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THE PRODUCTION AND CHARACTERIZATION OF HUMAN-HUMAN AND HUMAN-MOUSE Rh(D) SPECIFIC MONOCLONAL ANTIBODIES

by

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Abstract

Human monoclonal antibodies specific for the Rhesus(D) antigen were produced. Hybrid cell lines were generated by the fusion of pooled Epstein-Barr virus (EBV)-transformed B cells secreting Rh(D) antibodies with either the human lymphoblastoid cell line HOA.1 or the murine myeloma cell line NS.1. The selection of hybrids was achieved in RPMI 1640 medium containing hypoxanthine, aminopterin, thymidine (HAT) and ouabain. A higher fusion efficiency was obtained with the NS.1 cell line; however, a higher proportion of the hybrids, with HOA.1, secreted antibody which exhibited a greater clonal stability. The products of 4 clones (3 human-human and 1 human-mouse), that consistently secreted antibodies for over 11 months were tested for specificity with a panel of red cells of different Rh phenotypes. The supernatants of all 4 clones showed anti-Rh(D) specificity, but failed to react with the red cell D^{u} phenotypes categorized as $DV(D^{w}+)$ and DVI. Two of the three human-human clones secreted IgM(lambda) and the third IgG1(kappa) antibody. The human-mouse clone produced IgG1(kappa) antibody. None of the hybridomas produced ascites

tumours after injection into immunocompetent murine recipients that were pre-treated with cyclophosphamide. The Epstein-Barr nuclear antigen (EBNA) was detectable in all hybridomas generated by human-human hybridization. The human-mouse hybridoma did not express EBNA, indicating that the EBV genome was not maintained. The IgG monoclonal anti-Rh(D) specifically precipitated a 32-33KD protein from the membrane of Rh(D)-positive red cells, but not from red cells typed Rh(D) negative.

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List of Abbreviations

1. ABA.....azobenzenearsonate
2. AR.....azophenylarsonate
3. ARCO.....antigen-reactive cell opsonization
4. BSA.....bovine serum albumin
5. CFA.....complete Freund's adjuvant
6. CY.....cyclophosphamide
7. DOC.....deoxycholate
8. EBNA.....Epstein-Barr nuclear antigen
9. EBV.....Epstein-Barr virus
10. ELISA.....enzyme-linked immunosorbent assay
11. H-chain....immunoglobulin heavy chain
12. HAT.....hypoxanthine, aminopterin and thymidine
13. HDN.....hemolytic disease of the newborn
14. Id.....idiotype
15. HGPRT.....hypoxanthine guanine phosphoribosyl transferase
16. KD.....kilodaltons
17. L-chain....immunoglobulin light chain
18. LCL.....lymphoblastoid cell line
19. MoAb.....monoclonal antibody/antibodies
20. PBL.....peripheral blood lymphocyte(s)

21.PBS.....phosphate buffered saline

22.Pc.....phosphorylcholine

23.PEG.....polyethylene glycol

24.PFC.....plaque forming cell

25.Rh(D).....Rhesus D antigen

26.SDS-PAGE...sodium duodecyl-sulphate poly-

...acrylamide electrophoresis

27.6-TG.....6-thioguanine

28.Tg.....thyroglobulin

29.Th.....T helper cell

30.TNP.....trinitrophenyl

31.TPH.....transplacental hemorrhage

32.Ts.....T suppressor cell

33.TsF.....T suppressor cell factor

34.V.....variable

I. LITERATURE REVIEW

A. INTRODUCTION

Quite often it is those rare, yet unfortunate incidents that arise, even though standard medical protocols of the time have been adhered to, that most enlighten researchers to the etiology of a particular disease. Such was the case when Levine and Stetson [102] first described the association of severe anemia in a stillborn fetus with the presence of an atypical antibody in the serum of the mother. The mother was transfused with the father's red cells and despite ABO compatibility she quickly developed a severe hemolytic transfusion reaction. This led Levine and Stetson to suggest that the mother possessed an isoagglutinin in her serum capable of recognizing a foreign antigenic determinant on the father's red cells. They further speculated that this antigen was present in the blood and/or tissues of the fetus and that it was inherited from the father.

Within the year Landsteiner and Weiner [97] reported the production of immune rabbit and guinea pig antisera after immunization with Rhesus monkey red blood cells which were capable of agglutinating Group

O human red blood cells. The new agglutinable blood group antigen was subsequently designated as Rh.

Later, Wiener and Peters [196], using the same rabbit antisera and the serum of patients who had experienced hemolytic reactions following multiple intragroup (ABO) compatible transfusions, were able to show that blood group incompatibility did exist between the patient's and donor's red cells. The agglutination reactions found with the human antisera (Rho) paralleled those of the rabbit antisera, thereby suggesting that the incompatibility involved the Rh antigen.

The pathogenesis of hemolytic disease of the newborn (HDN) was later clariffied by Levine and co-workers [100]. They suggested that most cases are due to the alloimmunization of Rh-negative mothers by Rh-positive red cells from their fetuses. This was demonstrated by the presence of anti-Rh agglutinins in the mother's serum. These authors also took the first recorded step in reducing the incidence of HDN by advising that Rh-negative mothers should be transfused only with Rh-negative blood.

Both groups of researchers correctly attributed the hemolytic reactions to an antigen on the donor red cells to which the mother had an antibody. However,

the antigen originally defined by the animal antisera [102], was later determined to be coincidental and serologically not identical with the Rh antigen recognized by human sera. Blood samples typed Rh positive or Rh negative with the human antisera were both found to be agglutinable with the animal antisera [52]. As well, animal antisera with the same antigen specificity were obtained from guinea pigs immunized with Rh-negative red cells. These antibodies were removable from the serum following absorption with either Rh-positive or Rh-negative human red blood cells [122]. In light of these findings, the human red cell antigen responsible for the agglutination reactions caused by the animal antisera was later renamed LW in honour of its discoverers. In the years following these earlier serologic studies, the complexity of Rh had grown from a single antigen into a major blood group system comprised of 42, so far, identified antigens [19].

The increased understanding of the Rh blood group system (particularily the D antigen) in the pathogenesis of HDN, has aided in the development of procedures (eg. plasmapheresis and intrauterine transfusion) capable of reducing perinatal mortality from Rh erythroblastosis. The most important

development was the prevention of Rh(D) alloimmunization by administration of Rh IgG antibodies.

B. The Rh Blood Group System

Several genetic theories have been suggested by different investigators to explain the antigenic complexity of the Rh blood group system. Unfortunately, a different form of Rh nomenclature was introduced with each theory and since none of the notations has met with overwhelming favour, a basic understanding of each is required.

1. Rh-Hr nomenclature

Wiener originally described a series of six Rh allelic genes to account for the different Rh antigens [191,197] he observed using the three Rh antisera; Rho (reacts with 85 % of the caucasion population), Rh1 (reacts with 70 %) and Rh2 (reacts with 35 %).

Wiener suggested that a single one of these Rh genes at the *Rh* locus of the chromosome codes for a single Rh molecule or "agglutinogen". Each

agglutinogen is comprised of two or more "partial antigens" (or factors) which are recognized by a specific antibody.

The theory of multiple alleles was later expanded to include new phenotypes identified by new antisera. The eight most common *Rh* genes and the agglutinogens which they code for, as postulated by Wiener, are listed in Table I-1. Those antisera (Rho, Rh' and Rh") which exhibited reactivity with Rh-positive bloods were collectively designated Rh. The antisera (Hr' and Hr") which showed reactivity with red cells not agglutinated by one or more of the Rh antisera were denoted by Hr [195].

Due to the reciprocal relationship which exists between Rh and Hr antisera, only the reactivity of a particular agglutinogen with either of the antisera is required to identify the factors of which it is comprised [195]. Only two of the expected three Hr antisera have been found. The third antiserum, Hr^{o}, was hypothesized but has not been detected.

Also listed in Table I-1 are some of the serologically identified factors comprising each agglutinogen. Italics are used to denote genes, whereas Roman type is used to designate the agglutinogen and its corresponding serologic factors.

Wiener also suggested that the intermediate Rh reactivity of some red cells observed with the Rh antisera is the result of rare allelic genes which arose through the mutation of the more common *Rh* genes [193].

2. Fisher-Race Nomenclature

Fisher proposed that the single *Rh* gene, as suggested originally by Wiener, was actually three closely linked loci with two alleles at each gene locus, *C-c*, *D-d* and *E-e* [146].

The Rho antisera of Wiener reacts specifically with the D antigen. Antisera against antigens have been found for all the antigens except for that coded by the d allele. It is believed that if such an allele exists the gene is most probably amorphic. The eight possible gene combinations using the *CDE* notation are shown in Table I-2. The corresponding Rh-Hr nomenclature is also shown.

The most common gene combinations are *CDe* (43.61 %), *cde* (37.9 %) and *cDE* (12.80 %) [51]. Because these three gene combinations occur with a >90.0 % likelihood, Fisher suggested that the rare gene combinations are maintained in the population through

Table I-1. Wiener nomenclature

Gene	Agglutinogen	Serologic Factors
R^0	Rh_0	Rh_0, hr', hr"
R^1	Rh_1	Rh_0, rh', rh"
R^2	Rh_2	Rh_0, hr', rh"
R^z	Rh_z	Rh_0, rh', rh"
r	rh	hr', hr"
r'	rh'	rh', hr"
r''	rh"	hr', rh"
r^y	rh_y	rh', rh"

Taken from
Case, J. The Rh blood group system. Pp.127 in Pitti lio, D H. (eds) Modern Blood Banking and Transfusion Practices, Davis Co., 1983.

Table I-2. The eight "basic" *Rh* alleles (gene complexes) and a few of their products

CDE term	Rh-Hr term	Shorthand symbol	Antigens made
CDe	Rh^1	R^1	C, D and e
cDE	Rh^2	R^2	c, D and E
cDe	Rh^0	R^0	c, D and e
CDE	Rh^z	R^z	C, D and E
cde	*rh*	*r*	c and e
Cde	*rh'*	*r'*	C and e
cdE	*rh"*	*r"*	c and E
CdE	rh^y	r^y	C and E

Taken from
Issitt, P. D. Serology and Genetics of the Rhesus Blood Group System, Montgomery Scientific Publications, 1979.

crossing-over. In the case of *Cde*, the least frequent gene combination, a double cross-over would be required. He also predicted that the gene order within the chromosome is more likely *DCE*, as opposed to *CDE*, since the frequency ratio of cross-over observed between *d* and *e* was greater than that observed for between *c* and *e*.

3. Nomenclature of Rosenfield

The complexity of the Rh locus has grown with the discovery of new antisera reactive with determinants other than those first described, as well as from the variable reactivity of the same antiserum with different red cells. In an effort to simplify the Rh nomenclature, Rosenfield and co-workers [159] introduced a modified version of an Rh notation first suggested by Murray [121].

A number was assigned to each antigen in the order of its discovery or upon its admission to the Rh blood group system. The phenotype of a particular red cell sample could then be expressed as a series of numbers. For example, the phenotype $C+D+E-e+hr^{s}-$ is written as Rh:1,2,-3,5,-19. The negative sign indicates no reactivity with the specific antiserum and the absence

of a number implies that no test was performed for that specific antigen. Table I-3 shows all of the, as yet, identified Rh antigens expressed in the three nomenclatures.

4. Rh(D) Variants

a. Weak or absent Rho(anti-Rh(D)) reactivity

Wiener's [192,193] observation of intermediate reactivity of some red cell samples with anti-Rh(D) suggested the possibility of allelic forms of Rh(D). Stratton [171] also reported that some red cell samples, denoted D^u, gave positive reactions with some anti-sera and negative with others.

Red cells that show weak (intermediate) or no reactivity with some of the available saline-agglutinable Rh(D) antisera are referred to as high grade D^u. Those red cells which do not react with anti-Rh(D) directly in saline but are positive by an indirect antiglobulin test are called low-grade D^u.

Table I-3. Antigens of the Rh blood group system in three nomeclatures

Numerical	CDE	Rh-hr	Other	Numerical	CDE	Rh-hr	Other
Rh1	D	Rh_0		Rh22	CE	rh	
Rh2	C	rh'		Rh23	D^w		Wiel
Rh3	E	rh''		Rh24	E^T		
Rh4	c	hr'		Rh25			LW
Rh5	e	hr''		Rh26	"c-like"		Deal
Rh6	ce	hr	f	Rh27	cE	rh_{II}	
Rh7	Ce	rh_i		Rh28		hr^{II}	
Rh8	C^w	rh^{w1}		Rh29		HR	total Rh
Rh9	C^x	rh^x		Rh30	D^{Cor}		Go^a
Rh10	ce^s	hr^v	V	Rh31		hr^B	
Rh11	E^w	rh^{w2}		Rh32		$\overline{R}^N$	
Rh12	G	rh^G		Rh33	D^{Har}	R_0^{Har}	
Rh13		Rh^A		Rh34		Hr^B	Bas.
Rh14		Rh^B		Rh35			1114
Rh15		Rh^C		Rh36			Be^a
Rh16		Rh^D		Rh37			Evans
Rh17		Hr_0		Rh38			Duclos
Rh18		Hr		Rh39	"C-like"		
Rh19		hr^S		Rh40			Targett
Rh20	e^s		VS	Rh41	"Ce-like"		
Rh21	C^G			Rh42	Ce^s	rh_i^a	

Taken from

Case, J. The Rh blood group system. Pp.132 in Pittiglio, D. H. (eds) Modern Blood Banking and Transfusion Practices, Davis Co, 1983.

i. Genetic D^u

The D^u phenotype first described by Wiener [192] and Stratton [171] showed a weakened reactivity with Rho antisera due to a diminished expression of Rh(D). These quantitative variants of Rh(D) can be inherited in a mendelian fashion and are referred to as genetic D^u [61].

ii. D^u mosaics

Some people, who were originally typed as Rh(D) negative but were later typed as D^u, were shown to produce anti-Rh(D) after transfusion with Rh positive blood. Antibodies to Rh(D) were also detected in multiparous D^u women after a pregnancy involving an Rh-positive fetus [5].

It was first suggested that the Rh(D) antigen was comprised of three components: D-1, D-2 and D-3 [5]. Individuals not expressing the full complement of Rh(D) components would be capable of mounting an immune response to those which were missing.

The mosaic theory was introduced later by Wiener [198] to explain this type of D^u. He suggested that

Rh(D) was composed of four antigenic portions; Rh^{A}, Rh^{B}, Rh^{C} and Rh^{D}. The normal Rh(D) contained all four components ($Rh^{A,B,C,D}$), whereas mosaic D^{u} individuals were missing one to three portions of th Rh(D) antigen. A lower case letter denotes the absence of that antigen. Support for this theory lost favour when it was dicovered that it was possible for some D^{u} persons to make anti-Rh^{ABCD}.

Tippett and Sanger [182] have presented a more comprehensive classification for the mosaic D^{u}. The classification scheme contains six categories which have been arranged according to the reactivity of red cells from different D^{u} persons with the anti-Rh(D) produced by other D^{u} persons. Red cells from the D^{u} mosaic persons found in the first three categories show reactivity with all anti-Rh(D) sera except their own. Other features are used to separate these D^{u} mosaics into the most appropriate category. For example, the mosaic D^{u} found in category II appears to be most often associated with the antigens C and e.

Individuals found in category IV react with 96 % of the anti-Rh(D) made by persons phenotyped D^{u}. Most blacks, in this category, are also positive for the Go^{a} antigen (Rh30), whereas most Caucasians are

Go^a negative. A slightly enhanced expression of the D antigen is also associated with Rh(30). It was suggested that Go^a represents an antigen produced by an allele that codes for a portion of the Rh(D) antigen [104].

The red cells from the persons in category V react with approximately 74 % of the anti-Rh(D) produced by D^u mosaics. Most of the red cells in this category were shown to be positive for the D^w antigen (Rh23) [26]. Like Go^a, D^w is believed to be a replacement antigen for a portion of the D mosaic.

Only 35 % of anti-Rh(D) sera will react with red cells of category VI. Most of the D^u mosaics which eventually make anti-Rh(D), after the proper immunogenic stimulus, are from this category. Most D mosaics from this group behave like low-grade D^u.

Recently, a seventh D^u category had been proposed for those people whose red cells exhibited a weakened expression of a variant Rh(D) antigen designated D^T [105]. Red cells of this D^u phenotype react with more Rh(D) antisera than those classified in categories V and VI but less than those red cells found in category IV. The D^T antigen is usually found in association with the low frequency Rh antigen Tar (Rh 40).

iii. Positional D^u

It was shown that when the *C* gene is in the trans position (present on the other homologous chromosome) to the *D* gene a diminished expression of Rh(D) results [22]. For example, red cells with the genotype CDe/Cde or Cde/cDe will may type as D^u instead of as Rh(D) positive. D^u red cells of this phenotype show variable reactivity with agglutinating antibodies in saline and are therefore considered high-grade D^u. The patient is however genetically Rh(D) positive.

b. Enhanced Rh(D) expression.

Several Rh phenotypes that show an enhanced level of Rh(D) expression have been detected. Most notable is the red cell phenotype lacking the other common Rh antigens (C, c, E and e) [148]. This red cell phenotype results from homozygosity with the rare *-D-* gene at the *Rh* locus. Red cells of this phenotype are agglutinable with incomplete anti-Rh(D) in a test system requiring much lower than normal amounts of protein. It was postulated that the increased amounts

of Rh(D) were due to a lack of competition from the other *Rh* genes which had been removed by partial gene deletion.

Another rare gene, *.D.*, is known to be very similar to *-D-*, except that red cells carrying this genotype are also strongly positive for the Evan's (Rh37) antigen [34].

Category IV D^u mosaics, which are also carrying the Go^a gene, display an enhanced level of the variant D antigen. The amount of the D antigen expressed is not as high as that found on red cells lacking the other common Rh antigens (see above).

Slightly enhanced Rh(D) antigen levels are also associated with the appearence of a rare Rh antigen (Rh32) produced by a low frequency *Rh* gene, $\bar{R}^N$ [160]. The presence of the antigen is also associated with decreased amounts of C and e.

Increased levels of available D antigen are also observed on red cells bearing the phenotype R2R2 [77].

5. Rosenfield's Genetic Model for Rh

Rosenfield and co-workers have introduced a genetic model for the Rh blood group system that takes into account the qualitative as well as the

quantitative variants of Rh(D) [158]. The conjugated operon model proposed by these authors envisions a regulatory gene closely linked with each of three structural genes at the *Rh* locus. Normal Rh(D) antigen expression would be under the control of a specific regulatory gene. Alleles of the regulatory gene locus would function with a variable efficiency in switching on the structural genes. Therefore, variant forms of the Rh(D) antigen, such as genetic D^u red cells where there is a diminished amount of Rh(D), would be controlled by specific regulatory gene alleles.

Rosenfield suggests that the Rh(D) antigen is not a single constituent, but is a molecular structure comprised of a series of antigenic determinants. Deletion or mutation at one or more of these sites would create different antigenic components, thus leading to a variety of D mosaics. Such D mosaics (D^u categories I-VI), already described by Tippett and Sanger [182], could then be inherited as structural alleles of Rh(D).

Evidence in support of independently segregating suppressor genes was also incorporated into the model. Homozygosity at the gene loci of the suppressor gene ($X^{o}r$) [101] was shown to be

associated with the Rhnull phenotype in which no Rh antigens are expressed. Pseudo rh^{G}, a slightly less depressed Rh phenotype, results from the homozygosity at another suppressor gene x^{Q} [28]. Rosenfield suggests that the suppressive effect, of either one of the two genes, occurs at the site of a main regulatory *Rh* gene. The suppression of this gene, or any of its alleles, would inhibit the activation of any of the regulatory genes and the subsequent expression of their linked structural genes.

