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Anti-RhD-Mediated Immunosuppression: Can Monoclonal Antibodies Imitate the Action of Polyclonal Antibodies?

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1. Introduction

Passively administered IgG antibodies can temporary prevent the antibody response to the corresponding antigen. This phenomenon of antibody-mediated immune suppression has been successfully applied in clinical practice: administration of polyclonal anti-RhD immunoglobulin to Rh-negative women during and after pregnancy is a very effective measure for the preventing D immunization by D-positive fetal red blood cells and, as a result, the hemolytic disease of the next D-positive fetus or newborn. Anti-D immunoglobulin is derived from sera of immune donors. Plenty of human monoclonal and recombinant anti-D antibodies have been obtained around the world and some of them have passed initial stages of clinical trials; however, none of the monoclonal preparations can be used as a surrogate for polyclonal ones. Evaluation has revealed the two major obstacles that limit the development of an effective monoclonal preparation. They are a low clinical activity of monoclonal anti-D and the lack of a suitable cell line-producer that will be able to provide a "correct" glycosylation of monoclonal antibodies.

Despite a long period of anti-Rh immunoglobulin application we still fail to determine a precise list of the cellular and molecular participants involved in the mechanism for immunosuppression. To date, the overwhelming evidence points to the key role of immune complexes and the peculiarities of their interaction with Fcgamma-receptors (FcγR) on immune cells. The most convincing is the mechanism for a temporal switch-off of the immune response to the antigen due to co-ligation by immune complexes of the B cell receptor and inhibitory low-affinity receptor FcγRIIB on specific B cells (effect of clonal silencing). We investigated *in vitro* into the interaction of human monoclonal anti-D antibodies with different types of FcγR, as well as into the molecular structure of genes of anti-D antibodies and the composition of the sugar which, as known, does exert a significant influence on the efficiency of interaction between the antibody Fc fragment and FcγR. We have received a series of anti-D antibody counterparts and shown that the effector function crucially varies depending on the nature of the host cells. The original research data provide information valuable for developing a strategy of creation of monoclonal drugs with anti-inflammatory properties; moreover, they may help with clarifying some still elusive aspects of regulation of the humoral immune response in general. The data obtained make it possible to speculate that the immunosuppressive activity of polyclonal antibodies and

inability of peripheral B cells to produce antibodies with a similar property may be attributed to the fact that B cells of different subpopulations secrete antibodies with different functional properties. According to the hypothesis proposed here, only long-lived plasma cells are able to synthesize anti-inflammatory immunosuppressive antibodies that are an essential element of the feedback regulation of antibody production.

2. Prevention of D sensitization: Clinical application of antibody-mediated antigen-specific immunosuppression

Prevention of RhD-sensitization - a mandatory procedure in obstetrics now - is currently the only example of a conventional clinical application of antibody-mediated antigen-specific immune suppression. This procedure involves administration of anti-D immunoglobulin to Rh-negative women after delivery of an Rh-positive infant and is required for prophylaxis of the Rh hemolytic disease of the next Rh-positive fetus and newborn.

The erythrocyte D antigen determines the Rh phenotype of human blood: individuals with D are Rh-positive, those without D are Rh-negative. The D antigen is highly immunogenic and present in part of population (about 85% of the European ethnicity are D positive), which creates conditions for incompatible transfusions and immunization of Rh-negative women with fetal D positive (D+) red cells during or after pregnancy. Despite a very small volume of the fetal blood that enters a maternal organism during pregnancy or delivery (it is less than 1 ml at uncomplicated delivery), this amount is quite sufficient for about 16% of Rh negative women to be immunized after their first ABO-compatible Rh-positive pregnancy (Bowman, 1988). During next pregnancies immune anti-D IgG antibodies cross the placenta and destroy fetal D positive red cells, provoking a severe pathology of the fetus /newborn - hemolytic disease. About 10% of neonatal deaths were due to hemolytic disease of the newborn before the era of immunoprophylaxis had begun (Bowman, 2003).

The idea to apply anti-D antibodies for the prevention of D-sensitization was experimentally supported in the 1950s-60s by several groups of researchers. By that time, it had been known from clinical observations that the ABO incompatibility of an Rh-positive fetus with its Rh-negative mother could reduce the incidence of D-immunization (Levin, 1943). It was assumed that suppression might be related to the destruction of D+ red cells by natural anti-A or anti-B antibodies, as well as to their fast clearance in liver before they reached immunocompetent sites. This clinical observation was experimentally checked, and the experiment proved that the ABO incompatibility did provide a partial protection against D sensitization (Stern et al., 1956). The same study also demonstrated that the injection into Rh-negative men of Rh-positive red cells coated *in vitro* with anti-D antibodies was completely ineffective in inducing the anti-D immune response. Based on these findings, a group of British researchers from Liverpool carried out a study when D-negative volunteers were given an injection of D+ red cells followed by anti-D immune serum and showed that IgM anti-D was ineffective, whereas anti-D IgG had a high protective effectiveness (Clarke et al., 1963). At the same time, similar studies were undertaken in New York; however, their theoretical rationale was different (Freda et al., 1964; Gorman et al., 1966). The authors decided to apply a phenomenon that was described by the first Nobel Prize Laureate in Physiology or Medicine Emil Adolf von Behring over 100 years ago (von Behring, 1892) and is currently called the antibody-mediated immune suppression (AMIS). This phenomenon is based on the ability of antisera to the antigen to suppress the immune response to this antigen after their simultaneous administration. A specific antibody injected passively,

either with its antigen or separately, has been found to prevent the active immunity that follows injections of the antigen alone in many different antigen-antibody systems in various species of animals, including humans. Suppression of the immune response to sheep red cells in rabbits and mice by antiserum or monoclonal antibodies against sheep red cells is a classical example of AMIS (Heyman & Wigzell, 1984). Based on the AMIS phenomenon, it could be expected that anti-D antibodies introduced simultaneously with D+ red cells would also block the anti-D immune response. The remarkable result of the studies on both sides of the Atlantic and following successful clinical trials resulted in that prevention of D sensitization with anti-D immunoglobulin became a mandatory procedure in obstetrics, thus turning the hemolytic disease of the newborn into a rare pathology in developed countries (Bowman, 1988; 2003). At present, every D-negative unimmunized woman must be given one prophylactic dose of anti-D immunoglobulin (the dose ranges from 150 to 300 mcg of anti-D in different countries) after the delivery of a D-positive infant, irrespective of the ABO blood group of the infant.

The most striking characteristic of a polyclonal anti-D preparation is its ability to induce a fast clearance of D+ red cells and their sequestration in the spleen (Mollison et al., 1965). Unlike the liver where red cells are sequestered after exposure to natural anti-A and anti-B antibodies, spleen is an organ of the active immune response; nevertheless, D+ red cells entering this lymphoid organ do not cause sensitization but, instead, lead to the suppression of the immune response. Anti-D immunoglobulin is able to prevent the primary anti-D response, but it is ineffective or low effective in the case of the secondary response (Tovey & Robinson, 1975; de Silva et al, 1985). Despite a long history of anti-D immunoglobulin usage we still fail to fully understand which of the two assumptions – fast clearance or AMIS – is closer to the truth and what particular process (or a set of processes) is crucial and leads to immunosuppression. The answer to this question may be interesting not only from the general scientific standpoint but is also essential in terms of its practical usage. Widespread prophylaxis of D-sensitization requires a large amount of preparation that is administered not only after delivery, but also in the last trimester of pregnancy, after abortion, after therapeutic and diagnostic amniocentesis and other episodes of transplacental hemorrhage. Anti-D immunoglobulin is produced by isolating the IgG fraction from pooled immune donor plasma. Obviously, the development of biotechnologies for obtaining monoclonal anti-D human antibodies has generated great optimism and expectations that the alternative source for the anti-D immunoglobulin production may be found. So, what is the reason for why the effective monoclonal anti-D is not yet developed, while a whole range of therapeutic monoclonal antibodies of other specificities is now used in oncology, rheumatology, etc.?

3. The mechanism for antibody mediated immune suppression

Some mechanisms are proposed to clarify the phenomenon of AMIS; among the most discussed are the following:

- the mechanism of antigen camouflage, that is, masking of antigen determinants due to the excessive dose of antibodies;
- a fast clearance of the antigen-antibody complex before it can activate specific B cells;
- a selective suppression of antigen specific B cells and lack of anti-D antibody production.

While the first mechanism does not require Fc fragments of antibodies for its work, and masking of antigens can be induced by the Fab parts of antibodies, the other two

mechanisms depend on the properties of the Fc fragments of immunoglobulins and the type of their interplay with Fc receptors. The effect of a large number of immune preparations is mainly based on the bipolarity of antibodies, that is, the ability of the antigen-binding site of antibodies to bind to a relevant antigen and the ability of the Fc fragment to mediate both recognition of the antigen-antibody complex by the immunocompetent effector cells bearing Fcγ receptors and elimination of these complexes from the organism.
What arguments are there "pro" and "contra" the involvement of the above mechanisms in AMIS and preventing anti-D sensitization?

