In vitro assessment of recombinant, mutant immunoglobulin G anti-D devoid of hemolytic activity for treatment of ongoing hemolytic disease of the fetus and newborn

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BACKGROUND: A specific treatment for ongoing hemolytic disease of the fetus and newborn (HDFN) due to anti-D would be very attractive. One approach could be administration to the mother of nonhemolytic anti-D, which by crossing the placenta can block the binding of hemolytic maternal anti-D.

STUDY DESIGN AND METHODS: Two anti-D immunoglobulin G3 (IgG3) heavy-chain mutants were expressed in Chinese hamster ovary cells. To investigate whether these anti-D IgG3 mutants could inhibit the red blood cell–destructive activity of recombinant human (rHu)IgG1 with identical antigen-binding region as well as polyclonal anti-D having multiple D epitope specificities, two assays were used, antibody-dependent cell-mediated cytotoxicity (ADCC) and a chemiluminescence (CL)-based method for detection of respiratory burst in peripheral blood monocytes.

RESULTS: The two IgG3 anti-D heavy-chain mutants inhibited the ADCC and CL responses mediated by a rHuIgG1 anti-D with identical antigen-binding region as the mutant antibodies, as well as the destructive activity mediated by a polyclonal anti-D.

CONCLUSION: The use of nonhemolytic anti-D may be an effective countermeasure against hemolysis in HDFN due to anti-D.

Hemolytic disease of the fetus and newborn (HDFN) due to placenta transfer of maternal anti-D may result in the death of the fetus. HDFN typically occurs when a D– woman is pregnant with a D+ fetus. During pregnancy, and at delivery, red blood cells (RBCs) may enter the circulation of the mother resulting in an antibody response. This immunization event may result in formation of anti-D IgG, which is harmful for the fetus because maternal anti-D IgG is able to cross the placenta, bind to fetal RBCs, and cause destruction of fetal RBCs.

ABBREVIATIONS: ADCC = antibody-dependent cell-mediated cytotoxicity; CL = chemiluminescence; HDFN = hemolytic disease of the fetus and newborn; rHuIgG1 = recombinant human anti-D of the IgG1 subclass; V_H = variable region of heavy chain; V_κ = variable region of κ-chain.

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Thus far, the best-known treatment of affected fetuses is ultrasound-guided intrauterine transfusion of RBCs. Each transfusion carries a risk of fetal death, however, and the procedure has a perinatal loss rate of approximately 1 to 3 percent.[1]

Various types of therapy to target the maternal RBC alloimmunization in pregnancy have been attempted.[2] These include serial plasmapheresis procedures,[3] oral administration of D+ RBC stroma in an effort to induce D-specific immunologic tolerance in the mother,[4] or the use of promethazine to decrease phagocytosis by the fetal reticuloendothelial system.[5] None of these have proven consistently beneficial in subsequent clinical trials.[2] Administration of intravenous immune globulin has shown some benefit in severe cases of HDFN.[6] The mechanism of action has not been determined, although inhibition of maternal antibody synthesis, increased catabolic rate of IgG, partial blockade of antibody transport across the placenta, and Fc blockade at the level of the fetal reticuloendothelial system have been proposed.[7-10]

Administration of D-specific nondestructive antibody could be an attractive alternative. The idea is that the nondestructive antibody should be administered to the mother, cross the placenta, and block the binding of hemolytic maternal anti-D. This principle was demonstrated by Armour and coworkers[11] who designed human IgG molecules without effector functions and showed the ability of these antibodies to inhibit the activity of harmful anti-D. The antibodies were constructed either by replacing IgG2 residues 233 through 236 into IgG1 and IgG4 or by replacing IgG4 residues 327, 330, and 331 into IgG1 and IgG2 (EU numbering system).[12] Armour and colleagues[13] later showed intravascular survival of RBCs coated with antibodies similar to those described by Armour and coworkers.[11]

In this article, which is based on previous work by Michaelsen and colleagues,[14] we describe the construction of two IgG3 anti-D mutants without destructive activity toward RBCs due to the lack of a normal hinge region. We investigated whether these IgG3 anti-D mutants could inhibit the RBC-destructive activity of a recombinant human IgG1 (rHuIgG1) anti-D with identical antigen-binding region as the mutant antibodies, as well as the activity of a polyclonal anti-D having multiple D epitope specificities. Two assays were used for the investigation, antibody-dependent cell-mediated cytotoxicity (ADCC) and a chemiluminescence (CL)-based method for detection of respiratory burst in peripheral blood monocytes after opsonophagocytosis.