C. PATHOGENESIS OF Rh DISEASE

1. Rh(D) alloimmunization

Rh HDN (or Rh erythroblastosis) develops as a result of the transplacental passage of maternal anti-Rh antibodies into the circulation of the Rh-positive fetus. These antibodies, which are invariably of the IgG antibody class, are directed against fetal red cell Rh antigens not shared by the mother and cause red cell destruction in utero. This leads to anemia, as well as a variety of other sequelae depending on the extent of the disease. In the most severe conditions fetal death will occur as a result of hydrops fetalis.

Inspite of the complex polymorphism which exists within the *Rh* gene locus, by far the most clinically significant Rh red cell antigen is Rh(D). The high immunogenicity of the D antigen places Rh-negative mothers bearing an Rh(D)-positive fetus at risk of Rh(D) alloimmunization. Thus, the incidence of HDN in any given population is governed by the prevalence of the D antigen.

The incidence differs considerably from population

to population, since the prevalence of the Rh-negative genotype (r/r) varies greatly. The variability is dependent on the racial and ethnic backgrounds of the persons comprising the individual groups [145].

In the American Caucasian population the high prevalence of the Rh negative phenotype (15 %) as compared to the much lower Black and Oriental prevalence is attributable to the relatively high $r(cde)$ gene frequency in caucasions. Despite the relatively large number of pregnancies among whites involving an Rh-negative mother and an Rh-positive fetus (90 per 1000 pregnancies) the incidence of Rh(D) HDN in those pregnancies at risk is only 13 %. In cases involving HDN, 17.5 % will result in perinatal mortality, with 14 % stillbirths and 3.5 % neonatal death [207].

The lower than expected incidence of Rh(D) HDN in those pregnancies potentially at risk suggests the involvement of certain factors capable of providing a protective mechanism, thereby reducing the probability of Rh(D) alloimmunization occurring as aresult of pregnancy. These factors are discussed in greater detail below.

a. Transplacental Hemorrhage

Rh(D) sensitization results from the exposure of the Rh-negative mother to the red cells of her Rh-positive fetus. The leakage of fetal red cells into the maternal circulation was shown to occur during pregnancy and/or at the time of delivery. The passage of fetal red cells across the placenta was first suggested by Wiener [194] but direct evidence was not provided until Chown [24] detected the fetal red cells of an anemic infant in the mothers circulation following a large fetal-maternal hemorrage (of 160 ml). This observation was later confirmed by Gunson [69] who in a similar situation was able to detect fetal red cells in the mother's circulation for a period of up to 2 months following delivery.

Studies involving the transfusion of Rh-positive blood to Rh-negative volunteers showed a direct relationship between the incidence of anti-D antibody formation and the volume of blood transfused. As many as 50 % of Rh-negative persons transfused with 500 ml of Rh-positive blood will show detectable antibody in their serum [44]. This is in marked contrast to the 15 % Rh(D) immunized volunteers following a challenge

with 1 ml [116].

This dose relationship holds true as well during pregnancy where the risk of sensitization is directly related to the size of the TPH [182,183,188]. The incidence and the size of the TPH can be determined throughout the pregnancy and following delivery utilizing the Kleihauer-Betke (K-B) acid elution technique [81]. The K-B technique allows for the detection of fetal blood volumes as low as 0.01 ml in the maternal circulation. Fetal hemoglobin, due to its insolubility under the conditions of the technique, remains intracellular resulting in the dark refractile staining of the fetal red cells, a sharp contrast to the adult red cell ghosts.

Zipursky [210] had reported fetal red cells in the maternal circulation as early as the eighth to ninth week of gestation. However, this observation has been disputed on the basis of the glycine/alanine ratio of the gamma-15 peptide of hemoglobin, since the value falls into the range of maternal and not fetal hemoglobin [134]. Therefore the earliest time that fetal cells may enter the maternal circulation has yet to be resolved.

The incidence of TPH during pregnancy and at the time of delivery varies considerably from study to

study [31,172]. However, trends do exist. The incidence of TPH increases during the course of normal pregnancy and is comprised primarily of small fetal bleeds of 0.1 ml or less. The highest incidence of TPH occurs at the time of delivery with large fetal-maternal bleeds (> 0.20 ml) occurring at 5-10 times the frequency of that detected during gestation (1 %). As many as 63-69 % of all mothers tested following delivery have circulating fetal red cells [201].

Therefore, it was suggested that the Rh negative mother can become Rh immunized following TPH by one of two mechanisms; in the majority of the cases , from repeated small TPH's during pregnancy and to a lesser degree, from a large hemorrhage as a consequence of an abnormal delivery .

It is well known that multiple exposures to small immunizing doses of antigen can be as effective in eliciting an immune response as a single large antigenic dose. It should also be noted that small TPH's may result in Rh sensibilization, whereby Rh immunization only becomes detectable during a subsequent pregnancy involving an Rh positive fetus. Immunogenic TPH's can also result during spontaneous or induced abortion, ectopic pregnancy, cesarean

section, manual removal of the placenta, external version and abruptio placentae [12,82].

It was also suggested that the TPH of maternal Rh-positive red cells into the circulation of an Rh-negative fetus may result in Rh(D) alloimmunization [7,177]. Known as the Grandmother Theory, this form of Rh immunization , if it does occur at all, is considered to be an extremely rare event.

In summary, both the frequency and the size of the TPH serve to influence the degree of the Rh(D) antigen exposure of the Rh-negative mother during pregnancy and at the time of delivery, and subsequently the risk of Rh alloimmunization.

b. Immune Responsiveness

Previous studies [115] indicate that only a small proportion of the Rh-negative recipients develop antibodies following a single challenge with Rh-positive red cells and that some individuals may require repeated injections of antigen before immunization occurs. As many as 25-33 % will fail to become Rh immunized even after repeated challenge with Rh(D) antigen [30,115].

Thus, the immune responsiveness of an Rh-negative individual to Rh(D) has been categorized into one of four groups on the basis of antibody production [77]: (i) those Rh-negative persons who readily make antibody are considered "good responders"; (ii) those individuals who require further Rh(D) antigen exposure are called "responders"; (iii) persons making only low levels of antibody are "poor responders"; and (iv) those failing to produce anti-Rh(D) following repeated injections are considered "non-responders".

The most plausible explanation for the varied immune response to Rh(D) in man can be found in animal studies concerning the genetic control of specific immune responses. With the use of inbred animal populations it has been well documented that the immune response to a large number of simple antigens is controlled by genes mapping to the region coding for class II antigens in the MHC complex. The genes responsible for this control have been designated immune response (*Ir*) genes.

Studies using a murine model [109] have shown that the response of a given inbred mouse line to either the tyrosine or the histidine-containing branched amino acid polymer is determined by a single autosomal

dominant gene. This *Ir* gene was mapped to the I-A subregion of the Ia locus, one of two subregions (I-A and I-E) containing Ir genes.

In man, the human counterpart to the murine H-2 locus is the HLA complex and like the murine MHC locus the HLA complex contains a number of closely linked loci, all of which display a variable degree of allelic polymorphism. Because of the close linkage, alleles at each locus are usually inherited as a unit and this unit is referred to as a haplotype.

Recent evidence was presented [150] suggesting that the immune response to Rh(D) may also be controlled by an *Ir* gene(s) and that its inheritence could be marked by specific complement alleles. The complement genes, BF, C2, C4A and C4B are inherited as a single unit termed a complotype. Due to the proximity of the complotype to HLA-DR and the absence of cross-overs between these two gene regions the frequency of the complement alleles has been used to identify those complotypes associated with specific *Ir* gene controlled responses.

The allelic frequencies for each of the four complement genes were studied in both Rh-negative non-responders and responders. Significant increases were detected in only the three alleles BF*F1, C4A*Q0

and C4B*Q0, which when present together with either of the two alleles at the C2 locus, comprise the F1C30 and SC01 complotypes. Therefore it was suggested that the Rh(D) antibody response in an Rh-negative individual is under the control of a gene(s) within the HLA haplotype as marked by either one of two possible complotypes and that absence of these complotypes is associated with non-resposiveness.

c. ABO Incompatibility

The observation that ABO incompatibility between the mother and the fetus offered some measure of protection against Rh alloimmunization was first made by Levine [99]. He found a decreased incidence of Rh(D) HDN in those infants from ABO incompatible matings. This was later supported by the observation of Nevanilinna and Vaino [127], who noted that HDN was most likely to develop from ABO incompatible matings when the last healthy baby delivered by the mother was ABO compatible. It was therefore concluded that Rh immunization is more likely to occur following the delivery of an ABO compatible infant.

The partial protection conferred by ABO incompatibility was demonstrated following the deliberate immunization of Rh-negative volunteers with Rh-positive blood [170]. Anti-Rh(D) was detectable in 70 % of those ABO compatible subjects injected, whereas only 15 % of the ABO incompatible individuals became Rh immunized. Fetal red cells were detected more frequently and in higher quantities in the circulation of mothers delivering an ABO compatible fetus than those delivering ABO incompatible fetuses [32].

Woodrow and Donohoe [202] showed in Rh-negative primiparae, tested during a 6 month period after delivery, that 8 % of the women with ABO compatible and 1 % of those with an ABO incompatible fetus, had detectable D antibodies in their serum . When these women were followed through a second Rh-positive pregnancy, Rh(D) antibodies could be detected at the time of delivery in a further 9 and 2.2 %, respectively. It was therefore concluded that 17 % of women who deliver two Rh-positive babies, the first being ABO compatible, will become Rh immunized by the end of the second pregnancy. Half of these would result from Rh(D) sensibilization after the first pregnancy and would only be detectable following a

second exposure to Rh-positive red cells during the second pregnancy.

The mechanism by which ABO incompatibility affords protection is believed to result from naturally occurring anti-A and/or anti-B. These isohemagglutinins are thought to effect the rapid removal of fetal red cells from the maternal circulation by complement-mediated (intravascular) red cell destruction with the subsequent removal of the red cell debris by hepatic macrophages through C3b-mediated immune adherence [112,115]. It is believed that hepatic clearance deviates the red cells away from secondary lymphoid organs (eg. spleen), thereby reducing the risk of Rh alloimmunization [12,147].

Issitt [77] also suggests that the few Rh negative-women who do become Rh(D) sensitized, as a result of an Rh-positive ABO incompatible pregnancy, may do so because of their genetic background. The rapid intravascular removal of fetal red cells by anti-A and/or anti-B may reduce the amount of Rh(D) antigen exposure so that only "good responders" receive immunogenic doses of the Rh(D) antigen and become Rh immunized.

d. *Rh* Genotype of the Fetus

Fetal red cell D antigen expression was quantitated by surface iodination [155] and was found to vary according to the probable *Rh* genotypes present at the *Rh* gene locus of both chromosomes. The number of D sites also may vary considerably as a result of gene interaction. This interaction can occur within the *D* gene containing *Rh* locus (cis effect) or as a result of modifying effects from the genes of the *Rh* loci of the second chromosome (trans effect). Cepellini [22] has shown that the expression of a D^u phenotype in some individuals can be attributed to the trans-positional effect of *C* on *D* (eg. *CDe /Cde*) resulting in considerably diminished cell surface D antigen.

The relationship involving an increased risk of Rh immunization from fetal red cells possessing more D antigenic sites, as shown by their increased in vitro reactivity with various anti-D antibodies, was identified by Murray [123]. After studying the *Rh* genotypes of fetuses of mothers who had detectable serum anti-Rh(D), he was able to conclude that the red cells from an R2r fetus are more likely to immunize

the mother than those of an R1r infant. This increased susceptibility to Rh immunization was also found to be associated with a greater severity of Rh(D) HDN [124].

2. Rh(D) antibody formation

As already described above, of those women who do become Rh immunized following a first Rh-positive pregnancy, approximately 50 % will display detectable serum anti-Rh(D) antibodies postpartum. The first appearance of Rh(D) antibody in these women is quite variable, generally ranging from six weeks to as late as six months following primary sensitization. The incidence of Rh antibodies at or before delivery is relatively rare (2 % or less) [13], despite the fact that some of these women may have become sufficiently immunized early in their pregnancy. This late development of maternal Rh(D) antibodies during pregnancy therefore presents no threat to the present fetus.

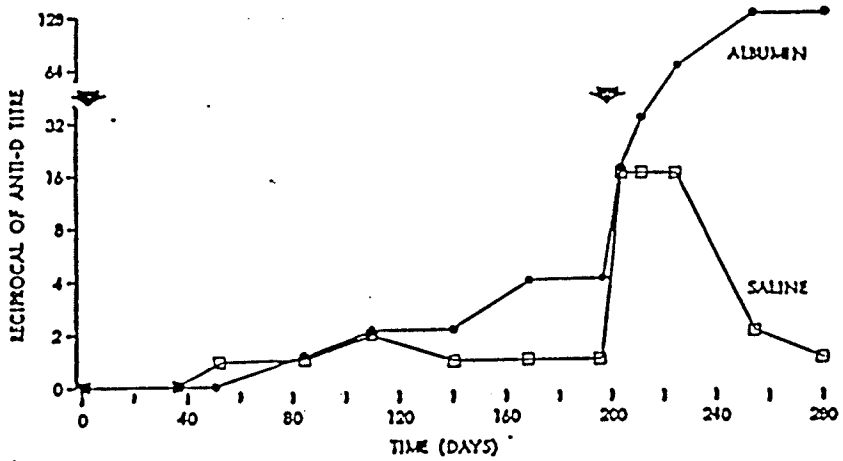
When Rh(D) antibodies develop during pregnancy, they usually develop late in gestation. The resulting HDN, therefore, is much less severe than that which is encountered in later pregnancies where maternal

antibody is present from the beginning. The long lag time prior to the development of a primary immune response was demonstrated in Rh-negative volunteers (Fig.I-1(A)) following injection of 2.0 ml of Rh-positive blood [207].

The first exposure to the Rh(D) antigen is a true primary response. Several months are required for the clonal expansion of the few progenitor antibody producing clones before an Rh-negative individual is capable of producing high levels of Rh(D) antibody [65]. Gorman suggests that the B cell repertoire in the Rh-negative individual is naive to the Rh antigen and that many of the antigens (eg. flagellar) studied by immunologists in establishing the pattern of a primary response are probably not true primary antigens so what is seen is a secondary response.

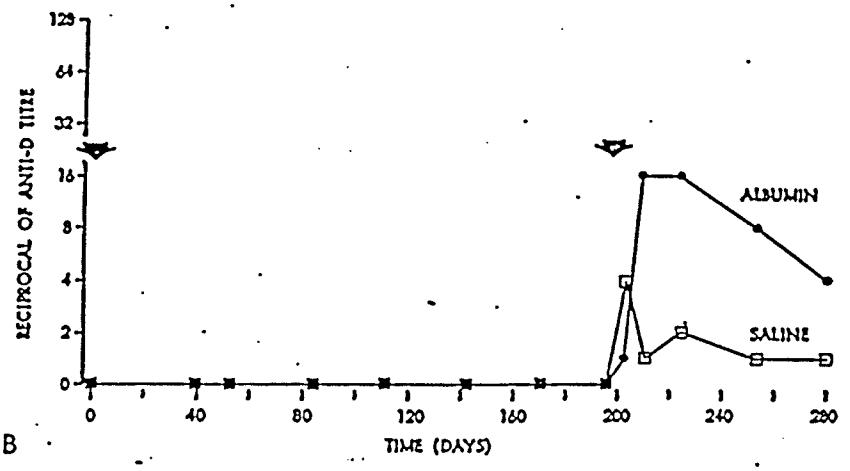
During pregnancy the relative refractory immune state may be due to a generalized suppression of T lymphocyte function and therefore a subsequent reduction in T cell dependent B cell activation. Except for a rapid increase in the very early stages, T lymphocytes were shown to be less responsive to phytohemagglutinin stimulation during pregnancy and

Fig. I-1(A). Rh(D) antibody response after immunization



A

Fig. I-1(B). Rh(D) Sensibilization



B

Arrows in both figures indicate the time Rh-negative volunteers were immunized with Rh-positive red cells. IgM anti-Rh(D) titres were measured with saline suspended red cells. Albumin was added to the red cell mixture to measure the anti-Rh(D) titre due to both IgG and IgM antibodies.

Both figures taken from
Zipursky, A. Isoimmune hemolytic disease. In Nathan D. G. & Oski, F. A. (eds) Hematology of Infancy and Childhood, W. B. Saunders, 1981.

remained suppressed until delivery [63] when mitogen-stimulated activity again increased.

The first antibody to appear in the primary immune response is usually IgM. It is commonly referred to as a complete or saline agglutinable antibody, since these antibodies are capable of agglutinating Rh-positive red cells suspended in physiologic saline. The IgM antibody, when present, is frequently only detectable for a short period of time and is usually of a relatively low titre. In some instances the IgM anti-Rh(D) may only be detectable when enzyme-modified red cells have been used for the testing.

In the primary response the appearance of IgM in the serum is often followed by a switch to IgG antibody production and a subsequent decline in IgM antibody levels. The IgG anti-Rh(D) antibodies produced are of the subclasses IgG1 and IgG3 with IgG1 being the most common. In those individuals who form mixtures of IgG1 and IgG3 antibodies it has been postulated that the IgG1 subclass is formed first with IgG3 appearing later and only after further antigen exposure [77].

The primary response may not always be detectable in those individuals exposed to the D antigen. This

form of Rh immunization is referred to as Rh sensibilization [126]. It is characterized by an increased rate of Rh-positive red cell clearance from the circulation and the apparent absence of Rh antibodies following primary immunization (Fig.I-1(B)). Small TPH's early in a second Rh-positive pregnancy are generally sufficient to elicit a prompt secondary immune response; evidence of previous Rh sensitization.

Secondary responses exhibit a rapid appearance in the serum of large amounts of D-specific antibody. The antigen dose required for this response is considerably smaller than that needed to initiate primary immunization. Unlike IgM anti-D whose presence in the serum may be transient and dependent upon recent antigen exposure, IgG Rh antibodies may persist at high titres for months and often are detected many years after Rh immunization.

The selective active transport of maternal Rh IgG antibodies, by their Fc component into the fetal circulation, identifies this antibody class as the mediator of red cell destruction in HDN [62,79]. The absence of placental Fc receptors for IgM and IgA prevents the passage of these antibodies into the fetal circulation. They therefore do not cause HDN.

In contrast to IgM, Rh(D) IgG antibodies are not able to cause the direct agglutination of Rh(D)-positive red cells suspended in saline and are thus referred to as incomplete antibodies. The number of available D antigen sites varies according to the *Rh* genotype and in the majority of cases ranges from $10\text{-}30\times10^{3}$ per cell [77]. Because of the low density of D on the red cell surface it would appear that bound Rh IgG is not capable of overcoming the electrostatic repulsive forces between negatively charged red cells, and thus bridging between cells and agglutination does not take place.

Several methods have been developed to facilitate the serologic detection of Rh(D) IgG by hemagglutination. Rh(D) IgG sensitized red cells can be made to agglutinate in an indirect antiglobulin test (IAT) following the addition of anti-IgG (indirect Coombs reaction) [35]. Direct hemagglutination can be effected either by the addition of albumin to the reaction mixture or by the enzymatic treatment of the cells before or during the test. The proteases papain, bromelin and ficin are all equally effective in facilitating the

agglutination of IgG sensitized red cells.

