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Impact on N-Glycosylation Profile of Monoclonal Anti-D Antibodies as a Way to Control Their Immunoregulatory and Cytotoxic Properties

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Abstract—Prophylaxis of hemolytic disease of newborns is based on the ability of polyclonal anti-D antibodies for suppressing maternal immune response against D-positive fetal red blood cells. The immunosuppressive effect of anti-D antibody is mediated by interaction between its Fc-fragment and low-affinity IgG Fc-receptor (FcγR) on the immune cell. No clinically effective monoclonal anti-D antibody (mAb) that can replace polyclonal anti-D immunoglobulin has been developed yet. The goals of this study were comparison of structural and functional properties of human anti-D polyclonal and monoclonal Abs and assessment of the possibility to manipulate the effector properties of the mAb. N-Glycosylation and particularly the content of nonfucosylated glycans are crucial for affinity of mAb to FcγRIIIA, which plays the key role in the clearance of sensitized cells. We studied and compared glycoprofiles and FcγRIIIA-mediated hemolytic ability of human polyclonal antibodies and anti-D mAbs produced by human B-cell lines, human–rodent heterohybridomas, and a human non-lymphoid cell line PER.C6. Replacement of producing cell line and use of glycosylation modulators can convert an inert mAb into an active one. Nevertheless, rodent cell lines, as well as human non-lymphoid cells, distort natural glycosylation of human IgG and could lead to the loss of immunosuppressive properties. All of the anti-D mAbs secreted by human B-cell lines have a glycoprofile close to human serum IgG. Hence, the constant ratio of IgG glycoforms in human serum is predetermined by glycosylation at the level of the individual antibody-producing cell. The anti-D fraction of polyclonal anti-D immunoglobulin compared to the total human IgG contains more nonfucosylated glycans. Thus, only human transformed B-cells are an appropriate source for efficient anti-D mAbs that can imitate the action of polyclonal anti-D IgG.

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Monoclonal and polyclonal immunoglobulins are used in clinical practice for neutralization of pathogens, destruction of tumor cells, blockage of molecules implicated in pathogenesis, and correction of immune response. An example of an immunosuppressive drug is anti-D immunoglobulin, which prevents Rh sensitization of D-negative women and development of hemolytic disease in their children [1]. The problem of Rh incompatibility is briefly as follows. Erythrocytic D-antigen of the Rhesus (Rh) blood group system is absent in 15% of Caucasians. The probability of Rhesus-positive (D^+) fetus in Rhesus-negative (D^-) pregnancy thus comprises about 60%. In the course of pregnancy and the act of delivery, fetal erythrocytes can strike the mother's bloodstream and cause her sensitization. The cells producing anti-D antibodies are sustained in the organism of the Rh-negative woman for many years, and the produced antibodies can pass through the placenta during subsequent pregnancies, resulting in immune-dependent hemolysis of fetal D^+ erythrocytes, the cause of hemolytic disease of the fetus and the newborn. In the epoch before prophylaxis, about 10% of cases of neonatal death resulted from the RhD hemolytic disease caused by anti-D antibodies [2]. Prevention of RhD-sensitization, a mandatory procedure in obstetrics

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; BCR, B cell receptor; CHO, Chinese hamster ovary cells; FcγR, Fcγ receptor; LBL, B-lymphoblastoid line cells; mAb, monoclonal antibody.

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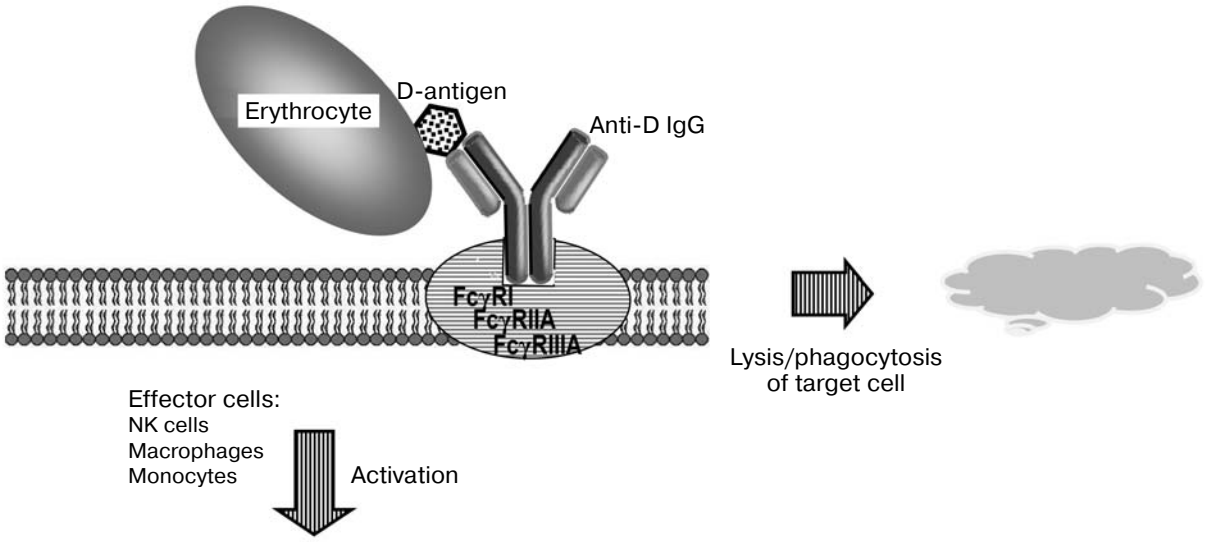