MATERIALS AND METHODS

Plasmids

The eukaryotic expression of intact antibodies was performed with plasmids, pLNOH2 and pLNOK.[15] pLNOH2 is a vector for expression of heavy-chain variable-region genes in combination with any constant region gene. In this study, three versions of the pLNOH2 vector were used. One version contains the human IgG1 allotype G1m(a,z), designated pLNOH2/Cγ1.[15] A second version contains the IgG3 m0/C131S mutant gene described by Michaelsen and coworkers[14] and is designated pLNOH2/HM5. A third version contains the IgG3 m0/C131S/R435H mutant gene (unpublished data) and is designated pLNOH2/HM5R435H. The R435H substitution was made assuming that this amino acid residue is crucial for binding to the neonatal Fc receptor, FcRn.[16,17] The change makes this region more IgG1-like, hopefully increasing the in vivo half-life as well as the ability to cross the placenta from mother to fetus. pLNOH2/HM5 was constructed by subcloning of the *Hin*dIII-*Bam*HI fragment from the vector $pSV2_{gpt}V_{NP}$ containing the IgG3 m0/C131S mutant gene into pLNOH2 generating pLNOH2/HM5. The IgG3 m0/C131S/R435H mutant gene was subcloned as a *Hin*dIII-*Bam*HI fragment into pLNOH2 generating pLNOH2/HM5R435H.

pLNOK is a vector for expression of Vκ genes in combination with the kappa constant domain gene. In this study, we used a version of the pLNOK vector that contained the Vκ gene of GAN4B.5.[18]

Cloning of variable region genes

Genes encoding the heavy-chain variable region of GAN4B.5 (GenBank Accession No. X79271) and the κ light chain genes of GAN4B.5 (GenBank Accession No. X79272) were obtained from the plasmid pFAB4HGAN4B.5.[18] The amplification and cloning of the heavy- and light-chain genes for GAN4B.5 are described by Nielsen and coworkers.[19]

The vectors for expression of the two mutated IgG3 anti-D were constructed by ligation of the *Bsm*I-*BsiW*I fragment from pG1 + GAN V_H, containing the variable heavy-chain gene of GAN4B.5,[19] to the vectors pLNOH2/HM5 and pLNOH2/HM5R435H previously digested with *Bsm*I and *Bsi*WI. The resulting vectors were designated pHM5 + GAN V_H and pHM5R435H + GAN V_H, respectively. Construction of the anti-D light-chain vector has previously been described.[19]

DNA sequencing

DNA was sequenced with a cycle sequencing kit (PRISM AmpliTaq FS, Big Dye Terminator, PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions were analyzed on a sequencing apparatus (ABI PRISM 3100, PE Applied Biosystems).

Transfection, expression, purification, and verification of specificity of recombinant anti-D

Transfection of Chinese hamster ovary (CHO) cells, expression, and purification were performed as previously described for IgG by Nielsen and coworkers.[20] The purification was monitored by ELISA for quantitation of recombinant IgG and by IAT gel card technique (DiaMed, Cressier sur Morrat, Switzerland) as previously described.[19] Purified recombinant antibodies were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Novex, San Diego, CA) followed by silver staining (Bio-Rad, Hercules, CA) and were also analyzed on a size-exclusion column (Superdex 200, Amersham Biosciences, Buckinghamshire, UK) to confirm size.