The mechanism by which proteolysis enhances the hemagglutination of Rh IgG sensitized cells is not fully understood. It has been suggested that the protease cleavage of sialopeptides reduces the net negative charge of the cell as well as altering the biophysical properties of the cell membrane (deformability and plasticity). The result is the weakening of repulsive forces and a increasing the amount of accessible D antigen [108]. The removal of sialic acid residues by neuraminidase though less effective than proteolysis, can also produce the same effect [108].

Following the detection of Rh(D) antibodies in the maternal circulation, the Rh antibody concentration is quantitated by titration. Saline titres, when present, are generally low, since only small amounts of Rh IgM are produced following primary immunization, since IgM anti-Rh(D) does not cross the placenta IgM saline titres are of no prognostic value.

With the use of standard tests (methods and reagents) maternal Rh IgG titers can be used with reasonable reliability in assessing the risk of severe HDN [6,12,60]. Some consider this an inaccurate method of assessment, since moderate or severe Rh

hemolytic disease may occur at low as well as at high antibody titres [131]. Parinaud and co-workers [131] have proposed that the prognostic value of antibody titres could be made more reliable by the inclusion of IgG subclass and allotype determinations [130] since the more severe cases of HDN appear to be correlated most often with the presence of the IgG1 antibody of the G1m(4) allotype.

D. PREVENTION OF Rh(D) ALLOIMMUNIZATION

1. History

Once the etiology of HDN was determined the attention of researchers became focused on developing methods for preventing Rh(D) sensitization during an Rh incompatible pregnancy. Through the research of three independent groups working simultaneously (in the United States, Britain and Canada) it became widely accepted that passive administration of Rh(D) specific antibody suppresses the primary immune response in the Rh-negative mother. Rh(D) IgG antibody was effective in reducing the incidence of Rh(D) HDN by 85 to 90 %. In those later studies where antepartum-postpartum prophylaxis programs were

also introduced a 97.5 % efficiency in reducing Rh(D) immunization was achieved [8,42,209]. It has been estimated that if such a preventive program were not in place today, as many as 10 % of all perinatal deaths in the United States would be caused by Rh(D) HDN [65].

Klemperer suggests that the effectiveness of the Rh prevention program has lead to complacency in the surveylance for antepartum women who have become sensitized to other blood group antigens [82]. The incidence of Rh(D) alloimmunization in areas of universal Rh prophylaxis implementation has fallen so low that HDN caused by non-Rh(D) antigens, principally anti-c, anti-Kell and anti-E [87], is now of growing concern.

Though Rh prophylaxis research groups arrived at the same conclusion concerning the effectiveness of passively administered Rh immune globulin, the reasoning for its application differed: (i) In the studies performed in Britain, the basis for suggesting Rh(D) specific antibody as a means of preventing Rh(D) sensitization arose from the observation that ABO incompatibility between mother and fetus partially protects the Rh negative mother from Rh(D) immunization. It was assumed that the introduction of

complete (IgM) Rh(D) antibodies would function analogous to the naturally occurring IgM anti-A and anti-B. The fetal red cells in the maternal circulation would be rapidly destroyed and eliminated from the circulation, thereby preventing Rh immunization.

Preliminary clearance studies involving ^{51}Cr labeled red cells (10 ml) injected into Rh-negative male volunteers appeared promising, since 50-60 % of the tagged cells were rapidly removed within 48 hrs [50]. From this it was assumed that a larger dose was required for complete elimination.

Encouraged by the clearance studies Clarke and co-workers attempted to prevent Rh(D) sensitization in 24 Rh-negative male volunteers. They administered high titre Rh plasma (10-20 ml) containing predominately "complete" (IgM) Rh(D) antibody 30 minutes after giving 5 ml of Rh-positive blood [29]. Of the thirteen volunteers in the treatment group receiving anti-Rh(D), 8 became Rh immunized as compared to only 1 in the control group, who had received only blood. These results indicated that the injection of complete Rh antibody, if anything, enhanced Rh immunization. Increases in the antibody titres of the antisera failed to clear Rh-positive

cells, even in the presence of detectable serum antibody, and was no more efficient than what was seen previously with lesser amounts of Rh(D) antibody.

In a subsequent experiment, complete Rh(D) antibody was replaced with plasma containing "incomplete" (IgG) Rh(D) antibody [29]. It was demonstrated that Rh immunization was prevented in Rh-negative individuals injected with Rh-positive erythrocytes that had been treated with "incomplete" anti-Rh(D) *in vitro* [169]. The incidence of Rh(D) immunization was significantly decreased in those receiving incomplete anti-Rh(D) as opposed to the control subjects: 3/21 and 11/21 respectively. Also, ^{51}Cr labelling studies showed that incomplete antibody was more efficient in red cell clearance [29].

Once Rh prophylaxis was demonstrated to be equally effective experimentally in men [201], a clinical trial involving Rh-negative primiparas was initiated [1]. The trial was restricted to women considered to be at "high risk" of Rh immunization because of the significant numbers of circulating fetal red cells. None of the women who received Rh immune globulin (0/78) within 36 hrs after delivery became Rh immunized. In comparison, 19 of 78 controls had

detectable Rh antibody in their serum, when tested 3-6 months postpartum.

(ii) In the United States, Freda and co-workers introduced the concept of antibody-mediated immune suppression for the prevention of Rh(D) sensitization from earlier observations concerning diptheria toxin [168]. Antitoxin, when in excess, inhibits the primary immune response to the toxin. The suppression of antibody formation by passive addition of antigen-specific antibody had also been demonstrated by Von Dungern [189], who showed that active immunization in rabbits to ox erythrocytes was inhibited by serum from previously immunized rabbits.

As in the experiments conducted in Liverpool, the effectiveness of passive Rh(D) antibody in preventing Rh alloimmunization was first noted in Rh-negative male volunteers [56]. Three to four days after receiving 10 ml of R1R2 blood, treatment groups were given 5 ml of incomplete Rh(D) antibody in the form of Rh immune globulin (6000-8000 μg of anti-Rh(D) per dose). In these studies, IgG anti-Rh(D) prevented primary Rh alloimmunization in the treatment groups.

In a further study, 11 male volunteers from each of the treatment and control groups received a second injection of Rh-positive blood plus Rh antibody and

Rh-positive blood, respectively. Again, no individuals in the treatment group became Rh immunized. When further challenged with 1 ml of Rh positive blood ten months later, nine of the treated volunteers failed to produce antibody. This suggested that none of the persons in the treatment group were sensibilized after earlier challenges with Rh-positive blood in the presence of Rh immune globulin. Treatment with IgG anti-Rh(D) did not permanently tolerize the persons in the treatment group, since 2/8 became Rh immunized after a further challenge with 10 ml Rh-positive blood. Therefore, it was proven that administration of Rh immune globulin effectively suppressed the primary immune response to the Rh(D) antigen. Furthermore, none of the individuals treated were in a sensibilized immune state, nor were they permanently protected against Rh antigen (red cell) exposure.

In a follow-up experimental study [143], the minimum dose of Rh immune globulin required to suppress the primary Rh immune response to 10 ml of Rh-positive blood (5 ml of Rh-positive red cells) was determined. Results from this study and the results of their first clinical trial [141] established that 300 μg of Rh IgG was as effective as larger antibody

doses in preventing Rh immunization. In this clinical trial, postpartum prophylaxis reduced the incidence of Rh sensitization from 12.94 % to 1.27 %, therefore protecting 90 % of Rh-negative women at risk. Though a standard dose of 300 µg is sufficient for the majority of the women at risk who will experience only small TPH's during pregnancy, it will be ineffective in suppressing Rh immunization resulting from large feto-maternal hemorrhages or accidental transfusions of Rh incompatible blood.

Rh-negative male volunteers were used to assess the relationship between protectivity and the potency of the Rh immune globulin [142]. This was done in order to establish the amount of Rh(D) antibody (µg) required to neutralize 1 ml of red blood cells . From this study, the effective dose of antibody required for larger volumes of Rh-positve blood could be calculated. Volunteers were divided into six groups with each group receiving a varying volume of packed Rh positive red cells (11.6 to 37.5 ml). Half of each group received either normal gamma globulin or 267 µg of Rh immune globulin. All individuals were injected 6 months later with 0.2 ml of Rh-positive blood and the serum of each was examined 2 weeks after to determine the incidence of Rh immunization in each

group. It was shown by linear regression analysis that 267 µg of Rh immune globulin was effective in protecting against a maximum volume of 15.1 ml of red cells. Therefore, the minimum protective dose required for each ml of Rh-positive red cells was calculated to be approximately 20 µg of Rh IgG anti-Rh(D).

For this relationship to be correct it must be assumed that all immune globulin preparations are equally effective, that is that the same number of micrograms of antibody are given. The preparations of anti-Rh(D) globulins are derived from the pooled sera of a number of hyperimmunized donors so that the average binding affinity will not vary significantly from one preparation to another. Pollack and co-workers [142] have also concluded that only 68 % of Rh-negative individuals can be immunized by a single large dose of Rh(D) antigen and that (among the individuals of a given group) there exists a variablity in susceptibility to the suppressive effects of a marginally effective dose of Rh immune globulin. The possible reasons for this observation have been discussed under section C. of this chapter.

The postpartum administration of Rh immune globulin fails to protect those women, 1.5 to 2.0 %,

who become Rh immunized during pregnancy. Programs which have introduced antepartum prophylaxis have been successful in preventing antepartum Rh sensitization by as much as 94 % and have been able to suppress Rh immunization in 97.5 % of women at risk [42,209]. The recommended protocol for antenatal prophylaxis is the administration of a single dose of 300 μg at 28 weeks [150].

The original fear that the injection of Rh(D)-specific antibody during pregnancy might pose a threat to the fetus is unfounded. The lifespan of Rh-positive ^{51}Cr-labelled erythrocytes injected into Rh-positive infants, who have also received Rh immune globulin, was shown to be normal [209]. There was no evidence of any hemolysis, even though all infants when tested showed serum detectable antibodies (weak positive papain reaction). Positive direct antiglobulin tests have been reported in healthy babies whose mothers have received antepartum Rh therapy [135].

Despite the obvious benefits of antepartum prophylaxis its application has not met with universal acceptance. Some suggest that the implementation of such a program is not cost-effective and that its wide acceptance in the absence of extensive scientific

scrutiny may subject healthy infants to as yet undefined but potentially harmful Rh(D) immunoglobulin-related conditions [3,71]. As one might expect in any controversy, evidence suggesting the contrary has also been presented in support of an antenatal program, thus leaving this issue open for continued debate [9,10,184,211].

Before Rh prophylaxis can be successful in reducing the incidence of Rh immunization to its lowest possible levels (0.1-0.2 % of those at risk) the Rh prophylaxis program must be extended to include all the already identified risk categories for Rh immunization. Additional problem areas which might result in the Rh immunization of the mother have been reviewed by Bowman [13] and are listed in Table I-4.

Once primary immunization has occurred in the Rh-negative mother, prophylaxis will be of no help in suppressing a secondary immune response following future Rh(D) antigen exposure [11,43]. It has been suggested that the absence of further antigen exposure, during a subsequent Rh-positive pregnancy (ie. no TPH), is the reason for the apparent reversal of Rh alloimmunization in Rh-negative women who had previously been immunized and had received immune globulin therapy during their present pregnancy [11].

Table 1-4. Problems remaining in Rh prevention

1. The overlooked Rh-negative women
2. The Rh-negative woman who aborts
3. The Rh-negative woman undergoing amniocentesis
4. The Rh-negative woman with massive transplacental hemorrhage—failure of a single prophylactic dose
5. The Rh-negative woman Rh immunized during pregnancy or within 3 days after delivery—failure of postdelivery prophylaxis
6. Maternal-fetal transplacental hemorrhage—the "grandmother" theory
7. The Rh-negative woman who reacts to Rh immune globulin
8. The Rh-negative woman who has a very weak Rh antibody—attempts at suppression

Taken from
Bowman, J. M. Suppression of Rh Isoimmunization.
Obstet. Gynec. 52,385,1978.

2. Proposed Mechanisms of Antibody-mediated Immune Suppression

The implementation of Rh prophylaxis has been successful in markedly reducing the incidence of Rh(D) immunization in Rh negative women, and therefore decreasing the incidence of Rh HDN. The mechanisms by which Rh immune globulin exerts its suppressive influence are still unidentified. Mechanisms proposed thus far are believed to work by either one of two possible routes: 1) by a direct effect on the afferent or peripheral arm of the immune response, thus preventing antigen accessibility of antigen-specific lymphocytes or 2) by the induction of an immunologic central inhibition mechanism resulting in the reduction of the number of antigen-reactive cells.

a. Afferent Suppressive Mechanisms

(i) Antigen blockade

Several possibilities have been postulated by which passively given antibody may effect suppression following afferent action, the simplest of these is

antigen blockade or competitive inhibition [45,113,176]. The binding of specific antibody physically blocks or masks the available antigen sites. This prevents the interactions of appropriate antigen with lymphoid cells which are required for antibody formation. If this were true, Fab and F(ab)2 fragments should be as effective in producing immune suppression as whole gamma globulin, thus suppression would not be an Fc-dependent phenomenon. Hoffmann [74] has presented supporting evidence for this theory, but excessively high concentrations of antibody were needed. Because of this difficulty, Hoffmann hypothesized that Fc-independent immune suppression was not the major mechanism, but that the major mechanism involved required an intact Fc site, (discussed below).

In the majority of the in vitro and in vivo studies concerning antibody induced suppression, the Fc fragment was shown to be an essential component in mediating suppression [57,74,86]. It was also shown that the amount of Rh(D) antibody sufficient to induce suppression, was capable of blocking only a small percentage of the D antigen sites available on the red cell surface [139]. Similar results were obtained with mice immunized with the human LCL, BALM-1, plus

varying amounts of antiserum directed against either one of two cell-surface determinants; one of which is shared by the human leukemic cell line K562 [57]. Antibody amounts sufficient to bind only 75 % and 10 % of the available antigenic sites were found to cause suppression. The above observations suggest that antigen blockade, or competitive inhibition, is not the major mechanism by which passive antibody induces suppression.

(ii) Immune deviation

Passive antibody might also induce immune suppression by immune deviation. By this mechanism, Rh immune globulin would act by diverting Rh(D)-positive red cells (and Rh antigen) from those anatomical sites favouring immunization (spleen and lymph nodes) to those regions (eg. liver) containing relatively few immunologically competent lymphocytes. In this case, specific Rh(D) antigen suppression, should coincide with non-specific suppression to other red cell allo-antigens.

Evidence in support of this theory comes from Clarke [30] who failed to observe any other irregular blood group antibodies (eg. anti-Kell) in 753

Rh-negative women treated with anti-D. In general, Rh-negative individuals who fail to produce anti-Rh(D), do not make antibodies to other red cell antigens either. Conversely, in Rh-negative individuals, when irregular antibodies to other erythrocyte surface antigens are present they are usually found in association with anti-D [77].

In contrast, Pollack [140], as well as other researchers [57,113] using different antigen-antibody systems, have shown passive antibody-mediated suppression to be determinant-specific. Pollack [139] suggests that anti-Rh(D) augments the immunologic response of the Rh-negative individual to other much less immunogenic red cell allo-antigens. The heightened responsiveness to these antigens appears to result from a similar mechanism responsible for the augmentation phenomenon when suboptimal doses of passive antibody are administrated. It is believed that low doses of Rh(D) antibody, not sufficient to induce suppression, increase the amount of antigen reaching the spleen and lymph nodes, so that immunization is favoured. Therefore, immuno-suppressive doses of Rh(D) passive antibody do not cause non-specific suppression, but rather, they fail to augment antibody responses to other red cell

antigens.

The mechanism of antigen deviation is analogous to that originally proposed by Race and Sanger [147] to explain the protection against Rh immunization afforded by ABO incompatibility. This was later used to form the basis for Finn and Clarkes [50] postulate to use passive Rh(D) antibody to prevent immunization. As mentioned earlier, anti-A and/or anti-B are thought to prevent Rh immunization by intravascular (complement-mediated) hemolysis with subsequent deviation and clearance of Rh-positive stroma through the liver.

Evidence to date fails to support a similar action for Rh immune globulin, since ^{51}Cr labelled red cells sensitized with Rh(D) antibody were shown to be destroyed by passage through the spleen [78]. Mollison has compared the rate at which labeled erythrocytes sensitized with anti-Rh(D) and a complement-fixing IgG antibody against another red cell antigen are cleared from the circulation [117]. He found that both are characterized by a biphasic process involving an initial rapid loss of label (hepatic clearance) followed by a much slower and prolonged decrease in radioactivity (splenic clearance). These rates of red cell destruction were

shown to be proportional to the amount of sensitizing antibody. High levels of Rh(D) sensitization involve hepatic clearance whereas lower antibody levels are associated with splenic removal.

Rh antibodies have always been considered non-complement-fixing, but Merryhew [112] has shown that Rh antibodies, though not capable of activating sufficient C1q to cause intravascular hemolysis, do possess sufficient complement-fixing activity at high levels of antibody concentration, to account for the initial hepatic clearance by immune (C3b) adherence.

Schrieber and Frank [163] have also proposed that the pattern of immune-mediated clearance is a function of immunoglobulin isotype, complement activation and specific antibody receptors. IgM sensitized red cells (eg. anti-A) are cleared from the circulation following intravascular lysis by hepatic macrophages through immune adherence. In contrast, erythrocytes sensitized with IgG antibody are sequestered primarily by splenic phagocytes possessing specific Fc receptors. IgG Rh(D) antibodies of the IgG3 subclass were shown to have a greater degree of Fc receptor binding than antibodies of the IgG1 subclass [213].

Since IgG sensitized red cells are primarily

cleared from the circulation by the spleen, the principal site for antibody production, it was proposed that immune deviation may still occur following erythrophagocytosis. The molecular structure of the native D antigen may become destroyed or altered so as to reduce its immunogenicity, thus preventing Rh immunization. In support of this hypothesis, stroma from lysed Rh-positive red cells has been observed to be less immunogenic than whole Rh positive red cells [140]. Studies illustrating the requirement of antigen presentation by macrophages for the development of a humoral immune response have shown antigen processing to be a necessary step and therefore fail to lend support to this hypothesis.

(iii) Rate of clearance

Because Rh alloimmunization generally does not develop for some time after delivery, the rate at which Rh immune globulin is able to clear the Rh positive red cells from the maternal circulation was also thought to be an important factor in producing suppression. The clearance rate of radiolabelled red cells was used as a yardstick by Finn and Clarke [29,50] in assessing the overall effectiveness of

different Rh(D) antibody isotypes to prevent Rh immunization. However, Pollack and co-workers [140] have shown, in both animal and human studies, that Rh immune suppression was achievable in all subjects when clearance rates were only marginally increased above those levels determined to be ineffective.

The second mechanism for antibody-mediated immune suppression, proposed by Hoffmann [74], involves lower antibody concentrations than those required for blocking and is Fc-dependent. Hoffmann has suggested that the immune complexes, formed following immunization with antigen and specific antibody, are removed from the circulation by binding to the many non-antigen-reactive cells expressing Fc receptors. The resulting suppression was not determinant specific. Therefore, Fc binding would still allow for antigen recognition by antigen-reactive precursor B cells and helper T cells, but would prevent B cell cooperation by the inhibition of bridge formation of the antigen-reactive B cell and helper cells (T cell and macrophages).

b. Mechanisms involving central inhibition

i. Antigen and Fc receptor bridging

Several investigators have demonstrated that the injection of diptheria toxin-antitoxin precipitates, formed in antitoxin excess, are capable of suppressing toxin-specific primary antibody formation [168,186].