Fig. 1. Mechanism of antibody-dependent cellular cytotoxicity (ADCC). The antibody Fc fragment "attracts" effector cells of the immune system (macrophages, monocytes, and natural killers) and directs them to attack the object the antibody is bound to. The Fc fragment is recognized by Fcγ receptor of the effector cell. The crucial role in ADCC *in vivo* belongs to FcγRIIIA, which can recognize Fc fragments of IgGs in a complex with antigen. In the body, high-affinity receptor FcγRI is saturated with free IgG and so minimally contributes to elimination of immune complexes.

now, involves administration of anti-D immunoglobulin to D-negative women after delivery of a D-positive infant. This is so effective against undesirable immunization that Rh D hemolytic disease of the fetus/newborn has become rare. Commercial anti-D immunoglobulin is an IgG fraction from pooled anti-D sera obtained from D-negative volunteers immunized artificially. The effect of this medication is based on a phenomenon described in the 19th century that antiserum to a distinct antigen can prevent immune response to this antigen when injected simultaneously [3]. Functional activity of anti-D immunoglobulin has been thoroughly studied both in experiment and clinic. Anti-D immunoglobulin *in vitro* can mediate destruction of target erythrocytes in the reaction of antibody-dependent cellular cytotoxicity (ADCC) (Fig. 1). In the body, anti-D immunoglobulin drastically accelerates elimination of D^+ erythrocytes from the bloodstream followed by their destruction in the spleen [4, 5]. However, as we reported earlier, rapid clearance of D^+ erythrocytes is not sufficient for efficient immunosuppression [6, 7]. The mechanism of the immunosuppressive action of anti-D immunoglobulin is not completely clear. Some data indicate that the antibody-dependent immunosuppression can be regulated by a specific mechanism mediated by the inhibitory receptor FcγRIIB [8, 9].

FcγR is a group of membrane-associated glycoproteins, members of the immunoglobulin superfamily, which are expressed on leucocytes, particularly monocytes, macrophages, lymphocytes, and natural killers (NK cells) [10, 11]. FcγR bind with constant fragment (Fc) of antibody, which triggers effector functions of the FcγR-bearing cell. There are three FcγR classes in humans: FcγRI, FcγRII (FcγRIIA, FcγRIIB, and FcγRIIC), and FcγRIII (FcγRIIIA and FcγRIIIB). FcγRI possesses high affinity to Fc; it is the only FcγR that can bind not only IgG of antigen–antibody complexes, but also free IgG molecules. The most important feature of FcγR is the character of transduced signal: either activating or inhibitory [12, 13]. The binding with activating receptors, such as all FcγR, except for FcγRIIB, leads to activation of the effector cell. Reactions of the activated cell are ADCC, phagocytosis, endocytosis, and release of cytokines or inflammatory factors, depending on the cell type [14, 15]. Cytotoxic reactions mediated by FcγRIIIA are the main destruction mechanism of sensitized target cells, including erythrocytes, *in vivo* [16-20]. In contrast, the inhibitory receptor FcγRIIB, when bound with IgG forming the immune complex, generates a signal that suppresses activation of the cell. In particular, co-ligation of FcγRIIB with the activated B-cell receptor leads to B-cell anergy, i.e. its unresponsiveness to an antigen stimulus (Fig. 2) [21]. FcγRIIB, the only FcγR receptor expressed on B-cells, plays a crucial role in regulation of B-cell proliferation and maintenance of immunoglobulin production level [8].

There are sufficient evidences in support of a correlation between affinities of antibody Fc fragment to low-affinity receptors FcγRIIIA and FcγRIIB, albeit the former is activating and the latter is inhibitory [22, 23]. Since there is no functional test for affinity to FcγRIIB, selection for FcγRIIIA activity is an indirect surrogate. This activity is estimated from ADCC-FcγRIIIA.

