METHODS

Isolation and -Analysis of Focus-Infiltrating Lymphocytes in Pulmonary Tuberculosis

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Lymphocytes affine for tuberculosis foci were prepared from sterile pulmonary resectates. It was demonstrated that these lymphocytes differ from lymphocytes isolated from normal lung tissue. The possibility of using cytotoxic cells ($CD8^+CD16^+$) and antigen-presenting cells ($CD4^+$) for prevention and treatment of recurrent tuberculosis is discussed.

Key Words: *lymphocyte; antituberculosis immunity; isolation and analysis; autoimmunotherapy*

Lymphocytes specifically determinated for the focus of inflammation can be isolated from patients with tuberculosis. These cells can be cultured with antimicrobial preparations and used for eradication of the remaining foci of the same genesis. Unfortunately, the information on this issue is scarce. Tumor-infiltrating lymphocytes isolated from surgery material have been used for prevention of metastases [2]. Specific T lymphocytes ($CD8^+$) reacting to mycobacteria have been identified [3]. Their activation is associated with detection of mycobacterial antigens and is mediated by interferon-γ and tumor necrosis factor-α. The possibility of using such lymphocytes in antituberculosis therapy has not been investigated. Our goal was to develop a technique for isolation of focus-infiltrating lymphocytes (FIL) in tuberculosis.

MATERIALS AND METHODS

Focus-infiltrating lymphocytes were isolated from lung resectates of 35 patients. Fifteen patients had tuberculoma, 13 patients had fibrous-cavernous tuberculosis in the stabilization phase, and 7 patients had progressing fibrous-cavernous tuberculosis. Fourteen segmental resections and one precision removal of tuberculoma were performed in patients with tuberculomas. Six segmental and 3 combined resections and 4 lobectiomias were performed in patients with fibrous-cavernous tuberculosis in the stabilization phase. Pneumonectomy was performed in all patients with progressing fibrous-cavernous tuberculosis.

The technique of FIL isolation is based on modified method [1]. Tissue from the tuberculoma capsule or from the wall of tuberculosis cavernae was obtained under sterile conditions during operation and subjected to "mild" mechanical homogenization. Cell suspension was fractionated by centrifugation on a Ficoll-Verografin gradient, washed several times in PRMI with rifampicin, which in bactericidal concentrations does not affect leukocytes *in vitro*. The lymphocyte suspension was incubated for 24 h at 37°C and then analyzed for cell morphology and the presence of *M. tuberculosis* (immunofluorescence method) and nonspecific microflora (growing on various culture media). Composition of the population was studied by the immunofluorescence method. Monoclonal antibodies LT (Institute of Immunology) were used for the immunofluorescent marker analysis. The data were processed by variational statistics methods and correlation analysis.

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TABLE 1. Immunofluorescent Analysis of Lymphocytes Isolated from the Focus of Tubercular Inflammation and Normal Lung Tissue

Lymphocytes subpopulation	Normal lung tissue, %	Tuberculoma, %	Fibrous-cavernous tuberculosis, %	
			Stable form	Progressing form
$CD3^+$	58.2	68.9	66.8	61.7
$CD4^+$	34.7	31.4	34.8	38.1
$CD8^+$	28.4	38.9	31.1	22.4
$CD16^+$	6.4	17.9	12.8	6.7
$CD21^+$	5.6	2.6	18.7	32.8
$CD8^+CD16^+$	0.0	28.4	15.8	8.3

RESULTS

The lymphocyte fraction isolated from one cubic centimeter of lung tissue contained $4.8\text{-}6.6\times10^6$ cells, 88-94% of which were viable by the Trypan Blue exclusion test. A negative correlation was established between the amount and viability of isolated lymphocytes, on the one hand, and duration of pathological process in the lungs, on the other, i.e., more viable cells are isolated from lung tissue with "fresh" inflammation. Morphologically, the fraction contained 4-6% of nonlymphocytic mononuclear cells.

Thus, up to 6×10^6 live lymphocytes affine for the focus of tubercular inflammation can be isolated from surgery material. The fraction contained no mycobacteria, as estimated by the luminescence method, and nonspecific microflora, as evidenced by the absence of microbial growth on culture media. Only one fraction was contaminated by *B. citrobacter*; however, the possibility of artefact cannot be ruled out.

It was interesting to study the composition of the fraction and compare it with lymphocyte fraction isolated from normal lungs. For this purpose, in 10 patients lymphocytes were isolated not only from tuberculoma but also from peripheral part of the resectate unaffected by tuberculosis. The results of immunofluorescent analysis are summarized in Table 1. The following markers were identified (the range within which the amount of cells bearing the marker varied in each for form of tuberculosis is given in parentheses): $CD3^+$ (61.7-68.9%), $CD4^+$ (31.4-38.1%), $CD8^+$ (22.4-38.9%), $CD16^+$ (6.7-17.9%), $CD21^+$ (2.6-32.8%), and a double marker $CD8^+CD16^+$ (8.3-28.4%). High contents of $CD8^+CD16^+$ (28.4 %), $CD3^+$ (68.9%), $CD8^+$ (38.9%), $CD16^+$ (17.9%) lymphocytes, $CD4^+/CD8^+$ ratio equal to 0.81, and low content of $CD21^+$ (2.6%) cells were typical of the stable forms of tuberculosis. Progressing fibrous-cavernous tuberculosis was characterized by high content of $CD21^+$ (32.8%) and low contents of $CD8^+$ $CD16^+$ (8.3%), $CD8^+$ (22.4%), $CD16^+$ (6.7%), and the $CD4^+/CD8^+$ ratio equal to 1.7. The absence of $CD8^+CD16^+$ and low contents of $CD16^+$ and $CD8^+$ lymphocytes were demonstrated for normal lung tissue.

Thus, the compositions of lymphocyte fractions isolated from the focus of tubercular inflammation and from normal lung tissue are different. Lymphocyte fraction isolated from diseased lung contains a population of cytotoxic lymphocytes ($CD8^+CD16^+$) and great amount of natural killer cells ($CD16^+$). This cells may act as effectors of antituberculosis immunity, which was confirmed by the fact that their relative content is different in stable and progressing foci. $CD4^+$ lymphocytes (presumably antigen-presenting cells) can be used for immunological "training" of peripheral blood lymphocytes *in vitro*.

Thus, we have developed a technique for isolation of lymphocytes from the foci of tubercular inflammation under sterile conditions. This technique yields up to 6×10^7 live lymphocytes from lung resectate. Upon density gradient fractionation, bacteria remain in the supernatant, and the resultant lymphocyte fraction is free from pathogenic and nonspecific microflora. The lymphocyte fraction contains specialized cells different from those isolated from normal lung tissue. The effectors of antituberculosis immunity ($CD8^+CD16^+$) and antigen-presenting cells ($CD4^+$) are prospective candidates for further investigations.

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