Normal murine spleen cells incubated with soluble sheep red blood cell (SRBC) antigen and anti-SRBC serum in vitro were also shown to suppress immune responsiveness to antigenic challenge with SRBC after adoptive transfer to irradiated syngeneic recipients [166]. Neither antigen nor antibody alone were shown to be suppressive. It was therefore proposed that the immune suppression of the primary anti-SRBC response with immune complexes was due either to one of two possible mechanisms.

The first mechanism involved the inactivation of antigen-reactive B cells by the cross-linking of their antigen-specific surface immunoglobulin and Fc receptors with antigen-antibody (IgG) complexes [165,166]. Kolsch and co-workers [87] have speculated

that the negative signal for plasma cell differentiation occurs either after the bridging of independent antigen and Fc receptors following the binding of the immune complexes Fig. I-2(A) or that the two receptors are linked and the occupation of both receptors activates the unit to provide the inhibitory signal (Fig. I-2(B)).

Fig. I-2. Kolsch's two models for the inhibition of B cell differentiation to plasma cells

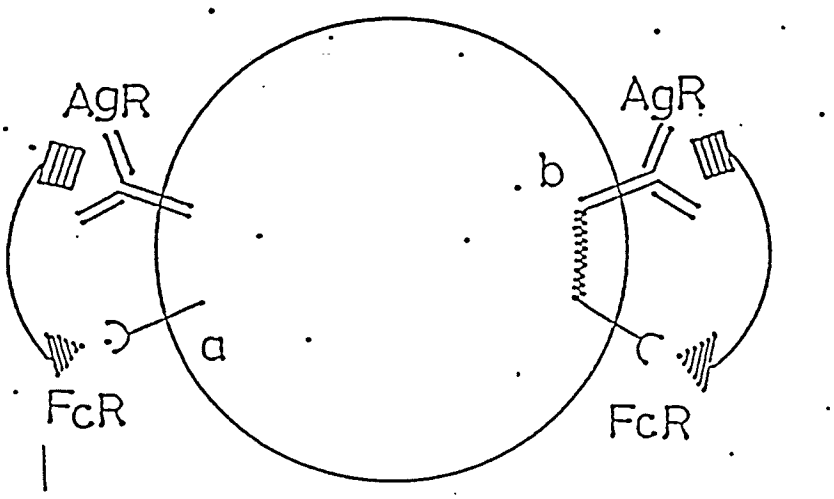


Figure 4. Schematic drawing of the two models for inhibition of B cell differentiation to plasma cells. (a) "bridge formation", (b) "active unit". See text for details.

Taken from
Kolsch, E. et al. The Fc-receptor: its role in the transmission of differentiation signals. Immunol. Rev. 49,61,1980.

Immune suppression was concluded to be independent of T cells or macrophages, since neither the addition of T cell replacing factor nor 2-mercaptoethanol, respectively, could reverse the suppression. Mice, that had recovered from the suppression and had been rechallenged with antigen, responded in a mannner characteristic of a secondary immune response.

Therefore, it was suggested that the negative signal, induced after immune complex cross-linking of B cell antigen and Fc receptors, may in fact be a positive signal for the differentiation to memory B cells. It is unlikely that this mechanism applies to Rh immunoglobulin protection, since Rh-negative individuals treated in the past with no visivle passive anti-Rh(D) display a primary immune response when rechallenged with Rh-positve red cells.

ii. Elimination of antigen-reactive lymphocytes

The second mechanism, as suggesled by Sinclair, [166] hypothesizes that the binding of immune complexes to antigen-reactive cells marks these cells for destruction. The administration of passive antibody has been shown to prevent the rejection of syngeneic and foreign (allogeneic and xenogeneic)

grafted tissues [76].

The mechanism of antibody-mediated immune suppression, proposed by Hutchinson [76], suggests that antigen-reactive cells (B and T) are opsonized by macrophages and possibly other Fc binding cells and subsequently destroyed (Fig. I-3).

Radioactively-labelled SRBC-reactive lymphocytes were shown to be readily opsonized in mice previously given SRBC-anti-SRBC complexes. Furthermore, mice depleted of macrophages, after treatment with carrageenan, failed to exhibit antibody induced suppression.

Hutchinson [76] has further speculated that antigen-reactive cell opsonization (ARCO) might explain the protection afforded by ABO incompatible Rh-positive pregnancies. If so, the immune suppression produced by ARCO should then not apply to that exhibited by passive Rh(D) antibody, since this was shown to differ from ABO antibody protection (discussed earlier in the text).

Fig. I-3. Antibody-induced suppression by antigen-reactive cell opsonization

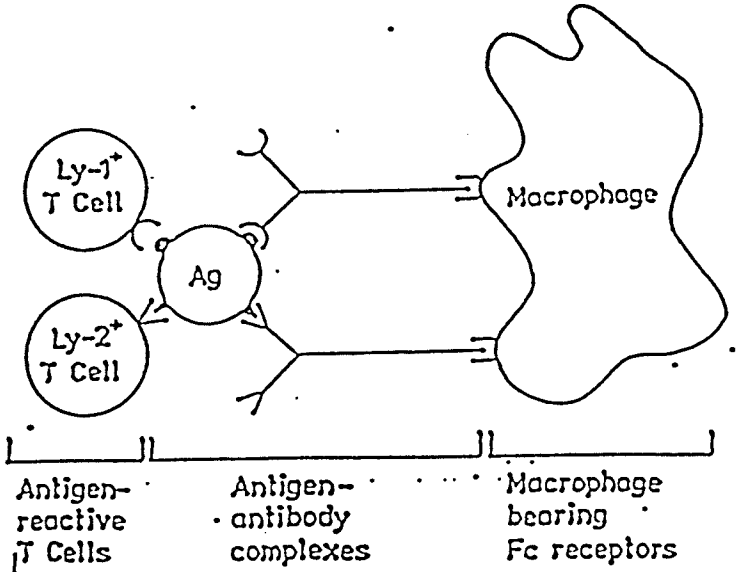


Figure X. Specific opsonization of antigen-reactive T lymphocytes by antigen-antibody complexes. T cells with receptors for MHC antigens bind to free SD (⊣) or LD (⋏) determinants in complexes between multideterminant antigen and anti-SD (⊱—) or anti-Ia (⊱—) antibodies. The Fc portion of the antibody is bound by the Fc receptor on macrophages leading to subsequent destruction of antigen-reactive cells. Note that anti-idiotype antibody may directly link T cell receptors to macrophage Fc receptors.

Taken from

Hutchison, I. V. Antigen-reactive cell opsonization (ARCO) and its role in antibody-mediated immune suppression. Immunol. Rev. 49,167,1980.

iii. Immunostat theory

Gorman [64,65] and Pollack [139] have introduced the "Immunostat theory" as a probable method by which passive antibody may mediate immune suppression of primary Rh immunization. Rh(D) antibody sensitized red cells are removed from the circulation and become trapped within the secondary lymphoid organs (predominantly the spleen). The increased local concentration of available epitopes is thought to function analogous to an "affinity column" capable of removing circulating Rh(D)-specific antibody as it passes through these regions of concentrated antigen. This process would therefore serve to increase both the percentage of the Rh antigen sites bound and bound Rh antibody.

Suppressive doses of antibody were shown to bind significantly higher levels of Rh(D) antigen than does an augmenting antibody dose [139]. Formation of these antigen-antibody complexes were then thought to activate T suppressor cells, which may mediate their suppressor influence through the release of soluble suppressor factors.

Pollack proposes that the formation of IgM-antigen

complexes would accelerate and augment the proliferation and differentiation of committed B cells towards the formation of plasma cells, whereas after the switch to IgG production, the formation of IgG-complexes limits the further expansion of plasma cell clones, but has no direct effect on antibody synthesized by already activated plasma cells [139]. In support of this hypothesis suppressor activity was identified in T cells with $Fc\gamma$, and helper activity in T cells with $Fc\mu$ receptors [120]. Therefore the development of a primary immune response or immune suppression depends essentially on the number of available antigen sites and on the class and availability of the antibody.

iv. Idiotype regulation

Antibody mediated immunosuppression of the anti-Rh(D) primary response may in fact be regulated through the variable (V) domains of molecular and cellular recognition molecules [65]. V regions of each of the recognition elements are capable of binding antigenic determinants through their combining site (anti-idiotype, or paratope), and as well they possess one or a number of immunogenic markers

(idiotypes, or idiotopes) by which other molecules may recognize them.

The theory proposed by Jerne [80] envisions the immune system to be regulated through a network of lymphocyte interactions involving idiotopes and complementary binding paratopes. Perturbation of this immune network by antigen would activate both antigen-specific lymphocytes, and regulatory cells interconnected with the activated clones through idiotype-anti-idiotype recognition.

Currently two mechanisms are believed to be responsible for idiotype-induced suppression: (i) Direct effect of auto-anti-idiotype on idiotype-bearing precursor B lymphocytes. Fig. I-4(A) presents a regulatory scheme by which auto-anti-idiotype may induce suppression during the course of an immune response to a T-independent antigen [73]. It was suggested that anti-idiotype prevents the proliferation and differentiation of precursor B cells by providing the necessary suppressive signal through cross-linking of surface Ig of idiotype-bearing antibody producing cells and their Fc receptors [73,83,181]. In Fig. I-4(A) it is assumed that auto-anti-idiotype develops in response to above threshold levels of antigen-specific antibody

Fig. I-4(A & B). The abbreviations Pre-B1 and Pre-B2 represent the precursor B cell clones which following activation differentiate into the mature lymphocytes B1 and B2, respectively. Antigen (Ag)-specific clones are idiotype positive (Id1), whereas idiotype-specific receptors are anti-Id1. The further differentiation of the mature B cells, B1 and B2, into antibody-secreting plasma cells is shown by Z1 and Z2, respectively. Precursor T helper cells are shown as Pre-Th and the more mature T helper as Th. In fig. I-4(B) T suppressor and their precursor cells are represented by Ts and Pre-Ts, respectively.

Fig. I-4 The suppression of idiotype positive B cell clones in a T cell-independent response by (A) autoant-idiotype antibody and (B) the generation of idiotype-specific T suppressor cells.

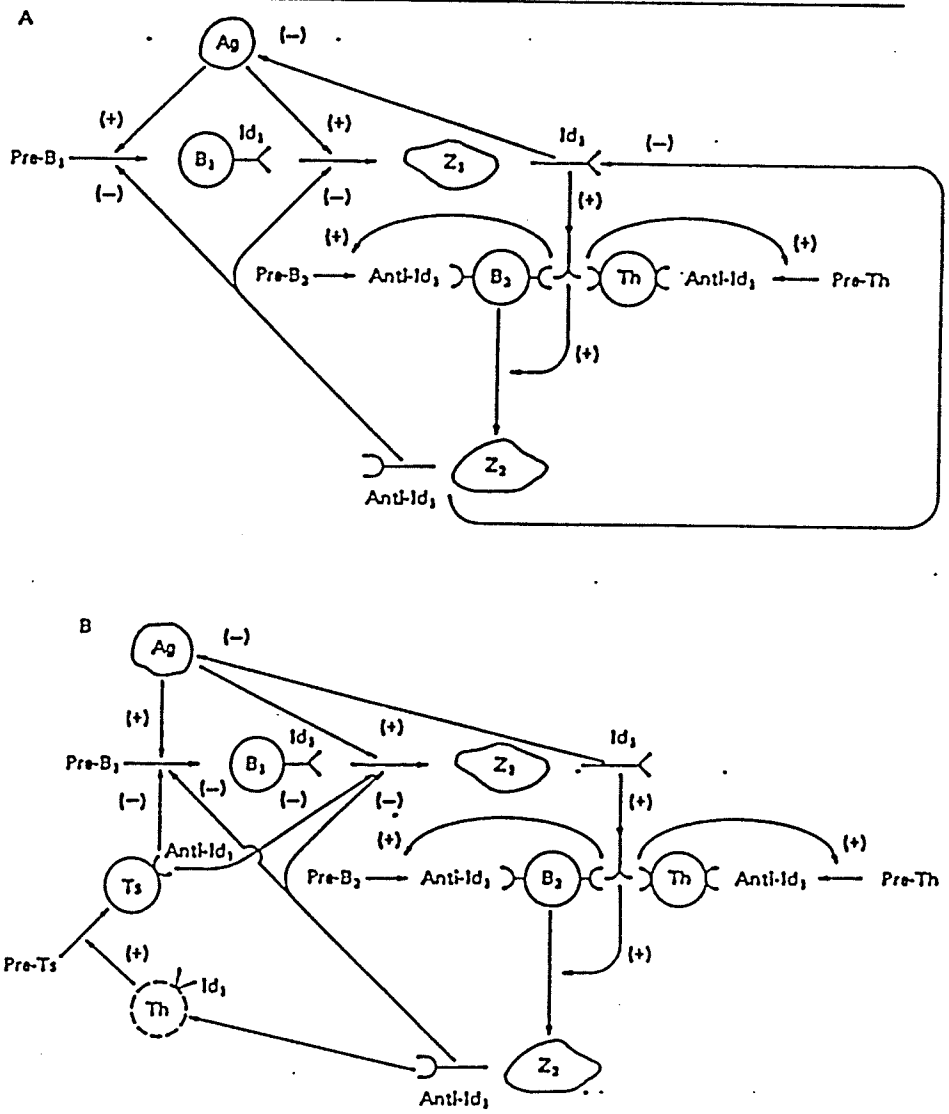


Figure 12–7. Regulatory interactions leading to the production of autoanti-idiotypic antibodies (anti-Id1), which suppress the proliferation and differentiation of Id+1 clones in the case of a T cell–independent response. Suppression can be achieved *(A)* without or *(B)* with the participation of suppressor T cells (see text). The sign (+) corresponds to activation and the sign (–) to inhibition. (Modified and reproduced, with permission, from Hiernaux J: Anti-idiotypic networks. *Fed Proc* 1981;40:1484.)

Taken from
Theofilopolous, A. N. Autoimmunity, in Stites, D. P., Stobo, J. D., Fudenberg, H. H. & Wells, J. V. (eds), Basic and Clinical Immunology, Pp.163, 1984. as cited from Hiernaux, J. Antiidiotypic networks. Fed. Proc. 40,1484,1981.

(idiotype-positive). Therefore, passive immunization with anti-Rh(D), at antibody concentrations known to be suppressive, may provide the necessary antigenic signal to activate the suppressive limb of the idiotype network through anti-idiotype production. (ii) Hiernaux [73] hypothesizes that anti-idiotype may also suppress antibody production by activating idiotype-specific Ts cells (Fig. I-4(B)). In this case it is assumed that anti-idiotype must first recruit an idiotype-positive precursor T helper (Th) cell in order to satisfy the need for complementary receptor recognition.

Balb/c mice repeatedly immunized with the Pneumococcus R36A vaccine produce antibodies specific for phosphorylcholine (Pc). The majority of these antibodies possess the same idiotype as the Pc-binding TEPC-15 murine myeloma [83]. Sera from mice who had received multiple injections of vaccine specifically suppressed the antibody response to Pc in vivo as well as in in vitro plaque forming cell (PFC) assays. The suppressive activity in the sera was removable by adsorption with TEPC-15-sepharose and the decline in the PFC response corresponded with the appearence of an antibody response directed towards the anti-Pc receptor. Mice immunized with antibody against Pc

(TEPC-15 in CFA) also displayed specific suppression of immune responsiveness when later challenged with Pc. Therefore, it was concluded that the suppression of the anti-Pc antibody response was attributed to an auto-anti-idiotype (anti-T15) antibody produced in response to the appearance of the Pc-binding immunoglobulins. An anti-(anti-idiotype) antibody response was also detected during the course of an immune response to Pc and was shown to inhibit specifically the production of anti-Pc antibodies bearing the original idiotype [144].

Immunization with idiotype bearing antibodies was used to demonstrate the idiotype-specific suppression of immune responsiveness to various foreign as well as self-antigens. For instance, lupus-prone female F_1 NZB/NZW mice were treated repeatedly with a murine monoclonal IgG to double stranded DNA which possessed a major idiotype in the anti-DNA response. Specific suppression of the antibody response to DNA and the subsequent suppression of glomerulonephritis was observed. The idiotype-specific suppression of anti-DNA antibody formation was associated with a serum detectable anti-idiotype antibody [70]. A similar suppression of auto-reactivity was displayed in Balb/c mice treated with an isologous murine

monoclonal antibody (Id62-positive) to thyroglobulin (Tg) [206], and in Brown Norway rats injected with idiotype-positive T lymphoblasts specific for tubular basement membrane antigen [125].

The second mechanism is related to the generation of suppressor T cells (Ts) stimulated by idiotype. The injection of anti-azophenylarsonate (AR) antibodies bearing the major idiotype characteristic of a humoral response in A/J mice, was shown to inhibit selectively the idiotype response [47]. Mice treated with the major idiotype still produced an anti-AR response, but none of the antibodies possessed the idiotype in question. Suppression was best observed when the idiotype-bearing anti-Ar antibodies were conjugated to either syngeneic or allogeneic thymocytes as opposed to antibody alone. Idiotype-induced suppression was not observed in mice which had already been primarily immunized with the Ar antigen conjugated to a carrier molecule. Greene and Sy [68], using anti-azobenzenearsonate (ABA) antibody coupled to lymphocytes also demonstrated idiotype-induced suppression of both anti-ABA antibody production as well as ABA-specific delayed-type hypersensitivity. In the studies described above the observed suppression was identified to be

cell-mediated, since it was demonstrated that the suppressed condition could be adoptively transfered to naive recipients with spleen cells (T cells only) from idiotype-primed mice. Suppressor T cells activated by idiotype were shown to possess anti-idiotype receptors and are known to release idiotype-specific T cell suppressor factors (TsF) [174]. The T cell subset population that is activated following immunization with anti-idiotype or antigen has been designated as second-order T suppressor cells (Ts2) [173]. The regulatory factors released by Ts2 may specifically inhibit idiotype-positive antibody production directly through antigen-specific receptors on precursor B cells and/or antigen-binding T helper cells bearing the cross-reactive idiotype [68].

The importance of immune complexes in regulating the suppression of the primary immune response has been already been described for a number of mechanisms (see above). Antigen-antibody complexes containing the TEPC-15 myeloma protein and Pc when incubated <u>in vitro</u> with responder spleen cells were shown to suppress the anti-Pc PFC response in a determinant-specific fashion [21]. The suppression could be transferred to fresh spleen cultures with washed cells from the spleen cells mixed with immune

complexes, therefore indicating that the suppression was cell-mediated. The cells were phenotyped and were identified as T lymphocytes. The inability of T15 specific-suppressor T cells to prevent immune responsiveness to Pc in Balb/c mice which lacked the T15-idiotype due to neonatal treatment with anti-T15 antibodies, clearly demonstrates the idiotype-specific nature of the suppression. Such idotype-specific suppressor cells are activated in 2 days.

Humoral immune responses to antigen are generally characterized by a heterogeneous antibody reactivity to a variety of epitopes (polyclonal response). It has been well documented that a significant portion of the polyclonal immunoglobulin population produced in response to an antigenic stimulus quite often possess identical or cross-reactive idiotypic structures [181]. It was hypothesized that those idiotypic determinants which are highly represented within the sera and/or on lymphocyte receptors provide the necessary immunoregulatory signals in the idiotype network and have been denoted as regulatory idiotopes [133]. Therefore, idiotype-mediated suppression of a polyclonal antibody response may likely be possible through the restricted heterogeneity of the regulatory idiotopes.