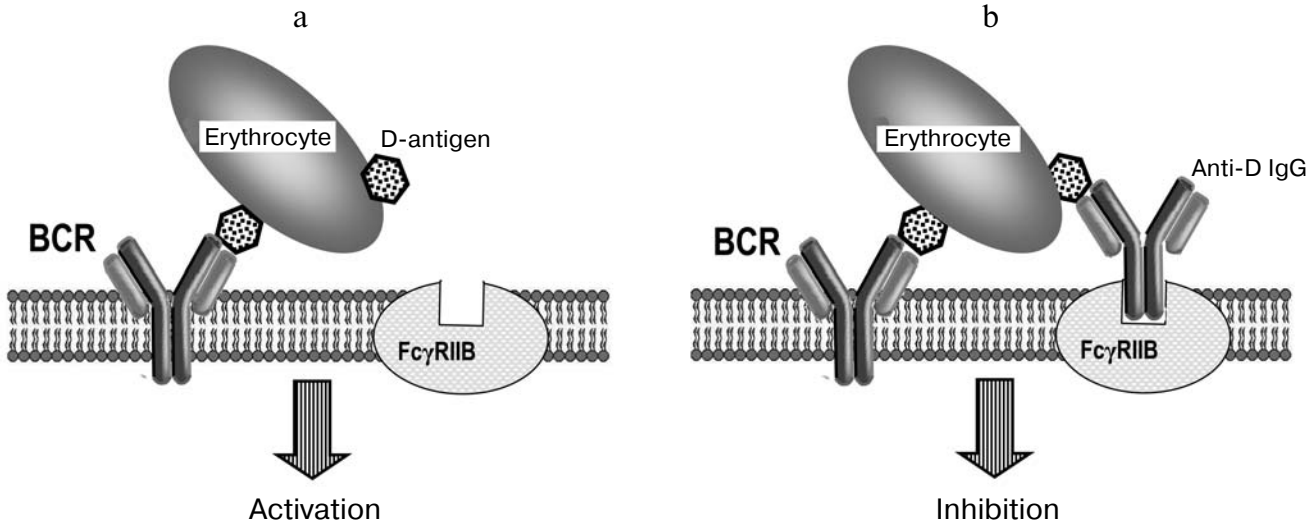

Fig. 2. Regulation of B-cell activity. a) BCR-dependent activation. The specialized B-cell expresses on its surface the antigen-recognizing receptor BCR (B cell receptor), which is a transmembrane immunoglobulin form. Interaction of BCR with corresponding antigen (in our case, erythrocyte D-antigen) is a signal initiating proliferation of this B-cell and propagation of a clone of plasma cells secreting antibodies specific to this antigen (this simplified scheme does not include cooperation with T-cells). b) FcγRIIB-dependent inhibition. The D^+ erythrocyte sensitized with anti-D antibodies interacts simultaneously with two receptor types on the B-cell: D-antigen binds to anti-D BCR, while the Fc-fragment of the antibody attaches to the erythrocyte – to FcγRIIB. Two simultaneous signals – from BCR and from FcγRIIB – lead to unresponsiveness of the B-cell to this antigen.

Mass prophylaxis of hemolytic disease of the newborn requires great volumes of medication, which is injected not only after delivery, but also in the last trimester of gestation and in the case of medical and spontaneous abortions, as well as therapeutic and diagnostic amniocentesis. Development of preparative technologies for human mAb production has generated great expectations that alternative source of anti-D immunoglobulin will be found soon.

Anti-D mAbs are only produced by cells of immune Rh-negative donors; laboratory animals do not respond to immunization with Rh blood system antigens. As a rule, cell lines producing anti-D mAbs are constructed by transformation of donor immune lymphocytes with Epstein–Barr virus. However, human lymphoblastoid cell lines cannot serve as a reliable basis for large-scale mAb production. To stabilize cell lines and elevate the yield of mAb, the cells are fused with mouse or rat myeloma cells to produce heterohybridomas secreting human antibodies or anti-D antibody-encoding genes are cloned and expressed in standard cell systems (for example, in Chinese hamster ovary (CHO) cells).

A series of anti-D mAbs was tested for their ability to prevent anti-D immunization. The test protocol was generally as follows: D^+ erythrocytes were injected into an Rh-volunteer, their initial level in the blood was determined, and then anti-D antibodies were injected. To estimate the effect of anti-D antibodies on the clearance of D^+ erythrocytes, their residual level in the blood was monitored during one day. In the next six months, blood samples were periodically taken to detect immune anti-D antibodies and determine their time of appearance, class, and titer (the case of inefficient medication).