3.1 The mechanism of antigen camouflage

Investigations of the primary immune response to the antigen in the presence of antibodies to this antigen used excessive dose of antibodies usually sufficient for binding all antigen determinants. "The determination of which effect of antibody will predominate probably depends on many factors, but if large amounts of antibody with high binding affinity are employed, suppression will usually occur" (Uhr & Möller, 1968). AMIS in transgenic mice lacking the known receptors for IgG (the fact that initially raised much confusion since the involvement of FcγR in regulation of the immune response had been thought unquestionable by that time) can most likely be accounted for by the masking of antigen (Heyman, 1999). The data indicating that F(ab)2 fragments as well as IgE are efficient suppressors of antibody responses in FcγR–deficient mice argue in favor of the antigen masking (Karlsson et al., 1999; Karlsson et al., 2001).
Interestingly, we accidentally found the masking effect of anti-D monoclonal antibodies during clinical trails of their efficacy (Olovnikova et al., 2000). One of the anti-D IgG1 monoclonal antibodies, G17, administered at the same dose as the other anti-D demonstrated the unique capability of binding *in vivo* the maximum D sites and making D+ red cells fully saturated. It was not possible to stain red cells sensitized *in vivo* by either other monoclonal or polyclonal anti-D antibodies. Irrespective of this property, G17 poorly accelerated the clearance of D+ red cells from the circulation in D-negative individuals, so we observed sensitized D+ red cells in the blood of the individuals during several months. None of the 5 subjects who had received G17 produced anti-D within 6 months; however, three in this group showed the secondary immune response after rechallenge with D+ red cells. Studies of AMIS, in FcR-deficient mice including, are often limited to investigations of the primary immune response (Heyman, 2000); meanwhile, it is quite possible that the results similar to ours may be obtained in the case of reimmunization.
These findings strongly suggest that IgG is able to efficiently suppress antibody response independently of the Fc part and argue in favor of an important role of antigen masking under some experimental conditions. However, the mechanism of antigen masking can not be relevant to explanation of the anti-D immune suppression since only about 10% of D antigen sites are found to be occupied after administration of an effective dose of a polyclonal or monoclonal anti-D antibodies. Moreover, approximately 200 anti-D IgG molecules per erythrocyte are sufficient to effectively suppress the anti-D immune response (Kumpel et al., 1995; 2006). It was shown that Fab can not prevent the anti-D immune response (Nicholas, 1969). The ability of antibodies to one blood group antigen to suppress the immune response to the other blood group antigen simultaneously expressing on the red cell also can not be accounted for by the mechanism of antigen blocking. For example, polyclonal IgG anti-K (Kell system antigen) was shown to prevent immunization against both K and D antigens after immunization of Rh-negative K-negative individuals with

D+K+ red cells (Woodrow et al., 1975). It is known that IgG can induce nonepitope-specific suppression; however, the effect requires a high epitope density (Heyman & Wigzell, 1984). D and K antigens have a relatively low density (approximately 20,000 molecules per erythrocyte), their positions in the membrane are not related to each other; this is the reason for why inhibition of the anti-D response by anti-K antibodies can not be explained simply by steric screening of D epitopes.

3.2 Fcγ receptors and assays for evaluation of the functional properties of anti-D antibodies

The immune response of an organism to a foreign agent is the process of activation and development of the system of specific protection, that is, recognition, neutralization, destruction and elimination of the foreign object. When antibodies form a complex with a soluble or membrane antigen, they activate the effector cells and molecules that destroy foreign cells and remove immune complexes from the organism. The interaction between a complement or Fc-receptors on effector cells and the Fc region of IgG forming complexes with the antigen plays the key role in the physiological response to the presence of immune complexes. Although the ability to activate the complement system underlies the action of many therapeutic cytotoxic antibodies, we do not consider this aspect here due to the fact that anti-D antibodies do not activate the complement, and this mechanism is not involved in the clearance of D+ red cells in the presence of anti-D.

Cellular receptors for IgG, FcγR, a group of surface glycoproteins belonging to the Ig superfamily and expressed mostly on immune cells, are divided into three classes: FcγRI (CD64), FcγR II (CD32) and FcγRIII (CD16). Some of the FcγR features that may be involved in the processing of sensitized red blood cells are presented in Table 1.

The important characterstics of FcγRs are their affinity for IgG and the nature of the signal transduced, i.e. whether they initiate activating or inhibitory signalling cascades. FcγRI has a high affinity for IgG and has the capacity to bind not only IgG within the complexes but also monomeric molecules. The other FcγRs are of low to medium affinity and recognize only the IgG in the form of immune complex (Aschermann et al., 2010). The reaction of an effector cell in response to binding the complex is determined by the cytoplasmic part of the receptor that bears the immunoreceptor tyrosine-based activation motif (ITAM) in activating receptors and the immunoreceptor tyrosine-based inhibitory motif (ITIM) in inhibitory receptors (Amigorena et al., 1992; Isakov, 1997). Interaction of the immune complex with the FcγRI, FcγRIIA and FcγRIII containing both a ligand-binding subunit and the associated signaling part ITAM leads to the cellular activation. The nature of the responses primarily depends on the cell type; these can be antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, endocytosis and cytokine or the inflammatory mediator release (Daëron, 1997; Clynes et al., 1999; Siberil et al., 2007). In contrast, the ITIM-containing receptor FcγRIIB, a transducer of inhibitory signals, down regulates the ITAM-mediated cellular activation when it co-ligates with the activating receptors (Ono et al., 1996). As an example, coligation of FcγRIIB and an ITAM-containing B cell receptor leads to aborted activation in B cells (Phillips &Parker, 1984).

The ADCC test using human peripheral blood mononuclear cells as effector cells is a multipurpose *in vitro* assay that makes it possible to determine the ability of antibodies to mediate cytolysis and to estimate the contribution of different types of receptors to this process (Engelfriet et al., 1994). The scheme of the ADCC assay adapted for the research of antibody-mediated hemolysis is shown in Fig. 1; the effector cells are listed in Table 1.

Receptor	Affinity	Affinity for IgG isotype	IgG bound form: monomeric/ complex	Signal	Mediating process	Lo ce or
FcγRI	High	IgG3 > IgG1>> IgG4>>> IgG2	Monomeric and within the complex	Activating	ADCC, endocytosis, phagocytosis	M m C
FcγRIIA	Low	IgG1> IgG2 (depends on FcγRIIA poly-morphism) >>> IgG3	Within the complex	Activating	ADCC, endocytosis, phagocytosis, inflammatory mediator release	M m ne pl
FcγRIIB	Low	IgG3 ≥ IgG1> IgG4 >>> IgG2	Within the complex	Inhibiting	Blockade of B cell activation, internalization of immune complexes	B m m m de
FcγRIIC	Low	IgG1, IgG3	Within the complex	Activating	ADCC (some isoform)	N ly
FcγRIIIA	Inter-mediate	IgG1= IgG3 >>>IgG2, IgG4	Within the complex	Activating	Cytotoxicity, endocytosis, phagocytosis, cytokine release	N T m m
FcγRIIIB	Inter-mediate	IgG1, IgG3	Within the complex	Activating	Generation of reactive oxygen species	N

Table 1. Human Fcγ receptors. The papers used in preparation of the table (Ravetch & Kinet, 1991; Engelfriet et al., 1994; de Haas et al., 1995; Ernst et al., 2002; Siberil et al., 2007; Kumpel, 2007)
*NK – natural killer lymphocytes

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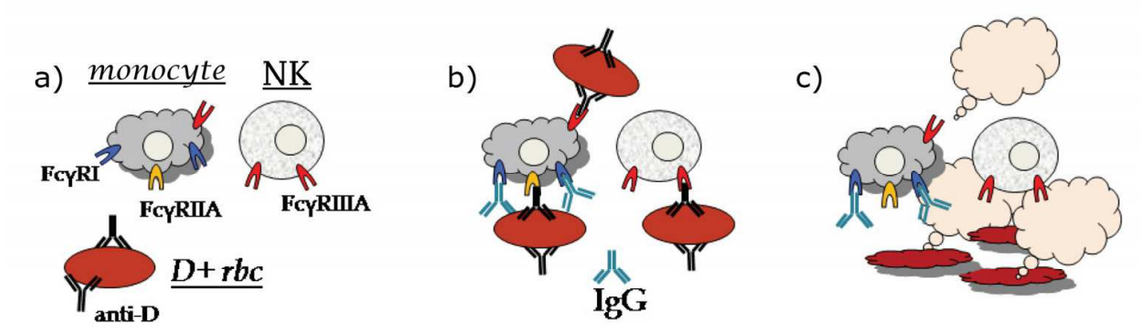

Fig. 1. Antibody-dependent cellular cytotoxicity assay. a) Participants of the ADCC assay are: effector cells bearing FcγRs (NK lymphocytes and monocytes); target cells (D+ red cells); anti-D antibodies. b) Intravenous Immunoglobulin (IG) is added as a source of monomeric IgG to block FcγRI. Only FcγRIIA and FcγRIIIA take part in the interaction with anti-D in this variant of the test that will be denoted as ADCC+IG. c) The concentration of free hemoglobin correlates with the hemolytic efficiency of anti-D in the standard formulation of the reaction

The concentration of free hemoglobin in the medium, being proportional to the number of destroyed target red blood cells, reflects the ability of antibodies to mediate ADCC. The non-immune IgG blocking the high-affinity FcγRI is added to the medium to estimate the ability of antibodies for triggering hemolysis via low-affinity FcγRIIA and FcγRIIIA (Fig. 1b). Antibody interactions with different types of FcγR can be studied using blocking antibodies against corresponding receptors (Kumpel et al., 2002a). Sometimes researchers apply a modification of the test, the so-called K-ADCC, which is designed to evaluate the interaction of antibodies with FcγRIIIA only. The fraction of nonadherent peripheral blood mononuclear cells containing FcγRIIIA-positive NK lymphocytes is used as effector cells in this test (Urbaniak, 1979a). It is worth noting that the test for the ability of anti-D to mediate lysis via FcγRIIIA is quite artificial: it is conducted with red cells treated with proteases only since native red cells do not work in this reaction (Urbaniak, 1979b). Irrespective of this, ADCC was found to be very useful for the evaluation and comparison of the functional activity of polyclonal and monoclonal anti-D antibodies. In the absence of a proper animal model of the Rh-conflict pregnancy, the efficacy of antibodies in ADCC serves a crucial factor before clinical trials.
Functional assay for the estimation of the IgG affinity for inhibitory receptor FcγRIIB is absent; the measurement of the IgG affinity for human FcγRIIB expressed on transfected cells or bound to beads may be used (Siberil et al., 2006; Lazar et al., 2006).

