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SUPPRESSION OF THE IMMUNE RESPONSE BY LUNG CELLS IN EXPERIMENTAL TUBERCULOSIS

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Infection of mice by virulent *Mycobacterium tuberculosis* cells is accompanied by generalized infection with bacteriemia, lymphadenopathy, and splenomegaly. The most marked pathological process develops in the lungs, which in tuberculosis are the site of direct interaction between pathogen and effector mechanisms of specific and nonspecific resistance [1].

Data which have now accumulated are evidence that lung tissue contains all the principal types of immunocompetent cells, including T and B lymphocytes, macrophages, dendritic cells, and natural killer cells [3, 5, 6], but their particular features in tuberculosis are unknown. This paper gives the results of a study of the immunologic properties of the interstitial cells of the lung in mice with experimental tuberculosis, evidence of activation, as a result of infection, both of T lymphocytes specific for mycobacterial antigens and of suppressor cells, adherent to plastic, which suppress proliferation of immune T cells.

EXPERIMENTAL METHOD

Mice of the inbred CBA/Sto line aged 3-4 months were obtained from the "Stolbovaya" Nursery, Academy of Medical Sciences of the USSR. The mice were infected intravenously with a virulent strain of *M. tuberculosis* H37Rv in a dose of 25 μg/mouse. The mice were used in the experiments 3-7 weeks after infection. Sterile suspensions of the interstitial cells of the lung were obtained by enzymic dissociation. For this purpose the vascular bed was perfused with an intravenous infusion of 7 ml of Hanks' solution with antibiotics, containing 10 U/ml of heparin, after which bronchoalveolar lavage was carried out with warm physiological saline containing antibiotics, 1-1.5 ml of solution being injected through a cannula inserted into the trachea, followed by aspiration of the contents, the procedure being repeated eight times. The lungs were removed from the chest, perfused another twice in medium 199 containing antibiotics, cut into small pieces measuring about 1-2 mm^3, and added to a solution containing 2 mg/ml of type I collagenase (260 IU/mg, from "Boehringer," West Germany) in medium L-15 containing 20 mM HEPES, 1% embryonic calf serum (ECS), 50 U/ml of kanamycin (all components from "Flow Laboratories," England), and 50 μg/ml of DNase (USSR). The sample was incubated at 37°C for 90 min on a planchet shaker (ABP-1).

A unicellular suspension was obtained by repeated pipetting of the suspension and passing it through a metal sieve. The cells were then washed 3 times by centrifugation at 150 g for 10 min and filtered through a cotton wool filter. The suspension thus obtained consisted of single cells with a viability of 80-90%. To remove cells adherent to plastic the suspension was incubated in complete nutrient medium (RPMI-1640, containing 10% ECS, 10 mM HEPES, 4 mM glutamine, 50 U/ml kanamycin, 1% of nonessential amino acids, 2.2 mM pyruvate, and $5 \cdot 10^{-5}$ 2-mercaptoethanol; all components from "Flow Laboratories," England), on plastic Petri dishes 90 mm in diameter for 2 h at 37°C in a CO_2-incubator in a dose of $(25\text{-}30) \cdot 10^6$ cells per dish. Adherent cells were removed mechanically after incubation of the monolayer with a cold solution containing 0.02% EDTA for 30 min at room temperature.

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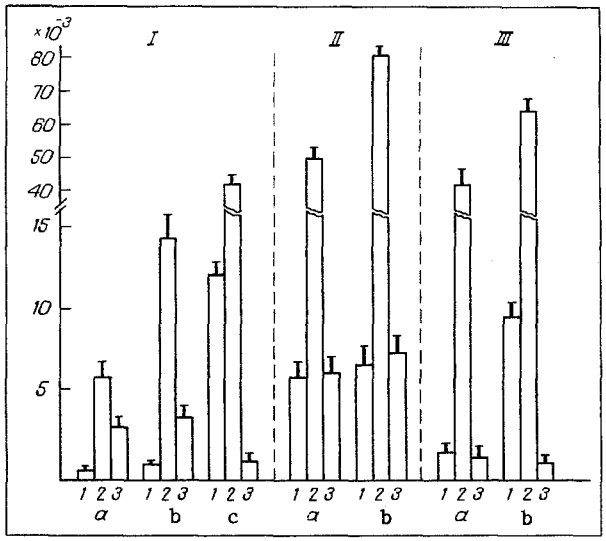

Fig. 1. Proliferative response of cells isolated from lungs (in CPM · 10^{-3}) of mice infected with tuberculosis (I), intact mice (II), or mice vaccinated with BCG (III). a) Unfractionated lung cells, b) lung cells without those adherent to plastic, c) fraction enriched with T lymphocytes; 1) in presence of PPD (10 μg/ml), 2) in presence of con A (2.5 μg/ml), 3) without stimulation.