Among all medications tested, the only effective (preventing sensitization) one was mAb IgG1 produced by virus-transformed human lymphoblastoid cells, which mediated hemolysis *in vitro* in ADCC-FcγRIIIA and accelerated clearance of D^+ erythrocytes from the bloodstream *in vivo* [24]. In contrast, anti-D mAbs produced by human–mouse heterohybridomas stimulated rather than inhibited anti-D response [6, 25, 26]. Recombinant anti-D antibodies produced in CHO cells were not tested for prevention of anti-D immune response because they very weakly accelerated clearance of D^+ erythrocytes [26]. One of possible causes of low, or even abnormal, activity of mAbs is unnatural glycosylation of human mAbs in rodent cells.

N-Glycosylation of antibodies plays a crucial role in their interaction with Fc receptors of effector cells [27]. The oligosaccharide attached to the Asp297 of each C_H2 domain is of the bi-antennary complex type carbohydrate, which is heterogeneous in human IgGs (Fig. 3). The effect of each oligosaccharide component on effector properties of IgG is considered in the review [27] in detail. Numerous studies have shown that the presence or absence of fucose dramatically influences the FcγRIIIA-binding capability of mAbs: nonfucosylated mAbs, unlike their fucosylated counterparts, demonstrate elevated activity in ADCC; this activity does not depend on FcγRIIIA polymorphism [28-30]. Induction of ADCC by

nonfucosylated mAb compared to the fucosylated one requires lower density of membrane-associated antigen [31]. Mutant cell lines with knocked out or decreased activity of fucosyltransferase FUT8 produce antibodies with low fucose content and high ADCC activity [28, 29, 32, 33]. Examples of these cell lines are the Lec13 CHO and the YB2/0 rat myeloma cell lines. Earlier, we demonstrated that IgG1 mAb, which is initially unable to interact with FcγRIIIA, becomes highly active when expressed in the YB2/0 cells [34].

The glycosylation machinery of mouse cells is dominant in heterohybridoma cells prepared by fusion of antibody-producing human cells with murine myelomas, so human IgGs secreted by heterohybridoma cells can contain glycans that are never found in normal human IgGs [35]. These glycans can be recognized by the human immune system as foreign and activate receptors and cells of innate immunity [36, 37], which results in stimulation of immune response. Appearance of a mAb expression platform based on human retinoblastoma cells PER.C6 [38] can be used to estimate glycosylation functionality in non-lymphoid cells.

The goal of the present work was to compare the structural and functional properties of human poly- and monoclonal anti-D antibodies and estimate the possibility of impact on effector properties of antibodies, primarily their affinity to FcγRIIIA, by their expression under different conditions.

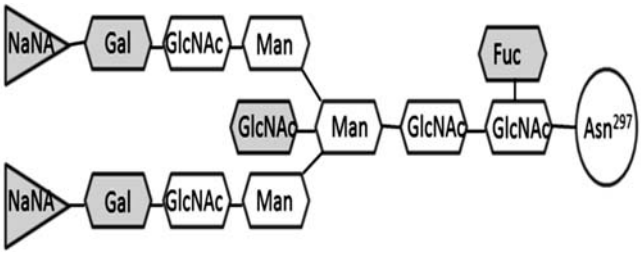

Fig. 3. Structure of human IgG Asn297-bound oligosaccharide. NaNA, *N*-acetylneuraminic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose; Asn297, asparagine of IgG Fc fragment. Carbohydrate moieties that can be absent (drawn in gray) give rise to heterogeneities of antibody glycoforms. Heterogeneity occurs because of presence or absence of sialic acids, bisecting *N*-acetylglucosamine, galactose and fucose residues. Despite high heterogeneity, the ratio between IgG glycoforms of healthy individuals is nearly constant, but they can vary significantly in various diseases, such as rheumatoid arthritis, immunoglobulin heavy chain disease, multiple myeloma, etc. [54-56]. IgG glycosylation is species specific. Since rodent cell lines are widely used in biopharmaceutical industry for recombinant protein production, the structures of immunoglobulin glycans are well studied. In particular, licensed recombinant antibodies produced by CHO cells and NS0 and Sp2/0 murine myelomas contain fucosylated oligosaccharides with low content of galactose [37, 57]. Oligosaccharides of recombinant IgG can contain *N*-glycolylneuraminic acid instead of *N*-acetylneuraminic acid, as well as represent mono-antennary and high-mannose oligosaccharides, i.e. structures that are never found in normal human IgG.

MATERIALS AND METHODS

Cell lines. Human B-lymphoblastoid cell lines producing anti-D antibodies were developed by transformation of human peripheral blood lymphocytes with Epstein–Barr virus. Human–mouse and human–rat heterohybridomas producing human anti-RhD antibodies were constructed by fusion of human B-lymphoblastoid line cells with the P3X63Ag8.653 murine myeloma and YB2/0 rat myeloma cells, respectively [34].

