STUDIES ON ANTIBODY-DEPENDENT CELL-MEDIATED
CYTOTOXICITY RESPONSES IN Rh(D) IMMUNIZATION

by

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Thesis presented for the Degree of Doctor of Philosophy
at the University of Edinburgh in the Faculty of Medicine

1984

TO MY FAMILY

DECLARATION

The work performed for this thesis is original and the thesis has been composed by myself and has not been submitted in any previous application for a higher degree.

The work in section 3.3 was done in collaboration with Dr G R Barclay and Miss M C McCann, with the technical help of Mr P Forouhi during summer vacation project.

Signed: ....

M A M Greiss

ACKNOWLEDGEMENTS

Parts of the work have appeared in the following publications:

1. URBANIAK SJ, GREISS M AYOUB: ADCC (K-cell) lysis of human erythrocytes sensitized with rhesus alloanti bodies. III. Comparison of IgG agglutinating and lytic (ADCC) activity and the role of IgG subclasses. Br J Haemat 1980, 46: 447-453.

2. BARCLAY GR, GREISS M AYOUB, URBANIAK SJ. Adverse effect of plasma exchange on anti-D production in rhesus immunization owing to removal of inhibitory factors. Br Med J 1980, 280: 1569-71.

3. URBANIAK SJ, GREISS M AYOUB, CRAWFORD RJ, FERGUSON MCJ. Prediction of the severity of rhesus haemolytic disease of the newborn by an ADCC assay (Letter). Lancet 1981, 280: 1569-71.

4. BARCLAY GR, GREISS MAM, McCANN MOIRA C, URBANIAK SJ. Rhesus immunization in male volunteers: changes in lymphocyte functions following secondary immunization in anti-D responders and non-responders. Br J Haemat 1983, 53: 629-40.

5. URBANIAK SJ, GRIESS M AYOUB, CRAWFORD RJ, FEREGUSON MCJ. Prediction of the outcome of rhesus haemolytic disease of the newborn. Additional information using an ADCC assay. Vox Sang 1984, 46: 323-29.

ABSTRACT

Standardisation of a homologous antibody-dependent cell-
mediated cytotoxicity (ADCC) assay was achieved by
evaluation in vitro of non-adherent peripheral blood
lymphocytes (PBL) from 120 healthy individuals for their
intrinsic activities in lysing group OR_1R_1 red cells
sensitized with a particular anti-D which had proved to
be highly active in ADCC despite having a low concen-
tration of antibody as measured by conventional haemag-
glutination methods.   The use of different ratios of
lymphocytes (effector cells) to ^{51}Cr-labelled OR_1R_1
red cells (target cells) allowed the construction of
dose response curves, where the ^{51}Cr release (% specific
lysis) was plotted against effector:target cell (E/T)
ratios.   In these curves % specific lysis started to
plateau at an E/T ratio of 10:1.   The results suggested
that in this system the intrinsic lymphocyte ADCC activ-
ity in healthy (non-immunized) individuals was indepen-
dent of age, sex and blood group of the donor.   It was
also found to be constant when tested sequentially in
individuals, although differences between individuals
were found to exist.

The standardised ADCC assay was used to study the lytic
potential of anti-D sera from different sources.   Anti-D
ADCC activity did not correlate with any of the conven-
tional haemagglutination assays used.   However, unexpec-
ted differences were found between the ADCC activity of
anti-D produced in deliberately immunized males and that

induced by pregnancy. Although the males produced high levels expressed in iu/ml, ADCC activity induced was much less than from females at equivalent concentrations. IgG subclass distribution between the different sera could not account for these differences. However, there was a tendency for IgG_3 titres to be higher where ADCC activity was seen, and in sera from males, IgG_1 titres were > IgG_3 and ADCC activity was very low.

Certain anti-D sera, selected on the basis of agglutination characteristics in vitro, failed to induce lysis of Rh(D) positive cells by ADCC and it appears that this anti-D blocked the effect of other anti-D sera (normally lytic in ADCC) in an antigen specific manner. Studies of a non-lytic anti-D showed that the blocking effect was associated with IgG anti-D. Antigen binding and Fc receptor binding studies indicated that the non-lytic anti-D was bound to Rh(D) positive red cells, but failed to mediate ADCC.

The ADCC assay was used successfully to predict the severity of haemolytic disease of the newborn (HDN) due to anti-D. An ADCC assay can in some cases indicate the outcome of pregnancy affected by HDN more accurately than can conventional methods.

In a study of changes in intrinsic lymphocyte ADCC activity in males participating in the Rh-immunization programme, responders (ie those who produced anti-D) showed marked depression of ADCC activity after secondary immu-

nization, and this was not seen amongst non-responders. This was also seen in patients undergoing plasma exchange therapy to reduce maternal concentration of anti-D, when fresh frozen plasma was used as a replacement fluid without regard to the Rh groups of the donors.

Suggestions are made for further studies on the basis of the findings reported.

CONTENTS

CHAPTER 2 - MATERIALS AND METHODS

CHAPTER 1

INTRODUCTION

1

1.1 <u>Mechanisms of human red cell destruction</u>

Immune red cell destruction without the presence of antibody, on an exclusively cell mediated basis, has not been demonstrated. It has been obvious for most of this century that two distinct types of red cell destruction seem to exist. Fairley (1940) used the term "intravascular" to describe the type of haemolysis associated with the destruction of red cells in the blood, and the term "extravascular" for the breakdown of red cells within the reticuloendothelial system (RES).

Of particular interest are the haemolytic conditions mediated by antibodies since these are often encountered in blood transfusion practice. The mechanisms of red cell lysis are basically the same whether mediated by transfusion alloantibodies (or host antibodies to transfused red cells) as in a haemolytic transfusion reaction, by transplacental transfer of alloantibodies as in haemolytic disease of the newborn (HDN), or mediated by autoantibodies (Table 1).

1.1.1 <u>Immunoglobulin class of red cell antibody</u>

The immunoglobulin class of most red cell antibodies is IgG or IgM, although IgA forms also exist. The term immunoglobulin is used to describe protein molecules that have the ability to complex specifically with antigen. Immunological differences between these classes are associated with the constant (C_H) region of the heavy chain (Fig 1). The heavy chains are

Table 1

Antibody-mediated RBC destruction

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1) RBC + antibody (IgM); C1-C9 complement activation; intravascular lysis

2) RBC + antibody (IgG or IgM); complement activation to C3; removal by reticulo-endothelial system (mainly liver)

3) RBC + antibody (IgG); directly removed by reticulo endothelial system (mainly spleen)

4) RBC + antibody (IgG); antibody dependent cell mediated lysis

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designated gamma (IgG), Mu (IgM) and alpha (IgA) (Table 2). Light chains occur as two immunologically distinct types designated kappa and lambda. The H chain classes share kappa and lambda chains. Approximately 70% of light chains are kappa and 30% lambda. Each antibody molecule possesses two light chains of the same type (ie two kappa or two lambda).

1.1.2 IgG Sub-classes

IgG is divided into sub-classes (IgG_1, $_2$, $_3$ and $_4$) which differ in their primary structure (Grey and Kunkel, 1964; Terry and Fahey, 1964). In all IgG molecules there are inter-heavy chain disulphide bonds in the hinge region but the number varies in the different sub-classes. There are two such bonds in IgG_1 and IgG_4, four in IgG_2 (Nisonoff et al, 1975) and eleven in IgG_3 (Michaelson (1977). The properties of IgG sub-classes which are important in the area of immunohaematology are presented in Table 3.

1.1.3 Role of antibody in destruction of human red cells

There is much evidence that binding of red cell antibodies to their antigenic determinants on the cell membrane does not cause direct damage to red cells (Mollison, 1972). When complement is present on the cell, either alone or together with IgG, the in vivo interactions differ from

SCHEMATIC DIAGRAM AND ASSOCIATED ACTIVITIES OF AN IgG MOLECULE
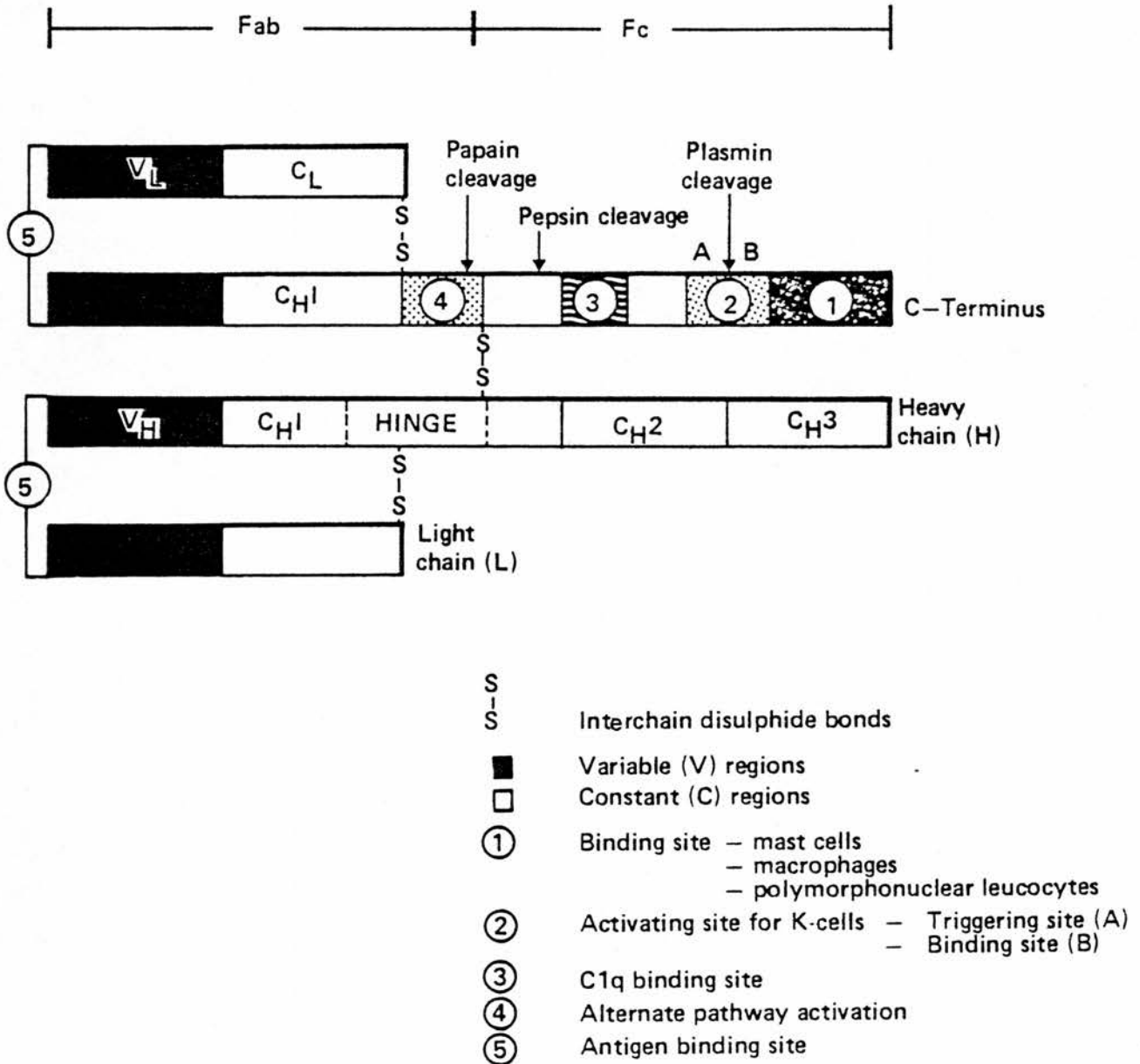
General structure, enzyme cleavage points and major fragments are shown.

Fig. 1

Table 2

Biological properties and activities of immunoglobulin in serum

Characteristic	IgG	IgM	IgA
Role	Protection of tissue fluids	Protection of circulation	Protection of mucosal surfaces
Heavy chains	γ	μ	α
Light chains	K or λ	K or λ	K or λ
Molecular weight (daltons)	150,000	900,000 (and polymers)	160,000 (and polymers)
J chain	No	Yes	Yes
Sedimentation coefficient (S)	7	19	7, 10, 14
Electrophoretic mobility	γ	γ-β	β
Concentration (mg/ml) - Adult	7-17	7-21	7-35
Concentration (mg/ml) - Newborn	> than adult	0.3-3	-
Catabolic rate T 1/2 (d)	23	5	6
% of total which is intravascular*	44	80	40
Synthesis (mg/Kg/day)	20-40	3-55	3-17
Complement fixation	Yes	Yes	No
Binding to Monocytes/macrophages	Yes	No	No
Number of sub-classes	4	1	2
Blood group antibody activity described	Yes	Yes	Yes
Placental transfer	Yes	No	No
Usual serological activity behaviour as red cell antibody	Incomplete antibody	Agglutinin	Agglutinin
Serological activity after heating to 56°C for 3h	Unaffected	Reduced	Unaffected
Effect of reducing agents on serological activity	May retain agglutinating activity	No longer agglutinates	Partially inactivated

* Percentage of intravascular poool destroyed daily

Adapted from:

Mollison (Blood Transfusion in Clinical Medicine 7th Edition, 1983)
Petz and Garratty (Acquired Immune Haemolytic Anemias, First Published, 1980)
Petz and Swisher (Clinical Practice of Blood Transfusion, First Published, 1981)

Table 3

Biological properties and activities of human IgG sub-classes

Characteristics	IgG_1	IgG_2	IgG_3	IgG_4
Serum (mg/ml)	5-12	2.6	0.5-1	0.2-1
Half-life in days	22	22	5-16	22
Fractional turnover (%)	7	7	17	7
Synthesis (mg/Kg/day)	25		3.4	
Placental transfer	+	+	+	+
Complement fixation	++	+	+++	-
Binding to macrophages	++	±	+++	±
Binding to neutrophils	+	+	+	+
Binding to lymphocytes	++	±	+++	±
Binding to platelets	+	+	+	+
Staphylococcal A	+	+	-	+
Allotypic (genetic) markers	4	1	12	0

Symbols used: -, negative; ±, weakly positive; +, positive; ++, +++ strongly positive; blank space, not tested.

Adapted from:

Speigelberg (Advances in Immunology 1974, vol 19).
Schanfield (AABB, American Association of Blood Banks, Nov 1977).

those observed when only IgG is present. Extra-vascular red cell removal is enhanced considerably when complement is present on red cells in addition to IgG. Mollison (1979) showed that red cells sensitized with 7 to 9 μg/ml of a complement-binding IgG allo anti-Fy^a were cleared from the circulation with a half time of 2 to 4 min (indicating almost complete clearance of the cells at a single passage through the liver), whereas red cells sensitized with a similar amount of non complement-binding IgG antibody were cleared with a half time of the order of 50 min, indicating negligible clearance by the liver and only partial clearance at each passage through the spleen. Schreiber and Frank (1972 a-b) injected normal guinea pigs and guinea pigs deficient in complement with IgG and IgM-sensitized guinea pig red cells in different experiments. The cells survived longer in animals lacking either C3 or C4 and terminal complement components, which suggests that complement played a significant role in determining the rate of destruction.

It has been known for some time that IgM antibodies are somewhat better at activating complement than IgG forms, only one molecule of IgM on a cell membrane being necessary to activate the complement system (Borsos and Rapp, 1965; Humphrey and Dourmashkin, 1965). In contrast, it

is thought that IgG needs to form a "doublet"; that is, two IgG molecules have to combine with antigens on the cell membrane as close together as 25-40 nm before they are able to activate C_1 (Humphrey and Durmashkin, 1965). IgG_1 and IgG_3 molecules are good at initiating complement activation; IgG_2 molecules have limited ability in this respect and IgG_4 molecules may be unable to activate complement at all (Müller-Eberhard, 1969).

It has been shown that most IgG anti-Rh anti-bodies are IgG_1 and IgG_3 (Natvig and Kunkel, 1967; Frame et al, 1970; Abramson and Schur, 1972) yet they very seldom cause complement to be bound. Mollison (1972) has suggested that this may be due to the location of Rh antigen sites on the red cell membrane. It has been suggested that Rh antigens are mobile within the membrane and may cluster during antigen-antibody reactions (Victoria et al, 1976). It is possible that the ability of antigens to cluster may be an important factor in immune red cell destruction as it may influence the ability of antibodies to activate complement (eg there is a greater likelihood of IgG forming "doublets") and it may alter the presentation of membrane-bound IgG to the macrophage (Garratty, 1982).

Those antibodies which fix complement react at $37^{\circ}C$ and cause rapid intravascular lysis of red cells (Table 1). The red cell membrane is damaged as a result of activation of the complement cascade C1 to C9 with holes being produced by enzyme action followed by osmotic lysis as a result of ions, fluxes and water entering the cell (Müller-Eberhard, 1969).

Although it was previously believed that C3b was primarily involved in bringing about attachment to macrophages whilst ingestion depended on IgG (Mantovani et al, 1972), it is now known that this conclusion applies only to inactivated macrophages. When macrophages are activated by a lymphokine, C3b receptors are able to migrate within the plane of the plasma membrane of the cell and the cells show C3b receptor-dependent phagocytosis (Griffin and Mullinax, 1981). This interaction was described as immune adherence and the receptor to which complement attaches is known as an IA receptor (Nelson, 1956). It is now known that the immune adherence receptor consists of three separate receptors CR1 (for C3b), CR2 (for C3d) and CR3 (for C3bi) (Ross, 1980). When C3b is present on red cells in addition to IgG, interaction with macrophages is enhanced; the amount of IgG antibody required for

immune adherence in the absence of complement is quite considerable (approximately 100 times more - Ehlenberger and Nussenzweig, 1977).

It has been postulated that this mechanism results in the _in vivo_ blood picture of spherocytosis, red cell fragmentation and intravascular haemoglobin seen in immune haemolytic anaemia. By the use of ^{51}Cr-labelled red cells it has been shown that C3-coated cells are removed more efficiently by the liver than by the spleen (Cookesley _et al_, 1973) as the liver has a larger blood supply. However, red cells sensitized by IgG only are destroyed predominantly in the spleen and it has been shown that the spleen is about 100 times more efficient than the liver at removing Rh (IgG) sensitized red cells (Mollison, 1972).

IgG-coated red cells will adhere to human monocytes leading to rosette formation (Lo Buglio _et al_, 1967; Cline and Lehrer, 1968; Huber _et al_, 1968). The attachment of IgG-coated cells to monocytes depends on a site on the Fc fragment; only IgG_1 and IgG_3 molecules bring about attachment (Huber and Fundenberg, 1968, Henderson 1976), the degree of which tends to be greater with IgG_3 than with IgG_1 (Abramson and Schur, 1972; Schanfield _et al_, 1980). Further, IgG_3 has greater affinity than IgG_1 for the Fc receptor on

monocytes (Engelfriet et al, 1981). Adherence to monocytes can be demonstrated with Rh (D) positive red cells coated with anti-D or with $CrCl_3$-treated red cells coated with IgG (Lo Buglio et al, 1967; Huber and Fudenberg, 1968). When examined under the electron microscope, red cells which have been bound to monocytes are seen to be adhering very closely with coarse interdigitation between the cells; red cells which break away have increased osmotic fragility (Lo Buglio et al, 1967; Abramson et al, 1970a).

Lo Buglio et al (1967) showed that the reactions between the Fc receptor on human monocytes, and macrophages with IgG-sensitized red cells, could be specifically inhibited with IgG or its Fc fragment in solution. It was suggested that the entrapment of antibody-coated red cells in vivo is enhanced by plasma skimming; the fact that the spleen is notable for both erythroconcentration and efficient trapping of IgG-coated red cells supported this interpretation. Huber et al (1968) have confirmed the inhibitory effect of even small amounts of normal plasma or serum. However, Scornick et al (1975) showed that IgG-sensitized red cells are rapidly cleared from the circulation of patients with myelomas in spite of serum IgG concentrations several times higher than normal. They concluded that current explanations derived from in vitro experiments

are insufficient to explain the IgG-dependent clearance of red cells in the presence of free IgG. Most alloantibodies (eg Rh, Kell, Duffy) other than anti-A and anti-B destroy red cells extravascularly. Most of the red cell destruction associated with autoimmune haemolytic anaemia (AIHA) is extravascular. In addition, almost all drug-induced haemolytic anaemias (eg those caused by methyldopa or penicillin) are associated with extravascular red cell destruction.

It has been demonstrated that red cells sensitized in vitro with increasing amounts of anti-Rh(D) antibody have rates of in vivo destruction which correlate directly with the degree of antibody sensitization (Mollison, 1979). There is a general correlation between the amount of antibody taken up on red cells with the amount of red cell destruction, but there are extreme examples of non-correlation at each end of the scale (Garratty, 1982). Cases of AIHA illustrate the dramatic lack of correlation which can be seen between in vitro tests and in vivo red cell destruction. Although some workers (Rosse, 1971; Petz and Garratty, 1980) have found that auto-antibodies (like the alloantibodies described above) show a general correlation

between the amount of antibody on red cells (ie the strength of the direct antiglobulin test, DAGT) with in vivo red cell destruction others (Constatoulakis et al, 1963) have found no correlation. Gilliland et al (1970, 1971) have explained this as sometimes occurring because of the small numbers of IgG molecules (eg 50-200) present per red cell, and that these molecules are too few to be detected by the routine direct antiglobulin test (DAGT): this was confirmed by Petz and Garratty (1980). Hughes-Jones et al (1964) and Dupuy et al (1964) have shown that approximately 200-500 molecules of IgG per red cell have to be present before a positive DAGT can be obtained.

Recent in vitro evidence has suggested that monocytes (Holm, 1972) and lymphocytes (Urbaniak, 1976) may destroy sensitized red cells by extracellular cytotoxicity in addition to phago-cytosis. It is possible that this is a more important mechanism in vivo than immune adherence, especially where antibody-coating of red cells is minimal. It may also be an alternative explanation for non-complement-mediated haemoglobinaemia and haemoglobinuria associated with extravascular lysis. This type of lysis has been described as antibody-dependent cell-mediated cytotoxicity (ADCC) and is a mechanism which has been shown to result in

damage to many types of cells including allogeneic and xenogeneic red cells, tumour cells and lymphocytes. It is described in detail in the following section.

1.2 Antibody-dependent cell-mediated cytotoxicity (ADCC)

The role of lymphocytes in immune red cell destruction is controversial. Lymphocytes are known to have receptors for IgG (Fc) and complement (C3b, C3d and C4). They have been described as being capable of destroying sensitized nucleated cells (Perlmann and Holm, 1969). Before the cell is damaged, contact between the lymphocyte and the target cell has to occur and complement may or may not be involved. In vitro cell-mediated lysis requires several hours to occur, even under optimal conditions. Cell-mediated cytotoxicity is antibody-dependent where the target cells have to be sensitized with antibody. The effector cells for this reaction have been described as possibly being "null" lymphocytes (ie lacking sheep cell receptors and surface Ig, but having IgG Fc and complement receptors). They are either a sub-set of B lymphocytes (Brier et al, 1975) - the so called "Killer" lymphocytes (K-cells) which are non-phagocytic and non-adherent lymphocytic cells (Perlmann and Holm, 1969) - or they represent a heterogenous family of effector cells (both B and T cells). Still another group of workers (Shore et al, 1977) believe that the effector cells are neither B nor T cells. However, all agree that the cells possess an Fc receptor (Reviewed by Sanderson, 1981).

1.2.1 The nature of effector and target cells - influence on ADCC

Great interest has been shown in the true identity of the cells with lymphocyte morphology

which mediate ADCC, as originally described by Perlmann et al (1972) and MacLennan (1972). A great deal of earlier confusion in the literature was due to failure to appreciate the importance of target cells and their antibodies, and that there might be inter-species differences. An important observation was that the type of target and the source of antibody are important in determining the nature of the effector cell-mediating lysis. In general, it appears that highly purified lymphoid cells are much more effective in promoting the lysis of nucleated target cells such as allogeneic lymphocytes and tumour cell lines, whereas monocytes are more effective in lysing red cell targets. It is interesting to note that chicken red cells are nucleated and appear to be equally susceptible to lysis by monocytes and lymphoid cells (Kovithavongs et al, 1975; Zeijlemaker et al, 1975). Differences in ADCC activity are seen when target cells, antibodies and effector cells from different species are tested in various combinations (Zighelboim and Gale, 1974). Urbaniak (1979a) emphasised the importance of using a homologous system when testing human ADCC activity in patients.

It has been shown that human cells other than purified lymphoid cells are active in ADCC, eg polymorphonuclear neutrophils (PMN) which require

greater amounts of antibody (Gale and Zigelboim, 1974, 1975; Oleske et al, 1977; Clark and Klebanoff, 1977; Levy et al, 1979; Fuson et al, 1981; Conkling et al, 1982). This may be because PMN-Fc receptors have lower avidity for IgG antibody. Monocytes have also been reported to lyse antibody-sensitized erythrocytes (Holm, 1972; Holm and Hammarström, 1973; Papamichael and Temple, 1975) but to be relatively inactive against nucleated target cells (MacDonald et al, 1977; Trinchieri et al, 1975). Others have indicated that monocytes may lyse nucleated cells as well (Kohl et al, 1977; Horwitz et al; 1979; Levy et al, 1979). Eosinophils have also been reported to lyse antibody-sensitized red blood cells (Hanepps and Bankhurst, 1978; Parillo and Fauci, 1978; Lopez and Sanderson, 1982). Although chicken red cells were lysed by human lymphocytes, Holm (1972) reported that human red cells were not directly lysed in the presence of anti-A or anti-Rh when the preparation was more than 99% pure lymphocytes. Hinz and Chickosky (1972), using a lymphocyte preparation containing less than 1% neutrophils and virtually no monocytes, were able to demonstrate cytotoxic reactions between lymphocytes and group A and Rh-positive red cells in the presence of anti-A and anti-Rh respectively. In the anti-A experiments, lymphocytes from group O, A or AB

donors were equally active but in the Rh experiments only lymphocytes from an Rh hyper-immune donor would cause lysis of the red cells. Normal lymphocytes from both Rh-positive and Rh-negative donors were completely without effect, even in the presence of a powerful anti-Rh. In contrast, Urbaniak (1976) was able to show that normal or autologous lymphoid cells would lyse Rh sensitized red cells and that killing occurred at very low antibody concentrations. Recently, other workers have also reported ADCC against human red cells mediated by anti-A (Northoff et al, 1978; Moore and Kimber, 1980) and anti-Rh (Handwerger et al, 1978; Milgrom and Shore, 1978; Northoff et al 1978; Moore and Kimber, 1980; Kimber and Moore, 1981).

Previous systems using anti-A/group A or anti-B/ group B target red cells (Holm and Hammarström, 1973; Poplack et al, 1976) have demonstrated that effector red cells include both monocytes (Holm et al, 1974) and a non-monocytic lymphoid K-cell (Urbaniak, 1976; Milgrom and Shore, 1978; Kurlander et al, 1978). Others have not been able to demonstrate such activity in vitro (Holm and Hammerström, 1973; MacDonald et al, 1975, Engelfriet et al, 1981). Although the lysis of such targets by the ADCC mechanism is well documented, evidence obtained in heterologous systems using either mononuclear target cells

(MacLennan, 1972; Ziegler et al, 1977) or chicken
red cells (Perlmann et al, 1972, Wisloff et al,
1974) and in homologous systems using human red
cell D/anti-D (Urbaniak, 1976) indicates that
intimate cell-to-cell contact is required and
that this is mediated via the Fc portion of
target bound antibody (IgG class) and an Fc
receptor on the effector cell.   Holm and
Hammarström (1973) reported that monocytes were
much more active than lymphocytes in a D/anti-D
ADCC assay, and found that trypsinisation of red
cells enhanced lysis.

Urbaniak (1976, 1979a, b) has achieved consider-
able enhancement by papainisation of human red
cells in a K-cell assay and this has been
confirmed by other workers (Shaw et al, 1978;
Milgrom and Shore, 1978; Kurlander et al, 1979).
The nature of the enhancing effect is unknown but
it is probably a combination of enhanced antibody
uptake by the cells (Masouredis, 1962;
Hughes-Jones et al, 1964) and increased exposure
of Rh antigens in the red cell membrane by
removal of surface glycoproteins (Romano et al
1975). Altered cell surface charges (Stratton et
al, 1973) produced by treatment with papain may
also be responsible for these findings. Recently
it has been suggested that papainisation of the
red cell target increases fluidity of red cell
membrane structures so that the anti-D can bring

about a re-distribution of the red cell surface
D-antigen/antibody complex in localised
aggregates, which appears to be necessary for the
interaction between target red cells and
lymphocytes in promotion of ADCC (Shaw et al,
1980). It has been adequately confirmed that the
surface receptor for the Fc portion of Ig (FcR)
is involved in effector functions such as ADCC
(Perlmann et al, 1976; Lamon et al, 1975, 1976,
1977; Urbaniak, 1979b, Perlmann and Cerottini,
1979) or inhibition of T or B cell proliferation
in certain in vitro systems (Moretta et al,
1977a, 1979). That lymphocytes have a strong
affinity for those complexes was first demon-
strated by Uhr and Phillips (1966) and Basten et
al (1972). Antigen-antibody complexes, aggre-
gated IgG and, at times, IgG itself were shown to
bind to lymphocytes by rosette formation with
antibody-coated erythrocytes, autoradiography and
immunofluorescence. Since a requirement for
binding was an intact immunoglobulin-contained Fc
piece it was assumed that the binding lymphocytes
contained Fc receptors (Basten et al, 1972; Grey
et al, 1972). Although it is clear that most
lymphocytes with Ig receptor are much more active
in binding than those without (Basten et al,
1972; Dickler and Kunkel, 1972; Cline et al,
1972; Yoshida and Henderson, 1972; Brown and
Greaves, 1974), the distribution of Fc receptors
on B cells, T cells and thymus cells varies from

one report to another (reviewed by Dickler, 1976).

The Fc portion is required in ADCC since its removal from the antibody results in the loss of ADCC activity (Ewald et al, 1976). Pape et al (1977 a, b) observed that effector cells (K) involved in ADCC are Ig negative and FcR positive and that the Fc receptors on Ig positive cells were qualitatively different from those on K cells. Similarly Kay et al (1977), working with peripheral human blood cells, reported that natural killer (NK) cells and effector K cells in ADCC are overlapping a population of FcR positive T lymphocytes. Horwitz et al (1977) have also shown that FcR positive cells are effective in ADCC, whereas FcR negative cells are ineffective. These observations are in agreement with those that heat aggregated (MacLennan, 1972; Wisloff et al, 1974; Urbaniak, 1979b) and chemically aggregated (Speigelberg et al, 1976) preparations of IgG markedly inhibit ADCC.

It has also been shown that ADCC lymphocytes lack detectable membrane Ig determinants (Perlmann et al, 1972) and expressed either T cell (Perlmann et al, 1975 and West et al, 1978) or null markers (Ozer and Strelkawskas, 1979). Further, Ross and Lambris (1982) suggested that the small number of Ig negative CR3 positive cells detected on the K

cell represent the cells functional in ADCC. It has been established that monocytes, macrophages (Lo Buglio et al, 1967) and lymphocytes (Urbaniak, 1976; Shaw et al, 1978; Kurlander et al, 1978) are capable of mediating lysis of human red cells coated with antibody. However, destruction by all these effectors is markedly inhibited by unbound fluid-phase IgG (Lo Buglio et al, 1967; Shaw et al, 1978; Kurlander et al, 1978) and this inhibition has engendered doubt about the role of these effectors in vivo, since the amount of IgG that effectively inhibits target cell destruction in vitro is far less than is present in serum (Shaw et al, 1978, Kurlander et al, 1978). However, Urbaniak (1979b) has achieved satisfactory lysis by lymphocytes of sensitized red cells in the presence of 40% human serum, whereas very low concentrations of human serum will inhibit monocyte-mediated ADCC of human red cells (Holm et al, 1974 and Shaw et al, 1978) and also inhibit monocyte immune adherence of antibody-coated red cells (Abramson and Schur, 1972). Kurlander and Rosse (1979) demonstrated that by the addition of purified monoclonal IgG_1 (1000 μg/ml) to the lymphocyte ADCC culture medium, the lysis was markedly inhibited. In contrast, in the presence of undiluted human serum lysis was equal to, or greater than, lysis in ADCC culture medium alone even in the presence

of IgG_1 at 1000 μg/ml - despite the high concentration (6000-19000 μg/ml) of IgG in human serum: ie the lymphocyte's ADCC system is inhibited by purified fluid-phase IgG, but not by serum IgG. Urbaniak (1979b) and Kurlander and Rosse (1979) have suggested that lymphocytes may play a more important role in in vivo destruction of red cells of patients with immune haemolysis than has previously been appreciated.

It has been noted recently that in vitro expression of ADCC may be influenced by suppressor lymphocytes (Pollack and Emmons, 1979) and there is some evidence that this effect may be mediated by soluble factors (Madhavan and Schwartz, 1981). There are also data available which indicate that ADCC activity can be influenced by cytokines such as interferon (Herberman et al, 1979), although no effect was seen by Kimber and Moore (1981) when they used purified lymphocytes in ADCC culture. A variety of agents that can influence intracellular levels of cyclic AMP or cyclic GMP are known to influence expression of Fc receptors by T cells (Gupta, 1979). Since it has been shown that the T cell bearing Fc receptors for IgG is capable of effecting ADCC (Shaw et al, 1979; Katz and Fauci, 1980) these agents may alter K-cell activity. Indeed recent work (personal communication M McCann, 1984) has shown

that agents which raise intracellular cyclic AMP depress ADCC while agents which raise cyclic GMP enhance.

Some cell mobility would appear to be necessary to account for the number of red cells lysed per effector cell. Estimates of K cell numbers are of the order of 1% to 10% of mononuclear cells (Biberfeild et al, 1975) and it has been calculated that up to 40 red cells could be lysed per K cell (Urbaniak 1979b). The divalent cations Mg^{2+} and Ca^{2+} are not required for rosette formation between IgG-sensitized red cells and mononuclear cells (Lay and Nussenzweig, 1969), and have also been shown to be unnecessary for K cell recognition and binding to the target (Ziegler et al, 1977). However, these cations are necessary for lysis but it is not clear why this is so: they are necessary for phagocytosis but this is not an essential pre-requisite for ADCC lysis of targets. It has been suggested that they may be required to trigger intra-cellular events analogous to secretion of proteins from cells or lymphocyte transformation (Urbaniak, 1979b).

1.2.2 Role of antibody in ADCC

It has been reported that monocyte-mediated antibody-dependent haemolysis was not only largely dependent upon the amount of antibody

fixed to the target, but also varied with yet undefined properties of the antigen, the antibody, or both (Kurlander et al, 1978). Likewise, there are data suggesting that the effectiveness of antisera in producing lymphocyte-mediated lysis may also depend upon the sub-class and strength of the antiserum, the number of available antigen sites on the red cell membrane, and on other qualitative properties (Handwerger et al, 1978). Kurlander et al (1979) have suggested that the degree of cell destruction by lymphocytes depends upon the concentration of antibody on the surface of the red cell (target cell); lysis may be detected at levels of anti-D coating less than 2000 molecules per red cell.

1.2.2.1 Requirement for various portions of the Ig molecule

Binding via the Fc receptor of lymphocytes depends on the immunoglobulin class (Basten et al, 1972). Removal of the Fc fragment by pepsin digestion of the sensitizing antibody was found to reduce ADCC significantly (Gelfand et al, 1972; Moller and Svehag, 1972). The nature of the trigger to lysis is unknown but the activated Fc-portion of cell-bound IgG is an essential factor

(Larsson et al, 1973; Ewald et al, 1976; Urbaniak, 1979b).

An obvious question is where on the Fc portion of the IgG is the peptide sequence responsible for the reaction with Fc receptors? (Fig 1). It is reasonably well established that the chains of IgG are folded into compact domains, each corresponding to one of the regions apparent in the primary structure (Cathou and Dorrington, 1975). Although the Fc-portion appears to be responsible for non-specific biological activities of the IgG molecules, the site involved in each of these may not be the same. Plasmin cleavage, which removes the terminal 107 amino acid residues, was shown by MacLennan (1972) to reduce the ability of antibody to mediate ADCC, suggesting that the $C\gamma 3$ domain is required. However, Wisloff et al (1974) found that the Fch fragment containing a portion of the N-terminal part of Fc-inhibited ADCC against chicken red cells was better than the C-terminal half of the Fc fragment, suggesting that the $C\gamma 2$ region is more important to the reaction. The reason for the discrepancy between these two

studies is not clear. MacLennan's study employed Chang target cells, while the latter report used chicken red cells as targets. Perhaps different portions of the Fc fragment activate different effector cell mechanisms.

Treatment of sensitising IgG with 2-mercaptoethanol to cleave disulphide bonds in the Fc portion of the heavy chain also reduced ADCC (Denk et al, 1974). Removal of one of the antigen-binding sites did not prevent cytolysis, however, when chicken red cells were used as targets. (Michaelsen et al, 1975).

It has been suggested that these studies indicate that the necessary structural features are not solely within the $C\gamma3$ domain, and that $C\gamma2$ structural integrity is also required, either to stabilise $C\gamma3$ or to enhance the affinity of $C\gamma3$ for the effector cell membrane receptor. (Reviewed by Lovchik and Hong, 1979). Similarly Spiegilberg et al (1976), who investigated the reactivity of human IgG portions with K-cells in ADCC suggested two binding sites, one having triggering (enhancing)

activity located on the Cγ2 domain and one having high affinity-binding located on the Cγ3 domain (Figure 1). The complexity of Fc regions expressing biological functions is further evidenced by the failure of either the Cγ2 or Cγ3 domains of IgG, to bind the Fc placental receptor. It was suggested that the Cγ2 and Cγ3 domains may contribute to the formation of a co-operative binding site through quaternary interdomain interaction. It may be that the Fc site responsible for different functions of lymphocytes is also different (reviewed by Weigle and Berman, 1979).

1.2.2.2 Antibody class and subclass

Antibodies capable of triggering the ADCC response were found to elute predominantly with the IgG-rich fraction rather than the macroglobulin fraction of a Sephadex column (MacLennan et al, 1969; Möller and Svehag, 1972). Recently, Urbaniak (1979b) has reported that only IgG is effective in inhibiting K cell lysis and that IgA and IgM at concentrations normally found in plasma are ineffective, and indicated that the lymphocyte K cell receptor - and not IgA or IgM - is specific for the Fc portion

of IgG. It has been shown that
aggregated IgG has "activated" Fc
regions to bind more firmly to Fc
receptors of mononuclear cells than does
monomeric IgG (Dickler and Kunkel, 1972;
Urbaniak, 1979b), and it has been seen
to inhibit more efficiently than
equivalent amounts of native IgG
(Urbaniak, 1979b). Further, all four
sub-classes of human IgG, when
aggregated, were able to inhibit the
reaction - presumably by competing with
the antibody complexed to target cells
for Fc receptors on the effector cells
(MacLennan et al, 1973; Perlmann et al,
1972). Attempts to rank the sub-classes
with respect to inhibitory capacity have
produced variable and conflicting
results (MacLennan et al, 1970; Chapuis
and Brunner, 1971; Greenberg et al,
1973; 1975; Larsson et al, 1975). Some
differences may be related to the use of
different species of antibody and
effector cells. For example, using
human myeloma proteins Wisloff et al
(1974) and Larsson et al (1975) found
chicken red cell lysis to be inhbited by
IgG_1 and IgG_3 to a much greater extent
than by IgG_2 and IgG_4. Using Chang

cells as targets, however, MacLennan et al (1973) found that all four subclasses inhibited lymphocyte cytotoxicity. Moreover, the same myeloma proteins ranked differently in inhibiting lymphocyte effector cells than they did when monocyte cells were the effectors (Larsson et al, 1975). Spiegelberg et al (1976) have reported that preparations of IgG_1 and IgG_3 markedly inhibited ADCC, while IgG_2 and IgG_4 were less effective. More recently Urbaniak (1979b) demonstrated that all three IgG sub-classes caused inhibition ranked in the order $IgG_1 > IgG_3 > IgG_2$, when a homologous system is used in the ADCC assay.

