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MONOCYTE SPECIFIC ANTIGENS IN HUMANS

by

© MOLLY JOHNY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled MONOCYTE SPECIFIC ANTIGENS IN HUMANS submitted by MOLLY JOHNY in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in IMMUNOLOGY in the Department of Medicine.

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ABSTRACT

The technique of antibody dependent cellular cytotoxicity (ADCC) was modified and standardized for the detection of monocyte specific antibodies. Radiolabelled (^{51}Cr as sodium chromate) subpopulations of peripheral blood leukocytes namely T lymphocytes, B lymphocytes and monocytes were used as targets. A comparative study of the ADCC technique with two complement dependent cytotoxicity methods, dye exclusion and fluorochromasia, indicated that ADCC was significantly more sensitive for the detection of monocyte specific antibodies. Hence this method was used in further studies. These are the only data in HLA immunogenetics in which this sensitive assay has been used for antigen detection on pure B lymphocyte and monocyte preparations.

Sera from renal transplant recipients (28), multiparous women (7) and systemic lupus erythematosus patients (5) were studied for the presence of monocyte specific antibodies. The sera were pooled platelet absorbed and tested with highly enriched T, B and monocyte subpopulations. Strong monocyte specific reactivity was seen in 2 transplant and 3 multiparous sera. These also showed B cell specific antibodies. Further absorptions of the sera with pooled B lymphocytes from appropriately

reactive donors or chronic lymphatic leukaemia cells removed B cell specific antibodies. But monocyte specific reactivity persisted in all the sera. Pooled monocyte absorption removed the reactivity completely in one serum tested, further confirming the monocyte specificity of the antibodies.

Studies in two informative families with one serum indicated that the monocyte specificity segregated with single parental HLA haplotype. Further studies are required for accurate definition of the allelic antigen system of monocytes and its linkage to the major histocompatibility complex.

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The author is particularly grateful to those friends and families for providing blood for the study, without which it would have been impossible to carry out this work.

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Abbreviations Used

1.	AMS	-	Anti-macrophage serum
2.	ADCC	-	Antibody dependent cellular cytotoxicity
3.	CDC	-	Complement dependent cytotoxicity
4.	CLL	-	Chronic lymphatic leukaemia
5.	CLLAB	-	CLL absorbed
6.	^{51}Cr	-	^{51}Cr as sodium chromate
7.	DR	-	D-related
8.	E-M	-	Endothelial-monocyte
9.	FDA	-	Fluorescein diacetate
10.	FCS	-	Fetal calf serum
11.	F-H	-	Ficoll hypaque
12.	FITC	-	Fluore cein isothiocyanate
13.	LD	-	Lymphocyte defined
14.	LMC	-	Lymphocyte mediated cytotoxicity
15.	LPS	-	Lipopolysaccharide
16.	M	-	Monocyte
17.	MLC	-	Mixed lymphocyte culture
18.	PBL	-	Peripheral blood leukocytes
19.	PL	-	Platelets
20.	PLT	-	Primed lymphocyte typing
21.	PPLAB	-	Pooled platelet absorbed
22.	SD	-	Serologically defined
23.	SLE	-	Systemic lupus erythematosus
24.	SRBC	-	Sheep red blood cell
25.	TD	-	T cell dependent
26.	TI	-	T independent
27.	UMLC	-	Unidirectional mixed lymphocyte culture

I. Introduction and Literature Review

A. Introduction.

Interest in the concept that the monocyte and its derivative macrophage play an important role in the initiation of the immune response has waxed and waned in the past decade (Nelson,1969). The macrophage first gained recognition in the early 1920's and 1930's as the cell responsible for clearing antigen (Sabin,1923). Its importance faded with the recognition that lymphocytes and plasma cells,and not macrophages produce antibody molecules. Recently the macrophage has become the centre of immunologic attention with the realization that the immune response to T cell dependent antigens (TD) requires the participation of macrophages, T lymphocytes and B lymphocytes (Claman and Mosier,1972; Katz and Benacerraf,1972; Unanue,1972; Pierce and Kapp,1976). In contrast T independent (TI) antigens like lipopolysaccharide (LPS) (Yoshinaga et al,1972; Coutinho and Moller,1975; Jacobs,1975; Lemke et al,1975) and pneumococcal polysaccharide (Rowley et al,1973) do not appear to require the participation of macrophages or only to a lesser extent than TD antigens. (Chused et al,1976; Lee et al,1976). The macrophage is intimately involved in antigen presentation to T and B lymphocytes (Rosenthal

and Shevac, 1973; Unanue, 1972) and in initiation of the response it acts as a focal point for cell interaction (Katz and Benacerraf, 1972; Taussig et al 1974, Erb and Feldman, 1975; Feldman et al, 1975). It is also responsible for production of a number of mediators which either enhance or suppress in vitro responses to antigens.

It is now well understood that specific immune response genes play an important role in regulation of immunocompetent cell interactions (Katz et al, 1973). Studies in mice show that the I region of murine H-2 complex particularly the I-J subregion is important in the control of these interactions (Murphy et al, 1976; Niederhuber, 1978). The Ia antigens of macrophages seem to be part of the signal for T helper cell activation by protein antigens (TD antigens), and recognition of Ia antigens of B cells seems to be involved in T-B cell cooperation (Kappler and Marrack, 1977).

As the macrophage is a principal site of expression of immune response gene function it is important to find out the effects of macrophage (monocyte) associated antibodies in the immune response to various antigens, especially in the case of organ transplantation. Studies

in mice with heterologous antimacrophage serum (AMS) give some evidence for this possibility. Macrophages treated with AMS in vitro have been shown to adhere poorly to glass, to exhibit decreased phagocytic activity and to be lysed in the presence of C' (Unanue, 1968; Hirsch et al,1969; Argyris and Plotkin, 1969; Gallily and Gornostansky, 1972). AMS treatment of mice has been shown to decrease resistance of mice to several viral and bacterial agents and to depress the antibody response to several antigens, (Cayeux et al, 1966; Hirsch et al,1969; Panijel and Cayeux, 1968; Zisman et al,1970). Reports on the effects of AMS on graft rejection have been conflicting and this has been attributed to poor specificity of the AMS used (Pearson and Osbold, 1974). Stinnet et al,(1976) characterized a mouse macrophage specific antigen (MSMA) in NP-40 extracts of ^{125}I radiolabelled mouse peritoneal macrophages using heterologous AMS. This antigen was shown to have a molecular wt. of 83,000 daltons and was present in normal and activated macrophages.

HLA-A,B,C antigens (Kourilsky et al,1971) and Ia like (DR) antigens (Wernet, 1976; Stastny, 1978a; Drew et al,1978) have been demonstrated on human monocytes.

Heterologous antiserum against human macrophages (from a case of pancreatic carcinoma) has been raised in rabbits (Greaves et al,1975). After appropriate absorptions, this serum appeared to mark a species specific antigen of the human monocyte-macrophage series. Recently there were reports suggesting the presence of alloantigens specific for human monocytes. (Stastny, 1977; Moraes and Stastny 1977a,b; Stastny 1978b,c; Cicciarelli et al, 1978).

Stastny,(1977) did parallel testing of monocytes and B cells from 40 normal donors with 7th Histocompatibility Workshop anti-Ia and local sera, using standard B cell cytotoxicity technique. Although most reactions were concordant (M+B+), extra reactions with monocytes (M+B-) were observed in 39 of the 180 7W sera. Extra reactions with B cells were rare. Some 2x2 correlations of the reactions with monocytes generated a number of clusters which were not observed in B cell reactions. They were reproducible and showed correlations with local sera known to contain monocyte antibodies and none of them appeared to correspond to Ia groups detected in the same cells. They also found that monocytes isolated from cord blood reacted in parallel with endothelial cells

from umbilical cord veins of the same donors (Moraes and Stastny, 1977a,b). Anti-endothelial sera absorbed by monocytes lost cytotoxicity for endothelial cells, suggesting that endothelial antigens are also expressed on the surface of monocytes. By antigen re-distribution experiments they showed that vascular endothelial antigens are distinct from HLA antigens. They named them endothelial-monocyte (E-M) antigens. The presence of additional antigenic structures on endothelial cells had been demonstrated earlier in mixed lymphocyte endothelial culture and inhibition studies (Hirschberg et al,1974, 1975). The importance of vascular endothelium in kidney allograft rejection has been reported in animals and in man (Porter et al,1963, 1964; Kincaid-Smith, 1964; Vetto and Burger, 1971; Moraes and Stastny 1975, 1976, 1977a,b; Hirschberg and Thorsby, 1975; Cerilli et al,1977).

Stastny (1978) reported that pre-treatment of monocytes and endothelial cells with anti-human B2-microglobulin made these cells resistant to lysis by E-M antibodies. He claims that E-M antigens are associated with B2-microglobulin on the cell surface unlike Ia antigens. It is also claimed that most but not all monocyte specific reactions segregate in linkage with HLA haplotypes

(Stastny 1977, 1978b,c).

Drew et al,(1978) from Terasaki's laboratory reported preferential lysis of monocytes compared to B lymphocytes by kidney recipient sera and attributed this to monocyte specific antibodies as a result of high degree of immunization by repeated blood transfusions and organ transplantation. Cicciarelli et al,(1978) reported the use of fowl anti-B cell serum to block DR antigens in the method for detecting monocyte specific antibodies. This is possible as the avian gamma globulin does not combine with mammalian complement.

Since these studies indicated the presence of allo-antigens specific for monocytes our aim was to demonstrate these antigens using multiparous and post transplant sera and to evaluate their possible role in organ transplant-ation. Though DR antigens are well characterized by cluster analysis which correspond quite closely and significantly to D locus antigens, most DR antisera showed specificity broader than the D locus specificity (Bodmer et al,1976). Since B lymphocyte enriched populations prepared by standard techniques contained a variable number of monocytes; these extra reactions might have been directed against alloantigens specific for monocytes

contaminating the preparation or due to an antigen moiety shared by many DR antigens: a public specificity.

B. The Major Histocompatibility Complex (MHC).

In all vertebrates studied thus far, the MHC has been described as a genetic system of closely linked genes (Dupont et al,1976). In the man the MHC is known as HLA and this encompasses a short segment of chromosome 6. This genetic region includes many loci controlling cell surface determinants immune response differences, some components of the complement system and perhaps other related functions (W.H.O. Terminology Committee,1975).

1) The HLA-A,B,C antigens.

These loci with multiple alleles code for the HLA-A,B,C antigens detectable by serological methods on all nucleated cells (Wernet,1976). These loci of HLA are located so close together on chromosome 6 that HLA haplotypes are generally transmitted from generation to generation without change. Antigens of HLA-loci show genetic linkage disequilibrium which means that some alleles of two loci can be found together on the same haplotype with a much higher (or lower) frequency than expected from the product of the two individual gene frequencies in the population (McDevitt and Bodmer,1974).

Antigens of the HLA-loci show serological cross reactivity. As in the H-2 system of mouse, this

phenomenon has been explained on the basis of private (narrow) and public (broad) specificities (Ceppellini, 1971; van Rood, 1973). This may be due to the presence of common determinant parts between allelic products of one locus and each allele having individual or unique antigenic determinants (Dupont et al,1976).

The HLA-A,B,C antigens consist of two polypeptide chains (Cresswell et al,1973; Peterson et al,1974; Cullen et al,1974). A larger chain, a glycopeptide with 30,000 molecular wt. and a smaller chain which appears to be identical with B2-microglobulin.

The most commonly used technique for detection of HLA-A,B,C antigens is complement dependent cytotoxicity (Mittal et al,1968).

2) HLA-D locus.

When lymphocytes from two genetically different individuals are co-cultured in vitro a blastogenic response with cell proliferation occurs (Bain et al,1964; Bach and Hirschhorn, 1964). The cell proliferation is measured by the extent of incorporation of radioactive thymidine into DNA on the 6th culture day. The response of each lymphocyte population is studied separately by doing one-way or unidirectional MLC (UMLC) cultures in which stimulating cells are treated with mitomycin C or irradiated. This

inhibits cell proliferation without interferring with their stimulating capacity. Cells that respond to MLC are mainly T cells, being stimulated principally by B cells (Lohrmann et al,1974).

In man HLA identical siblings are usually non-stimulatory in MLC (Amos and Bach 1968). However, most HLA identical unrelated individuals do stimulate each other in MLC (Mempel et al,1973). MLC experiments in families with recombination showed that MLC interaction is caused by disparity at another locus closely linked to the B locus of HLA (Ceppellini and van Rood,1974; Thorsby, 1974). This locus has been designated as HLA-D at the Histocompatibility Conference 1975. HLA-D determinants have a more restricted distribution compared to HLA-A,B,C antigens. They are strongly expressed on B cells and monocytes but not detectable on T cells by conventional methods. HLA-D determinants are present also on spermatozoa (Halim and Festenstein, 1975), epidermal cells and endothelial cells, but are absent from fibroblasts and platelets (Thorsby et al,1977). There is some evidence for a second MLC locus close to the HLA-A locus. Disparity at this locus is associated with a weaker MLC response than disparity at HLA-D.

On the basis of observed recombinants the map order

of the four well defined HLA-loci seems to be HLA-D, HLA-B, HLA-C, and HLA-A. (see Appendix A)

a) Homozygous cell typing.

Following the inital suggestion of Mempel et al (1973) identification of HLA-D determinants by means of stimulating lymphocytes from donors homozygous at HLA-D has become a widely used method. No response or a weak response by responding lymphocytes indicates that they have that HLA-D determinant which is homozygous on the stimulating cells. A strong response indicates HLA-D disparity. By this method and an international cell exchange eleven HLA-D determinants have been identified (Thorsby et al,1977). It is often difficult to obtain enough homozygous cells for each specificity and reproducibility of the typing is still imperfect thus two different typing cells or repeated investigations are usually necessary for assignment of each Dw determinant.

b) Primed lymphocyte typing (PLT).

This method derives from the original observations of Anderson and Hayry,(1973) in the mouse that responding lymphocytes which have been first primed in vitro in a primary MLC, respond with accelerated kinetics and increased magnitude when confronted with the same stimulating cells in a secondary MLC or with other cells

sharing their HLA-D determinants (Thorsby et al, 1977; Bach et al, 1977). This method requires much shorter culturing time for typing (24-48 hours) and priming need not be done by HLA-D homozygous cells. The homozygous cell typing and PLT method supplement each other, but technical improvements are highly desirable.

3) DR antigens (Ia-like).

In allograft survival it is now recognized that matching of the HLA-D antigens by MLC, gives greater success than that obtained from HLA-A,B,C matching. The time required to do HLA-D typing is a handicap and it is not yet possible to use this method in cadaver kidney transplantation. Hence development of less time consuming (serologic) methods became a necessity (van Leeuwen et al, 1973; Kovithavongs et al, 1974b).

In mice the I region of H-2 was shown to contain genes controlling a new set of alloantigens detectable by serological techniques on B cells; the Ia (I region associated) antigens (David et al, 1973; Hauptfeld et al, 1973; Sachs and Cone, 1973; Hammerling et al, 1974). Rapid progress has been made in this field because of the availability of inbred strains of mice. These antigens were shown to be identical or very closely associated with MLC activating determinants (Lozner et al, 1974;

Meo et al, 1975).

Following the observation of Ceppellini et al, (1971) of MLC inhibition by certain HLA antisera van Leeuwen et al, (1973) from van Rood's laboratory, reported on sera containing antibodies possibly directed against MLC determinants. An unrelated stimulator cell was used which was SD identical to the responder in the procedure of MLC inhibition. Inhibition was explained as due to an interaction of antibody with the MLC determinant of the stimulator cell. Fluorescent antibody techniques showed there was reaction only with about 15-25% of lymphocytes, later proved to be B lymphocytes. Following this observation many laboratories confirmed that HLA antisera from multiparous women, or multiply transfused individuals, contain antibodies that recognize alloantigens present on B lymphocytes, which are apparently absent from most T lymphocytes and platelets (Wernet et al 1975; Mann et al, 1975; van Rood, 1976; Thompson et al, 1976). A collaborative effort using B and T lymphocytes from peripheral blood, B cell populations from chronic lymphatic leukaemia (CLL) patients and established B cell lines led to the serological indentification of a B cell specific alloantigen system. Segregation studies in informative families showed

association to HLA-D locus determinants (Bodmer et al, 1978). Because of the analogies between murine Ia system and human B cell alloantigens the latter are often referred to as "Ia-like" antigens. Ia-like antigens in man have been more recently designated as DR (D related) antigens to indicate their relation to the D locus (Nomenclature C mittee for factors of HLA system - 1978). Antisera can now clearly characterize 8 DRw antigens. Definition of DR n igens faced the same problem due to cross reactions as was seen for HLA-A,B,C antigens. This could be due to the presence of "private" and "public" specificities on the antigenic molecule (Thorsby et al,1978). The fact that HLA-DR antigens are clearly separate and different form HLA-A,B,C antigens is shown in re-distribution and capping experiments. B2-microglobulin which is part of HLA-A,B,C molecules i not part of HLA-DR antigens (Solheim et al,1975). HLA-DR antigens appear to be composed of two polypeptide chains with molecular weights of approximately 23,000 and 30,000 (Humphreys et al,1976).

The standard technique for DR typing is CDC by dye exclusion with longer incubation (Bodmer et al,1978). The test is done at 20°C with 1 ul serum and 2,000 ul separated B cells. After 1 hour incubation 5 ul rabbit

complement is added and incubated for 2 hours. Cytotoxicity is judged by the percentage of cells killed in a given well. As B lymphocytes carry HLA-A,B,C antigens on their surface it is essential that sera have no HLA-A,B,C antibodies. This is achieved by absorption with pooled platelets. As target cells, B cell enriched lymphocyte suspensions are often produced from peripheral blood lymphocytes through gradient depletion of T lymphocytes that form rosettes with sheep red cells. Monocytes, lymphoblastoid B cell lines and CLL cells (B) may also be used. Other techniques like immunofluorescence and ADCC are ocassionally used.