Recombinant anti-RhD antibodies. The genes encoding heavy (H) and light (L) chains of anti-D IgG were cloned using reverse transcription of mRNA isolated from B-lymphoblastoid antibody-producing cells followed by PCR amplification with degenerate primers [39]. The H- and L-chains of mAbs G12 and G108 were cloned under the CMV-promotor in an original vector carrying the neomycin resistance gene followed by transfection of the PER.C6 cells by electroporation, selection, and cloning of the mAb-producing cells. Kifunensine (Sigma, USA) was taken at the concentration of 0.1 μg/ml [40].

The antibodies were isolated and purified by affinity chromatography on Protein A Sepharose (GE Healthcare, USA) followed by ion-exchange chromatography on ceramic hydroxyapatite CHT-I (Bio-Rad, USA). Eluate of polyclonal anti-D IgG1 obtained by acidic elution of antibodies adsorbed on D^+ erythrocytes [41] was separated on Protein A Sepharose. Eluates were isolated from either commercial therapeutic anti-D immunoglobulins or from anti-D antisera pooled from several immune Rh-donors.

ADCC-FcγRIIIA assay. Human peripheral blood mononuclear cells from non-immune donors were used as effector cells. The cells were isolated by centrifugation of whole blood samples with an anticoagulant on Ficoll (MP Biomedicals, France). The reaction was carried out in U-shaped 96-well plates (Greiner Bio-One, Germany). The following reagents were sequentially added into each well: $500 \cdot 10^3$ mononuclear cells in 50 μl RPMI 1640 medium (HyClone, USA) containing 1% fetal calf serum; 50 μl of serially diluted anti-D antibodies; 50 μl of normal human immunoglobulin (1 μg/μl; Sverdlovsk Regional Blood Transfusion Station, Russia); and $0.5 \cdot 10^6$ D^+ erythrocytes in 50 μl of the medium. Erythrocytes were preincubated for 10 min at room temperature in a solution of the proteolytic enzyme bromelain (Serlabo Technologies, France): 0.3 ml of D^+ erythrocytes washed with saline were suspended in 10 ml of 0.1% bromelain solution and, following the incubation, washed thrice. The plates were incubated for 18-20 h at 37°C, and free hemoglobin in the reaction medium was determined using a colorimetric test with 2,7-diaminofluorene [42].

Supernatant from the well containing no antibodies was used as the control for spontaneous lysis. Each antibody sample was analyzed in triplicate. Contents of anti-

D in tested samples were normalized by titration against a standard.

Addition of non-immune immunoglobulin that blocks high-affinity FcγRI receptors allows to estimate the contribution of low-affinity receptors, primarily FcγRIIIA.

Investigation of oligosaccharide structure. The glycosylation pattern of anti-D antibodies was determined by the GlycoSolutions (USA) using fluorescent labeling of neutral oligosaccharides and their chromatographic separation.

RESULTS

Manipulating the effector properties of antibodies. Figure 4 shows how producing cells influence the mAb activity in ADCC-FcγRIIIA. The anti-D mAb G12 belonging to the IgG1 subclass was used. We compared effector activities of mAb G12 counterparts produced by: a) human B-lymphoblastoid line cells (G12-LBL); b) human–mouse heterohybridoma (G12-P3X63Ag8.653); c) human–rat heterohybridoma (G12-YB2/0); d) human non-lymphoid line PER.C6 (G12-PER.C6) [43], and e) PER.C6 cells cultured in the presence of kifunensine (G12-PER.C6/kifunensine). Kifunensine is a potent inhibitor of α-mannosidase I; when taken at the concentration of 0.1 μg/ml, it leads to synthesis of antibodies carrying low-fucosylated and high-mannose glycans [40].

Figure 4 demonstrates crucial effect of glycosylation on effector properties of antibodies. Replacement of cells producing the same mAb or addition of substances influencing glycosylation not only enhances activity, but it even converts mAb that is inactive in ADCC-FcγRIIIA into a highly active form. In particular, the activity of mAb G12-YB2/0, which contains more than 70% nonfucosylated carbohydrates (detailed glycan structures are not shown), is comparable with that of polyclonal anti-D immunoglobulin, although its "prototype" G12-LBL has only minimal ADCC-FcγRIIIA activity. Another influence – addition of kifunensine – allows complete "mending" of the idle mAb G12-PER.C6 to give it hemolytic activity comparable with that of polyclonal anti-D antibodies.