The amount of antibody required to sensitize target cells is very small. Under standard conditions, 50% lysis of chicken red cells can be obtained with as little as 0.1-1.0 ng of Ig antibody (Perlmann et al, 1972). This amount is too low to activate complement and indicates the great sensitivity of ADCC. More recently Urbaniak (1979a) has reported significant lysis with an IgG anti-D concentration of 0.02 μg/ml (3.0 ng per culture) using human red cells as

targets in an ADCC assay. The demon-
stration of ADCC activation by non-
complement fixing antibodies IgG_2
(Larsson et al, 1974) and Rh blood group
antibodies (Holm et al, 1974; Urbaniak
1976, 1979a, 1979b; Kurlander et al,
1978, Kurlander and Rosse, 1979; Kimber
and Moore, 1981) increases the potential
biological spectrum of activity of such
antibodies in immune mechanisms.
Furthermore, Urbaniak (1979a) has noted
that there was no correlation between
anti-D lytic potential (in terms of %
specific lysis of red cells) and
haemagglutination titres, and that there
was no correlation between the source of
anti-D (ie pregnancy-induced or
deliberate immunization) and the %
specific lysis. He suggested that this
disparity between the agglutinating
ability of anti-D and lytic potential
may have importance in vivo, and it is
conceivable that the ADCC assay will
predict the severity of in vivo
haemolysis better than conventional
serological techniques. This discrep-
ancy between agglutinin titre and the
level of ADCC has been noted by other
workers (Lajos et al, 1979).

1.2.3   Participation of Complement

Perlmann and Holm (1969) found that concentra-
tions of anti-serum which were too dilute to
cause target cell lysis in the presence of
complement were even more effective in mediating
cell-induced lysis than were higher concen-
trations.   Also, complement-dependent lysis was
more rapid than ADCC.   These findings suggested
that complement is not involved in ADCC, at least
not in a conventional manner.   Others found that
caragheenan, which binds the first component of
complement, did not inhibit ADCC (Dennert and
Lennox, 1972; Dickmeiss, 1973; Pollack and
Nelson, 1973; Gale and Zighelboim, 1975).   This
did not rule out involvement of the alternative
complement pathway, however (Lovchik and Hong,
1979).   Van Boxel et al (1974) found that both
serum and cells from animals genetically defic-
ient in fourth, fifth and sixth components of
complement were fully effective in inducing ADCC.
However, co-operation between antibody and
complement in vivo is possible to make the
binding of effector to target cell more
efficient, since target-bound complement is
itself unable to induce cytotoxicity but enhances
ADCC at low antibody concentrations (Perlmann et
al, 1975; Lustig and Bianco, 1976).   In the
presence of antibody to the target cell, target
cell-bound C3 fragments strongly enhanced ADCC
(Ghevrehinet et al, 1979; Perlmann and Cerottini,

1979).   It was concluded that the target cell-associated   C3   amplified   ADCC   by   improving effector cell-target cell contact.   Recently, Perlmann et al (1981) have reported that the activity of lymphocytes functional in ADCC was greatly enhanced by target cell-bound complement in the order of C3bi > C3d > C3b.

1.3   Rh haemolytic disease of the newborn

In vivo haemolytic disease of the newborn (HDN) is the most complex of the three common forms of IgG-mediated red cell destruction (HDN, autoimmune haemolytic anaemia 'AIHA', haemolytic transfusion reactions) since it involves the production of antibody in one individual and cell destruction in another. The concentration of all four IgG subclasses has been found to be slightly higher in cord serum than in maternal serum (Mellbye and Natvig, 1973). However, it is known that IgG_1 crosses the placenta early in pregnancy; that by 20 weeks gestation maternal IgG_1 is detectable in cord serum; and that by 26 weeks gestation the cord serum level equals, or exceeds, the maternal serum level (Morell et al, 1971 and Schur et al, 1973). In contrast, the level of IgG_3 in cord serum does not reach the maternal level until 28 to 32 weeks gestation, and it may never rise higher than that of maternal serum during pregnancy.

Schanfield (1977) has reported that, in general, cases of HDN caused by IgG_1 anti-D involve more serious anaemia than those caused by IgG_3 anti-D. This presumably reflects the longer exposure of foetal red cells to IgG_1 than to IgG_3 anti-D. However, in cases of HDN caused by IgG_3 anti-D the infant's post delivery bilirubin level often exceeds that seen in infants suffering HDN caused by IgG_1 anti-D. This presumably reflects the fact that once it reaches the infant's circulation, IgG_3 anti-D causes a greater amount of red cell destruction than does the IgG_1 type. However, it

has been reported that there is no correlation between
the subclass patterns of IgG anti-D antibodies and the
degree of illness of the child (vant veer-Korthof et al,
1981)

1.3.1   Prediction of the outcome of Rh haemolytic
        disease of the newborn
        Prediction of the probable outcome is currently
        based on a number of factors:

        1)   The outcome of previous pregnancies
                    gives some indication of expected
                    severity, especially when the father is
                    homozygous for the Rh(D) antigen
                    (Bowman, 1978).

        2)   Estimation of maternal anti-D levels
                    during the course of pregnancy also has
                    predictive value, but antibody titres
                    are not always helpful and it is not
                    possible to compare results between
                    centres due to lack of standardisation.

                    Automated estimation of anti-D by
                    continuous-flow analyser gives more
                    accurate measurement of change, and
                    comparison with national or
                    international standards allows
                    standardisation of the assay (Morley et
                    al, 1977). Recent studies have shown

that Rh(D) positive babies will be
affected by HDN if the anti-D level is
greater than 5 iu/ml (equivalent to $1\mu g/$
ml), and thereafter the prognosis be-
comes worse with increasing anti-D
levels (Tovey and Haggas, 1971; Fraser
and Tovey, 1976; Tomlinson et al, 1981;
Bowell et al, 1982).

3)   Amniocentesis and optical density
measurements (ΔOD 450) of amniotic fluid
also give indications of the progress of
the pregnancy and when intrauterine
transfusions should be considered
(Liley, 1963). Combined estimates based
on previous history, anti-D levels and
serial amniocentesis can enable
prediction of the outcome in approxi-
mately 95% of cases (Bowman, 1978).
Nevertheless, unexpectedly mild (or
severe) cases can occur when using these
methods of prediction. When a high
level of anti-D justifies amniocentesis,
but the foetus is shown to be only
mildly affected, there is a risk of
stimulating further anti-D production as
a result of accidental foeto-maternal
haemorrhage. Conversely, a low level of
anti-D may allay suspicion until it
becomes apparent that the foetus is

already severely affected. Recently
Urbaniak (1978) suggested that the
ability of an anti-Rh(D) antibody to
induce K-cell lysis may be a more
important consideration than the
strength of the antibody as determined
by conventional titration methods, in
predicting the severity of HDN that the
antibody will cause.

1.3.2 Management of Rh HDN

The discovery of the cause of Rh haemolytic
disease of the newborn (HDN), and the development
of a world-wide prophylaxis programme, is a
unique illustration of modern day preventive
medicine at its best (Robinson, 1983). Between
1940 and 1970 dramatic progress was made in
reducing mortality from this disease from 50% to
5-9% of all perinatal deaths (Tovey, 1982).
Contributory events were the development of
Coombs' antiglobulin technique, the use of
exchange transfusion between 1940 and 1950, the
advent of amniocentesis and controlled premature
delivery between 1950 and 1960, and the
development of intrauterine transfusion (IUT) by
Liley (1963).

However, despite this dramatic reduction in
incidence, cases of Rh HDN still occur. These
are due to already immunized women embarking on a

subsequent pregnancy, failure to administer anti-D after an abortion, amniocentesis or normal delivery, or to failure of the anti-D immunoglobulin to protect when given correctly. Unfortunately, once sensitisation has occurred the alloimmunized pregnant woman has few therapeutic options open to her. Currently there are two therapeutic procedures available: plasma exchange and intrauterine transfusion (IUT). Intrauterine transfusion is not without risk, and with the reduced incidence of the disease the procedure is likely to become more hazardous as fewer skilled exponents of the art will be available (Palmer and Gordon, 1976; Robertson et al, 1976; Berkowitz, 1980). IUT is not possible before 24 weeks gestation and if attempted before 26 weeks the risks are high (Berkowitz, 1980). Plasma exchange (PE) is a relatively non-hazardous alternative, the objective being to remove specifically the causative antibody (IgG alloantibody) (Reviewed by Robinson, 1983). Whether or not this can be done successfully on a long-term basis during pregnancy without the aid of immunosuppression is another matter (Urbaniak, 1984). If PE is carried out with a large enough volume and often enough then reduction in anti-D levels can be achieved. However, this may require PE three or more times a week throughout the pregnancy (Fraser et al, 1976; Graham-Pole et al, 1977; Robinson and Tovey, 1980), and rapid

rebound of antibody levels cannot always be controlled even with daily PE, in which case foetal death results.

Although at first sight intensive PE in women immunized to Rh seems entirely rational, and might be expected to lead to substantial lowering of the concentration of anti-Rh in the foetus in utero, results have in fact been very variable (Mollison, 1983). PE is not to be undertaken lightly in cases of HDN because the mother is being subjected to an arduous procedure designed to protect the foetus, and a number of maternal deaths have been directly attributed to PE with a large volume of fresh frozen plasma (FFP) (Huestis, 1983). Robinson (1983) and Urbaniak (1984) have recommended that PE should only be considered under the following conditions:

(i)   There is a previous history of foetal loss or a very severely affected infant, and there is a strong possibility that the father is homozygous for the Rh(D) antigen;

(ii)   PE should be started early in pregnancy, and potentially immunizing effects (such as amniocentesis) should be avoided until 26-28 weeks, as IUT cannot be carried out safely before then;

(iii)   Consideration should be given to prema-
          ture delivery after 31-32 weeks, rather
          than attempting IUT (depending on the
          facilities available).

Because immunosuppression cannot be used there is
a risk of rebound synthesis of anti-D (Barclay et
al, 1980). In an attempt to reduce this, efforts
have been made to maintain total IgG levels with
transfusions of FFP, although this may be
associated with a high incidence of allergic
reactions,   including   severe   anaphylactoid
reactions (Urbaniak, 1984).   Paradoxically, it
has been reported that FFP may result in boosting
of anti-D levels if plasma from Rh (D) positive
donors is used, due to contaminating red cells
(McBride et al, 1978, 1983; Robinson, 1983).

1.4 Immunization of Rh negative male volunteers

The use of passively administered antibody to prevent primary immune induction has gained wide acceptance for the prophylaxis of Rh immunization. Since the number of sensitized women during pregnancy has diminished due to the successful programme of prophylaxis, it has become necessary to recruit and immunize male volunteers in order to maintain a satisfactory input of plasma for the production of anti-D immunoglobulin. It has long been apparent that it can be very difficult to stimulate formation of anti-Rh in some Rh-negative subjects, despite repeated injections of Rh positive red cells, about one third of Rh-negative subjects failing to form anti-Rh (Mollison, 1967). A large primary dose is chosen because a more satisfactory response is obtained with large doses of red cells (Cook I A, 1971; Cook K, Rush, 1974; Pollack et al, 1971). It has also been shown that Rh-negative subjects who have failed to respond to a small dose (0.01 ml) of Rh-positive cells will not subsequently respond to a larger one (10 ml) (Jakobowicz et al, 1972). A number of different immunization schedules have been investigated (Gunson et al, 1976; Ekland, 1978; Cook et al, 1980). One of the most successful, in terms of the proportion of subjects in which anti-D responses were induced (responders), is that used in the Edinburgh Regional Blood Transfusion Centre (Urbaniak and Robertson, 1981).

There still remain several unanswered questions concerning those individuals who fail to make any

detectable anti-D (non-responders) when subjected to this intensive immunization regimen. It would be a considerable practical advantage if it were possible to identify such non-responders before, rather than after, attempting immunization. The immunological basis of the response to the Rh(D) antigen is not known and currently there is no test which can distinguish responders from non-responders prospectively. Barclay (1980) observed certain changes in intrinsic lymphocyte transformation (LT) and also (Barclay et al, 1979) in the extrinsic effects of sera on LT, which are associated with immunization or regulation of immune responses. Preliminary observation also indicated that lymphocyte antibody-dependent cell-mediated cytotoxicity (ADCC) might also show changes following immunization (Urbaniak, 1977).

1.5 Aims of the study

Urbaniak's (1979a) observations suggested that varia-
tions exist in the degree of anti-D ADCC activity
related to the source of the anti-D (ie pregnancy-
induced or the result of deliberate immunization) and
that ADCC activity does not correlate with conventional
haemagglutination assays.

It was therefore decided:

i)   to study in detail the potential lytic activity
        of anti-D sera from different sources, employing
        a standardised homologous ADCC assay; and
ii)   to determine whether a correlation exists between
        this activity and the outcome of pregnancies
        affected by HDN.

Lymphocytes are the source of both humoral (antibody)
immune response and the regulatory activities which
govern the expression of specific immune responses, so
study of intrinsic lymphocyte ADCC activity in vitro
should give an insight into the mechanisms which operate
in vivo during the deliberate immunization of Rh(D)
negative male volunteers to produce anti-D immuno-
globulin for prophylactic use.

The intention was thus:

i)   to improve the accuracy of prediction and thus
        the management and treatment of severe haemolytic
        disease of the newborn resulting from Rh(D)
        isoimmunization in pregnancy; and

ii)   to determine whether any differences could be
        demonstrated between eventual responders and
        non-responders when seeking to stimulate
        production of anti-D.

CHAPTER 2

MATERIALS AND METHODS

2.1   Preparation of lymphocyte suspensions

2.1.1   Source of lymphocytes

Peripheral blood lymphocytes (PBL) were obtained from blood donors and laboratory personnel in good health and on no medication were used as normal K-cell donors. Other lymphocytes were obtained from male volunteers who were undergoing plasmapheresis for the production of anti-D for therapeutic use, pregnant women undergoing plasma exchange to reduce maternal anti-D concentrations in severe haemolytic disease of the newborn (HDN).

2.1.2   Mononuclear Separation

Blood collected in universal containers with 10 unit/ml preservative-free heparin was layered over an equal volume of Ficoll-Triosil (specific gravity 1.077 - see 2.1.3 below) and centrifuged at 400 xg for 40 min at room temperature. Mononuclear cells were collected at the interface of the plasma and the Ficoll-Triosil (Fig 2). The mononuclear suspension was diluted to a convenient volume with tissue culture medium 199 (TC 199 Gibco-Biocult) and centrifuged at 400 xg for 20 min. The supernatant was discarded and the cell pellet resuspended gently in 10-20 ml TC 199. This was then centrifuged at 200 xg for 10 min to reduce the number of platelets in the
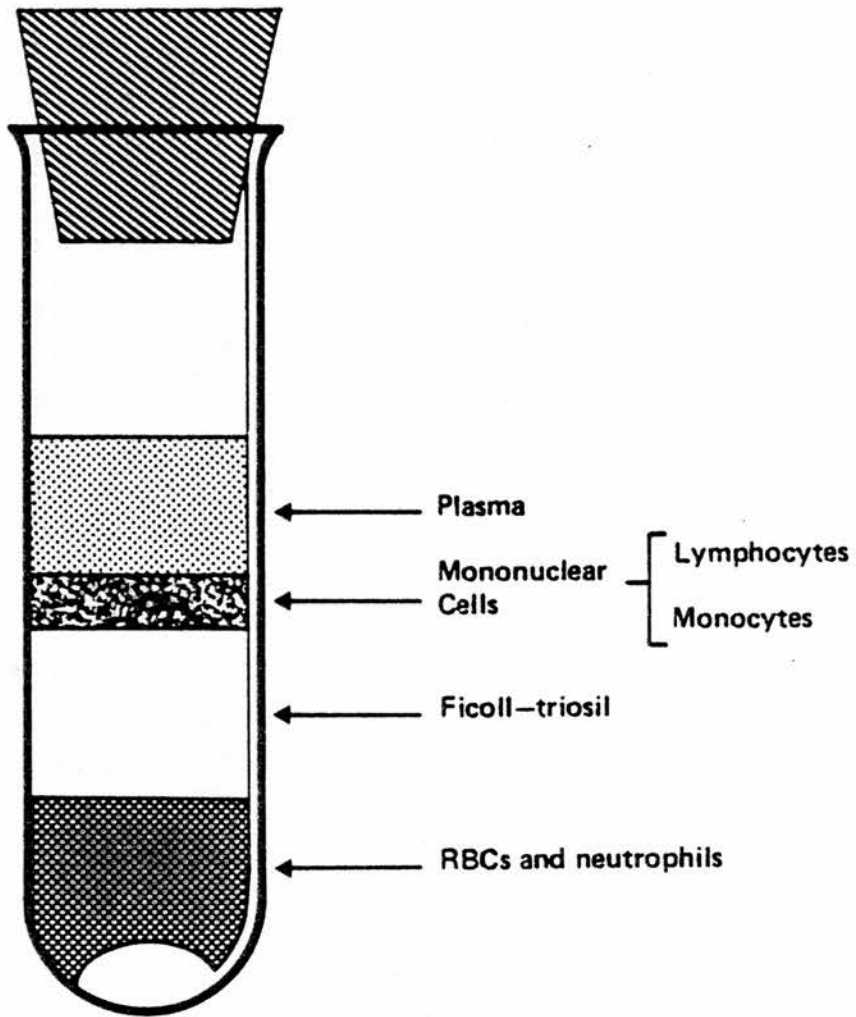
Fig.2   Separation of mononuclear cells

preparation. The cell pellet was then resus-
pended gently in TC 199 containing antibiotics
and 10% group AB serum to a convenient volume for
application to a nylon wool column (2.1.5 below).
The viability of the cells was checked by Trypan
blue dye exclusion and was always > 95% and
usually > 99%.

2.1.3   Preparation of Ficoll-Triosil
Solution A: 9% Ficoll
90g Ficoll (400000: Pharmacia) was dissolved in
1000 ml sterile distilled water and kept at $37^{o}C$
in a water bath.

Solution B: 33.9% Triosil
200 ml Triosil 440 (Nyegaard & Co) was made up to
445 ml with sterile distilled water.

960 ml solution A was mixed with 400 ml solution
B and the specific gravity adjusted to 1.076-
1.078.   The mixed solution was distributed into
medical flat bottles in volumes of approximately
75ml each.   These were sterilised by autoclaving
for 10 min at $115^{o}C$ and then stored in the dark
at $4^{o}C$.

2.1.4   Preparation of TC 199/10% AB serum
In all the experiments the basic tissue culture
medium (Gibco Biocult) contained Earle's salts,
25mmol/l Hepes buffer, .1 mg/ml L glutamine,

supplemented with 10% group AB serum + 100 μl penicillin G and 100 μg/ml streptomycin (Difco TC pen/strep).

Group AB serum was obtained from blood donors and checked for the absence of red cell antibodies by incubating 2 drops of serum with 1 drop of a 5-10% suspension of target cells at room temperature and at $37^{o}C$ for 1h, then centrifuging at low speed. Lack of haemolysis or aggluti- nation was observed macroscopically. The AB serum was then heat-inactivated at $56^{o}C$ for 30 min to destroy complement activity, and stored in 10 ml volumes at $-40^{o}C$ until required.

2.1.5   Preparation of nylon wool column

The required amount of nylon wool (Tuko-pak, Fenwal Laboratories Ltd, approx 500 mg) was soaked in TC 199 to exclude air and then packed into a 10 ml syringe (Plasti-pak) with a 3-way tap at the nozzle (Pharmaseal). The nylon wool was pushed tightly down the syringe to remove further any trapped air bubbles, then teased out to give a loosely-packed column. This was washed through with 20ml TC 199, followed by 20-30 ml TC 199/10% AB serum. The top of the 10ml syringe was sealed with parafilm (American Can Company) and then incubated vertically for 1h at $37^{o}C$ to allow equilibration. After washing with pre-warmed TC 199 the column was ready for use.

2.1.6   Removal of monocytes by nylon wool column

The mononuclear cell suspension in 2-3 ml of TC 199/10% AB serum was added to the column and the top sealed with Parafilm. The column was then incubated horizontally for 30 min at $37^{o}C$ in an incubator. It was then rotated through 180° and incubated for a further 30 min. The non-adherent cells were eluted in a vertical position with pre-warmed TC 199: 20 ml was found to be sufficient to recover more than 90% of the non-adherent cells (Fig 3).

The cells were recovered by centrifugation, washed twice in TC 199 and finally re-suspended at the desired concentration in TC 199/10% AB serum. The viability of the red cell suspensions was between 95-99%.

The average recovery of lymphocytes was 54% and the degree of monocyte contamination was 0-1.5% (Urbaniak, 1979a), as estimated by the non-specific esterase method using α-naphyl butyrate as substrate (Li et al, 1973).

PREPARATION OF LYMPHOCYTES FROM WHOLE BLOOD

VENOUS BLOOD

↓

LAYER ONTO FICOLL—TRIOSIL

↓ Centrifuge
(400 x g for 40 min)

HARVEST MONONUCLEAR CELLS

↓ Centrifuge
(400 x g for 20 min)

CONCENTRATE

↓ Resuspend (TC – 199)

REMOVE PLATELETS

↓ Centrifuge
(200 x g for 10 min)

RESUSPEND + COUNT

↓ (TC – 199/10% AB serum)

NYLON WOOL COLUMN TO REMOVE MONOCYTES

↓ 1 h Incubation

ELUTE NON—ADHERENT CELLS

↓ Centrifuge
(200 x g for 10 min)

RESUSPEND + COUNT
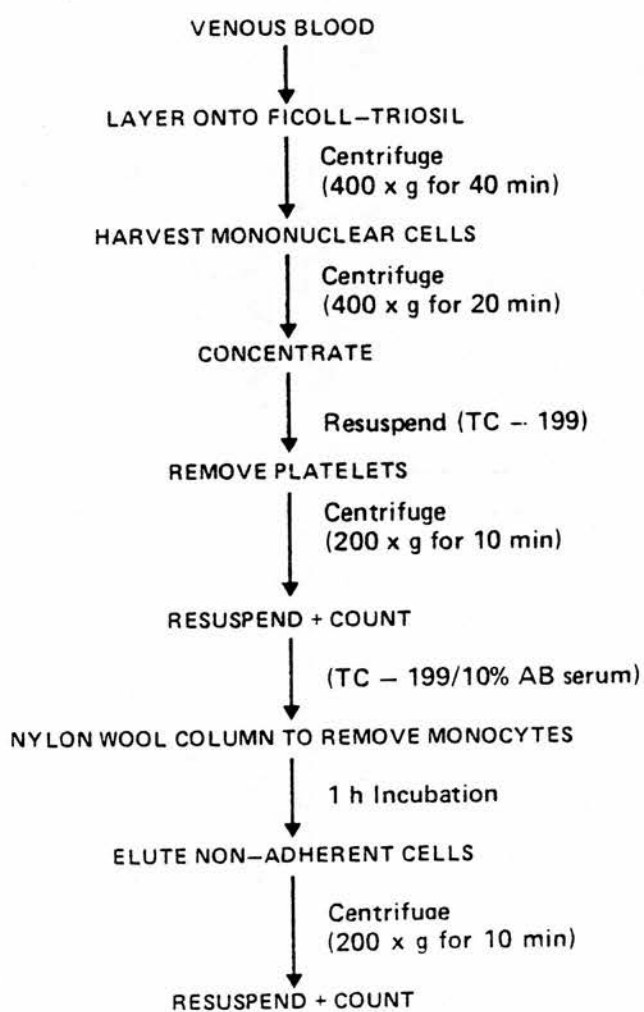
Fig 3

2.1.7 Lysis of contaminating red cells

Occasionally, when there was residual red cell contamination of the mononuclear cell suspensions prepared as above, it was important to remove these red cells. This was done by hypotonic lysis:

Composition of lysis medium:

Ammonium chloride 8g/l

Tri-sodium EDTA 1g/l

Potassium dihydrogen phosphate 0.1g/l

In sterile distilled water

The cell pellet was incubated with 2 ml of the lysis medium for 5 min at $4^{o}C$, followed by the addition of 10 ml TC 199 and then centrifugation. Two further washes in TC 199 were carried out prior to re-suspending the mononuclear cells in the appropriate medium for further experiments. Viability was always > 95%.

2.2 Identification of lymphocytes

The mature lymphocyte is normally seen in two forms in peripheral blood: the small lymphocyte (10μm) and the less numerous large lymphocyte (15-20μm). The nucleus is usually spherical but may show a slight indentation on the side of the greatest mass of cytoplasm. It can be difficult to distinguish large lymphocytes from monocytes (15-20μm) on the basis of morphology alone, although the monocyte's nucleus is larger, slightly eccentric, irregular and deeply indented or horseshoe-shaped, suggesting an aerial view of a mountain range. Three methods were applied to make sure of the absolute number of lymphocytes in the final ADCC culture. (Adherent monocytes were removed by a nylon wool column in all the experiments).

2.2.1 Phase-contrast microscopy

One drop of cell suspension (approx 5×10^6/ ml) was used to fill the chambers of a Neubauer haemocytometer which was examined under phase-contrast light at the highest convenient magnification. The granular cytoplasm of neutrophils and monocytes shows up clearly in contrast to that of lymphocytes, which have a much lighter and more uniform appearance. Since the nuclear morphology can also be seen, neutrophil polymorphs are easily recognised. The percentage of lymphocytes was estimated from a count of at least 200 cells.

2.2.2   Toluidine blue staining

The supravital stain toluidine blue is taken up
by living cells so that cytoplasmic and nuclear
morphology may be clearly distinguished.

Slides were prepared by making a 5 g/l suspension
of toluidine blue in absolute methanol, adding
one drop to a clean dry microscope slide,
followed by drying at 37°C in an incubator.   A
thin deposit of toluidine blue remained so that
one drop of cell suspension could be added
directly to the prepared dried slides, gently
mixed, a cover slip added, and the cell
suspension examined after allowing 2-3 min for
the cells to take up the dye.   The percentage
lymphocytes was assessed by counting at least 200
cells.

2.2.3   Latex particle phagocytosis

Monocytes can be identified following the
ingestion of a suitable indicator material.
Latex particles (1μm diameter; Dow-latex) are
convenient in that they are inert, readily
phagocytosed and can be easily identified within
the cell under light-microscopy.   Two or three
drops of a 1 in 100 dilution of latex suspension
(as obtained from the manufacturer) were added to
each 1 ml of TC 199/10% AB serum containing
mononuclear cells at 5-10 x 10^6/ml.   The mixture
was incubated at 37°C for 30-60 min with frequent

mixing. Excess latex particles were removed by washing in TC 199. The cells were examined under light microscopy, monocytes being identified by the presence of latex particles within the cytoplasm. Neutrophil polymorphs also ingest particles but they were distinguished from the monocytes on the basis of nuclear morphology, lymphocytes not showing ingested particles. The percentage of monocytes was calculated from a count of at least 200 cells.

2.3 Preparation of human red cells

Venous blood samples were obtained from normal donors or laboratory personnel of known ABO and Rh groups. The Rh types were confirmed by routine blood bank methods.

Blood was collected into storage medium (as used at the Central Laboratory of the Netherlands Red Cross, Amsterdam), 1 volume to 9 volumes of blood, and then stored at 4° until required. Blood stored in this way was suitable for the lytic assay for up to 3 weeks, and up to 5 weeks for other purposes (Urbaniak, 1979a).

2.3.1 "Amsterdam" storage medium

Na_2 EDTA	0.02 mol/l
Na_2 HPO_4	0.02 mol/l
Bovine albumin	10.0 g/l
Chloramphenicol	1.0 g/l

Dextrose 10.0 g/l

In distilled water

Nine volumes of whole blood were added to one volume of storage medium, mixed by inversion and stored at $4^O C$ until required.

2.3.2 Papainisation of red cells

Five drops of red cell suspension (stored as above) were mixed with an equal volume of 10 g/l papain in sterile saline (BDH Papain, papaya) for 4 min at room temperature. The cells were then washed four times in 10 ml saline, re-suspended

and counted. Papainisation of red cells was carried out prior to ^{51}Cr labelling to avoid unnecessary handling of radio-active material.

2.3.3 <u>Labelling of RBCs with ^{51}Cr</u>

20 x 10^6 papainised red cells in 100 μl normal saline were incubated at 37°C for 1h with 200 μCi ^{51}Cr (Na^{51}Cr 04-CJS4, Amersham, adjusted to 50 μCi per 10 μl with sterile saline) with frequent mixing to allow maximum ^{51}Cr uptake by the cells. After incubation, the red cells were washed x 4, re-suspended in 10 ml TC 199/10% AB serum and adjusted to an appropriate concentration for the ADCC assay.

^{51}Cr-labelled papainised red cells were used in all the ADCC assay experiments.

2.3.4 <u>Pre-sensitisation of red cells with anti-D</u>

Target cells pre-sensitized with anti-D were prepared as follows:

Labelled papainised red cells were suspended to a concentration of 8 x 10^5/ml, to give an effector/target cell ratio of 10:1 (see below). 50 μl of anti-D serum were added to target cells (50 μl) in a microplate (96-well round bottomed plates, "Removawell", Dynatech) and incubated for 1h at 37°C in an incubator. The cells were washed four times with TC 199, centrifuged in microplate tray holders, and the red cell pellets

resuspended using an orbital microplate shaker. Red cells were pelleted in the wells by centrifugation and the supernatants were discarded and blotted from the inverted plates. The pellets were re-suspended in 50 μl of TC 199/AB serum for the ADCC culture (see 2.5.1 below).

2.4   Preparation of antisera

    2.4.1   Source of anti-D sera

Several packs of citrated plasma were obtained by plasmapheresis from a donor SL (Mrs S Louden) who aborted due to haemolytic disease of the newborn as a result of anti-D immunisation. Despite the relatively low amount of anti-D in this material as determined by routine methods (see below) the biological potency of the antibody had been proven (Urbaniak, 1976-1979). This material was therefore used extensively as a biological control for the ADCC assay.

Other anti-D containing sera were obtained from routine samples received in blood transfusion centres for ante-natal screening during pregnancy, or from male and female volunteers who were undergoing plasmapheresis for the production of anti-D for therapeutic use or plasma exchange to reduce maternal anti-D concentrations in severe haemolytic disease of the newborn (HDN).

Plasma was converted to serum by the addition of one drop of thrombin solution (Parke-Davies) per ml of plasma. After incubation at $37^\circ C$ for one hour the material was centrifuged and the clear supernatant transferred into clean sterile containers. The serum was then heat-inactivated at $56^\circ C$ for 30 min to destroy complement activity. The specificity of the anti-D was

confirmed by testing against a panel of standardised red cells by conventional saline, enzyme and indirect antiglobulin methods and by an automated continuous-flow method using Technicon Auto Analyser[TM] apparatus and quantitation technique modified from the method of Gunson et al (1972) by pre-treatment with enzymes of the red cells and omission of the bromelin line, an equivalent volume being run along with the methyl cellulose in that line, which was increased in size. The National Institute of Biological Standards and Controls standard anti-D (code no 72/229) was used for calibration. A conversion factor of 5 iu=1 μg was used (Gunson and Thomas, 1979).

2.4.2   Other sera containing red cell antibodies

These sera were obtained from routine samples received in the blood transfusion centre for ante-natal screening, or from donors and patients with pre-formed antibodies of known specificity. In each instance the specificity of the antibodies was checked against a standard red cell panel using methods appropriate to that antibody, and the titre checked using a known positive-reacting red cell.

All sera were heat-inactivated at 56°C for 30 min and stored at -40°C until required.

2.4.3   Absorption of anti-D sera

Group O Rh negative (rr) and O Rh Positive (R_1R_1)
red cells were washed in normal saline and
hard-packed by centrifugation. One volume of
anti-D serum (2-3 ml) was added to an equal
volume of packed red cells of each phenotype,
incubated for 1h at 37^oC in a shaking water bath,
centrifuged and the supernatant removed. The
cells were washed three times in normal saline
and dispensed for subsequent recovery of anti-D
by elution and anti-D specific binding studies
(see 2.4.4 and 2.4.5 below). The bulk of the
supernatant was again incubated with an equal
volume of packed red cells in an attempt to
maximally deplete sera of residual anti-D.

2.4.4   Preparation of anti-D eluate

Eluates were prepared by adsorbing specific
anti-D on to group OR_1R_1 red cells (as in 2.4.3
above) and recovering the anti-D by acid elution
from red cell stroma, according to the method of
Jenkins and Moore (1977).

Briefly, this procedure follows the same general
principles as the standard method of Kochwa and
Rosenfield (1964) except that table-top
centrifugation is used in place of high-speed
spins, and pH and ionic strength are rapidly
corrected with concentrated phosphate buffers in
place of overnight dialysis.

Reagents:

Washed red cells (see above)

Digitonin suspension

0.1 mol/l glycine-HCl, pH 3.0

0.8 mol/l K_2 $NaPO_4$, pH 8.2

Procedure:

1.0 ml of packed red cells, 9.0 ml of normal saline and 0.5 ml of Digitonin were mixed well and allowed to stand for 3 min before centrifuging for 5 min and the supernatant removed. The stroma were washed with saline until free of haemoglobin (4 washes) and 2.0 ml of glycine-HCl added to the packed stroma which was mixed well and allowed to stand for 1 min. After further centrifugation for 5 min the supernatant was removed and 0.2 ml of K_2Na PO_4 added. After centrifugation to clear the precipitate the supernatant eluate was removed and tested.

2.4.5 <u>Anti-D specific binding to D-positive red cells</u>

This was measured by an immunoradiometric assay (IRMA) employing ^{125}I-labelled anti-human IgG.

Radiolabelled anti-IgG:

Affinity-purified rabbit anti-human IgG (a gift from Dr S Moore, Regional Blood Transfusion Centre, Edinburgh) was labelled with ^{125}I (Amersham International) after the method of

Hunter (1978), employing a 1:3:6 ratio of protein: chloramine-T:sodium metabi-sulphite, by weight in 0.02 mol/l Tris buffer at pH 7.4, purified on a Sephadex-A50 column (Pharmacia) and eluted with 0.01 mol/l Tris HCl.

Human group OR_1R_1 red cells were used for specific anti-D binding studies and group Orr red cells to measure non-specific background binding. Red cells in microplates (as above) were pre-incubated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at pH 7.2 with 1g/l sodium azide added, for a minimum of 4h at room temperature. Washes and dilutions were performed in 1% BSA/PBS/azide with 0.01% Tween-80 added. Red cells were pelleted in wells by centrifugation and supernatants were removed and blotted from inverted plates, followed by re-suspension of red cell pellets using an orbital microplate shaker after each wash and before adding anti-sera.

Red cells were dispensed at 10 million/ well on microplates and washed x 3 (100 μl/well). Anti-D dilutions were added at 100 μl/well, followed by thorough mixing, and the plates were incubated for 18h at room temperature. Following 4 washes, ^{125}I-labelled anti-IgG was added (100 μl/well) and the plates incubated for a further 90 min at room temperature followed by 5 washes. Residual

^{125}I radioactivity associated with red cell pellets was determined by counting individual wells in a gamma-counter. All tests were performed in triplicate and the results expressed as means of the three readings.

2.4.6   Anti-D Fc-receptor activity

Red cells (Group OR_1R_1) were washed in PBS. One volume of packed red cells was mixed with an equal volume of anti-D serum (serum from a group AB Rh positive donor being used as a control) and incubated at $37^\circ C$ for 1h. The sensitized red cells were washed 5 times in TC 199 and made up as a 2% suspension. Washed peripheral blood mononuclear cells were incubated with latex for phagocyte identification (Urbaniak et al, 1978) and made up to 3 million/ml in TC 199 for Fc-rosetting. Equal volumes of red cells, lymphocytes and heat-inactivated foetal calf serum were added to round-bottomed tubes (Luckham, LP3), pelleted by gentle centrifugation (150xg), incubated at $37^\circ C$ for 15 min, and incubated on ice for 60 min. Cell pellets were gently re-suspended by aspiration. Mononuclear cells were stained with dilute acridine orange and lymphocyte rosette proportions (at least 2-3 red cells adhering) were determined in wet (coverslip) preparations viewed by incident fluorescent microscopy with background-trans-mitted red illumination.

2.4.7   IgG subclasses of anti-sera

Antisera to IgG_1, IgG_2, IgG_3 and IgG_4, suitable for use in agglutination assays were purchased from the Central Laboratory of the Netherlands Red Cross, Amsterdam.

Anti-D sera:

Volumes of the same D-positive (0 CDe/ CDe) red cells used for ADCC assays were washed three times with normal saline, suspended to 5-10% then incubated with equal volumes of doubling dilutions of anti-D serum at $37^{\circ}C$ for 1h in a water bath or an incubator. After four washes in normal saline the sensitized cells were adjusted to a 2-5% suspension in low ionic strength saline. A 1 in 20 dilution of each anti-IgG subclass serum was prepared in 1% saline albumin. Equal volumes of red cell suspension and IgG subclass serum (20 μl) were incubated at $37^{\circ}C$ for 15-30 min in a 75 x 10 mm glass tube, pelleted by gentle centrifugation and the end-point agglutination determined macroscopically. Rh(D) negative (Orr) cells processed identically were used as a negative control.

Anti-c sera:

These were treated as for anti-D (see above) except that c-positive red cells (0 cde/cde) were sensitized by anti-c, and Rh(c) negative cells (0 CDe/CDe) used as the negative control.

2.4.8   Enrichment and depletion of serum IgG

Anti-Rh(D) sera were treated with ammonium sulphate (using the method of Heide and Schwick, 1978) or polyethylene-glycol (PEG) 4000 (using the methods of Iverius and Laurent, 1967 and Polson and Ruiz-Bravo, 1972) to precipitate serum IgG.

PEG Precipitation:

PEG 4000 (1.5g) was added to 50 ml of serum at $4^{\circ}C$, with constant stirring, to give a 3% (30 g/l) solution. After stirring for 60 min, the precipitate which formed was collected by centrifugation at 1800 xg. A further 6g of PEG was then added to the supernatant while stirring to give a 15% (150 g/l) solution. The precipitate which formed after 60 min was again collected by centrifugation. This IgG-enriched fraction was re-dissolved in 50 ml saline (9 g/l Nacl).

Ammonium sulphate precipitation:

Ammonium sulphate powder was added to 50 ml serum to give a final molarity of 1.82 (approximating to 45% saturation with ammonium sulphate). The solution was kept at room temperature overnight and the precipitate then collected by centrifugation (as with PEG precipitation) and dissolved in 50 ml saline. Both precipitate-solution and supernatant serum were dialysed against several

changes of saline at $4^{O}C$ for 3 days. The precipitate solution was labelled "IgG-enriched fraction" and the supernatant "IgG-depleted serum".

IgG-enriched fractions (PEG or ammonium sulphate methods) contained >90% of original serum IgG as measured by nephelometry.

2.4.9   Dilution of anti-Rh sera for culture

Anti-D or anti-c sera were diluted with heat-inactivated red cell antibody-free sera from normal group AB donors, so that final total immunoglobulin concentrations were comparable for each dilution of the anti-sera.

50 μl of diluted antibody was added at three times the desired concentration to ADCC cultures to produce in each case a final volume of 150 μl.

2.5 Culture conditions for ADCC assay

All the experiments were set up in triplicate and including the following controls:

i) ^{51}Cr-labelled papainised red cells in TC 199/ 10% AB serum, to assess spontaneous ^{51}Cr release.

ii) ^{51}Cr-labelled papainised red cells in the presence of anti-sera, to assess potential cytotoxicity.

iii) ^{51}Cr-labelled papainised red cells in distilled water or 1% Triton X, to assess maximum ^{51}Cr release possible.

iv) ^{51}Cr-labelled papainised red cells without sensitising antibody, in the presence of the effector cells to assess any direct cyto- toxicity.

The test cultures contained a known number of ^{51}Cr-labelled papainised target red cells (usually group O R_1R_1), a known number of lymphocyte effector cells with the effector/target (E/T) ratio usually 10:1, and anti-D sensitising antibodies. The anti-D was either added directly to the cultures at the beginning of the incubation period as a known amount of serum, or used to pre-sensitize the papainised target cells as described above.

The ADCC cultures were set up in microplates (96-well round-bottomed "Removawell" plates, Dynatech) which

could be processed and centrifuged as a single unit.

The culture conditions involved incubation of the cell mixture at $37^{\circ}C$ in a humidified atmosphere containing 5% CO_2/air for 15-18 h (overnight).

