Immunological Memory Induced by *Mycobacterium bovis* in Inbred Mice

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Immunological memory was reproduced and studied by subcutaneous vaccination of mice with attenuated strain of *Mycobacterium bovis*. Ionomycin-resistant cells of CBA mice were for the first time isolated from immune cells and studied as memory T-cells. Previously they were described for another experimental model. They are characterized by high expression of CD3, CD4, CD8, CD28, and α/β-T-cell receptor. The capacity of splenocytes and ionomycin-resistant cells to adoptive transfer of antituberculosis resistance to intact recipients is studied.

Key Words: *experimental tuberculosis; Mycobacterium bovis; immunological memory; ionomycin-resistant T-cells*

Vaccination with live Calmette—Guerin bacillus (BCG) and attenuated *Mycobacterium bovis* strain protects mice from subsequent infection with virulent *M. tuberculosis* H37Rv strain [1-3,6,7]. The protective effect is mediated mainly by T-cells and activated macrophages [7,11,12]. The formation of immunological memory is a remote result of contact of the organism with mycobacteria [4,5]. Immunological memory providing a rapid intense response of the host to a repeated contact with mycobacteria was studied using intravenous infection of mice with BCG [5] and *M. tuberculosis* [4]. This phenomenon is mediated by silent $CD4^+$ T-cells.

The immunological memory phenomenon was never studied on the model of subcutaneous BCG vaccination of mice [10].

We attempted to generate and study immunological memory by subcutaneous BCG vaccination of mice. Ionomycin-resistant (IR) cells, previously described for another experimental model [8], were for the first time isolated from immune mice and studied as memory T-cells in experimental tuberculosis.

MATERIALS AND METHODS

Previously described approaches [4,5,10] adapted for our model were used to induce immunological memory to mycobacteria. CBA mice (females aged 5-7 weeks) were subcutaneously vaccinated with BCG in a dose of 10^7 colony-forming units (CFU) per mouse. After 5 weeks, oral chemotherapy with rifampicin and isoniazid in a daily dose of 750 μg per mouse was started, which was carried out for 2 months. After therapy, the mice were left at rest for 4-6 months. Resistance to virulent tuberculous infection was assessed by the mean survival rate of mice infected intravenously with a lethal dose (10^7 CFU) of *M. tuberculosis* H37Rv and by the rate of mycobacteria isolation from the spleen of animals infected with a lower dose (10^5 CFU) [9].

Protective activities of cell populations were verified by adoptive transfer of antituberculosis immunity to syngeneic sublethally irradiated (500 rad) recipients of splenocytes (5×10^6 cells per mouse), T-cells (2×10^6 cells per mouse) [9], and IR-cells (5×

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10^5 cells per mouse) which were isolated as described previously [8]. One day after the transfer, mice were intravenously infected with 10^5 CFU *M. tuberculosis* H37Rv, and after 10 days the rate of mycobacteria isolation from the recipient spleen was assessed.

The cell population phenotype was determined by direct immunofluorescence with monoclonal antibodies to CD3, CD4, CD8, CD28, and α/β-T-cell receptor (TCR) labeled with phicoerythrin or fluorescein isothiocyanate (Pharmingen). Control cells were not treated with monoclonal antibodies. Fluorescence of stained cells was analyzed in an EPICS "ELITE" laser flow cytofluorimeter (Coulter). The results were statistically processed using MultiGraph software (Coulter).

RESULTS

The following groups were investigated: 1) intact mice; 2) mice vaccinated with BCG without subsequent chemotherapy; 3) chemotherapy after BCG vaccination (mice with "pure" immunological memory — memory mice, or m-mice). The latter group was characterized by the absence of mycobacteria; active immune processes in m-mice gradually faded during the period of rest. Survival rate of mice after infection with a lethal dose of *M. tuberculosis* H37Rv was 36.0±1.8 days (group 1), 73.2±5.9 days (group 2), and 52.2±4.1 days (group 3). The groups significantly differed in resistance determined by survival rate. According to this parameter, m-mice occupied an intermediate position between intact mice and mice with active antituberculosis immunity maintained by persistent BCG mycobacteria.