Caulfield [20] proposes that the observed augmented antibody response associated with the IgM isotype may be due to idiotype-specific T helper cells capable of recognizing idiotypic determinants not found on IgG antibody-antigen complexes. Therefore, different immunoglobulin isotypes may express different sets of regulatory idiotopes, one capable of enhancing, the other of suppressing the humoral immune response. In support of this hypothesis, Zoller [212] has recently shown that the intravenous injection of an IgM monoclonal anti-TNP specific antibody bearing a cross-reactive idiotype activated idiotype-specific T suppressor cells capable of down-regulating determinant-specific T cell suppression resulting in an increased number of PFC directed against the TNP hapten.

The requirement for an intact Fc domain for passive antibody to effect suppression implies Fc receptor involvement. This observation and the identification of IgG as the suppressive antibody isotype in a number of immune networks is in keeping with what has been documented previously in Rh(D) prophylaxis. Thorbecke [181] suggests that if auto-anti-Id antibody is capable of down-regulating only those B cells which possess surface Ig, then the

more mature B cells (surface IgD-negative, IgG secreting) such as plasma cells would be unresponsive. If this is shown to be true, this might explain the potency of Rh(D) prophylaxis in preventing only primary D sensitization and its ineffectiveness in suppressing secondary immune responses.

E. BIOCHEMICAL CHARACTERIZATION OF THE Rh(D) ANTIGEN

Despite the advances made by serologists and geneticists in the understanding of the Rh(D) antigen and its important clinical significance in HDN, little is known about its biochemical nature. Molecular characterization has been difficult because of the small amounts of available antigen per cell, and the difficulty in removing the Rh(D) molecule(s) from the membrane without loss of antigenicity.

The Rh(D) antigen is believed to be an integral membrane molecule. It is hydrophobic and membrane phospholipids are required for the expression of Rh(D) activity. Treatment of D positive red cell membranes with phospholipases (A_2 and C) or by n-butanol, results in a loss of Rh(D) antigen activity [67,138]. Partial Rh(D) activity can be restored in the enzyme-treated membranes by the addition of deoxycholate (DOC) [138]. These results suggest that phospholipids do not contribute to the antigenic determinants of the Rh(D) molecule but rather, they contribute to antigen expression by maintaining the native conformation of the molecule. The Rh(D)

molecule is completely degraded after protease treatment, which indicates that it is predominately protein in nature [2,58].

Unlike the majority of cell surface proteins, the Rh(D) antigen is not glycosylated, since it cannot be labelled with galactose oxidase NaB3H4 [58] and does not stain with periodic acid Schiff reagent [205]. In addition the Rh(D) antigen failed to bind any of the lectins tested, nor was it degraded when treated with glycosidases [59].

In the past, a number of molecular sizes ranging from 7KD-174±10KD have been reported for the Rh(D) antigen [2,53,137]. Recently, several groups working independently, have isolated by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) a 28.5-33KD polypeptide from non-ionic detergent solubilized Rh(D)-positive red cells by surface ^{125}I-labelling followed by immunoprecipitation with polyclonal IgG anti-Rh(D) [58,75,119]. SDS-PAGE analysis of the immunoprecipitate in the presence of 8 M urea has revealed two additional polypeptides of 50KD and 68KD to be associated with Rh(D) antigen expression [119]. In accordance with the results obtained with polyclonal Rh(D) antisera, Paire and co-workers [130] have identified two polypeptides of

33KD and 42KD from intact Rh(D)-positive red cell membranes by the use of two human monoclonal antibodies.

A single proteolipid of 35KD was also isolated from the red cell membranes of various Rh phenotypes (R1R1, R2R2 and rr) by chloroform-methanol extraction and subsequently reconstituted into micelles with native lipid [167]. Micelle preparations from both Rh(D)-positive and Rh(D)-negative red cell membranes proved to be equally effective in inhibiting the hemagglutination of Rh(D)-positive red cells by anti-Rh(D) serum. These micelles expressed antigenic determinants of common Rh antigens (eg. C and e). From these observations, it was suggested that the common Rh antigens (D, C & E and their respective alleles) reside on a single polypeptide of 35KD and that rr red cells may possess some other macromolecule which sterically inhibits Rh(D) antigen expression in vivo, but is removed during extraction.

Furthermore, the Rh(D) antigen was also found to be present on the cytoplasmic face of membrane vesicles constructed from Rh(D)-negative red cells [136]. In addition, Rh(D) antigenic determinants could be made available for binding following solubilization of Rh(D)-negative red cell membranes

with DOC [137].

These observations are in contrast to the generally accepted view that Rh(D)-negative red cell does not have the D antigen on or in its cell membrane. Also, a procedure which demonstrates the immunoprecipitation of Rh(D) antigen from ^{125}I-labelled Rh(D)-positive unsealed membranes following solubilization with a non-ionic detergent failed to isolate D antigen from Rh-negative (rr) red cells [59].

Recently, it has been demonstrated that the majority (80 %) of the Rh(D) antigen remains in association with the cytoskeleton (detergent insoluble matrix) following Triton X-100 solubilization [59,154]. It was proposed that the linkage of the Rh(D) antigen with the membrane skeleton may be effected through binding with one of the major red cell polypeptides, Band 3. This 90KD integral protein is believed to function in the transport of anions across the cell membrane [107]. It is also known to be intimately associated with the erythrocyte cytoskeleton and is capable of binding several red cell polypeptides [58]. This might explain the previous binding of anti-Rh(D) antibodies with Band 3 [188].

Rhnull, the red cell phenotype lacking all Rh blood group antigens is associated with the abnormal red cell syndrome, stomatocytosis. Red cells from individuals with this condition are known to have a shorter lifespan *in vivo*, and an increased osmotic fragility. Not surprisingly, this condition quite often is associated with hemolytic anemia. These observations, and the evidence linking the Rh(D) antigen with the red cell cytoskeleton [59,154], support the belief that the Rh(D) antigen plays an important role in maintaining the shape and integrity of the red cell membrane.

F. HUMAN MONOCLONAL ANTIBODIES

1. Introduction

Rh immune gamma globulin has proven to be successful in the prevention of Rh(D) sensitization and HDN in Rh-negative women. This has been one of the hallmark examples by which the benefits of immune antiserum therapy has been demonstrated. However, the production of any immune antiserum by conventional immunization protocols is not without some difficulties.

The main disadvantages encountered with conventional immunization for the production of immune antisera have been described by Shay [162] and are as follows: (i) unpredictability of the immune response (eg. low titres, weak or lack of antibody production to some antigens); (ii) heterogeneity of the antibody response (ie. cross-reactivity, variation in class and subclass isotype as well in affinity); (iii) supply is often limited; (iv) the identical antibody may not be made in a new or from a 2nd or 3rd bleeding from the same animal (or human donor); (v) production is relatively expensive and time consuming.

The introduction and advancements in monoclonal

antibody technology in recent years have provided a means by which the problems associated with the production of immune antiserum can be overcome. Monoclonal antibodies (MoAbs), particularily of murine origin, have provided a vast amount of information concerning immune regulation and immunoglobulin structure. As well, they have found a wide application in the diagnostic laboratory.

The potential therapeutic value of MoAbs is currently being directed towards the areas of: (i) targeting drugs to tumours, pathogenic microorganisms, lymphocytes mediating autoimmune and allergic reactions; (ii) diagnostic imaging of malignant cells, damaged tissues, embryologic abnormalities and infectious agents; (iii) active immunization with anti-idiotype antibodies to generate antibody responses against pathogens not suitable for conventional vaccines; (iv) modulation of autoimmune and endocrine conditions with anti-receptor antibody, and (v) passive immunization for the prevention of HDN, the neutralization of drugs and toxins and the opsonization of bacteria and viruses [18].

It is not likely that murine MoAbs will be beneficial in humans requiring multiple treatment, since antibody responses directed against the mouse

antibodies may neutralize their effect and as well as lead to immune-complex mediated diseases (eg. serum sickness). Allotypic and idiotypic markers on human MoAbs would be far less immunogenic and would therefore persist in the circulation longer, thereby increasing their effectiveness.

Also, specific effector functions have been identified with species-specific carbohydrate moieties on immunoglobulins [96,128]. It has been shown that carbohydrates in the CH2 domain of a mouse IgG2b MoAb have a profound effect on; (i) the binding of the antibody to Fc receptors on macrophages, (ii) complememt activation, (iii) their effectiveness during antibody-dependent cellular cytotoxicity, and (iv) the rapid elimination of immune complexes from the circulation.

In addition, antigenic determinants recognized on the immunogen by one species (eg. mouse) may not be the sites responsible for immune reactivity in those individuals of another species that have been immunized [33]. Antigenic determinants considered common within the human population may dominate the immune response when injected into another species, thus increasing the heterogeneity of the immune response as well as reducing the immunogenicity of the

antigen of importance. The development of human MoAbs should prove to be far more beneficial.

Human MoAbs of known specificity have been generated either by transformation with Epstein-Barr virus (EBV), a human lymphotropic herpes virus, or as a result of somatic cell hybridization.

2. EBV Transformation

EBV is the etiologic agent in infectious mononucleosis and is also associated with nasopharyngeal carcinoma and Burkitt's lymphoma. When mixed with peripheral blood lymphocytes (PBL) in vitro, EBV infects and transforms B cells into lymphoblastoid cells capable of continuous growth in culture [132]. These transformed or immortalized B cells secrete immunoglobulin [157]. The B cells susceptible to EBV infection are those that bear CR2 complement receptors [55]. Originally, only small resting B cells were thought to undergo transformation. Chan and co-workers [23] using limiting dilution analysis have identified a more heterogeneous susceptible B cell population. The vast majority of the EBV responsive B cells secreted IgM and were derived predominantly from large lymphocytes

indicative of cells entering the cell cycle. In contrast, cells committed to IgG or IgA were derived entirely from small resting B cells.

Steintz and co-workers [168] were the first to utilize the transforming and polyclonal activating properties of EBV for the production of human monoclonal antibodies. These authors used the marmoset cell line, B95-8, as a source of EBV. The transforming virus is released into the culture supernatants and can be harvested by centrifugation. If transformed by this virus, B cells do not go through the lytic cycle and can be identified by the expression of a nuclear antigen, EBNA [152]. Permanent lymphoblastoid cell lines (LCL) secreting human MoAbs to NNP (4-hydroxy-3,5 dinitrophenacetic acid) were established from individuals with naturally high titers to the hapten. This approach has since been used to generate B cell lines secreting human MoAbs against a wide variety of antigens, including tetanus toxoid [33,91] and the Rhesus factor [14,37,47,88,130].

Despite the simplicity of EBV transformation for the production of LCL secreting human Mabs, the technique is severely limited by a number of problems. Antibody production generally declines with time and is quite

often lost. Crawford [36] proposed several mechanisms which might account for the loss of antibody secretion: (i) stimulation of immunoglobulin may be transient and infected cells may revert to a stable state of low level antibody production; (ii) high level antibody secreting cells may be short-lived and may die out in culture, and (iii) antibody producing cells are overgrown by the faster growing non-producing cells which may have less stringent nutrient requirements. Recent evidence suggests that only a small percentage of those cells transformed have the capacity for continuous growth in vitro. The majority of EBV transformed B cells will continue differentiation along the B cell pathway and reach a state analogous to nonproliferating mortal plasma cells [111].

Enrichment of the antigen-reactive cells, coupled with early cloning of the EBV transformed B cells has been proposed for the development of stable antibody secreting cell lines [37]. However, transformed B cells are difficult to clone, since they grow poorly at low cell densities, even in the presence of a feeder layer and growth factors [17,18]. Also, the amount of immunoglobulin secreted by transformed B cells are generally low (<1 μg/ml) with the majority

of the antibodies being of the IgM isotype [17,162].

3. Somatic Cell Hybridization

Kohler and Milstein [84] were the first to utilize cell hybridization as a method of generating continuous cell lines secreting MoAb of predefined specificity. Hybrids were constructed by Sendai virus-induced fusion between a hypoxanthine-guanine phosphoribosyltransferase (HGPRT)-deficient murine myeloma (MOPC-21) and SRBC immune murine spleen cells.

HGPRT-deficient cells are capable of continuous cell growth under normal culture conditions. However, in the presence of a folic acid analogue, such as aminopterin (A), which blocks the pathway of de novo DNA synthesis, the mutant cells will die because the HGPRT-dependent salvage pathway of DNA synthesis is nonfunctional. Cells possessing a salvage pathway can utilize added hypoxanthine (H) and thymidine (T) to overcome the aminopterin block of purine metabolism. The myeloma cell confers continuous cell growth in culture whereas the splenic B cell, which dies in culture, provides the necessary salvage pathway and antibody specificity. Therefore, only the MOPC-21-spleen cell hybrids survive, when grown in a

selective culture medium containing HAT. Polyethylene glycol (PEG) has since become the fusing agent of choice.

The same principles were used to develop human hybridomas. Human MoAbs against the 2,4 dinitrophenyl hapten (DNP) were produced by the fusion of a HAT-sensitve mutant (SKO-007) of the human plasmacytoma U-266 with the splenic lymphocytes of a DNP-sensitized Hodgkins lyphoma patient [129]. Croce and co-workers [40] fused a HAT-sensitive LCL (GM1500-6TG-2; 6-thioguanine resistant) with the PBL of a patient with subacute sclerosing panencephalitis. The resulting hybrid cells produced MoAbs against the nucleocapsid antigen of measles virus.

Human MoAbs have also been produced to a number of different antigens, including the Rh(D) antigen, by interspecies hybridization [179]. However, the success of human hybridoma technology has been limited by the lack of optimal fusion partners as well as the difficulty in obtaining sufficient numbers of antigen primed lymphocytes.

It is difficult to establish cell lines from human myelomas and for this reason only two such lines are widely available, RPMI 8226 and U-266 [33]. Although

human plasmacytomas possess the desired cellular morphology consistent with high level antibody production and secretion (ie. rough endoplasmic reticulum, few free polysomes, well developed golgi apparatus and numerous mitochondria), they display extremely low fusion frequencies in comparison with their murine counterparts, and tend to grow slowly in culture [89]. Human LCL fusion partners do not possess the same cellular characteristics and their hybrids generally secrete less antibody. However, the LCL are easily adapted to tissue culture. They grow faster and fuse with a higher frequency than do plasmacytomas, and therefore have been used more extensively [89]. Both plasmacytoma and LCL fusion partners developed to date produce immunoglobulin. Antibodies secreted by human-human hybrids would initially contain heavy and light chains from both parents, therefore limiting the amount of antigen-specific antibody released.

Murine myelomas have been used as fusion partners with sensitized human lymphocytes [164] as well as specific antibody-secreting LCL [93] in an attempt to take advantage of their high fusion frequency and clonability. Some myelomas do not produce immunoglobulin at all, which is a desirable

characteristic for the generation of hybridomas secreting MoAb of high quality. Heterohybrids produced from the fusion of human LCL and murine myeloma did not retain the EBV genome as indicated by the absence of EBNA [152].

Stable antibody secretors are difficult to maintain, since human chromosomes are segregated preferentially in such heterohybrids. It is possible only through extensive and continuous cloning, and bulk culturing is not feasible. The loss of human chromosomes from the heteromyelomas does not appear to be random since chromosomes 14 (heavy chain) and 22 (lambda light chain) are preferentially retained, whereas chromosome 2 (kappa light chain) is preferentially lost [41,49]. However, stable heterohybridomas secreting human MoAb against Rh(D) [180] and tetanus toxoid [16], have been generated using the murine myeloma P3X63Ag8.653. Therefore, maybe not all murine-human hybrids exhibit extensive chromosomal segregation, and thus the heterohybridoma approach may be possible with suitable fusion partners.

Heteromyelomas have also been produced from the fusion of human plasmacytomas with mouse myelomas [178] in a an attempt to obtain a better fusion

partner for MoAb production. Hybrid fusion partners have also been constructed from the fusion of the human myeloma RPMI 8226 and the human LCL KR.4 in an effort to combine the desired characteristics of these two cell lines [94].

As mentioned earlier, the production of human-human hybrids has also been limited by the availability of antigen-specific B cells. Peripheral blood lymphocytes, containing sufficient quantities of antigen-specific B cells, are readily available if taken 5-10 days after immunization or at any time from donors with autoimmune disease.

With some antigens, the immunization of human subjects is not possible for ethical reasons. Therefore, rosetting and panning techniques were used to enrich antigen-specific B lymphocytes from blood [200]. Kozbor and Roder [91] have described a negative selection procedure to enrich B cells reactive with tetanus toxoid. PBL containing B cells specific for tetanus toxoid were mixed with a soluble preparation of tetanus toxoid. On tetanus-specific B cells that bound the antigen, cap formation was induced by subsequent exposure to anti-tetanus antibodies, which led to the removal of surface immunoglobulin. Surface immunoglobulin positive B

cells were then rosetted with anti-immunoglobulin coupled to red cells, and separated from immunoglobulin negative cells on a Ficoll density gradient.

In vitro immunization techniques are currently under investigation. The short term exposure of PBL to antigen in the presence of B cell mitogens was shown to increase the number of hybridomas several fold [18,162].

4. EBV Hybridoma Technique

This technique has been developed in an effort to combine the advantages of EBV transformation and somatic cell hybridization and to overcome the limitations of each method [90]. EBV transformation is particularly useful for expanding the B cell population for hybridization, especially when combined with an antigen-specific enrichment technique (eg. rosetting). The lymphoblastoid cells produced by EBV transformation may be stored frozen, which provides further flexibility for the handling of PBL samples.

A ouabain-resistant fusion partner, KR.4, has been developed by Kozbor et al. [90] by mutagenization of the HGPRT-deficient LCL GM1500 6TG-2, with low level

gamma-irradiation followed by selection in ouabain-containing medium. KR.4 fuses preferentially with lymphoblastoid cells, with a 100 fold increase in fusion frequency over those achieved with PBL [92]. Human cells, unlike murine cells, are highly sensitive to the inhibitory effects of ouabain, a potent inhibitor of Na^+, K^+-ATPase. Therefore, when the cell mixture is placed into selective medium after fusion only hybrid cells are able to overcome the toxic effects of both the ouabain and aminopterin. Hybridomas constructed with EBV transformed cells by this fusion procedure secrete elevated antibody levels, similar to those observed after direct human-human hybridization (1-10 μg/ml and higher).

In contrast to human plasmacytomas, human LCL, as well as hybrids generated from a fusion with or between LCL, are EBNA positive, indicating that the EBV genome has been retained. Therefore the generation of infectious virus is possible in culture, which would limit the in vivo use of antibody as produced. However, because of the ubiquitous nature of the EB virus in the adult population (80-90 % show serological evidence of prior infection), many transfused blood products must contain viral DNA [39]. Furthermore, the purification procedure for the

antibody lowers the amount of viral DNA below levels of detection [38]. Therefore the concern over the retention of the viral genome in human monoclonal antibodies may be unfounded.

5. Bulk production of human monoclonal antibodies

Antibody levels of murine hybridomas can be increased by up to 1000 fold by growing the cells as ascites tumours in syngeneic recipients. For obvious ethical reasons this is not possible with human hybridomas. Human antibody-secreting cell lines cannot be grown in immunocompetent mouse strains either.

However, limited success has been achieved with irradiated nude (athymic) mice [156,185]. Human hybridomas were first passaged as subcutaneous tumours in such mice, and then injected intraperitoneally into irradiated and pristane-primed nude mice. This procedure, and some others involving drug-induced immunosuppressed mice, led to the formation of ascites tumours in less than half of the animals treated. The volume of ascites was usually quite low (<2 ml).

Mice suffering from severe combined immunodeficiency disease (deficient in both T and B

lymphocytes) accept xenografts and are suitable for the induction of ascites tumours with human-human hybridomas [190]. However, the high cost and the difficulty of maintaining these mutant mouse strains will preclude their use for large scale antibody production.