To obtain a cell suspension enriched with T lymphocytes nonadherent to plastic, the lung cells were applied in a concentration of $50 \cdot 10^6$ cells/ml to a column containing 0.7 g nylon wadding ("Femoall," USA), incubated for 60 min at 37°C, after which the nonadherent cells were eluted with warm solid medium at the rate of 1 ml/min.

Cells adherent to the nylon wadding were obtained by rinsing the column with 30 ml of medium, after which the column was filled with cold phosphate-buffered saline without Ca^{2+} and Mg^{2+}, and the wadding was pressed 3 times with the piston from the syringe used with the column. To obtain immune lymphocytes, mice were immunized in the footpads with Freund's complete adjuvant ("Sigma," USA), containing *M. tuberculosis* H37Rv cells or ovalbumin (100 μg/ml) or corpuscular antigen of *Staphylococcus aureus* ($5 \cdot 10^6$ microbial particles in 1 ml) in Freund's incomplete adjuvant. After 9-14 days a unicellular suspension was obtained from the popliteal lymph nodes, and washed twice with medium 199 containing 2% ECS, 10 mM HEPES, and antibiotics. The proliferative response of lung and lymph node cells was assessed on the basis of uptake of ^{3}H-thymidine. For this purpose $4 \cdot 10^5$ cells (three repetitions for each version of the test) were introduced into wells of a flat-bottomed 96-well planchet ("Nunc," Denmark) in 0.2 ml of complete nutrient medium. Proliferation was stimulated (experiment) by adding purified tuberculin (PPD, Statens Serum Institute, Denmark), concanavalin A (con A, "Pharmacia," Sweden), ovalbumin ("Sigma"), and the cytoplasmic fraction of antigens of *Staphylococcus aureus,* generously provided by M. M. Averbakh. Wells without addition of the antigen served as the control. After 66 h of culture, 1 μCi of ^{3}H-thymidine was added to each well, and after a further 6 h the contents of the wells were transferred to a glass fiber filter with the aid of a cell harvester, dried, and their radioactivity counted on a "Beta-2" liquid scintillation counter.

The suppressor action of the lung cells was tested by adding 1, 0.5, 0.25, or $0.125 \cdot 10^5$ unfractionated lung cells or of the different fractions of these cells to $4 \cdot 10^5$ immune lymph node cells.

EXPERIMENTAL RESULTS

It was possible to obtain 8-10 million viable cells by the method described above from the lungs of one intact mouse. After infection of the mice with tuberculosis the number of cells began to rise with the 10th-14th day (30-40 million cells/mouse), to reach a peak (60-70 million/mouse) in the terminal stage of the disease. The development of infection was accompanied by the appearance of giant cells, accounting for 5-10% of the total.

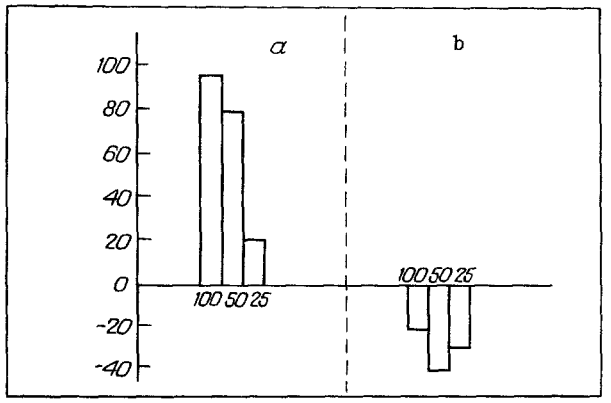

Fig. 2. Indices of suppression of proliferative response of immune lymphocytes to PPD during combined culture with unfractionated lung cells from mice infected with tuberculosis (a), and intact mice (b). Abscissa, number of lung cells (10^{-3}) per well; ordinate, index of suppression (IS) = $(a - b)/a \cdot 100\%$, where a is the difference between values of thymidine uptake by immune lymphocytes in the presence of antigen and in its absence, and b denotes the difference between the same values in the presence of suppressors.

