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Increase in Tumor Necrosis Factor Alpha- and Interleukin-6-Secreting Cells in Peripheral Blood Mononuclear Cells from Subjects Infected with *Mycobacterium tuberculosis*

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We detected and quantified tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) from monocytes/macrophages (Mφ) in the peripheral blood of subjects from three different population groups, i.e., tuberculin-negative healthy subjects, tuberculin-positive healthy subjects, and patients with active pulmonary tuberculosis. TNF-α or IL-6 activity in the culture supernatant of these cells was determined by the cytotoxicity of murine L-929 cells or by enzyme-linked immunosorbent assay, respectively. Detection and enumeration of cells secreting either TNF-α or IL-6 were performed by an adaptation of the enzyme-linked immunospot assay. Monocytes/Mφ from tuberculin-positive healthy subjects or patients with tuberculosis showed higher TNF-α- and IL-6-producing activities than those from tuberculin-negative healthy subjects. The number of TNF-α- and IL-6-secreting cells in either lipopolysaccharide- or muramyl dipeptide-stimulated mononuclear cells from tuberculin-positive healthy subjects and patients was significantly higher than that in cells from the tuberculin-negative healthy subjects.

Cellular immune reaction plays an important role in tuberculous infection, a chronic granulomatous disease. When cellular immunity develops, a large number of activated macrophages (Mφ) are accumulated in the tuberculous foci because of the chemotactic effect of certain lymphokines, and these Mφ actively phagocytize and suppress the multiplication of tubercle bacilli (12). On the other hand, many Mφ are killed during the process of phagocytosis, thus releasing large amounts of lysosomal enzymes, superoxide and hydrogen peroxide, neutral proteases, and cytotoxic substances such as tumor necrosis factor (TNF), which may cause tissue damage, e.g., caseous necrosis and softening and liquefaction, followed by cavity formation (4).

Mφ can be activated in the inflammatory processes in response to infection with *Mycobacterium tuberculosis* and other related *Mycobacterium* species, and they synthesize and release various cytokines, including interleukin-1 (IL-1), TNF, interferon, IL-6, and IL-8 (1, 2, 9, 16). These cytokines are regarded as inflammatory mediators, and they may be important modulators of local and systemic biological responses. Mycobacterial proteins, in addition to live organisms, have been reported to induce IL-1 and TNF in monocytes/Mφ (17, 20). However, no attempts to measure actual numbers of cells producing these cytokines with reference to the status of mycobacterial infections have been made. In this report, we demonstrate the presence of increased amounts of TNF-α and IL-6 in monocytes/Mφ from the peripheral blood of subjects infected with tuberculous bacilli by using a newly developed method.

MATERIALS AND METHODS

Reagents. Muramyl dipeptide (MDP) was kindly supplied by Daiichi Seiyaku Co., Ltd., Tokyo, Japan. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 was a product of List Biological Laboratories, Campbell, Calif. Human recombinant TNF-α (rTNF-α; specific activity, 3×10^6 U/mg) was a generous gift from Dainippon Pharmaceutical Co., Osaka, Japan. Human recombinant IL-6 (rIL-6; specific activity, 10^7 U/mg) and mouse anti-IL-6 monoclonal antibody were kindly provided by T. Kishimoto and T. Hirano, Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka, Japan.

Subjects. Three population groups were sampled; these groups included a tuberculosis group composed of 12 patients with recently diagnosed pulmonary tuberculosis, a healthy control group composed of 13 normal subjects with a positive tuberculin test, and another healthy control group composed of 11 normal subjects with a negative tuberculin test. The tuberculin test was done by intradermal injection of 0.05 μg of purified protein derivative (Nippon BCG Products Co. Ltd., Tokyo, Japan) into these subjects. The positive tuberculin test was defined as appearance of induration with a diameter of ≥10 mm 48 h after injection of purified protein derivative. The patients with tuberculosis were diagnosed on the basis of an abnormal chest X-ray radiograph, positive cultivation of *M. tuberculosis* from sputum, and febrile responses. They had been treated with drug regimens including streptomycin, isoniazid, ethambutol, or rifampin for ca. 50 days, and they showed no significant febrile responses when their blood samples were taken. The characteristics of the three experimental groups of subjects are summarized in Table 1.

