GENETICS

Preparation and Characterization of Mycobacterial Antigen-Specific T-Cell Clones from Mice with Genetically Different Sensitivity to Tuberculosis

V. V. Eremeev, I. V. Lyadova, K. B. Maiorov, S. V. Khaidukov,* A. M. Moroz, and A. S. Apt

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 123, No. 4, pp. 431-434, April, 1997
Original article submitted March 6, 1996

T-clones specific for purified protein derivative of tuberculin are derived from mice with genetically different sensitivity to tuberculosis. The absolute majority of resultant T-clones express the T-helper phenotype and are restricted for the class II H-2 gene products. T-clones exert different effects on the bacteriostatic activity of syngeneic macrophages. A relationship between the described characteristics of T-clones and the allele composition of their H-2 is hypothesized.

Key Words: *T-clones; tuberculosis; H-2 complex*

The leading role of specific immune reactions of T-cells in protection of the host organism from tuberculosis infection and in the formation of immunity after BCG vaccination is universally acknowledged [9,10]. The allele composition of H-2 complex (main histocompatibility complex of mice) is known to determine the sensitivity of mice to infection with *Mycobacterium tuberculosis* H37Rv and the level of antimycobacterial immune response [3-5]. Infected mice belonging to H-2 congeneic strains differ in the terms of survival, severity of pathological granulomatous changes in the lungs, and, less so, by the rate of isolation of mycobacteria from the organs. In addition, the efficacy of BCG vaccination from subsequent infection with virulent mycobacteria depends, besides other genes [1], on the H-2 complex haplotype [3]. The relationship between the efficacy of antituberculosis immune response and the H-2 complex may be as follows: the response to certain mycobacterial antigens is determined by the allele composition of this complex, because H-2 gene products, first, present the antigens to T-cells [7] and, second, determine the selection of the T-receptor repertoire in the thymus [2]. Moreover, the type of the forming T-cell response, namely, the predominant activation of types 1 or 2 T-helpers, may be related to the structure of H-2 [8]. We can answer these questions only after investigating the reactions to mycobacterial antigens at the level of different T-cell clones derived from mice with different H-2-determined sensitivity to the agent. This report characterizes the T-clones derived from mice resistant to infection (strains B10.D2 and B10.A) and strain B10.M with impaired efficacy of BCG vaccination.

MATERIALS AND METHODS

B10.D2 (H-2^d), B10.A (H-2^a), B10.M (H-2^f), B10.MBR (H-2^{bgl}), and B10.A(4R) (H-2^{h4}) mouse strains were used. All strains were bred at the vivarium of Central Research Institute of Tuberculosis. Purified protein derivative of tuberculin (PPD, Statens Serum In-

Central Research Institute of Tuberculosis, Russian Academy of Medical Sciences; M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow

0007-4888/97/0004-0373$18.00

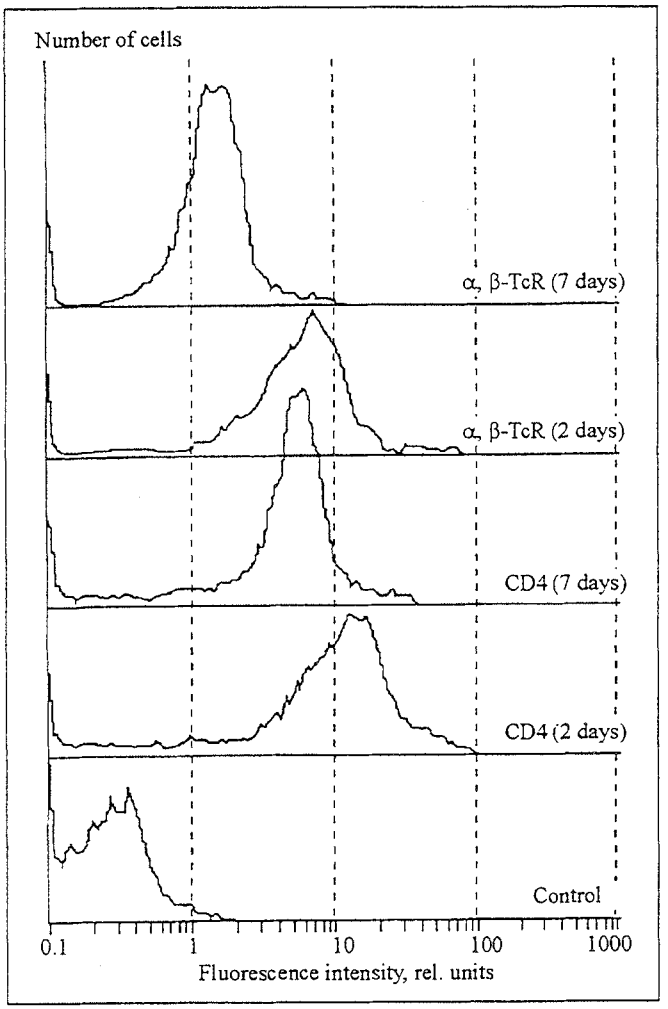

Fig. 1. Relationship between the level of expression of ab and CD4 markers with A1G10 T-clone and the time elapsed after restimulation of the clone.