Unfractionated lung cells from infected mice proliferated spontaneously in vitro only very weakly. Culture in the presence of PPD led to a significant reduction of proliferation of these cells. After removal of cells adherent to plastic, spontaneous proliferation increased and a weak proliferative response of the nonadherent cells to PPD was observed. The cell population enriched with T lymphocytes by purification on a column with nylon wadding differed in the increased intensity of "spontaneous" proliferation and the marked response to PPD (Fig. 1, I). Unfractionated lung cells of infected mice likewise did not respond to stimulation by con A, but after removal of the cells adherent to plastic, they began to respond to this mitogen. The population enriched with T lymphocytes responded to stimulation by con A by marked proliferation. The data given above indicate the presence of functionally active T lymphocytes in the lungs, including antigen-specific cells, whose proliferative response was probably suppressed by cells adherent to plastic and to nylon wadding. Unfractionated lung cells from intact mice proliferated in response to stimulation by con A, and removal of cells adherent to plastic and purification on a column with nylon wadding led to slight but not significant strengthening of the response. A response to PPD was absent in all groups (Fig. 1, II).

Unfractionated lung cells of animals vaccinated with BCG, like cells from healthy mice, responded to stimulation by con A but did not respond to PPD. Removal of cells adherent to plastic led to the appearance of a response to PPD and to enhancement of the response to con A. Additional purification on a column with nylon wadding very slightly increased the intensity of the response both to PPD and to con A (Fig. 1, III). Thus in this experimental model a response to PPD was observed only in mice in contact with mycobacteria, and it was absent in intact animals, i.e., it is an antigen-specific secondary response. The facts described above demonstrate, first, the presence of antigen-specific cells adherent to nylon wadding (T lymphocytes) in the lungs of infected and vaccinated mice, and second, marked suppression of the response of these cells to PPD and con A in tuberculosis, and third, dependence of suppression on cells adherent to plastic and to nylon wadding.

Proof of induction of suppressor cells in the infected lung was obtained by studying the effect of lung cells on the proliferative response of antigen-specific immune lymphocytes isolated from regional lymph nodes after immunization of the mice with Freund's complete adjuvant. In the absence of lung cells, cells of immune lymph nodes gave a high proliferative response to PPD in vitro. Addition of unfractionated lung cells obtained from infected mice to cells from immune lymph nodes in the ratio of 1:4 and 1:8 led to complete suppression of the response to PPD, and in this case the same numbers of lung cells from healthy mice did not suppress the response (Fig. 2). Removal of cells adherent to plastic from the population of suppressors led to a significant weakening of the suppressor effect (Fig. 3a). Adherent cells removed from plastic had the strongest suppressor effect, for in ratios of 1:4 and 1:8 they suppressed the proliferative response of immune lymph node cells to PPD and con A. The group in which adherent lung cells were added in the ratio of 1:16 is particularly interesting. In this case complete recovery of the response to con A took place but, as before, there was no response to PPD. With a ratio of 1:32 suppression of the response did not take place either to PPD or to con A (Fig. 3b). These results may be evidence that besides mechanisms of nonspecific suppression, a specific component may also be present in the lungs of mice infected with tuberculosis. An alternative

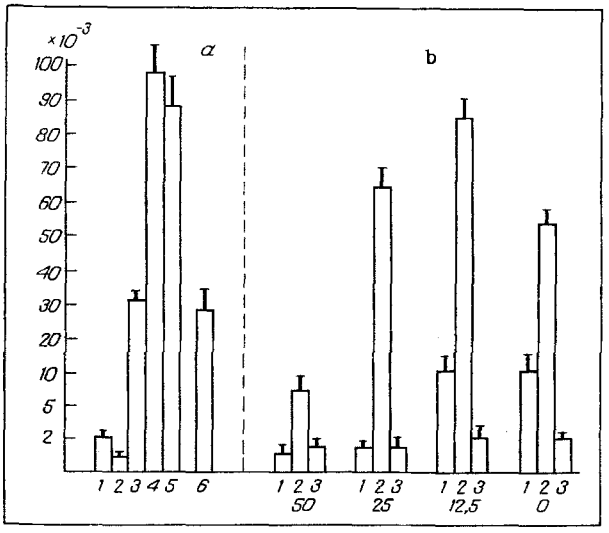

Fig. 3. Suppressor action of different fractions of lung cells from infected mice. a) Proliferative response (CPM · 10^{-3}) of immune lymphocytes to PPD in presence of: 1) unfractionated lung cells, 2) cells adherent to plastic, 3) cells after removal of those adherent to plastic, 4) fraction enriched with T lymphocytes, 5) taken from nylon wadding mechanically, 6) immune lymphocytes (control), b) proliferative response of immune lymphocytes to PPD (1) and con A (2) in presence of different numbers of lung cells adherent to plastic. 3) Spontaneous proliferation (control). Abscissa, number of suppressor cells (10^{-3}) per well.

