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Differences between the activities of human monoclonal IgG1 and IgG3 anti-D antibodies of the Rh blood group system in their abilities to mediate effector functions of monocytes

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SUMMARY

Seven IgG1 and seven IgG3 human monoclonal antibodies derived from heterohybridoma or Epstein–Barr virus-transformed lymphocytes and specific for the D antigen of the human Rh blood group system were tested for their ability to bring about red cell attachment to and phagocytosis by monocytes. The antibodies produced by the heterohybridomas were also investigated for their potency to mediate antibody-dependent cellular cytotoxicity (ADCC) by monocytes. When red cells were sensitized with any of the IgG1 anti-D antibodies, most of them were ingested by the phagocytes. By contrast, many of the red cells coated with any of the IgG3 antibodies remained attached to the monocyte surface while only few underwent phagocytosis. Some of the attached red cells remained on the phagocyte exterior for a considerable length of time. The ADCC activities of the IgG3 anti-D antibodies was greater than that of the IgG1 anti-D antibodies. The results mean that *in vitro* IgG1 anti-D mediates red cell destruction mainly by phagocytosis, while IgG3 anti-D causes their destruction predominantly by prolonged cytolysis. These differences between the effector functions of human monoclonal IgG1 and IgG3 anti-D antibodies might have important implications for their use in the prophylaxis of haemolytic disease of the new-born.

INTRODUCTION

The destruction by mononuclear phagocytes of red cells due to non-complement binding IgG antibodies, such as anti-D, has been studied extensively *in vitro*. Initially, the antibody-coated red cells attach to IgG Fc receptors on the phagocyte membrane. Subsequently they are destroyed by either or both of two effector functions expressed by mononuclear phagocytes, namely phagocytosis and cytolysis (Engelfriet *et al.*, 1981). The latter implies red cell lysis outside the effector cells. Phagocytosis of red cells is followed by their rapid digestion (Morita & Perkins, 1965). Degradation products of material engulfed by mononuclear phagocytes escape into the extracellular environment (Cohn, Hirsch & Wiener, 1963).

In vivo there is substantial evidence that anti-D-coated red cells are sequestered mainly in the spleen (Jandl, Jones & Castle 1957; Cutbush & Mollison, 1958; Crome & Mollison, 1964) and that the Fc-portion of the antibody plays an essential part in this process (Von dem Borne, Beckers & Engelfriet, 1977). However, little is known about mechanisms of their destruction on the cellular level.

Most anti-D immunoglobulins belong to the IgG1 and IgG3 subclasses (Natvig & Kunkel, 1968). Several studies with polyclonal (Engelfriet *et al.*, 1981; Douglas, Rowthorne & Schneider, 1985; Zupanska, Thomas & Merry, 1986), as well as with monoclonal (Wiener *et al.*, 1987; Armstrong *et al.*, 1987; B. M. Kumpel, E. Wiener, S. J. Urbaniak, and B. A. Bradley, manuscript submitted for publication) antibodies, have demonstrated that IgG3 anti-D is much more potent than IgG1 anti-D in mediating red cell interaction with mononuclear phagocytes *in vitro*. However, little is known about any differences between the two IgG subclasses in causing ingestion and cytolysis by the effector cells.

Employing monoclonal anti-D antibodies of subclass IgG1 or IgG3 we have shown recently that red cells require 100 times more molecules of IgG1 than of IgG3 to be bound to their surface in order to bring about an *in vitro* interaction with interferon gamma (IFN-γ)-stimulated macrophages (Wiener *et al.*, 1987). In this system, regardless of the IgG subclass, most red cells remain attached to the phagocyte surface while only a few undergo ingestion.

The present study delineates the activities of human monoclonal IgG1 and IgG3 anti-D antibodies in phagocytosis and cytolysis of red cells by monocytes. The results show a marked difference between the two subclasses in their ability to mediate these effector cell functions.