HLA-DR antigens are also reported to be present on other cells such as epidermal cells, endothelial cells and spermatozoa (Wernet, 1976). They have been more difficult to detect on T lymphocytes. Inhibition studies using HLA-DR sera suggest that HLA-DR antigens may be present on certain subpopulations of T cells as with Ia antigens in mice (Albrechtsen et al,1977a; Hirschberg and Thorsby, 1977).

HLA-DR antigens show striking association to HLA-D determinants (van Rood et al,1975b; Albrechtsen et al, 1977b). This association has been shown by antisera developed by planned immunization of volunteers (HLA-A,B,C

identical, LD disparate). These antisera specifically inhibit stimulating capacity of cells carrying the HLA-D determinant apparently recognized by the serum (Albrechtsen et al,1977b). Available data indicates that most serologically detectable antigens on B lymphocytes are identical or very closely linked, both genetically and in the cell membrane, to those carrying HLA-D determinants (Thorsby et al,1978).

As in the case of MLC a second locus close to HLA-A locus has been postulated for DR antigens (van Rood et al,1977; Whitsett et al,1977).

The I region of the murine H-2 complex determines genetic control of specific immune responsiveness (Shreffler and David, 1975; Klein,1975). Since HLA-DR determinants appear to be analogous to murine Ia antigens this genetic region may have a similar function in man. The striking association between certain HLA-D determinants and some diseases are in favour of this possibility (Thorsby et al,1978).

4) Significance of HLA-D and HLA-DR determinants in rejection.

Renal graft survival data have shown that HLA-D determinants are major histocompatibility antigens in man, and hence matching for LD may be more important than

conventional HLA-A,B,C matching (Sachs et al,1977; Uehling et al,1977; Solheim et al,1977). This has also been shown in clinical bone marrow transplantation (Copenhagen study group of immunodeficiencies, 1973; Goldman,1977). Based on murine data it has been suggested that HLA-D determinants play the greatest role in triggering off an immune response towards allogenic tissue possibly through activation of helper T cells (Thorsby 1974; Bach et al,1976).

Studies in mice have suggested that antibodies directed against Ia antigens may cause enhancement of H-2 disparate grafts (McKenzie and Henning,1977; Staines et al,1977). The explanation given is that anti-Ia antibodies might inhibit sensitization of T helper cells (Staines et al,1977). In man a positive CDC cross match only with donor B cells (and not T cells) apparently is not associated with hyperacute rejection of the kidney, in contrast to a positive CDC cross match involving HLA-A, B,C antibodies. A clear enhancing effect of preformed B cell specific antibodies on graft survival has not yet been demonstrated (Ettenger et al,1977; Lobo et,al,1977).

C. Human lymphocyte subpopulations.

The study of cellular interactions involved in generation of immune responses has been greatly facilitated by recent developments in cell separation based on surface markers for lymphocyte subpopulations (Chess and Schlossman, 1977).

Important cell surface markers include intrinsically bound surface Ig (Froland and Natvig, 1970; Grey et al, 1971; Siegal et al,1971), the receptor for sheep erythrocytes (E receptor) (Brain et al,1970, Coombs et al 1970; Lay et al,1971; Jondal et al,1972), receptors for C' components (Bianco et al,1970) and receptors for Fc fragment of antibody molecules (Dickler and Kunkel 1972; Basten et al,1972). Many studies clearly indicated that the subset of human lymphocytes forming rosettes with sheep erythrocytes were T cells (Froland 1972; Jondal et al 1972; Wybran et al,1972). In contrast, human B cells like their counterparts in rodents have intrinsically bound surface Ig and contained the receptor for C' components C3b and C3d (Moller,1973).

With respect to the Fc receptor, initial studies indicated that the predominant cells bearing Fc receptors for IgG were B cells and monocytes. Recent studies now indicate that a significant population of T cells have

receptors for Fc fragment of IgG and an even larger percentage have receptors for the Fc fragment of IgM (Moretta et al 1976, 1977). In addition a third population of cells exists in peripheral blood called "null" cells (Jondal et al 1973; Greenberg et al 1973), which is E rosette negative, surface Ig negative but Fc receptor positive. These cells have been shown to be the predominant lymphocytes effecting ADCC (Perlmann et al 1975, MacDermott et al 1975, Brier et al 1975). The Fc receptor, although perhaps useful in the discrimination of subclasses of T or B cells, is not particularly useful for the initial characterization of human lymphocytes into T or B subpopulations.

These cell surface markers have permitted development of methods for isolation of distinct receptor bearing lymphocytes. Despite the usefulness of conventional cell surface markers separation methods are still relatively crude and a number of technical factors account for variation in results of quantitation. Table 1 summarises present state of knowledge of membrane structures on human PBL.

Membrane Structure / PBL Sub-populations / Technique	HLA-A B,C	HLA DRw	HLA Dw	T cell receptor	*Fc Receptor			C_3 Receptor	Ig bearing			Monocyte*** specific Ag
	CDC-PBL	CDC-B ADCC-B	HTC in MLC	SRBC Rosetting	EA rosette			EAC rosetting	Ig	** allo-type	** idio-type	ADCC-M
					Ig	γ	μ					
T cells	+	-	-	++ high & low affinity	+ ?sub set	+ sub set	+ sub set	-	-	-	-	-
K cells (ADCC vs lymphoid or monocyte target	+	-	-	+ low affinity only	+	+	?	+	-	-	-	-
(B+M fraction)	+	+	+	-	+	+ sub set	+ ?sub set	+	+	+	+	+
B (pure)	+	+	+	-	+	+ sub set	+ ?sub set	+	+	+	+	-
Monocyte	+	+	+	-	+	+	?	+	-	-	-	+
Platelets	+	-	-	-	+	?	?	-	-	-	-	-
CLL	+	+	+	-	+	+	?	+	+	+?	+?	-

Table 1 Known membrane structures on PBL[a]

*Recently, Fc receptors have been characterized for IgG or IgM using OxRBC coated with IgG or IgM of Rabbit α-OXRBC

**Little data on these systems in human cells

***The technique developed to obtain data in this M.Sc. thesis

[a]Wernet, 1976; Moraes and Stastny, 1977; Moretta et al 1975,1976,1977; Ferrarini et al, 1975; West et al 1978; Bianco et al, 1970; Vitetta et al, 1975; Winchester and Ross, 1976; Dickler, 1976

D. Monocytes.

Monocytes are derived from bone marrow precursors. They leave the circulation randomly and enter various tissues to become macrophages (Nelson,1969). Transformation occurs without mitotic division (Bennet and Cohn,1966), nor do they undergo replication in blood under normal conditions. Replication occurs, however, in inflammed subcutaneous tissues (Ryan and Spector,1970).

1) Identification of monocytes.

Several techniques have been described for the quantitation of monocytes in mononuclear blood leukocyte preparations. Most methods use cytochemical staining based on the activity of such lysosomal enzymes as peroxidases, esterases and lysozyme (Briggs et al,1966; Yam et al,1971). Other methods are based on phagocytosis of particles which can be visualized by light or electron microscopy (Bennet and Cohn, 1966; Zucker-Franklin D., 1974). The most widely used staining methods are Acridine orange (Euchrysine) staining (Young and Smith, 1963; Allison, 1976) and non-specific esterase staining (Koski et al,1976). In living cells acridine orange is concentrated in lysosomes which fluoresce orange red because of molecular stacking of the dye molecules bound

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1) Identification of monocytes.

Several techniques have been described for the quantitation of monocytes in mononuclear blood leukocyte preparations. Most methods use cytochemical staining based on the activity of such lysosomal enzymes as peroxidases, esterases and lysozyme (Briggs et al, 1966; Yam et al, 1971). Other methods are based on phagocytosis of particles which can be visualized by light or electron microscopy (Bennet and Cohn, 1966; Zucker-Franklin D., 1974). The most widely used staining methods are Acridine orange (Euchrysine) staining (Young and Smith, 1963; Allison, 1976) and non-specific esterase staining (Koski et al, 1976). In living cells acridine orange is concentrated in lysosomes which fluoresce orange red because of molecular stacking of the dye molecules bound

to a specific glycolipid constituent of lysosomes. Lower concentrations of dye molecules accumulate in the nucleus which fluoresces green. The most intense lysosomal fluorescence is observed in mononuclear phagocytes.

2) Isolation of peripheral blood monocytes.

The method most commonly used for separation of monocytes takes advantage of the adhereing properties of monocytes (Rabinowitz,1964; Bennet and Cohn,1966; Broderson and Burns,1973; Ackerman and Douglas,1978), which enable them to stick to glass or plastic surfaces. Lymphocytes are not adherent. Adherent cells are usually recovered by gentle scraping with a rubber policeman (Broderson and Burns,1973). Koller et al,(1973) showed that this method could yield monocyte preparations of high purity provided that a high concentration of autologous or pooled AB sera were included in the suspending medium. In the absence of serum there was a great deal of contamination by B and T lymphocytes. Recently Sanderson et al,(1976, 1977) described a counterflow centrifugation method. In a Beckman J21B centrifuge equipped with the Elutriator rotor and a specially designed separation chamber cells having different physical properties (size, shape and density) equilibriate at different radii and are removed

sequentially by increasing the pump rate. Though this method has been reported to give highly purified preparations of monocytes counterflow centrifugation is not universally available.

E. Antibody dependent cellular cytotoxicity (ADCC).

Cytotoxic activity against antibody coated target cells by non-immune lymphocytes was first described by Moller, (1965) and has since been widely studied elsewhere (Reviewed by McLennan, 1972; Perlmann et al 1972; Cerottini and Brunner, 1974). This phenomenon has been referred to by different names (Wunderlich et al, 1971; Trinchieri et al, 1973; Kovithavongs and Dossetor, 1973), the most widely accepted one being antibody dependent cellular cytotoxicity (ADCC) introduced by Fahey and associates (Zighelboim et al, 1973). This form of cytotoxicity is independent of complement (Perlmann and Holm, 1969; McLennan and Loewi, 1968) and requires predominantly IgG antibodies with intact Fc region (Perlmann, 1970; McLennan et al, 1970), though IgM antibody has recently been reported to mediate ADCC (Fuson et al, 1978). The effector cell involved in ADCC has been characterized as having Fc receptors. Interaction of the Fc portion of IgG with the Fc receptor on the effector cell triggers lysis of the target cell (Fakri and Hobbs, 1972; Moller and Svehag, 1972). $F(ab)_2$ fragments when used to sensitize the target do not mediate ADCC (Larsson and Perlmann, 1972; Moller and Svehag, 1972; Gelfand et al, 1972; Hallberg, 1974,

McLennan et al,1974; Michaelsen et al,1975).

The target cell employed in ADCC is of critical importance in determining which effector cell(s) is required (Kovithavongs et al,1975; Nelson et al,1976). Cells of the monocyte macrophage system and polymorpho nuclear leukocytes are capable of lysing antibody coated erythroid targets, whereas non-erythroid targets are resistent to their cytotoxic potential. Non-erythroid targets are killed in ADCC by cells of the lymphocyte series (Kovithavongs et al,1975; Nelson et al, 1976). This subpopulation of lymphocytes has now been further characterized and the majority of evidence suggests that they lack the markers for B or T lymphocytes. Hence an operational term, K cell, has been widely accepted to indicate the effector cell. Other less popular terms such as UL cells (undefined lymphocyte like cells) and "null" cells have also been used (Greenberg et al,1973; Wisloff and Froland, 1973; Brier et al,1975; Nelsen et al 1976; Ramshaw and Parish, 1976; Dickler,1976).

K cells in human peripheral blood are non-phagocytic and non-adherent and do not have surface Ig. K cells do not form stable E rosettes with sheep erythrocytes (Spiegelberg et al,1976). More recently West et al,(1978) reported that majority of human K cells formed low

affinity E rosettes indicating a thymic origin. Studies in mice and in man using CDC and ADCC methods have further shown that K cells do not possess Ia antigens on their surface (Nelsen et al, 1977a, 1977b; Kovithavongs et al, 1978b).

ADCC is frequently performed by incubating effector cells from non-immune donors with antiserum directed against appropriate target cells. It has several advantages over the more commonly used cytotoxic assays such as complement dependent cytotoxicity (CDC). Radioactive sodium chromate, following diffusion through the cell membrane is retained in the cytoplasm for a relatively long period of time. Thus chromium release from a labelled target cell into the supernatant fluid does not occur unless the cell membrane is sufficiently damaged to allow the efflux of intracellular molecules. Release of ^{51}Cr following membranolysis from labelled target cells can be quantitated accurately in a radioimmuno assay system in ADCC. It has been clearly shown that the released material is not subsequently reincorporated by undamaged targets (Brunner et al, 1976). In contrast dye exclusion or fluorescein staining of viable cells, when used as end point indicator in CDC, is subject to errors of visual assessment. Being

complement independent, problems relating to potency of different batches of C' are also avoided in ADCC test.

Furthermore, ADCC is significantly more sensitive than CDC in that it requires only a very small amount of antibody. (Even as low as $1:10^7$ dilution of antiserum against erythroid targets, Moller and Svehag 1972 Cerottini and Brunner, 1974). This extraordinary sensitivity of the ADCC system has been especially useful in detection of antibodies against cell surface antigens.

Monocytes are known to have HLA-A,B,C and DR antigens. Hence identification of monocyte specific antibodies requires the removal of HLA-A,B,C and DR antibodies from test sera. These absorptions are unavoidably followed by significant dilution of test serum. Consequently monocyte specific antibodies have been only infrequently detected using less sensitive methods such as CDC (Cicciarelli et al, 1978). For these reasons we have elected to use ADCC for detection and quantitation of monocyte specific antibodies in test sera of transplant patients and multiparous women.

F. Significance of "broadly reactive" sera to peripheral blood leukocytes.

In developing serologic methods for identification of surface antigens on peripheral blood leukocytes emphasis has rightly been placed on more "narrowly" reacting sera. The terms "broad" and "narrow" refer to serum reactivity to varying proportions of a randomly selected panel of different individuals' cells; "broad" would be descriptive of a serum reacting with, say, 33% to 100% of the panel cells, "narrow" could be a serum with as low a reactivity as 1% of a panel provided that this reaction was strong and reproducible. With narrowly reactive lymphocytotoxic sera against PBL, and more recently B cells, 65 or more HLA-A,B,C and DRw antigens have been identified (see Appendix B)

"Broadly" reactive sera have usually been disregarded as being too polyspecific except when against a specificity such as HLA-A2 which is present on 50% of Caucasian cells. This is surprising in view of 1) van Rood's original demonstration of the broadly represented 4a/4b system (now known as Bw4/Bw6) using leuko-agglutinating antisera (van Rood, 1962) and 2) observations that broadly reactive sera may occasionally occur in multiparous women sensitized by no more than two paternal

haplotypes. The subject is complicated by the phenomenon of cross reactivity, often referred to as public specificity, probably due to presence of common determinant parts between allelic products of one locus.

It is therefore reasonable to look upon sera with broad reactivity with the frame-work shown in Fig. 1 and it was in such sera that a search was instituted for monocyte specific antibodies as reported in subsequent sections of this thesis.

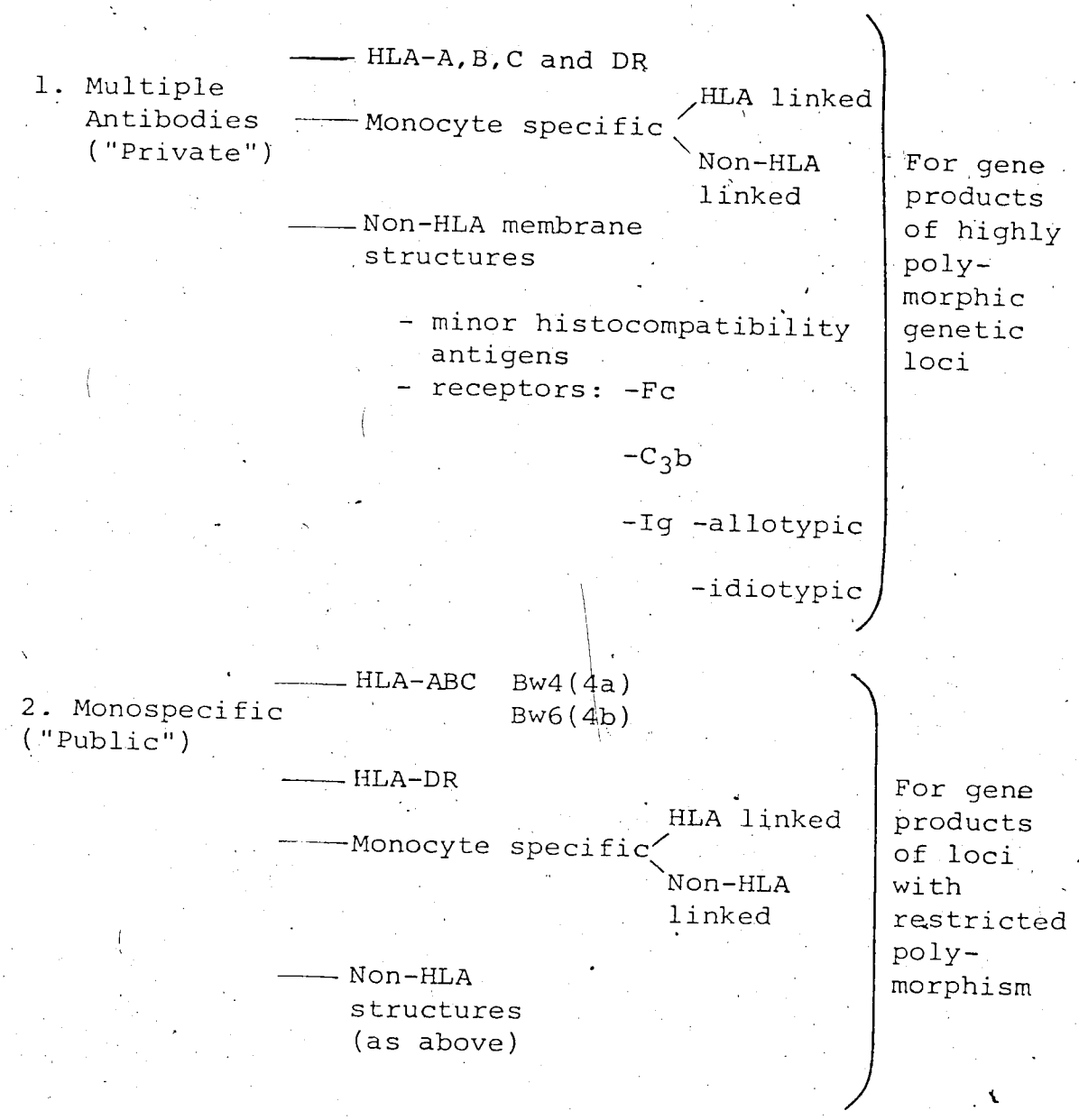

Figure 1 Classification of antibodies in broadly reactive sera

II. Materials and Methods

Cytotoxicity studies were performed using cell targets viz T and B lymphocytes and monocytes from peripheral blood of panel and family members. The test sera included those from transplant patients with rejection, multiparous women and systemic lupus erythematosus (SLE) patients. Both C' dependent (CDC, FDA) and independent (ADCC) methods were employed. Absorption studies were performed with pooled platelets to remove HLA-A,B,C; CLL cells and pooled B lymphocytes, D related (DR); and pooled monocytes, monocyte antibodies.

