CYTOKINES AND LOCAL CELLULAR IMMUNITY

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ABSTRACT

The characteristics and function of human lymphocytes at a morbid site were studied. Exudative-sensitized lymphocytes in tuberculous pleural fluid reacted to the specific antigen more effectively and produced higher titers of lymphokines including interferon γ (IFN-γ) and interleukin 2 (IL-2) than circulating lymphocytes. CD4$^+$/CD8$^-$ T-cell subset is responsible for the antigen-specific IFN-γ production in pleural T-lymphocytes of patients with tuberculous pleurisy. Thus, activated T-lymphocytes involve the production of lymphokines at the morbid site and they effectively exert local cellular immunity through the action of such lymphokines. Immunofluorescence study showed an increased production of inducible nitric oxide synthase (iNOS) and peroxynitrite in bacille de Calmette-Guérin (BCG)-inoculated human alveolar macrophages (AM). Reverse transcriptase-polymerase chain reaction methods also revealed the higher expression of iNOS-coding mRNA. Colony assay demonstrated that human AM effectively killed BCG in their cytoplasm. However, treatment of AM with N^G-monomethyl-L-arginine monoacetate resulted in markedly reduced killing activity. These results clearly show that BCG-induced nitric oxide (NO) and its reactive product with the oxygen radical, peroxynitrite, could play an important role in BCG killing in human AM.

Key Words: Interferon gamma, Interleukins, Cellular immunity, Tuberculosis, Nitric oxide

INTRODUCTION

Although the local immunity mediated by secretory IgA has been well known in the respiratory and digestive systems, that based on cellular immunity has not been elucidated in human diseases. This situation might be due to the difficulty of analyzing localized cellular immunity in human disorders. Understanding of the local cellular immunity in the respiratory system is obtained from studies in which the response of peripheral lymphocytes is compared to that of cells obtained from a bronchoalveolar lavage sample or pleural fluid. Cells obtained from these latter two sources presumably are representative of cells present at the site of inflammation (Fig. 1).

Tuberculous pleurisy is restricted to the pleural cavity, and profuse fluid, which contains numerous immunocompetent cells, is easily obtained. Moreover, the purified protein derivative (PPD) of tuberculin is easily obtainable as the specific antigen. The lymphocyte is the predominant cell in tuberculous pleural effusions. The high percentage of T-lymphocytes in these effusions accords well with the idea that T-cell-dependent cell-mediated immunity plays an important role in the local pathogenic mechanisms in this disorder.

In 1966, Glasgow[1] provided the first direct evidence that immunity and immune mechanisms are associated with interferon production. Subsequent experiments demonstrated that non-viral antigen (PPD) stimulated interferon production in cultures of lymphocytes from sensitized
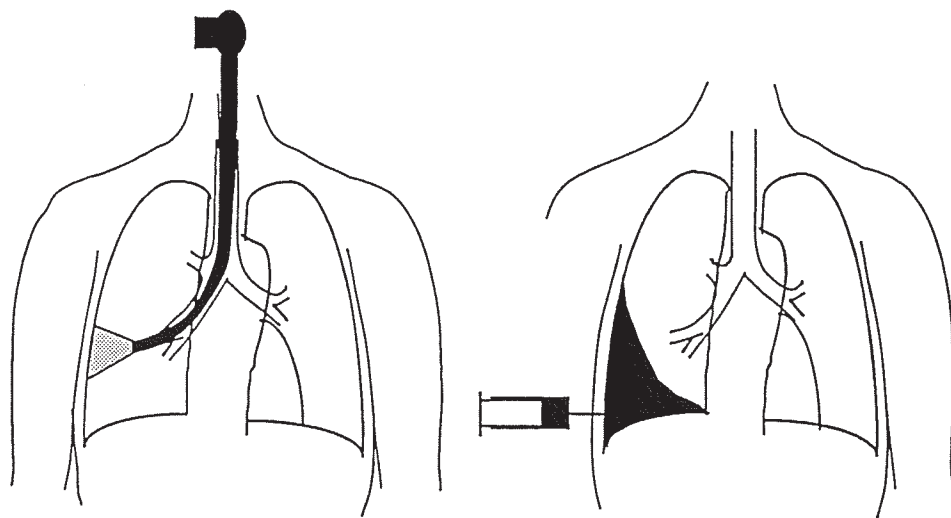
Fig. 1.   Approach to the Morbid Site

donors but not in cultures from donors who lacked sensitivity to this antigen.[2] These results suggested that the induction of interferon may be an essential part of the immune response. When lymphocytes sensitized with tubercule bacilli in patients with tuberculous pleurisy again encounter PPD, they may produce lymphokines, one of which is immune interferon, that is, interferon γ (IFN-γ).[3] Immune interferon may be appropriate for the estimation of sensitized lymphocyte function and, as we reported previously, useful as a quantitative indicator of cell-mediated immunity.[4]

