Lymphocyte Antibody-Dependent Cytotoxicity Test for Evaluation of Clinical Role of Monoclonal Anti-D-Antibodies for Prevention of Rhesus Sensitization

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Monoclonal antibodies to D antigen were studied in the reaction of antibody-dependent cytotoxicity for evaluation of the possibility of using these antibodies for preventing rhesus sensitization. High hemolytic activity of four anti-D-monoclonal antibodies in the antibody-dependent cytotoxicity test, mediated by their interaction with FcγRI, and the capacity to accelerate elimination of D^+ erythrocytes from circulation did not provide the immunosuppressive effect. It was hypothesized that monoclonal antibodies for prevention of rhesus sensitization should interact with FcγRIII on lymphocytes. These monoclonal antibodies are extremely rare: only 4 of 125 studied antibodies mediated hemolysis in the antibody-dependent cytotoxicity test with lymphocytes, while all polyclonal anti-D-preparations exhibited this activity.

Key Words: *D antigen; monoclonal antibodies; Ig; antibody-dependent cytotoxicity test; immunosuppression*

Erythrocytic D antigen determines rhesus appurtenance of human blood and is present in about 85% Caucasians. Antibodies to D antigen (anti-D) can form in rhesus-negative women in response to appearance of fetal rhesus-positive erythrocytes (D^+) in the circulation (mainly during childbirth). Anti-D-antibodies of IgG1 and IgG3 classes penetrate through the placenta and cause hemolytic disease of newborns during subsequent pregnancy. Injection of antirhesus Ig after delivery more than 10-fold reduces the probability of maternal rhesus sensitization and prevents hemolytic disease. Antirhesus Ig are derived from immune donor sera, which are extremely rare. Development of methods for obtaining human monoclonal antibodies (MAB) and their wide use for creation of diagnostic and therapeutic preparations suggest that monoclonal antirhesus Ig will soon replace polyclonal preparation. However, despite creation and introduction of numerous typing reagents based on anti-D MAB into immunohematological practice during the latest 15 years, there is still no monoclonal preparation for the prevention of rhesus sensitization. Two anti-D MAB preventing rhesus sensitization were described in available publications. One of them is based on BRAD5 MAB of IgG1 class secreted by human Epstein—Barr virus-transformed B-lymphoblastoid cells, the other (MonoRho) are recombinant human IgG1 MAB expressed in CHO cells [9,12]. Both preparations are now at the stage of trials. Four anti-D IgG1 MAB, selected by standard criteria (*in vitro* lysis of erythrocytes in antibody-dependent cytotoxicity (ADC) test and rapid elimination of D^+ erythrocytes from circulation in humans) failed to prevent rhesus sensitization [2].

We attempted to clear out what tests can be used for *in vitro* selection of anti-D MAB for prevention of rhesus sensitization.

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MATERIALS AND METHODS

Normal (nonimmune) human Ig (I. I. Metchnikov Biomed Company), Resogam antirhesus (immune) Ig (Behring), sera of immune anti-D donors, anti-D MAB obtained at our laboratory (G-7, G-12, G-17, and G-48 produced by heterohybrydomas; G-47, G-51, G-55, G-90, G-A6/H4 produced by human B-lymphoblastoid strains), and supernatants containing anti-D were used in the study. Human peripheral blood mononuclears were isolated from whole blood stabilized with heparin by centrifugation in Ficoll. Immune donor mononuclears were transformed with supernatant of B95-8 cell strain (Epstein—Barr virus producer) [1]; the cells were transferred in 96-well round-bottom plates (3×10^4 per well) in RPMI 1640 with 20% FCS and 2 μg/ml phytohemagglutinin. The supernatants were tested after 2-3 weeks, when large spherical colonies of transformed cells grew in the wells. Anti-D IgG were detected in indirect antiglobulin test. ADC test was carried out with two types of effector cells: nonfractionated mononuclears from nonimmune donors in flat-bottom 96-well plates and with non-adherent cells (lymphocytes) in round-bottom plates. Adherent cells were removed by adhesion (1.5 h at 37°C) in plastic flasks in a medium with 15% FCS. Each well contained 0.5×10^6 cells in 50 μl medium with 1% FCS, 50 μl supernatant or antibody dilution, 50 μl normal 0.1% Ig (50 μg/well), and 0.5×10^6 D^+ erythrocytes in 50 μl medium were put into each well. Erythrocytes were pretreated with 0.1% bromelin for 10 min at 20°C and washed 3 times. The plates were incubated for 18-20 h at 37°C and the content of free hemoglobin in wells was measured using colorimetric sample with 2,7-diaminofluorine [6]. Supernatant from the well without antibodies served as spontaneous lysis control. Maximum lysis was determined by adding 150 μl H_2O to 50 μl erythrocytes. The efficiency of lysis (%) was estimated by the formula:

$$(N\text{-}SL)/(ML\text{-}SL)\times100\%,$$

where N is optical density of the well at λ=620 nm, SL is spontaneous lysis, and ML maximum lysis. Each experimental point was repeated twice. Absorption/elution of anti-D on D^+ erythrocytes was carried out as described previously [15]. IgG were isolated by affinity chromatography on a column with protein-A- or protein-G-sepharose.