2.5.1 <u>ADCC assay</u>

Effector and target cell suspensions were prepared as above and adjusted so that the final concentration of effector cells was 8 x 10^6/ml and that of the ^{51}Cr-labelled papainised target cells was 8 x 10^5/ml. 50 μl of effector cell suspension was dispensed (using a micropipette) to give an E/T ratio of 10:1 with 4 x 10^5 effector cells and 4 x 10^4 target cells per culture. If anti-D pre-sensitized target cells had been prepared the culture volume was made up to 150 μl by the addition of 50 μl of TC 199/10% AB serum. If anti-D was to be added directly to the culture then the appropriate dilution of serum was added in 50 μl amounts to the appropriate cultures to give a final volume of 150 μl.

The lower limit of 4 x 10^4 red cells was chosen as the minimum number which gave satisfactory total counts of ^{51}Cr with minimum spontaneous ^{51}Cr release (Urbaniak, 1979a).

The spontaneous ^{51}Cr release and maximum ^{51}Cr release consisted of 4 x 10^4 red cells in 150 μl of TC 199/10% AB serum or distilled water. The microplates were sealed with self-adhesive sheet (Linbro) to minimise evaporation and then covered with a plastic lid (Removawell). Effector and target cells were brought into contact at the beginning of incubation by centrifuging the microplate at 200 xg for 10 min in microplate tray holders (MSE) followed by incubation at 37^oC in a humid 5% Co_2/Air incubator for 15-18h (overnight).

After incubation the plates were centrifuged for 5 min at 400 xg as above and 100 μl of super-natant removed by a micropipette into LP3/tubes. The radio-activity was assessed as below.

2.5.2 Assessment of cytotoxicity

The principle of the cytotoxic assay is that ^{51}Cr is incorporated into the red cell and bound irreversibly to intracellular proteins (Bunting et al, 1963). Furthermore, the ^{51}Chromate ion is converted to ^{51}Chromic ions which are then impermeable to the cell membrane. Extracellular release of ^{51}Cr therefore represents a breach in the red cell membrane and this has been taken to represent cell lysis. It has been shown that % red cell lysis correlates with % ^{51}Cr release under the culture conditions used (Urbaniak,

1979a). The various controls described above were used to estimate spontaneous ^{51}Cr release and maximum releasable ^{51}Cr. This was compared with the ^{51}Cr release from red cells in the presence of effector mononuclear cells, with or without antibody.

The % ^{51}Cr release was calculated as follows: volumes of cell-free supernatant collected as above were asssessed for ^{51}Cr activity using a gamma-counter adjusted to maximum sensitivity for this isotope. The total supernatant ^{51}Cr activity was assessed by multiplying the super- natant fluid count by a factor of 1.5 (100 μl volumes from a total of 150 μl culture).

Spontaneous release controls and maximum release controls were incorporated with every experiment and the degree of specific lysis of antibody-sensitized red cells was calculated and expressed as percent specific lysis as shown below.

% specific lysis =

$$\frac{(\% \ ^{51}Cr \ release \ in \ test \ culture)*-(\%^{51}Cr \ spontaneous \ release)+}{(\% \ maximum \ ^{51}Cr \ release) \ \ddagger-(\%^{51}Cr \ spontaneous \ release)} \times 100$$

The spontaenous ^{51}Cr release was usually similar in TC 199 alone or with anti-D containing material, in which case the average was used for calculations: if the spontaneous release was significantly higher in the presence of any material other than TC 199 the higher value was used in the calculation of % specific lysis.

*   Effector cells + red cells (with or without anti-D)

+   Red cells + TC 199 (or anti-sera) - see text

‡   red cells + distilled water, or 1% saponin

CHAPTER 3

RESULTS

3.1   Characteristics of standard ADCC assay

In a biological system one inevitably finds variation between one individual and another, and within the same individual on different occasions. In the present study it was necessary to minimise errors and variability due to the technique itself, and also to know the magnitude of these errors in order to interpret differences observed during in vitro investigations into the activity of the lymphocyte (K-cell) on the lytic potential of the antibody in the ADCC assay. Therefore the anti-D serum of S Louden (SL) was used as a biological control for all the experiments, since it has been shown that this antibody has high biological potency (Urbaniak 1979 a, b).

3.1.1   Specificity of lysis of D-positive red cells by anti-D (SL)

Anti-D serum (SL) was diluted with AB serum in an ADCC assay standardised at an E:T ratio of 10:1 and the results are shown in Fig 4.   A dose-response type curve was obtained with a gradual decrease of per cent specific lysis to a barely detectable level at 1 in 96 (the end point). This suggests that at this point there are in-sufficient anti-D molecules per red cell to in-duce K-cell lysis.   The anti-D concentration of this particular serum, when undiluted, was deter-mined by an automated continuous-flow method to be in the region of 6 iu/ml.   A dilution of 1 in 96 in the final culture therefore indicates

SPECIFICITY OF LOUDEN'S ANTI–D LYSIS OF D–POSITIVE
(OR$_1$R$_1$) AND D–NEGATIVE (Orr) RBC AT VARIOUS DILUTIONS
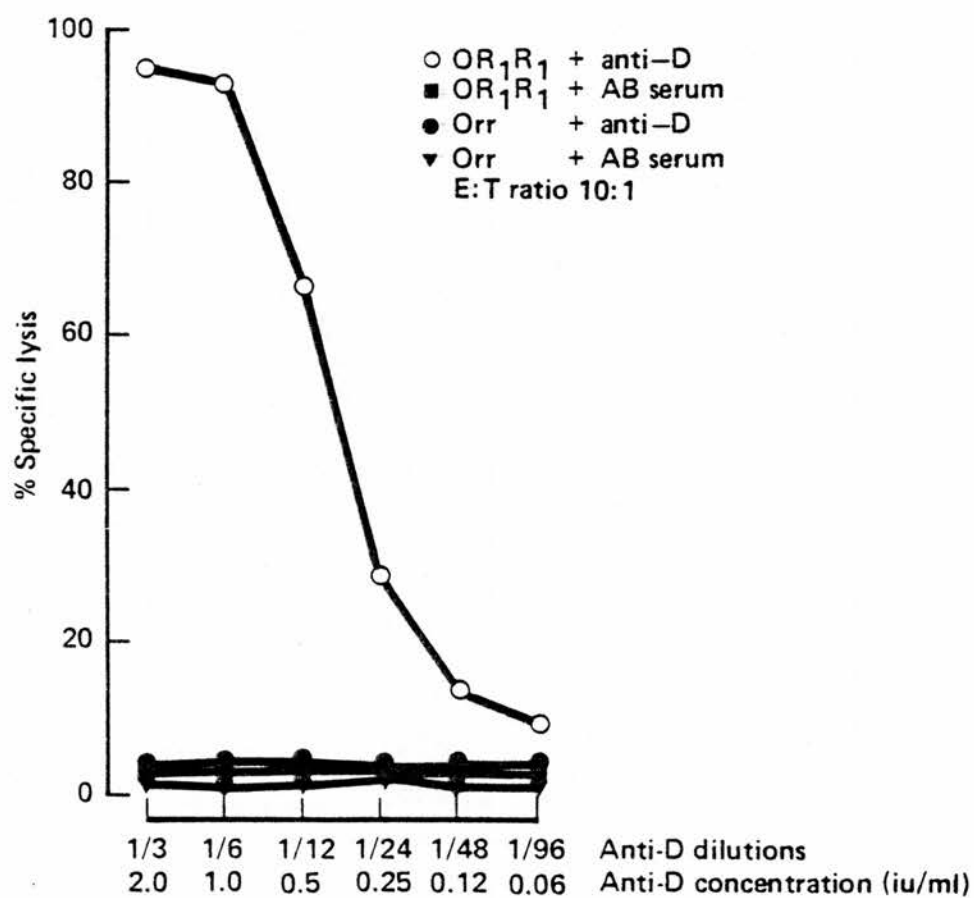
Fig. 4

activity at approximately 0.06 iu/ml (0.01 μg/ml) with a maximum of 97% specific lysis seen. It is interesting to note that the antiglobulin titre of this anti-D serum was only 1 in 16 but it could be diluted up to 1 in 96 in a test for K-cell lysis.

As shown in Fig 4, group Orr red cells were not lysed in the presence of anti-D (ie they gave the expected reactions of a negative control) and showed similar % specific lysis in anti-D or in antibody-free group AB serum. The degree of specific lysis was similar to that seen with effector cells and D positive red cells in the presence of antibody-free AB serum (ie only background release). Similar results were obtained when anti-D serum (SL) was used undiluted (ie 1 in 3 in the final culture) with effector:target ratios of 10:1, 5:1, 1:1, 1:5 and 1:10 using cells from the same lymphocyte donor (Table 4).

3.1.2 ADCC lytic activity of anti-D (SL) using various effector:target cell ratios

Effector cells were obtained from ten healthy individuals (members of the laboratory staff), five males and five females of different blood groups (Fig 5). The anti-D was used undiluted (giving a final dilution of 1 in 3 in the final culture) and this gave maximum % specific lysis

Table 4

Specificity of SL's Anti-D Lysis of D-positive and D-negative RBC in ADCC assay

	% Specific Lysis with							
	D Pos (OR_1R_1) RBC		D Pos (OR_1r) RBC		D Pos (OR_2R_2) RBC		D Neg (rr) RBC	
E:T Ratio ***	* + Anti-D	** + AB Serum	+ Anti-D	+ AB Serum	+ Anti-D	+ AB Serum	+ Anti-D	+ AB Serum
10:1	83.6	8	80.5	-4	83	7	-2.6	-8
5:1	63	4	59	3	67	7	-4	-5
1:1	47	3	45	7	48	4	-1.5	0
1:5	21	6	23	-2	24	-1	1	-2
1:10	17	2	14	1	11	2	-6.5	0

* Anti-D at final dilution 1/3
** AB Serum final dilution 1/3
*** The same K-cell donor (Fig 1) 4×10^4 RBC per culture at 10:1 E:T ratio

(A negative value indicates that ^{51}Cr release in the test sample was lower than the control value and the ADCC activity was effectively zero).

at E:T ratios of 10:1, 20:1, 30:1 and 40:1 respectively. The anti-D was therefore assumed to be present in excess. It can be seen that a sigmoidal dose-response type of curve was obtained from the mean % specific lysis at each E:T ratio for the ten individuals. There was a relatively linear increase in specific lysis with the increase in the number of effector cells, over the range of E:T ratios from 1:5 to 10:1 (0.8×10^4 to 40×10^4 mononuclear cells per culture). Very little lysis was seen below 1:5, where presumably the effector cells were unable to lyse sufficient numbers of red cells in the presence of anti-D (SL) to give ^{51}Cr release above background spontaneous release. A plateau effect began to appear at an E:T ratio of 10:1 with maximum % specific lysis at a mean of 89.9 ± 9.57. There was no significant difference between E:T ratios of 40:1, 30:1, 20:1 and 10:1 ($p > 0.05$), but there was a significant difference between E:T ratios of 10:1 and 5:1, 1:1, 1:5, 1:10 ($p < 0.000$ 1 Spearman rank correlation.

3.1.3 Specificity of antibody and antigen for ADCC lysis

To show that anti-D (SL) is specific for D-positive red cells, and that anti-c (obtained from a donor with a history of pregnancies affected by haemolytic disease of the newborn) is
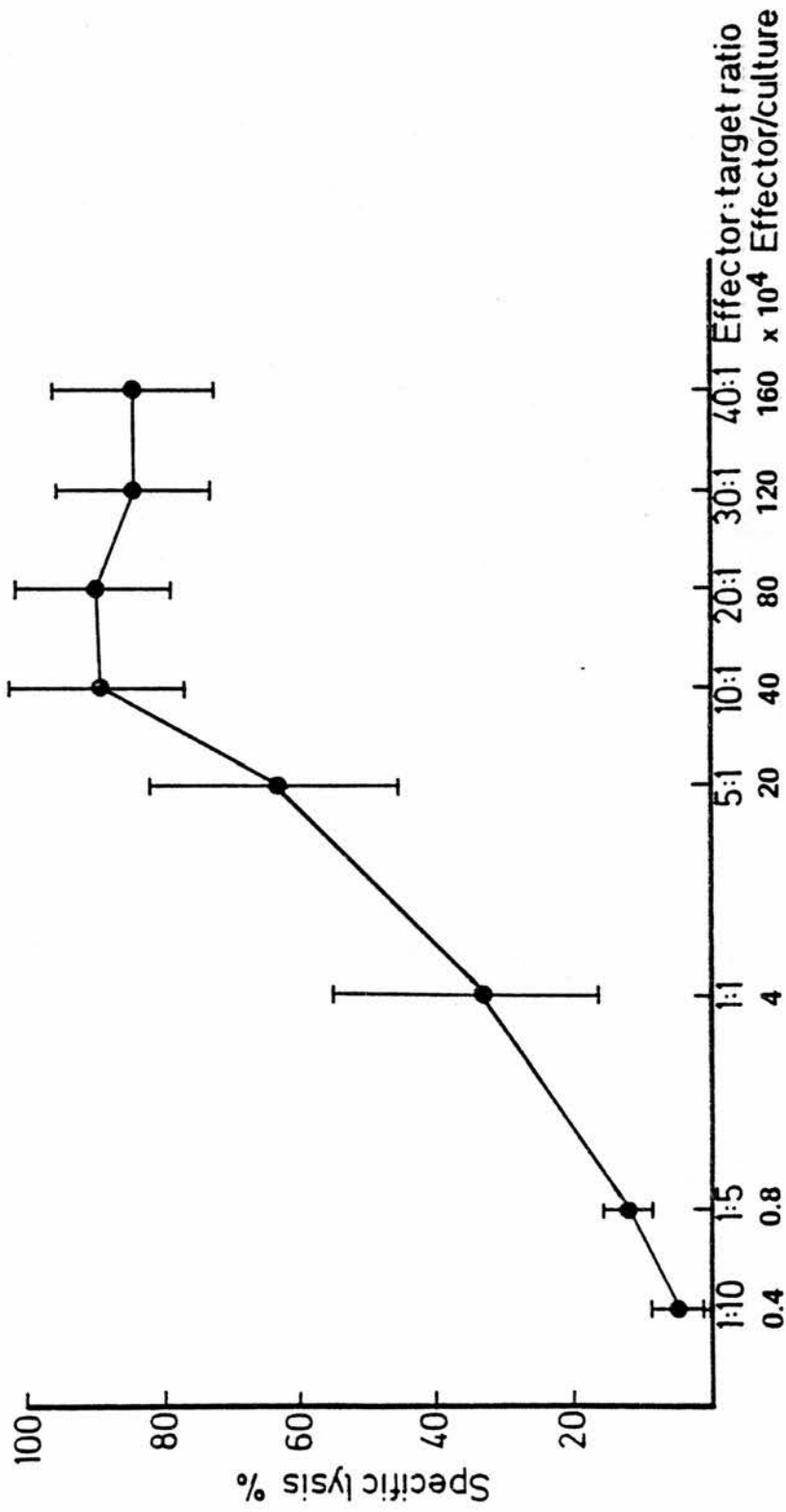
Anti-D (SL) Neat (1 in 3 in the final culture)

Healthy individuals (5 males and 5 females) tested at various effector/target ratios.

Fig. 5

specific for c-positive cells, controls set up were Orr (cde/cde) and OR_1R_1 (CDe/CDe) respectively (ie cells expected to give negative reactions with the two antibodies). Lymphocytes were obtained from four healthy individuals of different blood groups. The effector/target cell ratio was fixed at 10:1 (4×10^4 red cells in the final culture).

As shown in Tables 4 and 5, group Orr red cells were not lysed in the presence of anti-D and effector cells, and gave a similar ^{51}Cr release whether in anti-D or in antibody-free group AB serum. The degree of specific lysis was similar to that seen with effector cells and D-positive red cells in the presence of antibody-free AB serum (ie only background release). The same pattern of reaction (no lysis) was seen with anti-c when OR_1R_1 cells were used as a negative control.

It is also interesting to note that homozygous (D/D - c/c) red cells gave better specific lysis than did heterozygous cells (OR_1r, D/d - C/c) in the presence of the anti-D and anti-c respectively.

Table 5

Specificity of antibody for ADCC lysis

E:T Ratio *	Lymphocyte Donor Group	% Specific Lysis with					
		Anti-D			Anti-c		
		Orr	OR_1r	OR_1R_1	Orr	OR_1r	OR_1R_1
10:1	A rr	7	83	96	92	85	1
10:1	O rr	3	78	89	88	80	-1
10:1	B R_2r	4	69	75	79	70	2
10:1	O R_1R_1	6	81	90	87	78	5

E:T 10:1 (4×10^5 effector cells in the final culture)

(A negative value indicates that ^{51}Cr release in the test sample was lower than the control value and the ADCC activity was effectively zero).

3.1.4   Intrinsic K-cell activity of normal individuals
        in the ADCC assay

During the course of preliminary experiments
defining the effector cells in the ADCC assays it
was noted that certain individuals gave
consistently good or poor responses in terms of %
specific lysis obtained under standard
conditions. It has been noted that females have
lower K-cell activity than males when human
lymphocyte target cells are used (Kovithavongs et
al, 1974, Trinchieri et al, 1977) but there is no
sex-linked difference in intrinsic K-cell
activity with human red cell targets
(Kovithavongs et al, 1975).

3.1.5   Influence of donor sex and age on ADCC

Effector cells were obtained from 120 individuals
(68 males and 52 females). Laboratory personnel
and blood donors in good health and taking no
medication were used as normal donors, with ages
between 19 and 62 years and of different blood
groups. The assay systems were comparable with
E:T ratios of 20:1, 10:1, 5:1, 1:1 and 1:10, and
with an excess of anti-D (SL) serum and group
OR_1R_1 target cells. Results shown in Fig 6 were
obtained from the first test on each donor.
Individual variations were noted but no
significant differences in the mean values were
found when males were compared with females
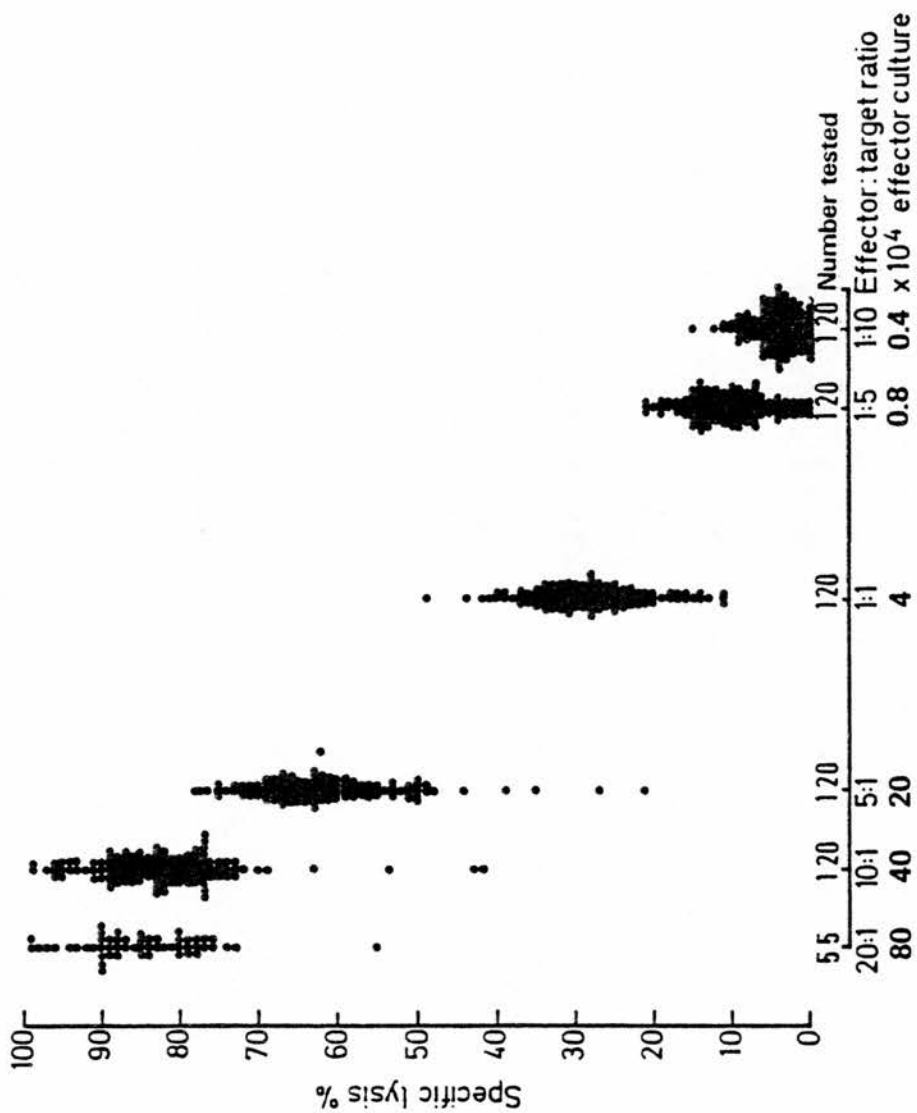
Anti-D (SL) Neat (1 in 3 in the final culture)

Healthy individuals (68 males and 52 females) tested on a single occasion.

Fig. 6

Table 6

Significance evaluation of lymphocyte ADCC between males and females

E:T Ratio	*Number of Males Tested	Mean	** SD	Number of Females Tested	Mean	S D	Significance Value
20:1	31	84.9	+- 7.87	24	85.75	+- 7.37	p > 0.05
10:1	68	82.34	+- 8.50	52	82.32	+- 9.46	p > 0.05
5:1	68	61.63	+- 8.81	52	60. 6	+- 9.57	p > 0.05
1:1	68	28.44	+- 7.75	52	28.76	+- 7.07	p > 0.05
1:5	68	14.25	+- 6.45	52	14.65	+- 6.05	p > 0.05
1:10	68	4.49	+- 3.10	52	4.21	+- 2.99	p > 0.05

* Total of 120 healthy individuals tested (68 males and 52 females, from Fig 6)
** Standard deviation

There is no significant difference in lymphocyte ADCC activity between males and females (Spearman rank correlation)

(Table 6), suggesting that effector cell capability in mediating cell lysis is independent of the donor's sex.

3.1.6 <u>Evaluation of K-cell activity in males and</u> <u>females over different periods of time</u>

Twenty females and 22 males (age range 25 to 44) were selected at random from the same group of 120 normal individuals. Sixteen of the females who were menstruating normally and with ages between 19 and 35 years were evaluated on a sequential basis to analyse the effects of time and hormonal changes on K-cell activity, each donor being tested at least once a week. The assays were standardised with E:T ratios of 20:1, 10:1, 5:1, 1:1, 1:5, 1:10 and an excess of anti-D (SL) serum, OR_1R_1 cells being used as target cells. Tables 7 and 8 present the mean percent specific lysis of each donor ($\pm$ standard deviations). From these data no obvious hormonal influence was discernable. Comparison between females and males of specific lysis demonstrated no significant difference ($p > 0.05$ - Spearman rank correlation). However, two out of the 42 donors (MG male and JW female) showed generally poor K-cell activity, never reaching values of 60% specific lysis at an E:T ratio of 10:1 (Figs 7 and 8).

SUBJECT	TIMES TESTED	AGE	* 20:1	10:1	5:1	1:1	1:5	1:10
J.D.	26	38	** 86 ±8.6	80 ±8.4	65 ±9.5	28 ±6.2	10 ±2.3	3 ±1.5
J.W.	23	42	88 ±5.7	79 ±9.2	59 ±8.3	28 ±5.7	10 ±2.7	4 ±2.2
G.P.	18	35	77 ±14.4	77 ±11.1	65 ±11.1	28 ±8.3	11 ±5.0	5 ±3.9
T.P.	10	36	80 ±12.9	77 ±8.2	58 ±7.7	25 ±7.3	10 ±6.6	5 ±4.9
A.A.	8	30	NT	78 ±9.6	68 ±9.5	34 ±8.7	10 ±4.1	4 ±2.2
H.B.	4	38	80 ±5.3	73 ±4.1	59 ±5.1	28 ±3.2	13 ±2.6	6 ±2.7
P.A.	9	25	79 ±7.7	78 ±7.4	63 ±5.9	34 ±8.0	19 ±6.7	9 ±5.7
R.W.	9	44	89 ±7.8	85 ±9.3	72 ±9.5	37 ±7.6	21 ±7.2	8 ±6.9
S.A/K.	15	29	90 ±1.5	83 ±5.6	69 ±11.7	28 ±9.6	7 ±6.0	3 ±2.7
I. EH.	17	27	85 ±10.1	78 ±9.8	67 ±12.7	27 ±9.3	7 ±5.8	3 ±2.6
K.L.	3	26	87 ±5.2	89 ±10.1	69 ±5.0	25 ±10	15 ±8.1	2 ±2.0
B.P.	11	26	88 ±6.0	77 ±8.5	61 ±8.5	27 ±6.2	13 ±7.4	3 ±2.8
J.B.	6	25	82 ±5.2	81 ±5.4	59 ±8.3	35 ±16.1	11 ±6.3	3 ±3.8
J.K.	5	35	NT	74 ±7.6	50 ±6.7	21 ±10.0	10 ±6.0	5 ±3.6
M.G.	30	34	55 ±5.0	43 ±6.3	35 ±3.5	12 ±4.7	6 ±3.6	2 ±2.1
P.T.	3	32	NT	79 ±6.0	61 ±5.2	29 ±7.5	14 ±3.2	9 ±6.8
A.W.	7	25	85 ±5.9	83 ±5.7	63 ±4.9	36 ±9.9	9 ±6.5	3 ±2.4
M.M.	7	27	NT	82 ±6.1	58 ±6.3	31 ±5.0	12 ±4.5	8 ±3.2
R.B.	9	29	NT	54 ±8.1	27 ±5.1	14 ±3.6	7 ±3.2	3 ±3.2
A.WT.	9	34	80 ±7.8	83 ±7.1	63 ±8.3	32 ±9.4	15 ±4.6	5 ±4.1
A.R.	21	45	84 ±3.8	86 ±7.5	75 ±9.4	44 ±13	13 ±4.4	6 ±2.9
J. BG.	18	34	86 ±7.1	86 ±8.0	72 ±10.8	41 ±9.5	14 ±3.3	7 ±2.3

* Effector : Target Ratio 20:1 (8×10^4 red cell per culture)

** Mean (± SD) Values

Mean (± SD) values of ADCC activity for 20 normal females randomly selected

SUBJECT	TIMES TESTED	AGE	* 20:1	10:1	5:1	1:1	1:5	1:10
J.M.	15	24	**92 ± 3.5	84 ± 5.6	65 ± 6.5	30 ± 6.5	9 ± 5.0	5 ± 5.1
A.L.	37	27	89 ± 7.4	89 ± 6.7	78 ± 10.2	42 ± 13.6	17 ± 6.6	15 ± 1.9
A.E.R.	11	62	80 ± 7.8	79 ± 7.3	63 ± 8.2	30 ± 7.8	14 ± 3.2	4 ± 3.2
M.Mc.	3	25	79 ± 2.2	75 ± 1.0	59 ± 5.0	29 ± 3.6	13 ± 5.5	3 ± 3.0
G.T.	14	56	85 ± 12.2	78 ± 7.8	66 ± 7.2	32 ± 6.8	18 ± 5.9	8 ± 5.3
S.A.	8	35	NT	77 ± 6.7	58 ± 9.7	31 ± 6.7	7 ± 1.3	4 ± 1.5
A.S.	4	29	74 ± 11.6	79 ± 11.5	61 ± 11.6	35 ± 3.2	8 ± 1.0	4 ± 0.5
P.F.	5	48	78 ± 1.7	77 ± 1.4	62 ± 3.5	33 ± 4.2	10 ± 2.1	4 ± 0.7
M.G.	4	32	81 ± 2.1	75 ± 6.0	56 ± 5.5	31 ± 5.8	14 ± 4.3	9 ± 6.5
C.E.	3	26	78 ± 6.7	77 ± 6.4	55 ± 10	22 ± 2.6	19 ± 5.2	2 ± 3.0
G.L.	11	24	76 ± 6.0	77 ± 6.9	56 ± 6.3	28 ± 6.8	14 ± 9.1	3.8 ± 4.4
C.W.	6	30	90 ± 9.2	89 ± 6.6	75 ± 3.7	49 ± 6.3	21 ± 5.0	8 ± 3.4
P.R.	7	31	79 ± 10.2	77 ± 7.9	53 ± 10.7	20 ± 9.4	10 ± 4.6	3 ± 3.4
J.D.	19	28	88 ± 6.0	83 ± 7.1	50 ± 10.2	24 ± 6.4	11 ± 4.5	3 ± 3.7
W.Mc.	3	19	NT	75 ± 1.0	53 ± 2.6	26 ± 9.5	6 ± 4.0	2 ± 3.4
L.Mc.	6	27	NT	77 ± 8.1	58 ± 5.0	29 ± 5.8	14 ± 4.2	4 ± 3.0
S.W.	9	25	NT	72 ± 3.2	50 ± 5.4	24 ± 7.7	13 ± 3.8	3 ± 2.4
J.W.	16	24	NT	42 ± 13.8	22 ± 10.0	12 ± 6.0	4 ± 4.1	2 ± 3.6
M.B.	3	60	NT	73 ± 3.2	49 ± 4.0	32 ± 3.0	12 ± 3.0	2 ± 2.6
I.D.	6	25	NT	63 ± 11.5	44 ± 9.1	11 ± 8.8	4 ± 6.0	3 ± 5.8

* Effector : Target Ratio 20:1 (8×10^4 red cells per culture)

** Mean (± SD) Values
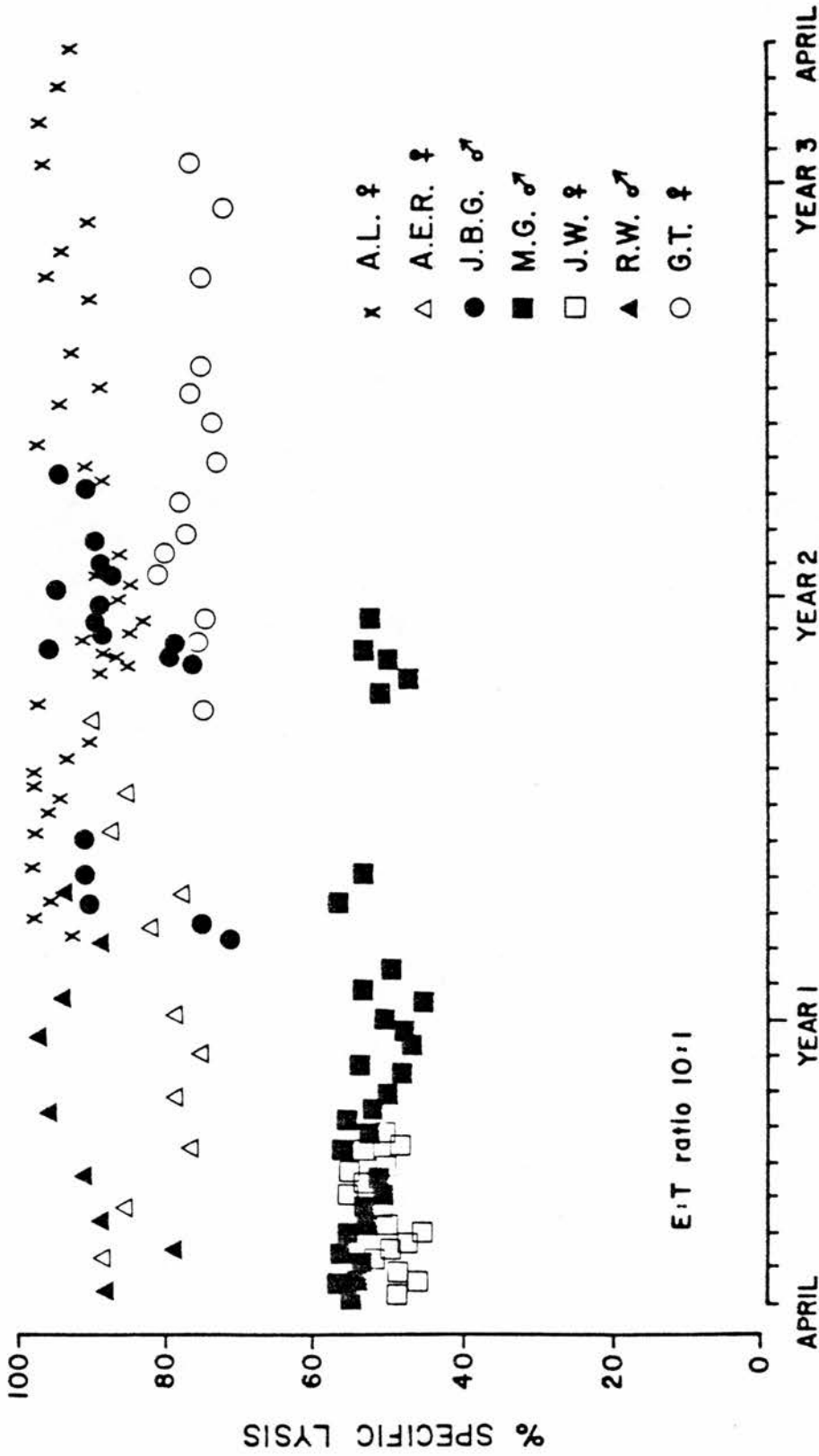
LONGITUDINAL STUDY OF SEVEN HEALTHY INDIVIDUALS SHOWING DIFFERENCES BETWEEN
GOOD AND POOR RESPONDERS (DATA FROM TABLES 7 & 8).

Fig. 7

3.1.7   Comparison between good and poor K-cell activity

Two K-cell donors were selected (Tables 7 and 8) to demonstrate the difference between good and poor K-cell activity in terms of specific lysis. The female (AL) and the male (MG) were tested at intervals of two days for eighteen days (Fig 8). Significant differences were found between good and poor K-cell cytotoxicity at each E:T ratio (p < 0.01 Spearman rank correlation). Also, testing of AL on the first day of the menstrual cycle again confirmed that there was no hormonal influence on ADCC activity.
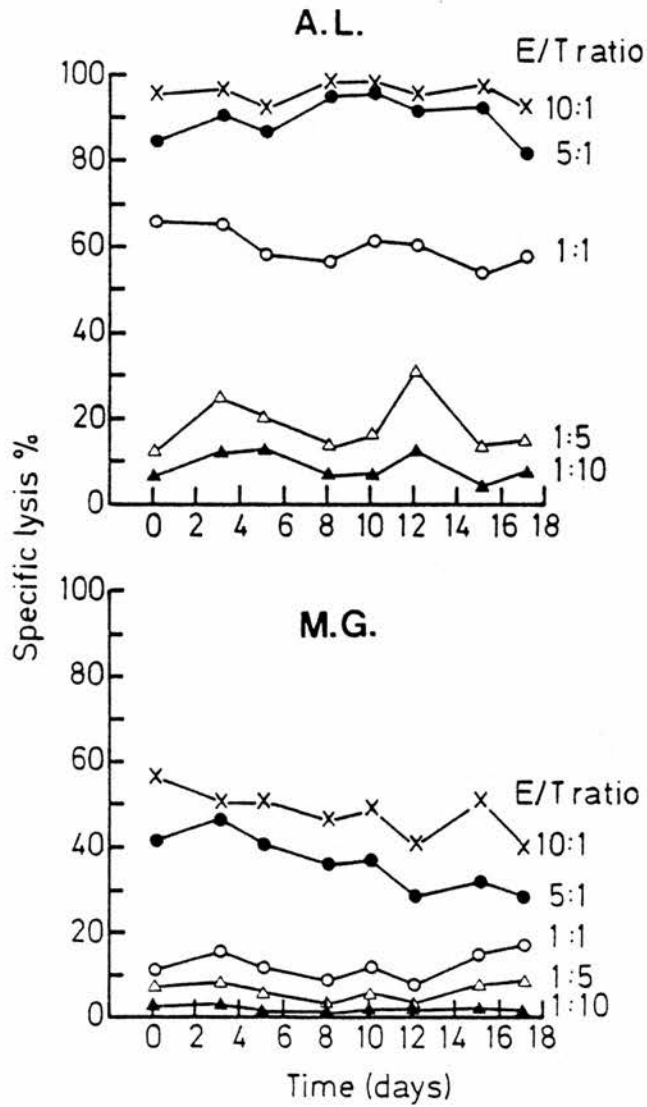
Anti-D (SL) Neat 1 in 3 in the final culture
E/T ratio 10:1 (4x10^4 OR$_1$R$_1$ red cells per culture)
Two healthy individuals (AL, good responder;
MG poor responder, from Table 7 & 8) studied
at different E/T ratios over a period of time.

Fig. 8

3.2 ADCC activity of anti-D from different sources

Since the preliminary experiments involved in demonstrating the existence of ADCC among normal individuals used only one anti-D serum (SL), it was important to demonstrate that other sources were capable of specific lysis of D-positive red cells.

A comparative experiment was performed with a single K-cell donor and anti-D (SL) as a control, the only culture variable being the source of anti-D. Eleven samples of anti-D from women immunized during pregnancy, and six anti-D sera from Rh(D) negative male volunteers who had been immunized for production of anti-D immunoglobulin, were tested for cytolytic activity. Anti-D from the same donor was numbered 1 or 2 to distinguish sera taken on different occasions at an interval of 6 weeks.

The standardized ADCC assay was used at an E:T ratio of 10:1 with the anti-D used undiluted (ie 1 in 3 in the final culture). Results are shown in Table 9. Specific lysis of D-positive red cells occurred with all samples tested, except those of GP and JDu (in both 1 and 2). However, the degree of lysis bore no obvious relationship to the concentration of the anti-D as determined by continuous-flow quantitation. Unexpectedly, it was also found that sera from females were more lytic than those from males, despite the findings using conventional methods.

Table 9

ADCC activity of different anti-D sera (1)

Source of Anti-D		ADCC activity % sp. lysis *	Continuous-flow quantitation (iu/ml) **	Manual titration	
				Enzyme	I A G T
MMc	(F)	69.0	5.35	16	256
SL	(F)	86.5	6.00	16	16
IM	(F)	89.0	6.25	32	64
HL	(F)	62.0	12.10	64	256
SH	(F)	65.0	13.50	64	64
LK 1	(F)	55.0	22.50	32	128
IP 1	(F)	82.0	24.40	32	128
LC 1	(F)	21.0	33.70	128	256
MG 1	(F)	75.0	35.50	128	256
VMc 1	(F)	62.0	35.55	64	128
KP	(F)	38.0	69.25	128	512
GW	(M)	39.2	84.95	128	512
GP 1	(M)	-1.8	95.45	256	2048
WR	(M)	76.6	98.55	512	1024
DS 1	(M)	75.1	117.20	256	512
JDu 1	(M)	-6.0	126.20	256	1024
GR	(M)	26.4	351.75	256	1024

* E:T ratio 10:1 (4 x 10^4 OR_1R_1 red cells per culture)
5 iu = 1 μg anti-D

Continuous-flow quantitation results in rank order.

(A negative value indicates that [51] Cr release in the test sample was lower than the control value and the ADCC activity was effectively zero).

The same comparison was repeated with a smaller number
of sera (4 from females, 5 from males) tested on the
same day with the same K-cell donor and under identical
culture conditions. Similar results were obtained, the
sera from female donors being more active than those
from males, despite their containing less anti-D as
measured by continuous-flow quantitation (Table 10). At
highest concentrations there was suggestion of prozone
phenomenon which might have caused the low ADCC activity
in male sera.

3.2.1   Comparison of male and female anti-D sera in the
        ADCC assay

Results from the previous experiment suggested
that sera from females may contain less anti-D
when assessed by a continuous-flow analyser, but
were more active than sera from males in the ADCC
assay. Urbaniak (1979a) suggested that when
using an IgG fraction of anti-D (SL) at high
concentrations a prozone phenomenon occurred
which could be due to saturation of both effector
and target cells by excess anti-D molecules. A
similar phenomenon was previously suggested by
Zeijlemaker et al (1975) when using transformed
cell lines in an ADCC assay. Three experiments
were carried out, therefore, to demonstrate that
differences between anti-D sera from different
sources when used in ADCC assays are not due to
an artefact.

