PREPARATION OF MONOCLONAL ANTIBODIES TO STAPHYLOCOCCAL α-TOXIN BY IN VITRO IMMUNIZATION

N. N. Belyaev, G. K. Zakir'yanova, G. O. Kostin, R. T. Tleulieva, A. O. Agashkin, D. G. Navasardyants, Yu. V. Ezepchuk, and A. B. Beklemishev

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Interest in staphylococcal α-toxin (SAT) is due to its essential role in the pathogenesis of staphylococcal infections. There is experimental justification for regarding it as one of the leading factors of virulence of *Staphylococcus aureus* [2, 9]. In the modern view SAT has three functional activities against the host: lethal, dermonecrotic, and hemolytic. The immunochemical identification of the regions responsible for exhibition of these properties involves the use of polyclonal monospecific sera [1, 4]. However, the use of monoclonal antibodies (MAB) for the epitopic analysis of the SAT molecule is unquestionably to be preferred. The investigation of hemolytic activity of SAT with the aid of MAB has been reported. Creation of a panel of hybridomas producing monoclonal antibodies to SAT would make for substantial progress in the study of functionally important epitopes of SAT, standardization of the conditions of it's immunochemical identification, and significant improvement of the purification of SAT on standard monospecific immunosorbents, and facilitate it.

EXPERIMENTAL METHOD

SAT was isolated from the culture fluid of *Staphylococcus aureus* strain S-15 by the method in [5] with modifications. The protein fraction was obtained from the culture fluid by precipitation with 75% saturation of ammonium sulfate, desalted on G-25, and subjected to column chromatography on DE-52, followed by elution with Tris-HCl buffer, pH 7.6. Further purification was carried out by chromatofocusing on PBE 94 by the method suggested by the firm of "Pharmacia." The fractions were tested for homogeneity by PAG-SDS electrophoresis [12]. SAT, purified to the homogeneous state, was conjugated with CH-sepharose 4B granules by the method suggested by "Pharmacia" at the rate of 10 μg SAT to 1 μl of CH-sepharose residue. The degree of adhesion was estimated by disappearance of hemolytic activity of the material not bound with the granules. SAT conjugated with sepharose was used to immunize splenocytes in an in vitro system as in [13] with modifications. Splenocytes ($2.5 \cdot 10^8$) obtained by "teasing" from the spleens of three nonimmune BALB/c mice were incubated with $15 \cdot 10^6$ peritoneal macrophages from the same mice in 15 ml of conditioned mixed lymphocyte culture medium (CMLCM) in the presence of conjugated SAT (2 μg/ml). The CMLCM were obtained by coculture of thymocytes of 4-week-old BALB/c and C57BL/6 mice, $5 \cdot 10^6$ of each cells, in 1 ml for 48 h [7] in medium containing 10% fetal calf serum. After incubation for 5 days in a CO_2 incubator (5% CO_2, 37°C) the splenocytes were harvested, living cells were counted with the aid of trypan blue, hybridized with myeloma P3 × 63Ag8.653, and cultured in 96-well planchets in medium RPMI-1640 containing 10% each of fetal calf and horse serum and standard concentrations of glutamine, 2-mercaptoethanol, hypoxanthine, aminopterin, thymidine, and antibiotics [9]. Selection of antibody-producing hybridomas and cloning by

M. A. Aitkhozhin Institute of Molecular Biology and Biochemistry, Academy of Sciences of the Kazakh SSR, Alma-Ata. N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR A. T. Skavronskaya.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 7, pp. 73-76, July, 1991. Original article submitted November 3, 1990.