RESULTS

Four anti-D IgG1 MAB, the most active in the ADC test with unfractionated nuclears, were studied on volunteers who gave their consent to rhesus immunization. All MAB induced rapid clearance of D^+ erythrocytes from circulation, but the duration of elimination varied. The capacity to accelerate erythrocyte elimination did not correlate with suppression of rhesus sensitization [2]. Only serum antirhesus Ig prevented sensitization, while after injection of MAB the incidence of sensitization was higher than in the control group (Table 1).

The mechanism of the immunosuppressive effect of antirhesus Ig remains unknown [5,10]. Anti-D antibodies stimulate phagocytosis or lysis of erythrocytes by cells carrying Fc receptors. Activation of monocytes/macrophages is realized through FcγRI, of lymphocytes through FcγRIII [7]. ADC test is the only method for *in vitro* study of antibody functions mediated by Fc fragment. The study of different anti-D in ADC test showed that addition of normal Ig sharply reduced activity of MAB, but not of polyclonal antirhesus Ig (Fig. 1). FcγRI presented on monocytes effectively bind nonspecific monomeric IgG present in Ig preparations [3,11]. Inhibition of ADC by normal Ig suggests parti-

TABLE 1. Incidence of Immunization of Rhesus-Negative Subjects after Injection of 5-15 ml D^+ Erythrocytes and Anti-D Antibodies

Antibody	Dose and route of injection	Number of volunteers	Subjects with anti-D, %
MAB G-17	1000-1200 μg i/v	16	63
MAB G-12	600-2000 μg i/v	17	40
MAB G-48	1200 μg i/v	7	75
MAB G-7	600-200 μg i/v	6	67
MAB (G-7+G-12)	(300+300) μg i/v	3	100
Antirhesus Ig	200 μg i/m	4	0
Control group	—	12	33

Note. i/v: intravenously; i/m: intramuscularly.

TABLE 2. Anti-D MAB Active in ADC Test with Lymphocytes

Anti-D MAB	Donor No.									
	1	2	3	4	5	6	7	8	9	10
MAB tested in ADC with lymphocytes	6	57	8	14	11	13	1	1	3	2
Active in ADC test with lymphocytes	—	—	1	—	1	2	—	—	—	—

cipation of monocytes in the lysis of MAB-sensitized erythrocytes. The same MAB only slightly stimulated erythrocyte destruction in ADC test with lymphocytes (maximum activity no more than 2% of antirhesus Ig activity) or did not modify it at all, which could be due to their incapacity to bind FcγRIII. These MAB can rapidly eliminate D^+ erythrocytes from circulation [2], but they do not prevent D-sensitization (Table 1). On the other hand, all polyclonal antirhesus Ig, as well as BRAD-5 [9] and Mono-Rho MAB [13] are active in ADC test with lymphocytes and inhibit anti-D response. These indirect data suggest that antibodies with immunosuppressive effect induce hemolysis in the "lymphocytic" variant of ADC test and are not inactivated by normal Ig. None of 9 anti-D MAB obtained at the laboratory possessed these characteristics.

A total of 116 anti-D MAB from 10 immune donors were studied. The data of a representative experiment with MAB from two donors are shown: of 16 antibodies, 2 (both from the same donor: clones 11 and 14) met the requirements (Fig. 2). Of 116 tested MAB only 4 caused hemolysis in ADC test with lymphocytes, including the test in the presence of Ig (Table 2).

Similar results were reported by other authors: of 37 MAB only 5 possessed hemolytic activity in the ADC test with lymphocytes, all of them were obtained from the same donor [8]. Of 64 anti-D IgG MAB only 8 caused erythrocyte lysis in ADC test with lymphocytes and only 4 of them were not inhibited by Ig [11]. These properties of MAB do not depend on the isotype or epitope specificity

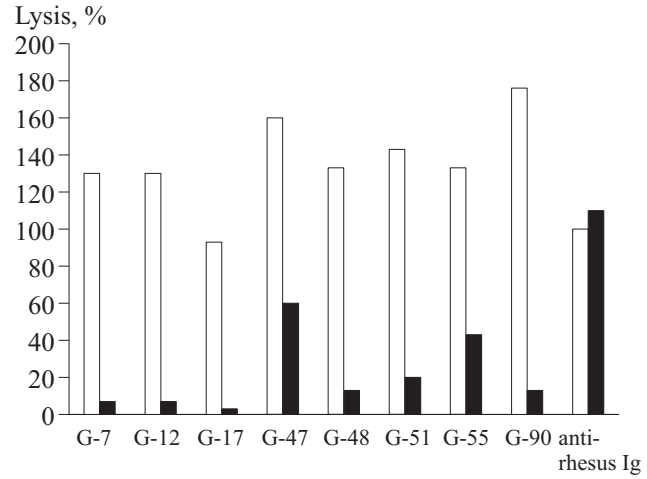

Fig. 1. Effect of normal Ig on activity of anti-D monoclonal antibodies (MAB) in antibody-dependent cytotoxicity test with undivided mononuclears. 100%: efficiency of D^+ erythrocyte lysis in the presence of antirhesus Ig without normal Ig. Concentrations of all antibodies were equilibrated by titer and were about 50 ng/ml. Here and in Figs. 2, 3: light bars: no Ig; dark bars: Ig. Abscissa: anti-D antibodies.