The concentration of purified rHuIgG1 and IgG3 mutant anti-D (HM5 + GAN and HM5R435H + GAN) was determined by absorbance at 280 nm with extinction coefficients calculated from a software package (Wisconsin Sequence Analysis, Genetics Computer Group, Madison, WI). For the calculation of concentration, molecular weights of 150 kDa were used for rHuIgG1, HM5 + GAN, and HM5R435H + GAN. The concentrations of the recombinant antibodies were adjusted to correct for contaminating bovine IgG as previously described.[20] The specificity of the produced antibody was verified by IAT technique in gel card as previously described.[19]

Purification of peripheral blood mononuclear cells and sensitization of RBCs for CL and ADCC

Blood was obtained from blood donors according to the approval issued by The Danish National Committee on Biomedical Research Ethics. Purification of peripheral blood mononuclear cells (PBMNCs) was performed as previously described.[20]

Sensitization of RBCs for CL and ADCC was performed essentially as previously described.[20] Briefly, D+ RBCs of the R1r phenotype (DCcee) no more than 2 days old were washed and counted. Inhibitory and active anti-D were mixed before being added to RBCs for sensitization. The hemolytic antibodies, rHuIgG1, or RhIG (Rhesogamma P, Aventis Behring GmbH, Marburg, Germany) were added in an amount corresponding to a stoichiometry of two antibodies per D antigen. IgG3 anti-D mutants were added either alone (at a stoichiometry of two antibodies/D antigen) or in 3×, 10×, 30×, or 90× molar excess of the hemolytic antibody as indicated in the figures. For each comparison of antibodies, we used a new batch of R1r RBC and for the calculations of antibody amounts to be added we used an estimated 12,000 epitopes per RBC.[21] The RBCs and antibody mixes were incubated for 1 hour, washed, and finally resuspended in assay buffer.

Detection of respiratory burst by CL

Interaction of antibody and Fc receptors was tested with a CL-based assay essentially as previously described.[20] Briefly, frozen PBMNCs were thawed, washed, resuspended in assay buffer, counted, and added to microtiter wells. After incubation of monocytes for 5 hours, sensitized RBCs and luminol were added to the wells. A RBC-to-PBMNC ratio of 6:1 was used. The light emission was monitored for five hours in a luminometer. All measurements were performed in quadruplicate. The result for a given measurement (m) was calculated from the integral of light measurements as:

$$\text{Relative activity} = (m - m_{neg})/(m_{pos} - m_{neg}) \times 100\%,$$

where m_{pos} is the mean of the positive control (rHuIgG1 or RhIG when appropriate), and m_{neg} is the mean of the negative control (nonsensitized RBCs). For illustration purposes the mean of n comparisons of antibody ± SD or antibody mixes ± SD are shown. The n comparisons of the antibody or antibody mixes were done with different batches of PBMNCs and RBCs, and each mean was determined in quadruplicate.

ADCC

ADCC assays and colorimetric assays with 2,7-diaminofluorene were performed as previously described.[20] RBC-to-PBMNC ratios of 1:1 and 5:1 were tested. PBMNCs and RBCs were incubated for 20 hours before assaying for released hemoglobin by the 2,7-diaminofluorene assay. All measurements were performed in octuplicate, and the results were calculated and illustrated as described above for detection of respiratory burst by CL except that OD values were used for the calculations.

Statistical analysis

Statistical analysis was performed on log-transformed raw data with computer software (SPSS version 13.0 with the general linear model, univariate option, SPSS, Inc., Chicago, IL). The data were analyzed with Dunnett's pairwise multiple-comparison t test post hoc test option to correct for multiple comparisons. p Values below 0.05 were considered significant.

RESULTS

Generation and purification of recombinant human anti-D

The three antibodies IgG1 + GAN, HM5GAN, and HM5R435H were cloned and expressed as described under Materials and Methods.[19] ELISA and gel card analy-

sis were used to measure antibody production levels in the supernatants. The results of the ELISA and the gel card assay—additionally—confirmed paring of heavy and light chain.