Segregation studies of monocyte specific reactivity were performed in selected families.

A. Preparation of peripheral blood leukocytes (PBL).

Mononuclear cells from peripheral blood were prepared by the method of Boyum (1968) with minor modifications, as follows:

Seven ml. tubes of heparinized venous blood were centrifuged for eight min. at 1500 rpm (500xg). About 1 ml. of buffy coat was collected from each tube and mixed with equal volumes of medium RPMI 1640. About 5 ml. of this buffy coat suspension were layered over 5 ml. of Ficoll-Hypaque (F-H) (Sp. Gr. 1.077) in 17 x 100 mm clear

Falcon plastic tubes and centrifuged at 1500 rpm (500xg) for 20 min. Mononuclear cells at the interface were collected with a Pasteur pipette and transferred into another plastic tube, washed three times and re-suspended in RPMI. This method regularly gave more that 95% mononuclear cells of which about 15% were monocytes. Viability was estimated at over 98% by dye exclusion.

B. Separation of different subpopulations of PBL.

PBL were prepared from about 40 ml. of heparinized blood as described above, subjected to rosetting with sheep red blood cells (SRBC) followed by centrifugation on F-H to separate the rosetted T cells. Non-rosetted cells were layered in a plastic petri dish to allow monocytes to adhere to the plastic surface. The non-adherent cells formed the B-enriched population. Adherent cells (monocytes) were removed by scraping with a rubber policeman.

1) Preparation of T lymphocytes by sheep erythrocyte (SRBC) rosetting technique.

The method of Ettenger et al,(1976) was used with modifications. The major requirements included preparation of neuraminidase treated SRBC, SRBC absorbed foetal calf serum (FCS-Abs) and hypotonic EDTA-saline.

a) Preparation of neuraminidase treated SRBC.

Only SRBC collected in Alsever's solution and stored less than four weeks in the cold were used. Of these, 0.2 ml. packed SRBC were washed three times with saline and re-suspended in 10 ml. of RPMl-1640 with 10% foetal calf serum (FCS). To this 25 units of neuraminidase (from Cl.perfringens) were added and the mixture was incubated at 37°C for ½ hour. Neuraminidase treatment enhances binding of SRBC to T lymphocytes. These neuraminidase treated SRBCs were washed three times with RPMl-1640 and re-suspended in RPMl-1640 at a concentration of 0.5%.

b) Preparation of SRBC absorbed FCS (FCS-Abs).

FCS was absorbed with SRBC for use in rosetting to remove naturally occurring anti-SRBC antibodies.

For this purpose FCS was first decomplemented at 56°C for ½ hour and mixed with SRBC at a ratio of 4:1 (20% suspension). The mixture was incubated at room temperature for ½ hour followed by ½ hour in the cold. The tubes were spun at 2,000 rpm (800xg) for 20 min. The serum was separated and stored at -70°C for future use.

c) Hypotonic EDTA-saline (Shocking solution)

Shocking solution for lysing SRBC adhering to the T cells contained 0.25% EDTA in 0.26% Sodium Chloride. Neutralizing solution (hypertonic) was made of 9% NaCl, 2.5%

EDTA and 5% Na_2HPO_4.

d) E-rosetting technique and separation of rosetted cells from non-rosetted cells.

PBL prepared as described above were adjusted to concentrations of 3 x 10^6/ml. and mixed with an equal volume of 0.5% neuraminidase treated SRBC. To this, SRBC absorbed foetal calf serum was added to a final concentration of 10%. The tubes were spun at 1000 rpm for 10 min. and left at 4°C for 1 hour. After gentle re-suspension, taking care not to dissociate the rosettes, a small amount was placed on a hemocytometer to determine the percentage of rosetted cells. The remaining cell suspension was immediately layered over cold F-H and centrifuged at 2000 rpm for 20 min. at 4°C. Rosetted T lymphocytes and free SRBC settled to the bottom of the tube while non-rosetted cells containing B cells and monocytes remained at the interphase, the latter were transferred into another test tube with a Pasteur pipette. T cells in the pellet were recovered by hypotonic lysis of the SRBC. For this purpose 10 ml. of shocking solution was added to the pellet, mixed, and left for 1 min. One ml. of neutralizing solution was then added and mixed, followed by centrifugation at 1500 rpm for 7 min. This T-enriched population was washed twice and re-suspended in 10 ml. of RPMI containing 10 mM

Hepes buffer and 10% FCS. Purity of the preparation was checked by re-rosetting with SRBC.

2. Preparation of B lymphocytes.

Non-rosetted cells from PBL were allowed to adhere on plastic petri dishes according to the method of Moraes and Stastny, (1977a). About 25% of PBL remained non-rosetted. Approximately 7-14 x 10^6 non-rosetted cells were obtained from 40 ml. of blood. This was washed once with RPMl-1640 and re-suspended in 20 ml. of RPMl-1640 containing 20% autologous plasma collected from the target cell donor. (Autologous plasma was inactivated at 56°C for ½ hour and centrifuged at 2000 rpm for 30 min.) The cell suspension was layered in a 10 cm. petri dish and incubated at 37°C in a humid atmosphere with 5% CO_2 for about 2 hours. Non-adherent cells were removed, washed once by spinning at 1000 rpm (200xg) for 7 min. and re-suspended in 10 ml. of RPMl-1640 with 10 mM Hepes buffer and 10% FCS. These were the B-enriched cell population. Purity was checked by demonstration of surface Ig (S.Ig) by immunofluorescence.

3. Preparation of monocytes.

The plates with adherent cells were thoroughly washed 5-6 times with 6-8 ml. of RPMl to ensure removal of non-adherent cells. Completeness of removal was checked by

inverted phase microscope examination of the petri dish. Ten ml. RPMl with 0.2% EDTA was then added and cells were released by gentle scraping with a sterile rubber policeman. The suspension was collected in a plastic tube washed once with RPMl (1000 rpm for 7 min.) and re-suspended in RPMl with 10 mM Hepes buffer and 10% FCS. Purity of the monocyte suspension was checked by Acridine orange (Euchrysine) staining.

C. Tests for checking purity of subpopulations of PBL.

1) Re-rosetting with SRBC.

Cells suspended in RPMl-1640 0.25 ml. ($3x10^6$/ml), were mixed with 0.25 ml. of 0.5% neuraminidase treated SRBC. To this 0.5 ml. of FCS-Abs was added and the mixture centrifuged at 1000 rpm for 5 min. and left at 4^oC for 2 hours. After gentle re-suspension the percentage of rosetting cells was checked.

2) Immunofluorescence for demonstration of surface Ig (S.Ig) on B lymphocytes.

The method of Lobo et al,(1975) was used.

To avoid non-specific fluorescence due to IgG that remain bound to Fc receptors cells were preincubated at 37^oC in serum free RPMl-1640 and washed with warm RPMl.

The technique was as follows: 2-5 x 10^6 B lymphocytes were incubated in 5 ml. RPMl at 37°C for ½ hour in the presence of 5% CO_2. Following incubation about 7 ml. of warm RPMl (37°) was added to this and the tubes were spun at 1500 rpm for 7 min. The supernatant was removed and 0.1 ml. of 1/10 dilution FITC conjugated polyvalent anti-human Ig was added. After mixing tubes were incubated at 4°C for ½ hour, washed twice with cold RPMl-1640 before re-suspension in RPMl-1640 at 5 x 10^6/ml. concentration. Membrane immunofluorescence was examined using a Zeiss fluorescence microscope with an excitor filter BP450-490, achromatic beam splitter FT510, a barrier filter LP520 and a high pressure mercury illuminator HB050. (see Appendix E)

3) Acridine orange staining.

Acridine orange 1/100,000 (0.1 mg./10 ml.) solution was prepared in the Hanks balanced salt solution. A pipette was wet with the solution and a small amount of cell suspension was drawn into the pipette. Monocytes were recognized by the presence of bright orange staining granules in the cytoplasm and pale green coloured characteristic large nucleus under a fluorescence microscope. (see Appendix F)

D. C' dependent cytotoxicity by dye exclusion for B lymphocytes (CDC-B).

The standard method adopted at the Seventh Histocompatibility Workshop (Bodmer et al, 1978) was followed.

Disposable microdroplet trays (Cooke Laboratory Products, Div. Dynatech Laboratories Inc., Alexandria, Virginia) with 72 wells (12x6), the bottom of each being optically clear, inner surface being wettable and exactly the size of a 10x objective field was used.

To 1 ul of serum, approximately 2000 B cells were added and incubated at room temperature (20°C) for 1 hour. Following incubation, rabbit complement (5 ul) was added and incubated at room temperature for 2 hours. Aqueous eosin (3 ul of 5%) was added to each well, using a multiple needle dispenser. After 3-5 min., neutralized formaldehyde (8 ul) was added, using a multiple needle dispenser, and 50 x 75 mm. coverslip was ov id. Reactions were read with an inverted phase contrast microscope using 10x objective on the same day or on the following day after leaving the trays in the refrigerator overnight.

E. Fluorochromasia method for monocytes (FDA).

For complement dependent cytotoxicity (CDC) using fluorescein diacetate as marker for live cells, the method of Bodmer and co-workers (1967), modified by Moraes and Stastny, (1975) was used.

Sera were dispensed in 1 ul amounts into plastic microtest trays (Cooke Laboratory Products) under droplets of mineral oil and frozen for future use. Fluorescein diacetate (ICN Nutritional Biochemicals Div. International Chemical and Nuclear Corp., Cleveland, Ohio) was dissolved in acetone, 5 mg/ml and stored at -20°C. Just before use, 0.1 ml. of this solution was diluted 1:100 with RPMI-1640 and 0.1 ml. of this working solution was added to each milliliter of cell suspension. Cells were incubated with fluorescein diacetate at room temperature for 15-20 min. and washed in RPMI-1640 at 4°C and re-suspended in Hanks balanced salt solution with 3% bovine serum albumin. Labelled cells were then adjusted to a concentration of 2 x 10^6/ml. and 1 ul (approximately 2000 cells) was added to each serum well. After incubation for ½ hour at room temperature rabbit C' (5 ul) was added to each well and incubated again for 1 hour. The trays were refrigerated and read with a Ziess

microscope with FITC excitor and 530 nM barrier filter as soon as possible. Live cells in control preparations showed bright green fluorescence whereas dead cells lost the dye. A decrease of 50% fluorescent cells was considered positive. If more than 80% were killed the reaction was scored "strong positive".

F. Antibody dependent cellular cytotoxicity (ADCC).

The method for the assay was that modified and standardized by Kovithavongs et al (1974a,c) in this laboratory.

1) Target cells.

Target cells were obtained from 30 panel members and members of six families. HLA-A,B,C typing results were available for all target cell donors. HLA-DR typing results were available for members of one family and 19 of 30 panel members (Table 8 and Fig.25).

Different targets were used: T lymphocytes, B lymphocytes and monocytes, purified B lymphocytes, monocytes and CLL cells.

2-5 x 10^6 target cells were suspended in 10 ml. of RPMI with 10 mM Hepes buffer and 10% FCS and labelled with 50-100 μci of ^{51}Cr as Sodium Chromate (Amersham,

Toronto, Ontario), overnight at 37°C in an atmosphere of 5% CO_2 and 95% air. Just before performing the test these cells were washed three times with RPMl containing 2% FCS. The cells were re-suspended at a concentration of 1 x 10^6 cells/ml. in RPMl containing 10% FCS.

2) Test sera

Sera from transplant patients undergoing rejection (28), multiparous women (7) and systemic lupus erythematosus patients (5) were used in this study. Sera from transplant patients were collected during periods of rejection and stored at -70°C.

All serum samples were initially inactivated and absorbed by pooled platelets. Six broadly reactive sera were then absorbed with CLL cells. One of these six sera with predominant monocyte reactivity was absorbed with pooled monocytes as well as pooled B cells from panel members.

3) Serum absorptions.

a) With pooled platelets(PPLAB).

Outdated platelets were obtained from the Red Cross, Toronto. Platelets from 75-100 individuals were pooled and washed four times with saline containing anti-microbial agents (penicillin, streptomycin, and fungisone)

1 volume of serum was absorbed with 1/3 volume of packed platelets and incubated for ½ hour at room temperature fo' owed by ½ hour at 4°C. The absorption was repeated 2 mes with freshly packed platelets to ensure complete removal of HLA-A,B,C reactivity.

b) With chronic lymphocytic leukaemia (CLL) cells (CLLAB). Since the majority of CLL are of B cell type, they pro--vide a source of large quantities of highly enriched B cells. Mononuclear cells were prepared by F-H sedimentation as described earlier. Contamination with monocytes and T cells was checked by Acridine orange staining and E-rosetting respectively. The monocyte counts were insignificant and T cells were less than 15%. In selecting CLL cells for absorption of a particular serum sample preliminary ADCC tests were performed with the CLL cells and the serum to be absorbed.

One ml. of ½ diluted serum that had already been absorbed with pooled platelets was incubated twice with approximately 150 x 10^6 CLL cells for 45 min. at room temperature. Absorption process was repeated whenever B cell reactivity remained detectable.

c) With panel monocytes and B cells.

Mononuclear cells were prepared from 100 ml. of blood from

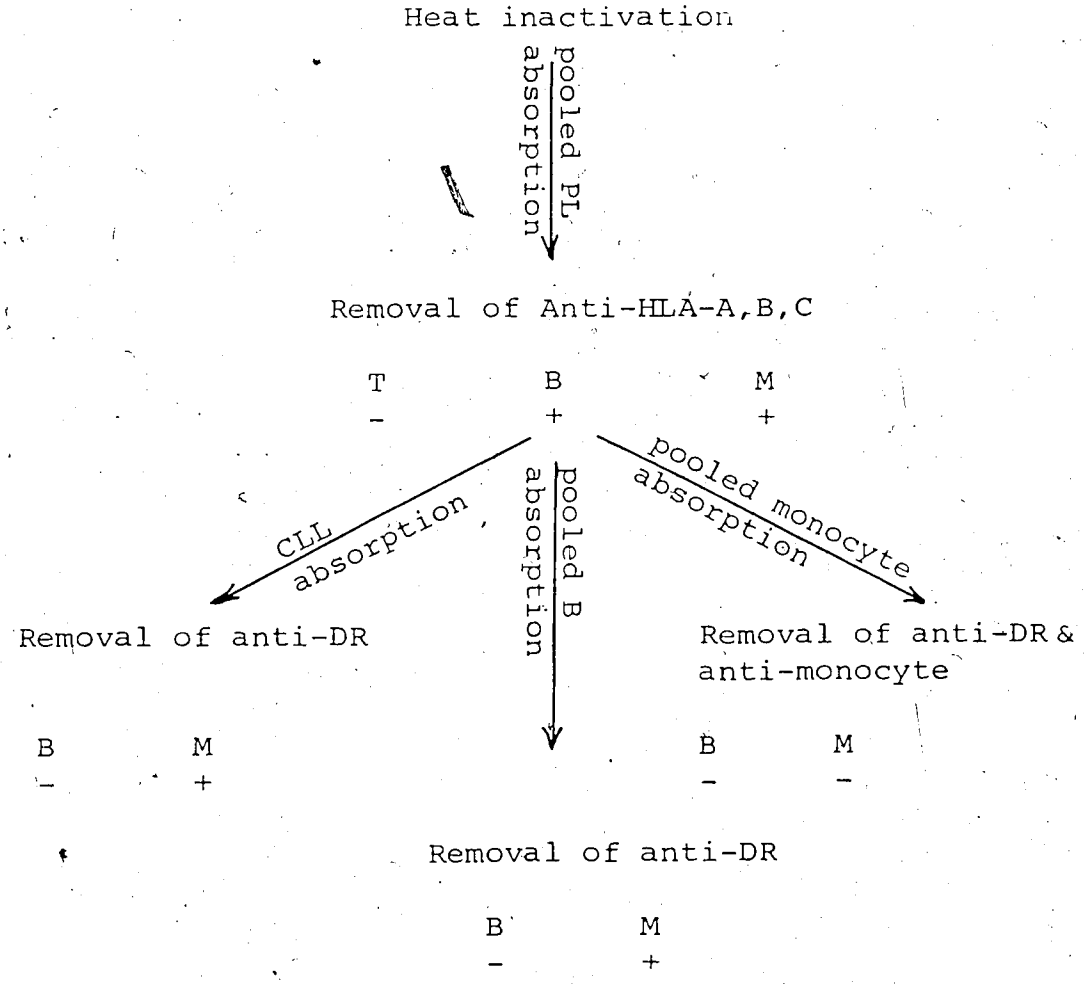

Figure 2 Procedures used for purification of test sera

each of 10 panel members that reacted with the serum in question which had been screened beforehand with many such members. Monocytes were then separated from non-adherent cells (mixture of B and T cells). Contaminating T cells in the non-adherent fraction do not interfere with B cell absorption.