THE RELATIONSHIP OF T-LYMPHOCYTES IN PLEURAL EFFUSIONS AND PERIPHERAL BLOOD TO INTERFERON PRODUCTION

The proportion of T- and B-lymphocytes in exudates relative to that in the peripheral blood may point to the presence of local immunologic phenomena in various pulmonary and pleural disorders. In tuberculous pleural effusions, both the proportion and the absolute number of T-lymphocytes were significantly higher than those in peripheral blood, which justifies some associations with the events in the cell-mediated immune response.[5]

Study of the percentage of T-lymphocytes in tuberculous pleural effusions and peripheral blood revealed the dominance of T-lymphocytes in the pleural effusions.[6] When lymphocytes in peripheral blood were cocultured with PPD, they produced IFN-γ; however, the titer was very low. When lymphocytes in the pleural effusions were cocultured with PPD, they reacted to PPD and produced IFN-γ. Titers were much higher than those of lymphocytes in peripheral blood.[6]

These findings appeared consistent with compartmentalization of specifically tuberculin-reactive lymphocytes in the pleural fluid with depletion of such cells from the circulating pool.[7] However, in view of the demonstration of suppressor adherent cells in the peripheral blood of anergic patients with pulmonary tuberculosis,[7–9] it is possible that potentially tuberculin-responsive T-lymphocytes continue to circulate, but their function is masked by suppressor cells.[7] It is

well known that when sensitized T-lymphocytes again encounter the specific antigen they show incorporation of ^{3}H-thymidine[10] or produce lymphokines. In fact, lymphocytes in pleural effusions cocultured with PPD produced far more immune interferons than did peripheral blood lymphocytes.[6] The compartmentalization of specifically tuberculin-reactive lymphocytes in the pleural fluid of patients with tuberculous pleurisy may indicate the local cellular immunity at the morbid site.

It would be very interesting to know which T-cell subset produces IFN-γ. The development of monoclonal antibodies reactive to cell surface antigens of human lymphocytes made it possible to identify the T-cell subsets with discrete functions.[11–14] Lymphocytes in the pleural effusions were treated with monoclonal antibodies against the human T-lymphocyte surface antigen and its complement. Treatment of lymphocytes with the CD4^{+} monoclonal antibody and its complement resulted in a significant decrease in the titers of IFN-γ, while treatment with the CD8^{+} monoclonal antibody and its complement did not affect the IFN-γ titers significantly. Therefore the production of IFN-γ derives not from CD4^{-}/CD8^{+} but from CD4^{+}/CD8^{-} lymphocytes.[15]

PPD-INDUCED IL-1 AND IL-2 ACTIVITIES

Blood monocytes or pleural fluid macrophages acquired from patients with tuberculous pleurisy showed significantly more IL-1 activity than blood monocytes from normal subjects when stimulated with PPD.[16] Purified tuberculous peripheral blood and pleural fluid T-lymphocytes were reconstituted with autologous purified blood monocytes and pleural fluid macrophages, respectively. The supportive effects of tuberculous blood monocytes and pleural fluid macrophages involved with PPD-induced IL-2 activity were compared in this attempt, in which both types of accessory cells were added to autologous blood and pleural fluid T-lymphocytes. Supernatants of reconstituted cells, which were cocultured with PPD, showed an increase of IL-2 activity. The combination of pleural fluid T-lymphocytes and pleural fluid macrophages appeared to be the most effective. Conversely, the supernatants acquired from combination of blood T-lymphocytes and blood monocytes showed the lowest IL-2 activity. The other two combinations ranked in between.[16]

Tuberculous pleural fluid macrophages promoted PPD-induced IL-2 activity of autologous T-lymphocytes more effectively than autologous blood monocytes, while there was no significant difference in the IL-1 activity of the macrophages and monocytes in the patients. Therefore, factors other than IL-1 may also contribute to IL-2 production in T-lymphocytes. Suppressive adherent cells may be present, for example, and suppressive factors may be released in greater amounts by monocytes than by macrophages.[8,17]

It is well known that T-lymphocytes play a major role in the defense of intracellular organisms. Both the proportion and the absolute number of T-lymphocytes in tuberculous pleural effusions were reported to be significantly higher than in peripheral blood,[5] and specific antigen PPD-reactive lymphocytes preferentially sequestrated in the pleural space.[18] When T-lymphocytes in tuberculous pleural effusions were cocultured with PPD, they showed a higher proliferative response to PPD[18] and produced far more immune interferons than autologous blood lymphocytes.[6] Sequestration of PPD-reactive T-lymphocytes in the pleural space in patients with tuberculous pleurisy seems to be critical for the defense of the lungs against invading acid-fast bacilli. In these cases, pleural fluid macrophages probably act more effectively as accessory cells than autologous blood monocytes and contribute to active local cellular immunity at the morbid site through the stimulation of PPD-reactive T-lymphocytes.

