Ex Vivo Production of Interferon-γ, Tumor Necrosis Factor-α, and Interleukin-6 by Mouse Macrophages during Infection with *M. bovis* and *M. tuberculosis* H37Rv

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 11, pp. 552-555, November, 2007
Original article submitted January 29, 2007

Ex vivo production of IFN-γ, TNF-α, and IL-6 by mouse peritoneal macrophages was studied during successive infection with the vaccine strain *M. bovis* BCG and virulent strain *M. tuberculosis* H37Rv. The increase in the concentrations of TNF-α and IL-6 did not depend on the sequence of macrophage infection with the vaccine or virulent strain, but was related to the presence of the vaccine strain *M. bovis* BCG in the medium. IFN-γ production depended on infection of macrophages with the virulent strain *M. tuberculosis* H37Rv. The concentration of IFN-γ was maximum during primary infection with the virulent strain and did not increase after successive infection with the virulent and vaccine strain.

Key Words: *IFN-γ; TNF-α; IL-6; M. bovis BCG; M. tuberculosis H37Rv; macrophages*

Vaccination of humans with attenuated strain *M. bovis* BCG is the major preventive treatment for tuberculosis. Vaccination with BCG is followed by stimulation of cell immunity, which provides protection from the disease. Special attention is paid to the "natural" immunity mediated by macrophage cells. Macrophages (MP) respond to mycobacterial infection by initiation of apoptosis, which serves as a component of effective antimycobacterial protection. Cytokines play an important role in the induction of apoptosis and bactericidal effect of phagocytizing cells. *In vitro* studies of MP infection with various mycobacterial strains showed that these cells produce TNF-α, IL-1, IL-6, IL-10, and other cytokines in response to mycobacterial infection [4,8]. Under natural conditions, the human organism is usually successively subjected to the vaccine and then to virulent strains. It remains unclear whether exposure of vaccinated organism to the virulent strain is accompanied by changes in cytokine concentration.

Here we studied *ex vivo* production of major cytokines during successive infection of MP with the vaccine and virulent strain of mycobacteria (BCG and *M. tuberculosis* H37Rv, respectively).

MATERIALS AND METHODS

Experiments were performed on 40 C57Bl/6 mice weighing 18-20 g.

H37Rv and BCG were obtained from the L. Pasteur Institute (France). Mycobacteria were passaged on Dubois broth with 0.5% bovine serum albumin (BSA, Difco) at 37°C (2 cycles of 7 days). Serial 5-fold dilutions of the suspension filtered through a 5-μ filter (Millipore) were placed (20 μl) on Petri dishes with Dubois broth to evaluate the number of mycobacterial colony-forming units. Microcolonies were counted after 4-day culturing at 37°C.

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0007-4888/07/1445-0709

Mouse peritoneal MP were isolated 5-6 days after intraperitoneal injection of 2 ml 3% peptone. Exudate cells were adhered to plastic Petri dishes. Adherent MP were removed with Versene.

MP were infected with mycobacteria using 24-well flat-bottom plates with RPMI-1640 medium and 5% BSA. The suspension of peritoneal MP (750,000 cells per well) was subjected to repeated adhesion in wells of the plate. H37Rv and/or BCG were added to monolayers of MP in the 1:5 ratio.

The concentrations of IFN-γ, TNF-α, and IL-6 were measured with a test system of Quantikine kit (R&D Systems) according to manufacturer's recommendations. The standard curve was constructed for each compound.

Primary infection of MP with H37Rv and BCG was performed immediately after isolation of MP culture. The cells were repeatedly infected 24 h after primary infection. H37Rv-infected MP were repeatedly infected with H37Rv (group 3) or BCG (group 4). BCG-infected MP were repeatedly infected with BCG (group 6) or H37Rv (group 7).

Cytokine concentration in the culture medium was measured 24 h after secondary infection of MP (*i.e.*, 48 h after the start of study). In the same period (48 h after the start of study), cytokine concentration was measured in the culture medium of MP infected with H37Rv (group 2) or BCG (group 5) and intact cells (group 1).