Table 10

ADCC activity of different anti-D sera (2)

Source of Anti-D	ADCC activity % sp. lysis*	Continuous-flow quantitation (iu/ml)	Manual titration	
			Enzyme	I A G T
Females				
SL	79	16.10	32	64
IP 2	75	32.20	64	256
MG 2	82	34.70	64	128
VMc 2	60.3	32.50	64	64
Males				
LH	12	36.0	64	256
JDu 2	-1	116.0	256	1024
GP 2	-3	130.0	256	2048
OH 1	36	2105.0	1024	62536
OH 2	42	4045.0	4096	125072

5 iu = 1 μg anti-D

* E:T ratio 10:1 (4 x 10^4 red cells per culture); anti D final concentration 1 in 3. The same K-cell donor as in experiments 1 and 2, all tested on the same day.

Continuous-flow quantitation results in rank order.

(A negative value indicates that ^{51}Cr release in the test sample was lower than the control value and the ADCC activity was effectively zero).

3.2.2   ADCC   activity   of   anti-D   sera   adjusted   to
equivalent concentrations

A comparison was made of the ADCC activity of a
number of anti-D sera (3 male and 5 female
donors).   The sera were diluted in AB serum to
give the same concentration in the standardized
ADCC assay with an E:T ratio of 10:1 (Table 11).
The anti-D from females were all highly active in
ADCC but that from the male volunteer OH was poor
at inducing ADCC at two high concentrations (as
expressed in iu/ml).

3.2.3   Dose-response of different anti-D sera in ADCC

Anti-D sera obtained from 4 females with bad
obstetric histories, and 5 males immunized for
anti-D immunoglobulin production, were diluted in
AB serum in an identical series of ADCC cultures
to give a dose-response curve for each anti-D.
The results (Fig 9) show that at a given
concentration the anti-D from females were more
active in ADCC than those from males.

3.2.4   Comparison of ADCC activity of anti-D on pre-
sensitized red cells and free anti-D in culture

With pre-sensitization of red cells by anti-D the
majority of D-antigen sites are likely to be
occupied by high-affinity anti-D, and it was
confirmed by a haemagglutination technique that

Table 11

ADCC activity of different anti-D sera adjusted to equivalent anti-D concentration in ADCC culture

Source of Anti-D		ADCC Activity % sp. lysis*	Continuous-flow quantitation	
			Original serum+ (iu/ml)	Diluted++ Equivalent to (iu/ml)
Expt 1				
HL	(F)	85.5	23.50	3.0
IMcC	(F)	85.9	33.0	3.0
SL	(F)	82.4	40.5	3.0
DH	(M)	64.4	194.50	3.0
OH 2	(M)	16.6	4045.0	3.0
Expt 2				
SL	(F)	66.0	16.10	5.35
IP 2	(F)	58.5	32.20	5.35
OH 1	(M)	-3.1	2105.0	5.35

* E:T ratio 10:1 (4 x 10^4 OR_1R_1 red cells per culture); same K-cell donor for all tests in expts 1 and 2.

5 iu = 1 μg anti-D

+ original anti-D concentration before dilution

++ actual anti-D concentration in culture after appropriate dilution

F = female source of anti-D

M = male source of anti-D

Continuous-flow quantitation results in rank order.

(a negative value indicates ^{51}Cr release in the test sample was lower than the control value and ADCC activity was effectively zero).

ADCC LYTIC POTENTIAL OF ANTI–D SERA FROM
DIFFERENT SOURCES

△ Female (pregnancy)

▲ Male (immunised volunteers)
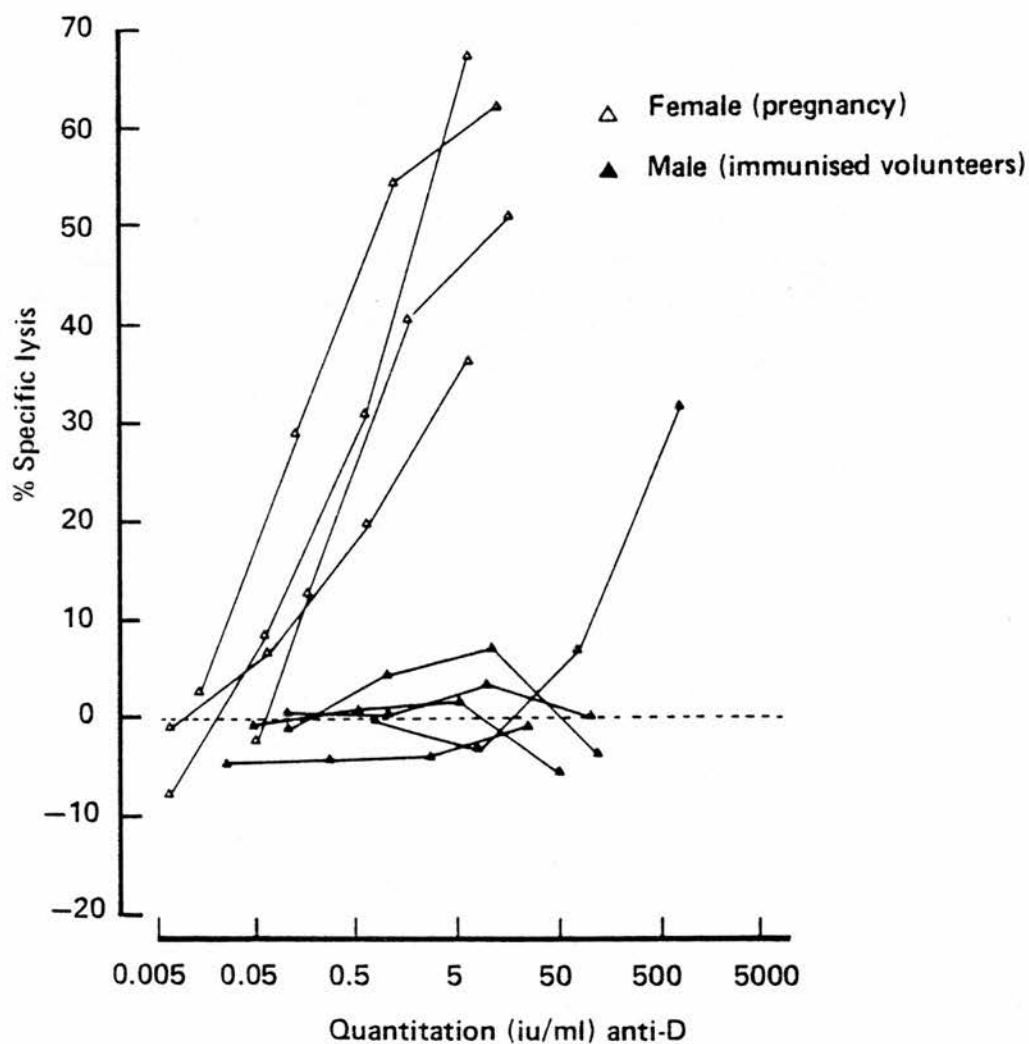
Anti-D added directly to give final concentrations
shown; E/T 10 : 1 (4 x 10^4 red cells per culture)

Fig. 9

anti-D was bound to these cells. This suggested "non-specific" blocking of the Fc receptors by "irrelevant" IgG molecules, similar to the inhibition of Fc-rosette binding by monocytes in the presence of a high concentration of serum containing IgG (Abramson et al, 1970).

Selected anti-D sera (from 8 females and from 8 males) were added directly to the culture, and were also used to pre-sensitize the same number of Rh(D) positive cells, before adding the effector cell (K-cell donor No 1). The results are given in Table 12. It can be seen that the free anti-D sera showed a slightly (but not significantly, $p > 0.05$) greater degree of specific lysis than the bound anti-D. However, the most potent sera were those from pregnancy-immunized females. The experiment was repeated under the same conditions but using a different K-cell donor (No 2) and a smaller number of anti-D sera (from 6 females and from 6 males) with similar results (Table 13). It can be seen that the first K-cell donor was, in general, more active than the second.

3.2.5 Comparison between pregnancy-induced anti-D and anti-D produced by deliberate immunization

Anti-D sera from 4 male volunteers undergoing an immunization schedule were compared with anti-D taken during pregnancy from 4 females. These

Table 12

ADCC activity of anti-D sera - pre-sensitized onto red
cells and free in the culture

Source of Anti-D		Continuous-flow Quantitation (iu/ml)+	* % Specific lysis with		IAGT
			Pre-sensitised red cells **	free serum anti-D***	
MMc	(F)	5.35	75.0	72.0	256
SL	(F)	6.00	77.0	80.0	16
IM	(F)	6.25	79.0	90.0	64
HL	(F)	12.10	45.0	52.0	256
IP 1	(F)	24.40	76.0	80.0	128
MG 2	(F)	34.70	72.0	70.0	256
VMG	(F)	35.55	55.0	61.0	128
GMcB	(F)	39.50	60.5	56.0	256
JA	(M)	32.80	0.0	-1.0	256
JM	(M)	80.0	23.0	30.0	512
GP	(M)	95.45	-3.0	-3.0	2048
WR	(M)	98.55	70.0	79.0	1024
DS	(M)	116.20	66.0	69.5	512
JDu	(M)	126.20	1.0	-1.0	1024
AW	(M)	283.0	0.0	14.6	512
OH 1	(M)	2105.0	32.8	64.0	62536

+ 5 iu = 1 μg anti-D
* E:T ratio 10:1 (4 x 10^4 0 R_1R_1 red cells per culture) K-cell donor No 1
** Target red cells pre-sensitized with anti-D sera prior to addition of effector cells to ADCC culture
*** Anti-D sera added directly to ADCC culture (1 in 3 in final culture)

F = female source of anti-D   M = male source of anti-D

Continuous-flow quantitation results in rank order.
Spearman Rank correlation:
Females: % specific lysis with, pre-sensitized vs free serum p > 0.05
Males: % specific lysis with, pre-sensitized vs free serum p > 0.05
(a negative value indicates ^{51}Cr release in the test sample was lower than the control value and the ADCC activity was effectively zero).

Table 13

ADCC activity of anti-D sera - pre-sensitized onto red
cells and free in the culture

| Source of Anti-D | Continuous-flow quantitation (iu/ml$\ddagger$ | * Specific Lysis | | IAGT |
		pre-sens exp (2)**	free serum (exp (2)***	
Females				
SL	16.10	53.0	68.7	64
IMcC	17.20	24.0	55.7	128
SHa	23.30	59.0	58.2	64
IP 2	32.20	34.0	68.6	256
LK	37.25	52.0	57.5	128
JMcB	39.50	27.0	30.6	256
Males				
JA	32.80	0.0	-1.6	256
JDu	69.0	0.0	1.0	512
GK	137.0	16.0	27.5	128
GH	259.0	0.0	10.5	512
AW	283.0	0.0	14.6	512
OH 1	2105.0	13.5	64.0	62536

$\ddagger$ 5 iu = 1 μg anti-D

* E:T 10:1 (4 x 10^4 O R_1R_1 red cells per culture) K-cell donor No 2

** Target red cells pre-sensitized with anti-D sera prior to addition of effector cells to ADCC culture

*** Anti-D sera added directly to ADCC culture (1 in 3 in final culture)

Continuous-flow quantitation results in rank order.

Spearman Rank correlation:
Females: % specific lysis with, pre-sensitized vs free serum $p > 0.05$
Males: % specific lysis with, pre-sensitized vs free serum $p > 0.05$

(a negative value indicates ^{51}Cr release in the test sample was lower than the control value and the ADCC activity was effectively zero).

females had had neither transfusion nor intra-
uterine transfusion and produced babies affected
by HDN which required exchange transfusions. The
highest anti-D levels amongst the four females
was that of ES (31.31 iu/ml). It can be seen
that the ADCC activities of anti-D from the four
females were consistently high compared to the
levels expressed as iu/ml (Fig 10). However, the
red cell specific lysis of anti-D sera from males
fluctuated (RG and GH) or were non-lytic (JDu and
GP). This again showed that anti-D lytic
activity does not correlate with anti-D
quantitation in terms of iu/ml, and also that
anti-D from females with bad obstetric histories
gave high red cell specific lysis in ADCC assays.

3.2.6   Comparison of ADCC with agglutination assays

One unexpected finding was the low ADCC activity
shown by several anti-D sera from immunized males
with very high antibody levels as determined by
continuous-flow quantitation. (Tables 9-13).
When a number of anti-D sera from immunized males
and from pregnancy-sensitized females were
diluted to equivalent continuous-flow concent-
rations the poor ADCC activity of several sera
from male donors was confirmed (Table 11 and Fig
9). Anti-D sera were quantitated by conventional
agglutination techniques (enzyme and antiglobulin
titres; continuous-flow Auto-Analyser quantit-
ation) and the results compared with the ability

COMPARISON BETWEEN PREGNANCY – INDUCED ANTI – D AND ANTI – D PRODUCED
BY DELIBERATE IMMUNIZATION

Quantitation of anti-D sera (iu/ml) (○)

● % Specific lysis. E/T ratio 10:1 (4 x 10⁴ O R₁R₁red cells per culture). Random K-cell donors

○ Anti–D level; 5 iu = 1μg. Boosts are indicated by vertical broken lines

▲ SL's Anti-D used as control. M = Male source of anti-D serum.

⇪ Amniocentesis
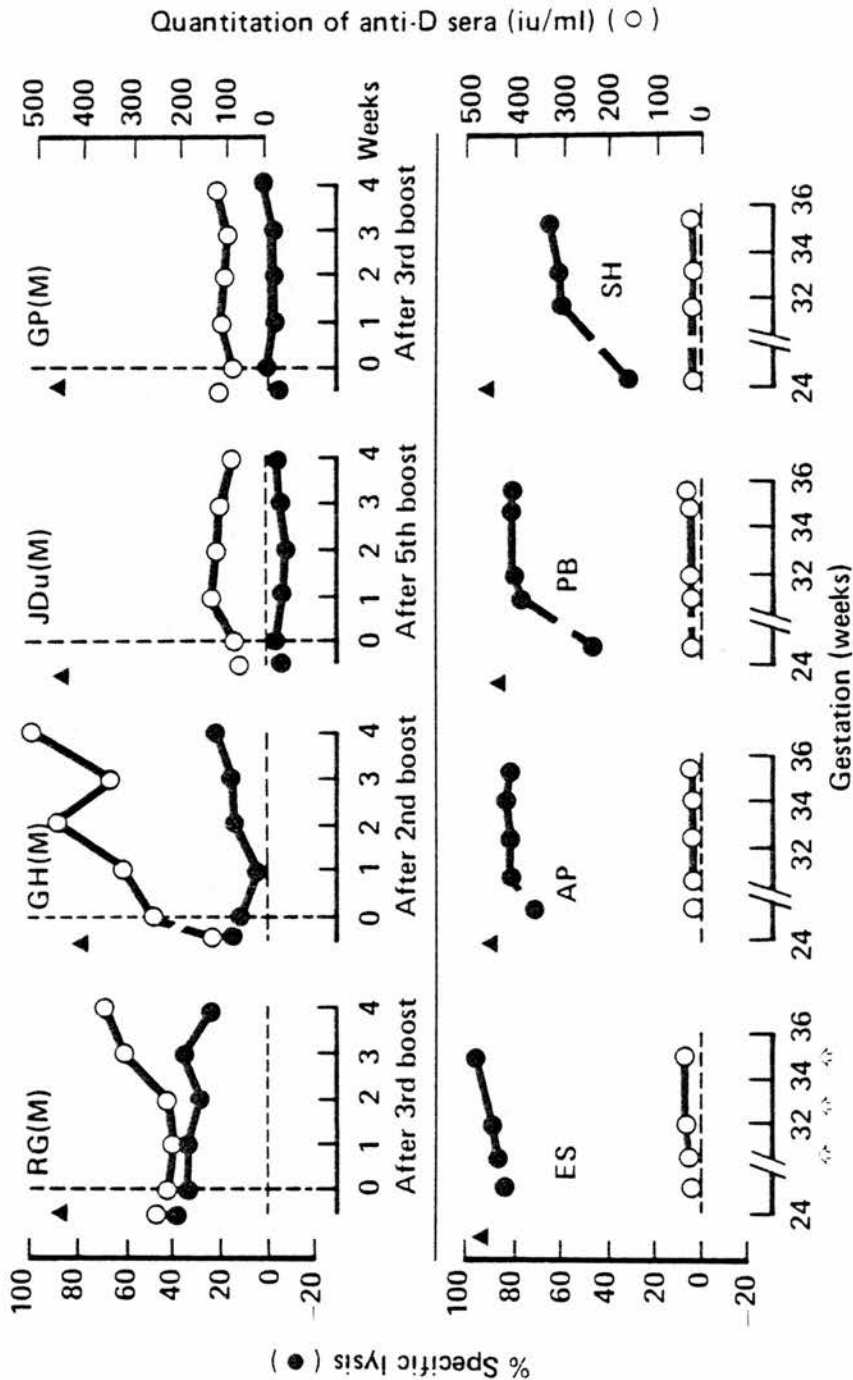
Fig. 10

of the same sera to mediate lysis of Rh(D) positive red cells in ADCC assays.

Sera from 19 Rh(D) negative women immunized during pregnancy and 17 Rh(D) negative male volunteers who had been immunized for the production of anti-D immunoglobulin were assayed by conventional assays and in the ADCC assay (Table 14). Although manual enzyme and antiglobulin titres do not allow accurate comparison of anti-D levels the results did show a statistically significant correlation (Spearman Rank correlation) with the continuous-flow quantitation (continuous-flow vs enzyme in all tests, $P < 0.006 > 0.005$; continuous-flow vs antiglobulin in all tests $P << 0.0001$). Similar results also could be obtained from Tables 10-13.

The ADCC activities of the anti-D sera did not correlate with any of the conventional assays (Spearman Rank correlation). Comparison of anti-D from males with that from females showed that in the males levels were significantly higher when assessed by continuous-flow estimation ($P = 0.002$ Wilcoxon test), yet were significantly lower in ADCC activity ($P = 0.00024$ Wilcoxon test).

Table 14

Comparison of ADCC of Anti-D from different sources
with conventional agglutination assays

Source of Anti-D	ADCC Activity* % Sp. lysis	Continuous-flow quantitation (iu/ml)‡	Manual titration Enzyme	Indirect Antiglobulin
Females				
McC	69.0	5.35	16	256
IM	89.0	6.25	32	64
SMcK	0.5	6.5	8	32
LC	10.0	11.9	16	256
HL	62.0	12.10	64	256
SH	65.0	13.5	64	64
SL	67.7	16.10	32	64
IMcC	36.5	17.2	64	128
SHa	58.0	23.30	16	64
SB	21.0	23.75	32	512
EMcK	15.7	28.90	32	128
IP 2	61.7	32.20	64	256
VMcC	60.3	32.5	64	64
MG 2	70.0	34.70	64	128
LK 2	57.5	37.25	128	128
GMcB	51.0	39.50	64	256
SHo	5.8	53.0	128	512
SU	20.0	54.50	256	512
MW	52.0	71.50	64	256
Males				
AB	-6.3	0.20	1	Nil
ND	-4.5	16.30	16	256
JA	-1.6	32.8	64	256
LH	0.0	56.0	64	256
MB	21.3	61.0	128	512
DS 2	2.5	73.0	64	128
JM	25.0	80.0	128	512
GW	39.2	84.95	128	512
GP 1	-1.8	95.45	256	2048
WR	76.6	98.55	512	1024
JDu 2	-3.0	116.0	256	512
GK	-5.5	137.0	32	128
RG	-0.7	206.5	512	2048
GH	0.23	259.0	256	512
AW	-3.6	283.0	64	512
GR	26.4	351.75	256	1024
OH 1	32.8	2105.0	1024	62536

* E:T ratio 10:1 (4×10^4 OR_1R_1 red cells per culture); anti-D final
   concentration in culture 1 in 3; random K-cell donors (a negative
   value indicate ^{51}Cr release in the test sample is lower than the
   control value and the ADCC activity is effectively zero).
‡ 5 iu = 1 μg anti-D

Continous-flow quantitation results in rank order
Spearman rank correlation:
quantitation vs enzyme (all test) 0.006 > P > 0.005
quantitation vs antiglobulin (all test) P << 0.0001
Wilcoxon test: quantitation results, males vs females P = 0.0024
ADCC results: males vs females P = 0.00023

3.2.7   ADCC activity and IgG subclasses

The previous experiments showed that the ADCC activity of anti-D sera did not correlate with any of the conventional haemagglutination assays. It has been shown that IgG_1 and IgG_3 red cell antibodies are the most efficient at inducing ADCC lysis (Holm et al, 1974; Urbaniak, 1979b) and at fixing complement and inducing immune adherence (Abramson and Schur, 1972). The same sera were therefore tested to determine their IgG subclasses, to show whether there was correlation between the lytic activity of anti-D with IgG subclass.

The IgG subclass distribution of the sera is shown in Table 15. In all the sera tested the subclasses known to be lytic in ADCC (IgG_1 and IgG_3) were present either together or singly, and in all the sera from males (except that of DS) the IgG_1 titre was higher than that of IgG_3 (where present).

Sera from females were more heterogeneous but there was a tendency for the IgG_3 titres to be higher than those of the males when related to the total anti-D level (iu/ml). In 18 of 23 sera (78%) in which IgG_1 was present, and to a level higher than IgG_3, the ADCC activity was less than 30% specific lysis, whereas in all twelve sera in which the IgG_3 level was greater than that of

Table 15

ADCC activity in relation to IgG sub-classes

Source of Anti-D	ADCC activity % Spec Lysis*	IAGT**	IgG sub-classes (titres)			
			IgG_1	IgG_2	IgG_3	IgG4
Females						
SMcK	0.5	32	128	0	0	0
SHo	5.8	512	512	16	16	8
LC	10.0	256	16	0	0	0
SU	20.0	512	256	32	4	4
SB	21.0	512	256	4	8	4
IMcC	36.5	128	64	32	256	32
KP	38.0	512	128	0	256	0
LK 2	42.0	128	64	0	0	0
GMcB	51.0	256	128	1024	2048	1024
MW	52.0	256	256	64	512	16
SHa	53.0	64	8	256	512	64
IP 2	61.7	256	128	64	256	128
VMc	62.0	128	64	4	128	0
HL	62.0	256	64	0	128	0
SH	65.0	64	32	0	64	0
SL	67.7	64	32	16	0	0
MMc	69.0	256	32	1	128	0
MG 1	75.0	256	64	0	128	0
Males						
AB	-6.3	Nil	4		0	0
GK	-5.5	128	2048	256	128	256
ND	-4.5	256	32	0	0	0
AW	-3.6	512	512	128	128	128
JDu 2	-3.0	512	512	64	128	32
GP	-1.8	2048	128	0	8	0
JA	-1.6	256	512	16	0	0
RG	-0.7	2048	512	0	32	128
GH	0.2	512	2048	256	0	256
LH	0	256	512	16	0	0
DS 2	2.5	128	128	32	128	16
MB	21.3	512	128	64	32	16
JM	25.0	512	256	0	8	16
GR	26.4	1024	512	0	128	0
OH 1	32.8	62536	4096	128	512	1024
GW	39.2	512	512	8	128	8
WR	76.6	1024	512	64	128	0

* E:T ratio 10:1
** IAGT = indirect antiglobulin test (broad spectrum)

(A negative value indicates ^{51}Cr release in the test sample was lower than the control value and the ADCC activity was effectively zero)

ADCC activity results in rank order.

IgG_1 the activity was greater than 30% specific lysis. IgG_2 and IgG_4 were present in anti-D sera from both males and females, but always in association with IgG_1 and/or IgG_3. There was no obvious correlation between IgG_2 and/or IgG_4.

3.3 Characterization of anti-D sera which are non lytic in ADCC

Conventional manual and automated quantitation of anti-Rh(D) antibodies relies upon their agglutinating properties. The ability of such antibodies to sensitize D-positive red cells for lysis by peripheral lymphocytes in vitro by ADCC may be a measure of the biological potency of anti-D in vivo: eg as in haemolytic disease of the newborn (Urbaniak, 1978). Previous studies have shown that the lytic activity of anti-D in ADCC is not related to the anti-D content as measured by conventional assays based on haemagglutination. It has also been found that anti-D from deliberately immunized males generally exhibits less ADCC activity in relation to its anti-D content (in terms of iu/ml) than do sera from women immunized during pregnancy. Those differences are not directly related to differences in the IgG subclasses of the antibodies. It is important, therefore, to attempt to characterize the factors which determine the lytic activity of anti-D. In this section, the characteristics and interactions of lytic and non-lytic anti-D sera in ADCC are described.

3.3.1 Comparison of anti-D quantitation, specific anti-D binding and specific lysis in ADCC of anti-D sera

Anti-D sera from male volunteers were investigated for anti-D content by conventional continuous-flow quantitation; anti-D specific red cell-bound IgG by IRMA; specific ADCC by the

standardised ADCC assay; and ADCC against anti-D pre-sensitized red cells. The results are shown in Table 16. Eleven anti-D donors were studied, one of them (JW) twice, on serum taken on two different occasions, two months apart.

Three sera (GP, JW1 and JW2) were found to lack ADCC activity in the conventional assay. However, JW1 and JW2 showed ADCC lytic activity in the pre-sensitization assay; only GP lacked such activity completely and it was selected for further study. Although JW's anti-D appeared to retain ADCC activity in the absence of other serum components - possibly indicating some non-specific "irrelevant" IgG molecules to block ADCC - further studies were carried out on this serum, and an anti-D serum with ADCC lytic activity from a male donor (JM Section 3.2) was selected for comparative study.

Analysis of the overall pattern of results (Table 16) showed no significant relationship between ADCC assays (standardised or pre-sensitization) and either anti-D content or specific anti-D binding to red cells (P > 0.05). However, a significant correlation was found between anti-D content and specific binding (P < 0.01) by both normal and non-parametric (Spearman) analysis of correlation.

Table 16

Comparison of anti-D quantitation, specific anti-D binding
and specific lysis in ADCC of anti-D sera

Serum	A D C C *(% specific lysis) free serum	pre-sensitised red cells	Continuous flow quantitation (iu/ml$\ddagger$	Specific binding (IgG IRMA) mean cpm
AA	27.0	28.2	6	6589
SW	64.3	52.9	12	6455
LK	31.7	50.3	25	8026
LH	41.5	30.4	39	23029
GP	0	0	75	35555
DH	90.2	83.9	136	43240
DH	97.4	76.9	145	59976
JM	93.5	62.0	157	47569
GM	78.1	71.9	210	46478
IM	58.4	87.0	214	52942
JW 2	0	69.9	533	69235
JW 1	0	53.3	578	67569

$\ddagger$ 5 iu = 1 μg anti-D
*   E:T 10:1 (4×10^4 O R_1R_1 red cells per culture) K-cell donor No 2
**   Target red cells pre-sensitized with anti-D sera prior to addition of effector cells to ADCC culture
***   Anti-D sera added directly to ADCC culture (1 in 3 in final culture)
Continuous-flow quantitation results in rank order.
Spearman Rank correlation:
% specific lysis with, presensitized vs free serum (P > 0.05)
ADCC assays vs anti-D sera quantitation (P > 0.05)
ADCC assays vs anti-D sera binding to red cells (P > 0.05)
Anti-D sera quantitation vs anti-D sera binding to red cells (P < 0.01)

3.2.2   The effect on ADCC of mixing lytic and non-lytic
        anti-D sera

        Non-lytic serum from donor GP was mixed with
        lytic serum from donor JM, and also with a second
        lytic serum SL (anti-D 6 iu/ml, ADCC of the order
        of 95% specific lysis). The results are shown in
        Fig 11.

        When equal amounts of the non-lytic serum (GP)
        and the lytic serum (JM) were mixed, ADCC
        specific lysis was as great as with JM's serum
        alone. Reduction of the proportion of GP's serum
        had no significant effect, but dilution of the
        lytic serum (JM) reduced ADCC lysis gradually (ie
        a dose response). However, dilution of the lytic
        serum (JM) in the presence of a constant amount
        of non-lytic serum (GP) demonstrated an
        inhibitory effect of serum GP on the ADCC lysis
        of JM's serum at lower concentrations,
        increasingly moving the "dilution curve" to the
        left with increasing dilution of the serum JM.

        In contrast, when the non-lytic serum (GP) was
        mixed with second lytic serum (SL), serum GP
        having a much greater anti-D content than serum
        SL, ADCC activity was completely abolished. In
        this case the anti-D ADCC activity gradually
        re-appeared on dilution of serum GP in the
        presence of a constant amount of serum SL.

110
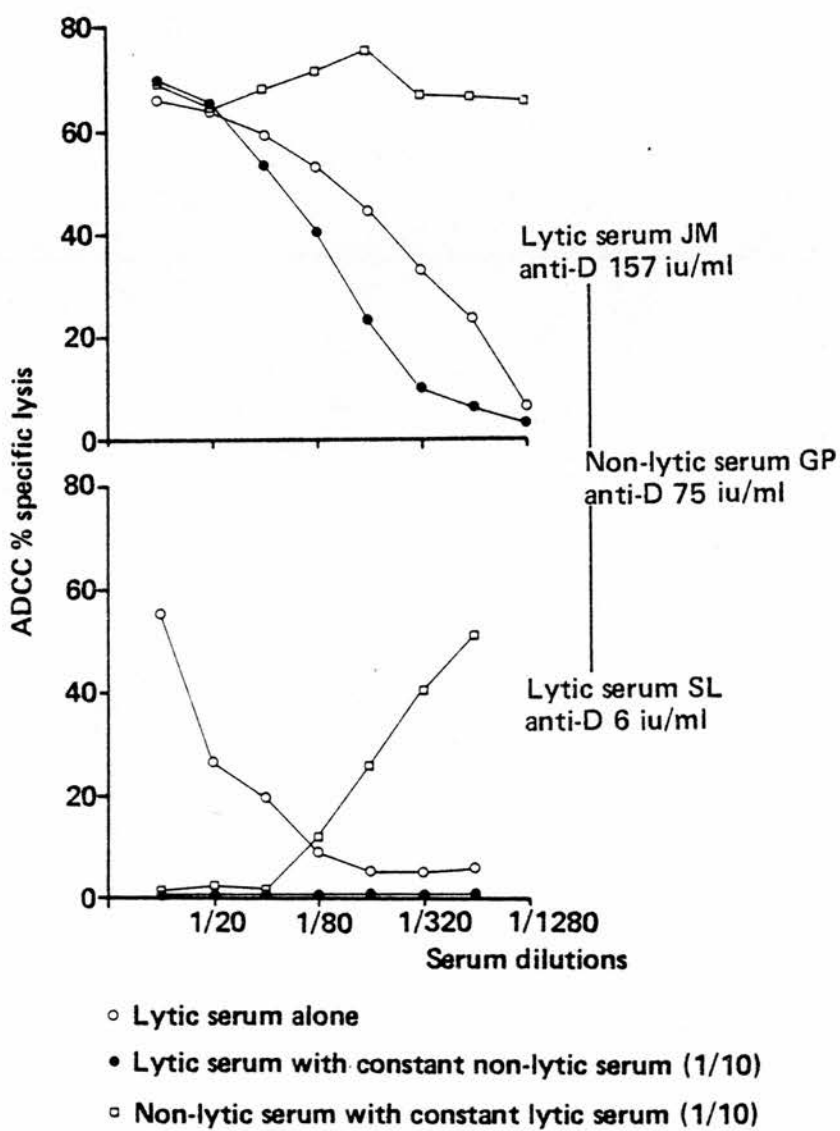
The effect on ADCC activity on mixing lytic and
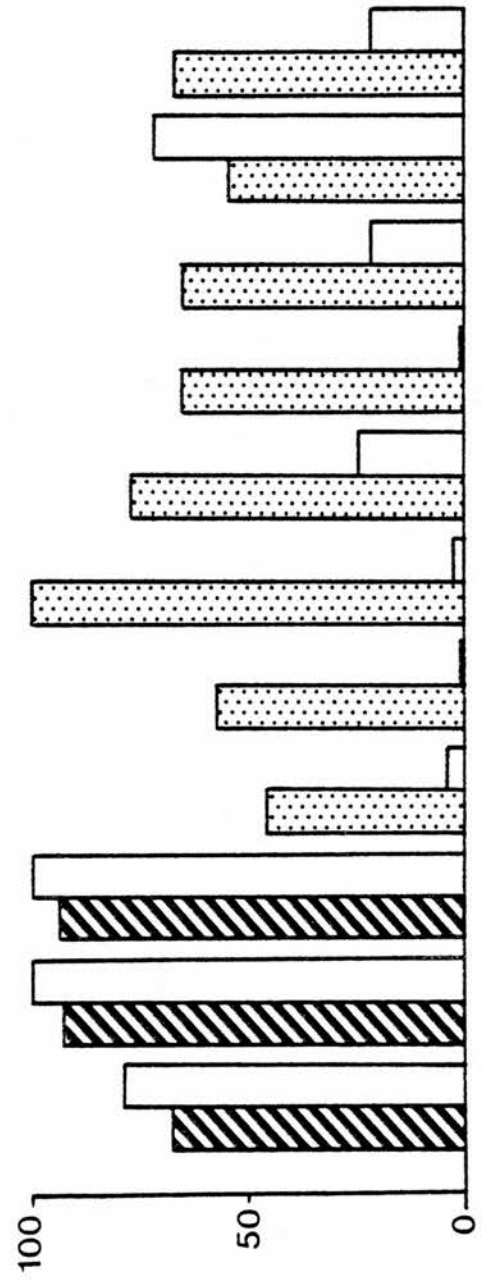non-lytic anti-D sera.

Fig. 11

Inhibition of the ADCC lytic activity of both anti-Ds was thus observed when they were mixed with an ADCC non-lytic anti-D in the appropriate proportions. The expression of the inhibitory effect appears to be related to the relative anti-D content of these sera.

3.3.3   Specificity of inhibition

To investigate the specificity of the inhibitory effects of the anti-D serum GP, 25 μl per well was pre-incubated with red cell targets at 37°C for 90 min, followed by addition of 25 μl of ADCC-lytic anti-c or anti-D and lymphocytes to give a final culture volume of 150 μl/well.   In this experiment (Fig 12) group OR_2R_2 (cDE/cDE) target red cells were used.   The standard ADCC assay was used as a control with anti-c or anti-D sera, target cells and lymphocytes, but without adding the non- lytic anti-D sera to the culture. It can be seen that the non-lytic serum (GP) had no inhibitory effect on the three lytic anti-c tested.   However, in 7 of 8 anti-D sera (87%) ADCC lytic activity was inhibited, and in 3 of 7 activity was virtually abolished.   The observed ADCC inhibiting effect of non-lytic anti-D thus appeared to be specific for the Rh(D) antigen. All anti-c sera tested were enhanced in the presence of non-lytic anti-D serum.

ADCC

% specific lysis of Group O, R₂R₂(cDE) red cells

The pattern of inhibitions on ADCC lytic potential of different Rh anti
sera (-c and -D) by ADCC non-lytic anti-D (GP): relationship to specificity.

Fig. 12

3.3.4   ADCC activity of anti-D eluates

Anti-D was specifically adsorbed onto group OR_1R_1 red cells from three sera from males (GP, JM and JW2 from Table 11) and recovered by elution after the first absorption. Absorption was repeated in an attempt to deplete sera maximally of residual anti-D.   The specific binding patterns of anti-D to red cells, as determined by IgG - IRMA for both sera and eluates, are shown in Figure 13. A prozone effect of reduced binding at high concentrations was found with all three sera and this probably reflects prevention of maximal anti-IgG binding due to rapid agglutination of the red cells. The anti-D content of each of the three eluates was of the same order of magnitude and gave similar binding curves.

Neither the serum nor the eluate of GP showed ADCC lytic activity (Figure 13), whereas both serum and eluate of JM showed such activity. As previously found (Table 11), serum JW2 did not show ADCC activity: however the eluate of JW2 was ADCC active. This appears to confirm the observations on the serum JW2 by both conventional and pre-sensitization ADCC assays (Table 11); ie anti-D JW2 is ADCC active and that other factors present in the whole serum inhibit its ADCC activity.

IgG IRMA: red cell bound anti-D
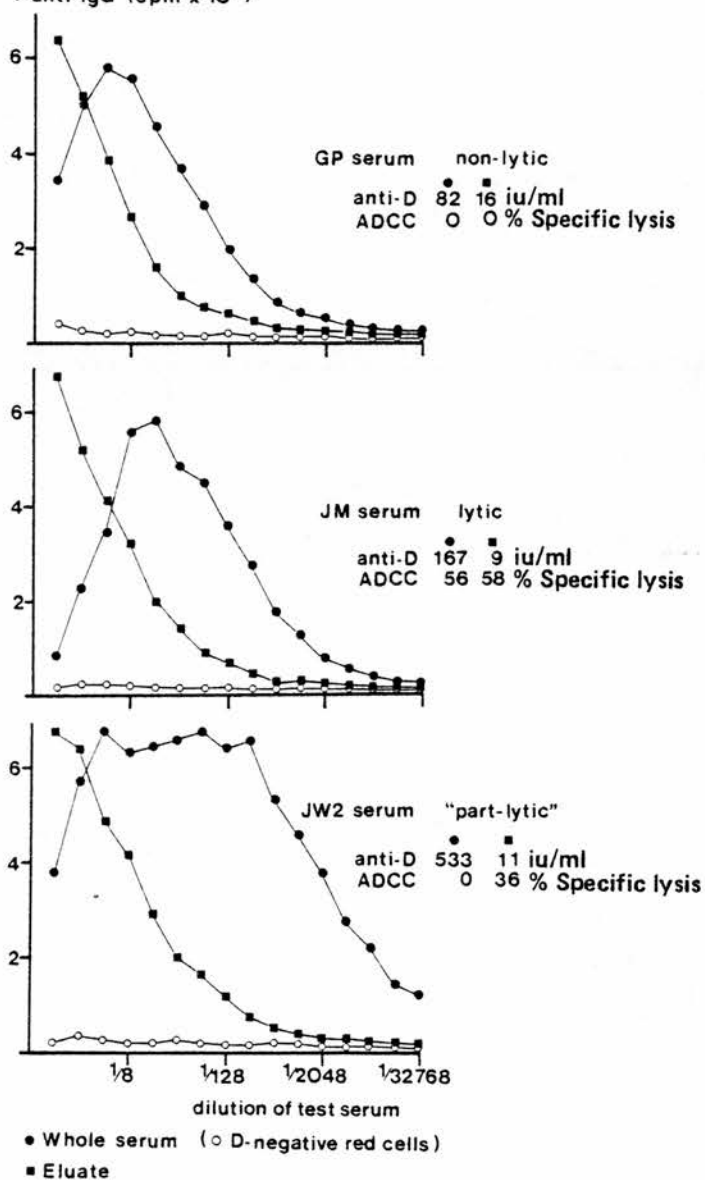
The specific binding of anti-D to red cells as determined by IgG IRMA for both sera and eluates; and ADCC lytic potential of sera and eluates.

Fig. 13

3.3.5   ADCC activity of IgG fractions of anti-D sera

IgG-enriched fractions of sera GP (ADCC non-lytic) and JM (ADCC lytic) were prepared by polyethylene glycol (PEG) and ammonium sulphate precipitation. IgG-depleted serum was prepared after removal of ammonium sulphate precipitates, followed by dialysis: (both < 1g/l IgG; < 1 iu/ml anti-D). The relative ADCC lytic activities of serum JM and its derivatives are shown in contrast to the relative ADCC inhibitory capacities of serum GP and its derivatives (Figure 14), where they reflect each other. Thus, in the (respective) lytic and inhibitory sera the lytic and inhibitory activities are reduced by (i) absorption of anti-D and (ii) depletion of IgG: both activities are recovered in (i) eluted anti-D and (ii) IgG-enriched fractions.

3.3.6   Anti-D Fc receptor rosetting activities

The ability of anti-D sera to sensitize Rh(D) positive red cells for rosetting through Fc receptors for IgG on lymphocytes was examined. All were capable of Fc-rosetting (Table 17). Red cells not sensitized with anti-D sera rosetted with less than 2% of the lymphocytes. ADCC-inactive serum (GP) did not differ significantly from other anti-D sera in their Fc-rosetting capacity.