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MATERIALS AND METHODS

Monoclonal anti-D antibodies

Culture supernatants of heterohybridomas prepared according to Melamed *et al.* (1987) and of Epstein–Barr virus (EBV)-transformed human lymphocytes prepared by Kumpel, Poole & Bradley (1988) were employed. The antibodies produced by the heterohybridomas and their functional affinity constants were Fog-1 (1×10^9/M) and Pag-1 (8×10^8/M), both of subclass IgG1; Fog-3 (2×10^9/M) and Gad-2 (7×10^8/M), both of subclass IgG3. The antibodies secreted by the EBV-transformed lymphocytes were CB6-1, 2B6-1, 1A3-1, FC3 and H26 (all of subclass IgG1) and 2B6-3, 1A3-3, 6D10, 6B7 and CB6-3 (all of subclass IgG3).

Anti-D quantification

This was carried out by the conventional Autoanalyzer technique (Gunson & Philips, 1972).

Red blood cells

These were obtained by collecting blood from a single donor of Rh phenotype R_2r into citrate-phosphate-dextrose solution and washing the cells four times in phosphate-buffered saline (PBS).

^{51}Cr labelling of red cells

This was performed by incubating 1×10^9 cells with 50 μCi $Na_2{}^{51}CrO_4$ (specific activity 450 mCi/mg Cr; Amersham International, Amersham, Bucks) for 90 min at 37°. After labelling, the cells were washed twice in PBS.

Sensitization of red cells

This was carried out by mixing 1×10^8 unlabelled or ^{51}Cr-labelled cells with 333 μl antibody culture supernatant and 667 μl 0·3 M glycine, pH 7·4. Red cells added to antibody-free culture medium and glycine served as negative controls. After incubation for 40 min at 37° the cells were washed four times in PBS at 4°.

Monocyte monolayers

These were prepared in Lab-Tek slide chambers (4 chambers/slide) (Miles Scientific, Slough, Berks) or in tissue culture dishes (30 mm diameter) (Sterilin Ltd, Feltham, Middx), as described previously (Wiener & Garner, 1987). Each chamber and dish were seeded with 1×10^6 and 5×10^6 mononuclear cells, respectively. After incubation at 37° for 1 hr, non-adherent cells were removed by washing the monolayers three times with Hank's balanced salt solution (HBSS; Gibco, Paisley, Renfrewshire). The number of monocytes per monolayer was then determined as described previously (Wiener & Garner, 1987). The numbers were usually of the order of 2×10^5 and 1×10^6 in slide chambers and dishes, respectively. Prior to use the monolayers were washed twice in HBSS.

Monocyte-binding (association) assay

This was performed as described previously (Wiener & Garner, 1987). The results were expressed (i) as an associaton index, i.e. the total number of red cells both attached to and ingested by 100 phagocytes; (ii) as an attachment index, i.e. the number of red cells attached to 100 phagocytes; and (iii) as a phagocytic index, i.e. the number of red cells ingested per 100 phagocytes. The indices were corrected for negative controls. All tests were done in quadruplicate.

The fate of monocyte-bound red cells following prolonged incubation

This was determined as follows: red cells sensitized with IgG1 or IgG3 anti-D were interacted with monocytes at a ratio of 100:1, as described under the monocyte association assay. After 1 hr of co-culture, the monocytes were washed four times with 1 ml HBSS to remove free red cells and reincubated in 1 ml of 10% fetal calf serum in RPMI-1640 (both from Gibco, Paisley, Renfrewshire). After 20 hr of incubation the medium was removed and the slides dissociated from the chambers. They were air-dried quickly, fixed in methanol, stained in May–Grunwald–Giemsa and examined under the light microscope. The results were expressed as described previously.

Antibody-dependent cellular cytotoxicity (ADCC) assay

This was carried out by adding 1×10^6 ^{51}Cr-labelled sensitized or non-sensitized red cells in 1 ml of RPMI-1640 containing 10% fetal calf serum to monolayers in culture dishes (red cell:monocyte ratio, 1:1). Spontaneous ^{51}Cr release from the cells was estimated by the addition of the same numbers of red cells to dishes without monocytes. After 20 hr of incubation at 37° in 5% CO_2 the culture supernatants were removed and centrifuged to sediment any intact red cells. Aliquots of the supernatants were assayed for radioactivity in an LKB-Univeral gamma-counter. Maximal lysis of the ^{51}Cr-labelled red cells was achieved by treatment with saponin (final conc. 2·5%, w/v). Test results were expressed as a percentage of the maximal lysis (percentage specific lysis). All tests were performed in triplicate.