These monocytes were then pooled and aliquoted into two portions of approximately 40 x 10^6 cells each. One ml. of ½ diluted test serum already platelet absorbed was mixed with one aliquot of pooled packed monocytes and left at room temperature for 45 min., twice for absorption.

The non-adherent cell fraction containing B and T lymphocytes were also pooled and used for absorption of another ml. of test serum as described for pooled monocyte absorption.

Figure 2 illustrates procedures used in purification of sera.

4) Effector cells.

Fifteen normal adults with high effector cell activity (ECA) in ADCC were selected as blood donors. All were males. Mononuclear cells were obtained by F.H. separation and cell count adjusted to 10 x 10^6 per ml. in RPMI-1640 with 10% FCS.

5) ADCC test.

Test serum was appropriately diluted (1/5 - 1/20) with RPMl; 0.025 ml. of diluted serum was pipetted into 13 x 100 mm. glass tubes and 0.025 ml. of target cells was added (concentration 1 x 10^6/ml.), followed by 0.1 ml. of effector cells already adjusted to a cell concentration of 10 x 10^6/ml. Control tubes were set up with RPMl containing 10% FCS of similar volume, but without test serum.

The cell mixture was incubated at 37°C for 3 hours in 5% CO_2 atmosphere. Following incubation cold saline (2 ml. at 4°C) was added and the tubes were centrifuged at 1500 rpm for 5 min. 4°C. The supernatant and pellet were separated and counted in a gamma counter.[a] All tests were performed in duplicates.

Maximum release of ^{51}Cr was measured by adding 0.5 ml. of haemolyte (Fisher Scientific Company ɔ 0.025 ml. of target cells. Background ^{51}Cr release was available from the control tubes.

The $\%^{51}Cr$ release was calculated from the following formula:

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[a] LKB-Wallace 80000 Automatic Gamma Sample Counter with 3" x 3" Crystal Size, Efficiency for ^{51}Cr 61%

^{51}Cr release% = $\frac{\text{cpm in supernatant}}{\text{cpm in supernatant \& cpm in pellet}} \times 100$

%Specific ^{51}Cr release was calculated from:

Specific ^{51}Cr release (%) =

$= \frac{\text{experimental } ^{51}Cr \text{ release} - \text{background } ^{51}Cr \text{ release}}{\text{maximal } ^{51}Cr \text{ release} - \text{background } ^{51}Cr \text{ release}} \times 100$

Specific release of $\geq$10% was considered positive.

G. Family segregation study.

Families with at least 3 children were used to study segregation of monocyte specific reactivity for linkage with HLA haplotypes. Parents were first screened for monocyte specific reactivity against sera known to contain monocyte antibodies. Families with only one parent showing monocyte specific reactivity were then selected for HLA haplotype segregation by determining HLA-A,B,C and DR antigens. Monocyte specific reactivity was then identified in children to determine whether it segregated in linkage with one or both HLA haplotypes of the monocyte reactive parent.

III. Results

A. Subpopulations of PBL.

1) T-enriched population.

The percentage of T cells in PBL measured in 21 normals by E rosetting method using neuraminidase treated SRBC ranged from 67-77% with a mean and S.D. of 72±5 (Table 2 and Fig.3). The purity of cells in the isolated fraction of T-enriched cell population was 90-95% when examined by re-rosetting (Table 4).

2) Non-rosetted cells (B+M).

The non-rosetted cells obtained from the interphase on F-H after T cell rosette sedimentation ranged from 18-30% of total PBL (mean and S.D. 24±6) (Table 3). This population (B+M) showed only 5-10% T cell contamination on re-rosetting.

3) Enriched monocytes (M).

Monocytes in non-rosetted fraction estimated by adherence method in 18 normal (Table 3 and Fig. 3) were 8±3% of total PBL. On purity checking with Acridine orange staining 86±6% of cells were identified as monocytes from the specific staining characteristics (Table 4). Addition of EDTA (0.2%) to RPMI prevented clumping of the monocytes in subsequent tests.

Table 2 Percentage of PBL forming E rosettes in normals

No:	PBL[b]	%E-rosette[a]
1.	DMAC	70
2.	KJOH	70
3.	TKOV	72
4.	CFAL	71
5.	DDRE	75
6.	MJOH	62
7.	JDOS	72
8.	SSAI	75
9.	SNAK	75
10.	EBRO	70
11.	NSHI	72
12.	JSLY	82
13.	LFAL	80
14.	JMUR	73
15.	KBET	75
16.	JSCH	77
17.	CMIL	68
18.	VLAO	76
19.	MDAS	66
20.	JPAZ	65
21.	VPAZ	62
	MEAN	72
	S.D.	5

[a]Rosetting done with neuraminidase treated SRBC

[b]Adherent cells not removed from PBL

Table 3 Percentage of different subpopulations of PBL in normals

No.	PBL	%B&M[a]	%B[b]	%M[c]
1.	DMAC	20	8	6
2.	KJOH	20	10	4
3.	MJOH	33	13	13
4.	DDRE	25	13	7
5.	SNAK	17	8	6
6.	JDOS	25	13	8
7.	TKOV	27	11	7
8.	CFAL	20	12	6
9.	SSAI	20	10	5
10.	CMIL	37	20	8
11.	EBRO	25	10	5
12.	JSCH	20	8	4
13.	NSHI	25	13	7
14.	DMAC	20	8	6
15.	JSLY	15	6	4
16.	JPAZ	34	14	11
17.	LMAR	25	10	10
18.	VPAZ	31	13	15
19.	JMUR	20	6	12
	MEAN	24	11	8
	S.D.	6	3	3

[a] B&M cells obtained by F-H gradient depletion of T cells after E-rosetting of PBL

[b] B lymphocytes obtained from B&M cells after removal of adherent monocytes

[c] Monocytes recovered from adhered plastic surfaces with rubber policeman

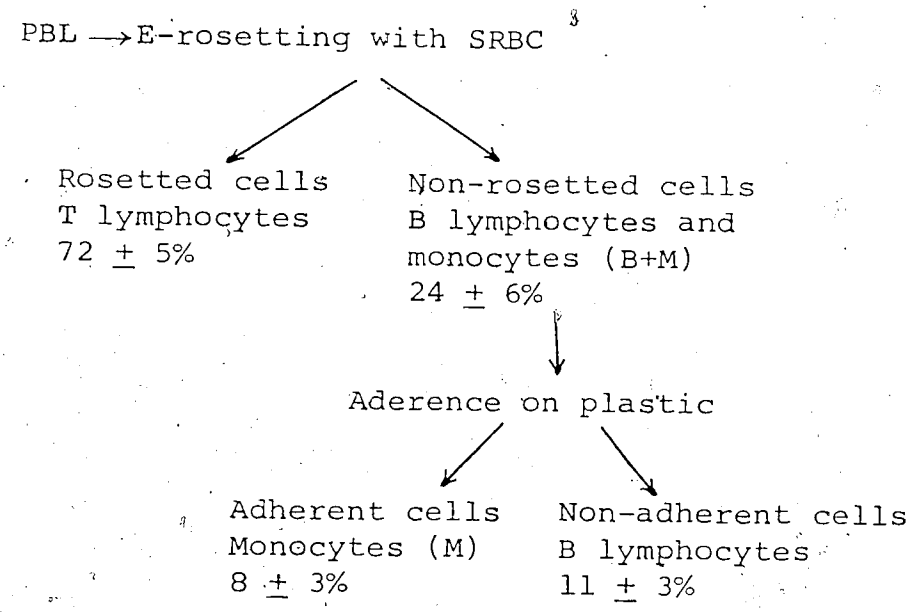


Figure 3 Results of separation of subpopulations of PBL

Table 4 Tests for purity of cell preparation

Methods	% T lymphocytes	% B lymphocytes	% monocytes
Re-rosetting	90 - 95	5 ± 4	2
Surface Ig (IF)	0	75 ± 4	5
Acridine orange staining	0	7 ± 1	84 ± 6

4) Enriched B lymphocytes.

The non-adherent population containing enriched B lymphocytes amounted to 8-14% of PBL in the 18 normals examined (Table 3 and Fig.3). 76±4% of these cells were positive for surface Ig (S.Ig) on immunofluorescence with FITC conjugated polyvalent anti-human Ig (Table 4).

B. Comparison of sensitivity of different methods.

Results of a comparative study of ADCC and two C' dependent methods, CDC by dye exclusion and by FDA, are shown in Table 5 and Fig.4. B+M targets from 19 panel members were tested with seven B reactive sera. Targets were monocytes only in FDA. All seven sera reacted in ADCC with panel cells, the number of reactions ranging from 7-16. In CDC, reactivity was seen only in the case of three sera, the number of reactions ranging from 2-9. Only two sera reacted in FDA. Because of the low sensitivity of the CDC methods, in further experiments only ADCC was used.

C. ADCC.

1) ^{51}Cr labelling of target cells.

Separation of the PBL into T, B and M subpopulations as targets required approximately 6 hours so the ADCC assay had

Table 5 Comparison of the sensitivity of different methods*

Methods	RCL			SWE			MJU			HAC			SCR			5108			ZOP		
Targets	ADCC B+M	CDC B+M	FDA M	ADCC B+M	CDC B+M	FDA M	ADCC B+M	CDC B+M	FDA M	ADCC B+M	CDC B+M	FDA M	ADCC B+M	CDC B+M	FDA M	ADCC B+M	CDC B+M	FDA M	ADCC B+M	CDC B+M	FDA M
LMAR	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−
JSCH	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	−	−	−	+	−	−
SSAI	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−
SSHI	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CMIL	+	−	±	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
VPAZ	+	+	−	+	−	−	+	−	−	−	−	−	+	−	−	+	±	−	−	−	−
CFAL	+	−	±	±	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	−	−
JSLY	+	±	±	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
MJOH	+	−	+	+	−	−	+	−	−	−	−	−	+	−	−	+	±	−	−	−	−
JPAZ	+	±	−	−	−	−	+	−	−	−	−	−	+	−	−	+	±	−	+	−	−
SNAK	+	−	+	+	−	−	+	−	−	−	−	−	+	−	−	+	−	−	+	−	−
KJOH	+	−	±	+	−	−	+	−	−	−	−	−	+	+	−	+	−	−	−	−	−
JMUR	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
JDOS	+	−	−	+	−	−	+	−	−	−	−	−	+	+	+	+	−	−	+	−	−
TKOV	+	±	+	+	−	−	+	−	−	+	−	−	+	−	−	−	−	−	+	−	−
KBET	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−
MHOL	+	−	−	−	−	−	+	−	−	+	−	−	±	−	−	±	−	−	+	−	−
ELIB	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	+	−	−	+	−	−
DDRE	+	±	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−

*ADCC - Target B+M, CDC - Target B+M, FDA - Target M. Target cells from 19 panel members were used. All sera were absorbed by pooled platelets. See also Fig. 4.

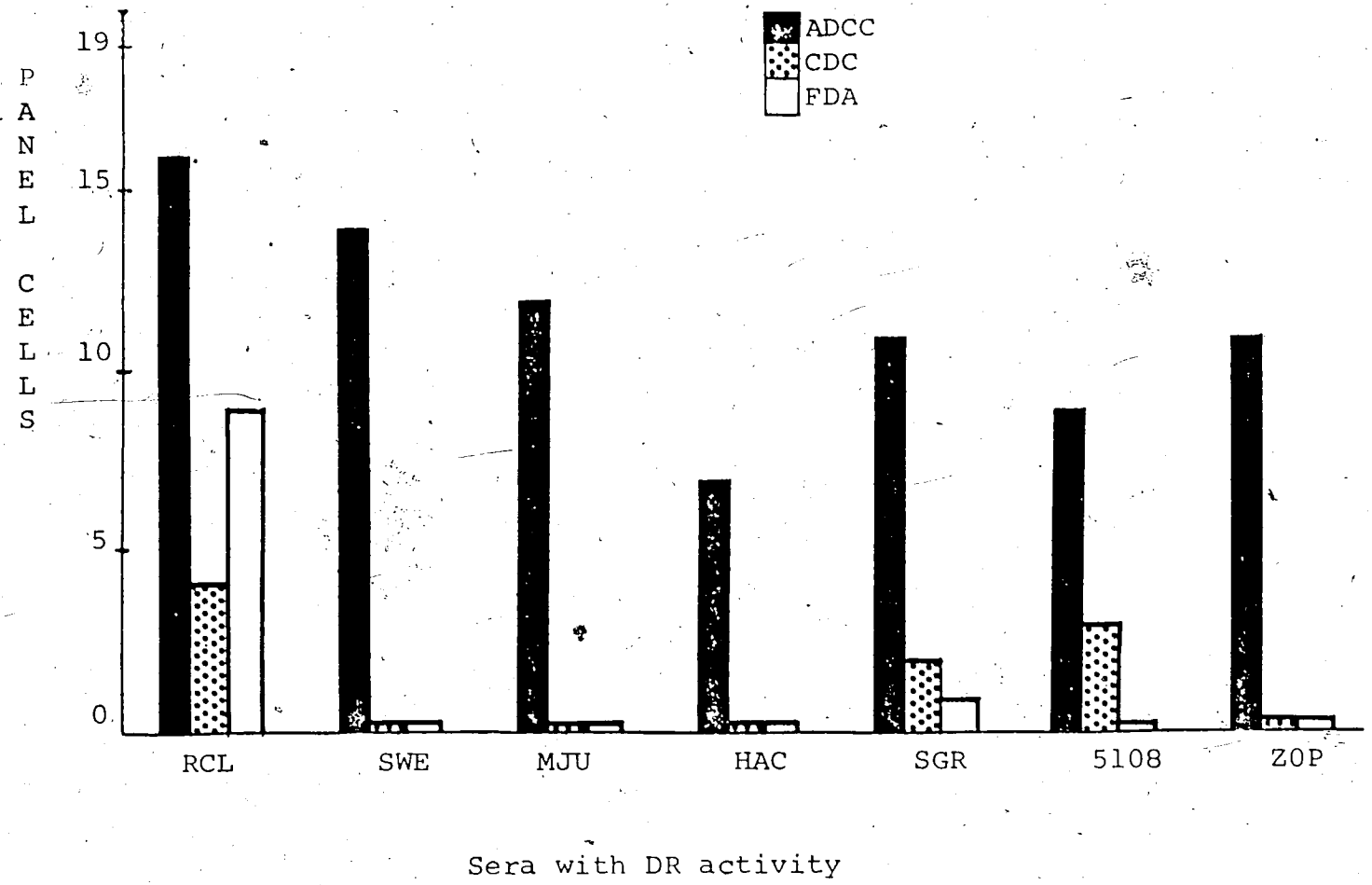


Figure 4 Comparative study of ADCC and C' dependent methods (CDC and fluorochromasia-FDA) B+M targets were used in ADCC and CDC and monocytes in FDA. All 7 sera reacted in ADCC, the number of panel cells reacted ranged from 7-16. Only 3 sera reacted in CDC and 2 in FDA.

to be performed on the following day. Target cells were labelled with ^{51}Cr by overnight incubation in 5% CO_2 atmosphere using larger quantities of media (10 ml.) compared with standard 1 hour labelling in 0.1 ml. volumes (Kovithavongs et al 1977). Background ^{51}Cr release from target cells in the presence of effector cells but no serum was 16±4.5% for T cells (71 experiments), 18±4% (130 experiments), for B cells and 20±5% (130 experiments) for monocytes (Table 6).

2) ^{51}Cr uptake by different subpopulation of cells. Uptake of ^{51}Cr varied for different subpopulations. Monocytes showing the highest uptake of ^{51}Cr (see Table 7) as much as three times that of T cells.

Table 8 shows HLA-A,B,C and DRw antigens of panel members.

3) Effector cells.

The HLA-A,B,C and DR antigens of the 15 effector cell donors used in this study are shown in Table 9. These 15 donors were pre-selected after prior testing for having satisfactory effector cell activity.

4) Purification of test sera.

Completeness of removal of HLA-A,B,C antibodies from test sera was tested by subsequent ADCC assay. An example is presented in Table 10. It was found that absorption had

Table 6 ADCC - background readings*

Targets	No. of experiments	$\%^{51}Cr$ release
T lymphocytes	71	16 ± 4.5%
B lymphocytes	130	18 ± 4%
Monocytes	130	20 ± 5%

* Cells were labelled in 10 ml of RPMI with 0.1 ml of ^{51}Cr as sodium chromate (Amersham, England) for 18 hours. Background readings expressed as $\%^{51}Cr$ release from targets after 3 hours incubation at 37°C, 5% C02 in the presence of FCS and effector cells (no test serum). Effector to target ratio was 40:1

Table 7 Comparison of ^{51}Cr uptake by monocytes, B lymphocytes and T lymphocytes[a]

	Monocytes cpm[b]	B lymphocytes cpm[b]	T lymphocytes cpm[b]
Mean	15,357	6250	4916
S.D.	6331	3840	1240

[a] 5×10^6 cells were labelled in 10 ml RPMI with 0.1 ml of ^{51}Cr as sodium chromate (Amersham, England) in 37°C incubator with 5% CO_2 for 18 hours. Cells were washed and adjusted to 1×10^6/ml prior to testing.