ADCC

%INHIBITION OF LYSIS

Whole serum — Absorbed serum — Eluate — 3% PEG precipitate — 15% PEG precipitate — Ammonium sulphate precipitate — Ammonium sulphate supernatant

" SPECIFIC LYSIS

Inhibition of ADCC by GP serum and fractions (preincubation)

ADCC by JM serum and fractions
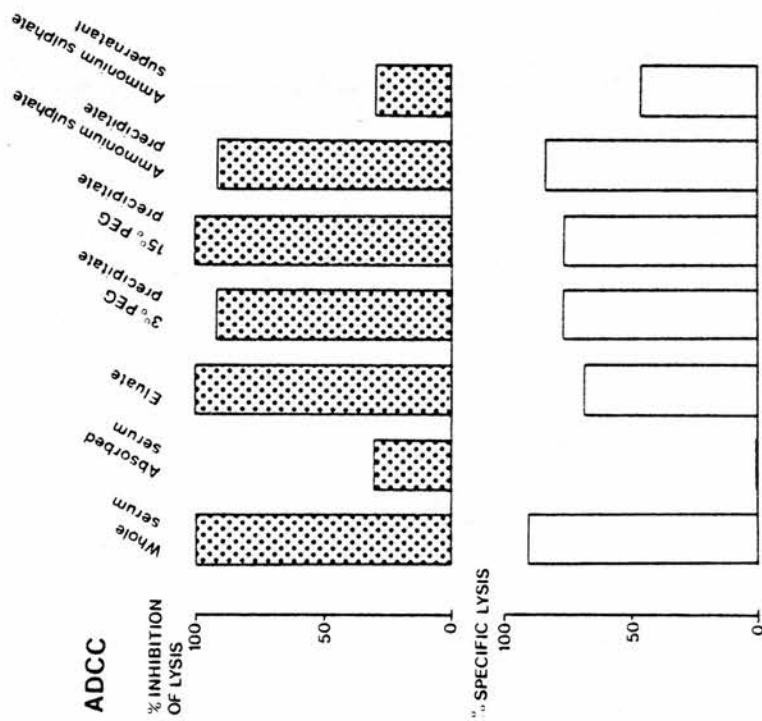
Fig. 14

ADCC inhibition and activity by IgG depleted and IgG enriched anti-D sera fraction, anti-D deplated sera and anti-D eluates.

Table 17

Anti-D Fc receptor rosetting activities

Serum	Lymphocyte-RFC (mean % Fc-RFC)	Continuous flow Quantitation (iu/ml)‡	% Sp. Lysis*
SL	24	6	97
GP	34	75	0
JM	31	157	94
JW 2	57	533	0**

* E:T ratio 10:1
** 70% specific lysis by presensitisation of red cells

$\ddagger$ 5 iu = 1 μg anti-D

Continuous-flow quantitation in rank order

3.3.7 IgG subclasses of ADCC-lytic and ADCC-non-lytic anti-D sera

The relative distributions of the IgG subclasses in ADCC-active sera and eluates from JM and SL were compared to those in the ADCC inactive serum GP and in the serum JW2 (anti-D active/whole serum inactive). The relative haemagglutination titre endpoints with anti-subclass antibodies on anti-D specifically-sensitized red cells are shown in Table 18. Anti-D IgG subclass distributions were similar in ADCC-active and ADCC-inactive sera. Eluates appeared to show relative enrichment of IgG_2 and depletion of IgG_3 compared with whole sera. It is improbable that any redistribution of subclasses of serum JW on elution was responsible for the ADCC-activity of this eluate in contrast to the inactivity of the serum in the standard ADCC assay, since serum JW showed ADCC activity in the pre-sensitization ADCC assay.

Table 18

Relative haemagglutination titre endpoints with anti-subclass antibodies on anti-D specifically sentitized red cells

| Test Serum | IgG Sub-classes (reciprocal of titre) | | | | Anti-D (iu/ml)‡ | % Sp. Lysis |
	IgG-1	IgG-2	IgG-3	IgG-4		
JM						
Serum	1024	16	32	0	167	+
Eluate	128	16	2	2	9	+
GP						
Serum	64	0	8	0	82	-
Eluate	128	4	1	0	16	-
JW						
Serum	> 2048	1024	512	512*	533	-
Eluate	128	8	0	0	11	+
SL						
Serum	8	2	0	0	6	+
Eluate			N O T D O N E			
Group AB D+ donor serum (control)	0	0	0	0	0	-

$\ddagger$ 5 iu = 1 μg anti-D

* JW serum agglutinated directly to a dilution of 1 in 32

3.4   ADCC and Rh Haemolytic Disease of the Newborn (HDN)

In pregnancies where Rh haemolytic disease is suspected, an indication of the prognosis can be obtained from the outcome of previous pregnancies (Bowman, 1978), from estimates of maternal levels of anti-D (Fraser and Tovey, 1976), and from optical density measurements of amniotic fluid which may indicate when intrauterine transfusion should be considered (Liley, 1963). Variations in the potency of anti-D sera in mediating ADCC of Rh(D) positive red cells have been noted previously (see Section 3.2) and it has been shown that there is little correlation between conventional agglutination assays and ADCC.

3.4.1 Anti-D lytic activity during pregnancy affected by HDN

Samples from 8 females were taken during the antenatal period where HDN was suspected, and the sera were stored frozen at $-40^{\circ}C$ until tested, when the anti-D content was estimated by continuous-flow quantitation. The samples were heat-inactivated ($56^{\circ}C$ for 30 min) and tested on separate occasions but the same lymphocyte (effector cell) and red cell (target cell) donor was used for each patient, with anti-D (SL) as a control to enable quantitative comparisons to be made. The clinical details of these cases are shown in Table 19.

Table 19

CLINICAL DETAILS AND LABORATORY FINDINGS IN CASES AFFECTED BY HDN DUE TO ANTI-D

Case	Previous history	OD 450 Modified Liley	Cord Findings			Transfusion	Outcome of Pregnancy
			Hb (g/dl)	Bilirubin (μm/l)	DAGT		
NC	Para 0 + 0	Not done	4.6 (34½)*	130	Positive	Exchange transfusion x 5	Survived
MD	Para 4 + 1 (affected by HDN)	Not done	13.0 (38½)	80	Positive	Exchange transfusion x 1	Survived
PB	Para 2 + 0 (no HDN)	Not done	15.0 (40)	43	Positive	None	Survived
CC	Para 2 + 0 (affected by HDN)	Not done	Not available (39)	55	Positive	2 red cell transfusions	Survived
A O'N	Para 4 + 1 (affected by HDN)	Low zone (27½)*	11.3 (38)	62	Positive	Exchange transfusion x 2	Survived
V McA	Para 1 + 0 (no HDN)	Mid zone (35) (36½)	12.9 (37½)	62	Positive	Exchange transfusion x 1 + 2 red cell transfusions	Survived
EB	Para 1 + 5 (affected by HDN)	High mid zone (29) (31) (32) (33) (34)	13.2 (34½)	80	Positive	Exchange transfusion x 2	Survived
FP	Para 4 + 1 (affected by HDN)	High mid zone (26) (28) (31) (32) (36)	11.3 (38)	123	Positive	Exchange transfusion x 3	Survived

DAGT = Direct antiglobulin test. OD 450 = optical density change at 450 nm, repeat amniocentesis predictions remained with the same zone.

* weeks of gestation

3.4.2   Cases   of   HDN   where   amniocentesis   was   not
performed

In the first four cases shown in Table 19 (NC,
MD, PB and CC) HDN was suspected from the
presence of anti-D only (Fig 15).   NC had neither
a previous history of pregnancy nor of blood
transfusion, but her anti-D level had risen
suddenly from 0.11 iu/ml to 7.26 iu/ml between
weeks 23 and 25 of pregnancy and then gradually
reached its highest level (15.81 iu/ml) at 32
weeks, labour being induced at 34.5 weeks.   This
rise was most probably due to transplacental
haemorrhage (TPH).

The anti-D of MD never reached 2 iu/ml (< $1\mu g/ml$)
during her pregnancy.   That of PB gradually rose
from 0.62 iu/ml at 12 weeks and reached 10.58
iu/ml at 40 weeks.   In patient CC the anti-D
level was 11.05 iu/ml at 12.5 weeks gestation and
then gradually tapered to 5.8 iu/ml at 39 weeks.
This was most probably due to the existence of
anti-D from her previous pregnancy, in which the
baby had been affected by HDN.

In general the anti-D level was not helpful in
prediction of the outcome of three of the four
pregnancies.   However, the ADCC assay gave very
good correlation with the outcome in the four
cases in relation to blood transfusion.   In two
of those cases (PB and CC) requiring just a red

ANTI – D LYTIC ACTIVITY DURING PREGNANCY
(without AMNIOCENTESIS)
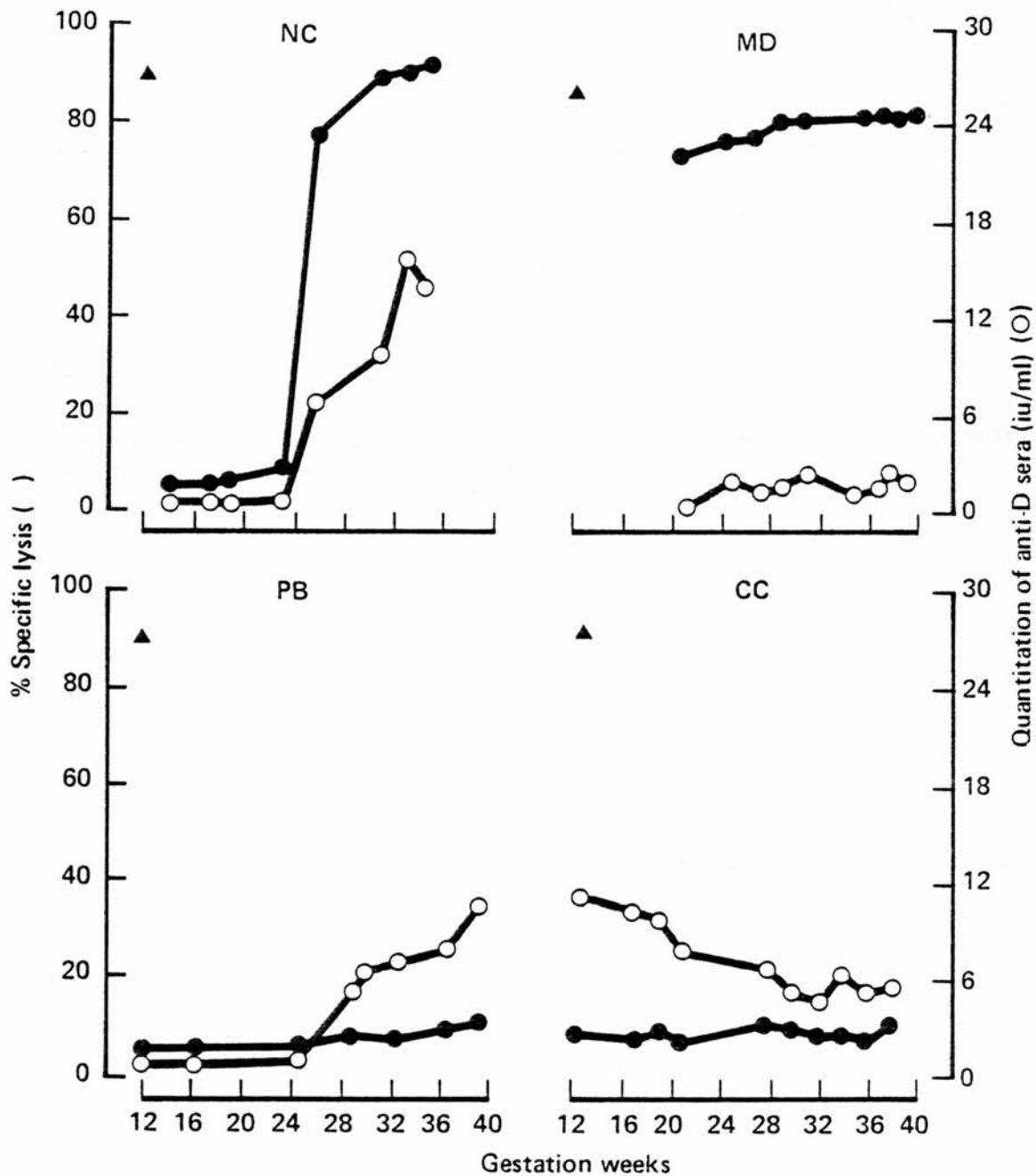
● % Specific lysis; E/T ratio 10:1 (4 x 10^4 O R_1 R_1 red cell per culture);
The same K-cell donor for all tests

○ Anti-D level; 5 iu = 1 μg

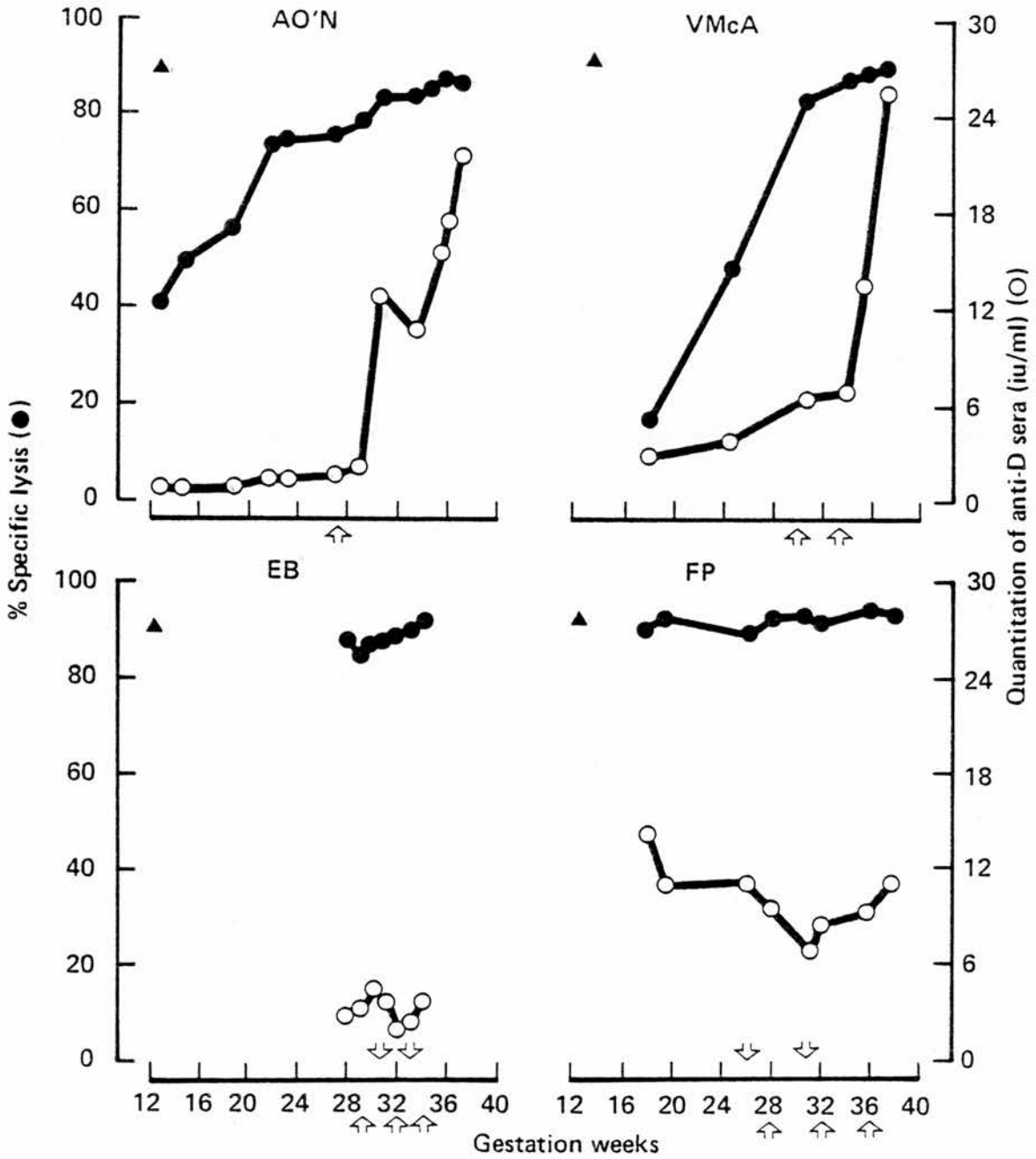▲ SL's anti-D used as control

Fig 15

cell transfusion (top-up) for anaemia, or no transfusion at all, the anti-D potential lytic activity in terms of specific lysis, was very low. The other two (NC and MD) had given birth to affected infants which required exchange transfusion and the ADCC activities of anti-D serum were high.

3.4.3   Cases of HDN where amniocentesis was performed

The second four cases of HDN shown in Table 19 (AO'N, VMcA, EB, FP) were tested to predict the outcome of pregnancy by amniocentesis and anti-D quantitation, and these results compared with ADCC assays (Fig 16).

The anti-D levels of AO'N remained at 1.35 iu until 27.5 weeks gestation, when amniocentesis was performed. The anti-D then rose suddenly to reach 13.50 iu/ml at 37 weeks and reached its highest level (21.50 iu/ml) at 37 weeks. Similarly, the anti-D of VMcA rose rapidly after amniocentesis, most probably indicating secondary immunization induced by amniocentesis. However, in cases EB and FP the anti-D level was not affected by the amniocentesis. Prediction of the outcome of pregnancy using amniocentesis was not accurate, and in the case of EB the highest measured level of anti-D was 4.32 iu (< $1\mu g/ml$) at 30 weeks gestation. However, ADCC assays predicted the outcome of pregnancy accurately

ANTI – D LYTIC ACTIVITY DURING PREGNANCY
(with AMNIOCENTESIS)

● % Specific lysis; E/T ratio 10:1 (4 x 10^4 O R_1 R_1 red cell per culture);
The same K-cell donor for all tests

○ Anti-D level; 5 iu = 1 μg

▲ SL's anti-D used as control

⇪ Amniocentesis

Fig 16

where a high potential lytic activity of the anti-D indicated a severely affected baby which subsequently required an exchange transfusion.

3.4.4   Series of anti-D sera tested blind

Samples taken during the course of pregnancy in cases of suspected HDN were also selected in Glasgow and tested "blind" in Edinburgh prior to the clinical details being revealed. Two series of samples (11 cases) were tested on separate occasions using the same effector cells, target cells and anti-D (SL) as described above.

Series 1:   Sera were selected from three cases where there were discrepancies between prediction based on the anti-D level and the amniocentesis results. The clinical details of these cases are shown in Table 20. The three sera each contained a high level of anti-D, indicating a poor prognosis, but the amniocentesis results suggested unaffected - or only mildly affected - babies in each instance. The clinical outcome showed that amniocentesis gave a misleading prediction in the cases of RMcG and AMcA.

The ability of the anti-D sera to mediate ADCC of ORh(D) positive target cells is shown in Figure 17. It can be seen that samples from RMcG and AMcA were highly active and resulted in lysis comparable to the control anti-D (SL), with a

Table 20

Clinical details and laboratory findings in:

SERIES 1

Case	Previous History	Anti-D (iu/ml)*	OD 450 Modified Liley	CORD FINDINGS			Transfusion	Outcome of Pregnancy
				Hb (g/dl)	Bilirubin (µm/l)	DAGT		
R McG	Para 1 + 0 (no HDN)	25.8** (38)	Low zone (31) (33)**	11.0 (38)	80	Pos	Exchange + red cell transfusion	Survived
A McA	Para 1 + 1 (no HDN)	49.7 (33)	Mid zone (28) (30) (32)	12.8 (33)	85	Pos	Exchange x 3	Survived
AM	Para 3 + 0 (2 with HDN)	19.7 (37)	Low zone (30) (33)	Not available (39)	20	Pos	2 red cell transfusion	Survived

* 5 iu/ml = 1 µg/ml anti-D − DAGT = Direct antiglobulin test

** Weeks gestation
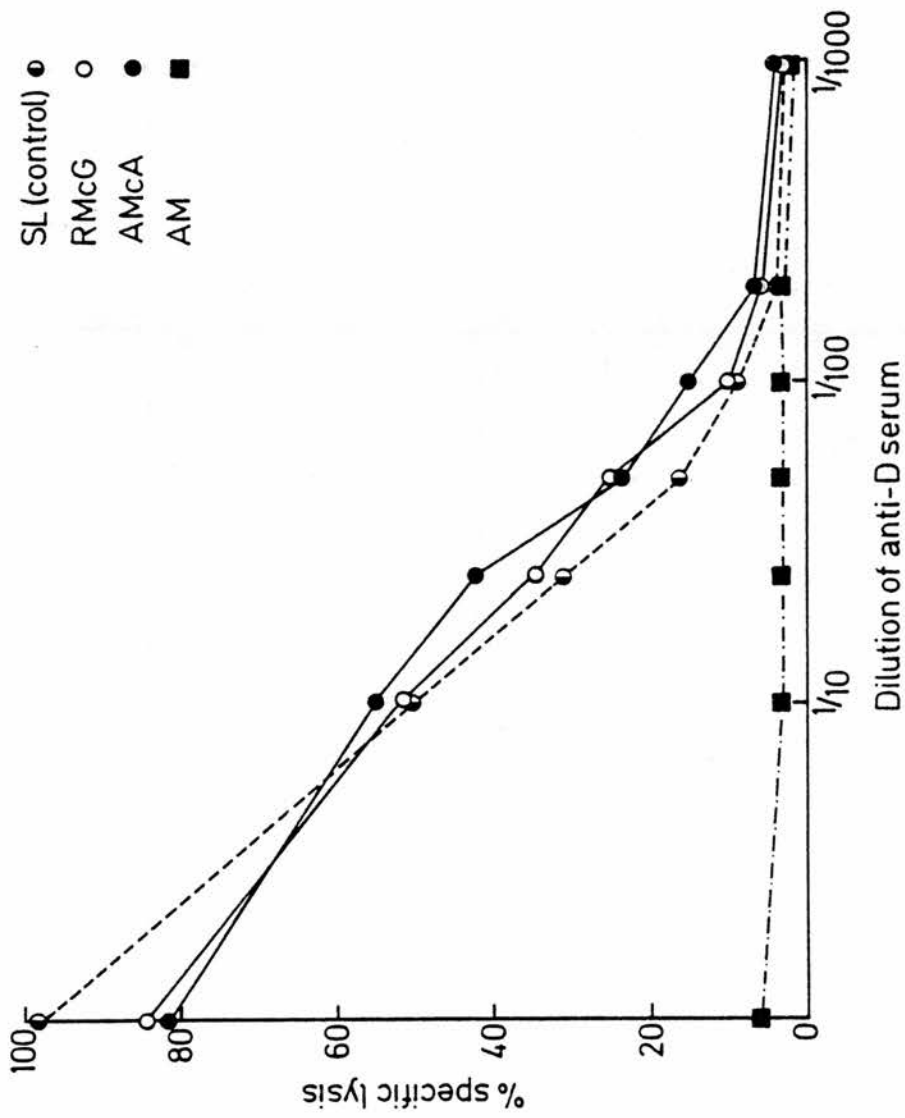
ADCC activity of anti-D sera from series-1 cases

E:T ratio 10:1 $(4 \times 10^4 \ OR_1R_1$ per culture),

anti-D dilution as shown

Fig 17

similar dose-response curve on dilution of anti-D in culture. The serum from AM was virtually inactive at all dilutions tested. The severity of HDN in each of the infants (Table 2) correlated with those findings with the babies of RMcG and AMcA requiring exchange transfusion, whereas that of AM required only a "top up" transfusion for anaemia.

Series 2: In the second series, a further eight cases were selected on the basis of high maternal anti-D levels (> 20 iu/ml) but these had variable clinical outcomes, including cases where this was better than expected on the basis of anti-D level alone.

The clinical details of the eight cases studied are shown in Table 21. For this experiment another two K-cell donors were used as well as the same effector cell donor (No 1) used in the previous study. Serum from each patient was used neat (ie 1 in 3 in the final culture, Table 22) and also diluted in inert (group AB) serum using donor No 1 (Figure 18).

In five cases with high anti-D levels, amnio-centesis correctly predicted the severity of the HDN. In two cases (MT, LM), despite high anti-D levels the outcome was favourable with no trans-fusions required. In retrospect, amniocentesis

Clinical details and laboratory findings in:

SERIES 2

Case	Previous History	Anti-D (iu/ml*)	OD 450 Modified Liley	Cord Findings Hb (g/dl)	Bilirubin (µm/l)	DAGT	Transfusions	Outcome of Pregnancy
JT	Para 1 + 0 (affected by HDN)	44.5** (20)	IUT zone (25) (28)			Stillbirth	after second IUT	
CR	Para 5 + 1 (affected by HDN)	42 (23)	Edge IUT zone (25)	12.2 (28)	197	Pos	IUT x 3 + Exch & Multiple red cell transfusions	Survived
FJ	Para 3 + 1 (affected by HDN)	24.5 (29½)	IUT zone (28½) (29½)			IUD after first IUT (30)		
CS	Para 3 + 0 (affected by HDN)	31 (30)	IUT zone (24)	11.2 (31½)	Not available	Pos	IUT x 2 + Multiple exchanges	Survived
MF	Para 1 + 0 (no HDN)	57.6 (34½)	IUT zone (33)	3.6 (34½)	Not available	Pos	Exchange x 2	Died 6 days after birth
EK	Para 1 + 0 (no details)	19.5 (23)	High mid zone (28) † (30) (33)	19.0 (37½)	37	Neg	None	Survived
MT	First pregnancy	20.4 (32)	Low mid zone (33)	14.4 (36)	80	Pos	None	Survived
LM	Para 2 + 0 (no HDN)	27.5 (38)	Not done	Not tested (38)	64	Pos	None	Survived

DAGT = Direct antiglobulin test; IUT = intrauterine transfusion; IUD = Intrauterine death

Case EK produced a Rh (D) - negative baby

*   5 iu = 1 µg anti-D

**   Weeks gestation

†   Discrepancy between reference and local lab results (low in local, high in reference lab)

131

Table 22

ADCC activity of anti-D sera from series 2 cases
using 3 K-cell donors

Source of K-Cell	SL Control	JT	CR	FJ	CS	MF	EK	MT	LM
Donor No 1	79*	99	97	94	91	91	50	28	7
Donor No 2	78	96	70	80	77	71	51	21	5
Donor No 3	90	92	83	80	91	89	54	21	0

* % specific lysis, anti-D "neat" (1 in 3 in the final culture)
E:T ratio 10:1 (4 x 10^4 red cells per culture)

SL(control) ●
JT ⊙ MF △
CR ◪ EK ◆
FJ ▲ MT ×
CS ▢ LM +

% specific lysis

Dilution of anti-D serum
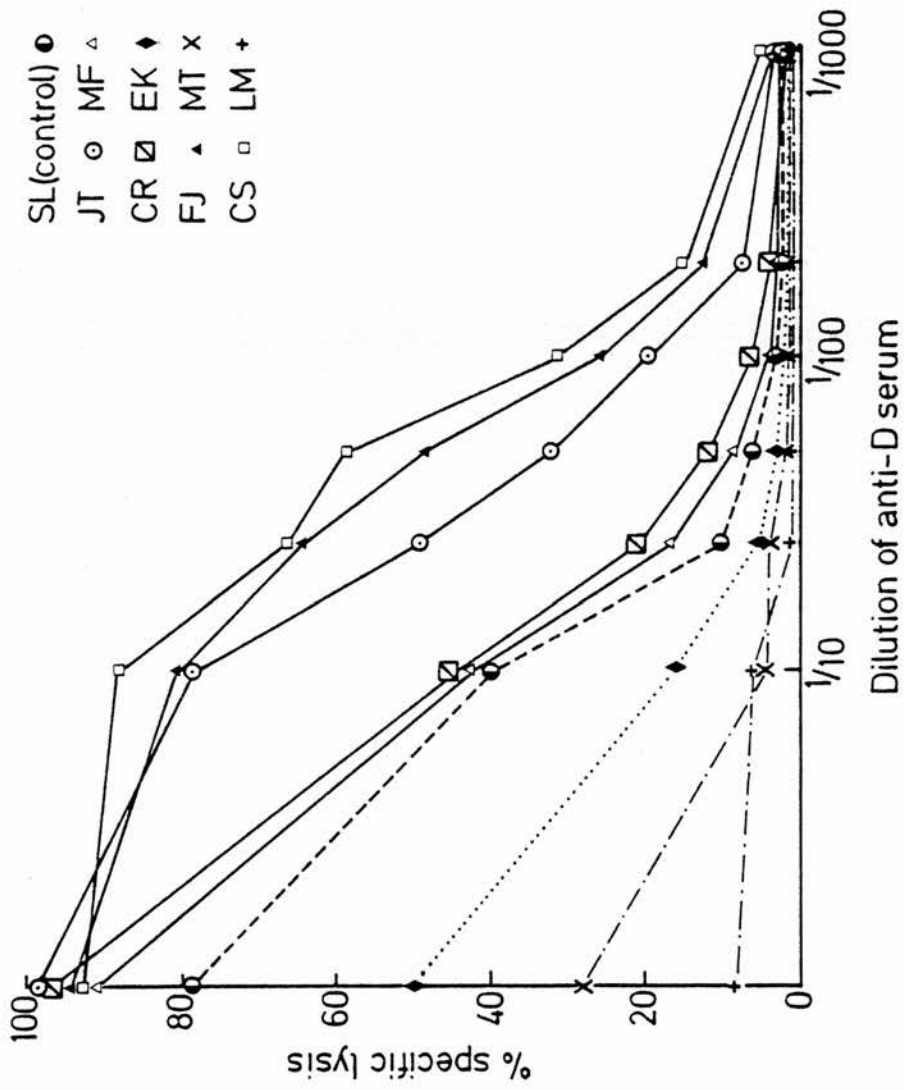
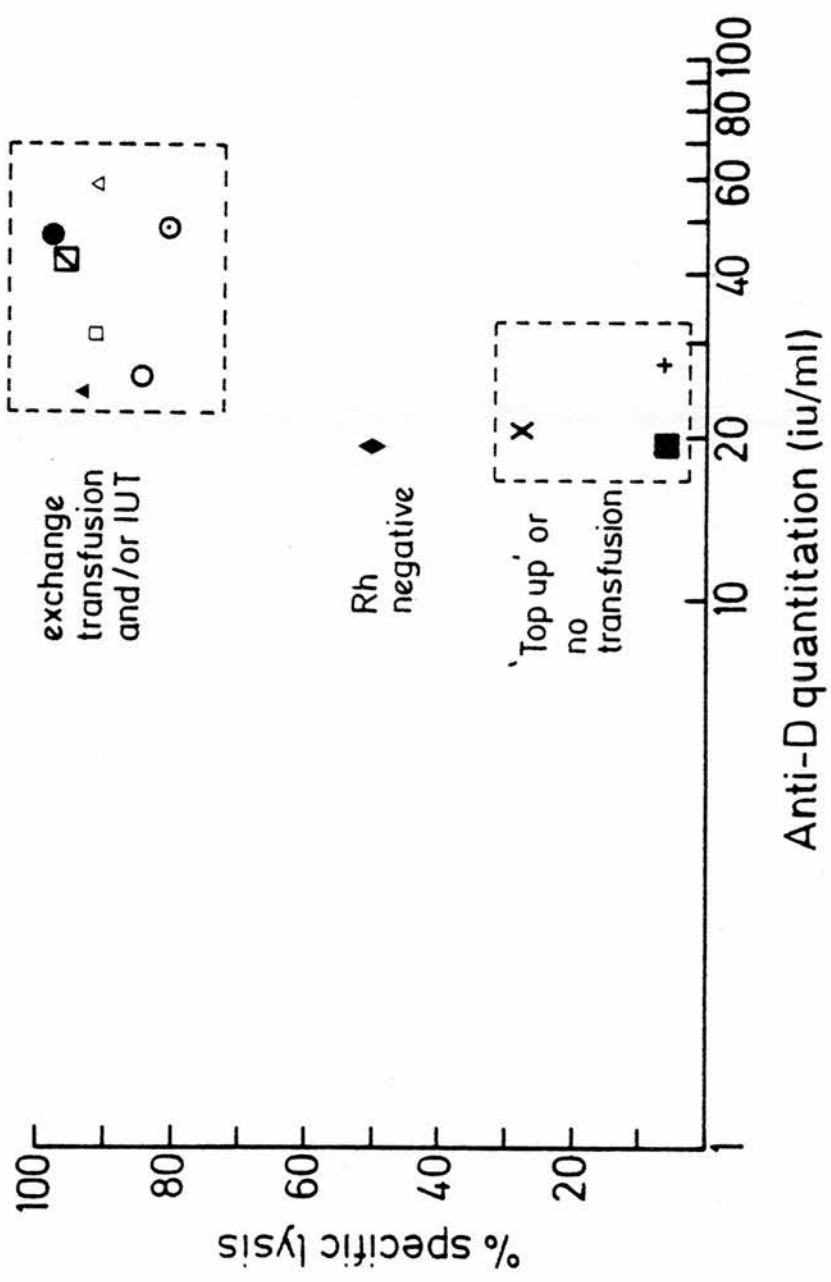ADCC activity of anti-D sera from series-2 cases

E:T ratio 10:1 (4 x 10⁴ OR₁R₁ per culture),

anti-D dilution as shown

Fig 18

would have been unnecessary. In one case (EK) a Rh(D) negative baby was delivered despite the amniocentesis prediction. The ADCC activities of these eight sera are shown in Figure 18.

Very high ADCC activity was seen in the five cases requiring intrauterine and/or exchange transfusion, and three of these five babies died. In all five cases the ADCC activity was greater than that of the control (SL). In the two cases where exchange transfusion was not required (MT, LM) extremely low ADCC activity was seen at all dilutions of anti-D serum and with the different K cell donors (Table 22). In the cases with an Rh(D) negative baby (EK) the moderate ADCC activity represented a "false-positive" reaction similar to the "prediction: given by amnio-centesis. As in the Series 1 cases, the active sera showed clear dose-response curves.

When the ADCC activity of the undiluted anti-D sera is correlated with the actual anti-D level (Fig 19) both Series 1 and Series 2 cases segregate into two well-defined groups on the basis of severity of the HDN. It can be seen that the % specific lysis gives better discrimin-ation between "good" and "poor" outcomes, than does anti-D quantitation alone.

Fig 19

Comparison of anti-D level (5 iu = 1 μg) with ADCC activity in relation to the severity of HDN. Cases from series-1 and series-2 combined (symbols refer to same cases from Figs 17 and 18). Values are those of "neat" anti-D with affector/target ratio 10:1).
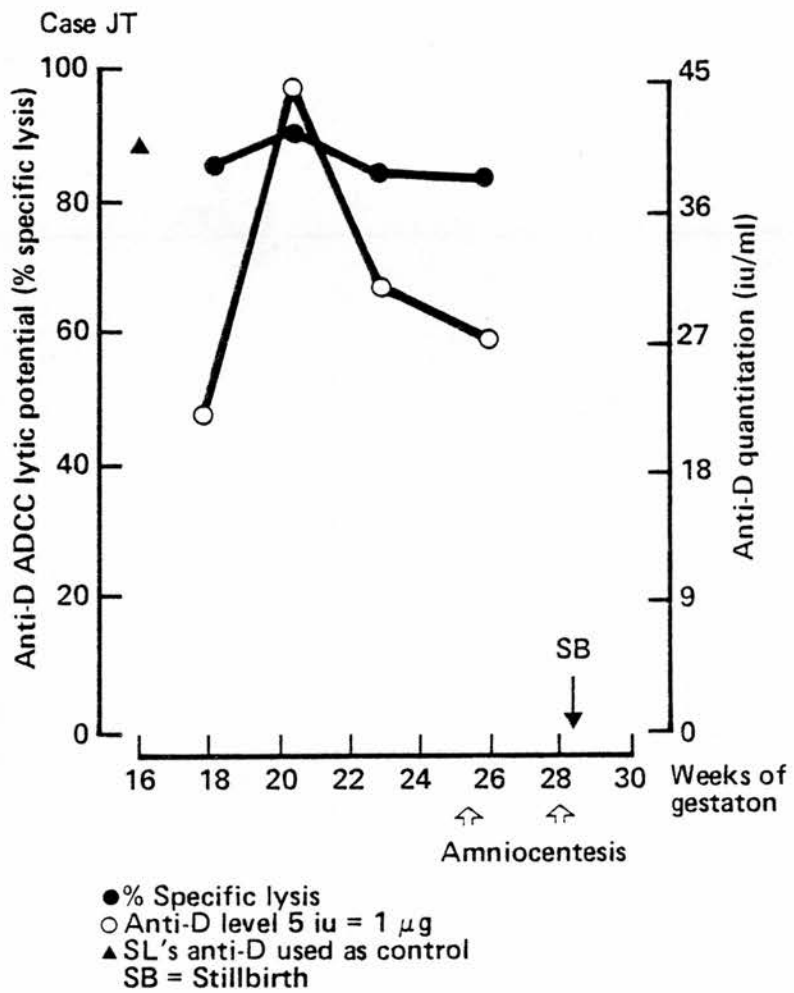
Further samples were provided from two cases JT
and FJ (in Table 21) during the same pregnancy
and these were tested with the same effector
cells (donor No 1). Results of the tests on
these are shown in Figs 20 and 21 respectively.
The ADCC activity of anti-D sera were consist-
ently high, confirming the previous observations.

3.4.5   Correlation of outcome with ADCC assays in HDN
        due to anti-c

Anti-c is the second most common cause of severe
HDN, after anti-D. Affected infants may require
exchange transfusion and perinatal deaths can
occur. As variations in the potency of anti-D
sera in mediating ADCC lysis correlate with the
outcome of HDN, and are therefore of value in
predicting severity of disease, it was of
interest to compare results obtained by
conventional assays with the ADCC assay in cases
of HDN due to anti-c, to determine whether a
similar correlation exists.