RESULTS

The association with monocytes and subsequent fate of red cells sensitized with IgG1 or IgG3 anti-D

Red cells were sensitized with heterohybridoma-derived IgG1 or IgG3 anti-D at different concentrations and tested in the monocyte-association assay at a red cell:monocyte ratio of 100:1. Approximately 50 times more IgG1 anti-D than IgG3 anti-D was required before detectable association could be seen. Moreover, at high anti-D concentrations IgG3 mediated three times more red cell–phagocyte interaction than IgG1 (Fig. 1a). The fate of the monocyte-associated red cells was greatly influenced by the anti-D subclass. The majority of IgG3-sensitized cells remained on the monocyte surface and it was only at high anti-D concentrations that a few cells underwent phagocytosis. Under these conditions some monocytes showed a few attached but no engulfed red cells. In contrast, most of the IgG1-coated red cells were ingested whatever the antibody concentration, while cells attached to the monocyte exterior were almost undetectable (Fig. 1b and c). The ability of these IgG1 and IgG3 antibodies to mediate both attachment and phagocytosis was also investigated at different red cell:monocyte ratios. Whether tested at the ratios of 100:1, 20:1 or 4:1, the majority of IgG3-coated red cells remained on the phagocyte surface, while most of the IgG1 sensitized cells were ingested (Fig. 2). Similar results were obtained when monocytes were interacted with red cells coated with either IgG1 or IgG3 anti-D antibodies produced by EBV-transformed lymphocytes (Fig. 3).

Red cells sensitized with IgG1 (Pag-1 or Fog-1) or IgG3 (Gad-2 or Fog-3) anti-D were added to monocytes. After 9 hr of incubation free red cells were removed. The fate of monocyte-

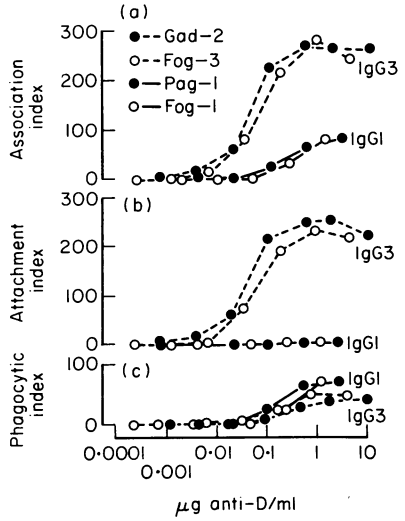

Figure 1. The interaction of red cells sensitized with IgG1 and IgG3 anti-D antibody derived from heterohybridomas; (a) association index, (b) attachment index (c) index; sensitized red cells were added to monocytes at a ratio of 100:1.

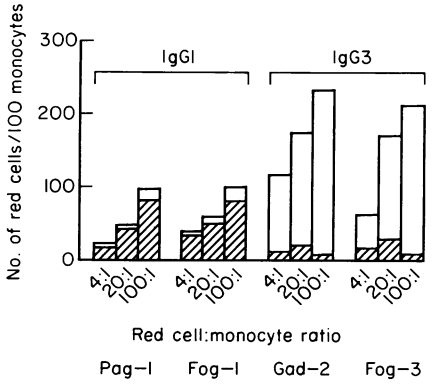

Figure 2. The attachment to and phagocytosis by monocytes of red cells sensitized with IgG1 or IgG3 anti-D derived from heterohybridoma at various red cell:monocyte ratios. Non-shaded area indicates extent of attachment; shaded area indicates extent of phagocytosis. The antibody concentrations in the culture supernatants used for sensitization of red cells, expressed as μg anti-D/ml, were as follows: Pag-1, 2·7; Fog-1, 1·2; Gad-2, 9·1; Fog-3, 3·9.

bound red cells was followed during further incubation. Even after 20 hr of incubation, 10–20% of the IgG3 anti-D-coated red cells originally bound to the monocytes could still be detected on the phagocyte exterior. At this time only approximately 5% of the IgGl anti-D-coated red cells were detected within phagolysosomes and none were visible on the monocyte surface (results not shown).