In vitro bulk culture techniques, utilizing serum-free media, are expected to evolve into an economically feasible alternative [17].

II. EXPERIMENTAL

A. Introduction

Rh immune globulin therapy has proven to be a very effective method for the prevention of Rh(D) alloimmunization and HDN [13]. Its success in reducing the incidence of perinatal mortality from Rh erythroblastosis is reflected in the low and falling numbers of available Rh immunized serum donors.

Currently, post-menopausal women, who became sensitized to Rh(D) during their child-bearing years, are periodically boosted with Rh(D)-positive red cells for the production of anti-Rh(D) immune globulin at the Winnipeg Rh Institute. It is feared that this procedure may lead to immunization to other red cell antigens and/or to infection by blood-borne pathogens.

With a view to satisfying future needs for Rh(D) antibodies, attempts have been made in recent years for production of Rh IgG <u>in vitro</u>. Human cell lines secreting monoclonal anti-Rh(D) antibodies were generated by EBV transformation of peripheral blood lymphocytes (PBL) from sensitized patients [14,88].

However, these cell lines failed to maintain stable antibody production in vitro. More recently, stable human hybridomas have been generated by somatic cell hybridization that produce MoAb against a variety of antigens, including the blood group antigens A and Rh(G) [54,149]. Lowe and co-workers [97] have also described the selection of a human-human hybridoma secreting IgM anti-Rh(D), after the direct fusion of D-sensitized PBLs with the lymphoblastoid fusion partner WI-L2-729-HF2.

This report describes the fusion of EBV transformed B cell lines with either a mouse myeloma or a human LCL for the production of human monoclonal IgG1(kappa) anti-Rh(D) antibodies by both human-human and human-mouse hybridomas and human IgM (lambda) anti-Rh(D) antibodies by human-human hybridomas.

B. Materials and Methods

1. Isolation of PBL

A 50 ml blood sample was obtained at the Winnipeg Rh Institute (Winnipeg, Canada) from a pregnant Group O, Rh(D)-negative woman after a sharp increase in her anti-Rh(D) titre had been detected.

The mother had been previously sensitized to the Rh(D) antigen during an earlier Rh(D)-positive pregnancy. The PBL were removed from the interphase following Ficoll-Paque gradient (Pharmacia, Dorval, Que.) centrifugation (2,000xg for 20 min.) and used for EBV transformation.

2. EBV production and concentration

A 2 ml starter culture of the B95-8 marmoset cell line (donated by Dr. L. Perelmutter, Bu. Microbiol., Hlth. Protection Br., Ottawa, Ont.), brought up from liquid nitrogen storage, was grown in RPMI 1640 medium (Gibco, Burlington, Ont.) containing 5.0 % fetal bovine serum (Bocknek, Rexdale, Ont.). The cell line was incubated at 37°C in 5.0 % CO2 and expanded to a final volume of 1000 ml over a 6-8 week period. Cells were removed by centrifugation at 400xg for 10 min at 4° C and the supernatant, containing the infectious virus, was sterilized by filtration using a 0.22 µm filter (Millipore Corp., Bedford, Mass.). The viral concentrates were prepared from the centrifugation (20,000 x g for 120 min at 4°C) of spent B95-8 culture supernatants. In our hands, concentrated virus preparations could be maintained at

-70 C for up to 6 months without loss of infectivity.

3. EBV Transformation

Transformed B-cell lines secreting IgM and IgG Rh(D) antibodies were developed by EBV activation. Briefly, PBL suspensions ($1.0x10^4$ or $1.0x10^5$ cells per well in round-bottom 96-well tissue culture plates) were incubated for a period of 2 hrs at 37°C in a transforming medium. The composition of this medium was as follows: RPMI 1640 (Gibco), 20 % fetal bovine serum (Bocknek), 0.005% sodium pyruvate (Gibco), 5 µg/ml insulin (Sigma, St.Louis, MO.), 10 µg/ml gentamycin (Gibco), 1 µg/ml Cyclosporin A (Sandoz Ltd., Dorval, Que.) and 40-fold concentrated EBV.

4. Fusion

Lymphoblastoid cell lines (LCL) secreting high levels of Rh(D) antibodies were rosetted with Group O, Rh(D)-positive red cells (R1R2), and the rosette-forming cells were expanded in culture prior to fusion.

Human-human and human-mouse hybridomas were

generated from the fusion of the LCLs with the cell lines HOA.1 and NS.1, respectively. The HOA.1 cell line was a ouabain-resistant (1 uM/ml) mutant developed in this laboratory from the human lymphoblastoid cell line WI-L2-729-HF2 [72]. The NS.1 cell line, a murine myeloma selected by Kohler and Milstein [85], was donated by Dr. L.D. Kohn (National Institute of Health, Bethesda, Maryland) and was found to be naturally ouabain-resistant (500 μM/ml).

The fusion protocol developed by Kozbor et al [94] was used. Fused cells (2.0×10^5) were distributed in 96-well Linbro tissue culture plates (Flow Laboratories) containing a feeder layer of murine peritoneal macrophages (1.0×10^4 cells/well) which had been plated on the previous day. Growing hybrids were selected in RPMI 1640 supplemented by hypoxanthine (100 μM, Sigma), thymidine (3.14 μM, Sigma), aminopterine (0.082 μM, Sigma), and ouabain (0.2 μM for HOA.1 and 10 uM for NS.1, Sigma) similar to that described in the previous section for EBV transformation, except that Cyclosporin A was omitted. Approximately every four days half of the culture fluid in each well was replaced with fresh culture medium. Aminopterin was omitted from the medium after 14 days.

5.Statistical Analysis

The number of wells not containing hybrids was counted and the fusion frequencies of NS.1 and HOA.1 were determined by the method described previously by Tong et al. [183]. The expected number of fusion events per well (x) was calculated using the Poisson equation

$$P_o = e^{-x}$$

where P_o is the proportion of negative wells and e is a constant with an approximate value of 2.71828. Fusion frequency was then expressed as the expected number of fusion events per 10^7 cells.

The Chi-square (χ^2) test

$$\chi^2 = \text{sum}\frac{(\text{observed}-\text{expected})^2}{(\text{expected})}$$

was used to determine if the fusion frequencies of the fusion partners, HOA.1 and NS.1, were significantly different. The χ^2 value was interpreted with one degree of freedom.

6. Screening

The culture supernatants were screened, 4-6 weeks post-transformation and 3-4 weeks post-fusion, for the presence of antibodies to Rh blood group antigens by capillary hemagglutination [25], using saline suspended and two-stage papainized [66] Group O, Rh(D)-positive red cells.

7. Stabilization of Clones

The hybrids secreting anti-Rh(D) were cloned by limiting dilution in 96-well round-bottom tissue culture plates (Flow Laboratories) containing murine (Balb/c) erythrocytes (1.0×10^6 cells/well) as feeder cells. Cloned hybrids were later placed back into HAT-medium to determine if cells were still resistant to aminopterin.

8. Ascites Production

Ascites tumours were produced in immunosuppressed mice from transplanted human-human or human-mouse hybridomas. Briefly, 4 groups of 6 female

mice (8-12 wks), were first primed with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Company,Inc., Milwaukee, Wis.) intraperitoneally. After 14 days, the mice received a subcutaneous injection of cyclophosphamide (CY: 160 mg/kg body weight) 18 hrs before and at the time of injection of the hybridoma cell lines (10^7 cells in 0.5 ml sterile PBS intraperitoneally) [151]. Half of the mice in each group received an additional dose of CY 2 days post-transplantation.

Ascitic fluid was removed through an 18 gauge needle 10 to 12 days later, and was clarified by centrifugation (400 x g for 10 min.) to remove cells and lipids. Ascitic fluid containing anti-Rh(D) antibody was identified and the Rh(D) antibody quantitated by the methods described earlier for culture supernatants.

9. Immmunoglobulin isotyping and quantitation

The isotypes of the immunoglobulin heavy and light chains were determined by an enzyme-linked immunosorbent assay (ELISA) [48]. Microtitre plates coated with an affinity-purified rabbit anti-human Fab, prepared from hyperimmune rabbit antisera [191],

were used to adsorb human hybridoma antibody from the culture supernatants.

To minimize the non-specific adsorption of the test antibodies, the wells were treated with 1 % gelatin (Knox, Thomas J. Lipton Inc., Toronto, Ont.) in phosphate buffered saline (PBS), ph 7.2, for one hour at 37°C. Culture supernatants (50 µl) were then added to duplicate wells containing an equal volume of PBS-Tween 20 (0.5 %). The plates were washed 3 times with PBS-Tween, after one hour at 37°C, and then re-incubated with goat antisera specific for the heavy (IgM or IgG) and light (kappa or lambda) chain isotypes (Tago, Burlingame, CA.). The antibodies against the heavy chains and the light chains were diluted in 100 µl of PBS-Tween at 1:500 and 1:100, respectively.

The plates were then washed and the bound isotype-specific antibody was detected with rabbit anti-goat peroxidase conjugated antibodies (Dako Corp., Santa Barbara, CA.) diluted 1:1000. The plates were washed after incubating for one hour at 37°C and a 100 µl volume of phosphate-citrate buffer (0.1 M Na2HPO4, 0.05M citrate: C6H10O8, ph 5.0) consisting of o-phenylenediamine (0.04 %, Sigma) and H2O2 (0.03 %, Sigma) was added to each well. The plates were then

incubated in the dark for 30 min at room temperature and the reaction stopped by the addition of 25 µl of 4N H2SO4 per well. Optical density readings were determined at 490 nm using a Dynatech MR600 microplate reader (Dynatech Instr., Santa Monica, CA.).

A similar assay was used to quantitate IgM Rh(D) antibodies secreted by the EBV transformed B cells. Immunoglobulin concentrations were determined by interpolation from a standard curve constructed by the titration of whole myeloma IgM (Bio/Can Scientific, Missisauga, Ont.) over the wide concentration range of 10 µg/ml to 10 ng/ml.

The immunoglobulin subclass of IgG Rh(D) antibodies was determined by the automated antiglobulin test using a Technicon autoanalyzer [175]. Typing antisera (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) were diluted 1:100 in saline containing 0.5 % bovine serum albumin. The autoanalyzer was also used to quantitate the IgG Rh(D) antibody concentrations from spent culture supernatants [118].

10. EBNA determination

The presence of the Epstein-Barr nuclear

antigen (EBNA) in hybridoma and EBV-transformed B-cell lines was determined by anti-complement immunofluorescence (ACIF) [152] utilizing the method and reagents supplied in an ACIF test kit (Litton Bionetics, Charleston, S.C.).

11. Chromosome analysis

The chromosomal composition of individual cell lines was determined from exponentially growing cultures by the method of Worton and Duff [204].

12. Anti-Rh(D) specificity and titre

Hybridoma supernatants found to be positive on initial screening with Group O, Rh(D)-positive red cells were assessed for Rh(D) antibody specificity with a variety of Group O red cell samples expressing common Rh phenotypes, by the method initially used to detect the antibody. An indirect anti-globulin (Coombs) test [35] was used to test Rh(D)-specific antibodies against red cells of the D^{u} phenotype. The absence or abnormal expression of the Rh(D) antigen on red cells associated with the expression of rare *Rh* genes was studied also.

For the determination of the titres of monoclonal antibodies directed against the Rh(D) antigen the culture supernatants were 2-fold serially diluted and tested with albumin suspended red cells by a tube method [103] and by saline capillary hemagglutination [25].

13. Biochemical analysis

The surface proteins of Group O, Rh-positive (R1R2) and Rh-negative (rr) red cells were iodinated using lactoperoxidase. Briefly, a 5 % red cell suspension ($2-3x10^9$ cells in PBS: 0.01 M PO4 + 0.14 M NaCl) for each test condition was labelled after the addition of 10 µl lactoperoxidase (166 units/ml in PBS) and 10 µl of H2O2 (10 % in PBS) at the beginning of each 1 min. interval over a 3 min. period at room temperature. Tubes were mixed by vortexing after each addition. The iodination reaction was then terminated by transferring the red cells to tubes containing ice cold PBS/BSA (0.05 %). The labelled red cells were then washed 3 times with 10 ml ice cold PBS/BSA. After the final wash, the red cell pellets were resuspended and then incubated at 37°C for 60 min. in the presence of: 1) culture supernatant from L.2.2 (9

ml), or 2) polyclonal anti-Rh(D) (D. Boake) (1.2 ml), or 3) pooled normal human serum from Group O, Rh-positive males (8 ml). The red cells were washed 3 times in PBS (10 ml) and the ghosts were isolated following the addition of a hypotonic buffer (40 ml of 5 mM NaH2PO4/Na2HPO4, PH 7.4) and centrifugation (13000 x g).

The red cell membranes were then solubilized in a 6 ml volume of 1 % Triton X-PBS for 15 min. at room temperature. The samples were then centrifuged (45000 x g) for 15 min. at 4° C and the immune complexes were adsorbed from the supernatants with 0.5 ml of a 10 % (v/v) suspension of protein-A-sepharose in 1 % Triton X-PBS during a 30 min incubation period at 4° C with continuous mixing. Immunoprecipitates were then analyzed by sodium duodecyl-sulphate polyacrylamide (10 %) gel electrophoresis (SDS-PAGE) using the method of Laemmli [95]. Autoradiographs were developed following the exposure of dried gels to X-ray films (Kodak X-MAT).

C. Results

1. Hybridoma production and clonal stability

EBV transformation followed by polyethylene glycol (PEG)-mediated fusion has yielded stable human-human and human-mouse hybridomas secreting human MoAb reactive with Group O,Rh(D) positive red cells. The production of human LCL secreting anti-Rh(D) is summarized in Table II-1.

Rh(D) antigen-specific B cells were enriched prior to fusion with the LCL by rosetting with papainized Group O, R1R2 red cells. Cells negatively selected (i.e. non-rosette-forming) by this technique did not produce any human-human hybrids secreting monoclonal anti-Rh(D) (Table II-2). In contrast, supernatants from 54 % of the wells containing human-human hybrids generated from rosette-forming cells were positive for anti-Rh(D) reactivity following the initial screening (Table II-2). Three of these clones (20 % of those initially selected) were stabilized for antibody secretion by limiting dilution and have been maintained (at the time of preparation of this manuscript) in culture for more than 11 months.

Table II-1. Rh(D)-positive LCL derived from EBV transformation

Plate density	Wells tested	Percentage of wells positive
10^4	884	7.3
10^5	186	48.0

Table II-2. Stabilization of EBV transformed Rh(D)-positive antibody secreting B cells by fusion with a human (HOA.1) or mouse (NS.1) ouabain-resistant cell line

EBV transformed B-cells*	Fusion partner	Plate density	Wells plated	Wells screened	Hybrids(%) secreting Anti-Rh(D) Initial @	Stable^
Rosette -ve	HOA.1	$5x10^4$	441	115	0	0
Rosette +ve	HOA.1	$2x10^5$	96	28	54.0	20.0
	NS.1	$2x10^5$	96	80	15.0	8.3

* A ratio of 1:1 was used in the fusion of EBV transformed B cells with the indicated fusion partner.

@ The proportion of wells positive for growth that contained hybrids secreting anti-Rh(D).

^ The proportion of hybrids initially positive for Rh(D) antibody secretion, some of which were stabilized by limiting dilution.

Following HOA.1-EBV transformed B-cell hybridization, hybridomas were detected in only 26-29 % of the wells plated. Hybrid colonies were detected in 83.5 % of the wells plated in the single fusion involving NS.1.

The expected number of fusion events which occurred per well, as determined by Poisson distribution analysis, were 1.79 for NS.1 and 0.35 and 0.30 for HOA.1 at plate cell densities of 2.0×10^5 and 5.0×10^4, respectively. The fusion frequency for NS.1 was 172 per 10^7 cells. The fusion frequencies that were observed for HOA.1 at the higher and lower cell densities were 33.6 per 10^7 and 66.6 per 10^7 cells, respectively.

The fusion frequency exhibited by NS.1 was significantly higher than that with HOA.1 ($\chi^2 = 92.4$, $p<0.0005$). The fusion frequency between the two HOA.1 fusions was also statistically significant ($\chi^2 = 10.7$, $p<0.005$).

Although NS.1 was capable of producing hybrids at a fusion frequency significantly higher than that obtained with HOA.1, a lower percentage of NS.1 hybrids (15 %) secreting anti-Rh(D) antibodies was detected upon initial screening. Within the first 3 weeks of their initial selection, the majority of

these heterohybrids lost their antibody producing capacity. Only one of these heterohybrids (L.2.2) has remained stable in culture for more than 11 months following cloning by limiting dilution.

L.2.2 was not able to grow in HAT-selective medium after cloning, but the cloned human-human hybrids were capable of continued growth in culture.

2. Characterization of human-human and human-mouse hybrids

The characterization of individual hybrids is summarized in Table II-3. Culture supernatants from parental and hybrid cell lines were used in an ELISA to determine immunoglobulin heavy and light chain isotypes. The parental human cell line HOA.1, a ouabain-resistant mutant of WI-L2-729-HF2, secretes an IgG(kappa) antibody, whereas the murine myeloma NS.1 is known to produce murine intracytoplasmic kappa light chains.

Representative EBV-transformed B-cell lines from those which were pooled at the time of fusion were shown to secrete either IgG(kappa) or IgM(kappa,lambda). The fact that multiple L-chains are present within the supernatants is indicative of

Table II-3. Characterization of EBV transformed B cells and hybrids

	H-chain Isotype	Subclass & L-chain	Anti-Rh(D) (µg/ml)	EBNA	Metaphase Chromosomes*
EBV Trans:					
39.R	IgG	IgG1(kappa)	0.5	+	46(34-46)
47.R	IgM	kappa & lambda	5.0	+	ND.
64.R	IgM	kappa & lambda	0.6	+	46(45-47)
Fusion Partners:					
HOA.1	IgG	kappa	0	+	46(44-48)
NS.1	-	murine kappa	0	-	96(69-106)
Patient Rh.60:					
serum	IgG & IgM	IgG1	109	ND.	ND.
Clones					
Human-Human					
G.11.1	IgM	kappa & lambda	5.1	+	93(71-93)
G.13.1	IgG	IgG1(kappa)	0.1	+	86(84-90)
G.14.3	IgM	kappa & lambda	1.9	+	87(84-89)
Human-Mouse					
L.2.2	IgG	IgG1(kappa)	6.5	-	92(84-96)

* Values represent the modal number of chromsomes from a minimum of 15 metaphases. Numbers in brackets indicate the range of values found. ND.:Not Determined.

the polyclonal nature of some of the individual cultures.

Rh(D) antibodies secreted by two of the three human stabilized hybridomas have been typed as IgM(lambda). In addition, heavy and light chain isotypes characteristically expressed by the parental line HOA.1 have also been detected. The third human hybridoma, G.13.1 and the heterohybridoma (L.2.2) both produced IgG(kappa), and each was further typed as IgG1. This was not surprising since the patient had only IgG1 and IgM anti-Rh(D) antibodies in her serum prior to the donation of the blood sample.

IgM and IgG anti-Rh(D) concentrations were quantitated from culture supernatants (1.0×10^6 cells/ml) and the patient's serum by ELISA and autoanalyzer techniques, respectively. Three of the hybridomas were found to secrete specific antibodies at levels ranging from 1.96-6.5 µg/ml, with a single clone (G.13.1) secreting nanogram levels per ml (0.1 µg/ml). In contrast, the majority of the EBV-activated clones tested secreted lower amounts of specific antibody (0.15-0.6 µg/ml). Only one EBV-derived clone (47.R) secreted anti-Rh(D) at levels (5.0 µg/ml) comparable to those obtained following human-human hybridization.