Anti-c sera from pregnant women were obtained
from blood samples which had been referred to the
laboratory undertaking antenatal testing. The
clinical details of the six cases selected for
study are given in Table 23. The IAGT did not
correlate well with subsequent severity of HDN.
In two cases where the titres were the same (ER
and AP) the outcome of pregnancy was different;

Case JT

ADCC activity of anti-D of JT during pregnancy

(from series-2)

Fig 20

Case FJ

● % Specific lysis E/T ratio 10:1
○ Anti-D level 5 iu = 1 μg anti-D
▲ SL's anti -D used as control
IUD = Intrauterine death
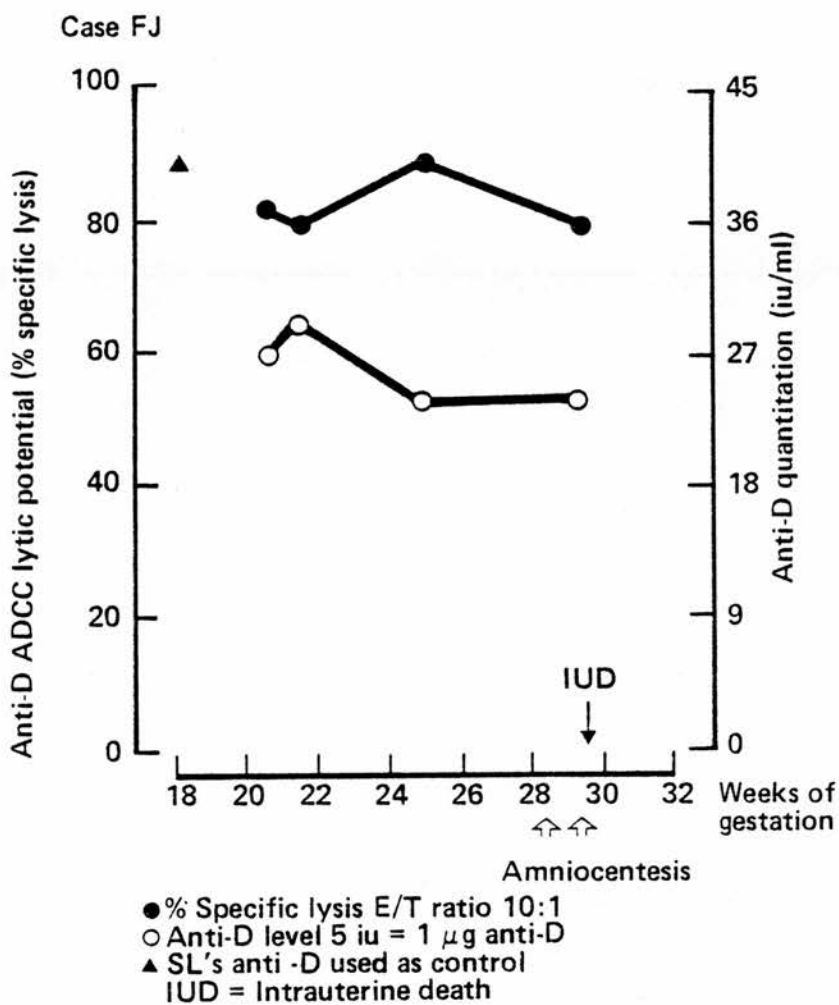
ADCC activity of anti-D of FJ during pregnancy

(from series-2)

Fig 21

Table 23

CLINICAL DETAILS AND LABORATORY FINDINGS IN CASES AFFECTED BY HDN DUE TO ANTI-c.

| Case | Previous History | Blood Group | | | ANTI-c IAGT (titre) | Min Hb (g/dl) | Max Bilirubin (μm/L) | DAGT | Transfusion | Outcome of Pregnancy |
		Mother	Father	Baby						
EY	Para 2 + 0 (no HDN)	AR_1R_1	O rr	OR_1r	256	11.0	308	Pos	Exchange transfusion x 1 + 2 red cell transfusions	Survived
JC	Para 3 + 0 (affected by HDN)	AR_1R_1	O rr	OR_1r	800	NT*	NT*	Pos	STILLBIRTH (38 Weeks)	
ER	Para 3 + 0 (no HDN)	OR_1R_1	OR_2r	OR_1r	8	12.8	380	Pos	Exchange transfusion x 2 + 1 red cell transfusion	Survived
NT	Para 1 + 1 (no HDN)	$A_2BR_1R_1$	OR_2r	BR_1r	32	13.0	395	Pos	Exchange transfusion x 1	Survived
AMc	Para 2 + 1 (no HDN)	OR_1R_1	O rr	OR_1r	32	9.8	349	Pos	Exchange transfusion x 2 + 2 red cell transfusions	Survived
AP	Para 2 + 1 (no HDN)	AR_1R_1	A rr	AR_1r	8	NT*	27	Neg	NONE	Survived

*NT = not tested; IAGT = indirect antiglobulin test; DAGT = direct antiglobulin test;
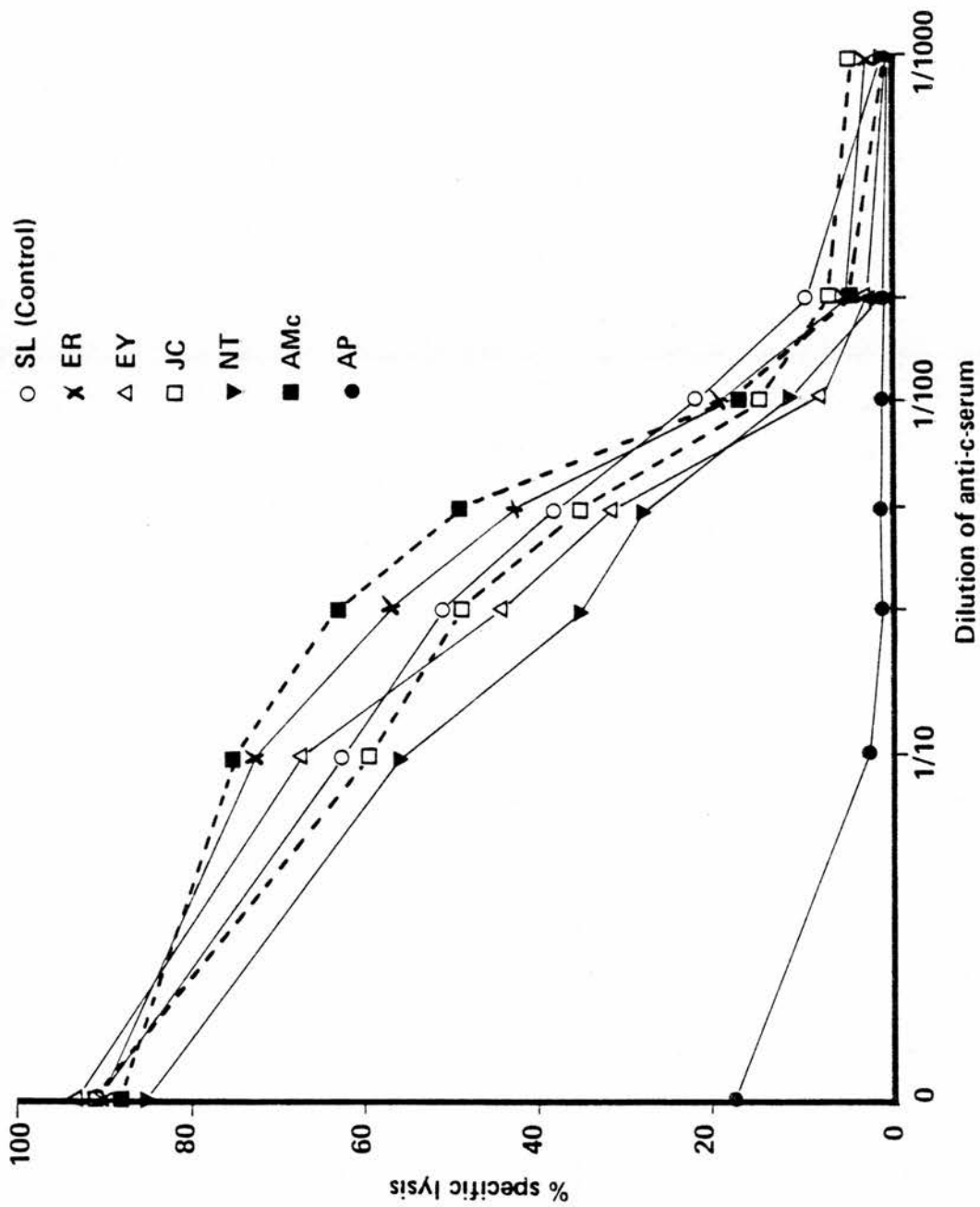minimum Hb and maximum bilirubin levels post delivery

AP producing a baby (A CDe/cde) not affected by HDN and ER having an affected baby which required exchange transfusion.

ADCC activity of anti-c sera

The same six anti-c sera (Table 23) were diluted in inert (Group AB) serum as shown in figure 22, and tested in an ADCC assay against a c-positive target red cell, using the same K-cell donor as above, the sera showing ADCC activity in a dose response manner on dilution. Very high anti-c lytic activity was seen in five cases, the infants of four of these subsequently requiring exchange transfusion and one (JC) resulted in a stillbirth. One patient (AP) had a healthy child and this was predicted by low activity in the ADCC assay.

3.4.6 ADCC activity and IgG subclasses of anti-c

With anti-D the IgG subclasses known to be lytic in ADCC are IgG_1 and IgG_3. (Section 3.2.7) The IgG sub-classes of anti-c in the six cases studied were heterogeneous (Table 24). However, there was a tendency for titres of IgG_3 antibodies to be higher in three of the five cases, which correlated with high lytic activity in the ADCC assay. It is also worth noting that case ER had a higher IgG_3 titre than AP. There were no obvious correlations between IgG_2 or IgG_4 anti-c and ADCC activity.

Fig 22

ADCC activity of anti-c

E:T ratio 10:1 (4×10^4 OR$_2$R$_2$ red cell per culture)

anti-c dilution as shown

% specific lysis

Dilution of anti-c-serum

○ SL (Control)
✗ ER
△ EY
□ JC
▼ NT
■ AMc
● AP

ADCC ACTIVITY OF ANTI—c SERA IN RELATION TO IAGT AND IgG SUBCLASSES TITRES

Case	Ab D	IAGT (titre)	IgG subclasses (titre)				ADCC activity (% Specific Lysis)*		
			IgG_1	IgG_2	IgG_3	IgG_4	Orr	OR_1R_1	OR_2R_2
SL	D	16	8	2	0	0	7	96	94
EY	c	256	16	4	64	0	94	1	93
JC	c	800	512	8	512	0	92	3	92
ER	c	8	4	8	16	4	90	6	93
NT	c	32	8	2	8	0	85	8	96
AMc	c	32	8	4	16	0	93	1	95
AP	c	8	8	2	8	0	10	5	16

* E:T 10:1 (4×10^4 RBC per culture); SL's anti-D used as control;

Ab = Antibody; IAGT = indirect antiglobulin test (broad spectrum)

Anti - c final concentration in culture 1 in 3

3.5   Rh immunization in male volunteers

In order to maintain a satisfactory supply of plasma for
the production of anti-D immunoglobulin it is necessary
to recruit and immunize male volunteers.   This is
primarily due to the diminishing numbers of women
sensitized during pregnancy as a result of the
successful programme of prophylaxis against Rh(D)
immunization.

3.5.1   Immunizing schedule

Individually matched Rh(D) positive frozen/
thawed red blood cells were used to immunize
Rh(D) negative male volunteers.   The immunizing
schedule (Fig 23) consisted of a single infusion
of 200 ml of frozen/thawed red cells followed,
after a six-month rest period, by six-monthly
booster doses of 0.5 to 1.0 ml using the same red
cell donor (Urbaniak and Robertson, 1981).

3.5.2   Normal values for lymphocyte ADCC

Six Rh(D) negative males were studied pros-
pectively, four of whom had produced anti-D
detectable by continous flow analysis, following
primary immunization, and two of whom had not.
Evaluation of lymphocyte ADCCs in healthy
individuals showed that while the majority
exhibited high specific cytotoxicity (75-100%
specific lysis) in this system at a 10:1 E/T
ratio, some individuals had a lower lytic
capacity.   There was no apparent correlation
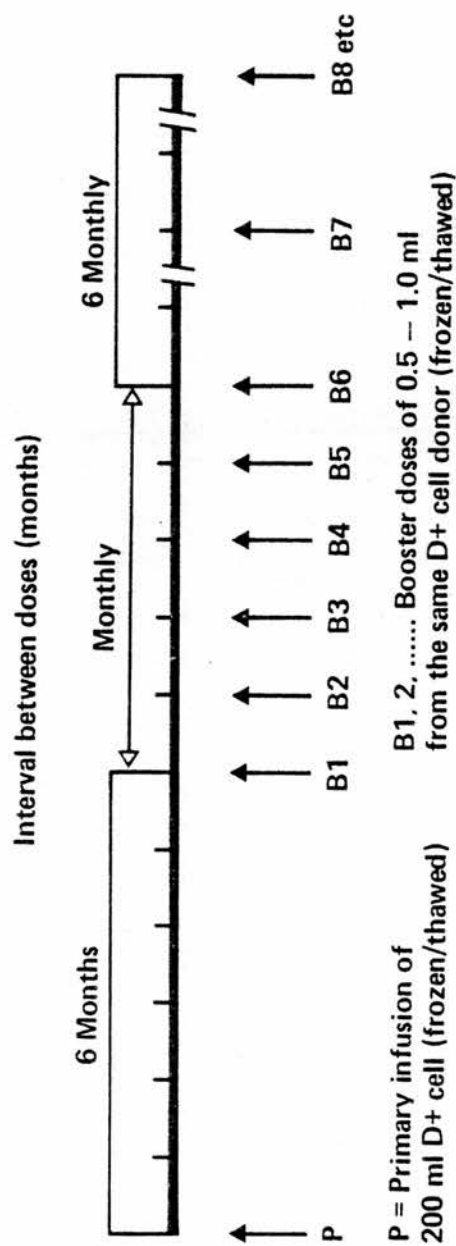
143

Fig. 23

between ADCC activity and ABO or Rh blood groups (Section 3.1.4). However, the lytic capacity of each individual appeared relatively constant and varied little on repeated evaluations over periods up to 2 years in some cases. The six volunteers were tested for their lymphocyte ADCC activity using E/T ratios of 10:1, 5:1, 1:1, 1:5 and 1:10. Normal ranges of ADCC were established for each subject by repeated testing during the 6 months prior to immunization, and these were taken as two standard deviations from the mean values (Table 25). In general, the cells of R2 showed higher lymphocyte ADCC activity than those of the other subjects. R1 and R3 were studied in greater detail (see below).

3.5.3 Changes in ADCC lymphocyte capacity during primary immunization

Four male volunteers 1R, R3, NR2 and NR2 (Table 25) were studied from the time of primary immunization with 200 ml frozen/thawed red cells (Figure 23). The E/T ratios were 10:1, 5:1, 1:1, 1:5 and 1:10. For simplicity, only the maximal specific lysis at 10:1 is given (Fig 24). Anti-D (SL) serum was used in all experiments to enable quantitative comparisons of the lymphocyte ADCC activity of each subject to be made. Subject R1 showed a slight transient fall in ADCC activity a week after immunization, and this lasted for 5 weeks. Anti-D was first detected at 8 weeks. In
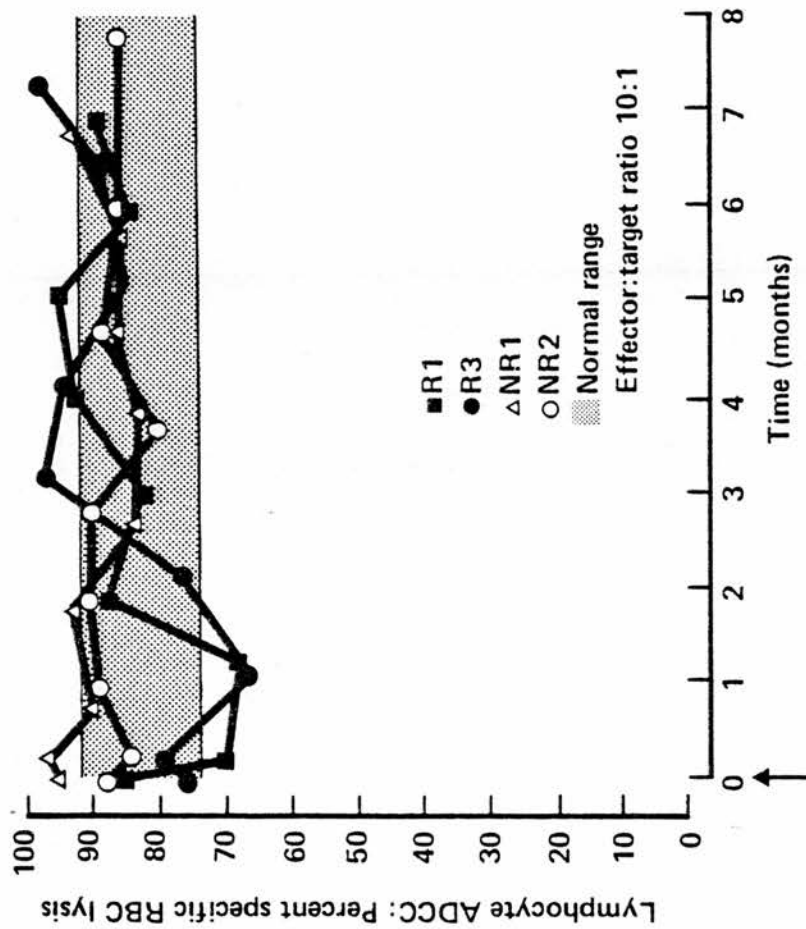
Table 25

Lymphocyte ADCC activity in responders (R) and non-responders (NR) studied at times when these volunteers were not being subjected to immunizations

Subject	No of Times Tested	Effector / Target Cell Ratios *				
		10:1	5:1	1:1 Values+	1:5	1:10
R 1 (B cde/cde)	14	84 ± 7.3	61 ± 6.1	$28. \pm 6.3$	10.3 ± 6.2	3.3 ± 2.2
R 2 (A2 cde/cde)	7	92.1 ± 6.1	72.3 ± 7.7	35.5 ± 5.7	21.5 ± 5.7	9.6 ± 3.0
R 3 (A1 cde/cde)	14	84.8 ± 9.2	65.0 ± 8.5	25.0 ± 5.5	13.2 ± 7.6	5.3 ± 3.9
R 4 (0 cde/cde)	7	88.4 ± 6.6	58.2 ± 7.4	27.3 ± 6.6	11.0 ± 6.2	6.3 ± 4.9
NR1 (0 cde/cde)	10	86.8 ± 8.1	63.5 ± 9.4	34.0 ± 8.0	8.8 ± 3.2	4.3 ± 2.2
NR2 (A2 cae/cde)	11	86.5 ± 9.4	59.3 ± 6.3	28.6 ± 9.6	12.3 ± 5.0	4.3 ± 3.3

* E:T ratio 10:1 (4×10^4 red cell per culture)

+ Mean $\pm$ standard deviation

LYMPHOCYTE ADCC ACTIVITY IN MALE VOLUNTEERS
AFTER PRIMARY IMMUNIZATION

Lymphocyte ADCC: Percent specific RBC lysis

Time (months)

■ R1
● R3
△ NR1
○ NR2
▨ Normal range

Effector:target ratio 10:1

P = Primary immunization 200 ml D + cells (frozen/thawed)

Fig. 24

subject R3 there was a slight short-lived drop of intrinsic ADCC activity at 4.5 weeks, and anti-D was first detected 13 weeks after immunization. However, subjects NR1 and NR2 showed no change in intrinsic ADCC activity.

3.5.4   Effect of booster doses on lymphocyte ADCC activity

The four Rh-negative volunteers who had detectable anti-D following primary immunization and responded to boosting with a rise in serum anti-D level were classed as responders (Fig 25, R1-R4). The remaining two subjects did not produce detectable anti-D in their serum at any time and these were classed as non-responders (NR1 and NR2). Peripheral blood samples for lymphocyte and serum studies were taken from subjects immediately prior to boosting and at various times thereafter (always including day 7).

In both non-responders studied, no changes in intrinsic ADCC activity were seen following booster injections of red cells (Fig 26, NR1 and NR2). Marked, but short-lived, depressions of ADCC activity were seen following booster injection in all four responders (Fig 26, R1 to R4). Examination of ADCC activity over a range of K-cell:target cell ratios following boosting confirmed that there was abolition of K-cell
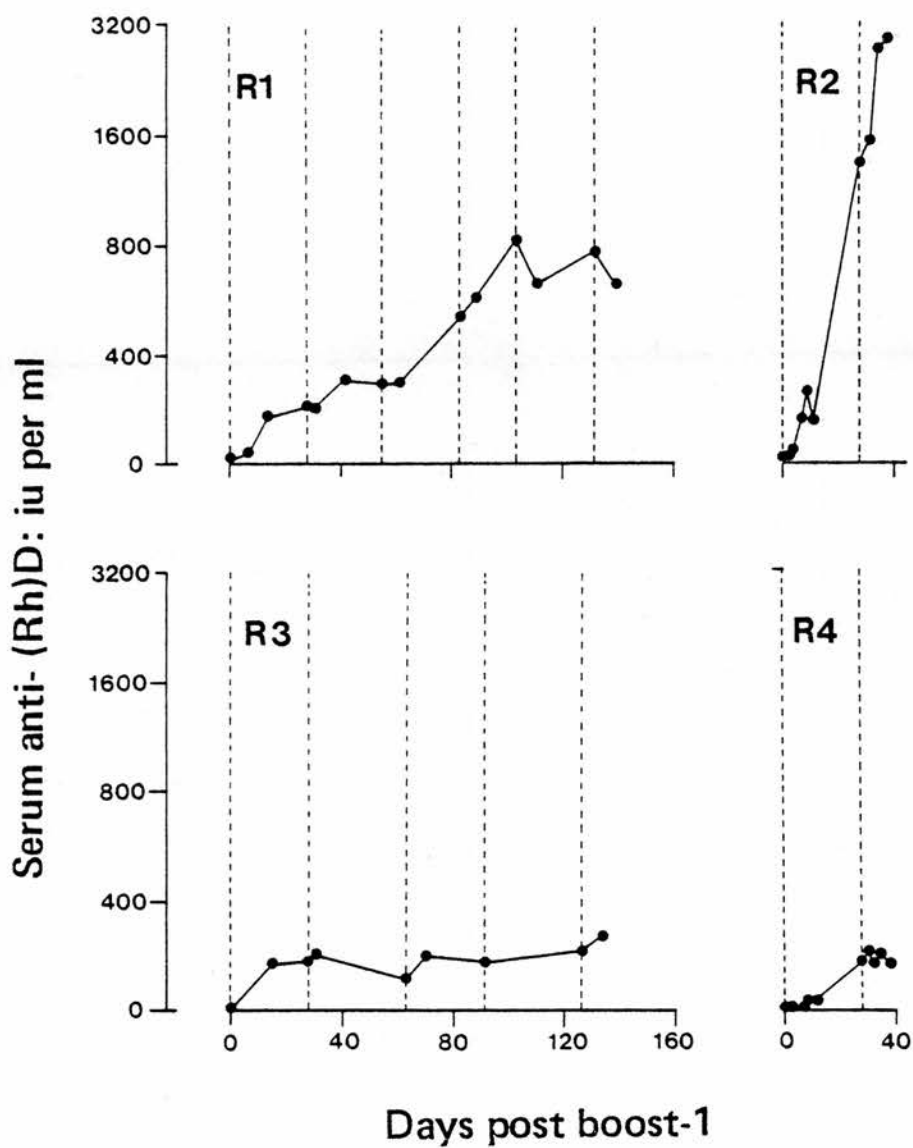
The effects of booster immunization on anti-D responses in subjects who were successfully immunized (responders R1-R4) Changes in serum anti-D are shown in relation to times of boosting by vertical broken lines.

Fig. 25

Fig. 26

The effects of booster immunization on lymphocyte
ADCC activity in responders (R1-R4) and non-responders
(NR1, NR2). Boosts are indicated by vertical broken
lines, and the normal ranges of each individual's
ADCC lymphocyte capacity are shaded (see normal values
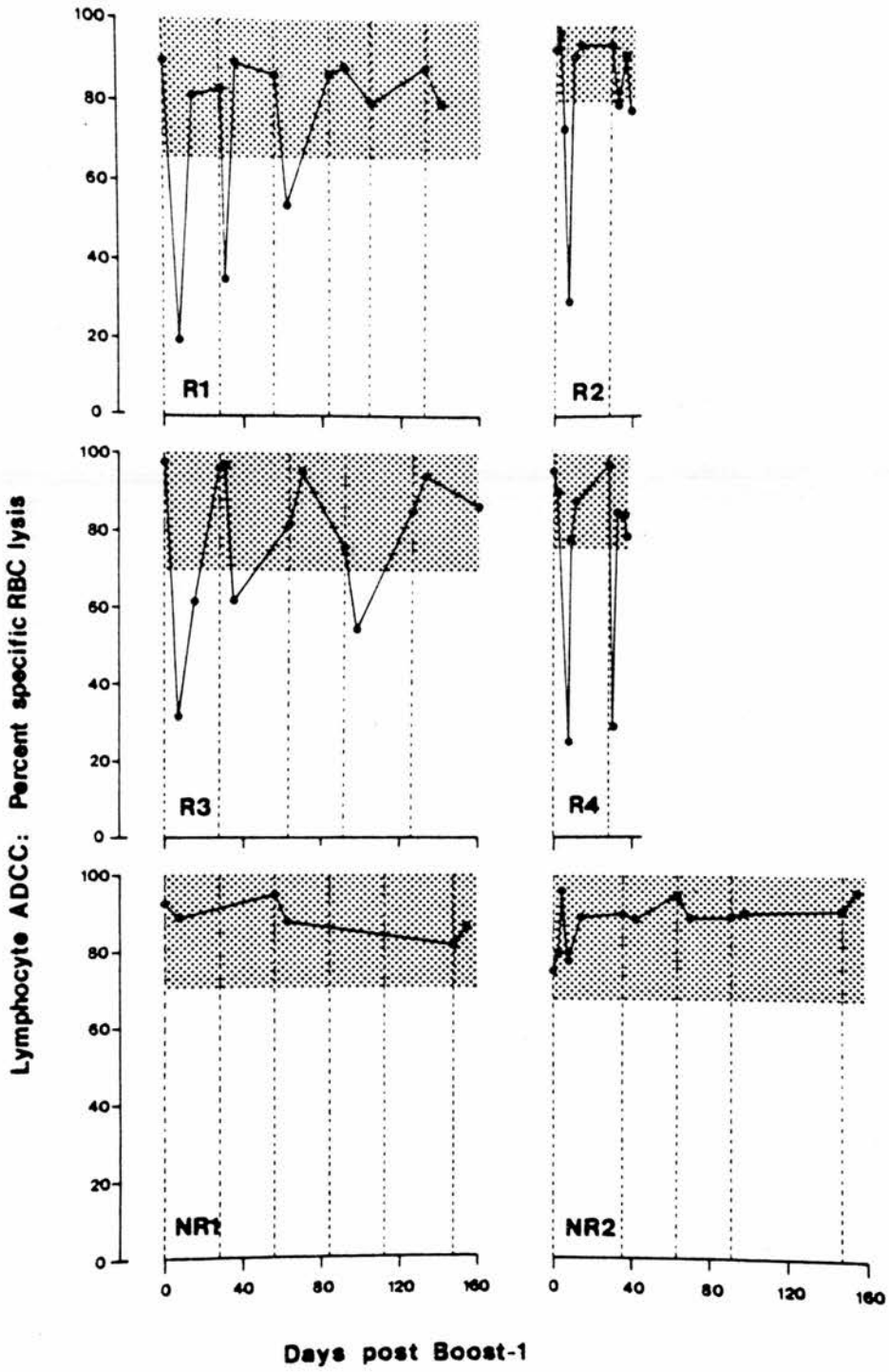Table 25).

Fig. 26

function rather than a shift in the "dose-response" curve, and that the effect of boosting was transient. The perturbation of ADCC induced by boosting appeared to diminish with subsequent successive boosts (Fig 26). However, detailed examination of the ADCC responses at frequent intervals following boosting indicated that with later boosts the depression of ADCC activity was of the same order of magnitude as with earlier boosts, but that loss of activity occurred earlier after the boost (Figs 27-30).

3.5.5   ADCC anti-D lytic activity

Using autologous cells:

In parallel experiments to determine intrinsic ADCC activity of the subjects R1 and R3 in an identical culture, each subject's K-cells were tested with their own serum instead of anti-D (SL). The pattern of intrinsic ADCC activity was similar to that when anti-D (SL) was used (31, 32). This was more obvious with subject R1 than with R3, who had a lower anti-D level than R1.

Using a normal K-cell donor:

Anti-D from subjects R1 and R3 was collected and stored at $-40^{o}C$ during the immunization schedule. These sera were tested against normal K-cells at an E/T ratio of 10:1. Both R1 and R3 showed marked ADCC anti-D activity after the first boost (Fig 31-32). However, in subject R1 the activity

started at the time of the first boost when his anti-D level was at 92 iu/ml. On the other hand the anti-D level of R3 at the same time was 13 iu/ml. In general, the ADCC anti-D activity of R1 was more lytic than that of R3.
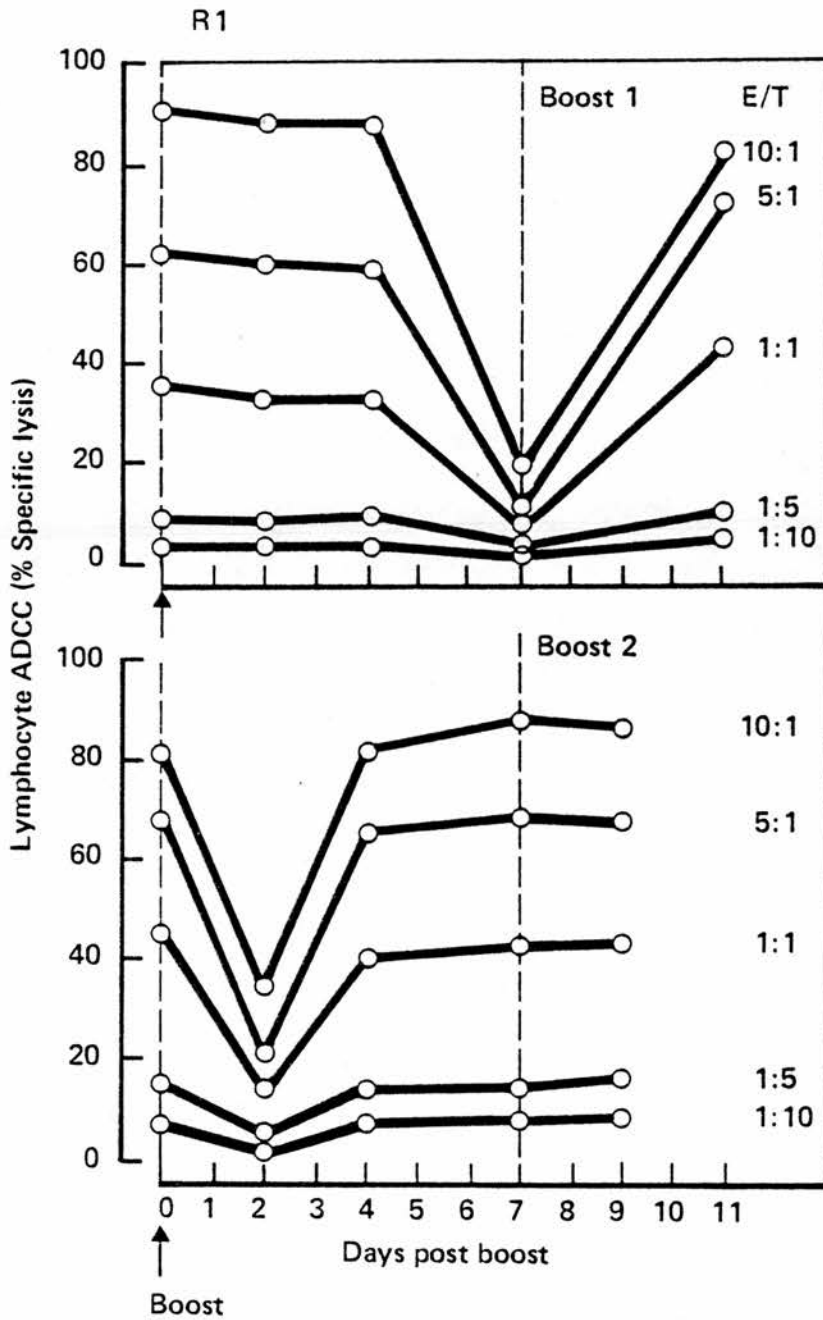
Lymphocyte ADCC activity in a responder (R 1):
effect of repeated boosts at various E/T ratio.
The usual time of assay (day 7) is indicated

Fig. 27

154
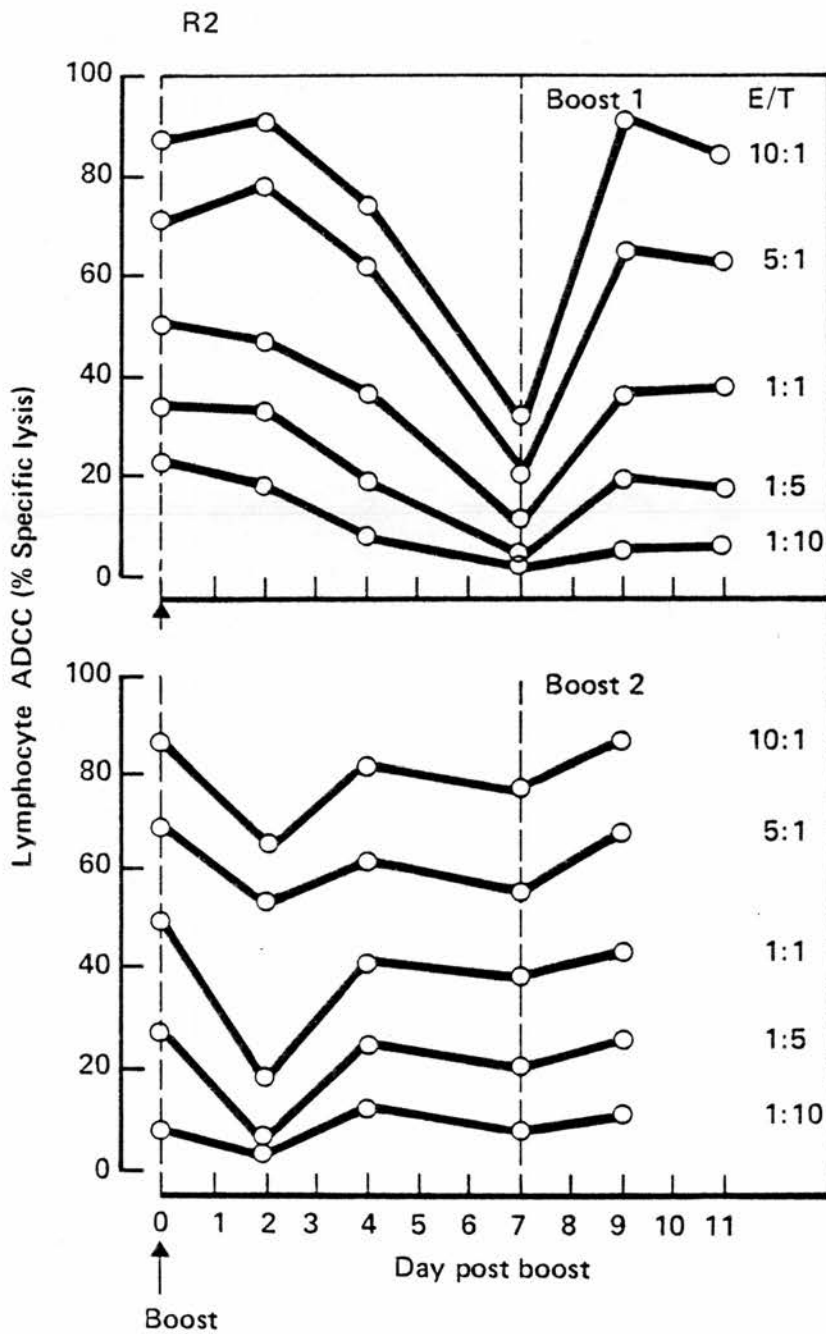
Lymphocyte ADCC activity in a responder (R2):
effect of repeated boost at various E/T ratio.
The usual time of assay (day 7) is indicated

Fig. 28

Lymphocyte ADCC activity in a responder (R3):
effect of repeated boosts at various E/T ratio.
The usual time of assay (day 7) is indicated

Fig. 29

156
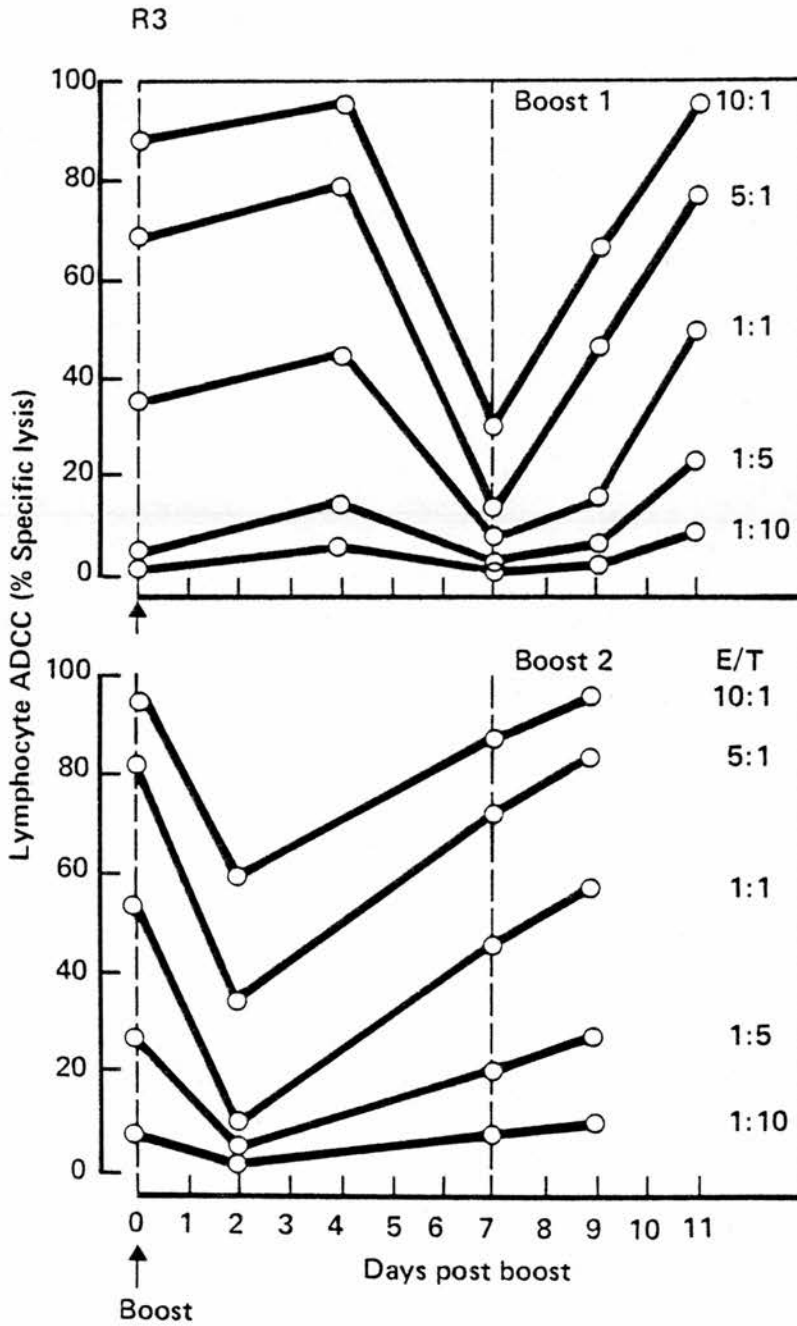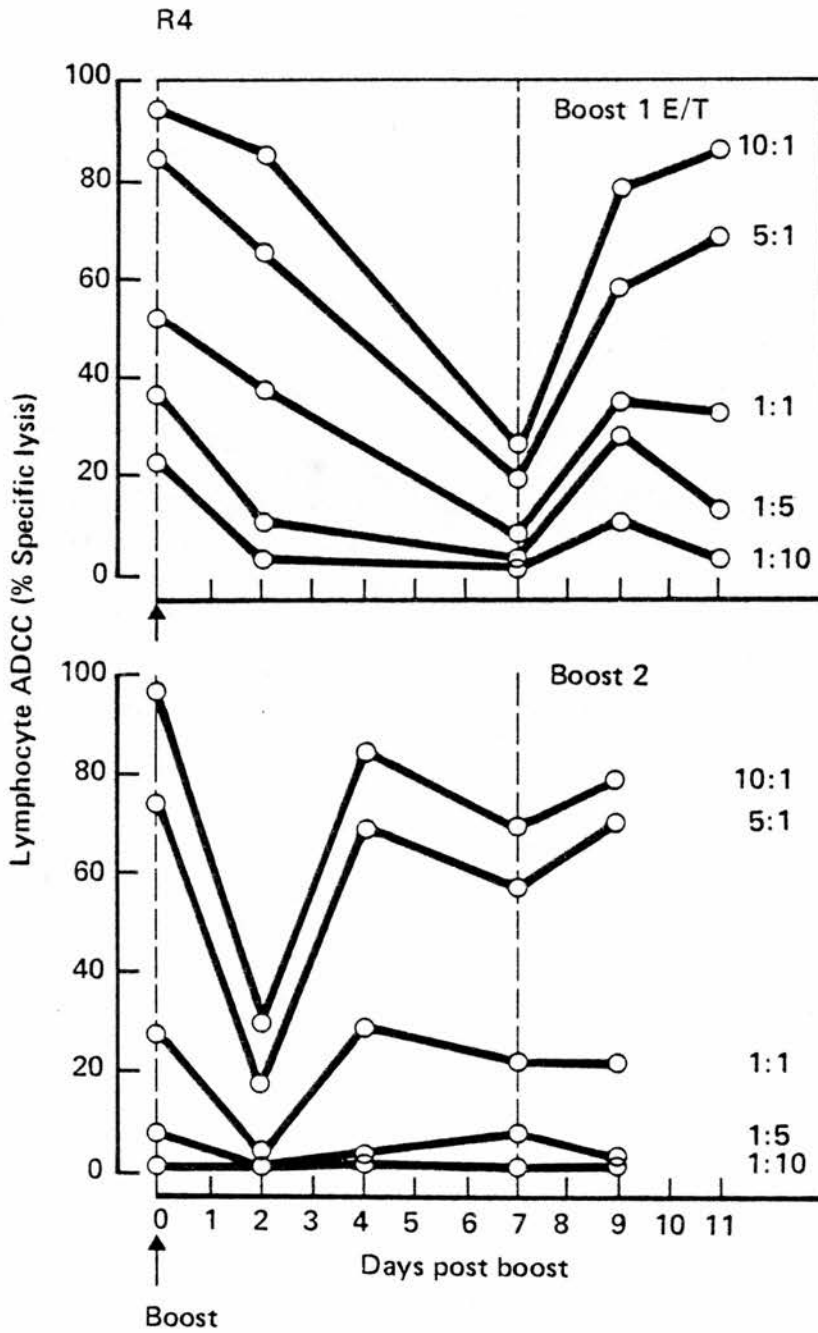

Lymphocyte ADCC activity in a responder (R4):
effect of repeated boosts at various E/T ratios.
The usual time of assay (day 7) is indicated

Fig. 30

Fig. 31

Intrinsic ADC activities (lymphocyte and anti-D) and anti-D level during immunization schedule in R1.