TABLE 1. Specific and Nonspecific Suppression of Proliferative Response of Immune Lymphocytes by Lung Cells Isolated from Mice Infected with Tuberculosis

Immunization by	Antigen (concentration)	Suppressor cells per well (× 10^{-3})	Proliferation (CPM×10^{-3}) with antigen	Proliferation (CPM×10^{-3}) without antigen	IS	Percentage suppression
FCA	PPD (10 μg/ml)	—	22,7±2,5	6,3±0,9	3,6	—
		A (100)	6,2±0,9	3,1±0,4	2,0	81
		A (50)	12,0±1,5	4,5±0,6	2,7	54
		A (25)	14,7±1,7	6,0±0,9	2,5	47
Staph. aureus + FICA	Cytoplasmic fraction (10 μg/ml)	—	42,7±6,5	9,1±1,2	6,7	—
		A (100)	16,6±1,9	7,3±1,4	2,3	72
		A (50)	39,4±5,2	6,4±0,8	6,1	2
		A (25)	52,8±7,4	7,5±1,8	7,0	34
FCA	PPD (10 μg/ml)	—	31,3±5,1	6,1±0,7	5,2	—
		B (100)	0,7±0,1	1,0±0,1	0,7	101
		B (50)	3,3±0,5	3,2±0,5	1,0	100
		B (10)	19,6±2,1	6,4±1,1	3,1	48
Staph. aureus + FICA	Cytoplasmic fraction (10 μg/ml)	—	53,3±7,2	12,4±1,6	4,3	—
		B (100)	4,4±0,3	1,1±0,2	4,0	99
		B (50)	13,3±2,0	3,3±0,4	4,0	98
		B (10)	40,1±5,5	6,2±1,0	6,5	17
Ovalbumin + FICA	Ovalbumin (100 μg/ml)	—	13,5±1,5	6,6±0,8	2,1	—
		B (50)	9,7±1,4	4,8±0,6	2,0	30
		B (10)	13,2±1,2	7,0±1,2	1,9	11

Legend. A) Unfractionated lung cells, B) lung cells adherent to plastic, FCA) Freund's complete adjuvant, FICA) Freund's incomplete adjuvant, IS) index of stimulation.

explanation would be that suppression depends on the strength and mechanism of action of the stimulating signal, for con A is a powerful mitogen, activating resting T cells nonspecifically.

To test these hypotheses, the suppressor action of lung cells was studied on the proliferative response of lymph node cells immune to ovalbumin and to *Staph. aureus* (Table 1). Unfractionated lung cells from mice infected with tuberculosis had a suppressor action in both cases on both spontaneous and antigen-induced proliferation of immune lymphocytes. However, with a ratio of suppressors to responders of 2:8 and 1:16, the response to staphylococcal antigen was restored; under these circumstances the response of the corresponding lymphocytes to PPD was suppressed as before.

Since the most marked suppressor activity in these experiments was connected with lung cells adherent to plastic, a comparative study was made of the effect of these cells on the antigen-specific proliferative response. In all the test systems spontaneous proliferation was considerably reduced on addition of adherent cells, but the response of the lymphocytes to stimulation by ovalbumin and staphylococcal antigen was preserved, and the response to PPD was completely absent.

The results thus confirmed the view that the lungs of mice infected with tuberculosis contain, not only a mechanism of nonspecific suppression, but also a mechanism responsible for predominant suppression of proliferation of T lymphocytes specific for mycobacterial antigens. The presence of radiation-sensitive adherent monocytelike cells, specifically suppressing the response of the peripheral blood lymphocytes of the patient to PPD, was demonstrated previously in the peripheral blood of persons with tuberculosis [7]. Antigen-specific and nonspecific suppression of the immune response, dependent on macrophages, has been described in experimental models of many infections [2, 4, 8]. However, mechanisms of suppression ensuring specificity of the effect have not yet been studied, and this is a task we are undertaking at the present time.

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