The destruction by monocytes of red cells sensitized with IgG1 or IgG3 anti-D

Red cell destruction by monocytes due to IgG1 or IgG3 anti-D was measured by an ADCC assay. Considerably less IgG3 anti-D than IgG1 anti-D was required for a similar ADCC activity.

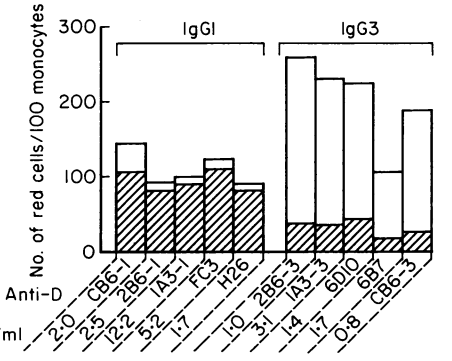

Figure 3. The attchment to and phagocytosis by monocytes of red cells sensitized with IgG1 or IgG3 anti-D derived from EBV-transformed lymphocytes. Sensitized red cells were added to monocytes at a ratio of 100:1. Non-shaded area indicates extent of attachment; shaded area indicates extent of phagocytosis. Values for the anti-D concentrations in the culture supernatants used for sensitization of the red cells are given below each histogram.

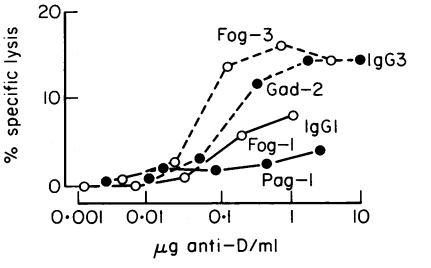

Figure 4. The ADCC activity of IgG1 and IgG3 anti-D derived from heterohybrodomas expressed as a percentage of the maximal possible lysis. Incubation time: 20 hr. Continuous lines, IgG1 antibodies; discontinuous lines, IgG3 antibodies. Abscissa; concentration of anti-D in the culture supernatants used for red cell sensitization.

Moreover, when the anti-D was used at the highest concentrations (1–10 μg/ml) the IgG1 anti-D only resulted in 3–8% lysis, whereas the IgG3 antibody resulted in about 15% lysis (Fig. 4).

DISCUSSION

Results of the present study confirm our earlier observations that human monoclonal IgG3 anti-D antibodies are considerably more potent than those of the IgG1 subclass in their ability to mediate red cell association with mononuclear phagocytes. In our previous investigations using IFN-γ-stimulated monocyte-derived macrophages as effector cells, the majority of IgG1- and IgG3-coated red cells remained attached externally to the phagocytes. This was probably due to the fact that IFN-γ augments the expression of IgG Fc-receptors but fails to stimulate the phagocytic activity of macrophages (Margolick *et al.*, 1986; Wiener & Garner, 1987). In the present experiments we used monocytes freshly obtained from donors and the results showed a considerable diversity in the fate of IgG1 and IgG3 anti-D-coated red cells. This difference was evident when using monoclonal anti-D derived from heterohybridomas as well as from EBV-transformed lymphocytes. The majority of IgG3-sensitized red cells remained on the phagocyte surface and, even

at high anti-D concentrations, relatively few were ingested. By contrast, most of the IgG1-coated red cells underwent phagocytosis, while only a minority were left attached to the monocyte exterior. In the ADCC assay, which measures red cell destruction by phagocytosis as well as by cytolysis of non-ingested cells (Holm & Hammarstrom, 1973, Beelen & Walker, 1984), IgG3 anti-D was more potent and caused greater lysis than IgG1 anti-D. These results imply that IgG3-coated red cells that remained attached to the phagocyte membrane did indeed undergo lysis and that IgG3 anti-D predominantly mediated red cell destruction by cytolysis.