Fig. 31

P = Primary immunization 200 mls D + cells (frozen/thawed)
B1–6 Boost from the same D+ cells (0.5 ml)
E:T ratio 10:1

159

Fig. 32

Intrinsic ADCC activities (lymphocyte and anti-D)
and anti-D level during immunization schedule in
R3.

Fig. 32

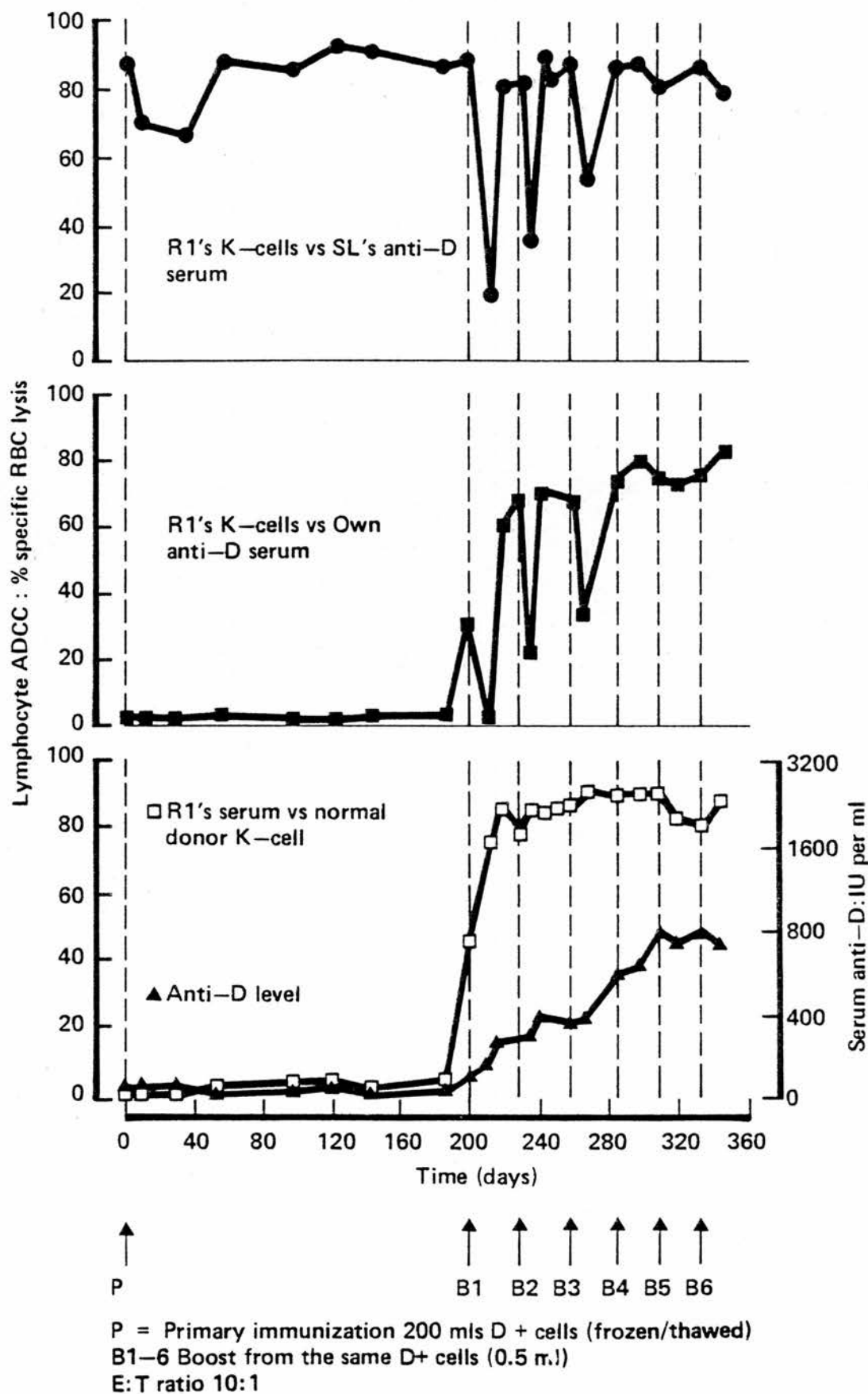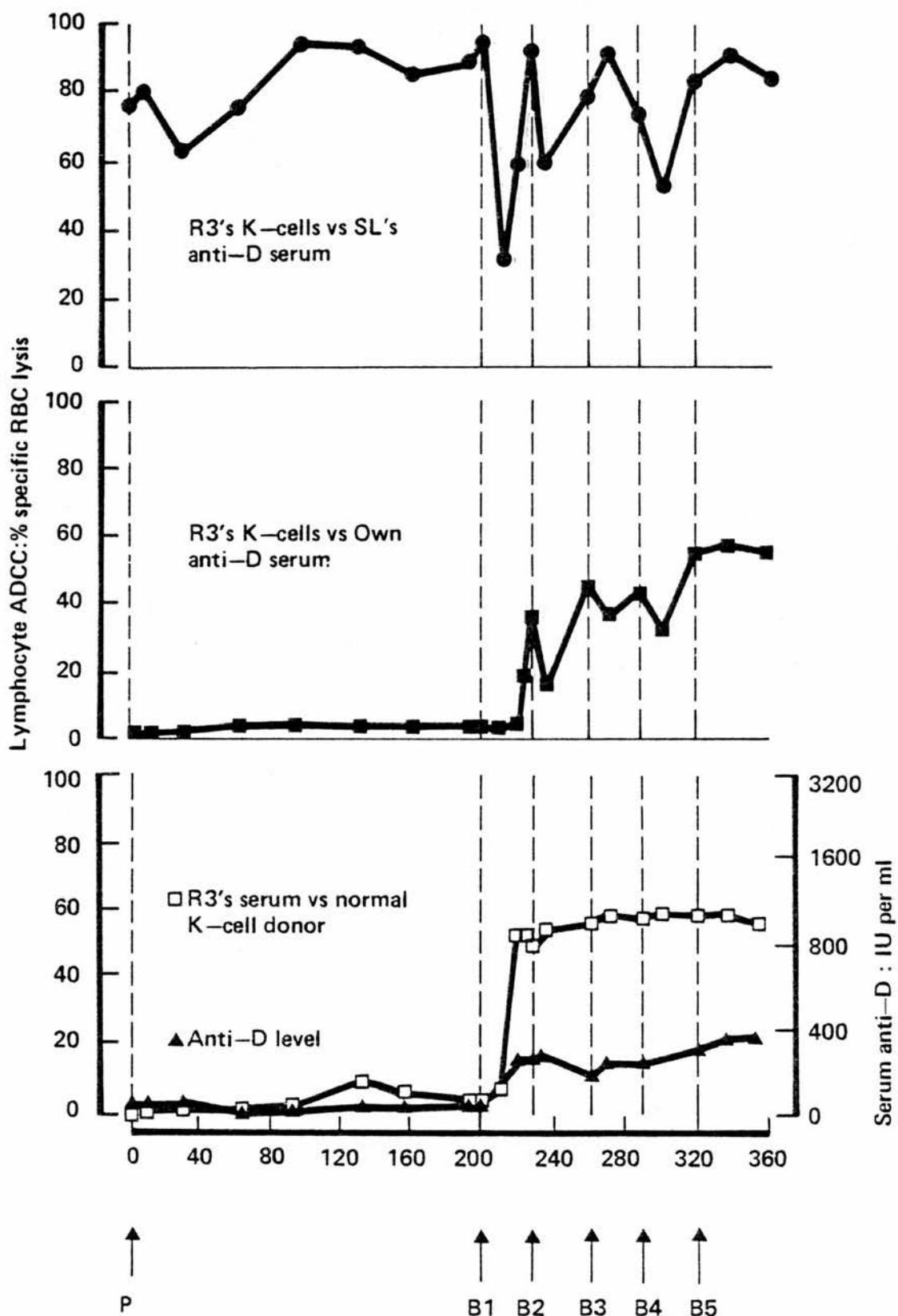## 3.6   Antenatal plasma exchange to reduce anti-D concentration

The technique of plasmapheresis has been used for many years to collect plasma from males and females iso-sensitized to the Rh(D) antigen, and with high antibody concentrations, for the preparation of anti-D immuno-globulin. The procedure has also been used in Rh immunized mothers during the antenatal period, in an attempt to reduce the level of antibody. Bowman et al (1969) and Powell (1968) tried antenatal plasmapheresis with equivocal results but Clark et al (1970) reported success after combining antenatal plasmapheresis with intra-uterine transfusion.

Plasma exchange may be more desirable than plasmapheresis alone, since plasmapheresis merely removes plasma while plasma exchange replaces the patient's plasma with normal plasma: this both replenishes plasma components and dilutes maternal IgG anti-D relative to the total plasma immunoglobulin levels (Powell, 1968 and Graham-Pole et al, 1977).

3.6.1   Effect of plasma exchange on maternal anti-D concentrations

The patients examined had been referred to the Regional Blood Transfusion Centre, Edinburgh, by local obstetricians and the clinical details are given in Table 26.

Three of the six mothers studied had experienced a foetal death in utero (AB, BL and MC), two had

Table 26

DETAILS OF PATIENTS SELECTED FOR PLASMA EXCHANGE

Case		Blood Group	Previous History	Father's Blood Group	Gestational Age (weeks at Presentation)	Presenting Anti-D Level	
						* IAGT titre	Analyser (iu/ml) **
1. AB	36 Yrs	O rr	Para 3 + 0 moderate HDN	B R_1R_2	27	1 : 64	30.70
2. BL	35 Yrs	O rr	Para 2 + 1 stillbirth	O R_1R_2	27	1 : 128	45.50
3. MC	45 Yrs	O rr	Para 5 + 2 intrauterine death	O R_1R_2	27	1 : 128	34.50
4. JM	25 Yrs	B rr	Para 4 + 0 moderate HDN	Father Uncertain	24	1 : 1024	89.50
5. ID	28 Yrs	O rr	Para 3 + 2 stillbirth	O R_2 r	25	1 : 32	26.50
6. HA	39 Yrs	A rr	Para 3 + 0 moderate HDN	O R_1R_2	12	1 : 1024	32.85

* IAGT = Indirect antiglobulin test; ** 5 iu = 1 μg anti–D

produced Rh(D) infants unaffected by Rh HDN (JM and ID) and one (HA) had a baby which had required two exchange transfusions and had survived (Table 27). In each of the cases, intensive plasma exchange was initiated at various stages of pregnancy (Table 26) and carried out with a continous-flow cell separator (Aminco Celltrifuge). During each exchange an average of 3-4 litres of maternal plasma was exchanged with an equal volume of normal frozen fresh plasma (FFP) and/or plasma protein solution (PPS 5% human albumin). Plasma exchange was undertaken approximately daily for the remainder of the pregnancy. FFP was used from the first day of exchange. In the first five cases the plasma used was from donors of the same ABO group, but not the same Rh group, as the patient; however HA was exchanged with ABO and Rh specific FFP. Throughout the study, anti-D levels were measured by conventional manual methods and by continous-flow quantitation.

It can be seen from Figs 33-37 that with daily exchange the first five cases each showed an initial reduction in the level of anti-D of the order that might be expected on theoretical grounds. After the eighth day, following initial exchange, despite continued exchange therapy there was an apparent increase in the level of anti-D, reaching its peak at or about the

Table 27

CLINICAL OUTCOME OF PLASMA EXCHANGE

Case	Number of Exchanges	Number of Weeks Treated	Litres Exchanged FFP [a] Rh +	Rh –	PPS [b]	Highest Anti–D Level (iu/ml)	Outcome of Pregnancy
1. AB	15	3	48.93	19.68	Nil	466.50 at day 15	IUT at 27 weeks, 28weeks; [c] IUD at 30 weeks [d]
2. BL	9	2	28.20	10.57	Nil	362.50 at day 12	IUT at 27 weeks, 29 weeks; IUD at 29 weeks
3. MC	9	3	25.55	8.76	Nil	134.50 at day 18	IUT at 27 weeks, 28 weeks, 28½ weeks, 30 weeks; IUD at 30½ weeks
4. JM	44	12	36.12	17.58	92.80	305.00 at day 10	ORh negative baby survived
5. ID	19	6	34.43	10.83	14.80	138.00 at day 13	ORh negative baby survived
6. HA	41	19.5	Nil	29.05	69.70	32.85 at day 1	ARh positive DAGT +++; [e] exchange transfusion x 2 survived

a. FFP = Fresh frozen plasma; b. PPS = plasma protein solution; c. IUT = intrauterine transfusion;

d. IUD = intrauterine death; e. DAGT = direct antiglobulin test

10th-18th day. In three cases (AB, BL and MC) amniocentesis and intrauterine transfusions were also carried out. In case 6 (HA, Fig 38) the anti-D level was reduced and remained around 5 iu/ml until the end of the plasma exchange therapy. Amniocentesis was performed at 32 and 36 weeks gestation, after the exchange therapy had ended after 41 exchanges (at 31.5 weeks gestation).

3.6.2 Effect of plasma exchange on anti-D IgG sub-classes (case AB)

Anti-D IgG sub-classes were studied during plasma exchange of AB. Samples were collected before and after exchange, as shown in Table 28, and Figure 33-A. As can be seen, there was a correlation between the IgG sub-class titres and anti-D concentration. However, there was no evidence of a qualitative difference.

3.6.3 Effect of plasma exchange on anti-D ADCC activity

Maternal sera obtained from clotted blood taken before and after each exchange was inactivated by heating at $56^{o}C$ for 30 min and stored at $-40^{o}C$ for retrospective investigation. The sera were tested on different occasions using the same K-cell donor (effector), the same target red cells (OR_1R_1) and anti-D (SL) as a control for all tests (% specific lysis = 86.6 ± 1.5). It has been shown that the ADCC of anti-D is

1:10.   For simplicity, the maximum specific lysis
at 10:1 is given.   Anti-D (SL) serum was used in
all experiments to enable quantitative compari-
son.

In two cases (JM and ID) a marked, but short-
lived, depression of ADCC activity was seen on
day 6 (Fig 36, 36A, 37A).   When the lymphocytes
of patient ID were tested with her own anti-D
(the first sample before plasma exchange) on the
same day and using the same target cells, the
depression of ADCC activity was of the same order
of magnitude as with anti-D (SL).

However, in patient HA, who was exchanged with
Rh(D) negative FFP, no changes in intrinsic ADCC
activity were seen (Fig 38).

Fig. 33

The effect of plasma exchange by FFP, unselected for Rh group, on anti-D ADCC lytic potential and maternal anti-D concentration.

Days on which amniocentesis and intrauterine transfusion were carried out are indicated (arrowed).

● Pre-

       plasma exchange

○ Post-

E:T ratio 10:1 (4×10^4 OR_1R_1 red cells/culture)

Anti-D concentration: Neat (1in 3 in final culture)

* intrauterine death

169
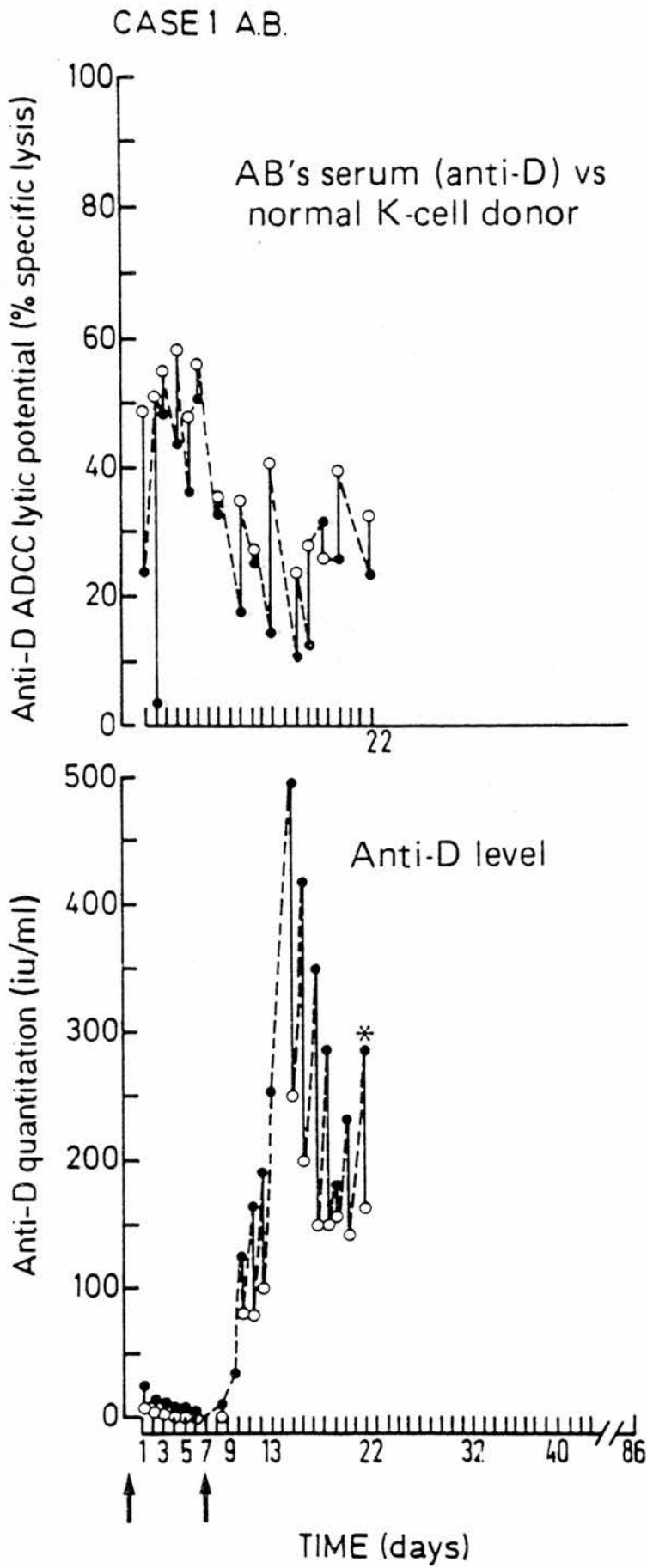
CASE 1 A.B.
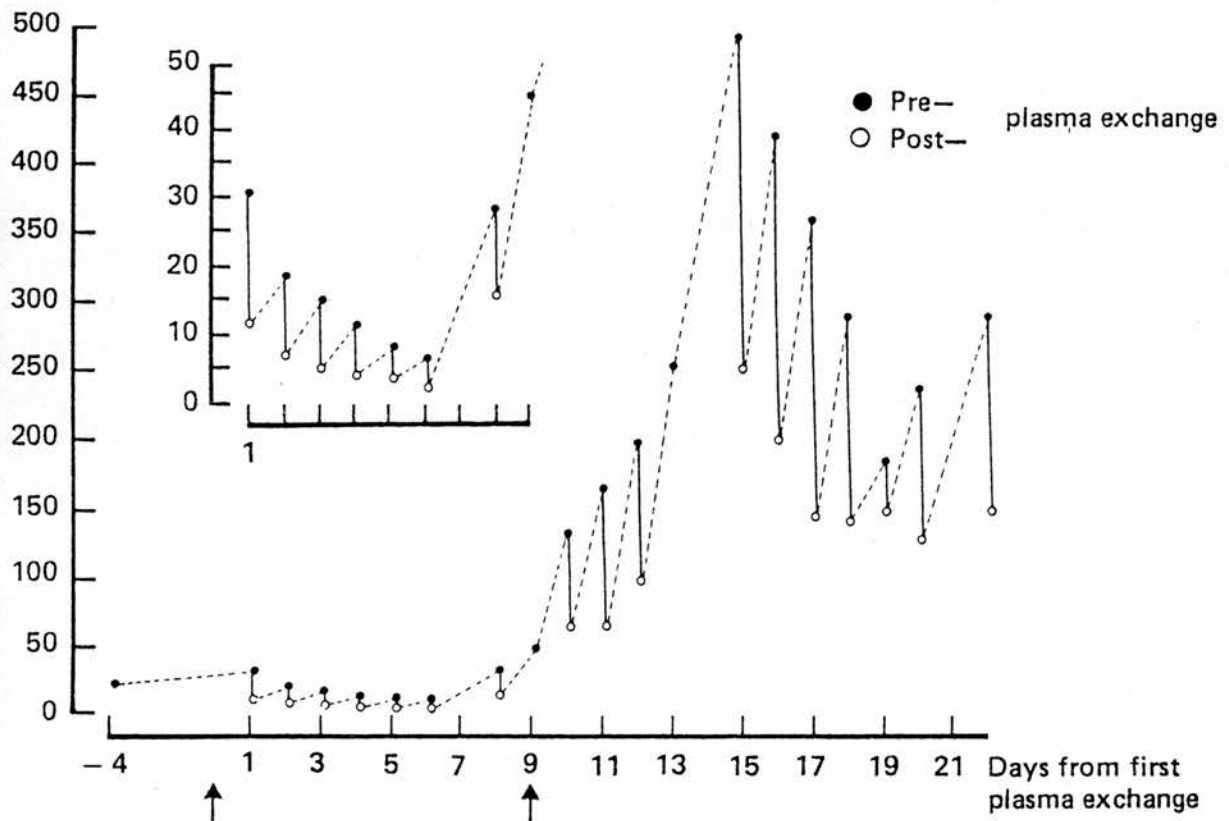
Fig. 33

Effect of plasma exchange on maternal anti-D concentrations
Days on which amniocentesis and intrauterine transfusion were
carried out are indicated (arrowed)

Fig. 33A

Table 28

Effect of Plasma Exchange on Anti-D IgG Sub-classes in Case   (AB)

Weeks of Gestation	Autoanalyser Anti-D *		IgG_1	IgG_2	IgG_3	IgG_4	IAGT
27	Pre:	30.7	128	16	64	0	64
	Post:	12.0	32	8	16	0	16
27.5	Pre:	10.6	16	2	8	0	32
	Post:	4.8	8	1	4	0	32
28	Pre:	6.1	32	4	16	0	64
	Post:	3.1	32	4	16	0	64
28.5	Pre:	129.0	128	16	128	0	512
	Post:	65.3	64	8	16	0	128
	Pre:	163.0	128	16	64	0	128
	Post:	65.3	64	8	32	0	64
29	Pre:	491.5	1024	128	512	0	4096
	Post:	249.5	512	64	256	0	512
30	Pre:	285.0	512	64	128	0	512
	Post:	147.2	256	64	128	0	128

Anti-D quantitation;   5 iu = 1 μg.
IAGT = Indirect antiglobulin test (broad spectrum)
Pre and post-plasma exchange

Fig. 34

The effect of plasma exchange by FFP, unselected for Rh group, on anti-D ADCC lytic potential and maternal anti-D concentration.

Days on which amniocentesis and intrauterine transfusion were carried out are indicated (arrowed).

● Pre-
          plasma exchange
○ Post-

E:T ratio 10:1 (4×10^4 OR$_1$R$_1$ red cells/culture)
Anti-D concentration: Neat (1 in 3 in final culture)
✻ intrauterine death

CASE 2 B.L.
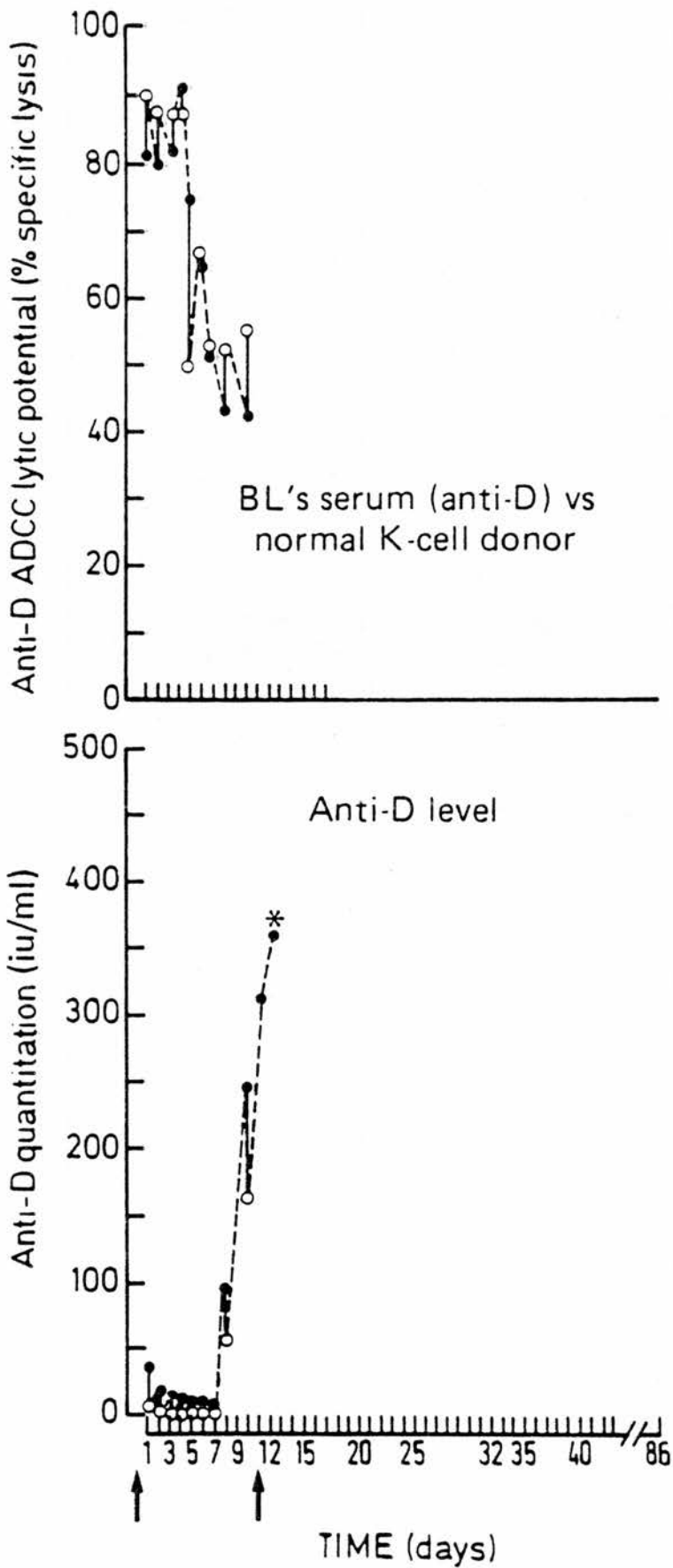
Fig. 34

Fig. 35

The effect of plasma exchange by FFP, unselected for Rh group, on anti-D ADCC lytic potential and maternal anti-D concentration.

Days on which amniocentesis and intrauterine transfusion were carried out are indicated (arrowed).

● Pre-

        plasma exchange

○ Post-

E:T ratio 10:1 (4×10^4 OR_1R_1 red cells/culture)

Anti-D concentration: Neat (1 in 3 in final culture)

* intrauterine death

CASE 3 M.C.

MC's serum (anti-D) vs normal K-cell donor

Anti-D level

Fig. 35

176
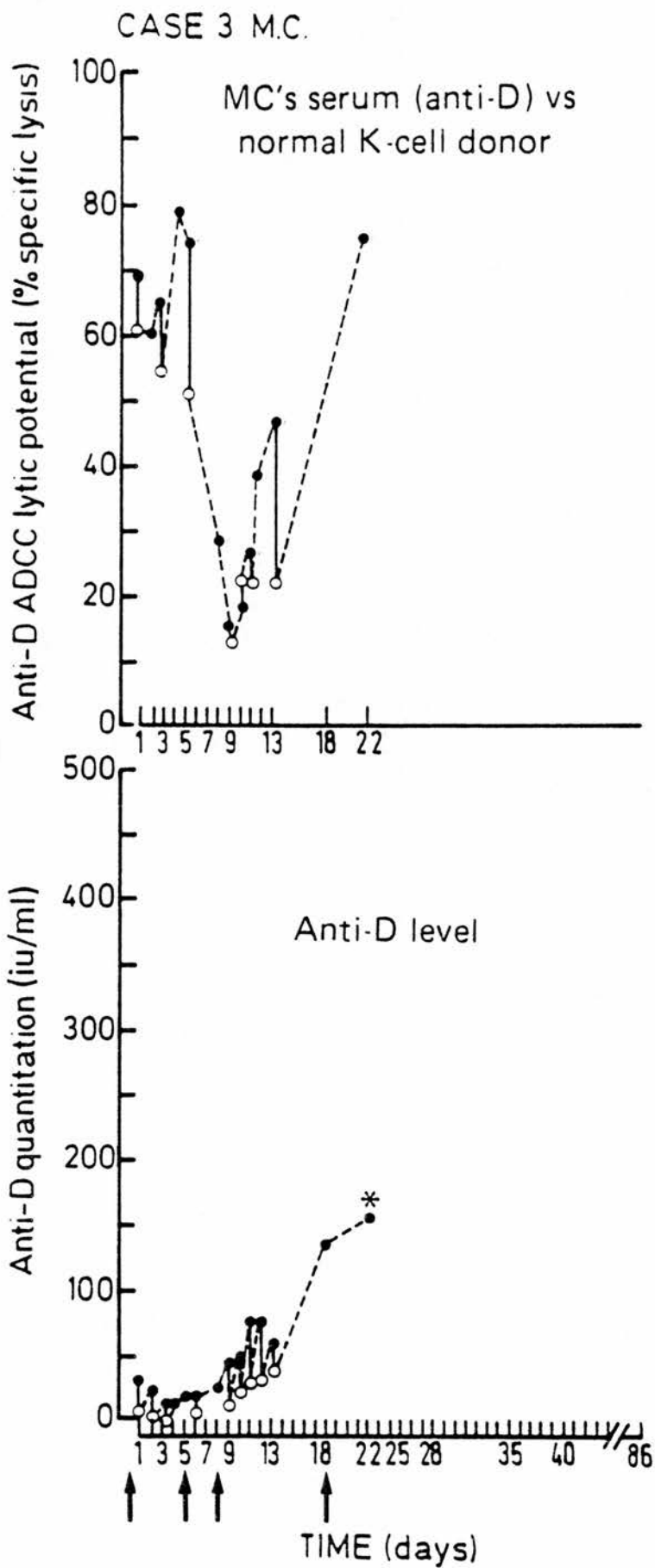
Fig. 36

The effect of plasma exchange by FFP, unselected for Rh group, on intrinsic ADCC activities (lymphocytes and anti-D) and maternal anti-D concentration.

● Pre-
          plasma exchange
○ Post-

E:T ratio 10:1 (4 x 10^4 OR$_1$R$_1$ red cells/culture)
Anti-D concentration, Neat (1 in 3 in final culture)
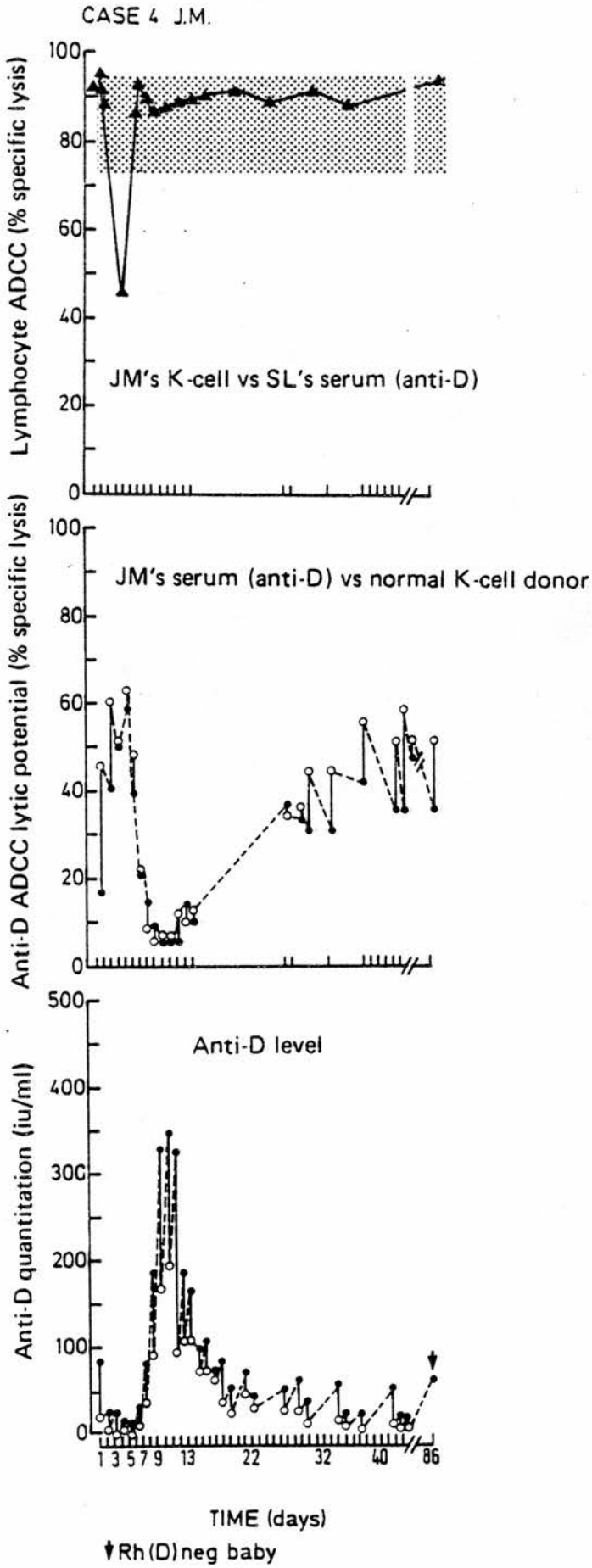
CASE 4 J.M.

Fig. 36

ADCC ACTIVITY IN CASE 4 (JM) DURING A
SERIES OF PLASMA EXCHANGES

K-cell (JM) vs anti-D (SL)
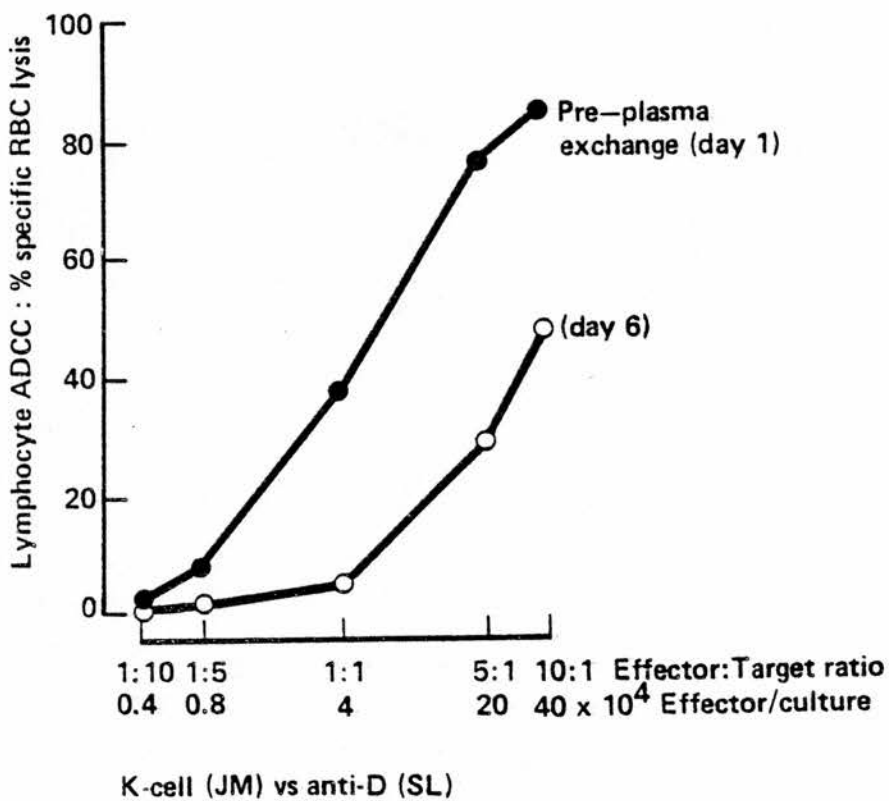
Fig. 36A

Fig. 37

The effect of plasma exchange by FFP, unselected for Rh group, on anti-D ADCC lytic potential and maternal concentration.

● Pre-

        plasma exchange

o Post-

E:T ratio. 10:1 (4×10^4 OR_1R_1 red cells/culture)

Anti-D concentration: neat (1 in 3 in final culture)

CASE 5 I.D.