[b] cpm, means of 12 experiments - ^{51}Cr uptake by 25,000 cells

Table 8. HLA-A, -B, -C and DRw antigens of panel members*

Panel members	Blood group	HLA-A		B		C		DRw	
1. KBET	0	2	31	40		3			
2. MDAS	B	24	11	7	17			2	7
3. DDRE	B	24	11	35	44	4	5	4	
4. JDOS	0	2	31	8	45			3	7
5. CFAL	0	25	29	39	45	5		5	2?
6. LFAL	A	1	24	17	22	1	6	5	
7. AJOH	0	2		40					n/d
8. MHIG		11	32	12	22	3			
9. MHOL		24	25	35	15	3			
10. KJOH	0	2	3	15	40			7	
11. MJOH	0	2	28	37	40			6?	new
12. TKOV	0	2		5	35	3		2	
13. VLAO	B	2		40	46	1	3	7	
14. JLI	A		11	15	35	4	6	2	
15. ELIB	0	30		39	50			2	
16. LMAR	0	28	32	14	22			6	
17. CMIL		2	29	45	15	3	6		
18. JMUR	A	2	31	17	40	3	6	2	7
19. SNAK	B	2	24	39	40		6		
20. JPAZ	0	2	33	8	17	3		3	
21. VPAZ	0	1	11	7	15	3	6	3	6
22. VJOH	0	2		40				n/d	
23. SSAI	0	2	26	7	27	2		4	6?
24. JSCH	0	11	32	35	44	4	5	3	4
25. SSHI	B		24	35	40	4	6	2	5
26. JSLY	AB	2	11	35	12	4	5	4	
27. CJAC		3	24	35	15	4	6	n/d	
28. AWIL	n/d	3	32	7	12			n/d	
29. MLON	n/d	1	2	15	37				
30. DMAC	n/d	2		7	14				

* Data supplied by Tissue Typing Lab, University of Alberta Hospital, Edmonton, Canada
n/d - not done

Table 9 List of effector cell donors and their HLA-A,B,C and DR antigens*

Effector cell donors**	HLA-A		B		C		DRw	
1. TKOV	2		5	35	3		2	
2. KJOH	2	3	15	40			7	
3. JDOS	2	31	8	45			3	7
4. MDAS	24	11	7	17			2	7
5. VLAO	2		40	46	1	3		7
6. GSCH	n/d							
7. DMET	n/d							
8. RDOU	n/d							
9. DDRE	24	11	35	44	4	5	4	
10. DMCA	2		7	14				
11. CJAC	3	24	35	15	4	6		
12. JLI		11	15	35	4	6	2	
13. JHAG	n/d							
14. EBRO	1	24	8	27				
15. AWIL	3	32	7	12				

*Data supplied by Tissue Typing Lab, University of Alberta Hospital, Edmonton

**All showed good effector cell activity on prior testing
n/d - not done

Table 10 ADCC results of serum RCL-PPLAB with panel T cel.

Targets (T cells)		specific ^{51}Cr release (%)
1.	LMAR	2
2.	SSHI	5
3.	JMUR	3
4.	KBET	-2
5.	AWIL	0
6.	ELIB	-3
7.	MDAS	0
8.	DMCA	0
9.	JSCH	1
10.	CFAL	0
11.	MJOH	1
12.	JPAZ	3
13.	SNAK	3
14.	JDOS	1
15.	TKOV	4
16.	MHIG	0
17.	CMIL	1
18.	SSAI	0
19.	KJOH	-3
20.	DDRE	2
21.	AJOH	-1
22.	VJOH	2
23.	JSLY	0

* Specific release $\geq$10% was considered as positive.
All 23 panel T cells were negative after pooled platelet absorption.

to be repeated 3-5 times for complete removal of HLA-A, B,C antibodies.

B cell reactivity in test sera could be removed by two absorptions using specific CLL cells.

Similarly, two absorptions with pooled B cells from 10 panel members removed B reactivity in test serum. Absorption with pooled M from 10 donors removed monocyte specific as well as B cell reactivity.

D. Sera used in the study.

Preliminary screening for monocyte specific antibody reactivity was performed in 40 serum samples obtained from, transplant patients (28), multiparous women (7), and patients with systemic lupus erythematosus (5) (Table 11). Sera from transplant patients were collected during episodes of rejection except in one case (details given in Table 12). Sera from multiparous women had been pre-selected and were known to react with panel targets (B+M) in ADCC and/or CDC. The sera were tested against different target cell subpopulations (T, B and M) from 30 panel members (Table 11).

As shown in Table 11, two transplant patients' sera showed strong monocyte specific reactivity (specific

Table 11 Sera screened for monocyte specific reactivity using ADCC technique

Serum source	No:	No. of panel members	No. of sera with anti-monocyte activity strong	weak
Transplant patients with rejection	28		2	7
*Multiparous (selected)	7	30	3	-
SLE	5		-	-
Total	40		5	7

*Multiparous sera were known to have broad reactivity in ADCC and/CDC

Table 12 Immunological details of transplant patients

	RAUT	JBUS	RCAM	KDAL	JDAR	LELL	BFIL	WGAL	RHAL	FJAC	PKLU	VLOU	KMOH	LOLE	LPAT	CROB	ESMI	HSTP	ESTO	JVAN	TWEI	AWIL	BSHA	SGR[b]	SHAR[b]	RCL[a b]	DFER[b]	BRAW[b]
HLA-A,B,C CDC/ADCC	+	-	+	+	-	-	+	-	-	+	+	+	-	-	-	+	+	-	+	-	+	+	-	+	+	+	+	+
LMC**	+	T.F	-	-	-	+	T.F	+	+	-	+	N.D	-	-	+	-	-	-	N.D	+	-	-	-	-	N.D	-	T.F	N.D
ADCC M*	±	-	-	-	±	-	±	-	-	-	±	-	-	±	-	-	-	±	-	±	-	-	-	+	-	+	(-)	-
ADCC B*	±	±	-	±	-	-	±	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	+	+	+	+

N.D. - not done T.F. - technical failure

[a]Not in rejection

[b]Transplant patients' sera known to have broad reactivity in CDC and/ADCC

* Cells from 7-29 panel members were used

**Lymphocyte mediated cytotoxicity

See text for discussion.

release of ^{51}Cr 15-40%) in addition to B cell specific reactivity. One of these sera (RCL) reacted principally with panel monocytes (Table 13 and Fig. 5 and 6). The other serum SGR showed both monocyte and B cell specific reactions with panel cells. B specific reactivity appeared to be predominating (Table 14 and Fig.7). Sera from 7 patients showed weak monocyte specific reactivity (specific ^{51}Cr release of 8-10%) (Table 12) and the sera reacted with only 1-2 panel cells.

Three multiparous sera showed strong monocyte specific reactivity in addition to B cell specificity. All 5 SLE sera were negative for both B specificity and M specificity.

Those 5 sera with strong broad reactivity (2 transplant, 3 multiparous) and another serum with known B reactivity (multiparous) were subjected to further detailed study using absorption methods with specific CLL cells, pooled B lymphocytes and monocytes. Relevant clinical and immunological data of those serum donors are shown in Table 15. Results of absorption studies of each of these serum samples are detailed below.

1) Serum RCL.

This serum was collected from a male patient about a month

Table 13 ADCC results of serum RCL-PPLAB with panel cells*

Targets		Specific ^{51}Cr release (%) M	B
1.	SNAK	37	10
2.	KJOH	17	11
3.	DDRE	18	14
4.	JSLY	32	22
5.	NSHI	17	11
6.	VLAO	13	18
7.	JLI	35	14
8.	JMUR	24	8
9.	KBET	17	7
10.	JSCH	28	6
11.	CFAL	20	8
12.	MJOH	21	4
13.	JPAZ	22	4
14.	JDOS	18	4
15.	TKOV	28	9
16.	MHIG	24	5
17.	SSAI	35	8
18.	AJOH	21	9
19.	VJOH	23	5
20.	CJAC	18	5
21.	DMAC	30	4
22.	LMAR	1	2
23.	AWIL	0	3
24.	SSHI	6	5
25.	ELIB	8	4
26.	MDAS	9	4
27.	CMIL	6	8

*Specific ^{51}Cr release of $\geq$10% was considered as positive.
Both M and B reactivity against 7 panel members
M alone against 14
M and B negative against 6

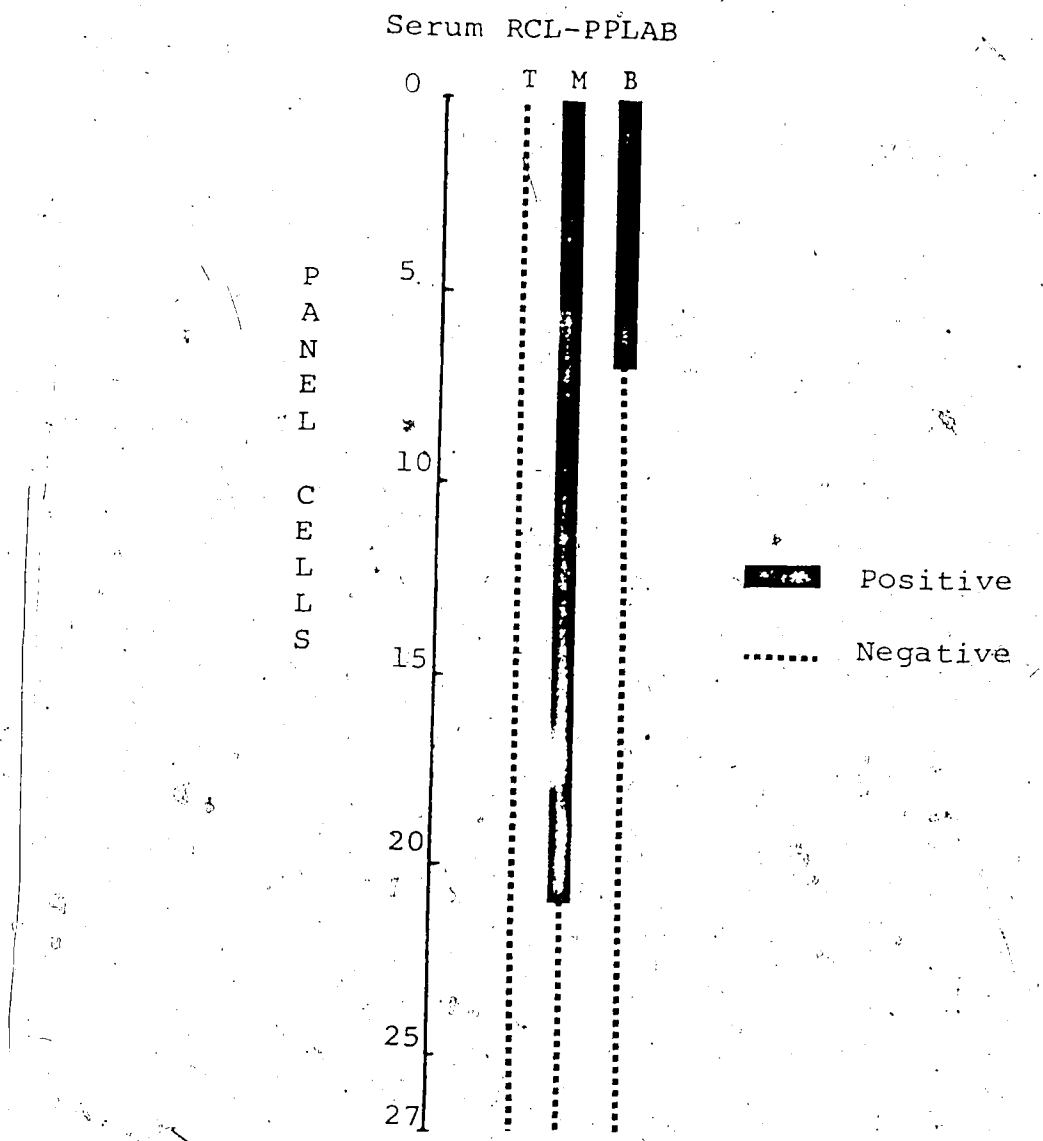


Figure 5 Histogram showing ADCC results of serum RCL (after pooled platelet absorption) with 27 panel cells - T,M and B: T reactivity was negative against all. 7 showed both M and B reactivity, 14 showed M alone and 6 negative with M and B

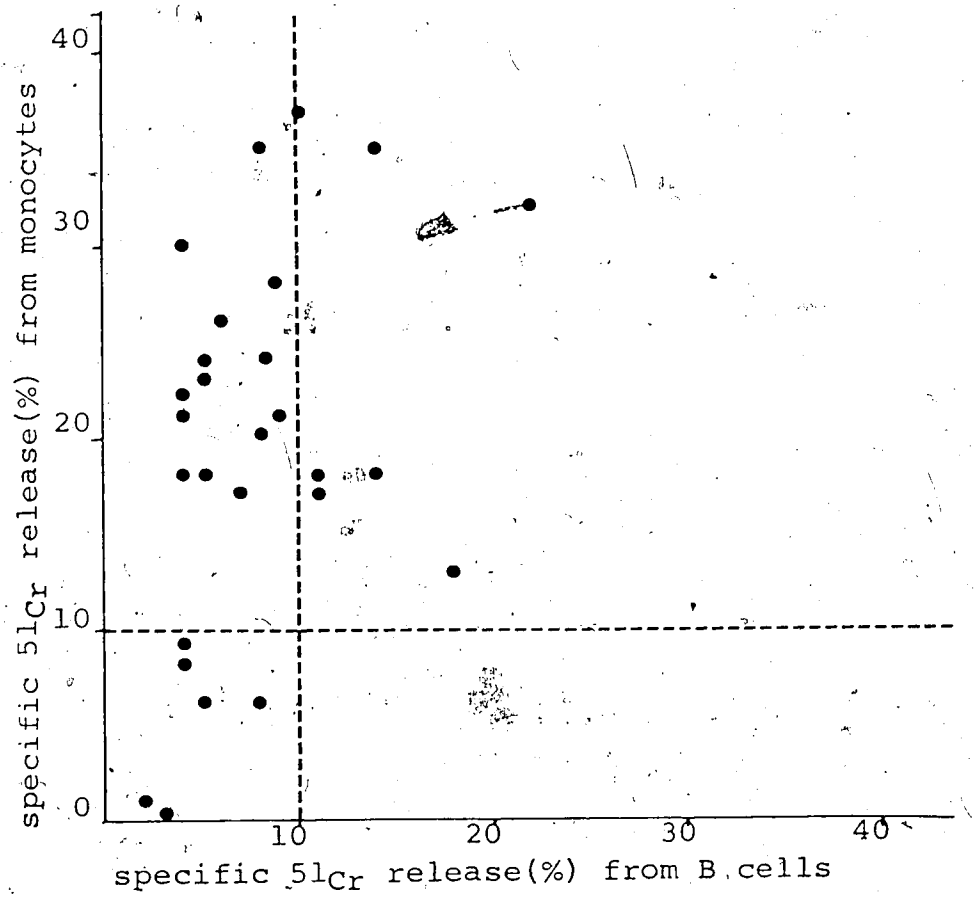


Figure 6 Scattergram showing specific ^{51}Cr release from 27 panel monocytes and B cells by serum RCL (after pooled platelet absorption) 6 showed both M and B reactivity with specific ^{51}Cr release of $\geq$ 10% (interrupted lines) 14 showed reactivity against M alone and 6 negative with both

Table 14 ADCC results of serum SGR-PPLAB with panel cells*

	Targets	specific ^{51}Cr release (%) M	B
1.	JPAZ	23	12
2.	SNAK	25	15
3.	TKOV	25	30
4.	VJOH	27	24
5.	AJOH	36	26
6.	DMAC	11	16
7.	MDAS	31	11
8.	VLAO	39	18
9.	KJOH	33	32
10.	JMUR	15	18
11.	JDDS	10	11
12.	NSHI	28	14
13.	LFAL	16	16
14.	VPAZ	8	15
15.	MJOH	15	9
16.	CJAC	27	5
17.	JSCH	12	4
18.	MHIG	29	4
19.	ELIB	20	9
20.	MLON	15	9
21.	SSAI	5	1
22.	SSHI	5	1
23.	CFAL	2	5
24.	LMAR	3	2
25.	CMIL	-1	3
26.	DDRE	3	3
27.	KBET	-4	5
28.	JLI	3	-2
29.	JSLY	3	1

* Specific ^{51}Cr release of $\geq$10% was considered as positive.
Both M&B reactivity against 13 panel members, M alone with 6,
B alone with 1
M and B negative with 9

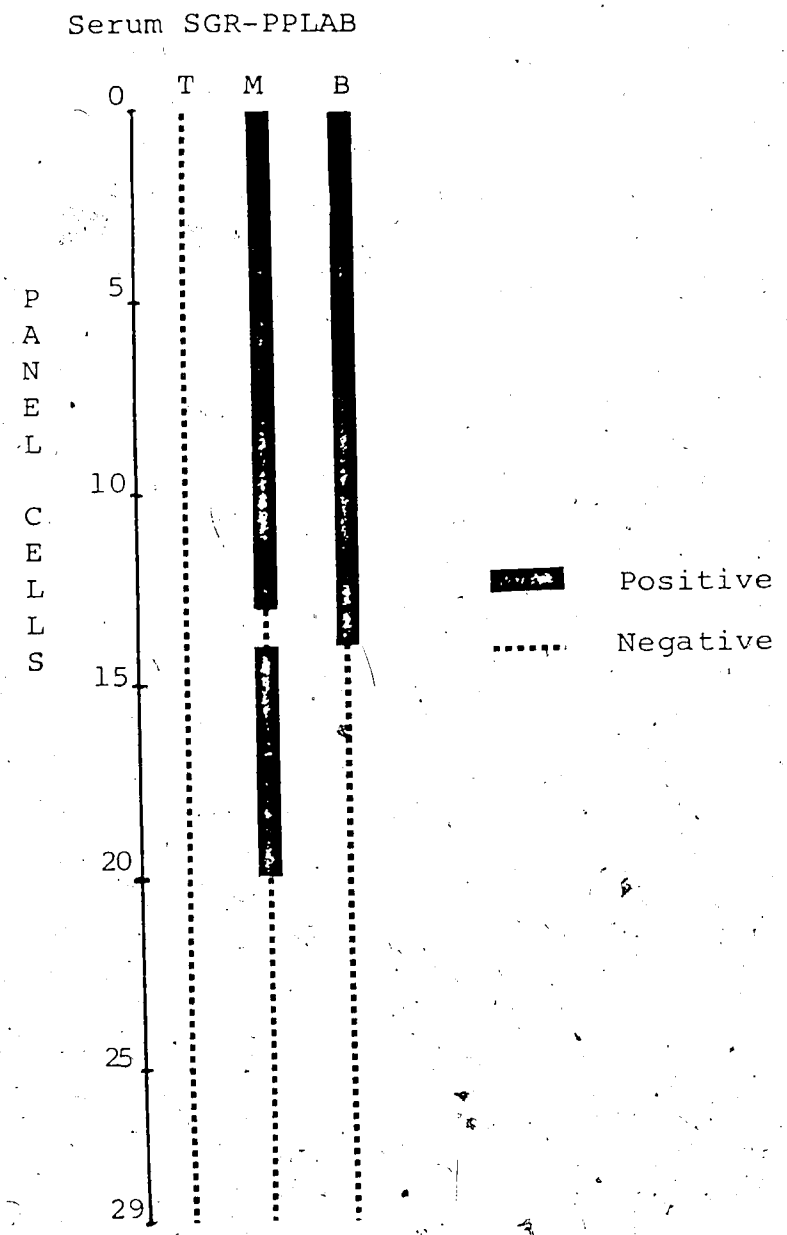


Figure 7 Histogram showing ADCC results of serum (SGR after pooled platelet absortpion) with 29 panel cells - T,M and B. T reactivity was negative with all. 13 showed both M and B reactivity, 1 showed reactivity with B alone and 6 with M alone. 9 were negative with both.