3.3 Clearance of D+ red cells *in vivo* upon the treatment with polyclonal anti-D immunoglobulin

The ability to accelerate the clearance of cells or target molecules is an important indicator for the effectiveness of the majority of immunopreparations. The ability of anti-D immunoglobulin to accelerate the clearance of coated D+ red cells is also considered to be its essential feature. Relation between the rate of clearance and the degree of red cell coating, on the one hand, and correlation between the rate of clearance and suppression, on the other hand, were found many years ago (Mollison et al., 1965; Mollison, 1984).

Anti-D prophylaxis was thought to be successful due to the efficient clearance of RhD-positive RBCs from the circulation and phagocytosis of anti-RhD-coated RBCs by macrophages in the spleen (Pollack, 1984). The role of spleen in the non-inflammatory removal of antibody-coated cells was reviewed in (Kumpel, 2006). The participation of the FcγRI in this process still remains elusive, since high-affinity FcγRI *in vivo* should be fully saturated with the circulating monomer IgG whose concentration in the serum is more than 10 mg/ml. One cannot exclude the possibility that the administered anti-D antibodies may be captured by FcγRI on macrophages along with other IgG. Sensitized ("arming") macrophages will in turn catch D+ red cells, thereby causing the *in vivo* destruction of the unsensitized red cells expressing the corresponding antigen (Griffiths et al., 1994). Nonetheless, it is highly probable that FcγRIII plays the key part in the clearance of sensitized cells. It was shown that intravenous infusion in chimpanzees of monoclonal antibodies which block 51-73 kD FcγRs that is similar to human FcγRIIIA dramatically prolongs the clearance of IgG-sensitized red cells (Clarkson et al., 1986). Strong association between the rate of removal of sensitized red cells and the allelic variant of human FcγRIIIA suggests, though indirectly, the engagement of this receptor in eliminating the antibody coated red cells. It has been demonstrated that the FcγRIIA polymorphism also has an effect on the rate of clearance (Miescher et al., 2004). However, FcγRIIA is considered to make no significant contribution to the red cells clearance *in vivo*, and FcγRIIIA is therefore likely to be the primary receptor utilized by macrophages *in vivo* for sequestration of anti-D-coated red cells (Kumpel, 2007). A set of data supports the role of FcγRIII in the cancer therapy with monoclonal antibodies and the relationship between the Fc - FcγRIII affinity and cytotoxic potency of antibody. For example, antitumor antibodies were unable to arrest tumor growth in FcγRIII-deficient mice (Clynes et al., 2000). A number of studies have documented a correlation between the clinical efficacy of anti-CD20 in humans and the allotype of their FcγRIIIA (Cartron et al., 2002; Weng & Levy, 2003).

The above data demonstrate that FcγR-dependent mechanisms substantially contribute to the action of cytotoxic antibodies and the clearance of target cells, but fail to answer the key question concerning the mechanism of AMIS: whether a fast clearance of the antigen can, by itself, make it possible to escape from the immunological surveillance and cause a temporal non-responsiveness to this antigen. On the one hand, two different processes such as an accelerated removal through the liver of the D+ red cells by natural IgM anti-A or anti-B antibodies and an accelerated removal through the spleen of the D+ red cells by IgG anti-D lead to the same result - the temporal tolerance to the D antigen, and this result indicates the importance of a fast removal of D+ red cells from the circulation. That is why the ability to interact with FcγRIIIA and induce a fast clearance of D+ red cells *in vivo* are the main features that should be taken into account during anti-D monoclonal antibodies selection. On the other hand, it was observed that the immune response could be suppressed if anti-D was given as late as two weeks after D+ red cells entered the circulation (Contreras, 1998), and that the initial rate of the red cells clearance did not appear to influence the effectiveness of protection (Kumpel et al., 1995). Our data providing evidence that a fast clearance is not sufficient for inducing immunosuppression will be discussed below (Olovnikova et al., 2000).

3.4 FcγRIIB is a negative regulator of the B cell differentiation and the antibody level

To date, there is a lot of data suggesting that AMIS can be regulated by a special mechanism mediated by the FcγRIIB inhibitory receptor. This is the only FcγR receptor expressed on B

cells, and it plays a major role in the negative feedback regulation of B cell responses (Heyman, 2003; Hjelm et al., 2006).

At the beginning of an immune response, the primary contact with antigen leads to the activation of B cells expressing the specific B-cell receptor (BCR). After the BCR activation, naïve B cells proliferate and differentiate rapidly into IgM-secreting plasma cells or mature after class-switching into IgG-B cells which differentiate into IgG-secreting plasma cells or join the memory B cell compartment (Igarashi et al., 2007; Fournier et al., 2008). The differentiation and expansion of B cells is tightly controlled, thus preventing inadequate levels of circulating antibodies, plasma cells, and memory B cells. The control is ensured through the IgG immune complexes that can bind simultaneously to the BCR and FcγRIIB, leading to the inhibition of the IgG-B cell response (Phillips & Parker, 1984). The cross-linking of FcγRIIB and BCR induces the ITIM-associated recruitment of the phosphatase SHIP (SH2 domain-containing inositol 5′-phosphatase) which dephosphorylates and thus inactivates mediators of the BCR signalling, thereby dampening the B cell activation (Ono et al., 1996). There is evidence that FcγRIIB can control the bone marrow plasma cell persistence also in the absence of BCR triggering (Xiang et al., 2007). The role of inhibitory FcγRIIB in the regulation of the BCR signalling has been convincingly shown by using FcγRIIB-deficient mice (Takai et al., 1996). For example, the mice lacking this receptor display elevated levels of antibodies after immunization with both thymus-dependent and thymus-independent antigens, as well as have increased anaphylactic reactions and more severe symptoms in various models for autoimmunity (Heyman, 2003). This perhaps is related to that FcγRIIB limits the activation of high affinity autoreactive B cells and can influence the activation of dendrite cells through an immune complex-mediated mechanism (Venkatesh et al., 2009). It has also been shown that the only FcγR that is important for the anti-inflammatory activity of IVIG is the inhibitory FcγRIIB. Mice deficient in FcγRIIB no longer respond to the IVIG therapy in models of idiopathic thrombocytopenic purpura, serum transfer arthritis and nephrotoxic nephritis (Aschermann et al., 2010). A lot of evidence has recently appeared in support of the disturbances of the FcγRIIB expression in human autoimmune diseases. FcγRIIB has been shown to be up-regulated on memory B cells in normal humans, but this upregulation is significantly decreased in systemic lupus erythematosus patients (Mackay et al., 2006). Accordingly, there is a decreased FcγRIIB-mediated suppression of the BCR activation in B cells from lupus erythematosus patients (Nashi et al., 2010). Studies of the receptor expression in healthy individuals compared with rheumatoid arthritis patients have demonstrated that rheumatoid arthritis patients have fewer FcγRIIB positive B cells and decreased receptor expressions in contrast to healthy subjects. Their B cells display a significantly increased proliferative response *in vitro*. Interestingly, healthy women have overall lower FcγRIIB expression on B cells than men and it significantly decreases with age. The reduced FcγRIIB expression on B cells in women may account for the increased frequency of autoimmunity in women in comparison to men (Prokopec et al., 2010).

As concerns prevention of the anti-D immune response, the involvement of FcγRIIB in this process still remains unclear. As discussed above, AMIS in FcγRIIB-deficient mice that can be accounted for by the antigen masking does not argue against the engagement of this receptor in the establishment of the antigen-specific immunosuppression. If we assume that the immune antibodies can block B cell maturation and limit their own production through the interaction of immune complexes with FcγRIIB, it follows that the polyclonal anti-D prepared from the plasma of hyperimmune donors should also have this property. Whereas

neither the antigen masking nor the accelerated clearance can explain the protective activity of anti-D immunoglobulin, the mechanism of a selective inactivation of antigen-specific B cells can elucidate all the known experimental facts related to AMIS. This mechanism, although being schematic, can give reasons for why antibodies to the Kell antigen prevent the immune response to the other antigens that are present on the erythrocyte, particularly the D antigen (Woodrow et al., 1975): while BCR specifically recognizes the D antigen, FcγRIIB interacts with the Fc region of the antibody bound to any antigen on the same red cell. Thus the cross-linking of "nonspecific"FcγRIIB and anti-D BCR leads to suppression of the anti-D response. D prophylaxis, in its turn, appears to prevent the synthesis of antibodies of other specificities (Pollack, 1984).