ID's serum (anti-D) vs
normal K-cell donor

Anti-D level

TIME (days)

↓ Rh(D) neg baby
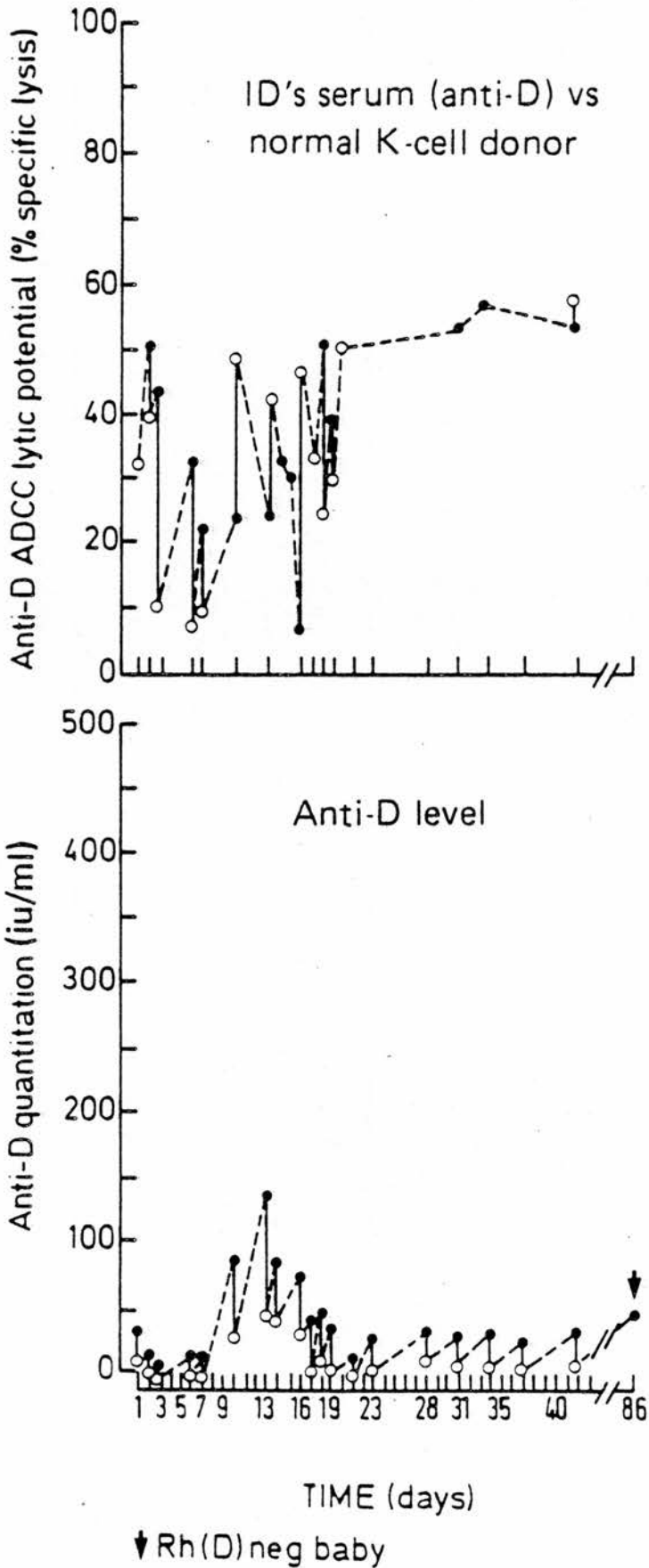
Fig. 37

ADCC ACTIVITY IN CASE 5 (ID) DURING A
SERIES OF PLASMA EXCHANGES

A K-cell (ID) vs anti-D serum (SL)
B K-cell (ID) vs own serum (anti-D)
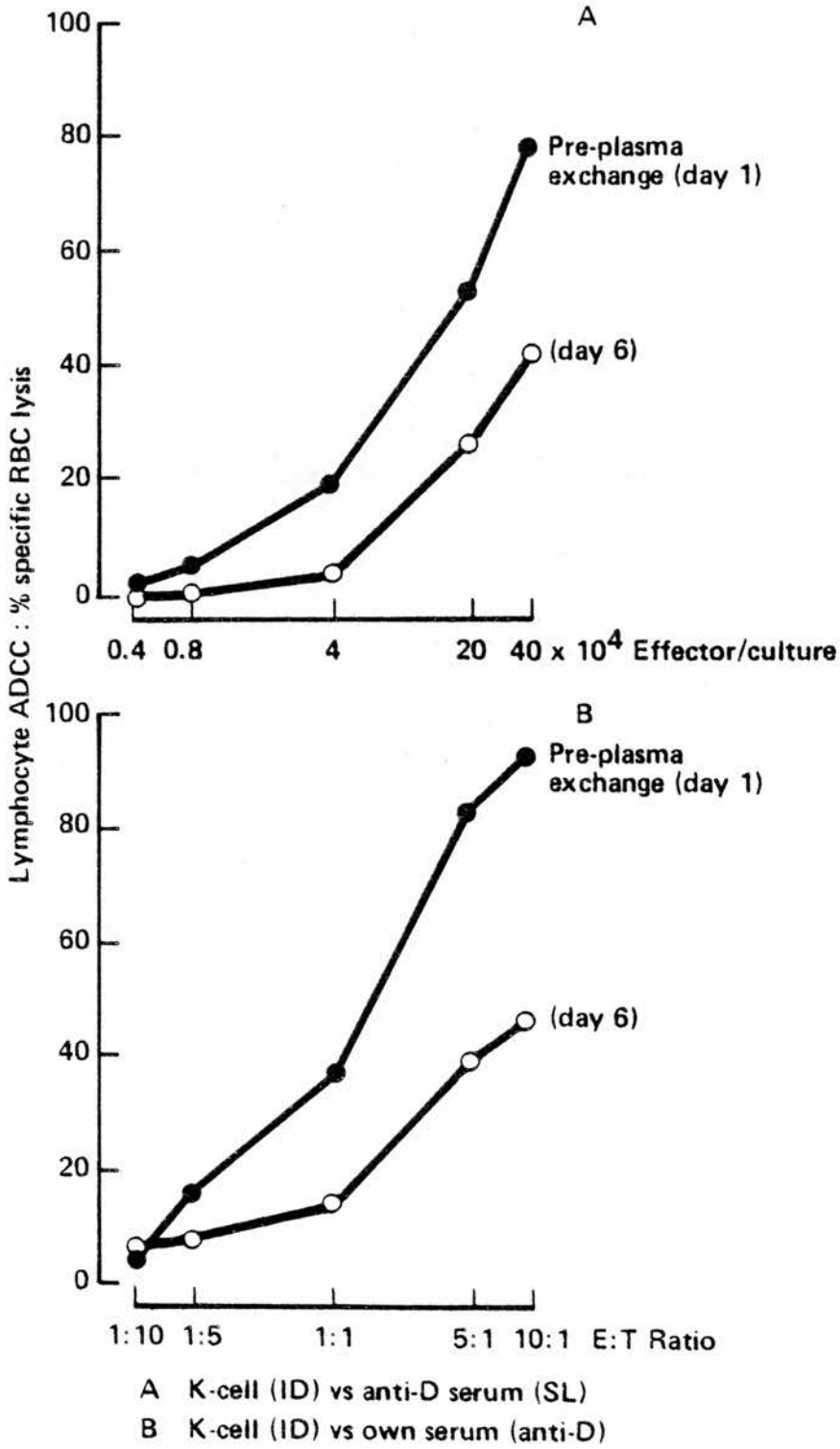
Fig. 37A

Fig. 38

Effect of plasma exchange by FFP, selected for Rh group (Rh negative), on intrinsic ADCC activities (lymphocytes and anti-D) and maternal anti-D concentration.

Days on which amniocentesis were carried out are indicated (arrowed).

● Pre-

        plasma exchange

○ Post-

E:T ratio 10:1 (4×10^4 OR_1R_1 red cells/culture)
Anti-D concentration: Neat (1 in 3 in final culture)
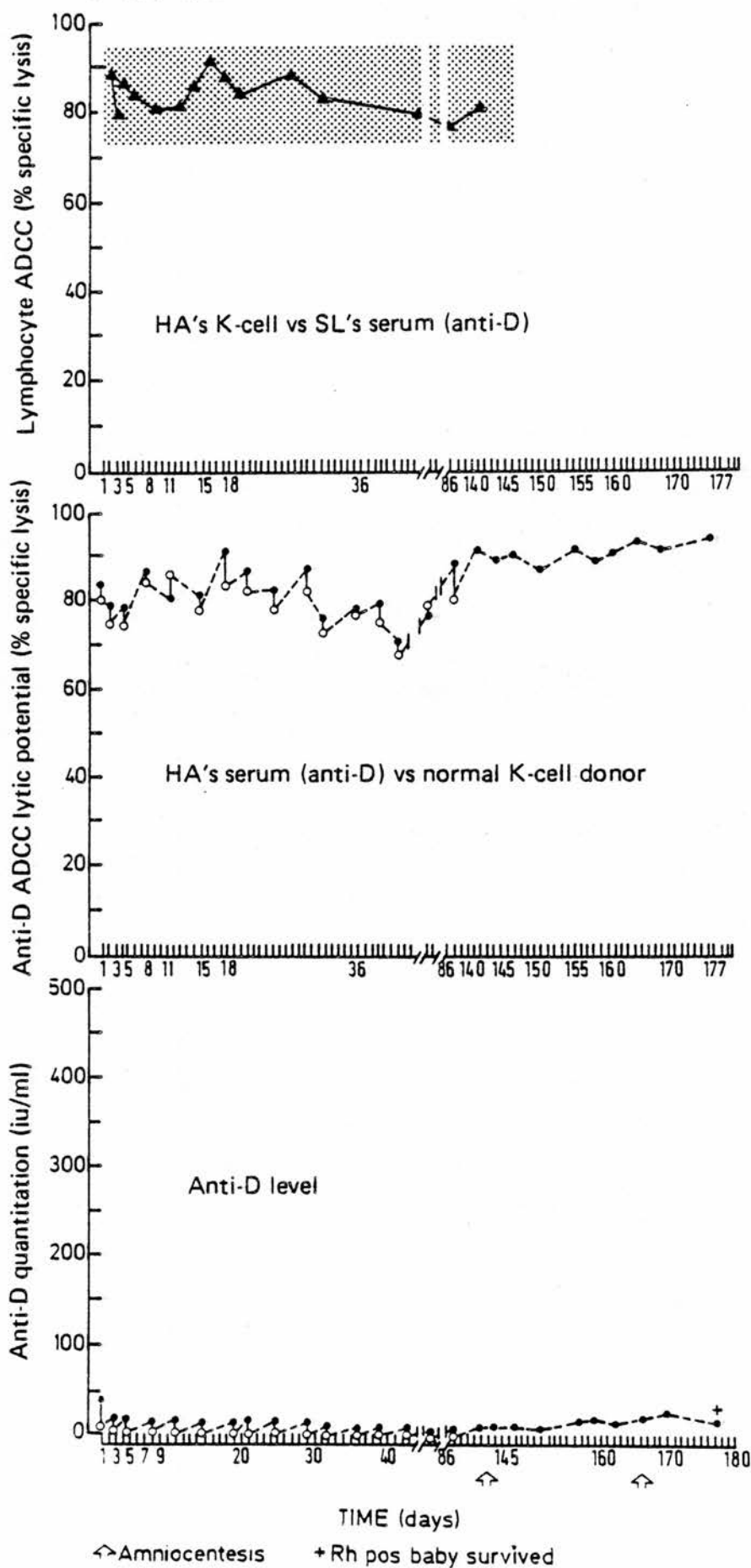
Fig. 38

CHAPTER 4

DISCUSSION AND CONCLUSIONS

4.1 Introduction

The original aim of this study was to standardise the ADCC assay in order to minimise the biological variability in intrinsic non-adherent lymphocyte (K-cell) activity in the general population. The 6 iu/ml anti-D (SL) was used as a control to enable quantitative comparison to be made. For the same reason a homologous system with group OR_1R_1 target red cells was used in most of the experiments.

This system was employed to study anti-D from different sources, and the results have thrown light on the discrepancies in ADCC lytic potential between anti-Ds, especially between those produced by deliberate immunization of male volunteers and those induced by pregnancy. These differences were confirmed by the correlation of high ADCC anti-D lytic potential, and severity of HDN. Changes were also found in the ADCC intrinsic lymphocyte activity of male volunteers during immunization, which distinguished between responders and non-responders of anti-D suitable for production of therapeutic immunoglobulin. Similar changes were noted in women undergoing therapeutic plasma exchange to reduce maternal anti-D when FFP from donors of different Rh groups was used.

4.2 K-Cell activity

Study of the control   anti-D serum SL has shown that it is able to lyse D-positive red cells at

different dilutions when a single effector cell donor is used, and whether the E/T ratio is fixed or changed (Fig 4 and Table 4). Significant lysis was obtained with anti-D concentrations as low as 0.06 iu/ml (= 0.01 μg/ml) per culture. Detection of activity at this degree of dilution of anti-D makes the ADCC assay much more sensitive than the conventional serological methods. Anti-D (SL) was equally effective when several donors of effector cells were employed at varying E/T ratios, with a plateau appearing at a ratio of 10:1 (Fig 5). For reproducibility an E/T ratio of 10:1 was therefore used for most of the experiments when the anti-D ADCC lytic potential was tested.

In this series of experiments it was demonstrated that Orr red cells are not resistant to K-cell lysis, an appropriate antibody specific for these cells (anti-c) having been included as a positive control (Table 5). This study revealed that the important factors (Urbaniak, 1979a) were: i) the quantity of antibody present in relation to the number of red cells; ii) the relative number of effector and target cells per culture (ie the E/T ratio); and iii) the appropriate combination of antigen and antibody (eg D/anti-D, c/anti-c). It was also noted that cells homozygous for D or for c give better lysis than heterozygous cells in the presence of anti-D or anti-c respectively, therefore OR_1R_1 cells were used in the standard assay.

Individual variation in intrinsic K-cell activity was seen amongst members of a healthy donor population. One hundred and twenty healthy individuals were tested in 496 experiments for K-cell intrinsic activity, in an ADCC assay with anti-D serum (SL) used in excess and OR_1R_1 red cells as targets (Fig 6). Titration of K-cells against a constant number of ^{51}Cr-labelled red cells allowed construction of dose-response cytotoxicity curves with % specific lysis plotted against the number of K-cells. Lymphocyte titration experiments were performed rather than single determinations of K-cell activity at fixed effector/target ratios, as the latter provides only limited information and comparison may be misleading. The use of varying effector/target ratios allowed genuine determination of a biological parameter (% specific lysis) which was directly comparable between individuals (Fig 6 and Tables 7 and 8).

Using the assay described in this thesis (Section 2.5.1, Urbaniak 1979a) no significant differences were found in the ADCC activity of lymphocytes when males and females were compared. These results appear to conflict with those of Kovithavongs et al (1974) and Trinchieri et al (1977), who reported depression of ADCC amongst normal females. The former group reported that menstruating females had depressed ADCC activity compared with age-matched males, but post-menopausal women were found to

exhibit ADCC activity equal to that of males. Kovithavongs et al (1974) suggested that the ability to mediate ADCC in vitro may be subject to hormonal influences, and also reported a positive correlation between increasing age of the donor and strength of ADCC activity. However, 80% of the females in the present study (Table 8) were tested close to the first day of their menstrual cycle. From these data neither obvious hormonal effect nor cyclic variation could be seen. A possible basis for the discrepancy between these results and those of Kovithavongs et al (1974) and Trinchieri et al (1977) may be either: i) the presence of two effector cells in peripheral blood which are capable of mediating ADCC (lymphocytes and mono- cyte); or ii) (more likely) a difference in target cells. Both groups of earlier workers used h uman lymphocytes as targets, but there was no sex difference when human red cells were used (Kovithavongs et al, 1975).

It has been demonstrated that K-cell activity of normal individuals is independent of sex and age when chicken red cells (Edwards and AVIS, 1979) and Chang-cells are used as targets (Penschow and Mackay, 1980). Differences in ADCC activity are seen when target cells, antibodies and effector cells from different species are tested in various combinations (Zighelboim and Gale, 1974). On this basis, Urbaniak (1979a) suggested the use of a

homologous system for comparison. The results in the present study suggest that the cytotoxic capability of K-cells from normal individuals is independent of the age, sex and blood group of the donors when an appropriate combination of antigen and antibody is used. The cytotoxic capability of K-cells was also found to be consistent in individuals when tested sequentially. However, significant differences were seen between different individuals in their capability to mediate ADCC in this system. Those who did not produce more than 50-60% specific lysis with anti-D (SL) at an E/T ratio of 10:1 were categorised as "poor" responders (< 2%), and those who produced more than 60% specific lysis as "good" responders (approx 98%).

In this study better results were achieved using OR_1R_1 (D/D) target cells at an E/T ratio of 10:1 and with an excess of anti-D (SL) serum, and this combination was used for the standard comparative experiments and the choice of good responders (as defined above). Thus in cases where K-cell activity was studied, lymphocytes were titrated in a standard ADCC assay. However, where antibodies were studied for their lytic potential activity, a fixed number of effector cells (good responders Figs 7 and 8) and target cells was used (E/T ratio 10:1).

It seemed possible that this mechanism may contribute to the in vitro lysis of red cells in haemolytic disease of the newborn (HDN) where anti-D is commonly implicated, and this is discussed later in this chapter.

4.3   IgG subclasses and ADCC activity

The lytic capacity of the effector cell suspension depends on a number of variables (Urbaniak, 1979a) and those were minimised in the present investigation by using standard assay conditions. On a number of occasions comparative experiments were done with a single K-cell donor, where the only culture variable was the source of the anti-D. Under these experimental conditions it was apparent that the "lytic potential" of anti-D sera for Rh(D) positive red cells in the ADCC assay did not correlate with any of the conventional methods of assaying anti-D (Table 14). The anti-D serum (SL) was included in all experiments as a control for ADCC activity of the K-cell donors.

An unexpected finding was the significantly low ADCC activity shown by several anti-Ds from immunized males with very potent antibodies in terms of iu/ml (Tables 9 and 10). When a number of anti-D sera from both immunized males and pregnancy-sensitized women were diluted to equivalent anti-D concentrations, the poor ADCC of several of the sera from male donors was confirmed (Table 11). The pregnancy-induced sera showed consistently high specific lysis and it is noteworthy that all of those women whose anti-D showed high lytic potential had borne infants affected by HDN and who had required exchange transfusions. The difference in lytic activity of

anti-D from males and that from females cannot be attributed to the absence of the IgG subclasses. IgG_1 and IgG_3, which are known to induce ADCC lysis of red cells (Holm et al, 1974; Urbaniak, 1979b, Hunt et al, 1980) since either or both were present in all sera. However, where the IgG_3 titre was greater than that of IgG_1 the anti-D was active in ADCC; conversely, where the IgG_1 titre was greater than that of IgG_3 the anti-D was inactive (or poor) in ADCC (Table 15). In five sera in which IgG_1 alone occurred, ADCC activity was low in two of three sera from females and in both those from males. The relative proportions of anti-D subclasses therefore appear to determine the degree of binding of any one subclass, assuming there are not wide differences in affinity. These results suggest that IgG_3 is more active than IgG_1 in ADCC, but the effectiveness of IgG_3 may be masked by other less active subclasses. The distribution of IgG subclasses between sera from males and from females provides a partial explanation for the observed discrepancies in ADCC activity. Although high titres of IgG_3 were seen in sera from males they occurred together with high titres of less active subclasses, which may have masked the ADCC activity. These findings are in accord with those of van der Meulen et al (1978), who found that IgG_3 "warm" autoantibodies to red cells were nearly always associated with demonstrable haemolysis, whereas IgG_1 was not infrequently associated with

normal red cell survival, and IgG_2 and IgG_4 were inactive.

In samples with very high levels of anti-D it was possible that the low ADCC activity could be due to inhibition of K-cells due to saturation of Fc receptors by an excess of anti-D molecules (a prozone effect). This was shown not to be the case (Fig 9), dose-response curves of ADCC activity correlating well with the single dilution results (Tables 9 and 10) and also in pre-sensitized red cell ADCC assays (Tables 12 and 13).

It is possible that "non-specific" blocking of the Fc receptors by "irrelevant" IgG molecules, similar to the inhibition of Fc-rosette binding by monocytes in the presence of high concentrations of serum containing IgG (Abramson et al, 1970), could have accounted for differences which were found in lytic potential. In these experiments the amount of immunoglobulin in the culture system was maintained at a constant level by making all dilutions of anti-D in inert (group AB) serum, thus effectively titrating the specific anti-D but maintaining a constant total immunoglobulin level. Non-specific blocking is improbable since for any given anti-D serum a constant level of K-cell lysis was observed whether the culture medium contained low (10%) or high (40%) proportions of the test serum (Urbaniak, 1979a), and ADCC lysis of anti-D

coated red cells by monocytes has been shown to proceed in the presence of undiluted human serum (Kurlander and Rosse, 1979).

4.4   Variations   in   activity   between   anti-D   from
      different sources

Different K-cell donors (good responders) were used
for each series of experiments and it can be seen
that consistently high or low ADCC results were
obtained with given anti-D sera, within the limits
of biological variation (eg sera of donors OH, AW,
RG, GH).   Interestingly, fluctuations were noted in
the ADCC activity of most male individuals during
the   course   of   immunization,   with   some   lytic
activity being shown on several occasions.

In this study anti-D sera from immunized male
volunteers were compared with different anti-D ADCC
activities   to   attempt   to   identify   differences
between   the   respective   anti-D   antibodies   which
might indicate a basis for their different ADCC
activities.   Experiments   were   conducted   to
investigate: i) differences in specific binding to
Rh(D) positive red cells; ii) differences in IgG
composition of anti-D antibodies;   iii) differences
in interaction of anti-D sensitized red cells with
lymphocytes;   and   iv) factors other than anti-D
antibodies which may affect ADCC.

No differences were found in the specific binding
of the anti-D sera to D-positive red cells when
assayed by IRMA (Table 16 and Figure 13).   At an
appropriate dilution of anti-D (1 in 50) falling on
the linear portion of each serum's anti-D binding

curve, highly significant correlation was found between bound IgG (IRMA) and anti-D content expressed as iu/ml. Neither IgG binding nor anti-D content showed any correlation with ADCC, either in the standard ADCC assay or when red cells were pre-sensitized with the anti-Ds (Table 16), confirming previous observations (Section 3.2.5). However, it was noted that one individual's anti-D which showed no ADCC activity in the standard assay (JW 1 and JW 2, Table 16) expressed ADCC activity when used to pre-sensitize red cells. This appears to indicate that the anti-D antibodies themselves are capable of supporting specific ADCC, but that other factors in this serum inhibited ADCC expression. This was supported by the finding that the anti-D of donor JW alone, recovered by elution after specific absorption on D-positive red cells, showed ADCC activity in the standard ADCC assay (Figure 13).

Two of the sera tested (JDu and GP, Section 3.2) were found to be consistently inactive in ADCC. the serum of donor GP was selected for further study, together with that of a male donor with similar content of anti-D, which showed high ADCC activity (JM, Table 16). Studies were also carried out on an anti-D serum which had high ADCC activity and low anti-D content (SL), and an anti-D from a male donor which was ADCC-inactive as serum, but the anti-D used for pre-sensitization or as an eluate was active (JW).

Serum mixing experiments were carried out to determine the effects of anti-D sera of different ADCC activities on the expression of ADCC. Only inhibition was observed (Fig 11), and this appeared to be related to the relative amounts of inactive and active anti-D in mixtures. This indicated that anti-D (GP) was intrinsically inactive in ADCC, and that it inhibited expression of ADCC by other anti-D sera by competing for D-antigen sites on red cells. This rules out insufficiency, either in the IgG subclasses or in the range of specificities, as a basis for ADCC-inactivity, since any insufficiency could have been restored by ADCC-active anti-D sera: such synergy might have been detected as enhanced ADCC in mixing experiments. Competition for D-antigen sites between ADCC-active and ADCC-inactive anti-D sera as the basis of the observed inhibition was confirmed by investigation of the ability of anti-D (GP) to inhibit ADCC activities of different anti-Rh antibodies (Figure 12). Inactive anti-D (GP) was unable to inhibit anti-c mediated ADCC, but inhibited anti-D mediated ADCC by seven out of eight anti-D sera. These results also show that the c-antigen is sufficiently independent of the D antigen in the red cell membrane to escape inhibition by the ADCC-inactive anti-D.

The subsequent experiments on anti-D isolated by absorption and elution, and on IgG-enriched and depleted serum fractions (Figures 13 and 14), support the conclusion that this ADCC-inactive anti-D (GP) has IgG anti-D antibodies which are intrinsically unable to collaborate with lymphocytes in mediation of ADCC of D-positive red cells, but in other respects behaves like anti-Ds which express normal ADCC activity. As well as showing comparable specific binding to the D-antigen on red cells, this serum was as capable of sensitizing red cells for rosetting with lymphocytes through Fc-receptors as were ADCC-active anti-D sera (Table 17), and contained comparable anti-D IgG subclasses (Table 18). This demonstrated that certain anti-D antibodies are intrinsically inactive in mediating lymphocyte ADCC or Rh(D) antibodies (whether from deliberately-immunized male volunteers or from pregnancy-immunized women) which show a range of ADCC activities.

Wide variation has been observed in the ability of anti-D sera from different sources to mediate the lysis of Rh(D) positive red cells in ADCC (Section 3.2), and there is no obvious correlation between anti-D levels (expressed as iu/ml) and ADCC activity (expressed as % specific lysis). The cases studied were selected to provide a range of unusual features which are encountered from time to time during the management of HDN.

4.5   The use of ADCC activity in predicting severity of
       HDN

It is not always possible to predict Rh-HDN by
conventional titration of maternal anti-D levels,
and in any case manual titration methods are
susceptible to large errors. Automated methods for
the quantitation of anti-D have been developed
using continuous-flow quantitation in an
Autoanalyser TM, and this is more accurate and
sensitive than manual titration (Gunson et al,
1972). Some workers (Fraser and Tovey, 1972) have
found that continuous-flow quantitation of anti-D
provides a more accurate basis for prediction of
the outcome of HDN than do manual methods, although
other workers have not agreed (Sturgeon and Kay,
1970; Cramer et al, 1970). The generally poor
correlation between manual and automated methods
noted by others (eg Fraser and Tovey, 1976) was
confirmed in this study. Although statistically
significant associations were seen between
continuous-flow quantitation and manual titration
methods (enzyme and antiglobulin techniques) the
results in individual cases did not allow accurate
comparisons to be made (Table 14). ADCC activity
of the anti-D sera did not correlate with any of
the conventional methods of assaying anti-D.

Recent studies have shown that Rh(D) positive
babies will be affected by HDN if the anti-D level
is greater than 5 iu/ml (= 1 μg/ml) (Tovey and

Haggas, 1971, Fraser and Tovey, 1978). However, in patient MD (Fig 15 and Table 19) the anti-D level never reached 2 iu/ml and the baby was affected by HDN, requiring exchange transfusion. Although the anti-D level had suggested prediction of a good outcome the anti-D ADCC had shown high lytic activity. On the other hand, the anti D levels in patients PB and CC were higher than 5 iu/ml at the end of the pregnancy, but neither baby required exchange transfusion (just a simple red cell transfusion for anaemia in the baby of CC). In the cases of patients AO'N and VMcA (Table 19 and Figure 16) the anti-D levels prior to amniocentesis suggested a good outcome, but following amniocentesis the levels rose markedly. However, ADCC assays gave reliable predictions throughout. In the three cases in Series 1, the liquor Δ OD 450 prediction (favourable outcome), was at variance with the serum anti-D level prediction (poor outcome). When these sera were examined "blind" for ADCC activity, this assay correctly identified the case where only mild HDN occurred (AM, Fig 17). In the Series 2 cases where high anti-D levels indicated a poor outcome, and the amniocentesis results concurred (IUT zone), the ADCC assays revealed highly active sera (Fig 18) and three of these five babies subsequently died of HDN (Table 21). The high ADCC activity of these anti-D sera therefore reflected the in vivo biological potential for lysis of Rh(D) positive red cells.

Two of the cases in Series 2 had favourable amniocentesis predictions but high anti-D levels, and again the ADCC assay identified these two sera as having low potential for Rh(D) positive red cell lysis (Fig 18). In one case (EK, Fig 18, Table 21), anti-D, amniocentesis and ADCC predictions were all incorrect in that each predicted an affected baby, yet a Rh(D) negative infant was born. In this case the activity of the anti-D presumably reflects events in a previous pregnancy, but no details were available to confirm this.

Generally, only single samples of anti-D were tested in each of these cases (Series 1 and 2), due to lack of sufficient sera. In previous studies no case was seen of a non-lytic anti-D which became lytic, or vice versa during the course of pregnancy (Table 19, Figures 9, 15 and 16). It is therefore likely that similar ADCC results would have been obtained in those cases where the anti-D sample was taken following amniocentesis (see Tables 20 and 21). Although sera highly active in ADCC can be correlated with a severely affected baby, the absolute level of ADCC activity does not necessarily distinguish between degrees of severity of HDN. In the in vivo situation the degree of lysis appears to depend on the ADCC activity of the foetal effector cells, the Rh(D) antigen density on the foetal red cells and the level of anti-D in the foetal circulation. In the test situation the

effector cells and the Rh(D) positive target cells were standardized, and the anti-D reflects the maternal (rather than the foetal) level of antibody. It is therefore to be expected that the ADCC assay may give "false positive" results in the case of a Rh(D) negative foetus, since the anti-D is being assessed in an artificial context. Similarly, differences in the activity of foetal cells to mediate ADCC influence the degree of in vivo lysis, and the in vitro ADCC assay may overestimate the severity of HDN if the effector cells of the foetus are unable to act efficiently.

Anti-c is the commonest cause of severe HDN, after anti-D. Affected infants may require exchange transfusion and perinatal deaths can occur (Table 23). In this study the aim was to test various anti-c sera for ADCC activity and to correlate any such activity with the IAGT titre, IgG subclasses distribution, and the clinical severity of HDN. In general, the IAGT titre does not correlate well with severity of HDN. Although two out of six of the patients tested had the same IAGT titre (ER and AP, Figure 22), one of these (ER) had an affected baby which required exchange transfusion, while the other (AP) had a healthy child, and this had been identified by low activity in the ADCC assay. There was a tendency towards higher IgG_3 titres of anti-c which were lytic by ADCC.

The present ADCC test system for assessing anti-D (or anti-c) activity employs lymphocyte "K-cells" but in principle there is not a priori reason why other effector cells (such as monocytes) should not be used. Provided that the effector cells and target cells are used under standardised conditions, the end result will be the same estimation of the lytic potential of the anti-D sera. Since the present assay allows addition of whole sera directly to the culture system it was an advantage over monocyte ADCC assays, which are inhibited by the presence of human serum (Shaw et al, 1978).

On the basis of the present observations, it would appear that the ADCC assay has a place in the identification of those cases of HDN which are going to have a satisfactory outcome, despite the level of anti-D as determined by conventional methods (manual titration in the case of anti-c). Amniocentesis, with the consequent danger of boosting antibodies could therefore be avoided. Although high lytic activity will generally correlate with a severely affected baby, "false positive" predictions may occur in the case of Rh(D) negative babies.

4.6   Rh immunization in male volunteers

In this study, changes in vitro lymphocyte function were detected during the course of deliberate immunization with Rh(D) positive red cells in vivo. Those individuals who eventuallly produced anti-D (responders) reacted differently from those who did not (non-responders).

Alterations in lymphocyte ADCC activity were seen following the primary immunization (Figure 23) of Rh(D) negative male volunteers. In this instance, a fall in K-cell activity was seen following infusion. Anti-D was not present at the time of infusion so that active haemolysis was not observed. Nevertheless, there was transient reduction in K-cell activity, followed some time later by greatly increased K-cell activity. It is possible that there is a redistribution of K-cells following infusion (eg) to the spleen.

Changes in K-cell activity were seen in each of two volunteer studies (R1 and R3) in relation to primary immunization. However, two of the male volunteers did not produce anti-D and showed no changes in K-cell activity (Figure 24). However, this latter effect may be an artefact introduced by the method of sampling at a fixed time (7 d) following primary immunization.

Following boosting, marked transient depression of lymphocyte ADCC during immunization was seen only in the four anti-D responders. This reduction in ADCC activity was due to abolition of K-cell function rather than a shift in the dose-response curve (Fig 26). Although the effect of immunization on ADCC appears consistent, the magnitude of the response apparently diminishes with successive immunizations (Fig 27). Examination of ADCC activity at more closely-spaced time intervals (Figs 27-30) showed that depression of ADCC may occur sooner, but is of similar magnitude, with later boosts.

It is interesting to note that reduction in ADCC was seen only in responders, and not non-responders. The ADCC assay can not distinguish between loss of function or loss of K-cell numbers, and it is not possible to determine directly whether the changes noted are due to inhibitory influences or to removal of K-cells from the circulation. Reduction in ADCC occurred when serum anti-D levels had risen considerably. It is therefore possible that injected Rh(D) positive red cells become sensitized with anti-D in vivo, resulting in removal or inactivation of K-cells in the circulation by interaction with the Fc receptor: the higher the anti-D level, the more efficiently the K-cells might be "neutralized". However, a maximum of 0.5 ml Rh(D) positive red

cells were injected at each boost and it is difficult to envisage this as being sufficient to remove most of the K-cells from the circulation effectively.

The changes seen could be due to immunoregulatory influences on the K-cell, secondary to the regulation of the immune responses in vivo. It has been noted recently that in vitro expression of ADCC may be influenced by suppressor lymphocytes (Pollack and Emmons, 1979) and there is some evidence that this effect may be mediated by soluble factors (Madhavan and Schwartz, 1981). There are also data available which indicate that ADCC activity can be influenced by cytokines such as interferon (Herberman et al, 1979), although no effect was seen by Kimber and Moore (1981) using an anti-D ADCC system similar to that used in this study. A variety of agents that can influence intracellular levels of cyclic AMP or cyclic GMP are known to influence expression of Fc receptors by T cells (Gupta, 1979). Since it has been shown that the T cell bearing Fc receptors is capable of effecting ADCC (Shaw et al, 1979; Katz and Fauci, 1980) these agents may alter K-cell activity. Those responders who developed anti-D showed normal ADCC lytic potential comparable to that of the control anti-D serum (SL) (ie they did not show evidence of inhibition of the control lymphocyte ADCC capacity (Figs 31, 32).

4.7   Secondary Rh immunization in females

Of the six women studied (Tables 26 and 27), three
lost their babies due to intrauterine death, the
others delivered live babies, two of which were
Rh(D) negative (and therefore not responsible for
the anti-D changes noted during plasma exchange)
and one which required exchange transfusion and
survived.   Interpretation   of   the   laboratory
findings associated with pregnancy is more complex
than with immunized male volunteers because: i)
the immunizing event can not be clearly documented;
ii) the possible effects of plasma exchange on
homeostatic immunoregulation can not be predicted;
and   iii) the status of the immune system in
pregnancy may differ from that in non-pregnant
subjects. HDN differs from other antibody-mediated
diseases in   that   the   foetus   can   benefit   only
indirectly from plasma exchange, effects depending
upon the free passage of antibody between foetus
and   mother.   IgG   levels   in   the   newborn   are
frequently   higher   than   in   the   maternal   serum
(Kohler et al, 1966) and the level of anti-D may
vary from 2% to 30% of that found in the mother
(Hughes-Jones et al,   1971).   Maternal   anti-D
concentrations   therefore   can   not   provide   an
accurate   prediction   but   only   a   guide   to   the
severity of the disease. Initially it appeared
that the maternal anti-D levels increased despite
intensive plasma exchange, and that the procedure
may   have   been   harmful   by   removing   maternal

"inhibitory" activity (Barclay et al, 1980). Whether plasma exchange alone provoked the anti-D response in patient AB, or whether further sensitization to foetal cells occurred at a vulnerable time in relation to the removal of the inhibition is not clear. Rh(D) positive foetal cells were not detected in the maternal circulation by the Kleihauer technique at the time of amniocentesis or of IUT at day 1, which would be likely in the event of immunization (Zipursky et al, 1963). However, failure to detect foetal cells in the maternal circulation does not exclude the possibility of foeto-maternal haemorrhage, as such cells are rapidly cleared in the presence of potent anti-D (Jandl et al, 1957). A more important additional factor may have been the presence of Rh(D) positive cell stroma in the FFP, since this was randomly selected as regards Rh group and was used in five of the six cases. It has been shown that red cells present in carefully prepared packs of plasma prior to freezing may average the equivalent of 0.06 ml blood per pack (range 0.005-0.25 ml), and that when a pack is frozen and thawed, 4% of the erythrocytes may survive intact (Wensley et al, 1980). Similarly, McBride (1983) demonstrated that an average bag of 220 ml plasma may contain approximately 10^{10} red cells; this is approximately equivalent to 2.5 ml of whole blood, or 1.0 ml of packed cells. If 20 bags of FFP are used for a 4 l plasma exchange, as much as 0.05 ml

in intact Rh(D) positive red cells might be transfused - an amount capable of stimulating an antibody response (Jakobowicz et al, 1972).

However, the placenta does not act as a simple filter, but bears Fc receptors and appears to be the rate-limiting point in antibody transfer (McNabb et al, 1976). It selectively and directionally transfers IgG against a substantial concentation gradient. The rate of transfer is relatively slow (Giltin et al, 1964) but is better from mother to foetus than from foetus to mother. Because of the active and selective transfer of IgG across the placenta it was elected to exchange with FFP rather than with PPS, in an effort to maintain total IgG levels while reducing the IgG anti-D component. However, long term transfusion of large amounts of FFP is associated with unacceptable side effects (Robinson, 1983; Urbaniak 1984) and one is obliged to use PPS during intensive plasma exchange (Table 27).

It has been shown in animal experiments by Branda et al (1975) that plasma exchange following secondary immunization results in a permanent lowering of the antibody level. However, it has recently been stated (Branda, 1979) that when antigen is continuously present only a temporary lowering of antibody can be achieved, with a prompt return to high levels.

Certain patterns emerged from the present studies
which become evident if one divides the patients
into three groups, according to the outcome of
their pregnancy (Table 27):

i)   those in whom intrauterine foetal death
     occurred;
ii) those who delivered an Rh-negative child;
iii) those who delivered a live Rh(D) positive
     child.

The changes in variables in groups i) and ii) can
be contrasted with group iii), and with the changes
which occurred in the same variables measured in
male volunteers.

Dramatic rises in anti-D production rates occurred
during plasma exchange therapy in 5 cases.   In
group i) these were not brought under control by
plasma exchange therapy, but in group ii) they were
brought down after apparent reductions in the rate
of anti-D production in vivo.   In the group ii)
cases, stimulation of anti-D production could have
been brought about by exposure to the exchange
(donor) plasma, and selected plasma from Rh(D)
negative donors was subsequently used for all
exchanges, to exclude any possibility of exposure
to Rh(D) positive red cell stroma which might
otherwise occur.   However, in the group iii) case,
no plasma from any Rh(D) positive donor was used

and the anti-D concentration was kept low, in the range of 5 iu/ml, until the end of the plasma exchange therapy. In three cases, the removal of anti-D by plasma exchange was accompanied by an increase in anti-D ADCC lytic potential, despite the net reduction of anti-D content (Figs 33, 34, 36). This may have been due to the joint removal of some lymphocyte K-cell (ADCC capacity) inhibiting factor from the sera, together with the anti-D. In turn, this could imply that observed serum anti-D ADCC lytic potentials depend on both serum anti-D and regulatory factors directed against K-cells in a given serum. However, in groups i and ii when the anti-D concentrations reached their highest level due to secondary anamnestic responses, unexpectedly, the anti-D lytic potential was markedly decreased. This may be due to the different route of immunization used (ie intravenous rather than transplacental) which may have peturbed the homeostatic regulation of the anti-D response which may indicate that the placenta plays a unique role in the immunization process. In group iii (HA), where there was no extrinsic antigenic stimulus, the anti-D lytic potential was consistently high. In general, no correlation was found between anti-D levels and anti-D ADCC lytic activity.

Only in the group ii) and iii) cases were intrinsic lymphocyte ADCC activities studied as for the immunized male volunteers. Two patients showed a dramatic short-lived drop in intrinsic lymphocyte ADCC capacity in the early stages of exchange therapy, which resembled that seen in the successfully immunized male volunteers following boosting. However, in one case (HA) no significant changes in intrinsic lymphocyte ADCC capacity occurred, which resembled the situation in non-responders amongst the male volunteers. The data in the first five cases (Table 26) could represent facets of a well-co-ordinated immune response rather than initiating factors of that response. The data are alike and confirm the findings made in secondary immunization (boosting) in male volunteers.

4.8   Conclusion and prospective studies

This work raises several important points calling for further research. The lytic capacity of various anti-D sera in ADCC has been investigated and unexpected differences were found between the ADCC of anti-D produced in male volunteers and that from pregnant females. Although the males produced high levels of anti-D, as measured in iu/ml, the ADCC activity induced was much less than that of the "female" anti-D at equivalent concentrations. Certain anti-D sera failed to induce lysis of Rh(D) positive red cells by ADCC, although they were capable of binding D-antigen, as measured by conventional haemagglutination techniques and by IRMA and Fc receptor binding studies. The IgG subclass distribution between different sera could not account for the differences seen, either IgG_1 or IgG_3, and/or both, were present in all sera.

As fewer women become immunized as a result of pregnancy, there is greater dependence on anti-D from male volunteers, for the production of specific immunoglobulin for therapeutic use. The demonstration that anti-D from male donors is not as potent as that from females in ADCC raises the intruiging possibility that anti-D from males may be less effective in the prophylaxis of Rh HDN.

Possible areas for further studies to distinguish between lytic and non-lytic anti-D are:

i) by study of the biochemistry of the components;

ii) chromatography, eg multi-step or gradient elution with phosphate solution and use of alternate eluants;

iii) in vivo study of the survival of ^{51}Cr-labelled Rh(D) positive red cells after injection of lytic and non-lytic anti-D, using different groups of volunteers, etc. However, the hazards of taking part in such a study must be fully explained to the volunteers;

iv) a search for a better reagent to determine the IgG subclasses (eg monoclonal antibodies).

When ADCC assays were used for predictions of the severity of HDN there was a problem with "false positive" reactions when Rh-negative infants were involved. It may be possible to overcome this by using the father's red cells as targets with maternal anti-D.

Lymphocytes are the source of both humoral (antibody) immune response and regulatory activities which govern the expression of specific immune responses, and therefore studies of lymphocyte functions in vitro will give an insight into the mechanisms which operate in vivo. It

would be important to study the lymphocyte intrinsic ADCC activity in male volunteers following primary immunization, in parallel with differential studies of lymphocyte subpopulations. It is possible that changes seen in lymphocyte ADCC activity in relation to boosting could be secondary to alteration in T-cell activity. It has been noted that the T-cell, with IgG receptors (T_G suspopulation) is active in the anti-D system (M McCann, 1984, personal communication).

Immunoregulation is seen as a homeostatic process. During an immune reasponse active regulatory mechanisms, termed "helper" and "suppressor" (T_S) determine the nature of the response, expressed by their balance, with restoration of homeostasis and de-activation of regulation after antigen elimination.

It has been shown that the intrinsic responsiveness of peripheral blood lymphocytes is depressed following in vivo immunization, and this has been attributed to activation of suppressor cells (T_S). Ts can also be activated in vitro by exposing normal PBL to antigen or mitogen (Barclay et al, 1979; Barclay, 1980). This may indicate that lymphocyte ADCC capacity is also susceptible to T_S suppression.

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- 222 -

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