MICROBIOLOGY AND IMMUNOLOGY

Effect of Producer Cell Line on Functional Activity of Anti-D Monoclonal Antibodies Destined for Prevention of Rhesus Sensitization

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 147, No. 4, pp. 430-434, April, 2009
Original article submitted December 26, 2007

The ability of anti-D antibodies to cause antigen-specific immunosuppression depends on their interaction with low-affinity Fcγ-receptors. Human monoclonal antibodies to D antigen of the rhesus system were investigated by antibody-dependent cytotoxicity assay in order to estimate their ability to induce hemolysis mediated by low-affinity Fcγ receptors. We demonstrate that affinity of monoclonal antibodies to receptors of this type does not depend on primary structure of Fc-fragment, but depends on the producer cell line which expresses the antibodies. Monoclonal IgG1 antibodies interacting with FcγRIIa and FcγRIII lost this property, if they were secreted by human-mouse heterohybridoma, but not by human B-cell line. On the opposite, monoclonal antibodies that could not activate low-affinity Fcγ receptors were highly active after human cells fusion with rat myeloma YB2/0. Hemolytic activity of IgG3 remained unchanged after fusion of human cells with rodent cells.

Key Words: *monoclonal antibodies; D antigen; antibody-dependent cytotoxicity; Fc-receptor; immunosuppression*

Antibody-dependent immunosuppression phenomenon consists in inhibition of the immune response to antigen by simultaneously injected IgG antibodies to the same antigen [7]. This effect is successfully used for prevention of D-sensitization in rhesus-negative women with anti-rhesus immunoglobulin (Ig) prepared from immune donor sera: administration of this preparation after delivery makes it possible to prevent immune response to fetal D^+-erythrocytes and hemolytic disease in neonate upon consequent childbearing. IgG class anti-D antibodies cause rapid excretion of D^+ erythrocytes mediated by interaction of antibody Fc fragments bound to D-antigen with Fcγ-receptors (FcγR) on splenic macrophages. FcγRI is a high-affinity receptor and is able to bind free IgG; low-affinity FcγRIIa and FcγRIII interact only with IgG-antigen complexes. Despite long-lasting experience in the use of specific and unspecific Ig as immunosuppressive drugs, the specific crucial process (or an ensemble of processes) leading to inhibition of the immune response remains unclear, while most facts attest to the key role of immune complexes and profile of their interaction with Fc-receptors on immunocompetent cells [4].

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0007-4888/09/1474-0448

Some compounds based on human anti-D-monoclonal antibodies (MCA) explicitly studied by *in vitro* tests were recently assessed on volunteers. It was demonstrated that recombinant human anti-D-MCA and the majority of MCA secreted by human-mouse heterohybridomas are not able to accelerate clearance of D^+-erythrocytes from circulation and to block immune response [10]. However, even those MCA that reproduced the effects of polyclonal anti-rhesus Ig *in vivo* and induced rapid clearance of D-erythrocytes, did not prevent immunization and in some cases even stimulated it [1]. These MCA demonstrated high hemolytic activity *in vitro* in the antibody-dependent cytotoxicity assay (ADC), they mediated hemolysis via interaction with FcγRI but unlike polyclonal anti-D-antibodies did not interact with FcγRIII [2].

In this study, functional activity of different anti-D-MCA was investigated and ADC was compared for the same MCA produced either by human B-lymphoblastoid cell line (LBL) or by heterohybridomas (HH) obtained by fusion of LCL with mouse and rat myelomas. This experiment was conducted basing on the data on different antibody glycosilation in cells from different species [13]. The aim of this study was to assess how changes of producer line affects MCA ability to interact with low-affinity FcγIII and FcγRIIa, because this property seems to be crucial for efficient prevention of rhesus-sensitization: MCA which were not able to bind FcγRIII were also not able to suppress anti-D immune response [2,10]. Since CH2 domain of the antibody is involved in binding with the receptor, we analyzed primary sequence of Fc-fragment genes of anti-D-MCA which differently interacted with low-affinity FcγR.