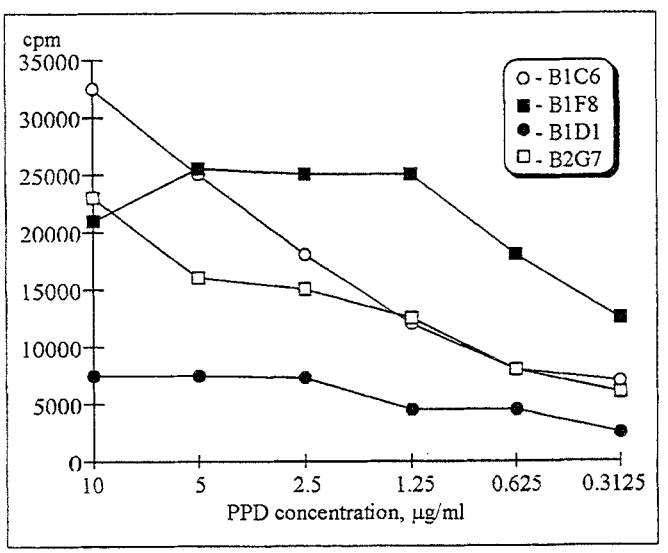

Fig. 2. Relationship between the level of proliferative response of T-clones B1C6, B1F8, B1D1, and B2G7 and the concentration of PPD in test medium.

stitute, Denmark) was used as antigen. In some experiments, *M. tuberculosis* H37Rv from the collection of the Institute of Tuberculosis was used. Mycobacteria grown in Dubos medium and washed in normal saline were resuspended in normal saline with 0.1% bovine serum albumin and 0.05% Tween-20 in a concentration of 1 mg/ml, which corresponds to 5×10^8 CFU/ml in B10.D2, B10.A, and B10.M mice were immunized subcutaneously in the paw with PPD in incomplete Freund's adjuvant in a dose of 50 μg of PPD per animal. Ten-fourteen days after immunization, the popliteal lymph nodes were collected under aseptic conditions, lymphocytes were isolated using a metal mesh, washed in Eagle's medium with 5% inactivated calf serum and antibiotics (washing medium), and resuspended ($4\text{-}5\times10^6$/ml) in RPMI-1640 containing 10% fetal calf serum, 5000 U/ml penicillin, 5000 μg/ml streptomycin, 8 μg/ml tylosin, 10 mM HEPES, 4 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 mM pyruvate, vitamins, essential amino acids (culture medium), and 10 μg/ml of PPD. All media and additives were from HyClone Laboratories. Lymphocytes were incubated at 37°C, 95% humidity, and 5% CO_2 for 3 days. Then blast cells were isolated by centrifuging in a Cedarlane Lympholite M gradient and, after three sedimentations in washing medium, cloned by limiting dilutions (3 to 10 cells per well) in culture medium with 10 μg/ml PPD, 10-15% interleukin-2-containing conditioned medium, and 2.5×10^6 syngeneic irradiated feeder cells per ml. The growing clones were restimulated in the same medium at 14-day intervals and, as the cell mass increased, used for tests. For the proliferative test cloned T-lymphocytes were purified from dead feeder cells in a Lympholite M density gradient no earlier than 7 days after the last restimulation and, after washing, resuspended in RPMI-1640 gradient with 2 mM of L-glutamine, 5×10^{-5} M mercaptoethanol, antibiotics, pyruvate, and 5% fetal calf serum (medium for tests). Cells of T-clone (3×10^4) and 3×10^5 cells of exposed feeder were transferred to 96-well plates. Antigen in various concentrations was added to experimental wells. Cells were incubated at 37°C, 95% humidity, and 5% CO_2 for 48 h. The intensity of T-clone proliferation was assessed from incorporation of ^{3}H-thymidine during the last 16 h of culturing.