The issue concerning whether a preventive effect of the polyclonal anti-D preparation results from the action of the anti-D antibody fraction or is associated with a nonspecific immunomodulating effect has arisen many times (Petri et al., 1984; Branch et al., 2006). The immunosuppressive and anti-inflammatory effect of a nonspecific intravenous immunoglobulin has been reliably evaluated in many autoimmune diseases (Bayry et al., 2002; Simon & Spath, 2003). Nonetheless, it is not quite justified to expect common mechanisms of anti-D and intravenous immunoglobulin action since intravenous immunoglobulin is administered repeatedly at a high dose (generally 1-2 grams per kg body weight), while anti-D is used as a single dose of 1-2 ml 10% IgG solution, that is, 100-200 mg IgG. The following facts argue in favor of anti-D being the main triggering factor. In the classical experiment that first showed the immunosuppressive effect of anti-D, Rh-negative recipients received red cells sensitized *in vitro* when all other components of the preparation had been removed (Stern et al., 1961). The ability of the monoclonal anti-D to prevent D-sensitization is one more piece of evidence that it is anti-D antibodies that play a key part in suppression of the anti-D immune response (Kumpel et al., 1995).

4. Effector activity of poly- and monoclonal antibodies in ADCC

4.1 Monoclonal anti-D antibodies

Lymphocytes from immune Rh-negative donors are the only source for generation of the antibody-producing cell lines and isolation of genes of anti-D antibodies because the animals conventionally intended for immunization do not respond to the human Rh antigens. Thus, the development of therapeutic anti-D antibodies does not encounter the bioengineering problem of humanization. The lymphoblastoid cell lines producing anti-D antibodies are usually established by the Epstein-Barr virus infection of human B cells (Koskimies, 1980). Epstein-Barr virus is a B-lymphotropic human herpesvirus which initiates the infection of B lymphocytes by binding to CD21, a complement receptor. A scheme of development of the cell lines producing anti-D monoclonal antibodies is as follows. An immune Rh-negative donor is given a booster injection of D+ red cells 7-10 days before blood collection to have a higher yield of the anti-D cell line (Deriugina et al., 1991). Mononuclear cells isolated from the peripheral blood are seeded into 96-well plates, followed by the addition to the medium of the virus and the agent, for example, cyclosporine A suppressing the cytotoxic attack against the virus-infected B-cells. After 2-3 weeks, when the colonies of transformed cells have grown in the wells, the supernatants are tested for the presence of anti-D antibodies in the agglutination tests with D+ red cells for the selection of anti-D producers. Lymphocytes immortalized by the Epstein-Barr virus may be cloned and then can grow for some period of time in the culture without ceasing

antibody secretion; however, for the purposes of stability and a higher yield of antibodies, they are usually fused with the mouse myeloma to derive stable heterohybridomas. Almost all anti-D monoclonal antibodies currently used in immunoserological testing are produced by heterohybridoma cell lines. An alternative approach to obtain anti-D is transfection and production of recombinant antibodies in rodent (Chinese hamster ovary - CHO, rat myeloma -YB2/0) or human (PER.C6) cell lines.

4.2 Characteristic features of poly- and monoclonal antibodies in ADCC

All polyclonal anti-D, either commercial products or individual sera from immune donors, as well as anti-D sera from pregnant women, have except rare instances the ability to mediate hemolysis in ADCC not only through FcγRI but also through FcγRIIIA (Armstrong-Fisher et al., 1995; Hadley et al.,1995). In contrast, only a few monoclonals have this property, which was demonstrated, for example, by a study of the functional activity of monoclonal anti-D IgGs submitted to the Fourth International Workshop on Monoclonal Antibodies against Human Red Blood Cells (Kumpel et al., 2002a). Only 8 out of 64 anti-D were shown to be able to mediate hemolysis in ADCC in the presence of monomeric IgG (ADCC+IG, Fig. 1b), but all of them could promote hemolysis through interaction with FcγRI.

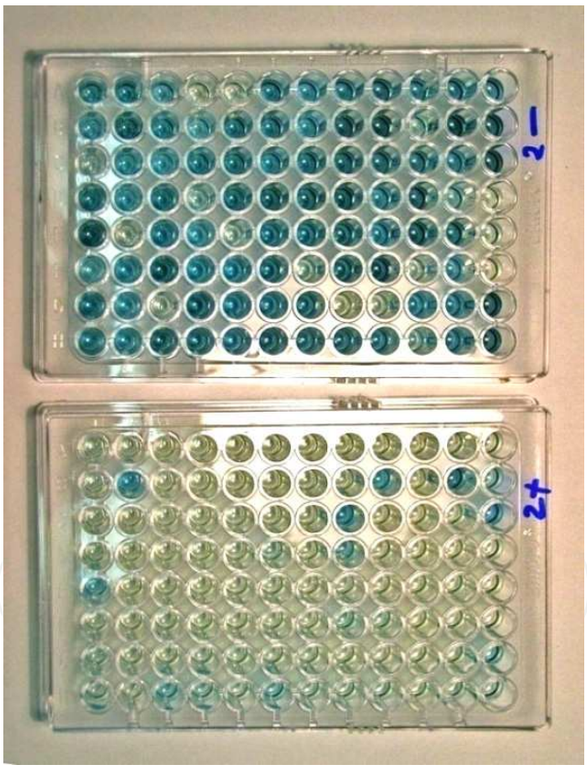

Fig. 2. Parallel testing of supernatants in ADCC (upper plate) and ADCC+IG (lower plate) three weeks after Epstein-Barr virus transformation of lymphocytes from anti-D donor. The effectiveness of hemolysis was estimated according to the concentration of free hemoglobin in the wells. The principle of this colorimetric assay is based on the oxydization of the 2.7-diaminofluorene by the pseudoperoxidase activity of free hemoglobin; the intensity of blue color is proportional to the hemoglobin concentration (Ducrot et al., 1996). Monomeric immunoglobulin is seen to fully or strongly inhibit ADCC (lower plate)

In order to find FcγRIIA / FcγRIIIA-binding antibodies and to evaluate the frequency of their occurrence, we performed ADCC+IG simultaneously with testing of anti-D in supernatants after transformation of lymphocytes by the Epstein-Barr virus (Olovnikova et al., 2006). The required anti-D were very rare: we could not detect them from some donors at all or detected only in 1-2% of wells with the anti-D, although the yield of FcγRI-active anti-D from the cells of the same donors was high (Fig. 2). Similar results were reported by Kumpel: only 5 out of 37 monoclonal anti-D IgG had a hemolytic activity in K-ADCC, and they were all obtained from one donor (Kumpel et al., 1989). A blend of the FcγRIIIA-nonactive monoclonal anti-D antibodies (pseudo-polyclonal anti-D) remained nonactive in ADCC+IG (data not shown). Possible reasons for this inconsistency, i.e., a high activity in ADCC+IG of all polyclonal anti-D antibodies and the absence of the monoclonals with the same hemolytic features, will be discussed in Section 8.
Nevertheless, we have received some lymphoblastoid cell lines that secrete anti-D IgG1 promoting hemolysis in ADCC+IG (Fig. 3) and compared them with anti-D IgG1 binding only FcγRI.

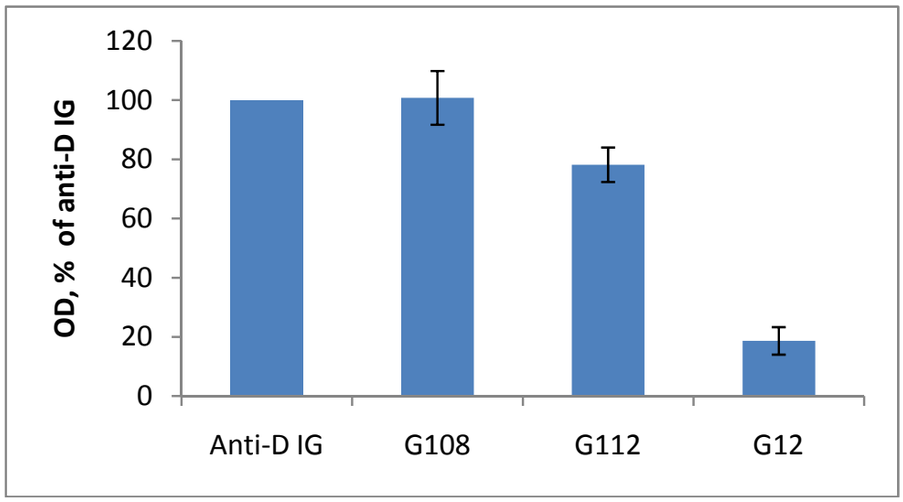

Fig. 3. ADCC+IG mediated by three anti-D IgG1 monoclonal antibodies (G108, G112, G12) produced by lymphoblastoid cell lines or the polyclonal product (anti-D IG). Concentration of anti-D in all the samples was 250 mcg/ml. Y axis: the optical density as percentage of the activity of polyclonal antibodies

Anti-D	Light chain	Isotype	Producing cell line	Activity in ADCC+IG (% of anti-D IG)	Sequencing of Fc
G108	κ	IgG1	Lymphoblastoid	101	IGHG1*03
G112	κ	IgG1	Lymphoblastoid	78	IGHG1*03
G12	κ	IgG1	Lymphoblastoid	19	IGHG1*03

Table 2. Structural and functional properties of anti-D IgG1 monoclonal antibodies
IGHG1*03 is the IgG1 allotype

The analysis of the primary sequence of genes of anti-D monoclonal antibodies with different activity in ADCC+IG did not reveal any differences in their Fc fragments (Olovnikova et al., 2009). Table 2 represents the characteristics of the three IgG1 anti-D having a different effector activity and obtained from lymphocytes of a single donor. We also did not find a correlation between the functional properties and the epitope specificity in the analysis of a set of IgG1 anti-D (data not shown).