Preparation of monocyte/Mφ culture supernatant. Heparinized venous blood drawn from each subject was fractionated by a modification of the Ficoll-Hypaque method (5), to obtain mononuclear leukocytes (MNL). A cell suspension containing 1.5×10^6 cells per ml of Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) was added to a 96-well plastic plate and incubated for 2 h at 37°C in 5% CO_2–95% air. After the plate was washed to remove the nonadherent cells, the adherent

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TABLE 1. Profiles of patients and control populations sampled for this study[a]

Subject group (*n*)	Age (yr) (mean ± SE)	Sex (male/female)	Mo of medication (mean ± SE)	BCG vaccination	Tuberculin test result
Tuberculosis (12)	30 ± 4	6/6	1.7 ± 0.5	Variable	Positive
Healthy control (13)	26 ± 2	8/5		Yes	Positive
Healthy control (11)	22 ± 2	6/5		No	Negative

[a] See Materials and Methods for details.

cells were supplemented with MEM containing 10% FCS with or without various concentrations of MDP or *E. coli* LPS. Monocytes made up 85 to 90% of the cells as determined by morphology and nonspecific esterase staining. The culture supernatant was collected after incubation at 37°C for 16 h.

TNF-α assay. TNF-α titers were determined by measuring cytolytic activity against murine fibroblastic L-929 cells, as described previously (11), with a slight modification. Aliquots (100 μl) of L-929 cell suspension (5×10^5/ml) were pipetted into 96-well flat-bottom microtiter plates (type 3072; Falcon, Becton Dickinson Labware, Lincoln Park, N.J.) and incubated for 24 h to allow adherence. Actinomycin D (2 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and dilutions (100 μl) of the monocyte supernatant or various concentrations of human rTNF-α were added to L-929 cells in MEM with 10% FCS to obtain a standard curve. After incubation at 37°C for 24 h, the supernatant was discarded and the cells were fixed with 5% formaldehyde and stained with 0.2% crystal violet. The stained plate was washed and dried. Viable cells retained the dye. The A_{595} of each well was read with a Titertek Multiskan MC photometer. Values obtained from triplicate wells were averaged to obtain the mean values. The TNF-α titer in a culture supernatant was calculated from the standard curve obtained by using rTNF-α and was expressed as the mean number of TNF-α units ± standard error of each subject group.

IL-6 assay. The IL-6 assay was an enzyme-linked immunosorbent assay (ELISA) (19). Briefly, 96-well ELISA plates (M129; Dynatech Laboratories Ltd., Billingshurst, United Kingdom) were coated with 100 μl of monoclonal antibody specific for IL-6 (1 μg/ml) at 4°C overnight in 0.1 M carbonate buffer (pH 9.6). The wells were then blocked with 100 μl of 1% bovine serum albumin–phosphate-buffered saline (PBS) at 4°C overnight and washed with PBS containing 0.05% Tween 20 (PBS-T). Test samples were added to each well. After overnight incubation at 4°C, the wells were washed with PBS-T, and rabbit anti-IL-6 antibodies (10 ng in 100 μl of PBS; Genzyme Corp., Boston, Mass.) biotinylated with NHS-X-Biotin (Pierce, Rockford, Ill.) were added. The plates were incubated at 4°C overnight and washed with PBS-T. Next, ABC-AP kit reagent (alkaline phosphatase standard AK-5000, Vector Laboratories, Burlingame, Calif.) was added to each well, and the A_{405} was read with a Titertek Multiskan MC photometer. Values obtained from triplicate wells were averaged to obtain the mean values and their standard errors. IL-6 titers in culture supernatants were determined with a standard IL-6 curve under the assay conditions described above, and titers were expressed as the mean number of IL-6 units ± standard error of each subject group.

Neutralization of TNF-α or IL-6 by anti-TNF-α or anti-IL-6 antibody. The culture supernatants were serially diluted twofold, and an equal volume of diluted mouse monoclonal antibody to human rTNF-α (clone E43; Olympus Immunochemicals, Tokyo, Japan), which neutralized TNF-α activity of 10^7 U/ml, or rabbit anti-rIL-6 antibody (Genzyme), which completely neutralized 10,000 U of human rIL-6 per ml, was added to each diluent. The mixtures were incubated at 37°C for 2 h before being assayed for TNF-α or IL-6 activity as described above.