Glycosylation of polyclonal anti-D antibodies. Table 1 shows the testing data of three polyclonal anti-D antibodies: commercial anti-D immunoglobulin, pooled human anti-D serum, and polyclonal anti-D IgG1 isolated from polyclonal antibody samples by affinity chromatography (eluates). The eluates contain chiefly anti-D IgG1, because polyclonal anti-D antibodies mainly represent IgG1 and IgG3 subclasses [44, 45], and Protein A allows isolation of IgG1 fraction. Such samples of purified anti-D antibodies are more adequate for studying biochemical peculiarities of immune antibodies because anti-D immunoglobulin contains a vast majority of other serum immunoglobulins and no more than 0.02% anti-D antibodies.

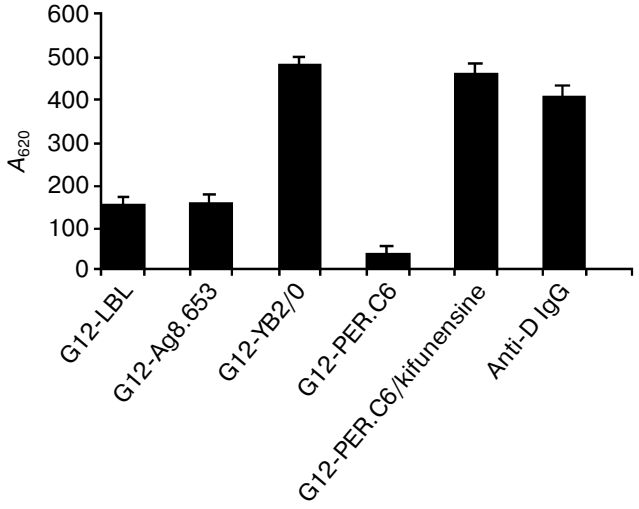

Fig. 4. Effect of producing cells and glycosylation modulators on hemolytic activity of anti-D mAb in ADCC-FcγRIIIA. Concentration of free hemoglobin in the medium is directly proportional to hemolytic activity of anti-D antibodies. Hemoglobin was determined by colorimetric test (see "Materials and Methods"). Polyclonal anti-RhD immunoglobulin was used as a control. The concentration of all antibodies was 100 ng/ml.

Table 1 shows that more than a quarter of serum IgG carbohydrates are sialylated; agalactosyl G0 type represents the minimal fraction. Purified polyclonal anti-D IgG1 antibodies compared with initial anti-D immunoglobulin samples demonstrate a shift to full-size sugars (G2), as well as nearly twofold elevated contents of nonfucosylated forms, both among neutral and sialylated sugars (fucosylation of sialylated sugars was only analyzed in eluate I).

Glycosylation of monoclonal anti-D antibodies. To evaluate what (if any) peculiarity in glycan composition correlates with FcγRIIIA-mediated hemolytic activity of antibodies, we compared carbohydrate compositions of four IgG1 mAb-LBL having different ADCC-FcγRIIIA activity (lowly-active G12 and G17, moderately active G112, and highly active G108). None of these mAbs are uniform in terms of bound glycan: each mAb demonstrate a broad spectrum of glycoforms (Table 2). It is worth noting that all mAbs produced by B-lymphoblastoid cell lines are characterized by a carbohydrate pattern characteristic of polyclonal anti-G IgG (Table 1).

Independently of the activity of antibodies in the ADCC-FcγRIIIA assay, all mAb-LBL compared with polyclonal antibodies (eluate II) contain less neutral nonfucosylated carbohydrates. It was previously reported that sialic acids have a negative effect on the affinity of Fc to FcγRIIIA [46, 47]. We do not confirm this dependence: as Table 2 indicates, both inactive and active mAb-LBL, as well as purified polyclonal anti-D antibodies, are sialy-

Table 1. Carbohydrate composition of polyclonal anti-D medications, %

Oligosaccharide type		Rhesogam anti-D immunoglobulin (Aventis Behring GmbH, Germany)	Anti-D immunoglobulin (Ivanovo Regional Blood Transfusion Center, Russia)	Anti-D serum (pooled)	Anti-D IgG1 eluate I**	Anti-D IgG1 eluate II
ADCC-FcγRIIIA activity		++++	++++	++++	++++	++++
Fucosylated						
	G0F*	14.3	16.5	16.2	12.7	6.0
	G1F	32.9	34.3	32.4	25.5	21.3
	G2F	18.6	16.5	16.2	23.1	33
Nonfucosylated						
	G0	0.6	0.8	0.6	0	0.7
	G1	2.0	2.3	2.4	3.9	1.3
	G2	1.3	1.6	1.2	6.5	7.8
	Σ	3.9	4.7	4.2	10.4	9.8
Sialylated						
	F	28.6	27.5	29.3	23.1	29.9***
	–	1.7	0.5	1.7	4.6	

* G2, G1, and G0 – neutral (asialylated) sugars containing two, one, and no galactose residues, respectively, at the end of the chain; F, fucose.
** Eluates I and II were prepared from pooled anti-D antisera and commercial anti-D immunoglobulin, respectively.
*** Portions of fucosylated and nonfucosylated oligosaccharides were not determined.