MATERIALS AND METHODS

Normal (non-immune) human Ig (Mechnikov Biomed Company), anti-rhesus Ig (Hemotransfusion Station, Ivanovo) and anti-D-MCA were used in the study. Anti-D-MCA-producing LBL were obtained by transformation of blood lymphocytes from immune donors with Epstein-Barr virus. HH were obtained by fusion of LBL with mouse myeloma P3-X63-Ag8.653 and rat myeloma YB2/0. Thus, the same MCA was investigated in different variants depending on the producer cell type. Anti-D antibody concentration was estimated by comparing its titer with the preparation (Behring) containing a known amount of D antigen or by spectrophotometry (after purification of MCA). RNA separation from MCA-producing cells and cDNA synthesis at olygo-dT-primer were performed using standard techniques. Heavy IgG chains were amplified using primers, which were degenerate for 5'-ends and universal for 3'-ends of all IgG (hsG-R3-ggatctagaggtgctttatttccatgctg). The cloned amplification product was sequenced in the multiple-access inter-institutional center GENOM of Institute of Molecular Biology of the Russian Academy of Sciences. ADC on mononuclear cells used as effector cells was conducted as follows: 0.5×10^6 mononuclear cells in 50 μl RPMI-1640 medium containing 1% fetal calf serum, 0.1×10^6 D^+ erythrocytes treated with bromelin, antigen D sample in 50 μl medium and 50 μl normal 0.1% Ig (50 μg per well) were placed in a well of 96-well round-bottom plate. Three repeats were done for each point. Plates were incubated at 37°C for 18-20 h, then samples of the supernatant were collected and free hemoglobin was assessed colorimetrically [5]. Hemolytic activity of MCA relative to antirhesus Ig activity taken as 100% was determined by the formula:

$$[(OD_{MCA}\text{-}SH)/(OD_{antirhesus\ Ig}\text{-}SH)]\times100\%,$$

where OD_{MCA} and $OD_{antirhesus\ Ig}$ are optical densities in the well containing D antigen at λ=620 nm and SH is the level of spontaneous hemolysis in the antibody-free well.

RESULTS

Anti-D-MCA interacting with FcγRIII are very occasionally obtained by lymphocyte transformation with Epstein—Barr virus, although all polyclonal anti-D preparations possess this property [2]. In order to assess MCA affinity to low-affinity FcγR, ADC assay was performed in the presence of normal Ig (ADC-Ig) which blocks high-affinity FcγRI on monocytes (Fig. 1). Results of estimation of hemolytic activity of three IgG1 MCA (G-108, G-112 and G-12; Fig. 2, *a*) are demonstrated. Each MCA had two sources: LBL or HH. G-108/LBL MCA demonstrated the same hemolytic activity as anti-rhesus Ig, at low concentrations it even surpassed activity of anti-rhesus Ig (Fig. 2, *b*); MCA G-112/LBL activity was about 80% of the control level, lysis efficacy in the presence of G-12 MCA was very low (Fig. 2, *a*). Fusion with mouse myeloma substantially reduced hemolytic activity of MCA G-108 and G-112 (Fig. 2, *a*, *b*): it was one half as high in MCA/HH than in their "counterparts" produced by LBL at the same concentration. Primary sequence analysis revealed no differences in the base composition of Fc-fragments of MCA G-108, G-112 and G-12 genes from LBL and G-12 gene from human—mouse HH, all of them belonged to the