Table 15. Clinical and immunological details of braodly reactive sera

No.	Name	Sex	Transplant/ Multiparous	No. of Transplants/ No. of pregnancies	Prior Transfusions	HLA-A,B,C ADCC/CDC	*B cell specific antibodies	*Monocyte specific antibodies
1.	RCL	M	Transplant	3	Yes	+	+	++
2.	SGR	F	Transplant	1	Yes	+	+	+
3.	SWE	F	Multiparous	10	No	weak	+	+
4.	MJU	F	Multiparous	5	No	weak	+	+
5.	G256	F	Multiparous	2	No	weak	++	+
6.	5018	F	Multiparous	2	No	weak	+	-

* A summary of the B cell specific and M specific reactions of the 6 broadly reactive sera with panel cells are shown. 5 sera showed both B and M specific reactivity. Serum RCL showed predominant M specificity. Serum G256 showed predominant B specificity. Serum 5018 had only B specificity.

after the third transplantation. The patient had received two previous transplants in the preceding six years. The first transplant was from a cadaver, the second from patient's father. Both allografts had been rejected. He had also received many blood transfusions during previous surgeries and while on haemodialysis. As a result he had developed wide spread sensitization against tissue antigens.

Initial cytotoxic screening of his sera against panel PBL showed broad reactivity in CDC. After five absorptions with pooled platelets, HLA-A,B,C reactivity was not demonstrable (see Table 10), but there remained broad reactivity with B and M targets in ADCC and CDC. The serum was then tested with enriched subpopulations of B and M from 27 panel members and the results are represented in Table 13 and Fig. 5 and 6. ADCC against monocytes alone observed in 14 panel members, B cells alone with none, and both B and M with seven. With six panel members there was no demonstrable reactivity against either B or M.

CLL cells from two patients known to react with RCL serum were pooled. The serum was absorbed with pooled CLL cells twice and then tested by ADCC assay for

monocyte and B specific reactivities against 17 panel members (6 with M and B reactivity, 11 with M reactivity alone). Table 16 and Fig. 8 shows that B cell reactivity was removed with all except one, where absorption was possibly incomplete. In contrast M reactivity persisted with all 17 indicative of the presence of monocyte antibodies in RCL serum.

In a different experiment RCL serum was absorbed twice with pooled B cells from 10 of the panel members with M and B reactivity (6) or M alone (4). Subsequent assay demonstrated complete removal of B reactivity from the serum whereas M reactivity persisted against all panel members tested (7) (Fig.8 and Table 17). This further supported the presence of M antibodies in RCL serum.

Pooled monocytes were prepared from the same 10 panel members used for pooled B cell absorption. RCL serum was then absorbed twice with pooled monocytes and tested for M and B specific reactivities to the seven panel members used in pooled B absorption study. Pooled monocyte absorption removed both M and B reactivity to all seven panel cells tested (Table 17 and Fig. 8).

It is inferred from these experiments that: 1) both

Table 16 ADCC reactions of serum RCL -PPLAB[b] and CLLAB[c] with panel cells

Panel	Target	specific ^{51}Cr release (%) PPLAB 1/10	1/20	1/40	CLLAB 1/10	1/20	1/40	PPLAB	CLLAB
1. SNAK	M	33	28		43	44		+	+
	B	3	15		13	9		+	-a
2. KJOH	M	17	17	19	16	17	14	+	+
	B	5	11	14	9	9	8	+	-
3. DDRE	M	17	21		17	22		+	+
	B	22	23		10	1		+	-a
4. JSLY	M	17	9		15	14		+	-
	B	27	21		19	12		+	+
5. NSHI	M	17	20	17	18	13	13	+	+
	B	11	12	8	5	4	5	+	-
6. JLI	M	35	36	31	24	19	16	+	+
	B	14	15	10	10	10	7	+	-
7. JMUR	M	18	18		19	20		+	+
	B	9	4		1	7		-	-
8. KBET	M	6	17		21	20		+	+
	B	6	7		9	9		-	-
9. JSCH	M	15	17		13	9		+	+
	B	1	1		-5	2		-	-
10. CFAL	M	25	34	33	37	36	27	+	+
	B	0	1	3	1	4	4	-	-
11. MJOH	M	11	7		14	7		+	+
	B	2	2		1	2		-	-
12. JDOS	M	9	18	13	8	8	17	+	+
	B	1	4	2	1	4	1	-	-
13. TKOV	M	28	30		30	34		+	+
	B	9	10		10	8		-a	-a
14. SSAI	M	33	35	31	36	35	30	+	+
	B	12	8	7	7	2	5	-a	-
15. AJOH	M	29	31		35	28		+	+
	B	8	7		8	8			-
16. VJDH	M	16	15		15	12		+	+
	B	5	9		7	7		-	-
17. DMAC	M	10	13	12	14	20	9	+	+
	B	3	3	4	5	4	3	-	-

[a]Considered as negative based on the results of previous experiments

[b]RCL-PPLAB - 6 panel members showed both M and B reactivity
11 showed M reactivity alone

[c]RCL-CCLAB - B reactivity removed against all except one
M reactivity persisted against all 17

Table 17 ADCC reactions[c] of serum RCL-PPLAB, pooled B-ABS[a] and pooled M-ABS[b] with panel cells

			PPLAB					Pooled B-ABSx2					Pooled M-ABSx2				
			1/5	1/10	1/20	1/40	1/80	1/5	1/10	1/20	1/40	1/80	1/5	1/10	1/20	1/40	1/80
1.	SNAK	M	32	37	42	42		36	37	35	22		4	3	1	4	
		B	9	9	9	8		6	8	6	4		-1	0	1	-2	
2.	KJOH	M	6	17	17	19		15	14	14	14		-3	3	0	1	
		B	8	5	11	14		5	8	5	6		2	0	7	5	
3.	DDRE	M	13	17	15	20		13	15	17	15		4	9	7	7	
		B	18	23	20	21		9	10	10	6[d]		8	7	7	8	
4.	SSAI	M	11	17	15	17		15	10	4	9		0	5	4	9	
		B	12	8	7	7		7	8	4	6		5	4	3	4	
5.	MJOH	M	6	10	16	15	13	24	10	9	7	13	-1	0	4	7	4
		B	2	1	4	-1	-1	-1	0	2	0	0	1	-3	-1	-2	-1
6.	JDOS	M	4	9	18	13	11	14	8	13	17	13	-4	3	5	6	3
		B	4	1	4	2	1	1	3	1	1	1	1	0	1	1	4
7.	TKOV	M	-1	5	8	15		11	9	12	19		-3	5	6	6	
		B	4	7	8	9		4	9	6	8		2	1	-1	2	

[a]Pooled B-ABS - B reactivity removed against all
M reactivity presisted against all

[b]Pooled M-ABS - Both M and B reactivity against all 7 removed

[c]Expressed as specific ^{51}Cr release(%)

[d]Considered as negative

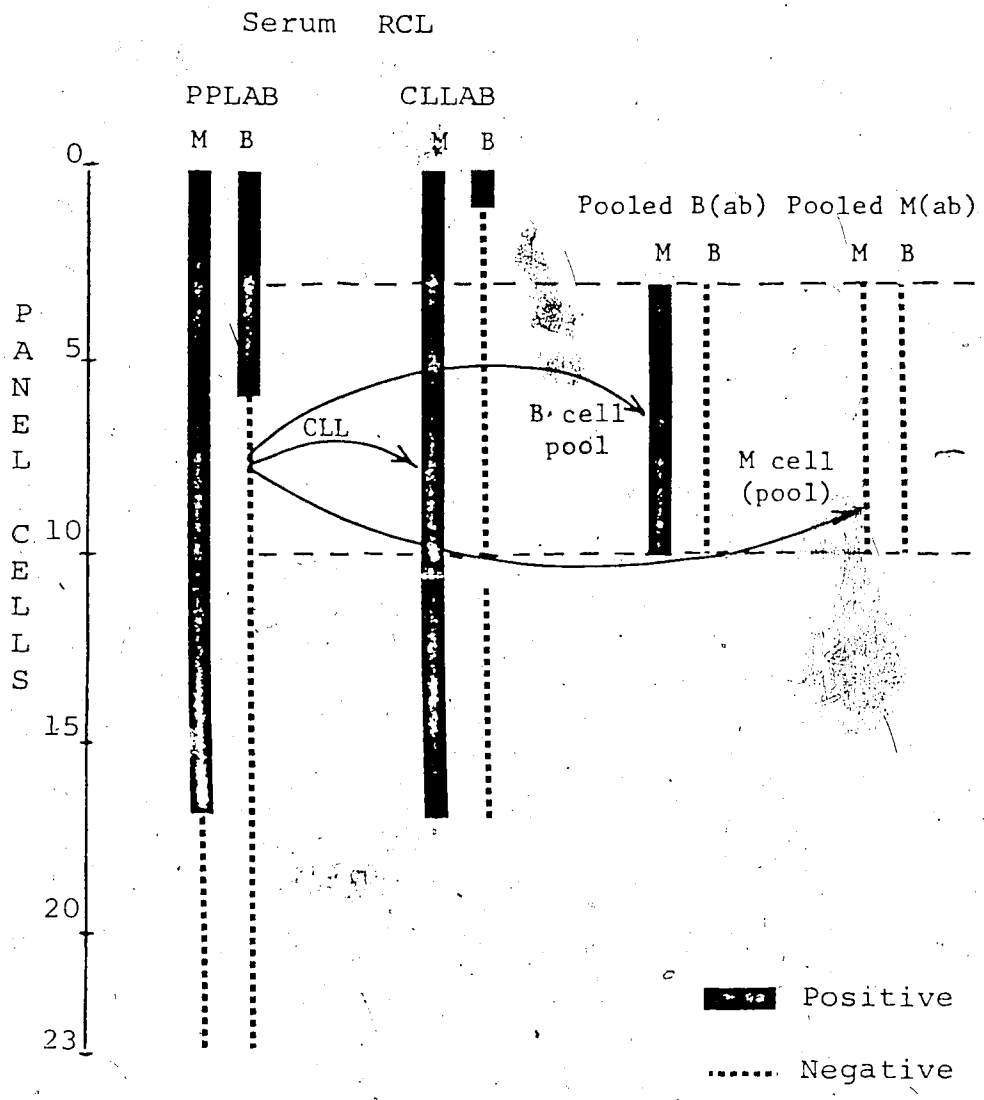


Figure 8 Histogram showing results of absorption analysis of serum RCL (after pooled platelet, CLL, pooled B and pooled M absorption) by ADCC method. Pooled platelet absorbed RCL serum when tested with 23 panel members showed both M and B reactivity against 6, M alone with 11 and negative with both in 6. CLL absorption removed B reactivity against all except one whereas M reactivity persisted with all 17. Similarly pooled B absorption removed B reactivity against all (7) whereas M reactivity persisted. Pooled M absorption removed both M and B reactivity.

M and B cell antibodies were present in RCL serum and could be removed by monocyte absorption alone, because monocytes bear both B (DR) and monocyte specific antigens; 2) pooled B cells or CLL cells removed only B (DR) reactivity, whereas the monocyte specific antibodies persisted in the serum; and 3) M reactivity appeared to be the predominant finding in RCL serum since ADCC against monocytes alone was observed in over 50% of panel members studied. Moreover the ^{51}Cr release was also notably higher in the monocyte assays.

2) Serum SGR.

This serum was collected from a female patient about two months after transplantation during an episode of rejection.

After pooled platelet absorption and removal of HLA-A,B,C antibodies, the serum was tested against 29 panel cells. Both M and B reactivity was demonstrable against 13 panel members, M alone against 6 and B alone against one. In the other nine instances both M and B reactivity were not demonstrated (Table 14 and Fig.7).

CLL cells from three patients reacted with the serum on preliminary testing, hence were pooled for subsequent absorption of the serum. The CLL absorbed serum,

Table 18 ADCC reactions of serum SGR-PPLAB[a] and CLLAB[b] with panel cells

Targets		specific ^{51}Cr release % PPLAB 1/5	PPLAB 1/10	CLLAB 1/5	CLLAB 1/10	PPLAB	CLLAB
1. MJOH	M	15	21	16	12	+	+
	B	9	9	6	7	-	-
2. JSCH	M	24	20	14	5	+	+
	B	1	3	1	1	-	-
3. CJAC	M	27	28	28	26	+	+
	B	5	1	3	2	-	-
4. KJOH	M		18	14	11	+	+
	B	11	12	3	2	+	-
5. SNAK	M	43	40	31	22	+	+
	B	25	18	15	13	+	+
6. TKOV	M	39	39	8	4	+	-
	B	17	19	4	3	+	-
7. MDAS	M	19	24	1	0	+	-
	B	13	11	0	1	+	-
8. JMUR	M	15	19	4	-1	+	-
	B	18	23	6	0	+	-
9. NSHI	M	28	22	9	6	+	-
	B	13	15	15	15	+	+

[a]SGR-PPLAB - 6 panel members showed both M and B reactivity
3 showed M alone

[b]SGR-CLLAB - Complete removal of B reactivity against 4 and incomplete with 2
M reactivity presisted with 4 (3 with prior M alone and 1 with both M and B)

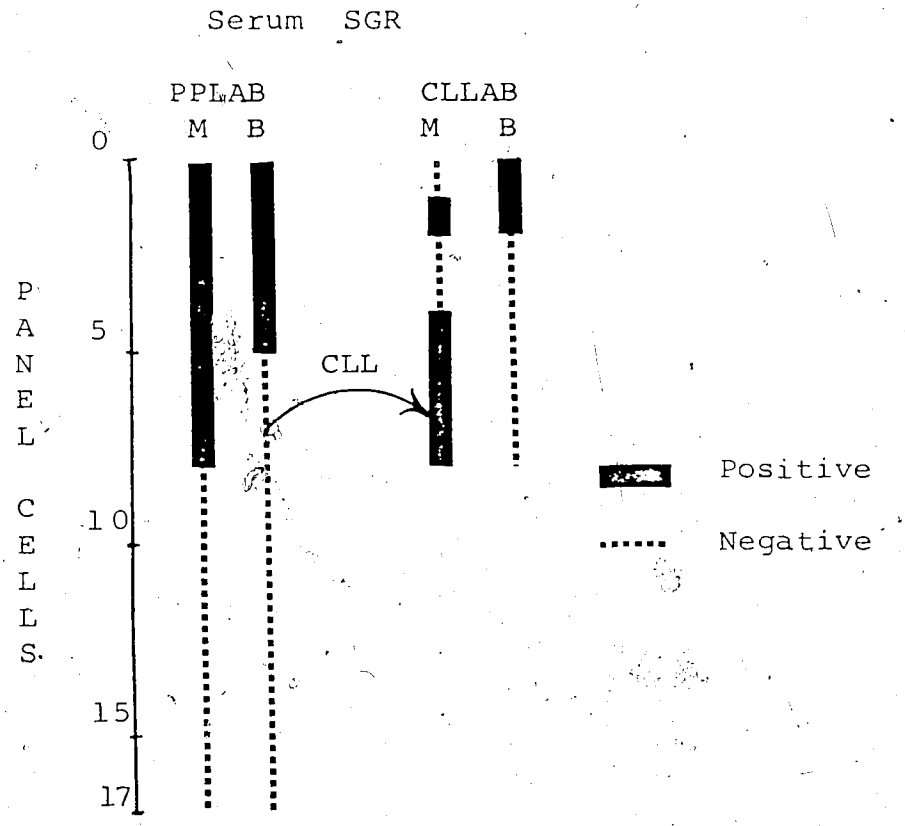


Figure 9 Histogram showing absorption analysis of serum SGR (platelet absorbed and CLL absorbed) by ADCC method. Pooled platelet absorbed serum SGR showed both M and B reactivity against 6 and M alone with 3. Both M and B were negative with 9. CLL absorption removed B reactivity against all (4) except 2. M reactivity persisted against 4 (all 3 with prior M alone and one with both M and B).

when tested against nine panel members (both M and B positive with 6 and M alone with 3) (Table 18 and Fig. 9) showed complete removal of B reactivity with 4 and incomplete with 2. Monocyte specific reactivity persisted in all three instances with pure M reactivity and in another with prior M&B reactivity. It is again inferred that serum from this transplant patient had monocyte specific antibodies, though B reactivity appeared to be broader.