Previous work on the effector functions of monocytes towards IgG anti-D-sensitized targets suggested that the degree of red cell sensitization rather than the IgG subclass was responsible for the relative importance of phagocytosis and cytolysis *in vitro* (Engelfriet *et al.*, 1981). Employing sera containing predominantly polyclonal IgG1 or IgG3 anti-D, these authors demonstrated that with increasing degrees of red cell sensitization phagocytosis becomes more prominent than cytolysis. By contrast, in the present study monoclonal IgG1 anti-D mediated mainly phagocytosis, even at low concentrations. Possibly the IgG1 anti-D serum used by Engelfriet *et al.* (1981) contained traces of IgG3 anti-D that escaped detection by the subclassing reagent but caused cytolysis at low degrees of red cell sensitization. Our results are in line with those of Engelfriet *et al.* (1981) in showing that relatively high concentrations of IgG3 anti-D are required to mediate phagocytosis of red cells by monocytes.

Monoclonal IgG3 anti-D caused more red cell association with monocytes than IgG1 anti-D when used at similar concentrations. It is possible that any ingested IgG3-coated targets were concealed by attached red cells and their number under-estimated. This does not seem to be the case for two reasons. First, even at high IgG3 anti-D concentrations, some monocytes showed no ingested and only a few attached cells which would not have hidden any engulfed targets. Second, the difference in the fate of IgG1 and IgG3 anti-D-coated red cells was evident when similar numbers of targets were associated with monocytes (Figs 1 and 3), and when low red cell: phagocyte ratios were employed (Fig. 2).

Thus the effector functions of monocytes towards anti-D-coated red cells seem to be determined mainly by the IgG subclass of the antibody. We have suggested previously that the greater potency of IgG3 than IgG1 anti-D in mediating red cell binding to mononuclear phagocytes is due to its relatively long hinge, which provides greater accessibility to the Fc-receptor binding site on the Fc-piece (Wiener *et al.*, 1987). During the ingestion of antibody-coated red cells by mononuclear phagocytes, continuous increments in pseudopod formation requires the repeated triggering of Fc-receptors by target-bound IgG molecules (Shaw & Griffin, 1981). This process might be facilitated by the relative proximity between the antigen and Fc-receptor binding sites of the IgG1 molecule (Woof *et al.*, 1986).

The destruction of red cells by monocytes due to IgG3 anti-D appeared to continue over a greater length of time than that mediated by IgG1 anti-D. As late as 20 hr following monocyte binding of IgG3-coated red cells, 10–20% of the targets were still identifiable on the phagocyte surface. At this time no IgG1 sensitized red cells were detected on the monocyte exterior and only a few were visible within phago-lysosomes.

Human monocytes express two classes of IgG Fc-receptors, namely FcR1 and FcR11. There is evidence that only FcR1 is involved in the association with mononuclear phagocytes of human red cells coated with human IgG anti-D (Looney, Abraham & Anderson, 1986). Therefore it is likely that in the present study both phagocytosis and cytolysis of red cells coated with IgG1 and IgG3 anti-D respectively, are mediated by FcR1.

The present finding that IgG3 anti-D mediates mainly prolonged extracellular cytolysis of red cells could have important implications for its possible use in replacing polyclonal anti-D preparations in the prophylaxis of haemolytic disease of the new-born. The action of polyclonal anti-D immunoglobulin to prevent immunization to the D antigen *in vivo* is probably due to its ability to mediate rapid extravascular destruction of D-positive red cells by the mononuclear phagocyte system. If the *in vivo* activity of IgG3 anti-D were the same as that observed *in vitro*, that is, that red cells remain extracellular, then antibody could become disassociated from the antigen and immunogenic red cells released back into the circulation. Such a mechanism would be in keeping with the recent observation of damaged but not destroyed red cells in the blood of a fetus with Rh haemolytic disease (Pollack *et al.*, 1987). In spite of its potency in bringing about the association with macrophages of red cells, the activity of IgG3 anti-D to mediate their destruction mainly externally to the phagocytes might be disadvantageous for its potential use in the prevention of Rh immunization.

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