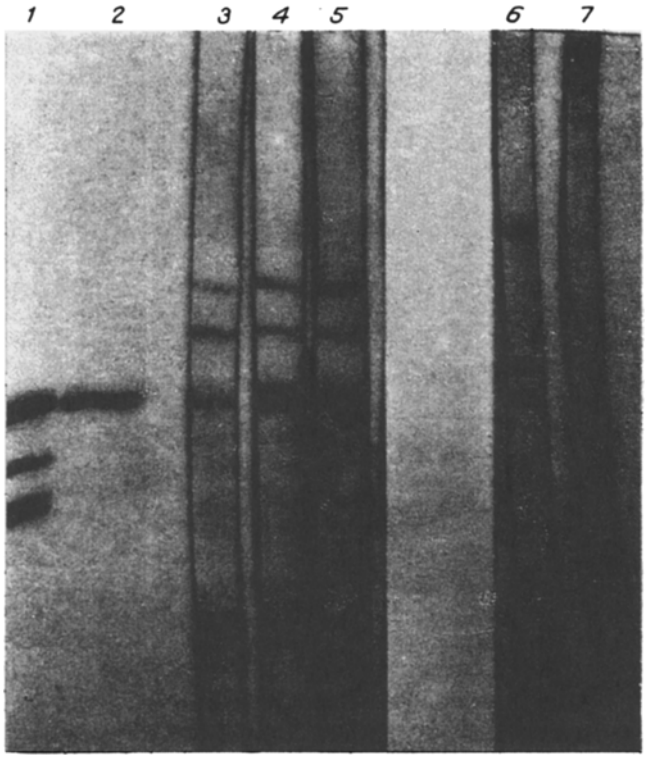

Fig. 1. Immunoblotting of MAB to SAT. 1) Electrophoresis of sulfate fraction of SAT after ion-exchange chromatography; 2) electrophoresis of SAT after chromatofocusing; 3, 4, 5) immunoblotting of monoclonal antibodies (5E10, 2D7, 2B9) from sulfate fractions of ascited fluids; 6, 7) immunoblotting of monoclonal antibodies (3G7, 2B9) from culture media.

TABLE 1. Growth Properties of Different Culture Media for Nonimmune Splenocytes

Parameter	CM	CMLCM	CMT	CMLCM + CMT
Incorporation of ^{14}C-thymidine, cpm × 10^3	3,5	24,5	4,0	18,0
Number of licing cells, % of original number	133	73	67	80

TABLE 2. Effective Hybridomas after Immunization in vitro

Cells containing hybridomas, % of total number of cells	Cells with antibody production, % of number of cells with hybridomas	Number of monoclones left for testing
47,5	62,5	14

the limiting dilutions method were carried out after testing of the culture media by enzyme immunoassay (EIA) [9]. Positive monoclonal antibody production was confirmed by the immunoblotting test [14], cloning was repeated another twice in order to obtain stable lines and the conversion was made to the ascites form by injecting $5 \cdot 10^6$-10^7 cells into BALB/c females, primed with pristane. The immunoglobulin fraction was separated from the ascites fluid by salting out with sodium sulfate [15], dialyzed against phosphate-salt buffer (5 mM NaH_2PO_4, 150 mM NaCl, pH 7.4), and kept at

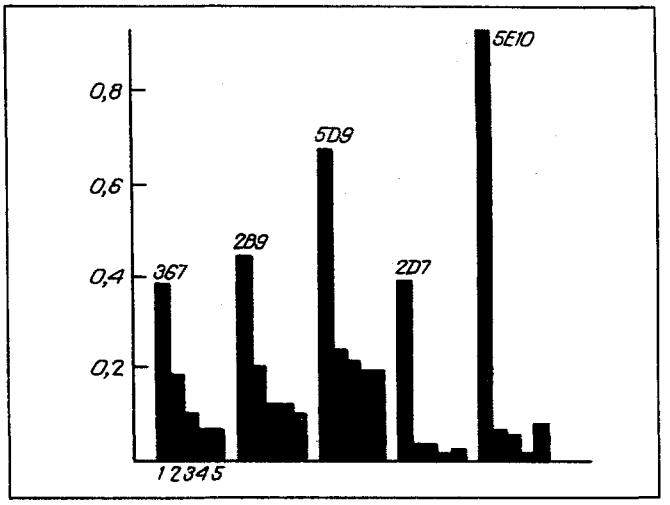

Fig. 2. Determination of isotopes of heavy chains of MAB to SAT in EIA. Ordinate, light absorbance at λ = 492 nm; abscissa, EIA with different antiisotopic sera: 1) anti-IgM; 2) anti-IgG_1; 3) anti-IgG_{2a}; 4) anti-IgG_2; 5) anti-IgG_3.