Similar regularities were observed upon isolation of mycobacteria from the spleen 10 days after infection with lower doses (10^5 CFU) *M. tuberculosis* H37Rv (immunological memory manifests itself functionally more intensely at the early periods after secondary contact with infecting agent [4]). The number of CFU per mouse spleen was 17×10^4 in group 1, 12×10^3 in group 2, and 25×10^2 in group 3. Mice vaccinated with BCG suppressed the growth of H37Rv mycobacteria most actively (by the moment of infection, BCG were virtually no longer isolated from the spleen). m-Mice were notably inferior to group 2 animals by this parameter, but were much more active than intact mice from whose spleen 10 times more mycobacteria were isolated than from m-mice.

Splenocyte capacity to adoptive transfer of antituberculosis resistance was studied. The differences between vaccinated BCG donors, m-mice, and intact donors were also observed in these experiments. Transfer of splenocytes from m-mice ensured intermediate resistance of the recipients. T-cells isolated from m-mice were also capable of rendering antituberculosis resistance, but IR-cells were the most active in this respect, regarding the number of cells transferred (Fig. 1).

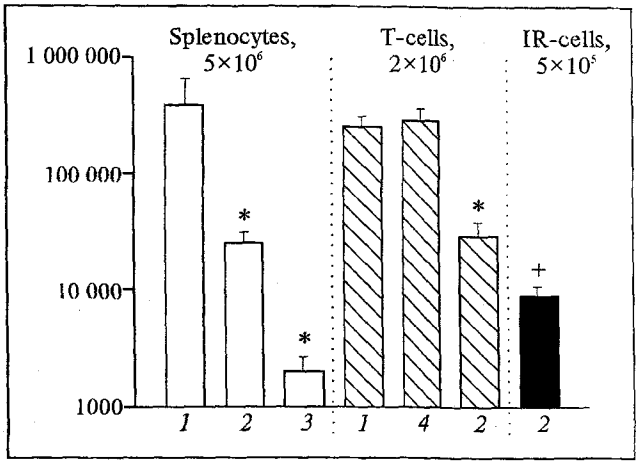

Fig. 1. Adoptive transfer of antituberculosis resistance by immune splenocytes of CBA mice to intact recipients. Donors: *1*) intact mice; *2*) m-mice; *3*) BCG-vaccinated mice; *4*) no adoptive transfer of cells. Ordinate: number of colony-forming cells per spleen. **p*<0.001 *vs.* *1*, +*p*<0.01 *vs.* group 2 T-cells.

Thus, our data permit us to distinguish active BCG-induced immunity ensuring the most effective protection against subsequent infection with mycobacteria and long-term immunological memory mediating increase (although not very great) of resistance to a repeated contact with mycobacterial infection. In our experiment, "pure" immunological memory was attained by using chemical drugs. Such a methodological approach can serve as experimental model of antituberculosis immunity in humans, which is formed for years after BCG vaccination, as the organism is gradually released from mycobacteria.

Change in the phenotype of immunocompetent cells during formation of the studied types of antituberculosis immunity attracted our special attention. Table 1 presents the phenotypes of splenocytes of intact, BCG-vaccinated, and m-mice 10 days after vaccination with a low dose of *M. tuberculosis* H37Rv. BCG vaccination led to a slight increase in the proportion of T-cells in the total splenocyte population.

TABLE 1. Phenotypical Characteristics of Splenocytes of CBA Mice Infected with *M. tuberculosis* H27Rv

Group of mice	% of positive cells		
	CD4	CD8	α/β-TCR
1 (intact)	27.4	5.4	26.4
2 (vaccinated BCG, no chemotherapy)	23.0	12.2	20.5
3 (m-mice)	26.1	12.4	10.9

This was paralleled by an increase in the count of $CD8^+$-cells in mice, for whom contact with *M. tuberculosis* H37Rv was secondary, and by a decrease in the count of $CD4^+$-cells in BCG vaccinated mice. The decrease in cells expressing α/β-TCR in m-mice was the most pronounced. This can hardly be explained by accumulation of memory T-cells, because these cells represent a negligible portion of the total count of T-cells [8]. Apparently, in this case a long process of formation of immunological memory in mice led to alteration of phenotype of T-cell populations other than memory cells. It is noteworthy that the density of α/β-TCR on cells of m-mice was much higher than in native mice (data not shown). According to our data, IR-cells of m-mice are characterized by a high level of expression of CD3, CD4, CD8, CD28, and α/β-TCR markers.

Thus, we induced and studied immunological memory developing after subcutaneous BCG vaccination. The differences in protection from *M. tuberculosis* in mice with active antituberculosis immunity and immunological memory are demonstrated. IR-cells were for the first time isolated from immune mice and studied as T-cells.

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