5. Glycosylation of IgG. Ways of modifying the effector properties of antibodies

Interaction with the FcγR on an effector cell is the prerogative of the Fc fragment of IgG. IgG is a glycopeptide; it is known that oligosaccharide bound to the Fc fragment of an immunoglobulin molecule through Asn^{297} influences pharmacokinetics and plays an important role in the interaction of antibodies with the Fc receptors on effector cells (Raju, 2008), although the sugar does not, by itself, directly contact the receptor (Radaev & Sun, 2001). Aglycosylated antibodies lose the possibility to interact with Fc receptors (Nose & Wigzell, 1983; Walker et al., 1989). The largest sugar chain of a human IgG is shown in Fig. 4. About 25% of the sugar chains are sialylated; the high heterogeneity of neutral glycans is produced by the presence or absence of the two terminal galactoses, the bisecting N-acetylglucosamin and the fucose residue. Despite this high multiplicity, the molar ratio of each oligosaccharide in IgG of a healthy individual is quite constant (Kobata, 1990) but can vary widely in different diseases: the rheumatoid arthritis, heavy-chain deposition disease, multiple myeloma (Furukawa & Kobata, 1991; Omtvedt et al., 2006).

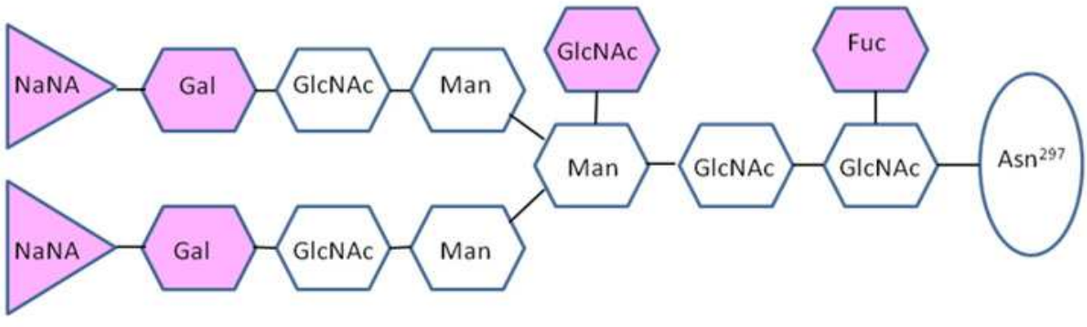

Fig. 4. The structure of the Asn ^{297}-bound oligosaccharide of human IgG
NaNA -- N-acetylneuraminic acid; Gal - galactose; GlcNAc – N-acetylglucosamine;
Man - mannose; Fuc - fucose; Asn^{297} - asparagine of the Fc fragment of IgG
Neutral sugars do not contain sialic acids (NaNA); G2, G1 and G0 are neutral sugars with two, one and without terminal galactose. Carbohydrate residues that can be absent are color-marked

The structure of glycans that are synthesized in cells of various species of rodents has well been investigated due to a wide usage of rodent cell lines in the biopharmaceutical industry to produce recombinant proteins (Hossler et al., 2009). The glycosylation machinery of the mouse cells is dominant in heterohybridomas obtained by the fusion of antibody-producing human cells with myelomas; human IgGs from heterohybridomas contain monoantennary

complex-type and high mannose-type oligosaccharides which have never been detected in human serum IgGs (Tandai et al., 1991). In hamster cells, as well as in murine cells, antibodies contain N-Glycolylneuraminic acid or some types of glycan that are not normally found, or found at low levels, in human IgG and can be immunogenic for humans (Hossler et al., 2009). For example, mannose can be bound by the circulating mannose receptor and recognized as being foreign by natural killer and macrophage cells (Rademacher, 1993).

At present, it is the carbohydrate moiety of IgG that is the main target for the modulation of the antibody effector properties. Numerous studies have provided evidence that fucose has a significant impact on the ability of monoclonal antibodies to interact with FcγRIIIA; defucosylated antibodies display an enhanced ADCC independent of the polymorphism of FcγRIIIA compared with their fucosylated counterparts (Shields et al., 2002; Shinkawa et al., 2003; Niwa et al., 2004). It has been shown that the antigenic density required to induce an efficient ADCC is lower for the low-fucose IgG1 as compared to a highly fucosylated antibodies (Niwa et al., 2005). This field of engineering is being actively elaborated due to the fact that it is the cytotoxic activity that underlies the action of many anti-cancer monoclonal antibodies. Antibodies with low fucose can be produced by the cell lines with a reduced fucosylation activity such as rat myeloma YB2/0 cells or the CHO variant cell line, Lec13 (Shields et al., 2002; Shinkawa et al., 2003). New host cell lines which produce completely defucosylated antibodies with enhanced effector functions were generated by knockout of the fucosyltransferase gene (Yamane-Ohnuki et al., 2004; Kanda et al., 2006). A number of other approaches to improve the FcγRIIIA affinity are being developed: for example, human IgG1 bearing immature oligomannose-type glycans also display an increased ADCC (Crispin et al., 2009).

An example of one anti-D given below shows a considerable magnitude at which the functional activity of its counterparts can vary both in different host cells and in the presence of the substances affecting the pattern of glycosylation. G12, anti-D IgG1, had a low activity in ADCC+IG when produced by the human lymphoblastoid cells (Fig. 5). Lymphoblastoid cells were fused with the mouse myeloma X63.Ag8.653 or the rat myeloma YB2/0; recombinant G12 were expressed in the retinal human cell line PER.C6® (Jones et al., 2003). Our special goal was to test the applicability of non-lymphoid human cell line PER.C6® (Olovnikova et al., in press) since the majority of industrial host cell lines have rodents cells origin.

Fig. 5 shows that the G12 expression in human-mouse heterohybridoma as well as in PER.C6® did not affect the activity in ADCC+IG, but the fusion with YB2/0 dramatically changed G12 properties: an inactive G12/LBL became highly active under the influence of the rat myeloma cells (Olovnikova et al., 2009). The same effect was attained by adding kifunensine to the G12/PER.C6® cell culture. Kifunensine is a potent inhibitor of α-mannosidase I, which leads to the synthesis of non-fucosylated oligomannose-type glycans (Zhou et al., 2008). This type of glycosylation totally transformed G12/PER.C6®, providing them with improved ability to trigger ADCC via low-affinity receptors. The effect of kifunensine can be explained by the two factors: the absence of fucose and a high content of olygomannoses that also enhances the affinity of the Fc fragment for FcγRIII (Raju, 2008; Zhou et al., 2008). However, a similar effect of the YB2/0 myeloma indirectly suggests the crucial contribution made by fucose.

Obviously, it is reasonable to increase the affinity of anti-tumor antibodies to FcγRIIIA since a high cytotoxicity of antibodies leads to a better anti-tumor effect. However, D antibodies

are intended for another purpose. The tendency to improve FcγRIII binding is explained not only by our desire to achieve the fastest possible clearance of red cells, but also by the hope that the binding of other low-affinity receptors, FcγRIIB including, will also be enhanced. There is sufficient evidence in support of this correlation. Thus, low fucosylated anti-D produced by YB2/0 binds strongly to both activating FcγRIII and inhibitory FcγRIIB, as opposed to its highly fucosylated counterpart produced by CHO (Siberil et al., 2006). Fc variants of anti-tumor MoAb trastuzumab with the greatest enhancements in the FcγRIIIA affinity also significantly increased binding to FcγRIIB (Lazar et al., 2006).