Table 2. Carbohydrate composition of monoclonal anti-D antibodies, %

		G12-LBL	G17-LBL	G112-LBL	G108-LBL	G12-PER.C6	Anti-D IgG1 eluate II
ADCC-FcγRIIIA activity		–	–	++	++++	–	++++
Fucosylated							
	G0F	2.3	5.0	2.4	4.8	62.8	6.0
	G1F	14.9	26.9	23.4	28.9	30.1	21.3
	G2F	47.2	29.4	44.3	35.3	5.9	33
Nonfucosylated							
	G0	0.2	0.0	0.0	0.9	0.2	0.7
	G1	0.7	0.8	0.6	1.4	0.5	1.3
	G2	1.6	2.6	1.8	2.5	0.0	7.8
	Σ	2.5	3.4	2.4	4.8	0.7	9.8
Sialylated*		33.1	35.3	27.5	26.2	0.5	29.9

* Detailed structural analysis of sialylated glycans was not performed.

lated to a nearly equal extent. The data on glycosylation of four mAb-LBL do not suggest significant difference in their glycan composition and does not allow a conclusion about what factor determines high hemolytic activity of G108-LBL. It is worth noting that eluates contain higher level of neutral nonfucosylated sugars (9.8%) in comparison to the mAb samples. The glycosylation pattern of IgG1 produced by PER.C6 human cells is highly distorted in comparison to that of normal IgGs. For instance, the recombinant antibody G12-PER.C6 does not contain sialic acids, and it is almost completely fucosylated. The mAb G12-PER.C6 is absolutely inactive in ADCC-FcγRIIIA (Fig. 4). Moreover, the mAb G108, when expressed in PER.C6 cells, has completely lost its hemolytic activity [43].

DISCUSSION

Medication inducing temporary tolerance to D-antigen occupies a special place among therapeutic mAbs. First, it is designed for injection into healthy individuals, i.e. it is a vaccine. Second, anti-D mAbs have a highly efficient polyclonal prototype, so they must also be

highly active. Despite multiyear practice, the mechanism of anti-D immunoglobulin activity is not yet known in detail. Polyclonal anti-D medications ensure quick removal of D^+ erythrocytes from the bloodstream due to interaction with FcγRIIIA. However, quick elimination of D^+ erythrocytes is an important, but not sufficient condition for anti-D response prophylaxis. The low-affinity B-cellular inhibitory receptor FcγRIIB seems to be involved in development of tolerance. This receptor suppresses activation of specialized B-cell, when the immune complex simultaneously interacts with BCR and FcγRIIB, and their receptors co-ligate (Fig. 2).

Since there is no experimental animal model of rhesus-incompatible pregnancy, anti-D antibodies tested *in vitro* are directly evaluated in volunteers, which, naturally, limits the number of antibodies tested *in vivo*. To date, the only rational strategy for developing an efficient anti-D mAb is the maximum possible copying of the functional and biochemical features of polyclonal anti-D antibodies. We analyzed previously the primary sequences of cDNAs encoding FcγRIIIA-active and inactive anti-D mAb IgGs secreted by different cell lines, and we did not find difference in structures of their Fc fragments, which is indicative of the role of posttranslational modifications on effector properties of the antibodies [34].

Analysis of glycans of mAbs produced by human lymphoblastoid cells revealed an interesting fact: each cloned mAb is a mixture of glycoforms, that closely resemble the composition of human serum IgG (Tables 1 and 2). The spectrum of human serum IgG glycoforms is known to be relatively constant. The common explanation is the constant ratio between clones of antibody-producing cells in which antibodies are differently glycosylated [48]. Our data suggest that the ratio between serum IgG glycoforms is determined by antibody glycosylation at the level of the individual antibody-producing cell.