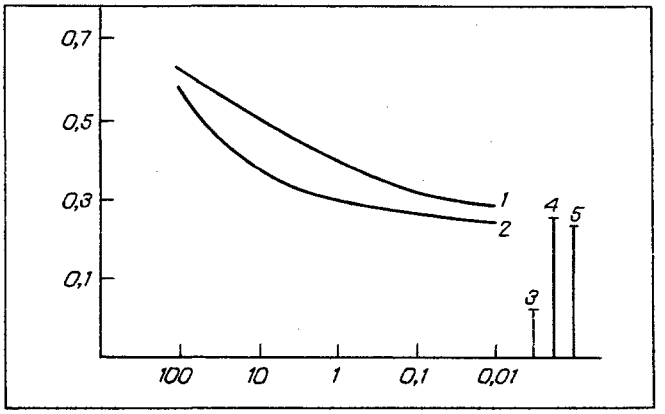

Fig. 3. Titration of monoclonal anti-SAT antibodies in ELISA. Ordinate, absorbance of light at λ = 492 nm; abscissa, concentration of antibodies (in μg/ml). 1) 2B9; 2) 5E10; 3) peroxidase conjugate of "rabbit anti-mouse" antibodies; background binding with plastic: 4) 2B9, 5) 5E10.

–20°C. Isotopes of the heavy chains of the MAB were determined by EIA using antiisotopic rabbit sera and a peroxidase conjugate of "goat – antirabbit" antibodies [9].

EXPERIMENTAL RESULTS

On isolation of SAT by the known method [5] difficulties arose on account of loss of hemolytic activity during purification. These difficulties were overcome by carrying out the purification procedures in the cold (0-4°C) and replacing dialysis by deionization on G-25. After ion-exchange chromatography virtually all the hemolytic activity and, consequently, the SAT proper was contained in the "breakthrough." Electrophoretic analysis of the preparation obtained at this stage of purification revealed the presence of three additional minor contaminating proteins besides SAT (Fig. 1, lane 1). This preparation was used to obtain the immunogenic conjugate. The incompletely purified preparation was used as antigen for screening the culture media of the hybridomas in EIA.

Although SAT is a sufficiently powerful immunogen, because of its high toxicity it is usually used for immunization after preliminary inactivation by heating or by treatment with formalin, which denatures the SAT molecule and, consequently, modifies the functional epitopes. We therefore used a technique of immunization in vitro, the essence of which is that lymphocytes isolated from the spleen of nonimmune mice are incubated with antigen in the presence of accessory cells (syngeneic macrophages), various lymphokines, and interleukins. To choose optimal conditions of incubation a preliminary experiment was carried out. Spleen cells were incubated for 5 days in 96-well planchets, with a density of $2 \cdot 10^5$ cells per well, in the following culture media: 1) RPMI-1640 with 10% fetal calf serum — complete culture medium (CCM), 2) CMLCM, 3) conditioned medium of thymocytes (CMT) of C57BL/6 mice aged 1 month, 4) a mixture of CMLCM and CMT in the ratio of 1:1. All media contained 0.1 mM glutamine and antibiotics (penicillin and streptomycin, 100 IU/ml of each). Proliferation was assessed on the basis of incorporation of ^{14}C-thymidine into DNA of the dividing cells [3]. The results are given in Table 1.

As Table 1 shows, CMLCM possessed the best growth properties. This was evidently due to the fact that CMLCM contains B-cell growth factor, interleukin 2, and allogeneic T-helper factor [13]. Accordingly, CMLCM was used later in the work as culture medium for splenocytes during immunization in vitro. At the same time, counting the cells surviving after incubation for 5 days with antigen revealed that only 20% of the original number of cells remained viable. However, despite the high mortality among splenocytes during incubation and the absence of the antisuppressor agent cimetidine from the culture medium, as was used by Brams [8], a sufficiently high yield of antibody-producing hybridomas was achieved (Table 2). The hybridomas obtained were cloned at least twice. Six monoclones were converted into the ascites form. Sulfate fractions from three ascites fluids were tested for binding with SAT in the immunoblotting test. Binding with the band corresponding to SAT can be clearly seen in Fig. 1 (lanes 3, 4, and 5). Two additional stained bands appeared, evidently as a result of specific binding of certain minor components of the partially purified SAT preparation, not revealed by electrophoresis, with immunoglobulins of the mice receiving the ascites tumor. In fact, when the immunoblotting test was carried out with culture fluid containing only mouse MAB, a band corresponding only to SAT was discovered (Fig. 1, lanes 6 and 7).