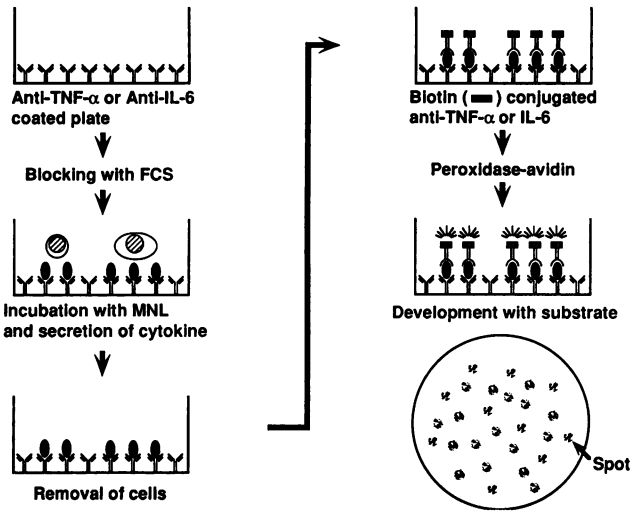

FIG. 1. Diagram of ELISPOT method for enumeration of specific cytokine-secreting cells among MNL (~10^4 to 10^6 per well). Individual wells were coated with monoclonal anti-TNF-α or anti-IL-6 antibody and incubated with MNL for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. After being washed with PBS-T to remove the cells, the plates were incubated with biotinylated anti-TNF-α or anti-IL-6. Spots were developed with avidin-peroxidase by using 3-amino-9-ethylcarbazole as the substrate.

ELISPOT assay for cytokine-secreting cells. An adaptation of the enzyme-linked immunospot (ELISPOT) assay originally developed by Czerkinsky et al. (8) was used to enumerate TNF-α- or IL-6-specific spot-forming cells (SFC) as outlined in Fig. 1. To detect individual cytokine-secreting cells, 96-well nitrocellulose-based plates (Millititer HA; Millipore Corp., Bedford, Mass.) were coated with monoclonal anti-TNF-α (clone E43; Olympus Immunochemicals) or anti-IL-6 antibody at a concentration of 1 or 10 μg/ml (100 μl per well), respectively. Control wells were coated with PBS containing 10% FCS. All wells were then blocked with MEM containing 10% FCS overnight at 4°C. Peripheral blood MNL (10^4 to 10^6 cells per 100 μl per well) stimulated with or without MDP or *E. coli* LPS (10 μg/100 μl per well) were added to individual wells and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The plates were thoroughly washed with PBS-T to remove the cells, and then the plates were incubated overnight at 4°C with 100 μl of rabbit anti-TNF-α (diluted 1:50) or anti-IL-6 (diluted 1:500) antibody biotinylated with NHS-X-Biotin (Pierce). After being washed, the plates were incubated with avidin-peroxidase (diluted 1:500; 100 μl per well) in PBS-T. Spots representing single cytokine-secreting cells were developed with a 3-amino-9-ethylcarbazole substrate (Aldrich Chemical Co., Milwaukee, Wis.) in 0.05 M sodium acetate buffer (pH 5.0). The spots were counted with the aid of a dissecting microscope at a magnification of ×150, and the results were expressed as the number of cytokine-forming spots per 10^6 MNL.

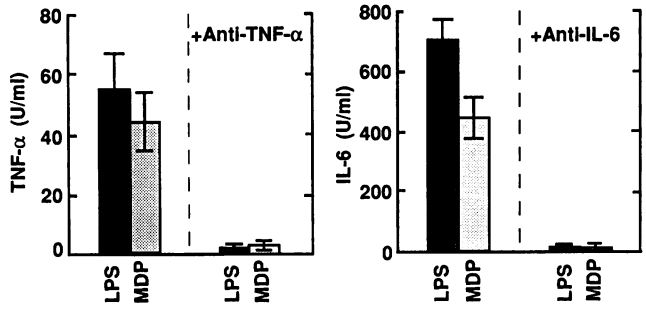

FIG. 2. Neutralization of TNF-α and IL-6 activity in the culture supernatant of LPS- or MDP-stimulated peripheral blood monocytes/Mϕ. Aliquots of the serially diluted culture supernatant were mixed with monoclonal anti-TNF-α or anti-IL-6 antibody and incubated at 37°C for 2 h before being assayed for TNF-α or IL-6 activity.

Statistics. Comparisons between groups were done by Student's *t* test for independent samples.

RESULTS

The specificity of antibodies to TNF-α and IL-6 was demonstrated by the neutralization of TNF-α and IL-6 activity in the culture supernatant of LPS- or MDP-stimulated peripheral blood monocytes/Mϕ. The activity of these cytokines was found to be almost completely neutralized by rabbit anti-human rTNF-α or rabbit anti-human rIL-6 antibody, respectively (Fig. 2).