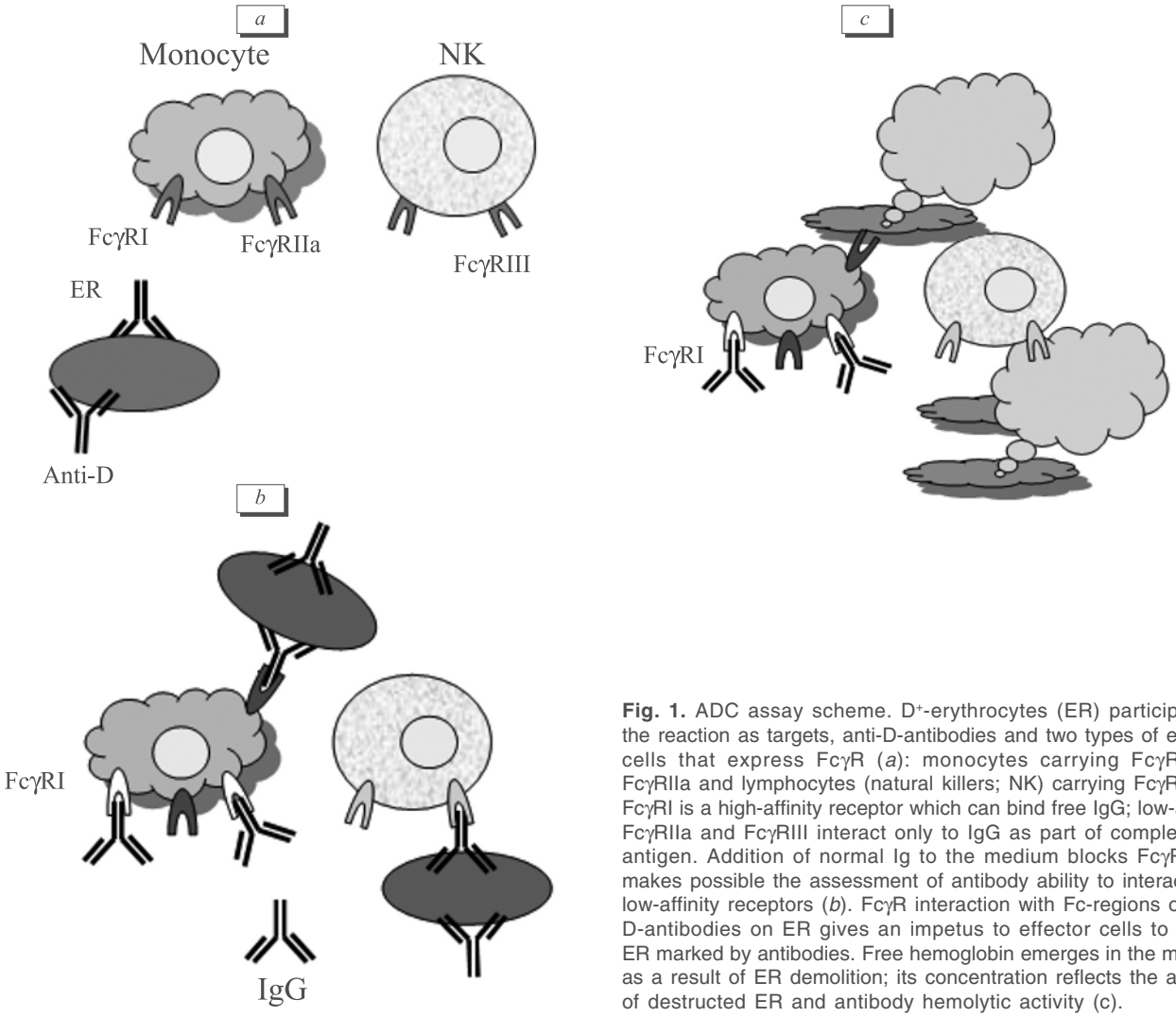

Fig. 1. ADC assay scheme. D^+-erythrocytes (ER) participate in the reaction as targets, anti-D-antibodies and two types of effector cells that express FcγR (*a*): monocytes carrying FcγRI and FcγRIIa and lymphocytes (natural killers; NK) carrying FcγRIII [6]. FcγRI is a high-affinity receptor which can bind free IgG; low-affinity FcγRIIa and FcγRIII interact only to IgG as part of complex with antigen. Addition of normal Ig to the medium blocks FcγRI and makes possible the assessment of antibody ability to interact with low-affinity receptors (*b*). FcγR interaction with Fc-regions of anti-D-antibodies on ER gives an impetus to effector cells to attack ER marked by antibodies. Free hemoglobin emerges in the medium as a result of ER demolition; its concentration reflects the amount of destructed ER and antibody hemolytic activity (c).

IGHG1×03 allotype [11]. Data on 3 studied IgG1 underclasses are shown (Table 1). Two more anti-D IgG1 MCA/HH which are not discussed in the paper (G-7 and G-47) belonged to the IGHG1×03 allotype.

Thus, Fc-fragments of both active in ADC-Ig and non-active MCA produced by human cells had 100% homology. Fusion with mouse myeloma did not introduce any mutations either (Table 1). These facts directly indicate that functional differences between MCA are determined by posttranslational modification of the antibody molecule, rather than their primary structure. It is known that the structure of oligosaccharide bound to Ig molecule through Asp^{297} plays an important role in antibody interaction with Fc-receptors on effector cells, although the oligosaccharide itself does not directly contact with the receptor [12]. The nature of Ig glycosylation is different in human and mouse cells; the type of glycosylation of human MCA in HH is determined by the specific origin of myeloma used for fusion [15]. Human FcγRIIa and FcγRIII less effectively recognize IgG1 MCA produced by human—mouse HH, because of altered oligosaccharide structure, which determines low activity of such MCA in ADC-Ig assay.