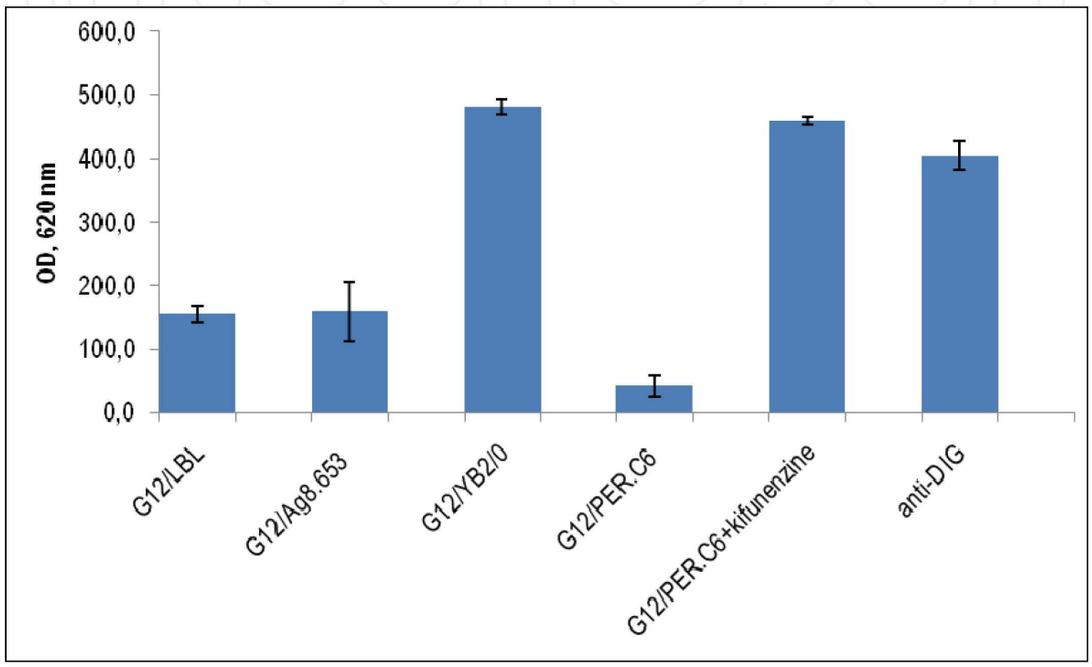

Fig. 5. The effect of host cells and metabolic modulation on the hemolytic activity of anti-D G12 in the ADCC+IG

Y axis: optical density at 620 nm; the concentration of all anti-D is 100 mcg /ml. G12/LBL – G12 from lymphoblastoid B-cell line; G12/Ag8.653 and G12/YB2/0 – G12 from corresponding heterhybridomas; G12/PER.C6® – recombinant G12 from PER.C6®; G12/PER.C6® + kifunensine - recombinant G12 from PER.C6 ®with 1 mcg/ml of kifunensine in culture medium; anti-D IG – anti-D immunoglobulin

We have shown that a pattern of interaction with FcγRs of low-fucosylated G12/YB2/0 was different from polyclonal anti-D and the G108 produced by lymphoblastoid cells. Polyclonal anti-D and the G108/LBL utilized both FcγRIIA and FcγRIIIA in ADCC+IG. The hemolytic activity of G12/YB2/0 in ADCC+IG was predominantly mediated through FcγRIIIA; the low fucose content had a negative effect on the affinity to FcγRIIA (Fig. 6).

Despite a high affinity for FcγRIIIA, anti-D/YB2/0 and anti-D/PER.C6 ®synthesized in the presence of kifunensine have an uncommon for human IgG oligosaccharide structure: anti-D /YB2/0 may contain rodent IgG sugar moieties whereas the cultivation with kifunensine leads to the synthesis of oligomannoses-type glycans. Such structures can be recognized in the organism not only by FcγRs, but also by the receptors of the innate immunity, which can lead to immunization, rather than immunosuppression.

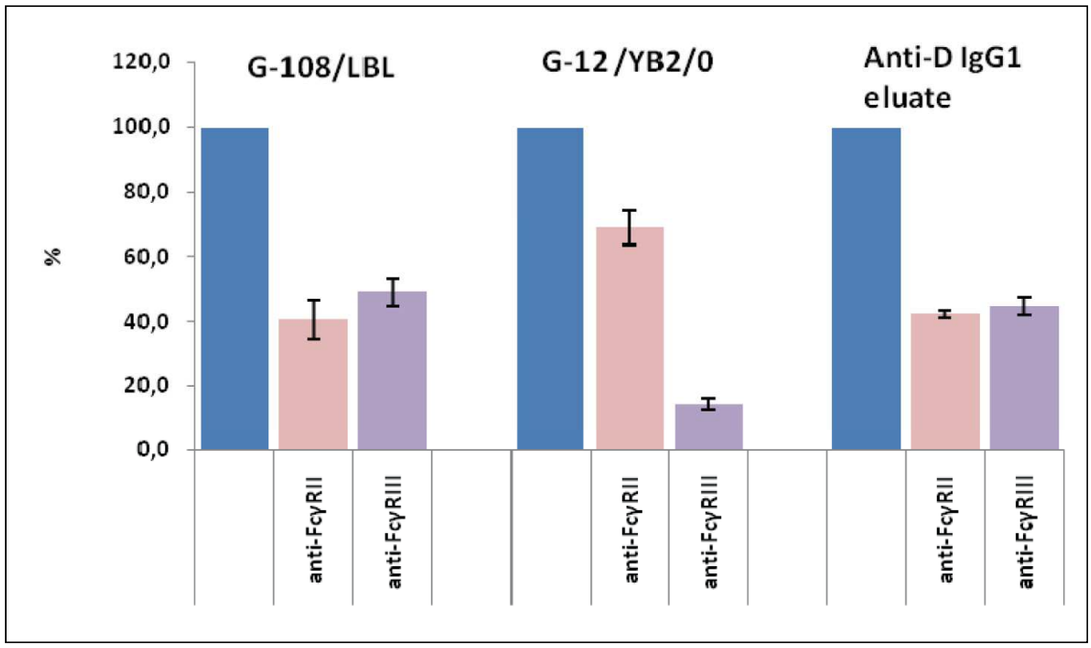

Fig. 6. ADCC+IG with the blocking antibodies to CD16 (FcγRII) and CD32 (FcγRIII) (Dako, Denmark). The activity of each anti-D in ADCC+IG (the first column) is taken as 100%. The second and third columns show the contribution of FcγRIII and FcγRII to ADCC+IG. Purified polyclonal anti-D IgG antibodies were prepared by adsorption of anti-D immunoglobulin on D+ red cells, followed by elution and isolation on Protein A. This control polyclonal preparation containing predominantly anti-D IgG1 is more adequate than total anti-D immunoglobulin in which the fraction of anti-D antibodies is about 0,1% (1 dose of anti-D is usually 1-2 ml of 10% IgG solution containing 200-300 mcg of anti-D antibodies). The concentration of each anti-D was 250 mcg/ml

6. The analysis of the glycan structure of anti-D antibodies

Comparison of the four anti-D IgG1, produced by human lymphoblastoid cell lines and having a different activity in ADCC+IG (G12 and G17 with a low activity, G112 and G108 with an intermediate and high activity, correspondingly), was performed to estimate whether the hemolytic activity correlates with the peculiarities of the composition of sugars (Table 3).

Earlier studies reported about the correlation between the ADCC activity and sialylation of monoclonal anti-D (Kumpel et al., 1994). Sialic acids were shown to have negative effect on the affinity of Fc fragments for FcγRIII (Scallon et al., 2007). However, we found no such connection. Apparently, both inactive and active monoclonal and purified polyclonal anti-D antibodies are almost equally sialylated (Table 3). Study of sugar moieties of the four anti-D/LBLs did not reveal any significant differences between the structures of FcγRIIIA-active and FcγRIIIA-nonactive anti-D. Table 3 allows comparison of glycosylation of the two anti-D polyclonal antibody products. One can see that the content of sialylated sugars and the neutral G2F form in anti-D IgG eluate is higher than in the total anti-D IG, while the G0F fraction is on the contrary sufficiently higher in the total anti-D IG. It is notable that the content of non-fucosylated neutral sugars in the eluate is higher than in other preparations:

9.2% vs. 2.6% in anti-D IG. As concerns glycosylation of IgG1 in human PER.C6® cells, there is a significant deviation in its profile in relation to a normal range of IgG glycans. An example of G12/PER.C6® shows that PER.C6® produces antibodies without sialic acids and completely fucosylated. This structure of glycan transformed FcγRIIIA-active anti-D G108/LBL into FcγRIIIA-inactive G108/PER.C6® (data not shown), despite the absence of sialic acids, which again points out the key role of fucose.

		G12/ PER.C6®	G12/ LBL	G17/ LBL	G112/ LBL	G108/ LBL	Anti-D IG	Anti-D IgG eluate
ADCC+IG		low	low	low	Inter-mediate	high	high	high
Fucosylated	G2F	5,7*	41,1	28,7	41,3	33,1	24,0	31
	G1F	29,1	13,0	26,2	21,8	27,1	35,0	20
	G0F	60,9	2,0	4,9	2,2	4,5	20,0	5,6
	total	95,7	56,1	59,8	65,3	64,7	79	56,6
Not fucosylated	G2	0,0	1,4	2,5	1,7	2,4	1,5	7,3
	G1	0,5	0,6	0,8	0,6	1,3	0,8	1,2
	G0	0,2	0,2	0,0	0,0	0,8	0,3	0,7
	total	0,7	2,2	3,3	2,3	4,5	2,6	9,2
Sialylated**		0,5	28,8	34,4	25,7	24,6	15	28
Unassigned		3,1	12,9	2,5	6,7	6,2	3,4	6,2

Table 3. Oligosaccharides of mono- and polyclonal D antibodies
The samples were analyzed following the SOP "AA Fluorescent N-linked Oligosaccharide Profiling of Neutral Monosaccharides using an Aqueous Chromatographic Separation;
* In % of all glycans;
** The structure of sialylated glycans was not analyzed.

Interestingly, none of the cloned lines secretes a really monoclonal antibody in terms of glycosylation: each monoclonal anti-D demonstrates a wide range of glycoforms (Table 3).