It would seem that any B-lymphoblastoid cell line producing a standard glycan pattern of immunoglobulin would also reproduce its functional properties. However, the vast majority of cell lines constructed by viral transformation produce anti-D mAbs that, unlike polyclonal anti-D antibodies, are unable to interact with FcγRIIIA. The mAb G108-LBL studied in this work is a rare exception [49]. What is the cause of low activity of anti-D mAbs expressed by the majority of B-lymphoblastoid cell lines? It has been hypothesized that only long-lived plasma cells secrete antibodies that are capable of suppressing activation of memory B-cells, thus regulating production of the antibody of the given specificity, and exactly these cells in the body of the immune donor are the main source of the immunosuppressive antibodies [50]. As mentioned above, construction of anti-D antibody-producing cells includes the treatment of Rh-donor lymphocytes with the Epstein–Barr virus that infects and immortalizes $CD21^+$ B-cells, using this B-cellular surface marker as the receptor [51]. Peripheral $CD21^+$ B-cells transformed with the virus are at an earlier differentiation stage and cannot secrete immunosuppressive antibodies. Different stages of B-cell differentiation are probably associated with differing activity of glycosyltransferase genes, so immunosuppressive properties of antibodies can depend on alterations in their glycosylation pattern in long-lived plasma cells. Our data show that, although all glycoforms inherent to polyclonal antibodies are present in mAb-LBL, the percentage of nonfucosylated G2 forms is higher in polyclonal anti-D antibodies (Table 2). At first glance, glycosylation patterns of FcγRIIIA active and FcγRIIIA inactive anti-D mAbs do not differ significantly (Table 2). No doubt, more detailed analysis of the carbohydrate composition (for instance, refinement of nonfucosylated form contents in the fraction of charged oligosaccharides) will help us with clarification of what difference is crucial between FcγRIIIA active and FcγRIIIA inactive anti-D antibodies.

The B-lymphoblastoid cell lines constructed using viral transformation can produce immunosuppressive antibodies, but they cannot provide for large-scale production because of unstable growth and low productivity. Replacement of expression system by means of fusion with myelomas or construction of recombinant anti-D mAb-producing cells on the basis of CHO or PER.C6 cells enables construction of stable high-yield cell lines, but it alters the antibody glycosylation pattern. It is obvious now that traditional cell lines, such as murine myelomas and CHO cells, are unlikely to become the source of effective anti-D medications. Alien glycan structure of anti-D mAbs synthesized in rodent cells can cause an opposite effect: stimulation of immune response instead of its suppression. Cells defective in fucosylation seemed to be attractive anti-D mAb-producers. In fact, decrease in contents of fucosyl forms expressed in YB2/0 led to drastic increase in mAb activity, which achieved the level of polyclonal anti-D medication (Fig. 4). It was reported that anti-D antibodies produced by YB2/0 sharply accelerated the clearance of D^+ erythrocytes *in vivo* [52], however there are no publications till now about clinical trials of the therapeutic drug based on these antibodies. It is possible that antibodies possessing high hemolytic activity, but carrying alien sugars, can produce side effects like described above.

There are virtually no producers on the basis of human cells in biopharmaceutical industry. The more interesting was to check applicability of PER.C6 human line for constructing recombinant immunotherapeutic antibodies. We succeeded in constructing high-yield clones producing up to 100 μg/ml mAbs under laboratory conditions. However, the glycan pattern of mAb-PER.C6 dramatically differed from that of normal IgG, so the mAb-PER.C6 was completely unable to interact with

FcγRIIIA. This activity drastically increases when the producing cells were cultured in the presence of kifunensine, an inhibitor of α-mannosidase. Application of kifunensine is the simplest way to obtain FcγRIIIA-activating antibodies, because it induces modifications enforcing affinity of IgG to FcγRIIIA. In its presence, low-fucosylated oligomannose type N-glycans are synthesized. Despite high affinity to FcγRIIIA, the glycans synthesized in the presence of kifunensine have the structure atypical of human IgGs; there are data indicating that antibodies enriched with oligomannose can be recognized by receptors of innate immunity and are quickly eliminated from the circulation [53].

Thus, having a broad spectrum of possibilities of manipulating effector properties of anti-D antibodies, *in vivo* we are restricted those stimulating anti-D response instead of suppression it if the antibodies carry non-canonical oligosaccharide.

In this work, we have first analyzed the carbohydrate composition of polyclonal IgG1 anti-D antibodies and compared it with that of anti-D mAbs possessing different affinity to FcγRIIIA. We have found that the oligosaccharide patterns of all studied mAbs secreted by cloned human B-lymphoblastoid cell lines are very similar both among themselves and with the glycans of serum IgG. The data suggest that biotechnological fitting of human lymphoblastoid cell line secreting anti-D IgG antibodies can solve the problem of anti-D production. This fitting should be in: 1) immortalization of a cell line, for instance, using telomerase (hTERT), instead of fusion with myelomas or mAb transition into the recombinant form; 2) manipulating expression of fucosyltransferase FUT8, which is now possible. Moreover, a strategy of anti-D antibody production could be applied in the future in development of immunosuppressive medications directly inhibiting a pathological immune response in autoimmune diseases.

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