Initially, protein G purification was attempted but the yield was low and it was thought to be due to partial degradation of recombinant antibody caused by the low pH of the elution buffer. Therefore, we used precipitation by ammonium sulfate, DEAE, ABx ion-exchange chromatography, and if necessary size-exclusion chromatography to purify the recombinant antibodies.[20]

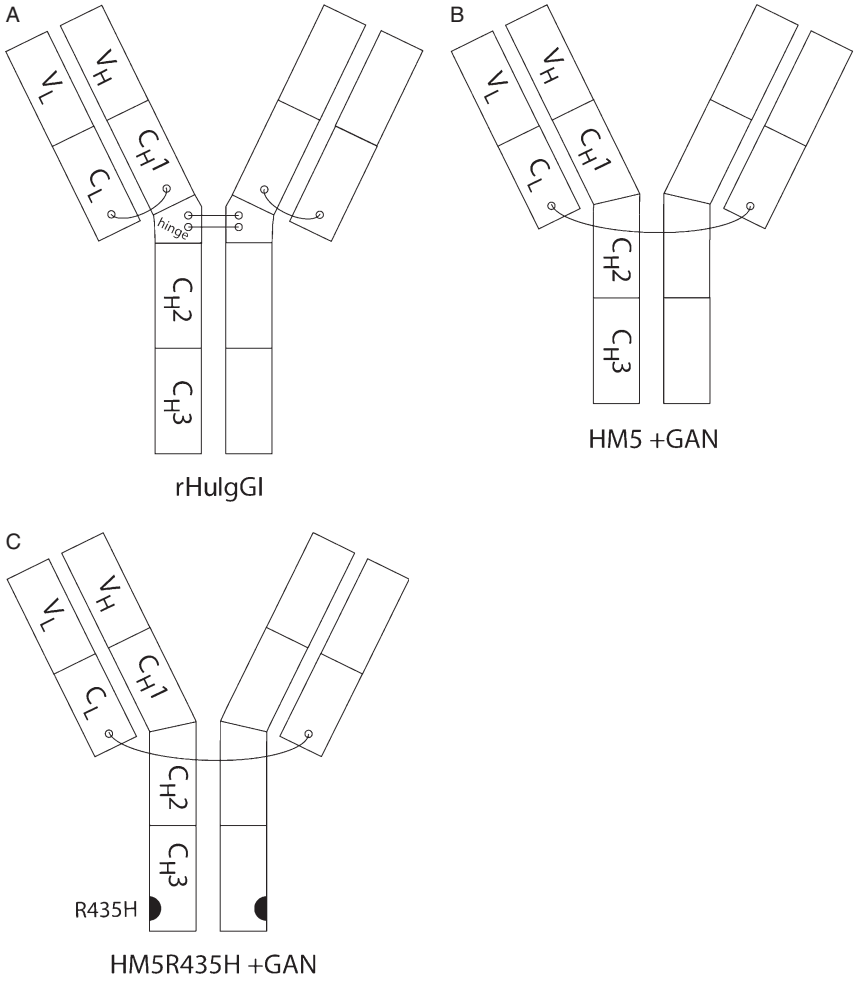

Fig. 1. Schematic drawing of the IgG structure of the wild-type IgG1 with anti-D variable regions (rHuIgG1) and the mutant IgG3 molecules with anti-D variable regions (HM5 + GAN and HM5R435H + GAN).

Characterization of recombinant anti-D

The three recombinant human anti-D were analyzed by SDS-PAGE. Under reducing conditions, all antibodies gave two bands: one band of approximately 50 kDa corresponding to the heavy chains and one band of approximately 27 kDa corresponding to the light chains (data not shown).

Under nonreducing conditions, a single band of approximately 150 kDa was detected for rHuIgG1, corresponding to the expected size of monomeric IgG, whereas for HM5 + GAN and HM5R435H + GAN two bands of approximately 50 kDa were detected, corresponding to heavy-chain monomers and κ-chain dimers, respectively. This was consistent with the presumed structure and confirmed the presence of a disulfide bond between the light chains as previously described for IgG3 m0/C131S mutant (= HM5) by Michaelsen and coworkers.[14] When analyzed under nondenaturing conditions by size-exclusion chromatography, however, rHuIgG1 as well as the IgG3 mutants eluted as one peak corresponding to approximately 150 kDa, indicating that the IgG3 mutants were made up of noncovalently associated heavy-chain dimers with disulfide-linked light-chain dimers (data not shown; see Fig. 1 for a schematic drawing of the three recombinant antibodies used in this study). Gel card analysis confirmed that the recombinant anti-D only reacted with D+ RBCs and did not bind other antigens on RBCs (data not shown).