Isotopic analysis of the five MAB showed that all monoclones studied secreted antibodies of the μ-isotype (Fig. 2). Our results are in full agreement with data in the literature indicating that during immunization in vitro the immune response develops in accordance with the primary type, i.e., with production of IgM-antibodies [9, 13].

Two ascites MAB (2B9 and 5E10) were tested for binding of SAT and titrated by ELISA (Fig. 3). As Fig. 3 shows, antibody 2B9 had greater affinity than 5E10 for SAT. Both antibodies titrated well within the range from 100 to 1 μg/ml.

The use of a new immunogenic form of SAT in the form of a conjugate with CN-sepharose 4B thus proved an effective way of obtaining monoclonal antibodies to the whole toxin molecule. By the use of the in vitro immunization technique monoclonal antibodies of the IgM-class were obtained.

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NATURAL KILLER CELLS IN PATIENTS WITH PULMONARY TUBERCULOSIS

I. S. Khomenko, V. Ya. Gergert, and N. S. Kolodyazhnaya

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The close study of immune reactivity in patients with tuberculosis has led to the creation of a sufficiently sound system of ideas on the connection between the time course of the disease and the level of T-lymphocytes ($CD3^+$-cells) and their principal subpopulations, namely helper and suppressor cells ($CD4^+$- and $CD8^+$-cells) [1-3, 7, 9-11]. Meanwhile, few studies of yet another lymphocyte subpopulation, namely natural killer (NK) cells, in tuberculosis have been published. According to data in the literature NK cells are polypotent: they play an important role in antiviral protection, they carry out antibody-independent lysis of tumor and virus transformed cells; they have also an Fc-receptor, which enables them to perform cytolytic functions with the involvement of antibodies, and they can directly kill certain bacteria [4-6, 13, 14].

Considering the mainly intracellular parasitism of *Mycobacterium tuberculosis,* involvement of NK cells in the mechanisms of resistance to mycobacterial infection cannot be ruled out. According to data in the literature, in some cases an increase in the number of NK cells is observed in the blood and pleural exudate of patients with tuberculosis compared with healthy individuals [8]. Activity of NK cells in the blood and exudate, according to these same workers, does not extend beyond normal limits. Onwubalili and co-workers [9] also noted the absence of changes in the corresponding parameters of NK cells in tuberculosis, which they analyzed in relation to the form of the disease or treatment given. They point out that the NK cell system evidently does not play an active role in protective mechanisms in tuberculosis. In other cases [12], a certain tendency was nevertheless found toward an increase in the activity of these cells, associated more with the duration of the disease. Similar views are held by Yoneda and co-workers [15], who observed correlation between a low level of NK cell activity and worsening of the roentgenologic data and progression of the disease.

The aim of the investigation described below was to determine the number of NK cells in the blood of patients with pulmonary tuberculosis, to establish correlation between their number and various other clinically important parameters, studied previously, relating to the mean level of $CD3^+$-, $CD4^+$-, and $CD8^+$-lymphocytes, and determination of the intensity of fluorescence of NK cells and of $CD3^+$-, $CD4^+$-, and $CD8^+$-lymphocytes.

EXPERIMENTAL METHOD

In order to carry out this task we undertook immunologic testing of the blood of patients with pulmonary tuberculosis and healthy volunteers. For immune phenotyping of the lymphocytes we used monoclonal antibodies from the firm Behringwerke (West Germany) ($CD3^+$ — BW264/56, $CD4^+$ — BW264/123, and $CD8^+$ — BW135/80, NK cells BMA070) in

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