We then developed a sensitive and specific system for the detection of individual cytokine-producing human cells which secrete either TNF-α or IL-6. The specificity of the spot developed was confirmed as follows. Active secretion specific for TNF-α in the ELISPOT assay was demonstrated by the addition of cycloheximide (25 μg/ml), which reduced the number of SFC by 70 to 95%. Preincubation of rabbit anti-TNF-α or anti-IL-6 antibody with the MNL culture before addition of biotinylated antibody specific for either cytokine in the ELISPOT assay reduced the development of spots by 75 to 97%, whereas normal rabbit immunoglobulin G had no effect (data not shown).

Figure 3 shows that LPS- or MDP-stimulated monocytes/Mϕ from patients with pulmonary tuberculosis and tuberculin-positive healthy subjects released higher levels of TNF-α and IL-6 than those from tuberculin-negative healthy subjects. No significant levels of TNF-α or IL-6 activity were detected in the culture supernatant of unstimulated monocytes/Mϕ from either tuberculin-positive or tuberculin-negative subjects. Furthermore, the monocytes/Mϕ from patients with tuberculosis produced more TNF-α and IL-6 than those from tuberculin-positive healthy subjects. Maximum TNF-α and IL-6 activity occurred at LPS and MDP concentrations of 10 and 100 μg, respectively.

Activated monocytes/Mϕ from tuberculin-positive subjects with LPS or MDP induced higher numbers of cytokine-secreting cells than inactivated controls in all population groups (Fig. 4). The number of SFC that produced cytokines in either LPS- or MDP-stimulated MNL from tuberculin-positive healthy subjects and patients with tuberculosis was 3,300 to 3,600 TNF-α-specific SFC per 10^6 MNL and 4,100 to 4,600 IL-6-specific SFC per 10^6 MNL. However, no significant difference in the number of cytokine-secreting cells from patients with tuberculosis and tuberculin-positive

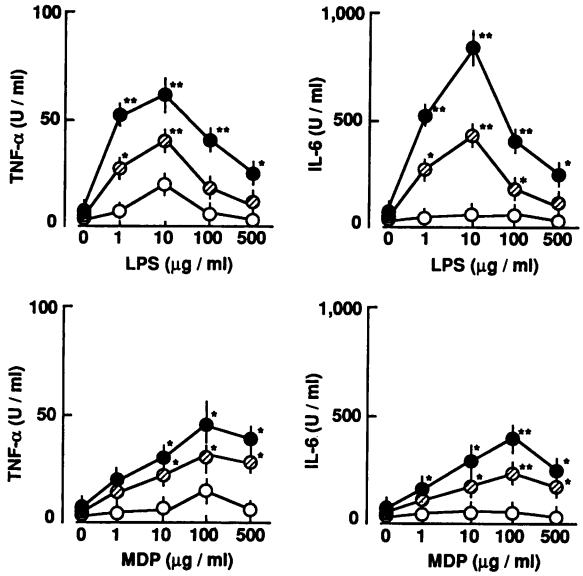

FIG. 3. Induction of TNF-α and IL-6 in monocytes/Mϕ from patients with tuberculosis (●), tuberculin-positive healthy subjects (⊘), and tuberculin-negative healthy subjects (○). The amounts of cytokines produced were determined by TNF-α or IL-6 assay, respectively. Values (mean ± standard error) are expressed as units per milliter of culture supernatant. Symbols: * and **, statistical difference from the value for the tuberculin-negative subject group with or without stimulation of LPS or MDP at $P < 0.05$ and $P < 0.01$, respectively.

healthy subjects was noted. In contrast, the numbers of TNF-α- and IL-6-secreting SFC in MNL from tuberculin-negative healthy subjects were markedly lower than the numbers of these SFC in MNL from tuberculin-positive healthy subjects (Fig. 4).

DISCUSSION

We have clearly demonstrated here that the monocytes/Mϕ of tuberculin-positive subjects produced enhanced levels of TNF-α and IL-6 when stimulated with either MDP or LPS. On the other hand, monocytes/Mϕ from tuberculin-negative healthy subjects released TNF-α but not IL-6 upon stimulation by LPS or MDP. We also found that not only *E. coli* LPS but also synthetic lipid A and its analogs stimulated human monocytes/Mϕ of tuberculin-positive and -negative subjects to exhibit TNF-α activity (data not shown). It is of interest to note that both MDP and LPS are parts of bacterial cell structural components and that infection with *M. tuberculosis* appears to enhance the ability of monocytes/Mϕ to synthesize certain cytokines such as TNF-α and IL-6. In this regard, Klimpel et al. (10) have recently reported that fibroblasts infected with bacteria such as *Shigella*, *Salmonella*, or *Listeria* spp. exhibited enhanced susceptibility to the cytotoxic activity of TNF-α.