Functional characterization of recombinant anti-D

Mutant IgG3 anti-D were tested in two assays (CL and ADCC) for determination of their ability to inhibit the responses of two active anti-D (rHuIgG1 and RhIG). Total inhibition was defined as reactivity not significantly different from the reactivity obtained with nonsensitized RBCs.

In the CL assay, the responses from rHuIgG1 mixed with 30 and 90 times excess of inhibitory antibodies were totally inhibited ($p > 0.8$; Fig. 2). With the polyclonal RhIG (Fig. 3) even a modest surplus of 10 led to total inhibition ($p > 0.2$) suggesting a lower affinity of the polyclonal anti-D compared with rHuIgG1.

In the ADCC assay with RBC-to-PBMNC ratios of 1:1 and 5:1 (Fig. 4), the responses from rHuIgG1 mixed with 90 times surplus of inhibitory antibodies were totally inhibited ($p > 0.05$ for all). Total inhibition was not observed at 30 times surplus of HM5 + GAN or HM5R435H + GAN. HM5 + GAN tested alone at a RBC-to-PBMNC ratio of 5:1 surprisingly gave a significantly lower response than background ($p < 0.03$). HM5 + GAN tested alone at a RBC-to-PBMNC ratio of 1:1 and

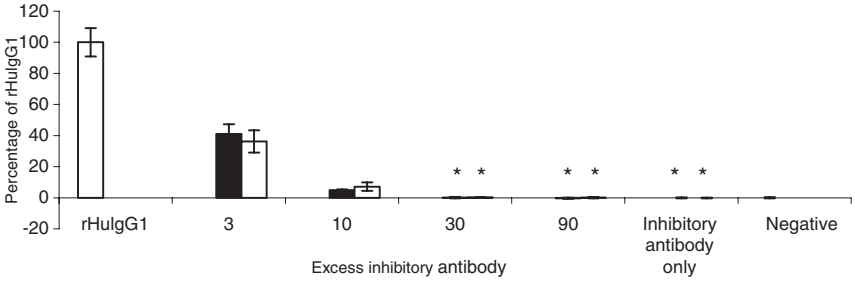

Fig. 2. CL tests with rHuIgG1, HM5 + GAN (■), HM5R435H + GAN (□), and mixes thereof. CL responses are shown relative to rHuIgG1 and were calculated as described under Materials and Methods. Quadruplicates were performed to estimate the mean and are shown as mean ± SD. *Results not significantly higher than the negative control (nonsensitized RBCs).

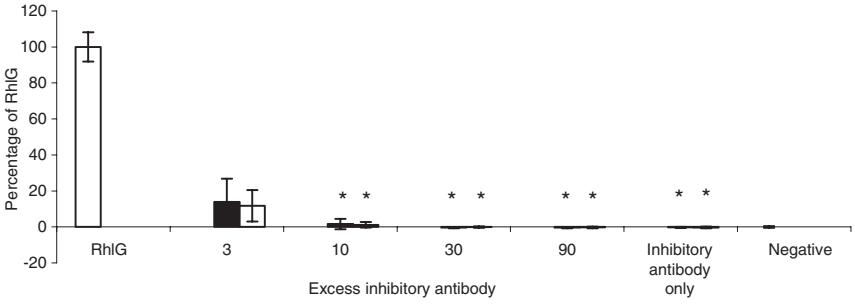

Fig. 3. CL tests with RhIG, HM5 + GAN (■), HM5R435H + GAN (□), and mixes thereof. CL responses are shown relative to RhIG and were calculated as described under Materials and Methods. The results are shown as the mean of three comparisons ± SD. The three comparisons were performed with separate batches of PBMNCs and RBCs. For each batch, quadruplicates were performed. *Not significantly higher than the negative control (nonsensitized RBCs).