CYTOKINE CONTENTS IN TUBERCULOUS PLEURAL EFFUSIONS

The observation that cytokines, including IL-1, IL-2 and IFN-γ, could be produced by pleural fluid immunocompetent cells *in vitro* on an immune-specific basis suggested that they might also be produced *in vivo* in tuberculous pleurisy. Although IL-1β levels were significantly higher in tuberculous pleural fluid than in carcinomatous pleural fluid, the difference was modest. IL-2 levels in tuberculous pleural fluids were significantly higher than those in carcinomatous pleural fluids. There was a significant difference between the pleural fluid IFN-γ levels in patients with tuberculous pleurisy and those in patients with carcinomatous pleurisy.[19] Thus, tuberculous pleural effusions contain significantly higher levels of IL-1, IL-2 and IFN-γ than carcinomatous pleural effusions, although the difference in IL-1 levels is modest. This result agrees in part with the results of another report, which studied IFN-γ levels in pleural fluids and reported high IFN-γ concentrations in tuberculous pleural effusions.[20] Patients with malignant pleural effusions, nonspecific pleural effusions, parapneumonic effusions, and pleural transdates had low levels of IFN-γ in this study.[20]

The mechanism causing the lymphoproliferative effect of the macrophage product IL-1 appears to be mediated by the stimulation of the the 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.[21] Monocytes from patients with tuberculosis produced significantly higher IL-1 activity than did those from healthy tuberculin reactors when stimulated with lipopolysaccharide or PPD.[17] 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 in IL-1 levels between tuberculous and carcinomatous pleural effusions, but the difference was small and there was a complete overlap in the IL-1 pleural fluid levels between the two groups.[19] It has been reported that membrane-associate IL-1 in macrophages is sufficient for the antigen-presenting capacity and that no IL-1 secretion is needed.[22] Therefore, the difference in 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 that observed in IL-1 levels.[19] In fact, pleural fluids from patients with tuberculous pleurisy showed higher frequencies of PPD-reactive T-lymphocytes than the peripheral blood from the same patients or tuberculin-positive healthy control subjects analyzed with a limiting dilution assay.[23] Human IL-2 induced peripheral T-lymphocytes could produce IFN-γ.[24] The fact that IL-2 contributes to the production of IFN-γ by human lymphocytes in addition to IFN-γ production by antigenic stimulation may explain the high level of IFN-γ in tuberculous pleural effusions. IFN-γ, in turn, activates macrophages, increasing their bactericidal capacity against *Mycobacterium tuberculosis*. Thus, local cellular immunity at the morbid site is established effectively in tuberculous pleurisy.

INTRACELLULAR KILLING OF BCG IN HUMAN ALVEOLAR MACROPHAGES

Macrophages play an important role in the first and essential line of defense against mycobacteria.[25] Several observations about the antimicrobial functions of macrophages have emphasized their ability to produce reactive nitrogen radicals[26,27] as well as reactive oxygen radicals[28] and various cytokines[29,30] in rodent immune systems. However, experiments performed with

human macrophages suggested little or no nitric oxide (NO) production in macrophages[31–33] and a different response to stimuli as compared with rodent macrophages.[32,34] The role of reactive NO in human monocytes/macrophages remains a matter of considerable debate.[32]

In our experiments, activated human alveolar macrophages (AM) collected from bronchoalveolar lavage (BAL) showed a marked increase in intracellular killing activity against bacille de Calmette-Guerin (BCG).[35,36] When N^G-monomethyl-L-arginine (L-NMMA) which is an analogue of L-arginine was added to human AM cultures, it caused almost complete inhibition of the intracellular killing activity against BCG, suggesting that NO products assessed by the L-arginine-dependent pathway play an important role in intracellular BCG killing in activated human AM. Inducible NO synthase (iNOS) mRNA expression of activated AM was significantly increased after inoculation with BCG. Furthermore, in an immunofluorescent stain study using the anti-human iNOS antibody, the iNOS expression was detected only in BCG-inoculated AM. These results indicate that BCG-inoculated AM, which had been activated *in vivo*, could induce both mRNA and protein expression in iNOS, which in turn may be associated with enhanced NO production.[36] Peroxynitrite, a reaction product of NO and super oxide,[37] is known to react with phenolic rings of tyrosine to produce nitrotyrosine.[38] The expression of nitrotyrosine confirms the production of peroxynitrite, which itself could be a strong bactericidal agent.[39] The immunofluorescent staining with the anti-nitrotyrosine monoclonal antibody showed a high expression of nitrotyrosine in BCG-inoculated AM which had been activated *in vivo*. These findings indicate the production of peroxynitrite and its source, NO, in human AM.[36]