EBNA was detected in all LCL and hybridomas resulting from hybridization with HOA.1, indicating the presence of the viral genome. The heterohybridoma L.2.2 showed no reactivity in the ACIF test, similar to that found with NS.1, indicating that the EBV genome was lost as a result of chromosome segregation.

The chromosomal content of the hybridomas and the parental cell lines was determined by karyotype analysis. HOA.1 and EBV transformed B-cells possess a diploid number of chromosomes (46) (Fig. II-1 A & B). The human-human hybridomas, G.11.1, G.13.1 and G.14.3, possessed modal chromosome numbers of 93, 86 and 87, respectively (Fig. II-2 A & B). This indicates that these Rh(D) reactive clones were the result of hybridization and not due to the selection of ouabain-resistant EBV transformed cells.

In contrast to the above observed pseudotetraploidy resulting from the hybridization of two human diploid cells, extensive chromosomal segregation occurred following heterohybridization with NS.1. The modal chromosome number of L.2.2 was 92, only slighty lower than that found in NS.1 (96) (Fig. II-3(A)) prior to fusion. An average of four human (metacentric) chromosomes was observed among the mouse (acrocentric) chromosomes in each L.2.2

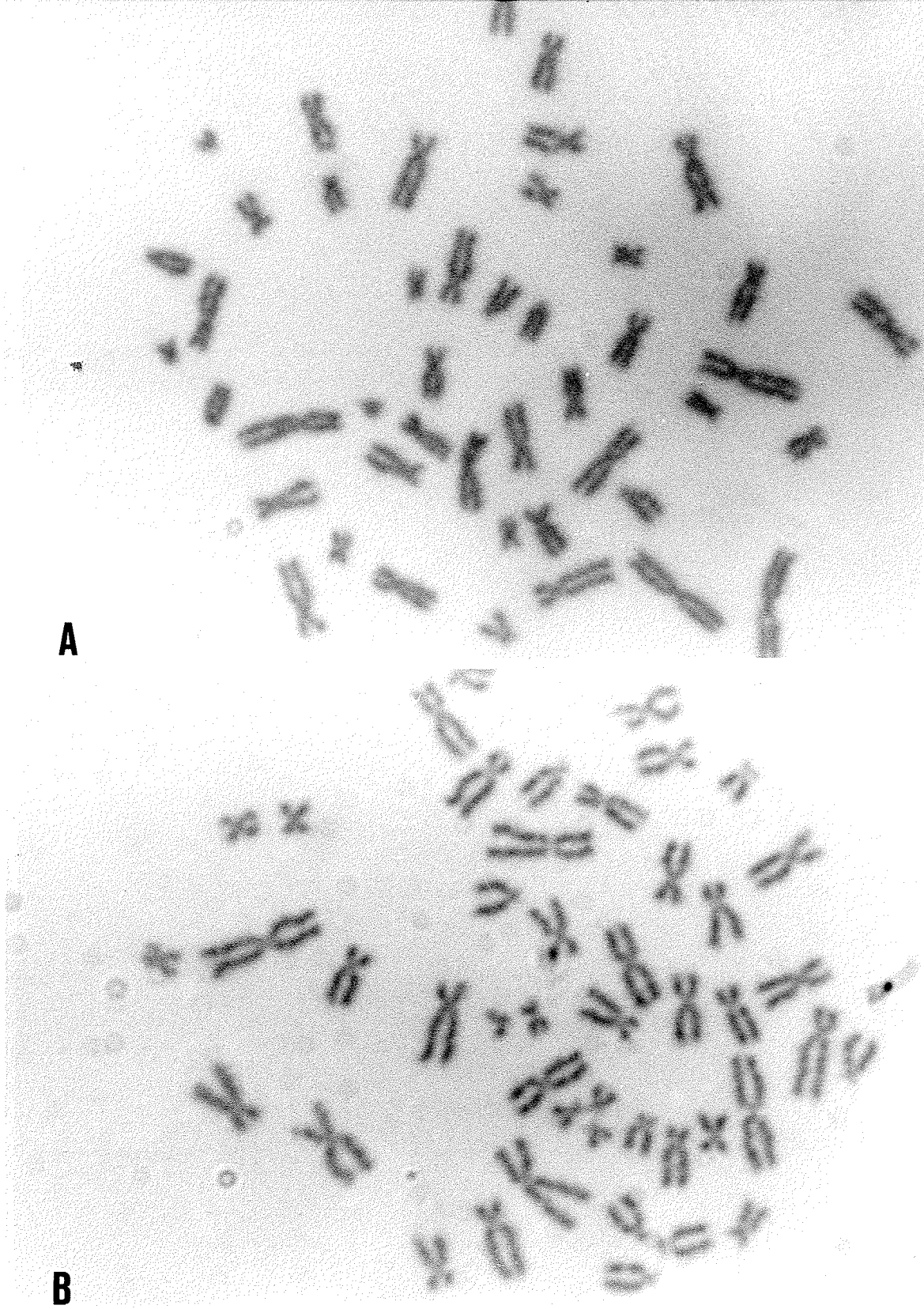


Fig. II-1. Metaphase chromosome spread of (A) HOA.1 and a represenative EBV transformed B cell (B) LCL 39.

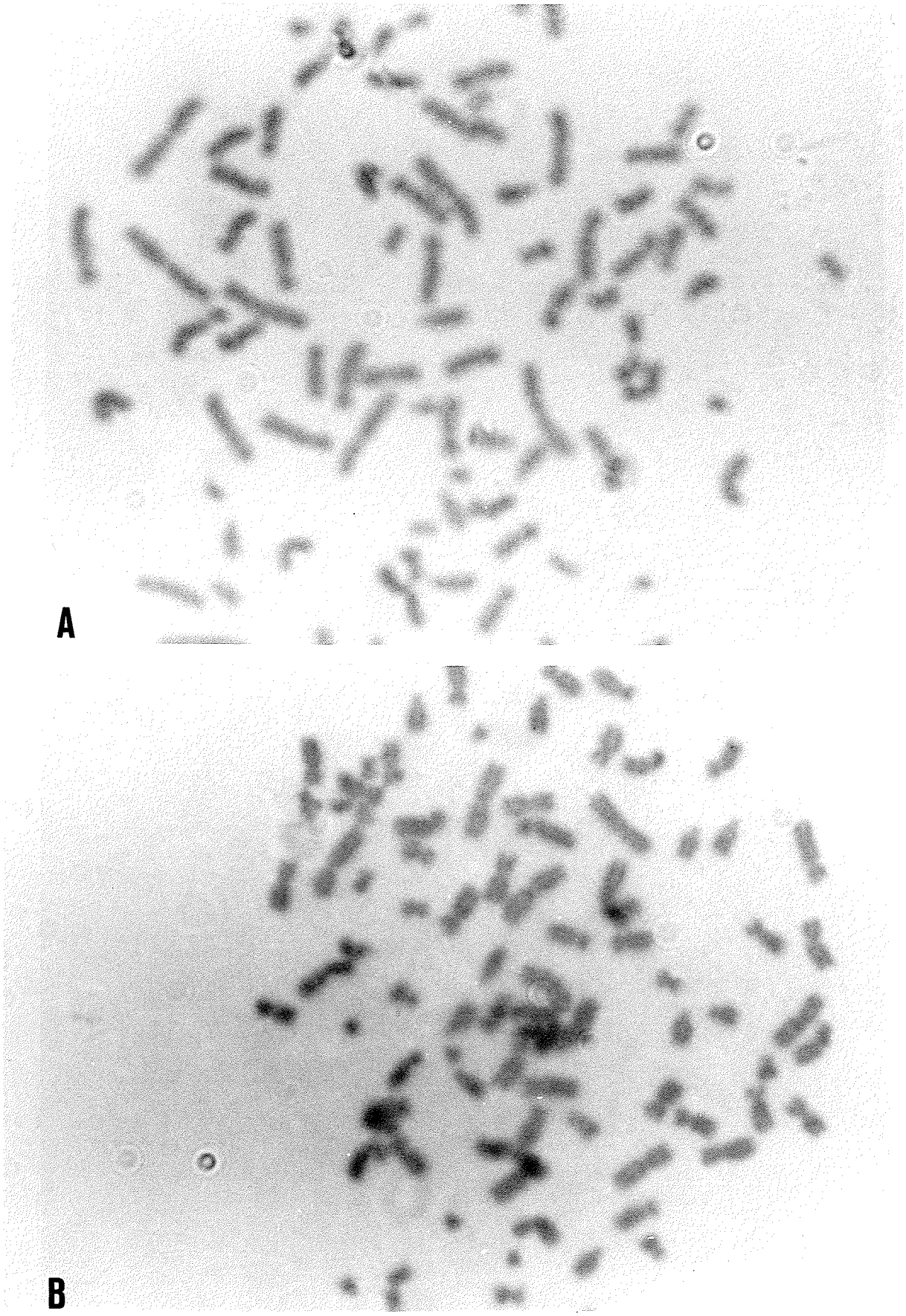


Fig. II-2. Metaphase chromosome spreads of the human-human hybrids (A) G.13.1 and (B) G.14.3 generated from the fusion of LCL with the ouabain-resistant fusion partner HOA1.

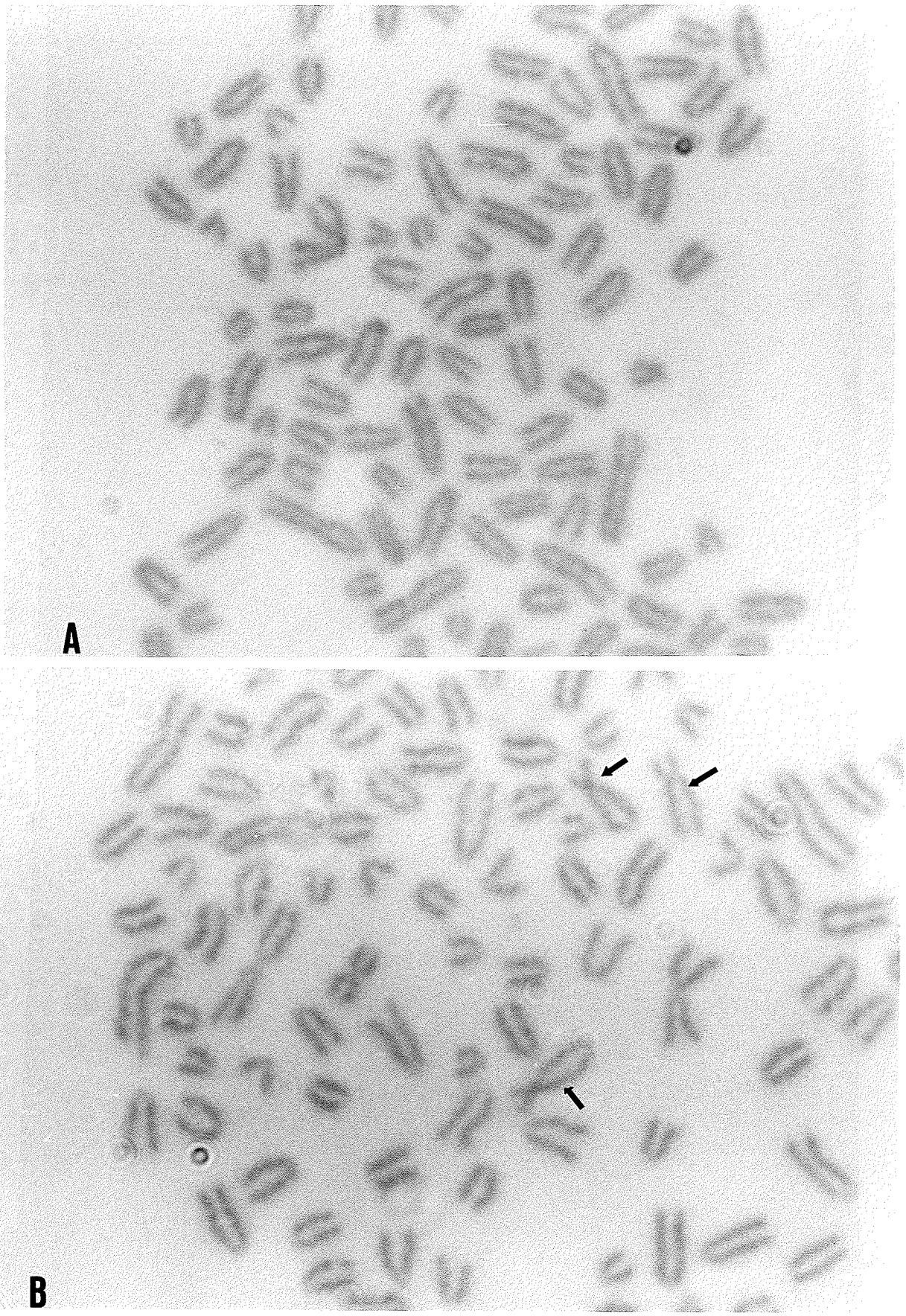


Fig. II-3. Metaphase chromosome spread of the murine fusion partner (A) NS.1 and of the heterohybridoma (B) L.2.2. Examples of the human metacentric chromosomes (arrowed) are visibly mixed with the murine(acrocentric) chromosomes.

metaphase (data not shown) which further confirmed the hybrid nature of the clone (Fig. II-3(B)).

3. Monoclonal Antibody Rh(D) Specificity

Anti-Rh(D) titres of spent supernatants were measured in doubling dilutions by hemagglutination of Group O, Rh-positive red cells. Culture supernatants from the four hybridomas G.11.1, G.13.1, G.14.3 and L.2.2 agglutinated Rh-positive red cells in the presence of albumin at the following titrations 1/128, 1/16, 1/64 and 1/256, respectively. The G.13.1 and L.2.2 lines failed to react in saline alone, which is consistent with the IgG nature of these antibodies.

All hybridoma antibodies, when tested against a panel of red cells expressing a variety of Rh phenotypes, agglutinated all Rh(D)-positive red cells and showed no reactivity against other common Rh antigens (Table II-4).

It was shown previously that G antigen expression is closely associated with a gene or gene complex carrying the *C* and/or *D* genes [4]. Anti-G reactivity can be routinely detected in potent antisera containing anti-C, anti-D or anti-(C+D) from Rh-negative women sensitized during pregnancy [77].

Table II-4. Human monoclonal antibody reactivity against red cells expressing different Rh phenotypes

Rh Phenotype	Panel	Clone G.11.1	G.13.1	G.14.3	L.2.2
R1R2	(*CDe/cDE*)	+	+	+	+
R1R1	(*CDe/CDe*)	+	+	+	+
R2R2	(*cDE/cDE*)	+	+	+	+
r'r	(*Cde/cde*)	–	–	–	–
r"r	(*cdE/cde*)	–	–	–	–
rr	(*cde/cde*)	–	–	–	–
R1wR1	($C^{W}De/CDe$)	+	+	+	+

Probable *Rh* genotypes are shown in brackets.

Table II-5. The reactivity of human monoclonal Rh(D) antibody against a panel of red cells bearing Rh antigens in association with or without Rh(D) antigen expression and red cells categorized as DU

	Saline				Indirect anti-globulin (anti-IgG)			
	G.11.1	G.13.1	G.14.3	L.2.2	G.11.1	G.13.1	G.14.3	L.2.2
Rh Antigens								
$\bar{R}^{N}$ (D+ve)^	+	–	+	–	–	C	–	C
G (D-ve)*	–	–	–	–	–	–	–	–
Du Categories								
DIV(Go(a+))	+	–	+	–	–	C	–	C
DV (Dw+)	–	–	–	–	–	–	–	–
DVI	–	–	–	–	–	–	–	–

^The $\bar{R}^{N}$ antigen is the product of the rare *Rh* gene $\bar{R}^{N}$.
*Red cells of this phenotype carry the rare *Rh* gene r^{G}.
Test results were scored after a 20 min. incubation period. C denotes a complete Coombs reaction (positive in 1 min).

To ensure that antibody specificity was directed against Rh(D) and not the G antigen, MoAb were tested for reactivity against red cells bearing the phenotype encoded by the rare gene complex r^G defined as G-positive, D-negative (Table I-5). None of the MoAbs reacted with the r^G cells directly in saline or by an indirect anti-globulin (Coombs) test. However, all MoAbs exhibited strong reactivity with red cells defined by the $\bar{\bar{R}}^N$ complex, an uncommon gene complex which codes for reduced C and e, but at least normal levels of D antigen [27].

The same serological techniques were used to evaluate MoAb reactivity against a panel of red cells categorized as D^u according to the original classification of Tippett & Sanger [182] (Table I-5). Both IgM and IgG MoAb agglutinated red cells of the Go(a+) phenotype, but showed no reactivity when tested against red cell samples of the D^u categories DV(D^w +)and DVI.

4. Ascites

Ascites tumours developed in only those immunosuppressed mice (Treatment Group 1) which did not received the additional dosage of CY 18hrs after

Table II-6. Ascites production from xenogeneic hybridomas in cyclophoshamide (CY)-treated mice

	Hybridoma cell line injected(i.p.) G.11.1	G.13.1	G.14.3	L.2.2
CY Treatment:				
Group 1^	3/3	1/3	2/3	2/3*
Group 2@	0/3	0/3	0/3	0/3

The number of animals developing ascites/total injected are given in the table.

^Received CY 18 hrs prior to and at the time of injection of the hybridoma cell lines.

@Received an additional dosage of CY 48 hrs after the injection of the hybridoma cell lines.

*Ascites containing anti-Rh(D) activity.

the injection of the xenogeneic hybridomas (Table I-6). All four hybridoma cell lines yielded ascitic fluid, but only the ascites produced by the heterohybrid L.2.2 contained Rh(D)-specific antibody. The concentrations of the human IgG anti-Rh(D) in the ascites from the two mice were 0.2 and 0.3 μg/ml. The volume of ascitic fluid removed from each of the tumour-bearing mice in treatment group 1 averaged less than 1 ml.