Activity of MCA G-303 did not depend on cell source (Fig. 2, *c*); this indicates that IgG3-antibody glycosyl residue is likely to produce a weaker effect on their interaction with FcγR.

Fusion with rat myeloma YB2/0 produced an opposite effect on anti-D-MCA activity: G-12 MCA became highly-active if human—rat HH served as producer cells (Fig. 2, *d*). The reason is that myeloma YB2/0 cells is characterized by impaired mechanism of fucosylation and MCA affinity to FcγRIII increases upon fucose level reduction in the MCA glycosyl residue [8,14]. It was demonstrated that MCA towards tumor antigens secreted by producers with impaired fucosilation in fact

possess stronger cytotoxic activity due to active recruitment of natural killers carrying FcγRIII [8], and recombinant anti-D-MCA expressed by YB2/0 myeloma accelerate excretion of autologous sensitized erythrocytes more efficiently than antirhesus Ig [3]. However, it should be taken into consideration that function of anti-D-MCA consists not only in target cell destruction as for antitumor MCA, but primarily in the development of immunological tolerance to D antigen. Thus, the effects of MCA with "rat" olygosachharide might also appear to be unpredictable *in vivo* similarly to the effects of investigated MCA with "mouse" oligosaccharide. Volunteer trials demonstrated that anti-D IgG1 MCA produced by human—mouse HH did not prevent immunization, and even paradoxically stimulated it in some groups of subjects and upon that dramatically accelerated erythrocyte excretion; immune response developed more rapidly and IgM antibodies appeared in the majority of patients, which is nonstandard [1]. It was suggested that MCA with "unfamiliar" oligosaccharides can activate innate immunity mechanisms and antibody presentation via other types of Fc-receptors [10]. MCA produced by LBL demonstrated the best results in volunteer trials although they were less effective than polyclonal anti-rhesus Ig [9]. The compound that included two MCA (IgG1 that caused FcγRIII-mediated hemolysis in ADC, and IgG3 inactive in this assay), accelerated D^+-erythrocyte clearance in rhe-

