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Role of Various Murine B-Lymphocyte Subpopulations in the Immune Response to T-Independent Antigens

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Polyclonal antibodies to Lyb 5^+ antigen of murine B lymphocytes are obtained and a methodological approach to the detection of cells carrying this antigen is developed with the aim of investigating the role of various subpopulations of mouse B lymphocytes in polyclonal activation induced by T-independent type 2 antigen. Hybridomas producing anti-Lyb 5.1 antibodies are obtained.

Key Words: *B lymphocytes; surface antigens; antibodies to Lyb 5 antigen*

As a rule, upon challenge an antigen not only induces antibody production in an animal organism, but intensifies the synthesis of nonspecific immunoglobulins (Ig) and increases the number of cells producing them. During a T-dependent response this latter circumstance depends largely on the nonspecific stimulating action of T lymphokines produced by antigen-activated T helpers. The mechanism of the production of antigen-induced nonspecific Ig in a T-independent response is unknown.

T-independent antigens are subdivided into type 1 antigens, polyclonal activators of B lymphocytes stimulating antibody production in both Lyb 5^- and Lyb 5^+ B cells, and type 2 antigens, which are not mitogenic and are capable of inducing antibody production only in mature B cells carrying the Lyb 5^+ marker [4]. That is why the appearance of not only antibody producers, but of numerous nonspecific Ig producers under the effect of some T-independent type 2 antigens has piqued the curiosity of scientists [3].

It is not clear whether only mature B lymphocytes with a surface Lyb 5^+ marker are "triggered" to undergo proliferation and differentiation, or whether the B cells with the Lyb 5^- phenotype are also involved. Polyclonal activation induced by T-independent antigens shows some specific features distinguishing it from polyclonal activation caused by T-dependent antigens. It is possible that

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B-cell factors also contribute to the immune response to T-independent antigens.

Identification of the role of various B-lymphocyte subpopulations in polyclonal activation (and suppression) of an immune response boosted by T-independent antigens is a task of basic immunology, although such studies are difficult because the relevant "tools", primarily antibodies identifying various B-lymphocyte subpopulations, are lacking.

This research was aimed at the production of polyclonal antibodies to mouse B-lymphocyte Lyb 5^+ antigen and the development of a methodology for the detection of cells carrying these antibodies.

MATERIALS AND METHODS

Experiments were carried out with CBA, C57Bl/6, DBA/2, and BALB/c mice from the Stolbovaya Breeding Center, Russian Academy of Medical Sciences, and CBA/N mice kindly supplied by Dr. B. V. Nikonenko from the Institute of Tuberculosis, Russian Academy of Medical Sciences.

F_1-hybrids were obtained by mating CBA/N females with DBA/2, BALB/c, and C57Bl/6 males.

Antiallotypical serum to Ig of C57Bl/6 mice (Igh-b allotype) was prepared by immunizing BALB/c mice (Igh-a allotype) as described previously [2] with an immunoadsorbent containing Ig of C57Bl/6 mice. The animals were intraperitoneally injected 100 μg of Ig immobilized on cellulose and after a month reimmunized with 100 μg of immobilized (intraperitoneally) and 10 μg of soluble Ig (intravenously); after 8 or 9 days blood was collected and serum prepared.

Polyclonal antiserum to Lyb 5.1 antigen was prepared as described previously [7]. Splenocytes of DBA/2 mice (Lyb 5.1 allotype) in a dose of 50×10^6 in complete Freund adjuvant were injected in pads to C57Bl/6 mice (Lyb 5.2 allotype). The animals were then intraperitoneally injected 25×10^6 splenocytes of DBA/2 mice every week for 5 weeks, and blood was collected on day 5 after the last immunization.

The antiserum was successively depleted by hepatocytes, splenocytes, and thymocytes of F_1 (CBA/N×DBA/2) males.

Anti-Lyb 5.1 antibodies were detected in the cytotoxic test. The tested antisera in various dilutions and guinea pig complement in final dilution 1:9 were added to 5×10^8 splenocytes of mice of different strains. To control samples the complement and normal murine serum were added and cell viability was assessed after 1-h incubation at 37°C in an atmosphere with 6% CO_2 by the incorporation of trypan blue.

The cytotoxicity index (CI) was calculated according to the formula: $CI=(A-B)/(100-B)\times100$, where A is the number of live cells in the tested culture and B the percentage of dead cells in the control.