5. SDS-PAGE Analysis

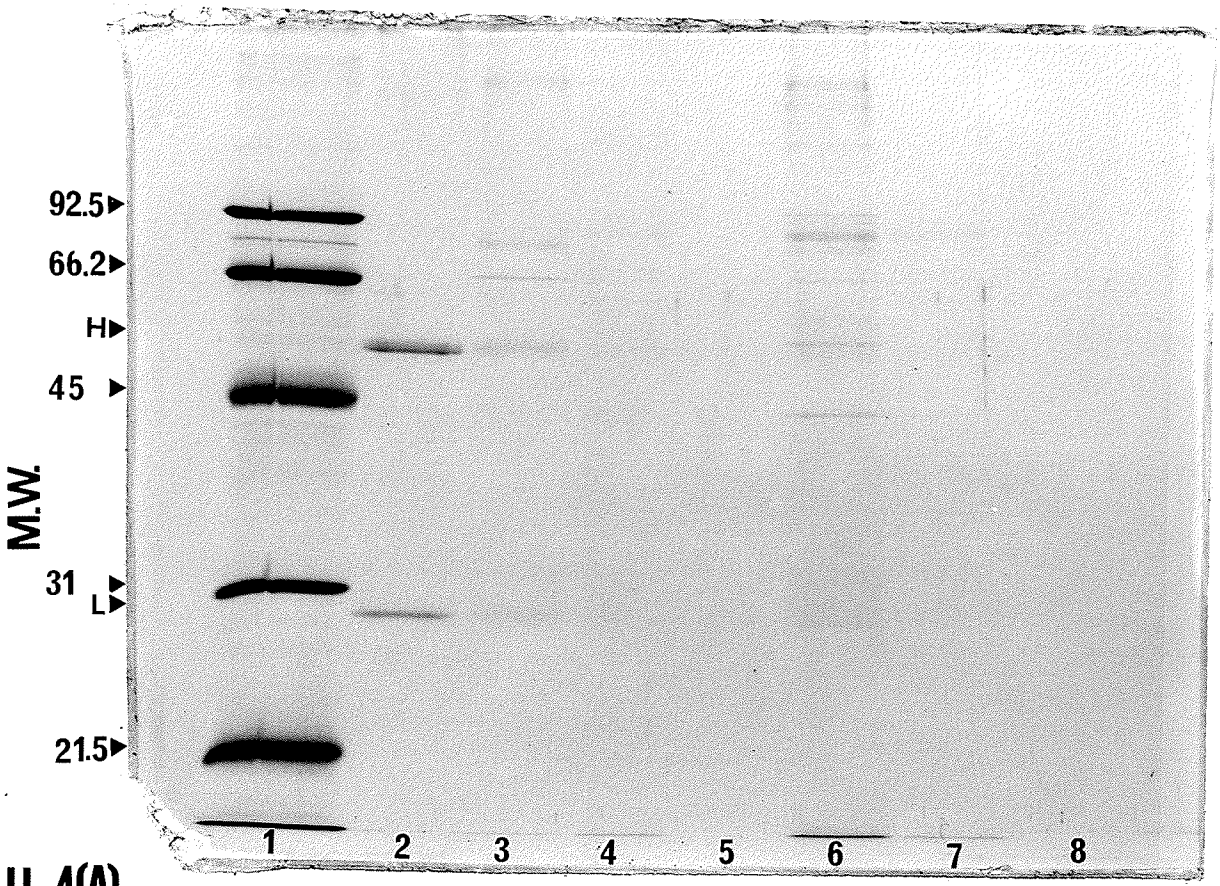
Immunoprecipitates were chromatographed by SDS-PAGE and the Coomassie stain banding patterns are shown in Fig. II-4(A). The autoradiograph of the dried gel (exposed for 72hrs) is shown in Fig. II-4(B). Supernatant from L.2.2, containing human IgG MoAb with anti-Rh(D) activity, precipitated a single protein with an apparent molecular weight of 32-33KD along with high molecular weight aggregates from Rh-positve red cells (lane 2, Fig. II-4(B)). Neither the 32-33KD peptide nor the high molecular weight aggregates were precipitated from Rh-negative red cells with the monoclonal anti-Rh(D) (lane 5, Fig. 4(B)).

The amount of protein isolated by the MoAb was quite small, since the banding pattern was only detectable by autoradiography and not by Coomassie staining (lane 2, Fig. II-4(A)). Only the H- and L-chains of the MoAb were visualized with the Coomassie stain. Similar peptide banding patterns were not observed when Rh(D)-positive or negative polyclonal antiserum was added to either Rh-positive or negative red cell lysates (lanes 3,4,6 & 7, Fig. II-4(B)). The absence of Rh(D) antigen peptide(s) when Rh(D)-positive polyclonal antiserum was used may possibly be due to an insufficient amount of Rh(D)-specific protein-A binding immunoglobulin in the volume of serum added and a much longer film exposure may be required to see the banding pattern. The barely visible staining of the H- and L-chains in lane 3, Fig. II-4(A) supports this hypothesis.

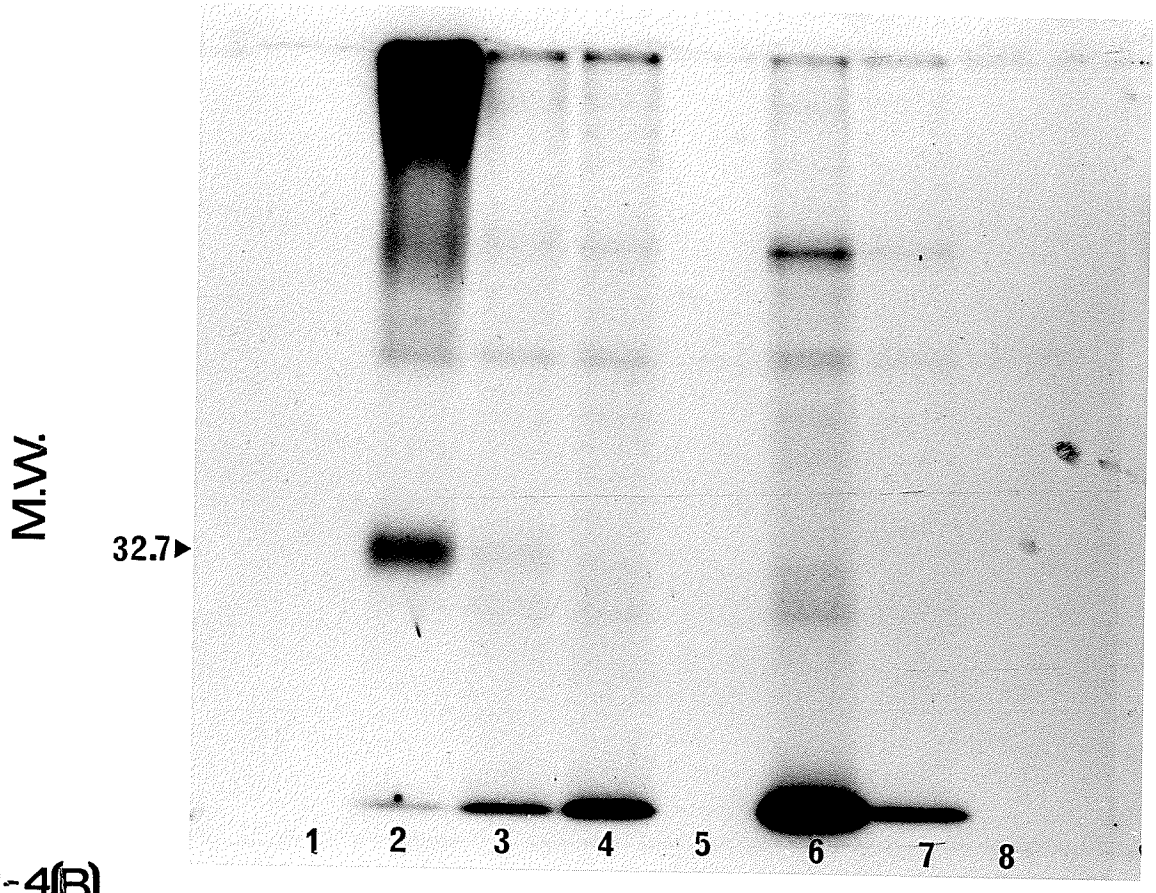
Peptides of approximately 25, 50 & 90KD were also detected in lysates of both Rh-positive and negative red cells (lanes 2,3,4,6 & 7, Fig. II-4(B)). It is unlikely that the 25KD and the 50KD peptide correspond to L- and H-chains of antibody since the iodinated red cells are washed before the additon of the antibodies. More likely, these two peptides, along with the 90KD peptide, are common components of the

Fig. II-4(A). Coomassie staining of SDS-PAGE displaying immune complexes after elution from protein-A sepharose. Immune complexes were formed after ^{125}I-labelled Rh(D)-positive (lanes 2,3 and 4) and Rh(D)-negative (lanes 5,6,7 and 8) red cells were mixed with either a monoclonal or polyclonal antibody preparation and solubilized with Triton X-100. Lane 1, molecular weight protein standards (Bio-Rad, low molecular weight standards); lanes 2 and 5, supernatant from L.2.2 (heavy (H) and light (L) chain bands in lane 2 and 3 are marked with arrows); lanes 3 and 6, polyclonal anti-Rh(D) serum; lanes 4 and 7, pooled negative serum and lane 8, protein-A sepharose only.

Fig. II-4(B). Autoradiograph of the dried gel shown in Fig. II-4(A).



II-4(A)



II-4(B)

red cell surface membrane whether Rh(D) positive or negative and therefore are not part of the Rh(D) antigen. Precipitation of these peptides was not due to non-specific adsorption to the protein-A-sepharose, since no radioactivity was detected from the lysate treated with beads alone (lane 8, Fig. II-4(B)). Therefore, it is probable that the antibodies are reacting with other red cell components through immunoglobulin structures other than their binding sites (ie. constant regions). It is not likely that these are hidden determinants, since antibody was reacted with the red cells prior to solubilization. Therefore, no unbound antibody would be available for reactivity with any cryptic sites, since all free antibody would have been removed during washing.

III. DISCUSSION

The introduction of somatic cell hybridization [84] and EBV transformation [169] made the in vitro production of human MoAb possible. Some monoclonal cell lines secreting antibody against human blood group antigens, such as Rh(D), have been established following EBV transformation of sensitized lymphocytes (37,47,130]. However, despite occasional

successes, long-term stable antibody producing LCLs resulting from EBV transformation alone appear to be the exception. Most clones rapidly lose the capacity for antibody secretion and continued growth in culture [111]. This inherent clonal instability and the relatively low levels of immunoglobulin (<1.0 μg/ml) produced, suggest that EBV transformation alone does not meet the requirements of large scale production of human MoAb.

Similarly, human hybridoma production by conventional fusion protocols has met with limited success due to the lack of acceptable fusion partners and the inability to isolate large numbers of sensitized lymphocytes. EBV transformation has been used to expand antigen-specific PBLs prior to fusion with the human LCL KR.4, the resulting hybrids secreted anti-tetanus toxoid antibody [90].

More recently, the heteromyeloma SHM-D33 and the murine myeloma X63-Ag8.653 have been used as fusion partners to generate Rh(D) antibodies (IgG and IgM) following EBV transformation [15,180].

Earlier experiments in this laboratory involving the direct fusion of Rh(D) sensitized donor PBLs with a variety of known fusion partners failed to generate Rh(D) antigen-specific hybrids (data not shown).

In this study, a two-step procedure was used to reduce the heterogeneity of the transformed B cell population after EBV polyclonal activation, and to further enrich the Rh(D) antigen-specific B cells prior to fusion. The procedure involved the distribution of the PBL into 96-well plates immediately after transformation, followed by a single rosetting with Rh(D)-positive red cells of those LCL secreting Rh(D)-specific antibody. Though it was not done in this study, antibodies from LCL could have been isotyped prior to rosetting so as to generate hybrids secreting only IgG anti-Rh(D), since this immunoglobulin is useful for immunosuppression.

In addition to the versatility of selecting functionally important clones prior to fusion, the procedure also allows for flexibility in handling, since the EBV transformed cells may be frozen in liquid nitrogen and brought back into tissue culture at convenience.

Fusion of the enriched B cell population led to the generation of human-human and human-mouse hybridomas secreting monoclonal Rh(D) antibody. A higher fusion frequency was attained with NS.1 in comparison with HOA.1. However, human-human hybridomas exhibited a greater clonal stability than

those produced by heterohybridization. This is not surprising, since karyotype analysis revealed extensive segregation of human chromosomes from L.2.2, a phenomenon well documented with interspecies hybridization [49,161]. The loss of human chromosomes from the heterohybrid was further evidenced by the clones inability to grow when placed back into HAT-selective medium, indicating the removal of the human X chromosome bearing the HGPRT gene region.

More human-human hybrids were generated with HOA.1 when the cells were plated at a lower cell density after fusion. It is possible that this cell density is optimal for this fusion partner when compared with higher cell numbers per well. It might also be possible that the cells which did not rosette with the RH(D)-positive red cells may represent a population of cells which are at a different but more favourable time in the cell cycle for fusion with HOA.1 than the antibody secreting cells.

Human hybrids possessed modal chromosome numbers more consistent with those of stable fusion products. In a previous study, NS.1 was found to function inefficiently as a fusion partner with human PBLs and the resulting hybridomas showed a rapid loss of antibody production following hybridization [16].

EBNA, a viral nuclear antigen found in close association with the chromosomes of EBV transformed B-cells, was detected in all LCL prior to fusion. Its presence in the resulting stable human-human hybrids indicates that the viral genome has been retained and that the potential for the release of infectious viral particles exists. However, it has been shown that infectious EBV can be removed from culture supernatants during antibody purification [38]. Therefore, the use of these antibodies in immune prophylaxis should be safe after purification. The absence of EBNA in L.2.2 was not unexpected, since it was shown previously that heterohybridization would result in the preferential removal of human chromosomes from these clones, including those associated with the viral genome [152].

All established hybridoma clones secreted MoAb (IgG or IgM) specific for the Rh(D) antigen as shown by standard serological tests. These clones remained stable for antibody production in culture for more than 11 months. Both IgM Rh(D) MoAb strongly agglutinated Rh(D) positive red cells suspended in saline and would, therefore, be of value as blood-typing reagents.

D^u was originally described as a red cell

phenotype with a weakened D antigen expression, which is only detectable by an indirect anti-globulin test. It is now also used to refer to the group of D^u mosaic red cells, which lack portions of the common Rh(D) antigen [61,77]. D^u mosaic individuals whose red cells display variable reactivity with different preparations of anti-Rh(D) are capable of producing on rare occasions anti-Rh(D) antibodies after exposure to normal Rh(D) antigen. To explain this phenomenon, it has been postulated that the D^u mosaic red cells possess rare allelic substitutions.

In this study, all MoAb strongly agglutinated D^u mosaic red cells of the DIV category phenotyped as Go(a+), but failed to agglutinate red cell samples categorized as DV or DVI. These results suggest that the gene region coding for the antigenic determinants of the common Rh(D) antigen, as recognized by the human MoAb is present and expressed in the red cells of the D^u phenotype Go(a+) but not in those samples of D^u categorized as DV or DVI.

The inability of any of the MoAb to agglutinate red cells carrying the genetic DV or DVI phenotype is not of major concern in blood-typing. D^u mosaic phenotypes are rarely, if ever, detected with standard laboratory Rh screening tests and only become known

when previously typed Rh-positive patients have detectable anti-Rh(D) in their serum. When the D^u phenotype is known, it is best to consider mothers and transfusion recipients of these phenotypes as Rh-negative in order to prevent unnecessary exposure to the Rh(D) antigen.

The growth of murine hybridomas in syngeneic recipients has become an established method by which large quantities of murine monoclonal antibody can be produced. Antibody levels contained in ascites tumour fluids are 100-1000 fold higher than those produced in tissue culture.

Unfortunately, hybridomas of xenogeneic origin fail to establish ascites tumours when transplanted into mice because of histoincompatibility. Mice with severe combined immunodeficiency disease (SCID) readily accept xenografts, but these mice are expensive and difficult to maintain [190]. In order to prevent the rejection of xenotransplanted hybridomas, recipients immunosuppressed with hydroxycortisone [199] and athymic mice treated with antilymphocyte serum and irradiation [110] have been with some success. Thus small amounts of ascitic fluid containing a high concentration of a bovine monoclonal antibody was produced following the

transplantation of a bovine-murine heterohybridoma into CY immunosuppressed mice [151].

The cytolytic drug, CY, is thought to have its greatest effect on rapidly proliferating cells, especially B cells. Studies on delayed-type hypersensitivity (DTH) reactions showed that CY exerts its greatest cytotoxic effect on the rapidly dividing T suppressor cell population, if given 1-3 days prior to antigen sensitization. This leads to the release of effector cells from immunoregulatory control, and thereby enhances the immune response. When CY is administered 3-5 days after challenge with the antigen the expanding effector T cell populations are the most sensitive to drug induced-cytolysis, and suppression of effector T cell function is the result [187].

In this study, mice that had received CY treatment (group 1), using a protocol described previously for the generation of bovine MoAb from mouse ascites [151], failed to produce ascites tumours after being injected with either the human-mouse or the human-human hybridomas. In treatment group 2 there was a complete absence of tumour formation in any of the animals. This is probably due to the direct cytolytic effect of the additional CY on the

transplanted hybridomas. These results agree with the limited success of other researchers in growing xenotransplanted hybridomas in partially immunosuppressed mouse strains, a very costly procedure.

In vitro bulk culturing may offer an alternative method for obtaining large amounts of MoAb against the RH(D) antigen. The LCL, WI-L2-729-HF2, from which the parental fusion partner HOA.1 was derived, was adapted for growth in serum-free medium [72]. Therefore, those hybridomas generated by fusion with HOA.1, may also be cultured under serum-free conditions.

Our preliminary results concerning the biochemical characterization of the Rh(D) antigen are in agreement with those performed previously with polyclonal Rh(D)-specific antiserum [58,75,119]. The human monoclonal IgG anti-Rh(D) (secreted by the clone L.2.2) precipitated specifically a 32-33KD peptide from the membrane of Rh(D)-positive red cells only. As well, the MoAb co-precipitated a high molecular weight component. Similar observations were made by Moore and co-workers [119] who showed that the high molecular weight component consisted of an aggregate of peptides which could be separated by electrophoresis under denaturing conditions (8 M urea)

into two peptides of 68KD and 50KD that are associated with Rh(D) antigen expression. Precipitates derived with our MoAb would have to be run under similar conditions to confirm if the high molecular weight component is identical in nature to that identified with the polyclonal antiserum. Monoclonal anti-Rh(D) secreted by EBV transformed B cell lines have been used recently to identify by immunoprecipitation and SDS-PAGE analysis two peptides of 42KD and 32KD associated with Rh(D) antigen expression. These results therefore support the previous observation that the Rh(D) antigen is a complex surface membrane molecule comprised of several peptide subunits one of which is in the order of 32-33KD. The antibodies produced in this study should prove to be of further value in elucidating the biochemical nature of the Rh(D) antigen.

The IgM MoAb produced in this study could also find immediate application in blood typing laboratories. In theory, the saline-agglutinable MoAb represents an infinite source of antibodies by which blood samples could be typed Rh positive or Rh-negative. As well, because of their monospecificity, the MoAb (IgG and IgM) may be useful as reference antibodies for the estimation of the

amount of each serum anti-Rh(D) isotype or for comparing the relative strengths of different Rho antisera.

However, most importantly, these IgG Rh(D) MoAb producing hybridomas represent an unlimited in vitro source of Rh immune globulin, which will prove to be useful for the suppression of Rh alloimmunization and the prevention of Rh erythroblastosis fetalis.

IV. CLAIMS TO ORIGINALITY

1. This thesis describes the generation of the first human (IgM) monoclonal antibodies against the Rh(D) antigen by human-human hybridization with a ouabain-resistant LCL. The only other successful human-human hybridoma secreting (IgM) anti-Rh(D), previous to this study, was a single clone generated after the direct fusion of PBL with WI-L2-729-Hf2.

2. In this study, the use of plating in combination with the rosetting of EBV transformed B cells was used for the enrichment of the RH(D)-specific cells prior to fusion. Both techniques have been used separately, by other investigators, either for the selection of EBV clones secreting specific antibody (plating) or for the enrichment (rosetting) of antigen specific lymphocytes from peripheral blood.

3. The double mutant LCL, HOA.1 (6-thioguannine and ouabain-resistant), developed in this laboratory, has proven to be a suitable fusion partner for the generation of human-human hybridomas.

4. All of the MoAb produced in this study were shown to recognize a determinant common to both the common Rh(D) antigen and the Rh(D) molecule of a D^u red cell antigen (category DIV), which is associated with the low incidence antigen Go^a.

5. Several polypeptides have been isolated from Rh(D)-positive red cells with polyclonal anti-Rh(D) by immunoprecipitation and SDS-PAGE. Biochemical data presented in this text, using the IgG MoAb secreted by the heterohybridoma L.2.2, have found one of these molecules, the 32-33KD protein, to be specifically precipitated from Rh(D)-positive red cells. This result is also in agreement with a previous report that identified a 32KD protein as one of two polypeptides isolated from solubilized Rh(D)-positive red cell membranes using human MoAb against Rh(D) developed by EBV transformation.

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