HM5R435H + GAN tested alone at RBC-to-PBMNC ratios at 1:1 and 5:1 were not significantly different from background ($p > 0.08$ for all).

In the ADCC assay with a RBC-to-PBMNC ratio of 5:1 (Fig. 5A), the responses from HM5 + GAN alone and from RhIG mixed with 90 times surplus of HM5 + GAN were totally inhibited ($p > 0.2$), whereas the response from HM5R435H + GAN alone and from RhIG mixed with 90 times surplus of HM5R435H + GAN were not totally inhibited ($p = 0.04$ and $p < 0.001$, respectively). This is in contrast to the experiments presented in Fig. 4A where HM5R435H + GAN alone totally inhibited hemolysis.

In the ADCC experiments with a RBC-to-PBMNC ratio of 1:1 (Fig. 5B), the responses from inhibitory antibody alone and from RhIG mixed with 90 times surplus of inhibitory antibody were totally inhibited ($p > 0.5$). At 30 times surplus of HM5 + GAN, hemolysis was totally inhibited ($p = 0.2$), whereas at 30 times surplus of HM5R435H + GAN hemolysis was not totally inhibited ($p < 0.001$).

DISCUSSION

The aim of this work was to investigate whether admixing one of two IgG3 anti-D heavy-chain mutants could inhibit the hemolytic activity mediated by wild-type monoclonal as well as polyclonal anti-D. Previously, one of the IgG heavy-chain mutants (HM5 = IgG3 m0/C131S) has been tested.[14] In that test it had specificity for the hapten 5-iodo-4-hydroxy-3-nitrophenacetyl and was negative for complement-mediated lysis, ADCC, and phagocytosis by U937 cells and polymorphonuclear leukocytes. This prompted us to investigate the use of this IgG mutant constant region expressed with a variable region recognizing D in the treatment of HDFN. A novel IgG3 mutant, HM5 + GAN with a R435H substitution (HM5R435H + GAN), was made assuming that this amino acid residue is crucial for binding to the neonatal Fc receptor, FcRn.[16,17] The change makes this region more IgG1-like, hopefully increasing the in vivo half-life as well as the ability to cross the placenta from mother to fetus.

Two assays were used for the evaluation of the anti-D IgG3 heavy-chain mutants: ADCC and a CL-based method for detection of respiratory burst. Initially, experiments were made with rHuIgG1 to confirm the concept of inhibiting antibodies (Figs. 2, 4). HM5 + GAN and HM5R435H + GAN effectively inhibited the response from rHuIgG1 in CL and ADCC. The amount of inhibiting antibody necessary to block the activity of rHuIgG1 in CL was less than in ADCC, indicating that ADCC may be more sensitive than CL or that different FcγR are involved in the assays.

Having confirmed the inhibitory effect of the IgG3 mutants, experiments were carried out with a polyclonal anti-D (RhIG, Rhesogamma P) as the active antibody. In the CL assay the response from RhIG was totally inhibited at 10 times surplus of inhibitory antibodies (Fig. 3). To reach the same level of inhibition of rHuIgG1, 30 times surplus of inhibitory antibody was needed. The reason for the different amount of antibody needed to inhibit monoclonal and polyclonal antibodies may be different methods of determining antibody concentration or different affinities of the active antibody or may simply be an uncertainty due to the low number of experiments. The surplus antibody needed to inhibit the response from rHuIgG1 and polyclonal anti-D,