7. What *in vivo* investigations of monoclonal and recombinant anti-D antibodies have shown

Study of immunosuppressive properties of monoclonal and recombinant anti-D antibodies was carried out following evaluations of their safety, pharmacokinetics, as well as the ability to accelerate the clearance of sensitized red cells (Thomson et al., 1990; Blancher et al., 1993; Goodrick et al., 1994; Bichler et al., 2004). To date, over ten anti-D IgG1 and two IgG3 of different origin (lymphoblastoid cell lines, human-mouse heterohybridomas, CHO, YB2/0) have been tested on volunteers. Kumpel in the detailed reviews reported on the results of the research into anti-D monoclonal antibodies on volunteers (Kumpel, 2007; 2008). Although the design of testing varied in details in different laboratories, its general outline was as follows. After the administration of 1-10 ml D+ red cells to a Rh-negative recipient, a blood sample is taken to measure the initial content of D+ red cells. Following intravenous or intramuscular injection of the anti-D antibody, blood samples are collected every few

hours during the first three days. The level of residual radioactivity, taking the initial sample as 100%, is evaluated when using red blood cells labeled with a radioactive isotope; in the case of injection of native unlabeled red cells, a number of D+ red cells is directly calculated using flow cytometry after labeling red cells with antibodies conjugated with fluorescein isothiocyanate. These data allow researchers to estimate the effect of anti-D on the clearance of D+ red cells, as well as the number of anti-D molecules bound to the D+ red cells if flow cytometry is used. Subsequent blood samples are regularly taken approximately every 2 weeks for the period of time up to 6 months in order to find the immune anti-D antibodies and to detect the time of their appearance, their class and titer. The boost, that is, unprotected red cells challenge, is eventually given no earlier than after 6 months to determine the state of sensitization in those subjects in whom antibodies were not detected: the secondary-type immune response indicates a state of sensitization and non-effectiveness of anti-D prophylaxis. Although the studies on volunteers have left many questions unanswered, they permitted detection of some features of monoclonal anti-D antibodies that were impossible to predict neither theoretically nor based on their properties in tests *in vitro*.

1. Although acceleration of the clearance is the most distinctive characteristic of the polyclonal anti-D, and designers of anti-D preparations initially tried to get just this property, a fast clearance is not the only requirement for the prevention of anti-D sensitization. Our study of the preventive activity of the four monoclonal IgG1 anti-D antibodies of the human-mouse heterohybridoma origin and the blends of these anti-Ds has found that the clearance of D+ red cells in the group of recipients given a blend of G7 and G12 anti-Ds was as fast as that in the group given polyclonal anti-D IG. Nonetheless, all the subjects treated with the G7 + G12 blend rapidly became D immunized (Olovnikova et al., 2000).
2. Acceleration of the red cell clearance usually directly correlates with the ability of antibodies to mediate ADCC through FcγRIIIA. This rule may have exceptions: our anti-Ds G7 and G12 that caused an effective clearance of D+ red cells had a low activity in ADCC + IG.
3. In the groups of volunteers given anti-Ds produced by human-mouse heterohybridomas, a percentage of sensitized subjects was higher than in the control unprotected group, irrespective of the rate of clearance of the red cells injected. Moreover, unusually for the anti-D response, most of the responders formed IgM anti-D and developed them more rapidly than would be expected after red cells alone. In other words, monoclonal anti-Ds had an adjuvant effect enhancing the immune response instead of suppressing it (Olovnikova et al., 2000; Beliard, 2006). It seems quite reasonable to agree with the opinion that monoclonals of the heterohybridoma origin containing foreign glycans may be recognized by Toll-like receptors of the innate immunity and direct the immune response in the pro-inflammatory way that stimulates the antibody production (Kumpel, 2007). These receptors do not participate in the destruction of the red cells sensitized by polyclonal anti-D or anti-D from human lymphoblastoid cells. In light of this assumption, a high *in vitro* and *in vivo* hemolytic activity of low-fucosylated anti-Ds secreted by rodent cells does not ensure their immunosuppressive effectiveness. Clinical trials of anti-D expressed in the YB2/0 myeloma that were announced in France are to give an answer to this question (Urbain et al., 2009).
4. Only anti-Ds produced by human lymphoblastoid lines that are able to mediate hemolysis via FcγRIIIA in ADCC showed a high protective activity among any of the

monoclonal preparations tested *in vivo*. Their immunosuppressive effectiveness was the same as that of polyclonal antibodies, and the absence of anti-D response in a large percentage of subjects after the next unprotected immunization indicated a long-term character of the anti-D suppression (Kumpel, 2002b).

5. There is no reliable answer to the question of which class of anti-D, IgG1 or IgG3, is more effective and whether it is essential to mix monoclonal antibodies of different classes. The experimental data did not allow conclusions about the benefits of the IgG1 + IgG3 mixture containing antibodies in a physiological proportion compared with a single IgG1 (Kumpel et al., 1995, 2002b).
6. The question of whether the oligoclonal mixture of antibodies of the same class can be more effective than a single antibody still remains unanswered. The first pseudo-polyclonal preparation Rozrolimupab that comprises 25 recombinant unique IgG1 antibodies expressed in CHO cells (Symphogen) is being investigated for the prevention of hemolytic disease of the fetus and newborn and for the treatment of idiopathic thrombocytopenic purpura (Stasi, 2010).
7. We lack a sustainable cell line that can stably grow in the culture and give a high-yield output of rightly glycosylated antibodies. Although some lymphoblastoid lines secrete the "right" antibodies, their unstable growth make them inadequate to allow a large-scale production.
8. As concerns a polyclonal anti-D preparation, it is still unclear whether it is a fraction of anti-D antibodies that is responsible for its immunosuppressive impact, or it is due to a cumulative effect of anti-Ds with different FcγR affinity.

8. Differential glycosylation of antibodies produced by different subpopulations of B cells as a mechanism for regulating the humoral immune response: A hypothesis

The amount of antibodies formed to a particular antigen can not rise for a limitlessly long period of time; in general, it appears to be restricted over a wide range of antigen dosage and a variety of immunization regimens and as a rule reaches a predictable maximal level, despite continued immunization (Uhr & Möller, 1968). The regulation of the antibody synthesis is assumed to be consistent with the feedback principle, and the ability of antibodies to enhance or suppress the immune response reflects this physiological mechanism. An insightful hypothesis of the mechanism for anti-D AMIS was proposed by Gorman and Pollack (Pollack, 1984). The authors speculated that, in particular, in the presence of early IgM antibodies the formation of IgM - antigen complexes stimulates the committed B cells, whereas after the switching to IgG production, the formation of IgG – antigen complexes limits further expansion of the plasma cell clones.

A primary contact with an antigen leads to the formation of Ab-secreting plasmablasts with a lifespan of less than one week and results in short-term Ab responses, but most Ab-secreting cells are generated during the secondary immune response (Smith et al., 1996, Odendahl et al., 2005). As a result of the secondary contact with the antigen, memory B cells undergo a massive expansion and differentiation toward short-lived plasma cells. Some plasma cells become long-lived if rescued in available niches such as bone marrow. These cells can survive, continue to secrete antibodies and sustain serum antibody levels for extended periods of time (> 1 year) even in the absence of any detectable memory B cells (Slifka et al., 1998; Bernasconi et al., 2002). Overall, kinetics of the antibody concentration in

serum is defined by the two factors: a rapid increase of the titer after boosting is associated with the activity of short-lived plasma cells, whereas a population of long-lived plasma cells insensitive to the antigen maintains a level of high affinity antibodies for an extended period of time (Manz et al., 1998; Manz et al., 2005).

The role of inhibitory FcγRIIB in the regulation of proliferation and differentiation of B cells into plasma cells, antibody affinity maturation and plasma cell numbers has already been discussed in Section 2.4. Given all findings, one can suggest that long-lived plasma cells produce antibodies with immunosuppressive properties that can inhibit the activation and differentiation of B cells through FcγRIIB. First, anti-inflammatory antibodies can serve to arrest naïve B cells, i.e., to suppress the activation of the primary immune response upon the secondary contact with the antigen. This inhibition prevents secretion of low-affinity antibodies, thus directing the immune response along the way of a rapid and effective secondary (memory) response, which allows production of high-affinity antibodies. It is unlikely that suppression of the primary response requires high titers of immunosuppressive antibodies. Second, after a pool of long-lived plasma cells has already formed, inhibitory properties of the antibodies which they produce make it possible to restrict the activation of antigen-specific memory B cells and overproduction of antibodies in the case of the next antigen stimulus. However, this process should take place only against the background of a high level of corresponding antibodies. This is why the anti-D polyclonal immunoglobulin prepared from the plasma of repeatedly immunized donors with high-titer anti-D contains antibodies that, in the first place, are capable of suppressing effectively the primary immune response.

In contrast, short-lived plasma cells which are generated at the stage of accelerating the immune response should not produce antibodies capable of interacting with FcγRIIB and inhibiting proliferation of B cells until a pool of long-lived plasma cells and that of memory B cells have formed. Obviously, it is this regularity that we face when receiving anti-D producers by viral transformation of lymphocytes. As mentioned above, when we tried to receive anti-D IgGs having the same pattern of interaction with FcγR as polyclonal antibodies, we found that only rare monoclonal anti-D antibodies had the capability of utilizing the low-affinity FcγRs. The Epstein-Barr virus, a B-lymphotropic human herpesvirus, infects B cells, including memory B cells, by using CD21 as a receptor (Cooper et al., 1988) and drives them out of the resting state to become activated proliferating lymphoblasts that produce and secrete Ig of any isotype (Miyawaki et al., 1988; Rickinson & Lane, 2000; Thorley-Lawson, 2001). The Epstein-Barr virus infection deregulates multiple differentiation factors and processes in B cells, promoting their growth and differentiation towards plasma cells (Miyawaki et al., 1991; Siemer et al., 2008). However, the process of differentiation fails to proceed to the terminal stage, to the plasma cell, thereby yielding cell lines with an immature "lymphoblastoid" phenotype; all virus-transformed cell lines that we obtained were CD19 +, CD20 +, CD38 +, $Ig^{membrane}$ +. It seems that this is the answer to the question why monoclonal antibodies with the properties of polyclonal antibodies can be so rarely received: because serum polyclonal antibodies and monoclonal antibodies are produced by different B cell subsets, namely, serum anti-Ds by long-lived plasma cells, and monoclonal anti-Ds by B-lymphoblasts. Low affinity for FcγRIIIA of anti-D antibodies produced by virus-transformed peripheral B cells of an immune donor, and a high affinity of polyclonal antibodies from the same donor indicate that the effector activity of the antibodies produced by different subpopulations of B lymphocytes is different (no data suggesting that the Epstein-Barr virus in itself may alter the pattern of glycosylation are available).