Production of TNF-α and IL-6 in LPS- or MDP-stimulated monocytes/Mϕ from tuberculin-positive subjects, including patients with active tuberculosis, was shown to be significantly enhanced (Fig. 3). It is of interest that Carswell et al. (6) originally found TNF as an antitumor substance in sera from *Mycobacterium bovis* BCG-primed mice injected with LPS. Recently, it has been reported that in vitro TNF-α production by LPS- or BCG-stimulated monocytes from

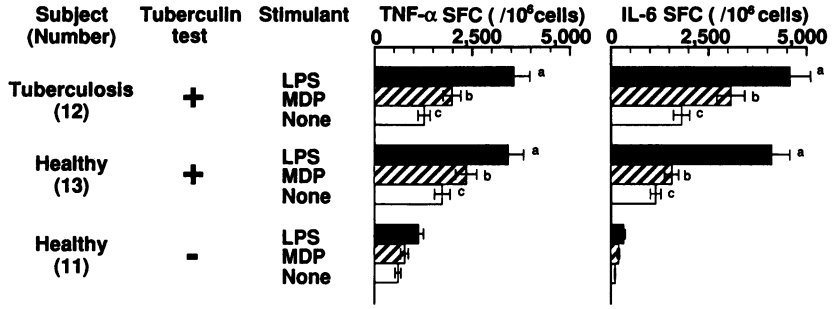

FIG. 4. Enumeration of TNF-α- or IL-6-specific SFC in monocytes/Mϕ from subjects with positive and negative tuberculin tests. The numbers of cytokine-specific SFC were determined by ELISPOT assay. Values (mean ± standard error) are expressed as spots per 10^6 MNL. Symbols: a, b, and c, statistical difference from the value for the tuberculin-negative healthy subject group with or without stimulation of LPS or MDP at $P < 0.05$.

patients with newly diagnosed tuberculosis was significantly higher than that in tuberculin-negative donors, whereas in patients with chronic refractory tuberculosis it was depressed (15). It is also true that TNF was directly induced in peripheral blood monocytes and alveolar Mϕ in tuberculin-negative subjects by mycobacterial whole cells and their cell preparations (17). Wallis et al. (20) showed that two protein fractions of *M. tuberculosis* with molecular weights of 46,000 and 20,000 directly induced IL-1 and TNF production by blood mononuclear cells in tuberculin-negative donors.

In addition to the higher amounts of TNF-α produced, we have found higher IL-6 production in monocytes/Mϕ from tuberculin-positive healthy subjects than in those from tuberculin-negative healthy subjects (Fig. 3). It is reasonable to hypothesize that cytokine production is enhanced in the peripheral blood cells and in body fluids, such as pleural fluid, of patients with tuberculosis. In fact, gamma interferon, as well as TNF, was produced in greater amounts in tuberculous patients than in healthy subjects, and greater amounts of both were found in the pleural fluid than in the blood of patients with tuberculous pleuritis (3, 13).

It was shown that the number of TNF-α- and IL-6-specific SFC in MNL of tuberculin-positive subjects was markedly higher than their number in the MNL of tuberculin-negative healthy subjects (Fig. 4). LPS (and to a lesser extent MDP) stimulation of MNL from tuberculin-positive subjects did enhance the number of SFC-secreting TNF-α and IL-6 (Fig. 4). It is suggested that the cytokine-secreting cells in patients infected with *M. tuberculosis* or in healthy subjects immunized with *M. bovis* BCG are activated in vitro by LPS or MDP, resulting in increases not only in the amounts of products secreted from the cytokine-secreting cells but also in the number of such cells. Thus, the results (Fig. 3 and 4) obtained in this study clearly show the correlation between frequencies of cytokine-secreting cells and levels of cytokine production upon stimulation with either LPS or MDP within the three subject groups. It may be speculated that mycobacterial infection in the lung sensitize monocytes/Mϕ locally to produce TNF-α upon priming with LPS or MDP or that TNF-α is produced and concentrated at the sites of tuberculous lesions. The TNF-α thus synthesized may play a role in human antimycobacterial defenses (3). IL-6 production by monocytes/Mϕ from patients with tuberculosis or tuberculin-positive healthy subjects is of interest. Enhanced production of IL-6 may be implicated in localized proliferative diseases (18). Further, these cytokines may trigger an autoamplification process that results in higher cytokine production, contributing to granuloma formation and defense against local invasion by pathogens (3). It should be mentioned here that methods of enumerating TNF-α-, gamma interferon-, and IL-1β-secreting cells in mouse and human lymphoid organs have been developed recently by Skidmore et al. (14) and Czerkinsky et al. (7).

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