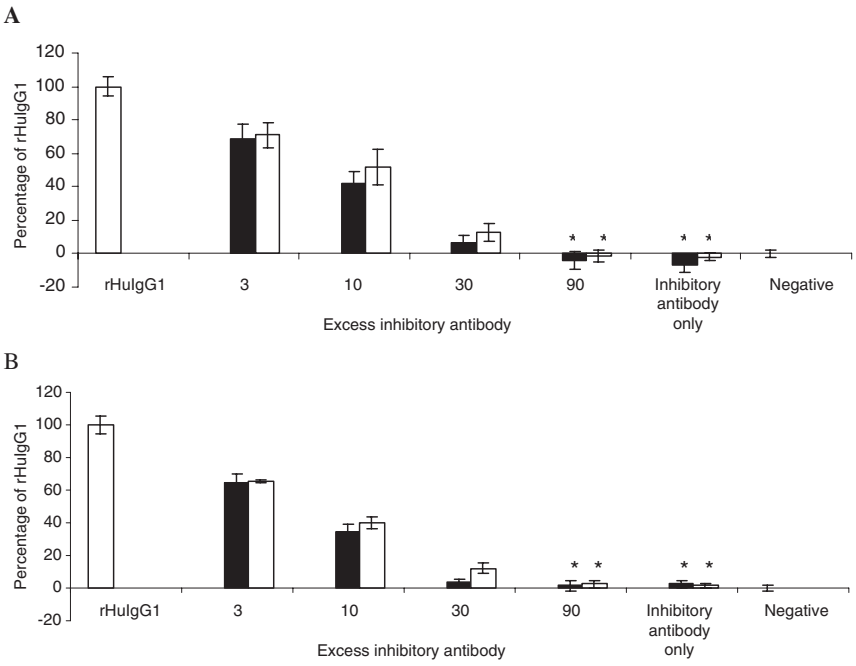

Fig. 4. ADCC tests with rHuIgG1, HM5 + GAN (■), HM5R435H + GAN (□), and mixes thereof. Percentages of lysis are shown relative to rHuIgG1 and were calculated as described under Materials and Methods. Octuplicates were performed to estimate the mean and are shown as mean ± SD. Two RBC-to-PBMNC ratios were used: (A) RBC-to-PBMNC ratio of 5:1; (B) RBC-to-PBMNC ratio of 1:1. *Results not significantly higher than the negative control (nonsensitized RBCs).

however, is comparable to that seen by Armour and colleagues.[11]

In the ADCC with RhIG and a RBC-to-PBMNC ratio of 5:1, more inhibitory antibody was needed to inhibit the response than at a RBC-to-PBMNC ratio of 1:1. The reason for the difference is not clear but it may, however, be due to a higher number of stimulatory interactions between RBCs and PBMNCs at a RBC-to-PBMNC ratio of 5:1 compared to a RBC-to-PBMNC ratio of 1:1.

In the same experiments more HM5R435H + GAN than HM5 + GAN is needed to inhibit the response from RhIG. The reason for this difference between HM5 + GAN and HM5R435H + GAN is not clear, however, but may be due to the amino acid substitution at position 435 because this is the only known difference between the two antibodies. The reason for the lack of replication of HM5R435H + GAN tested alone in Figs. 4A and 5A is not clear but may be due to statistical uncertainty due to the low number of experiments performed.

Because the ADCC and CL assays used in this study have not yet been tested in a clinical setting, it is not known to what level the active antibody should be inhibited to prevent HDFN. We have used a level "not significantly different from background" to describe a complete inhibition of active antibody, but the level relevant for treatment, where further intervention to prevent HDFN is not necessary, may very well be higher than that. For comparison, the CL assay used by Armour and coworkers[11] has a cutoff value of 30 percent. This indicates that even lower amounts of inhibiting antibody than estimated from our results can prevent the destruction of the fetal RBCs to a degree where further intervention to prevent HDFN is not necessary. After all, success of treatment with inhibitory antibody will be achieved when the rate of RBC destruction is below the rate of production of RBCs in the fetal marrow. Thus zero destruction is probably not necessary.

Armour and coworkers[11] gave a number of arguments as to the relevance of their results, arguments that are also relevant for our study. They argued that their results are likely to be relevant in the fetal setting since fetal white blood cells bear the same classes of FcγR found on equivalent adult cells, albeit at generally reduced densities.[22] In vitro experiments show that phagocytosis by fetal monocytes was inhibited more than 93 percent by anti-FcγRI.[23] This supports the relevance of the results obtained in our CL assay, because this assay is thought to reflect the interaction with FcγRI.[24-27] Although it has been shown that FcγRIIa interacts with IgG1 and IgG3 anti-D[28] and can participate in the phagocytosis of RBCs coated with IgG3 anti-D, FcγRIIa is not thought to play an important role in the hemolysis in vivo.[26] The role of FcγRIII was also investigated by Wiener and colleagues[23] and they found that anti-FcγRIII significantly inhibited the phagocytosis by fetal spleen mononuclear phagocytes but not as much as seen by inhibition by anti-FcγRI.[23]