The question of when a pool of the future long-lived plasma cells is generated and what factors are responsible for this branch point in the B cell differentiation still remains unanswered. Does this choice happen at the level of the memory B cell or does any B cell, once occurring in the right environment, trigger this program? Perhaps, the frequency of the clones secreting a monoclonal anti-D with the properties of polyclonal antibodies reflects the frequency of "pre-long-lived" plasma cells in the peripheral blood when migrating after the antigen boosting.

The way of regulating the immunomodulatory properties of antibodies may include some variations in their glycosylation that is defined by a set of glycosyltransferases expressing in the cells of different stages of the B cell ontogeny, in particular, in short-lived plasma cells and long-lived plasma cells. It is well known that the molar ratio of each glycoform of IgG from the sera of healthy individuals is quite constant (Kobata, 1990). It was suggested that a ratio of different clones of B cells which are equipped with different sets of glycosyltransferases is relatively constant in healthy individuals (Kobata, 1990; 2008). In light of the hypothesis proposed here, the constant ratio of IgG olygosaccharides from the sera may be explained by the programmed different patterns of IgG glycosylation in B cells at different developmental stages. The ratio of all the types of cells in the organism is approximately constant, which implies a stable ratio of IgG glycoforms. Moreover, B cell clones do not produce a monoclonal IgG with a unique sugar moiety, but with a wide range of olygosaccharides whose ratio is close to that of IgG glycoforms of a normal serum (Table 3).The question of whether glycosylation can be intended for regulation of the immunity is currently being discussed in the literature. For example, IgGs have been shown to acquire anti-inflammatory properties upon Fc sialylation (Kaneco et al., 2006); however, it is non-specific mechanisms that are likely to underlie their anti-inflammatory effect (Medzhitov & Janeway, 2002; Anthony & Ravetch, 2010). The anti-D response may serve as a model of how the antigen-specific feedback regulation works.

9. Conclusion

The two observations that had led to the application of anti-D antibodies for preventing D sensitization were: 1) a lower probability of D sensitization of an Rh-negative mother after the delivery of the ABO incompatible infant, and 2) the antigen-specific immunosuppressive effect of the antibodies injected simultaneously with the corresponding antigen. Paradoxically, none of these mechanisms is likely to play a crucial part in the anti-D immunoglobulin action. 1) Red cells coated with natural antibodies against A and B blood groups may be preferentially caught in the liver, where they are less likely to stimulate an immune response against the Rh antigens, whereas D+ red cells coated with anti-D antibodies are withdrawn through the spleen, i.e., through the organ of an active immune response. In addition, a fast clearance of the antigen from circulation did not appear to be a sufficient condition for preventing immunization. 2) Classical AMIS at which antibodies are abundant and saturate all antigen determinants may be accounted for by the antigen camouflage. However, this mechanism is inadequate in the case of the anti-D immune suppression since binding only a small portion of D sites is known to be sufficient for the immunosuppressive effect of the injected antibodies. Overall, the mechanism for an antibody-mediated suppression of the anti-D immune response by down-regulation of the specific B cells seems most relevant.

One can assume that anti-D antibodies secreted by long-lived plasma cells should have immunosuppressive properties to provide down-regulation of the immune response. When a pool of long-lived plasma cells has been formed, and the organism is sure to have a reliable protection by a sustainable production of high affinity antibodies, the immunosuppressive antibodies that have reached a threshold concentration begin to suppress the maturation of naïve and memory B cells following administration of the antigen. These protective measures are needed to prevent secretion of low-affinity antibodies of the primary response after repeated immunizations and avoid overproduction of the plasma cells of this specificity as the number of niches for long-lived plasma cells in the bone marrow is limited. A likely distinctive characteristic of antibodies with immunosuppressive properties is a unique pattern of glycosylation that provides opportunities for the interaction with low-affinity receptors. Thus, immunosuppressive anti-Ds are capable of binding both to an inhibitory FcγRIIB and an activating FcγRIIIA, which provides an effective clearance of sensitized D+ red cells. The proposed hypothesis explains the mechanism of a high efficiency of the polyclonal anti-D immunoglobulin derived from the sera of immune donors with a high titer of anti-D antibodies in the prevention of the primary anti-D response, as well as their low efficiency in the case of the secondary response.

The situation is different at the initial, accelerating, stage of the immune response when the antibodies produced by short-lived plasma cells should not suppress the immune response but instead enhance it. Apparently, we observed these antibodies when transforming the B cells derived from peripheral blood by the Epstein-Barr virus. The virus infects CD21+ B cells, thus evoking in them the process of differentiation towards plasma cells; however, a phenotype of the lymphoblastoid lines derived indicates a non-terminated process of differentiation. Analysis of a huge set of anti-D monoclonal antibodies has shown that the lymphoblastoid lines derived from peripheral B cells of large numbers of donors only rarely secrete antibodies that are able to interact with low-affinity receptors in contrast to the polyclonal antibodies simultaneously collected from the same donors.

Nevertheless, viral transformation sometimes makes it possible to obtain the lines secreting anti-D monoclonal antibodies with the properties of polyclonal antibodies. Further detailed analysis of their biochemical structure will, undoubtedly, provide information about peculiarities of their composition that make them different from inactive ones. We have found no differences in the primary nucleotide sequences of genes encoding for Fc fragments of active and non-active anti-Ds, which indicates the effect of posttranslational modifications on the functional properties of antibodies. It is known that the mode of glycosylation is associated with the level of affinity of the antibody Fc fragment for FcγRIIIA. We have also shown that the FcγR binding activity of antibodies can be significantly modified through their expression in different producer lines, and, furthermore, it is possible to transform any non-active anti-D into a highly active one *in vitro*. However, it is important to note that a structure of sugar should not be foreign, or, otherwise, antibodies may evoke stimulation rather than immunosuppression of the immune response. Such adjuvant-like effect was observed when we treated volunteers with monoclonal anti-Ds produced by human-mouse heterohybridomas.

Nevertheless, the answer to the question raised in the headline of this paper should, undoubtedly, be positive. Thus, the development of a relevant producer cell line that maintains the "right" glycosylation of antibodies is the most important challenge at present. It is already clear that traditional producers such as mouse myelomas or CHO cells are

unlikely to become a source of efficient anti-D preparations. A human myeloma with defective fucosylation could be potentially attractive for the production of not only anti-D but also other therapeutic antibodies. Another approach may utilize a genetic engineering tuning of any lymphoblastoid cell line secreting anti-D IgG. This process should involve immortalization of the cell line, for example, with the help of hTERT (human telomerase reverse transcriptase) and regulation of the expression of glycosyltransferases, which is presently possible. The development of a monoclonal anti-D product not only will make it possible to replace a serum anti-D but will also serve a model for inducing an antibody-mediated immunosuppression at autoimmune diseases. The history of attempts to create a biotechnological anti-D immunoglobulin that would replace serum preparations is, on the one hand, the way of disappointments and failures; on the other hand, it is an example of how our approaches to solve this complex and fascinating task with so many unknowns have been evolving while new information becomes available in this area.

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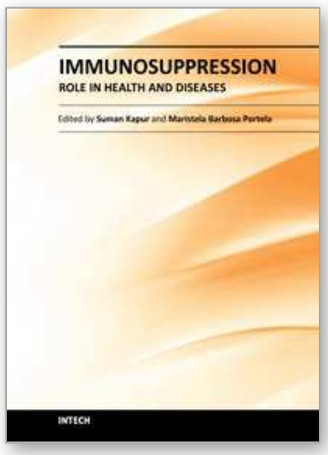

Immunosuppression - Role in Health and Diseases

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A need for a book on immunology which primarily focuses on the needs of medical and clinical research students was recognized. This book, "Immunosuppression - Role in Health and Diseases" is relatively short and contains topics relevant to the understanding of human immune system and its role in health and diseases. Immunosuppression involves an act that reduces the activation or efficacy of the immune system. Therapeutic immunosuppression has applications in clinical medicine, ranging from prevention and treatment of organ/bone marrow transplant rejection, management of autoimmune and inflammatory disorders. It brings important developments both in the field of molecular mechanisms involved and active therapeutic approaches employed for immunosuppression in various human disease conditions. There was a need to bring this information together in a single volume, as much of the recent developments are dispersed throughout biomedical literature, largely in specialized journals. This book will serve well the practicing physicians, surgeons and biomedical scientists as it provides an insight into various approaches to immunosuppression and reviews current developments in each area.

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