Previous reports demonstrated that IFN-γ and reactive nitrogen intermediates (RNI) are important mediators or final effective molecules against the intracellular bactericidal mechanism in
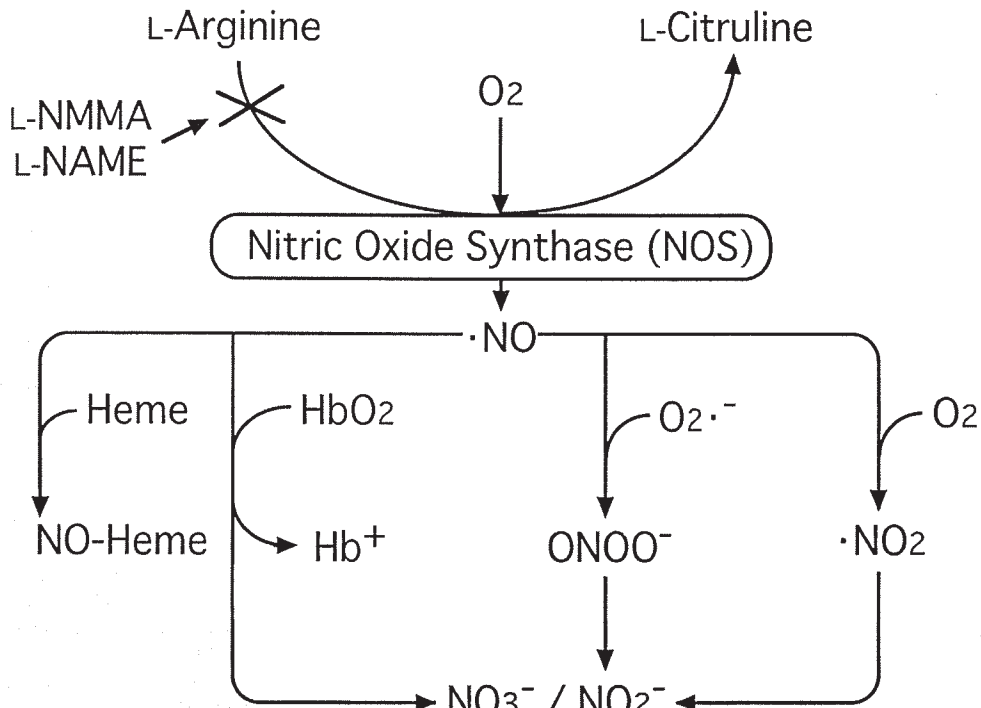

Fig. 2.   Nitric Oxide Pathway

rodent species.[26] The contribution of reactive oxygen intermediates (ROI) such as super oxide or hydroxyl radical remains unclear. ROI are considered to be insufficient to kill mycobacteria, while the combination of RNI and ROI could be enough to kill mycobacteria.[37,39] As for human macrophages, the final effecter molecules remain a matter of considerable debate. Although we could demonstrate the production of NO from human AM as well as rodent AM, the mechanism of iNOS activation may be different in the human and rodent systems.[32] IFN-γ and the tumor necrosis factor (TNF) have been reported to be potent activators of iNOS,[40] while interleukin-4 (IL-4), IL-10, and transforming growth factor-β suppress this enzyme activity in rodent macrophages.[41] However, there is a lack of response to NO production induced by cytokines such as IFN-γ, TNF, and the granulocyte-macrophage colony-stimulating factor in human macrophages.[42]

Although there have been controversial reports about the NO-dependent cytotoxic pathway in the human system, previous investigators suggested that human macrophages might require additional signals for iNOS induction[43,44] or that human cells might produce autocrine inhibitors or toxic molecules which suppress iNOS induction[41] in higher amounts than do rodent cells. We had previously reported that human AM from patients with a positive PPD skin test could kill BCG in vitro more effectively compared with AM from patients with a negative PPD skin test, and that these two categorized AM had different responses to stimulation by IFN-γ.[35] Macrophages pre-activated in vivo by various kinds of inflammatory mediators or cytokines, could more easily produce RNI following inoculation with BCG.

More effective use of the L-arginine-dependent mycobactericidal pathway would provide us with a new immune-therapeutic means to contain drug resistant mycobacterial infections (Fig. 2).

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