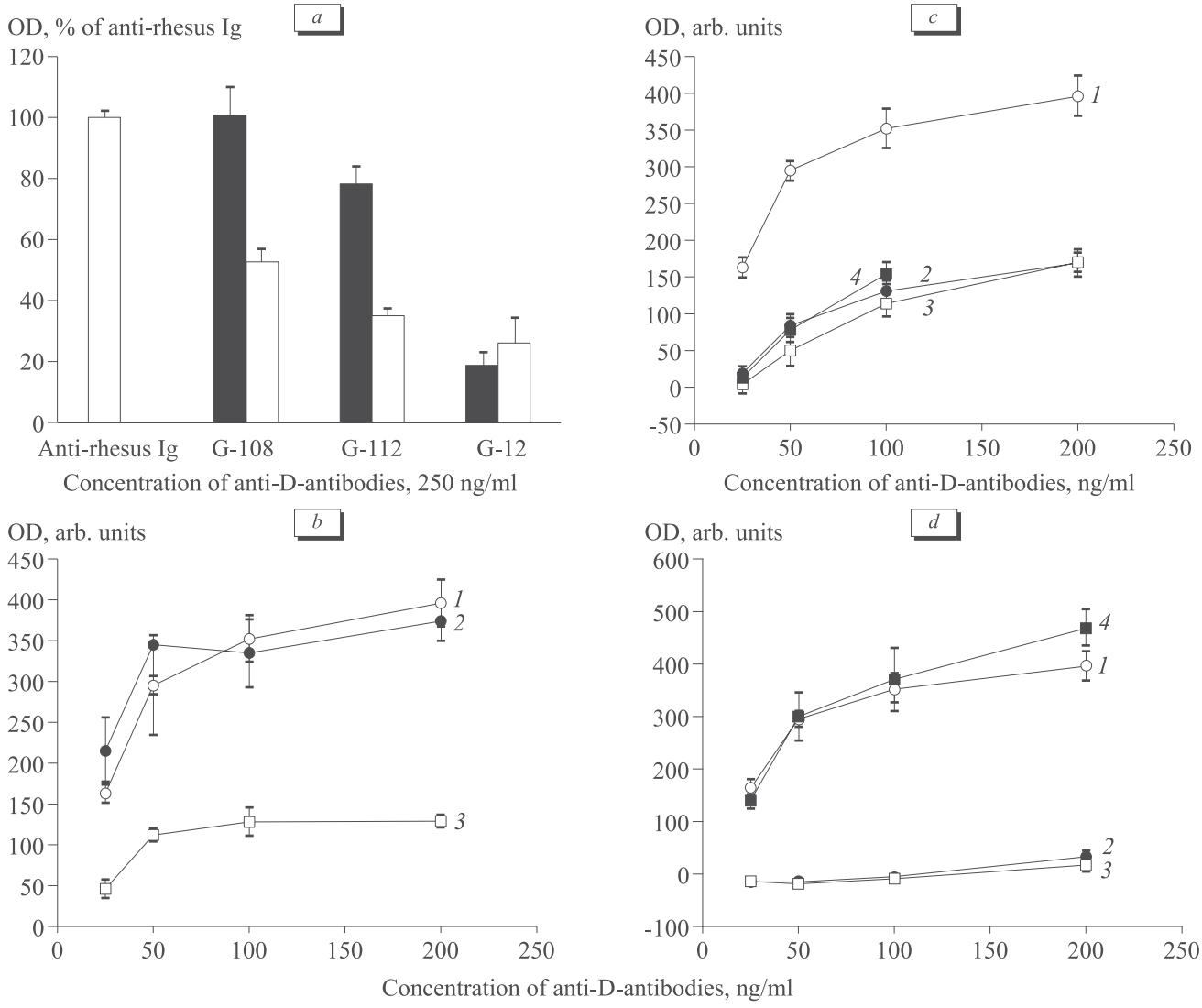

Fig. 2. Effect of producer cell line on hemolytic activity of anti-D-MCA mediated by interaction with low affinity FcγR in the ADC-Ig assay. Light bars: human HH, dark bars: LBL. OD: optical density. *a*) 100%: lysis efficacy in the presence of anti-rhesus Ig. *b*) anti-rhesus Ig (*1*), G-108, LBL (*2*), G-108, human—mouse HH (*1*). *c*) anti-rhesus Ig (*1*), G-303, LBL (*2*), G-303, human—mouse HH (*3*), G-303, human—rat HH (*4*). *d*) anti-rhesus Ig (*1*), G-12, LBL (*2*), G-12, human—mouse HH (*3*), G12, human—rat HH (*4*).

TABLE 1. Structural and Functional Properties of Anti-D IgG1 MCA

MCA	Light chain	Underclass	Producer line	Activity in ADC-Ig (% of antirhesus Ig)	Fc sequencing
G-108	k	IgG1	LBL	101	IGHG1×03
			human—mouse HH	53	Was not performed
G-112	k	IgG1	LBL	78	IGHG1×03
			human—mouse HH	35	Was not performed
G-12	k	IgG1	LBL	19	IGHG1×03
			human—mouse HH	26	IGHG1×03

Note. IGHG1×03 — IgG1 allotype.

sus-negative subjects, and reduced the rate of rhesus-immunization (2 of 96 subjects had response, 20 of subjects appeared to be non-responders). Unfortunately human LBL cannot serve as reliable producer by virtue of unstable growth, complications in cloning, and Epstein—Barr virus expression.

In vivo trials revealed MCA properties that could not be predicted basing on *in vitro* studies, and the collected data represents valuable information to be used upon strategy formulation for anti-D-MCA selection for antigen-specific immunosuppression. It was found that rapid D^+ erythrocyte clearance under the influence of MCA is not a sufficient condition for efficient prevention of sensibilization; the ability of MCA to interact with low-affinity FcγR during ADC is likely to be the critical criterion. This is the property of all polyclonal anti-D-antibodies and very rare MCA. It cannot be ruled out that this feature is indirect as far as antigen-specific B-cell inactivation in the body is mediated by antibody interaction with inhibiting FcγRIIb on B-lymphocytes, but there is no *in vitro* method for assessment of antibody negative effects. The analysis of primary sequences of anti-D-MCA with different affinity to low-affinity FcγR, that has been carried out, did not reveal differences in the Fc-domain structure, which confirms the key role of posttranslational modifications in the antibody effector function. Thus, the problem of search for appropriate producer cell line emerges into prominence; in future any human anti-D-antibodies in the cells of this producer might take on the properties of antibodies expressed in the organism.

The study was supported by Gematolog Company and Russian Foundation for Basic Research (grant No. 06-04-48910).

Authors express their cordial gratitude to Prof. I. L. Chertkov for ideological supervision and to employees of Moscow Hemotransfusion Station for the provided immune donor blood samples.

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