The effect of T-clones on the bacteriostatic activity of macrophages was assessed by culturing purified peritoneal macrophages *in vitro* in medium for tests without antibiotics, loading them with live H37Rv bacteria for 2 h in various macrophage/mycobacterium ratios, intense washing from microorganisms which failed to be phagocytosed, and culturing for 72 h with or without syngeneic T-clones in the T-

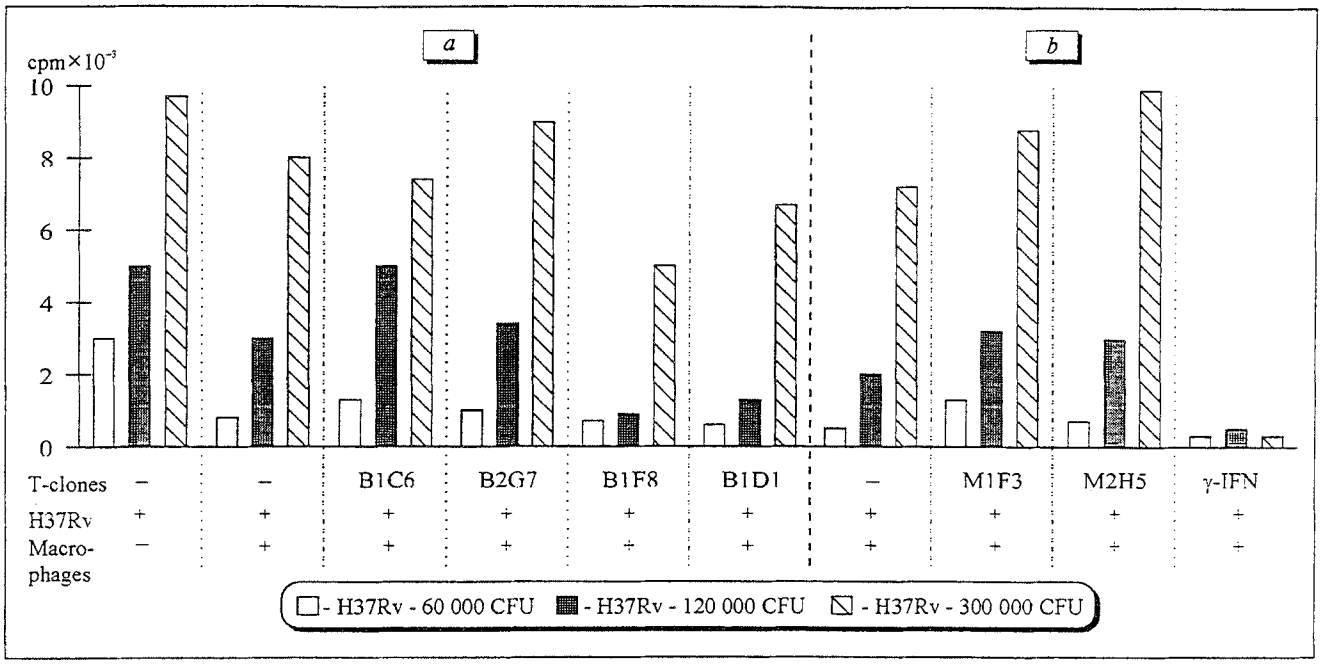

Fig. 3. Bacteriostatic activity of macrophages of haplotypes H-2^d (*a*) and H-2^f (*b*) with or without syngeneic T-clone cells.

clone/macrophage ratios of 0.5:1 or 1:1. The activity of proliferation of mycobacteria was assessed from incorporation of ^{3}H-uracil during 16 h of culturing.

T-clones were phenotyped by direct immunofluorescence with monoclonal antibodies to CD3, CD4, CD8, and α,β-TcR markers labeled with phycoerythrin or fluorescein isothiocyanate (Pharmingen). Washed cells (1×10^6) were resuspended in 10 μl phosphate buffer with 0.5% bovine serum albumin; monoclonal antibodies were added in amounts recommended by the manufacturer, and then incubated for 30 min on ice, after which the cells were three times washed in phosphate buffer with bovine serum albumin and fixed in 1% paraformaldehyde. Cells not treated with monoclonal antibodies served as the control. The fluorescence of stained cells was analyzed using a Coulter Electronics EPICS "ELITE" laser flow cytofluorometer. The particles differing in size and granularity from live cells were disregarded; for this purpose logical limitations were introduced in histograms of particle distribution by the small-angle and 90° light diffusion; the results were statistically processed using a Multigraph software.

RESULTS

We have characterized 10 resultant T-clones. Seven of them (B1F8, B1C6, B2G7, B3A2, B1D1, B3B3, and B1D12) belong to H-2^d haplotype, one to H-2^a haplotype (A1G10), and two to H-2^f haplotype (M2H5 and M1F3). Flow cytofluorometry showed that all the clones express CD3 marker, i. e., carry T-receptor and are T-lymphocytes. The phenotype of the overwhelming majority of T-clones is CD3$^+$, CD4$^+$, CD8$^-$, and αβ$^+$. The only exception is clone B1D12, which has phenotype CD3$^+$, CD4$^-$, CD8$^+$, and αβ$^-$. We suppose that its antigen-recognizing receptor includes the γ and δ chains.