3) Serum SWE.

The serum was obtained from a multiparous woman who had had 10 pregnancies. The last one was two years previously. She had received no blood transfusions. Serum was collected about one year after the last baby was born.

Weak T cell reactivity was initially absorbed with pooled platelets. Subsequently both B and M reactivity persisted against 13 panel members, M alone with 6 and B alone with one. Against 8 panel members there was no demonstrable M or B reactivity (table 19 and Fig. 10 and 11).

CLL cells from one patient with strong reactivity with SWE serum was used for absorption. Serum was tested

Table 19 ADCC reactions of serum SWE-PPLAB with panel cells*

Targets	specific ^{51}Cr release % M	B
1. SSAI	25	11
2. DDRE	29	20
3. SNAK	40	19
4. MJOH	41	16
5. KBET	25	26
6. JSLY	20	30
7. TKOV	40	22
8. VLAO	33	27
9. KJOH	31	35
10. JDOS	17	30
11. CMIL	17	20
12. AJOH	35	22
13. VJOH	35	19
14. LMAR	1	13
15. DMCA	25	9
16. CFAL	12	4
17. NSHI	15	7
18. JSCH	25	9
19. JLI	26	8
20. AWIL	11	7
21. JPAZ	4	2
22. VPAZ	-3	1
23. SSHI	-2	1
24. MDAS	3	2
25. JMUR	4	2
26. MHIG	2	1
27. MLON	2	1
28. ELIB	9	2

* specific ^{51}Cr release of $\geq$10% considered as positive
Both M and B reactivity against 13 panel members
M alone with 6 and B alone with 1
M and B negative against 8

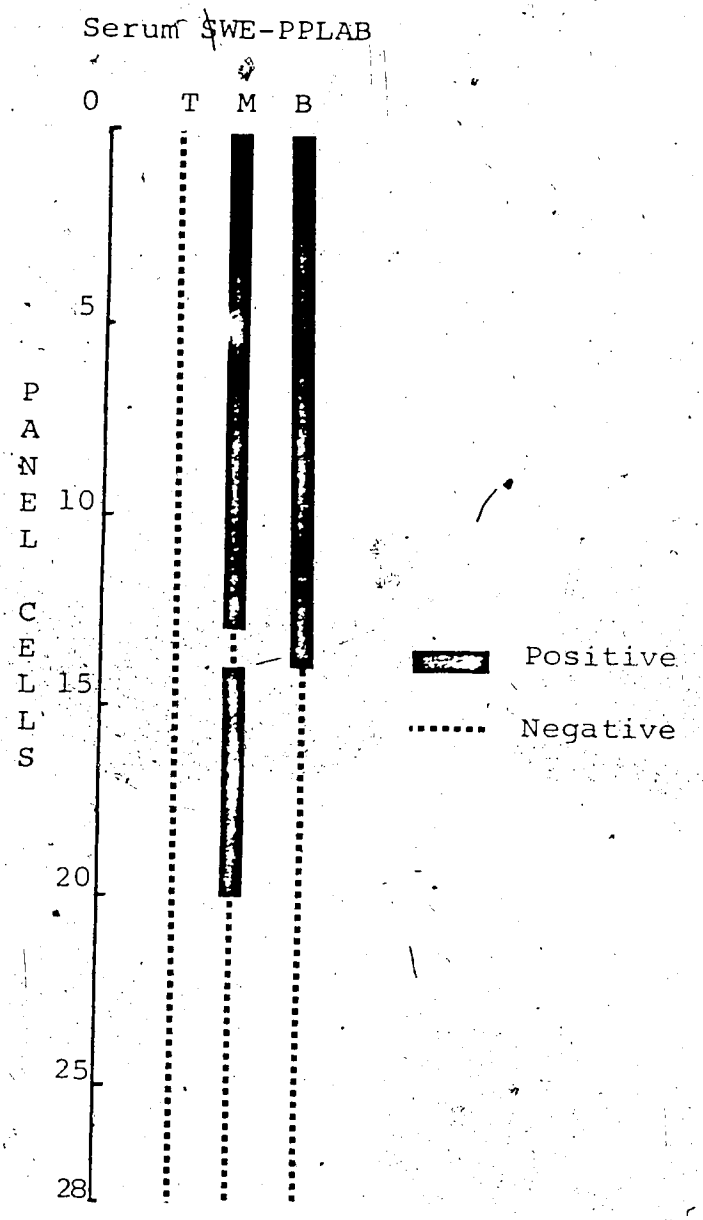


Figure 10 Histogram showing ADCC results of serum SWE (after platelet absorption) with 28 panel cells - T,M and B. T reactivity was negative with all. 13 showed both M and B reactivity, one showed reactivity against B alone and 6 with M alone. Both B and M were negative against 8.

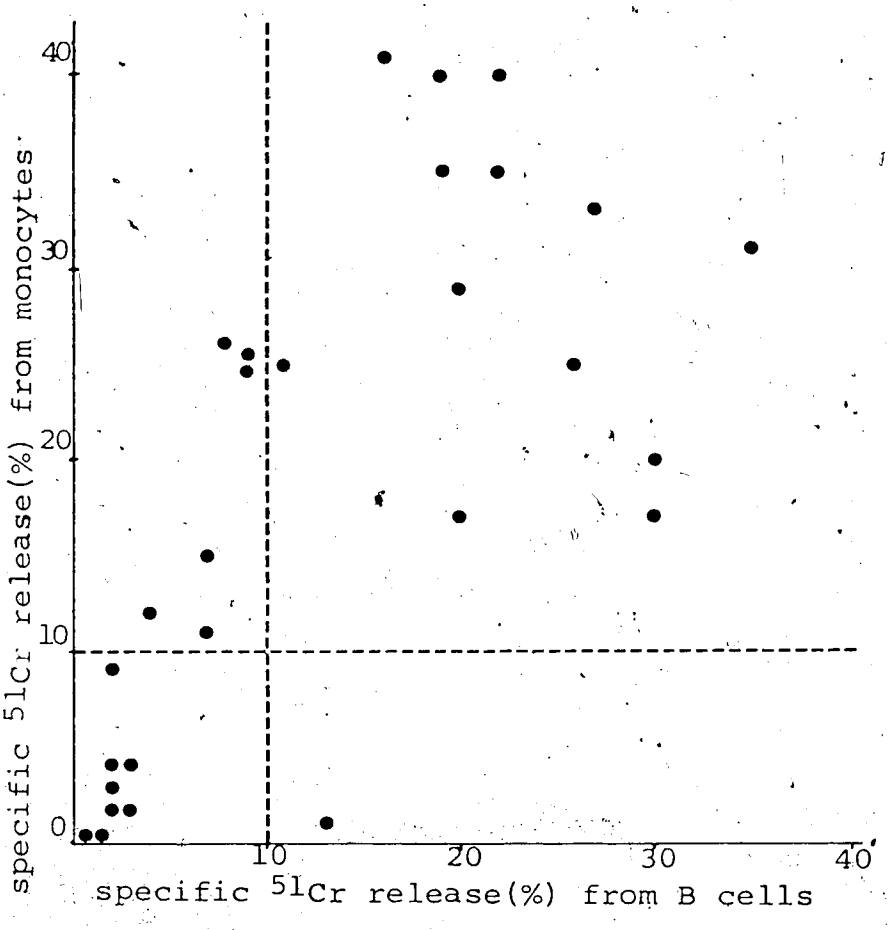


Figure 11 Scattergram showing specific ^{51}Cr release from 28 panel monocytes and B cells by serum SWE (after platelet absorption). 13 showed both M and B reactivity with specific ^{51}Cr release of $\geq$ 10%. One showed reactivity with B alone and 6 with M alone. Both M and B were negative nst 8.

Table 20 ADCC reactions of serum SWE-PPLAB[a] and CLLAB[b] with panel cells

Targets		Specific ^{51}Cr release(%) PPLAB 1/6	1/12	CLLAB 1/6	1/12	PPLAB	CLLAB
1. LMAR	M	1	1	2	-1	-	-
	B	12	10	3	1	+	-
2. SNAK	M	39	32	19	5	+	+
	B	19	14	7	7	+	-
3. MJOH	M	32	28	22	21	+	+
	B	13	10	5	5	+	-
4. TKOV	M	40	38	32	27	+	+
	B	22	23	3	6	+	-
5. SSAI	M	26	17	7	7	+	-
	B	11	10	-1	-2	+	-
6. AJOH	M	35	33	9	5	+	-
	B	22	13	6	3	+	-
7. VJOH	M	35	22	5	3	+	-
	B	19	20	6	7	+	-
8. DDRE	M	29	17	6	3	+	-
	B	21	13	6	1	+	-
9. KBET	M	25	27	13	5	+	-
	B	26	21	6	6	+	-
10. JSLY	M	20	20	-3	10	+	-
	B	30	24	2	3	+	-
11. VLAO	M	14	13	7	9	+	-
	B	28	20	14	8	+	+
12. KJOH	M	31	28	7	2	+	-
	B	35	35	11	7	+	+
13. JDOS	M	17	16	9	3	+	-
	B	30	23	10	6	+	+

Table 20 continued

Targets		Specific ^{51}Cr release(%) PPLAB 1/6	1/12	CLLAB 1/6	1/12	PPLAB	CLLAB
14. JLI	M	26	22	18	12	+	+
	B	8	7	2	6	-	-
15. DMAC	M	12		12		+	+
	B	8		2		-	-
16. NSHI	M	15	15	10	6	+	+
	B	7	6	1	-1	-	-
17. JSCH	M	26	24	28	24	+	+
	B	9	5	3	6	-	-
18. CFAL	M	8	12	7	3	+	-
	B	4	0	-1	-1	-	-

[a]SWE-PPLAB - 12 panel members had M and B reactivity
- 5 showed M alone and one with B alone

[b]SWE-CLLAB - Complete removal of B reactivity with all except 3
- M reactivity persisted with 7 (4 with prior M alone and 3 with prior M and B)

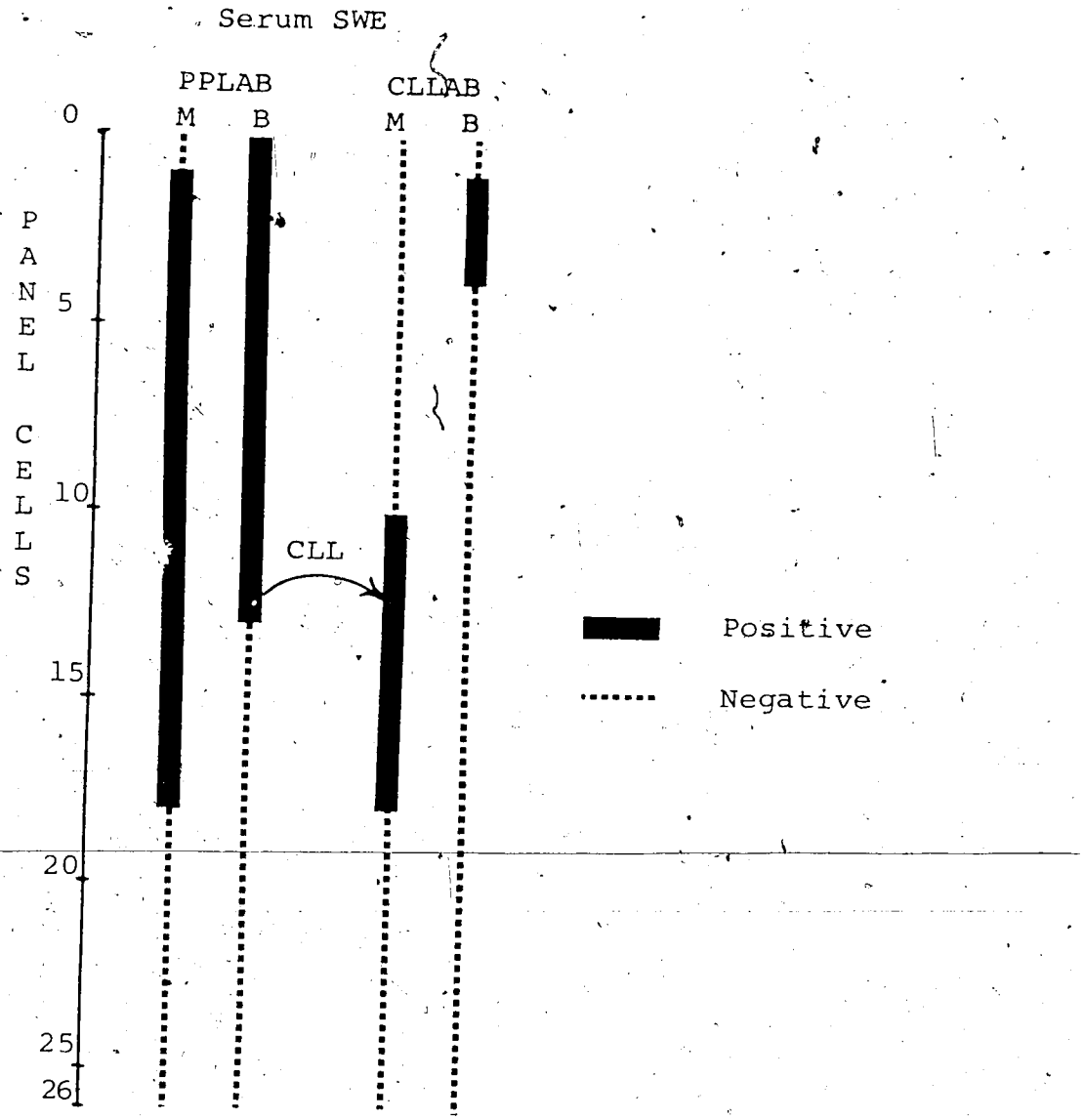


Figure 12 Histogram showing absorption analysis of serum SWE (after platelet and CLL absorption) by ADCC method. Pooled platelet absorbed SWE serum when tested with 26 panel members, showed both M and B reactivity against 12, reactivity with B alone in one and M alone in 5. Both M and B were negative against 8. CLL absorption removed B reactivity against all except 3. Monocyte specific reactivity persisted against 7 (4 with prior M reactivity alone and 3 with both M and B).

in 1/5 and 1/10 dilution in ADCC with 18 panel members, 12 with M and B reactivity, 5 with M alone and one with B alone. Post absorption serum failed to react with all but three B cells. For these three cells absorption appeared to have been incomplete, the residual B reactivity being weak (Table 20 and Fig. 12). Monocyte specific reactivity persisted in seven instances, four with prior M reactivity alone and three with prior M and B reactivity. The latter three had much stronger M reactivity compared to B reactivity on prior testing. It is concluded that this multiparous serum contained both monocyte specific and B specific reactivity, perhaps of equal magnitude.

4) Serum MJU.

This multiparous serum was tested with peripheral blood subpopulations from 28 panel members after removal of HLA-A,B,C antibodies by pooled platelet absorption. There was B and monocyte reactivity with 13 panel members; monocytes alone with 7 and B alone with none. In 8 anel members there was no demonstrable M or B reactivity (Table 21 and Fig. 13 and 14).

CLL cells from two patients which reacted with the serum were used for absorption. GLL absorbed serum

Table 21 ADCC reactions of serum MJU-PPLAB with panel cells*

	Targets	Specific ^{51}Cr release (%) M	B
1.	MJOH	30	33
2.	DMAC	21	21
3.	KJOH	25	23
4.	MHIG	18	11
5.	VPAZ	30	14
6.	TKOV	36	12
7.	AJOH	11	12
8.	NSHI	11	12
9.	VLAO	36	21
10.	JDOS	15	14
11.	MLON	39	36
12.	MDAS	11	15
13.	CJAC	16	11
14.	SNAK	29	5
15.	CFAL	26	5
16.	JPAZ	19	6
17.	JSCH	15	6
18.	VJOH	15	8
19.	ELIB	26	6
20.	LMAR	22	7
21.	JSLY	-4	-13
22.	KBET	-10	-6
23.	SSAI	2	3
24.	CMIL	0	1
25.	DDRE	5	5
26.	JMUR	-1	1
27.	SSHI	1	3
28.	AWIL	-2	3

* Specific ^{51}Cr release of $\geq$10% was considered as positive.
1/10 dilution of serum was used in these experiments
13 panel members showed M and B reactivity
7 showed M alone
M and B negative with 8

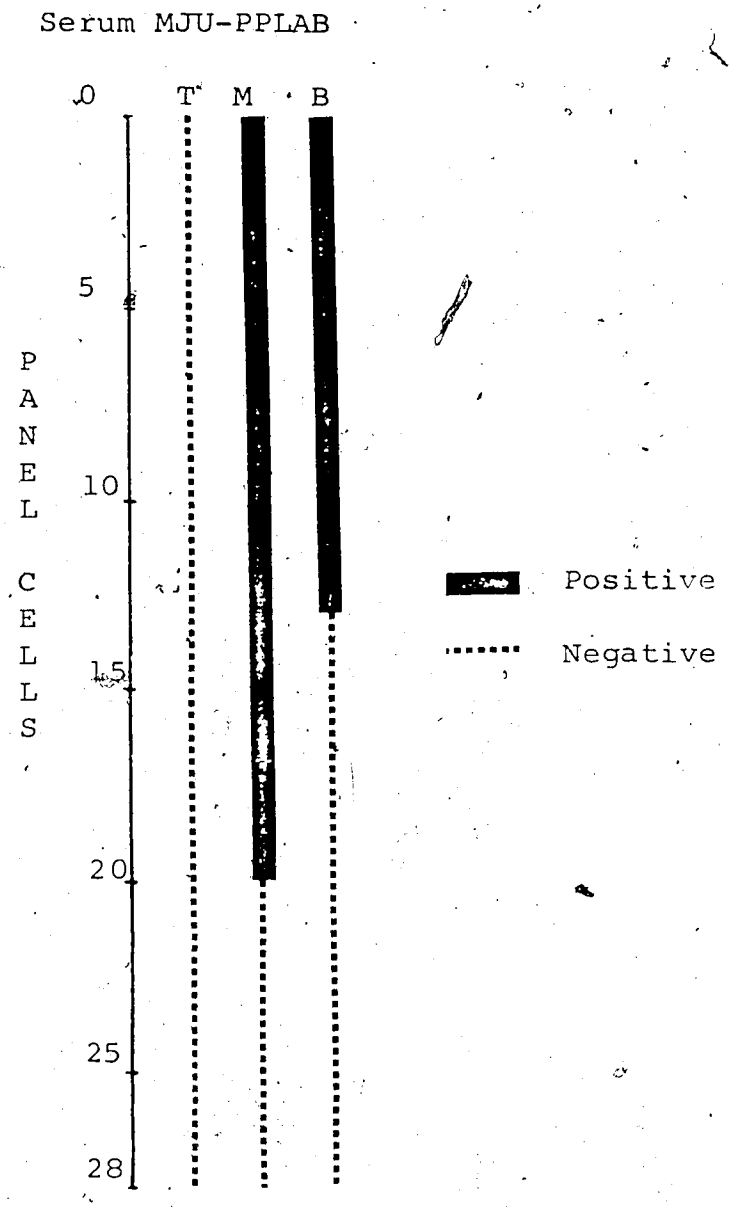


Figure 13 Histogram showing ADCC results of serum MJU (after platelet absorption) with 28 panel cells - T,M and B. T reactivity was negative with all. 13 showed both M and B reactivity; 7 showed reactivity with M alone. M and B were negative against 8.