Protein A (Pharmacia), rabbit antibodies to mouse Ig γ-chains, monoclonal antibodies to allotypical determinant of mouse Ig μ-chains (Ig-μ^a), (RS3.1), a gift of Dr. V. L. Yurin (Medicogenetic Research Center, Russian Academy of Medical Sciences) and polyclonal antiallotypical antibodies to Ig of C57Bl/6 mice were labeled with fluorescein isothiocyanate (FITC) after Forni [5]. Fresh FITC solution in 0.1 M carbonate (12.5 μg FITC per mg protein) was added to preparations dialyzed against 0.1 M carbonate-bicarbonate buffer. The reaction was carried out at 0°C and pH 9.0, which was maintained by adding 0.05 M carbonate solution if necessary. When a constant (unchanged for 15 min) pH was attained, the reaction mixture was left for 18 h in ice in a place shielded from light, the pellet was removed by centrifugation, and labeled proteins were sedimented from the supernatant with a saturated solution of ammonium sulfate. After one hour the precipitated sediment of labeled protein was removed by centrifugation, dissolved in normal saline buffered with phosphates, and dialyzed against normal buffered saline. In some cases FITC conjugates were fractionated on DE-52 cellulose. For this purpose labeled preparations in 0.01 M phosphate buffer, pH 7.5, were layered onto anion ion exchanger and step-by-step elution was carried out using the same buffer with ascending NaCl concentrations (0.05, 0.1, 0.2, 0.3, and 0.4 M).

Live cells adhering to slides by poly-L-lysine (900 kD, Sigma) were fluorescent-stained [6]. The studied antibodies were added to the cells and incubated for 40 min at 37°C in an atmosphere with 5% CO_2. After washing in Hanks' solution with lactalbumin, FITC-antibodies or FITC-protein A were added, the slides were incubated for 40 min at room temperature, washed again, and examined under a LYuMAM fluorescence microscope.

Murine Ig secreted by the cells were detected by solid-phase enzyme immunoassay with a Calbiochem kit for detection of murine Ig.

RESULTS

Preparation of anti-Lyb serum and detection of B cells carrying Lyb 5 antigen. The problem with antibodies to Lyb 5^+ surface antigen is that their production cannot be induced by direct immunization of xid CBA/N mice devoid of Lyb 3^+, 5^+,

7^+ B-lymphocyte subpopulations with the cells of a congenic CBA mouse strain possessing the same subpopulation. Hence, allotypical differences for Lyb 5 antigen were used to obtain anti-Lyb 5 antibodies in mice of different strains. Two Lyb 5 allotypes are known: Lyb 5.1 (DBA/2 mouse B cells carry it) and Lyb 5.2 (in CBA, BALB/c, and C57Bl/6 mice). Mutual immunization helps obtain anti-Lyb 5 antibodies.

As a result of immunization of C57Bl/6 mice with splenocytes of DBA/2 mice, an alloantiserum was obtained that was characterized by a high cytotoxic activity toward DBA/2 mouse splenocytes. For the removal of antibodies to H-2 antigens from this antiserum and the production of Lyb 5.1-specific antiserum, it was successively depleted of hepatocytes, splenocytes, and thymocytes of male F_1 hybrids (CBA/N×DBA/2). (The xid mutation being linked to the X chromosome, the use of lymphocytes from F_1 male hybrids without Lyb 3, 5, 7 surface markers on B cells helps remove antibodies to all DBA/2 antigens except antibodies to these markers.) As a result of such treatment, we obtained an antiserum exhibiting cytotoxic activity to DBA/2 ($H\text{-}2^d$, Lyb 5.1) mouse lymphocytes, but not to lymphocytes of BALB/c ($H\text{-}2^d$, Lyb 5.2) or CBA/N ($H\text{-}2^k$, Lyb 5^-) mice; treatment of cells with antiserum in the presence of complement led to the death of 22% of splenocytes of DBA/2 mice, but virtually did not lessen the viability of BALB/c mouse splenocytes.

Since B lymphocytes account for about 50% of murine splenocytes and Lyb 5^+ cells for about 60% of all murine B-lymphocytes [8], these data permit us to consider the alloantiserum a specific anti-Lyb 5 (3, 7) serum.

Allotype Lyb 5.1 is far less common in mice than allotype Lyb 5.2. Specific antibodies to Lyb 5.2 allotype could be of special interest to scientists, for they would extend the range of application of anti-Lyb 5 antibodies. We therefore attempted to prepare an antiserum to allotype Lyb 5.2 using a reciprocal immunization protocol, that is, by immunizing DBA/2 mice with splenocytes of C57Bl/6 mice. However, contrary to published reports [8], we failed to obtain antibodies to Lyb 5.2 marker by this method. Depleting the alloantiserum of hepatic, splenic, and thymic cells of male F_1 hybrids (CBA/N×C57Bl/6) resulted in the loss of its capacity to specifically react with B cells carrying the Lyb 5.2 surface marker.