The results were analyzed by means of Biostat and Microsoft Excel software.

RESULTS

By the 24th hour of the study, IFN-γ production in intact MP was practically undetectable and was much lower than after infection of peritoneal MP with any combination of mycobacterial strains (Table 1).

IFN-γ concentration in the medium significantly increased after 48-h culturing of MP primarily infected with H37Rv. Primary infection of MP with the vaccine strain BCG did not induce such increase in IFN-γ concentration. IFN-γ concentration in the culture fluid was 4-fold lower under these conditions (p=0.003, Table 1).

Repeated infection of H37Rv-infected MP with the same strain had little effect on IFN-γ production (compared to single infection with H37Rv). Similarly, infection of H37Rv-preinfected MP with the vaccine strain BCG did not modulate IFN-γ production. IFN-γ concentration in the medium under these conditions did not differ from that in groups 2 and 3.

IFN-γ production by MP after primary infection with the vaccine strain BCG was lower compared to that in primary infection of MP with the virulent strain H37Rv (p=0.03). However, successive infection of BCG-phagocytizing MP with various combinations of mycobacterial strains was followed by increased induction of IFN-γ. IFN-γ concentration reached 879±119 and 1001.0±76.2 pg/ml after secondary infection of MP with the same vaccine strain (p=0.05) and virulent strain H37Rv (p=0.004), respectively. The increased production of IFN-γ by BCG-preinfected MP did not depend on the nature of strain for secondary infection (vaccine or virulent strain). The differences between groups 6 and 7 were statistically insignificant.

Study of TNF-α production by peritoneal MP showed that the concentration of this cytokine in the supernatant of MP under various conditions of infection was much higher than in intact MP.

Primary infection of MP with the virulent strain H37Rv stimulated the production of TNF-α. TNF-α concentration in the culture medium increased to 1817.14±547.16 pg/ml by the 48th hour after infection. Secondary infection of MP with this virulent strain was followed by a greater increase in TNF-α concentration in the culture medium (up to 4795.71±272.74 pg/ml, p=0.02). Infection of H37Rv-preinfected MP with the vaccine strain BCG resulted in a sharp increase in the induction of TNF-α. The concentration of this cytokine in the culture

TABLE 1. Cytokine Concentration in the Culture Medium after Infection of MP with Vaccine Strain BCG and Virulent Strain H37Rv in Various Combinations (pg/ml, $M \pm m$)

Group	IFN-γ	TNF-α	IL-6
1, intact MP	0.57±0.45	1.01±0.78	0.45±0.23
2, H37Rv	2175.00±560.03	1817.14±547.16	71.07±1.15
3, H37Rv+H37Rv	1743.00±110.31	4795.71±272.74	120.40±2.83
4, H37Rv+BCG	2083.00±559.46	25,238.57±5606.00	385.40±49.50
5, BCG	581.00±97.5	21,831.00±1758.00	430.00±42.40
6, BCG+BCG	879.00±119.00	44,052.90±3788.00	941.40±7.07
7, BCG+H37Rv	1001.00±76.20	30,702.90±363.65	614.40±14.14

medium was 5.26-fold higher compared to that after secondary infection of MP with the virulent strain H37Rv (p=0.016).

Infection of MP with the vaccine strain BCG (primary or successive infection in various combinations with the virulent strain H37Rv) was followed by a sharp increase in TNF-α production compared to infection without vaccine strain BCG.

TNF-α concentration after primary infection of MP with BCG was much higher than after primary infection of MP with the virulent strain H37Rv. Secondary infection of BCG-preinfected MP with the same vaccine strain was followed by a significant increase in TNF-α concentration in the medium (p=0.02). Secondary infection of BCG-preinfected MP with the virulent strain H37Rv induced increased production of TNF-α compared to primary infection of MP with BCG (p=0.02). However, TNF-α concentration in the culture medium under these conditions was much lower compared to that after secondary infection of MP with the vaccine strain BCG (p=0.007).

On the 2nd day, IL-6 concentration in the culture medium of intact MP approached zero and was much lower than in any variant of PH infection with mycobacteria.