The expression of T-cell markers directly depends on the activation of T-clone. Analysis of the expression of markers αβ and CD4 by clone A1G10 clearly demonstrates a weaker fluorescence of cells obtained 7 days after restimulation of the clone with the antigen and interleukin-2 in comparison with cells isolated 2 days after restimulation (Fig. 1).

All the resultant T-clones except B1D12 are restricted for class II H-2 gene products (clone A1G10 is restricted for Ia^k) and are antigen (PPD)-specific. Figure 2 shows the titration curves for some clones indicating that clone B1F8 specifically responds to very low concentrations of PPD. We failed to detect antigenic specificity of clone B1D12 T-receptor; it probably recognizes an autologous determinant constitutively expressed by murine cells of H-2^d and H-2^a haplotypes, because B1D12 proliferates in the presence of presenting cells of these haplotypes and does not proliferate on cells of haplotypes H-2^{h4}, H-2^{bg1}, and H-2^f, no matter whether PPD is present or not in the culture. Hence, the restriction of B1D12 for allele d of complex H-2 D-terminal, common for haplotypes H-2^d and H-2^a, appears to be the most probable.

The results of one experiment studying the effects of T-clones on the bacteriostatic activity of

macrophages of H-2^d and H-2^f haplotypes are shown in Fig. 3. *M. tuberculosis* H37Rv rapidly multiplies in test medium without antibiotics, incorporating ^{3}H-uracil proportionally to the initial number of bacteria in a well. After the addition of H37Rv to 6×10^4 peritoneal macrophages in 1:1, 2:1, or 5:1 ratio, the incorporation of the label with phagocytized mycobacteria is also proportional to their initial number. The B10.M macrophages (Fig. 3, *b*) are characterized by slightly higher bactericidal activity compared with that of B10.D2 macrophages (Fig. 3, *a*). Macrophages of both genotypes completely suppress the proliferation of mycobacteria at 0.5:1 ratio of H37Rv to macrophage (data not shown). The addition of recombinant murine γ-interferon in a dose of 10 IU/ml increased bacteriostatic activity of macrophages and completely suppressed the growth of mycobacteria irrespective of the H37Rv to macrophage ratio, which agrees with results of others [6].

Figure 3, *a* shows that the presence of T-clones B1F8 and B1D1 in cell culture at 1:1 ratio of T-clones and macrophages results in suppression of mycobacteria growth. On the other hand, clones B1C6 and B2G7 exert no effects of this kind, and the addition of H-2^f haplotype T-clones M1F3 and M2H5, the only two clones we have today, even decreases the bactericidal effect of macrophages (Fig. 3, *b*).

The described characteristics of PPD-specific T-clones from mice with genetically different sensitivity to tuberculous infection are obviously regulated by the allele composition of H-2 complex. These data can be confirmed after studies on a panel of T-clones including T-clones derived from C57Bl/6 mouse strain sensitive to *M. tuberculosis* infection and investigation of their fine antigenic specificity and the spectrum of lymphokins produced by them.

This study was partially supported by the WHO and INTAS program (grant No. 94-1996).

REFERENCES

1. B. V. Nikonenko, M. B. Mezhlumova, and A. M. Moroz, *Byull. Eksp. Biol. Med.*, **110**, No. 11, 526-529 (1990).
2. P. M. Allen, *Cell*, **76**, 593 (1994).
3. A. S. Apt, V. G. Avdienko, B. V. Nikonenko, *et al.*, *Clin. Exp. Immunol.*, **94**, 322 (1993).
4. S. J. Brett and J. Ivanyi, *Immunology*, **71**, 113 (1990).
5. S. J. Brett, J. M. Orrell, J. Swanson-Beck, and J. Ivanyi, *Ibid.*, **76**, 129 (1992).
6. I. E. A. Flesch and S. H. E. Kaufmann, *Infect. Immun.*, **56**, 1464 (1988).
7. R. N. Germain and D. H. Margulies, *Annu. Rev. Immunol.*, **11**, 403 (1993).
8. K. Huygen, D. Abramovicz, P. Vandenbussche, *et al.*, *Infect. Immun.*, **60**, 2880 (1992).
9. M. J. Lefford, *Ibid.*, **18**, 646 (1975).
10. I. M. Orme, P. Andersen, and W. H. Boom, *J. Infect. Dis.*, **167**, 1481 (1993).