It is noteworthy that besides the method we used to obtain polyclonal antibodies to cells carrying Lyb 3, 5, and 7 antigens, another method has been described [9], consisting in the immunization of xid mutation-carrying male F_1 hybrids (CBA/N×BALB/c) with splenocytes of BALB/c mice. C57Bl/6 splenocytes (Igh-b allotype) may be suitable for the detection of such antibodies carrying the Igh-a allotype; other antibodies in this case might evidently be antibodies to the Igh-a allotype, ideally antibodies of C57Bl/6 mice to BALB/c Ig. We should not forget, however, that it is difficult to reproduce highly active antibodies to the Igh-a allotype of BALB/c Ig from C57Bl/6 mice [1]. Attempts at the preparation of such antibodies using various immunization protocols, including immunization on the adsorbent, have so far failed.

Antibodies to μ^a-chains of murine Ig were the only anti-Igh-a antibodies available to us. We tested whether such antibodies might be used for the detection of IgM antibodies to Lyb 5.2 antigen. With this in mind, we obtained hybridomas from splenocytes of male F_1 hybrids (CBA/N× ×BALB/c) after a single immunization thereof with BALB/c splenocytes. The bulk of antibodies after such an immunization was expected to belong to the IgM isotype and be detectable with antibodies to μ^a. Unfortunately, no positive results ensued. This could have been due either to the low activity of labeled antibodies or to the lack of production of anti-Lyb 5.2 antibodies.

The resultant anti-Lyb 5.1 serum was used to develop methods of detecting Lyb 5.1^+ B lymphocytes by indirect immunofluorescence. FITC conjugates with protein A, antibodies to γ-chains of murine Ig, and antiallotypical antibodies to C57Bl/6 Ig were used as fluorescent reagents.

In a model system with affinity purified rabbit antibodies to mouse Ig as the primary antibodies the FITC-protein A conjugate yielded a clearly defined specific staining of B lymphocytes binding antibodies against a low nonspecific background. If anti-Lyb 5.1 antibodies were used as the primary ones, the sensitivity of the method was insufficient for the detection of cells specifically binding anti-Lyb 5.1 antibodies.

Since the Ig receptors on the surface of B lymphocytes are represented mainly by the IgM isotype, we tried to use the FITC-anti-γ-chains conjugate for the detection of IgG-anti-Lyb 5.1 antibodies (obtained by multiple immunizations of animals) bound by B cells. However, the fluorescence of FITC-anti-γ-antibodies was highly nonspecific, making interpretation of the results difficult. Evidently, this was due to the presence of Fc-receptors, binding the rabbit IgG, on the cells. For this reason we propose to use in the future Fab- or $(Fab)_2$ fragments of FITC-labeled anti-γ-antibodies for the detection of anti-Lyb 5.1 antibodies.

The best results were achieved with FITC-antibody conjugates to the Igh-b allotype of C57Bl/6 mouse Ig. Such antibodies were obtained by immunizing BALB/c mice with C57Bl/c Ig as described previously [2]. FITC conjugates of antibodies yielded a low nonspecific background (staining of DBA/2 splenocytes pretreated with normal serum of C57Bl/6 mice) and permitted the specific detection of anti-Lyb 5.1 antibodies bound to cells expressing this marker. The working dilutions of the resultant conjugates were 1:8 to 1:16.

The development of a method for detecting antibodies to Lyb 5.1^+ cells by immunofluorescent analysis allowed us to go on to prepare monoclonal antibodies to Lyb 5.1.

Preparation of hybridomas producing anti-Lyb 5.1 antibodies. C57Bl/6 mice were immunized with DBA/2 splenocytes according to the above protocol. On day 3 after the last immunization the immune splenocytes were fused with the cells of mouse myeloma X63.Ag8.653, and thus cultures were prepared, 107 of which were Ig producers, as shown by immunofluorescent analysis. Nine of these cultures produced Ig not binding the splenocytes of male F_1 hybrids (CBA/N×DBA/2) devoid of the B-cell Lyb 5.1^+ subpopulation, but staining the cells of female F_1 hybrids and of DBA/2 mice expressing the Lyb 5.1 marker. These data prove that monoclonal antibodies to Lyb 5 antigens can be prepared, but the final assessment of the specificity of secreted antibodies will be possible only after further studies.

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