A therapeutic antibody without hemolytic activity for prevention of HDFN should be able to cross the placenta and therefore the next step in the investigation of the IgG3 mutant antibody includes in vitro placenta transfer studies. In vitro assays have been developed to measure this transport by several research groups.[29-31]

The amount of inhibitory antibody needed for therapy depends on several factors such as placenta transfer, affinity to antigen, and half-life of the antibody. Each of these parameters, however, must be determined experimentally.

If given to a pregnant woman, the two mutant IgG3 constant regions used in this study may be immunogenic and therefore result in an immune response toward RBCs

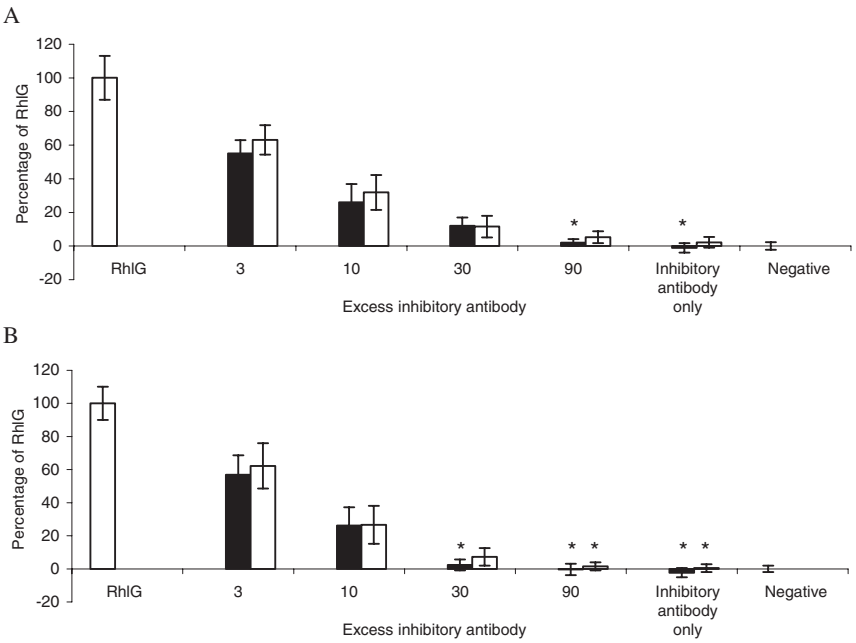

Fig. 5. ADCC test with RhIG, HM5 + GAN (■), HM5R435H + GAN (□), and mixes thereof. Percentages of lysis are shown relative to RhIG and were calculated as described under Materials and Methods. The results are shown as the mean of three comparisons ± SD. The three comparisons were performed with separate batches of PBMNCs and RBCs. For each batch octuplicates were performed. Two RBC-to-PBMNC ratios were used: (A) RBC-to-PBMNC ratio of 5:1; (B) RBC-to-PBMNC ratio of 1:1. *Results not significantly higher than the negative control (non-sensitized RBCs).

coated with these antibodies. In vivo studies must be performed to answer that question.

The two IgG3 mutants have a nonconventional structure in that a disulfide bridge covalently links the two light chains. Furthermore, the heavy chains are not covalently associated and are not covalently associated to the light chains. This could lead to an unstable molecule but the size-exclusion chromatography data indicated that the IgG3 mutants were stable. Further experiments need to be performed to verify the stability of the two IgG3 mutants. The two IgG3 mutant constant regions described in this article may also be used for treatment of other diseases where inhibition of an established immune response is needed, for example, alloimmune thrombocytopenia.

In summary, we have described the construction of two IgG3 mutant anti-D and demonstrated their ability to inhibit the CL and ADCC response mediated by monoclonal as well as polyclonal anti-D. The two antibodies may therefore be useful for the treatment of ongoing HDFN.

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