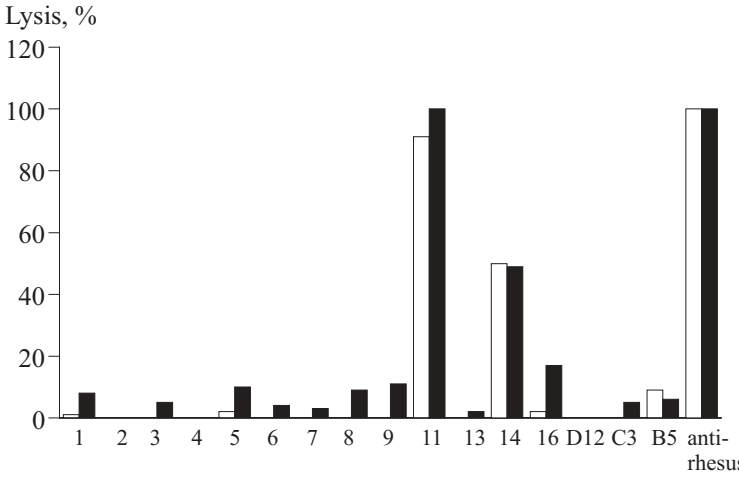

Fig. 2. Screening of anti-D MAB in antibody-dependent cytotoxicity test with lymphocytes. Abscissa: anti-D (clone No.).

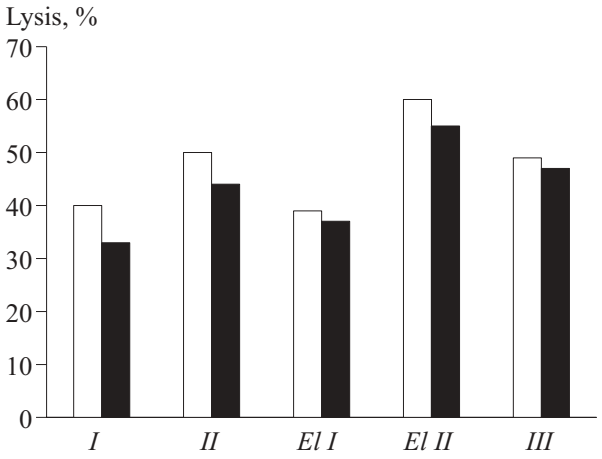

Fig. 3. Effect of normal Ig on activity of polyclonal anti-D antibodies in antibody-dependent cytotoxicity test with lymphocytes. *I, II*: donor sera; *El I, El II*: eluted and purified anti-D from the sera of corresponding donors; *III*: antirhesus Ig.

[3,8]. It is unknown whether functional activity of antibodies depends on the primary structure or post-translation modification of antibodies (*e.g.* glycosylation) [10,14]. On the other hand, in contrast to MAB, antirhesus Ig and individual donor immune anti-D sera tested in our studies were active in the ADC test with lymphocytes. In order to rule out possible effect of foreign antibodies which might modify ADC results, pure anti-D antibodies were isolated from the sera by adsorption/elution on D^+ erythrocytes with subsequent purification on protein-G-sepharose. Purified polyclonal anti-D retained activity in the ADC test with lymphocytes, including antibodies isolated from the sera of donors from whom no needed MAB could be obtained (Fig. 3).

Thus, the majority of immune polyclonal serum anti-D antibodies, similarly as MAB, mediate erythrocyte clearance by monocytes/macrophages, active antigen-presenting cells carrying FcγRI, and this route seems to stimulate the immune response. On the other hand, the polyclonal preparation contains anti-D antibodies reacting with lymphocyte FcγRIII receptors, which can inhibit the immune response. Immunosuppression of anti-D response is the summary effect of these reactions. Hence, antibodies active in the ADC test with lymphocytes, not inhibited by normal Ig, seem to be an obligatory component of a preparation for the prevention of rhesus sensitization. It remains unclear whether the effect of polyclonal antibodies can be completely reproduced by only one MAB. Presumably, cooperation of several MABs with different properties of Fc fragments is needed. Unfortunately, it is impossible to investigate the reaction of MAB with B lymphocytes carrying FcgRIIb in ADC test. This is the only inhibitory receptor type leading to B-cell apoptosis, which can underlie antibody-dependent immunosuppression [4]. Nonetheless, evaluation of the effector properties of MAB in the ADC test variants helps to limit the number of MABs selected for *in vivo* trials.

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