural fluid. In a bioassay, pleural fluid samples were not toxic for FL cells and we could evaluate IFN-γ levels. There was a significant correlation between IFN-γ levels in RIA and bioassay (r = 0.945, p<0.01). There was a significant correlation (p<0.01) between IL-2 and IFN-γ values in tuberculous effusions (Fig 4).

Pleural fluid ADA levels in 13 patients with tuberculous pleurisy were 56 IU/L in median and 16 to 108 IU/L in range, and those in 16 patients with malignant pleurisy were 13 IU/L in median and 6 to 53 IU/L in range (Fig 5). There was a significant difference between the two groups (p<0.01). There was no significant correlation between ADA levels and IFN-γ levels in pleural fluid.
DISCUSSION

Understanding of the local cellular immunity in the respiratory system is obtained from studies in which the response of peripheral lymphocytes is compared with that of cells obtained from bronchoalveolar lavage sample or pleural fluid. Cells obtained from these latter two sources presumably are representative of cells present at the site of inflammation.\(^9\) The lymphocyte is the predominant cell in tuberculous pleural effusions. The high percentage of T lymphocytes in these effusions is in good accordance with the idea that T-cell-dependent cell-mediated immunity plays an important role in the local pathogenic mechanisms in this disorder.

We have reported that tuberculous pleural fluid had more T lymphocytes than the peripheral blood, and that T lymphocytes in pleural fluid reacted to purified protein derivative (PPD) and produced higher levels of IFN-\(\gamma\) than those in peripheral blood.\(^9\) Our findings were confirmed by the report recently presented.\(^1\) These observations suggest that exudative-sensitized lymphocytes in morbid sites reacted to the specific antigen more effectively and produced higher titers of lymphokines than circulating lymphocytes. We have also indicated that at least the CD4\(^+\)/CD8\(^-\) T-cell subset is responsible for the antigen-specific IFN-\(\gamma\) production in pleural T lymphocytes of patients with tuberculous pleurisy.\(^8\) In addition, we have observed that peripheral blood monocytes and pleural fluid macrophages from patients with tuberculous pleurisy released more IL-1 than peripheral blood monocytes from normal controls when stimulated with PPD (no published data). We have also recognized that T lymphocytes in tuberculous pleural fluid produced a higher level of IL-2 than in peripheral blood using PPD as an antigen (no published data).

The observation that cytokines, including IL-1, IL-2, and IFN-\(\gamma\), could be produced by pleural fluid immunocompetent cells \textit{in vitro} on an immune-specific basis suggested that they also might be produced \textit{in vivo} in tuberculous pleurisy. The results of the present study clearly show that tuberculous pleural effusions contain significantly higher levels of IL-1, IL-2, and IFN-\(\gamma\) than carcinomatous pleural effusions, although the difference of IL-1 levels is modest. Our observation agrees in part with the report by Ribera.
et al., who studied IFN-γ levels in pleural fluid and demonstrated high IFN-γ concentrations in tuberculous pleural effusions. Patients with malignant pleural effusions, nonspecific pleural effusion, parapneumonic effusions, and pleural transudates, they reported, had low levels of IFN-γ. However, little is known about IL-1 and IL-2 levels in pleural fluid.

The mechanism of the lymphoproliferative effect of the macrophage product IL-1 appears to be mediated by the stimulation of the release of IL-2 by T cells. The magnitude of the resultant T-cell proliferative clonal expansion is thus dependent on the quantity of both IL-1 and IL-2 induced by antigen or lectin stimulation. Fujitwara et al. reported that monocytes from patients with tuberculosis produced significantly higher activities of IL-1 than did those from healthy tuberculin reactors when stimulated with lipopolysaccharide or PPD. We expected that macrophages in tuberculous pleural fluid would produce higher levels of IL-1 than those in carcinomatous pleural fluid and that tuberculous pleural fluid would contain higher levels of IL-1. There was a significant difference of IL-1 levels between tuberculous and carcinomatous pleural effusions, but the difference was small and there was complete overlap in the pleural fluid IL-1 levels between the two groups. It has been reported that membrane-associated IL-1 in macrophages is sufficient for the antigen-presenting capacity and no IL-1 secretion is needed. Therefore, the difference of IL-1 levels in pleural effusions could have been small between both pleural fluids studied.

As for IL-2 levels, the difference between tuberculous and malignant pleural fluids was greater than in IL-1 levels. In fact, pleural fluid from patients with tuberculous pleurisy showed higher frequencies of PPD-reactive T lymphocytes than peripheral blood from the same patients or tuberculin-positive healthy control subjects by using a limiting dilution assay. Kasahara et al. reported that human IL-2 induced peripheral T lymphocytes to produce IFN-γ. The fact that IL-2 contributes to the production of IFN-γ by human lymphocytes in addition with IFN-γ production by antigenic stimulation may explain the high level of IFN-γ in tuberculous pleural effusion. IFN-γ, in turn, activates macrophages, increasing their bactericidal capacity against Mycobacterium tuberculosis. Thus, local cellular immunity at the morbid sites is established effectively in tuberculous pleurisy.

Pleural tuberculosis has become a disease of older adults. This shift in age has led to problems in diagnosis, since many of these older patients have suffered from underlying or coexisting diseases that could have caused a pleural effusion. An elevated ADA level has been shown to be specific and sensitive in identifying tuberculous pleurisy. ADA has a crucial function in the differentiation and/or proliferation of lymphoid cells. We reconfirmed that ADA levels in tuberculous pleural effusions are high, but we could not find a significant correlation between ADA and IFN-γ levels in such effusions. Therefore, some different mechanism of the production of these two factors might be present in activated T lymphocytes.

REFERENCES