Primary infection of MP with the virulent strain H37Rv was followed by a significant increase in IL-6 concentration in the culture medium compared to intact MP. Secondary infection of MP significantly increased the synthesis of IL-6 by MP. Secondary infection of MP with the vaccine strain BCG induced a greater increase in IL-6 concentration in the culture medium than secondary infection with H37Rv (p=0.017).

Infection of MP with the vaccine strain BCG in various combinations was followed by a significant increase in IL-6 production compared to infection with no vaccine strain.

Primary infection of MP with the vaccine strain BCG induced a greater increase in IL-6 production than primary infection of MP with the virulent strain H37Rv. Secondary infection of MP with the same vaccine strain increased the production of IL-6 (p=0.04). Secondary infection of BCG-preinfected MP with the virulent strain H37Rv also stimulated the production of IL-6. However, the concentration of IL-6 on day 2 of culturing was much lower compared to that in secondary infection of MP with the vaccine strain BCG (p=0.001).

Our results show that primary or successive infection of MP with various combinations of virulent and vaccine mycobacterial strains stimulated the production of cytokines compared to intact MP. Infection of MP with the vaccine strain BCG (primary or successive infection in various combinations with the virulent strain H37Rv) induced a greater increase in the production of TNF-α and IL-6 compared to infection with no strain BCG. Production of TNF-α and IL-6 by peritoneal MP successively infected with the vaccine strain BCG and virulent strain H37Rv increased less significantly compared to that after successive infection of MP with the same vaccine strain.

IFN-γ concentration in the culture medium was highest after infection of MP with the virulent strain H37Rv in various combinations, but not with the vaccine strain BCG. IFN-γ concentration in the medium reached maximum after primary infection of MP with H37Rv (primary or successive infection in various combinations with BCG). Secondary infection of H37Rv-preinfected MP had no effect on IFN-γ production. IFN-γ concentration in the medium under these conditions did not differ from that in primary infection of MP with H37Rv. Infection of MP with the vaccine strain BCG induced a less significant increase in IFN-γ production than infection with the virulent strain H37Rv. However, this treatment was followed by induction of IFN-γ after secondary infection with various combinations of mycobacterial strains.

We conclude that production of TNF-α and IL-6 depends on the presence of the vaccine strain BCG in the medium of MP, but not on the sequence of infection with the vaccine or virulent strain.

IFN-γ production depended on infection of MP with the virulent strain H37Rv. IFN-γ concentration reached maximum after primary infection with the virulent strain. However, IFN-γ production did not increase after secondary infection with the virulent or vaccine strain.

Hence, cytokine synthesis is determined by infection with any strain. MP respond to the vaccine strain by increased production of TNF-α and IL-6, which causes apoptosis in these cells. Apoptosis in infected MP probably serves as a defense mechanism of the host organism from intracellular infection. Before death, MP kill or weaken mycobacteria. Mycobacteria are engulfed by newly formed phagocytes after cell destruction [2]. As differentiated from necrosis, apoptosis is not accompanied by inflammation or death of adjacent cells and tissues [1]. Experiments on the culture of BCG-infected MP showed that cell apoptosis plays an important role in the protection from mycobacteria. As distinct from necrosis, activation of apoptosis was followed by a decrease in the number of viable bacteria in the culture [5]. Further studied showed that apoptosis in mycobacterium-infected MP causes death of intracellular bacteria. However, ne-

crosis is followed by the release of viable bacteria into the medium. These bacteria can infect surrounding cells, which results in dissemination of infection. Induction of apoptosis in MP with TNF-α decreases the number of persisting tuberculous mycobacteria in the culture [6].

IFN-γ synthesis was determined by infection of MP with the virulent strain. Increased production of IFN-γ is more typical of tuberculosis patients than secretion of TNF-α and IL-6. The synthesis of these cytokines increases under various conditions of chronic bacterial infection [7].

IFN-γ is an essential component for protection from tuberculous mycobacteria and can modulate cellular response [3].

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