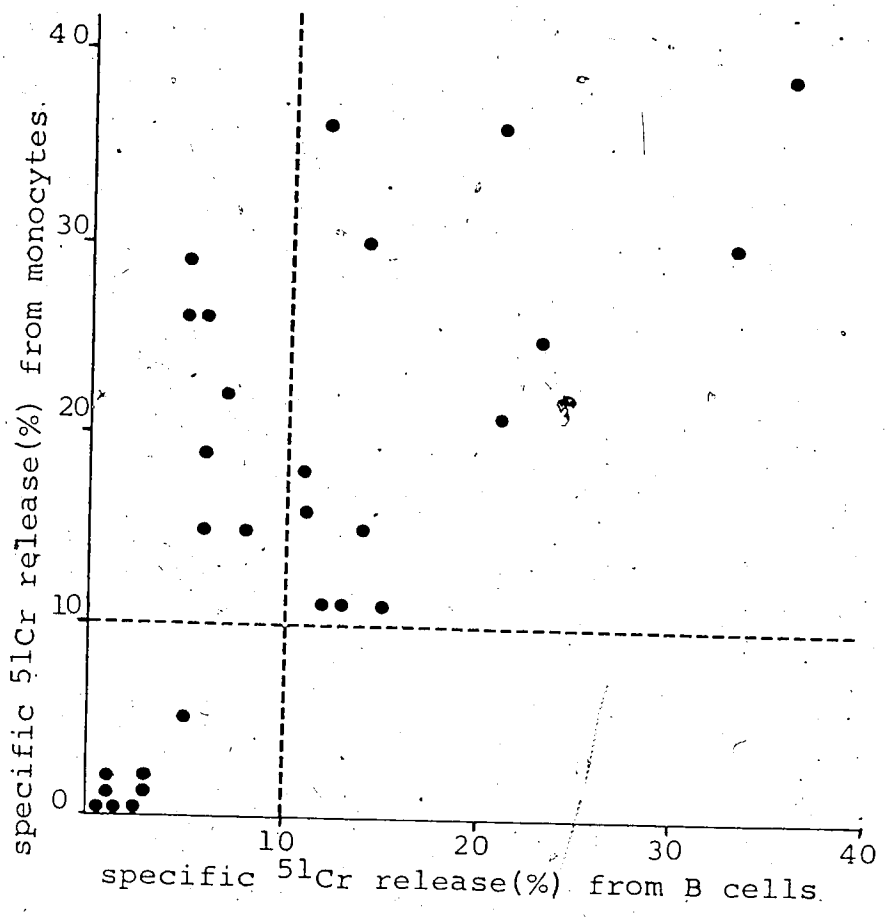


Figure 14 Scattergram showing specific ^{51}Cr release from panel monocytes and B cells (28) by serum MJU (platelet absorbed). 13 showed both M and B reactivity with specific ^{51}Cr release of $\geq$ 10% (interrupted lines). 7 showed reactivity against M alone. Both M and B were negative with 8.

Table 22 ADCC reactions of serum MJU-PPLAB[a] and CLLAB[b] with panel cells

Targets		Specific ^{51}Cr release (%) PPLAB 1/10	PPLAB 1/20	CLLAB 1/10	CLLAB 1/20	PPLAB	CLLAB
1. CFAL	M	26	22	20	5	+	+
	B	5	2	1	5	-	-
2. SNAK	M	29	26	12	4	+	+
	B	5	5	6	3	-	-
3. DMAC	M	21		7		+	-
	B	21		0		+	-
4. NSHI	M	11	7	-1	-1	+	-
	B	12	5	7	-4	+	-
5. JSCH	M	9	11	-3	5	+	-
	B	1	5	-7	-8	-	-
6. MJOH	M	21	21	8	6	+	-
	B	12	13	5	1	+	-
7. TKOV	M	36	35	7	7	+	-
	B	12	14	7	1	+	-
8. KJOH	M	30		8		+	-
	B	31		9		+	-
9. JDOS	M	15	13	3	3	+	-
	B	14	14	3	1	+	-
10. LMAR	M	22		1		+	-
	B	7		2		-	-
11. MDAS	M	11	7	5	1	+	-
	B	15	-3	0	-1	+	-
12. CJAC	M	16	7	5	2	+	-
	B	11	9	-1	-1	+	-

[a]MJU-PPLAB - 8 panel members had M and B reactivity
4 had M alone

[b]MJU-CLLAB - Complete removal of B reactivity with all M reactivity persisted with 2

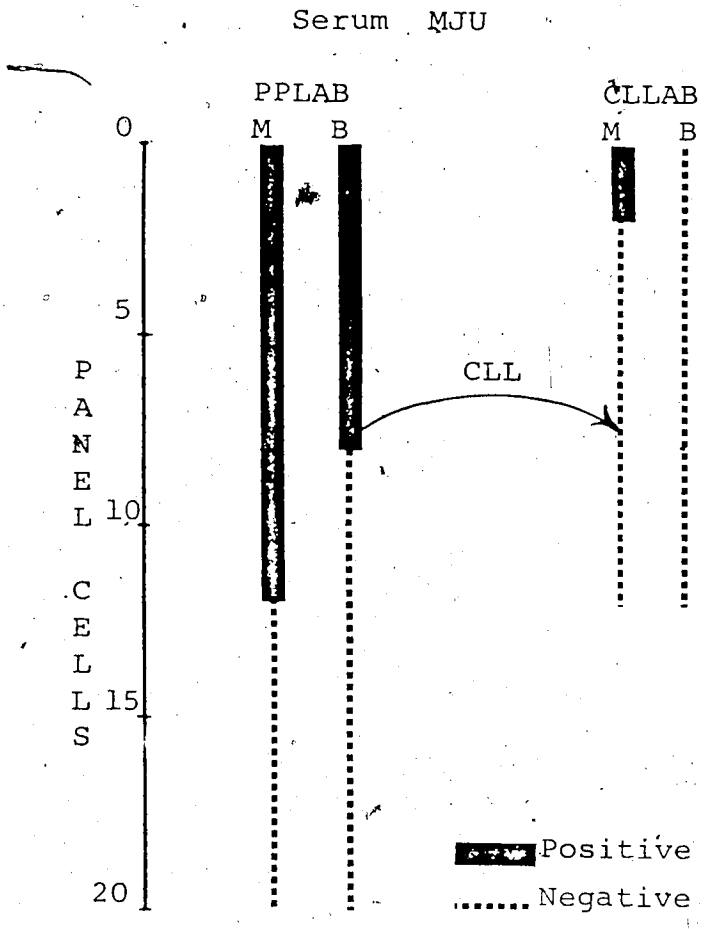


Figure 15 Histogram showing absorption analysis of serum MJU (pooled platelet absorbed and CLL absorbed) by ADCC method. Pooled platelet absorbed MJU serum when tested with 20 panel members showed both M and B reactivity with 8 and 4 showed reactivity against M alone. 8 were negative with both M and B. CLL absorption removed B reactivity against all whereas M reactivity persisted with 2.

failed to react with any of the tested B cell panel, whereas M reactivity persisted with two (Table 22 and Fig.15). The 12 panel members used in repeat testing included 8 with M and B reactivity and 4 with M alone in prior testing. It is inferred that this serum contained both M and B specific reactivity, predominantly the latter.

5) Serum G256.

This serum from a multiparous woman was tested with B lymphocytes and monocytes from 20 panel members after pooled platelet absorption. Both M and B reactivity was present with 17 panel members, M alone with one and B alone with none. There was no demonstrable M or B reactivity with 2 panel members (Table 23 and Fig.16 and 17).

CLL cells from two patients with strong reactivity with this serum were used for absorption. CLL absorbed serum was tested with 13 panel members, all having M and B reactivity. CLL absorbed serum failed to react with all panel B cells (Table 24 and Fig.18). M reactivity persisted with two. This serum contained monocyte and B specific reactivity, but predominantly B specific reactivity.

Table 23 ADCC reactions of serum G256 B-PPLAB with panel cells*

Targets	specific ^{51}Cr release (%) M	B
1. TKOV	42	26
2. JSLY	18	18
3. SNAK	29	16
4. MJOH	16	14
5. KJOH	33	20
6. VLAO	38	18
7. JMUR	31	16
8. CMIL	11	14
9. LMAR	21	30
10. JLI	21	22
11. SSAI	16	16
12. NSHI	26	21
13. KBET	36	14
14. CJAC	19	10
15. VJOH	30	17
16. AJOH	32	25
17. JSCH	35	11
18. JPAZ	12	4
19. CFAL	0	5
20. DDRE	9	9

* Specific ^{51}Cr release of $\geq$10% was considered positive. 1/10 dilution of the serum was used in these experiments. 17 panel members showed M and B reactivity. M alone with 1, M and B negative with 2.

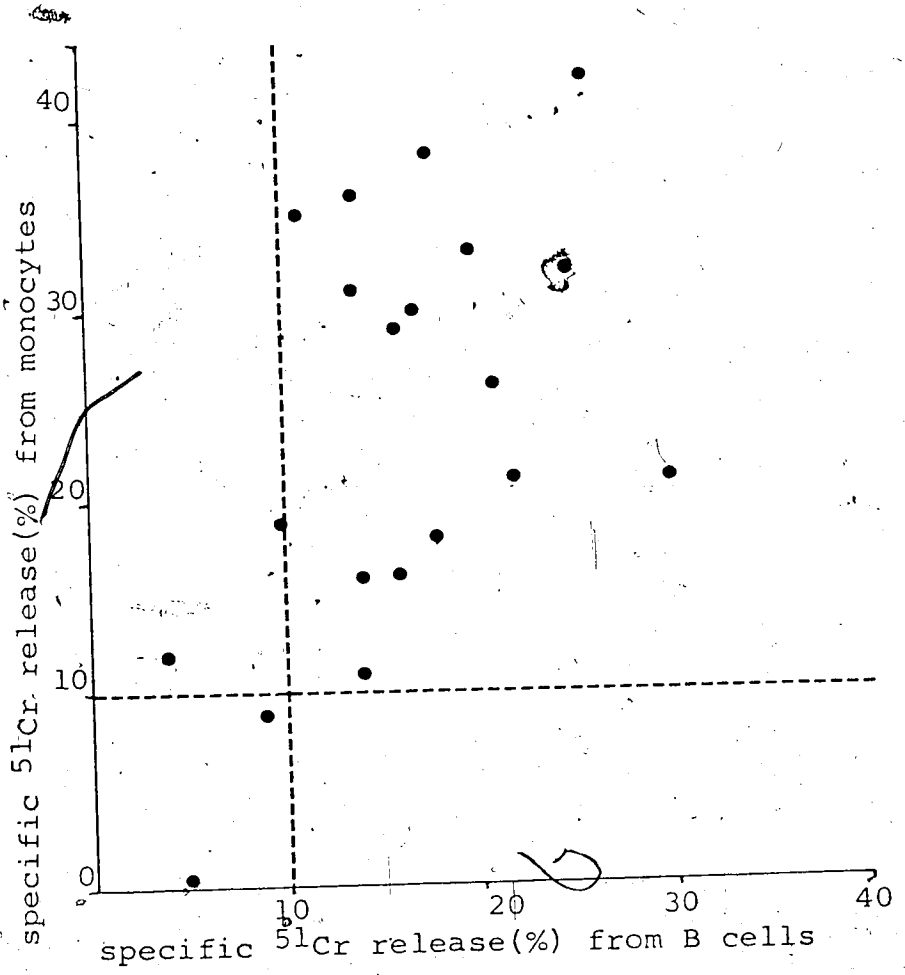


Figure 16 Scattergram showing specific ^{51}Cr release from 20 panel monocytes and B cells by serum G256 (platelet absorbed). 17 showed both M and B reactivity with specific ^{51}Cr release of $\geq$ 10% (interrupted lines), 1 showed reactivity against M alone and both M and B negative with 2.

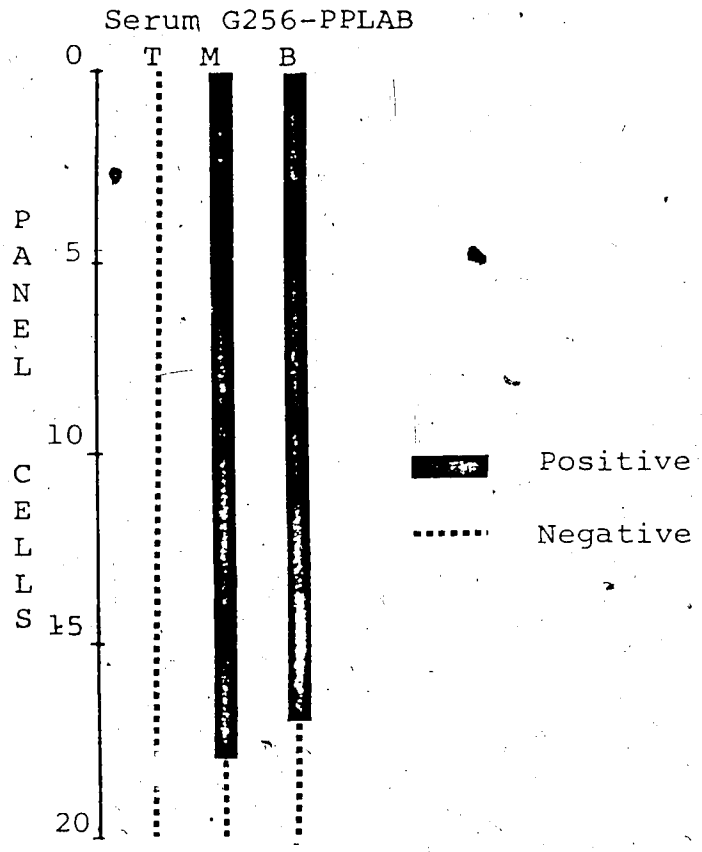


Figure 17 Histogram showing ADCC results of serum G256 (after platelet absorption) with 20 panel cells- T,M and B. T reactivity was negative with all. 17 showed both M and B reactivity, 1 showed reactivity against M alone and both M and B were negative with 2.

Table 24 ADCC reactions of serum G256 B-PPLAB[a] and CLLAB[b] with panel cells

Targets		specific ^{51}Cr release (%) PPLAB 1/10	PPLAB 1/20	CLLAB 1/10	CLLAB 1/20	PPLAB	CCLAB
1. KBET	M	36	27	14	14	+	+
	B	14	11	-1	-1	+	-
2. TKOV	M	42	45	15	8	+	+
	B	26	23	10	3	+	-
3. JLI	M	21	14	1	5	+	-
	B	22	16	-3	-3	+	-
4. NSHI	M	26	28	1	4	+	-
	B	21	40	4	5	+	-
5. SSAI	M	16	13	-1	-3	+	-
	B	16	14	-6	-3	+	-
6. JSCH	M	35	28	4	3	+	-
	B	11	11	-1	-1	+	-
7. SNAK	M	31	44	4	6	+	-
	B	25	17	8	10	+	-
8. MJOH	M	16	15	-1	3	+	-
	B	14	11	3	1	+	-
9. JSLY	M	18	21	7	2	+	-
	B	18	19	6	6	+	-
10. KJOH	M	21	16	-6	-10	+	-
	B	11	19	-1	1	+	-
11. LMAR	M	21	16	11	9[c]	+	-
	B	30	28	11	8[c]	+	-
12. JMUR	M	31	33	6	4	+	-
	B	16	26	1	-1	+	-
13. CJAC	M	19	17	2	3	+	-
	B	10	8	-8	-6	+	-

[a] G256B PPLAB - All 13 panel members had M and B reactivity

[b] G256B CLLAB - Complete removal of B reactivity against all M reactivity persisted against two

[c] Considered as negative

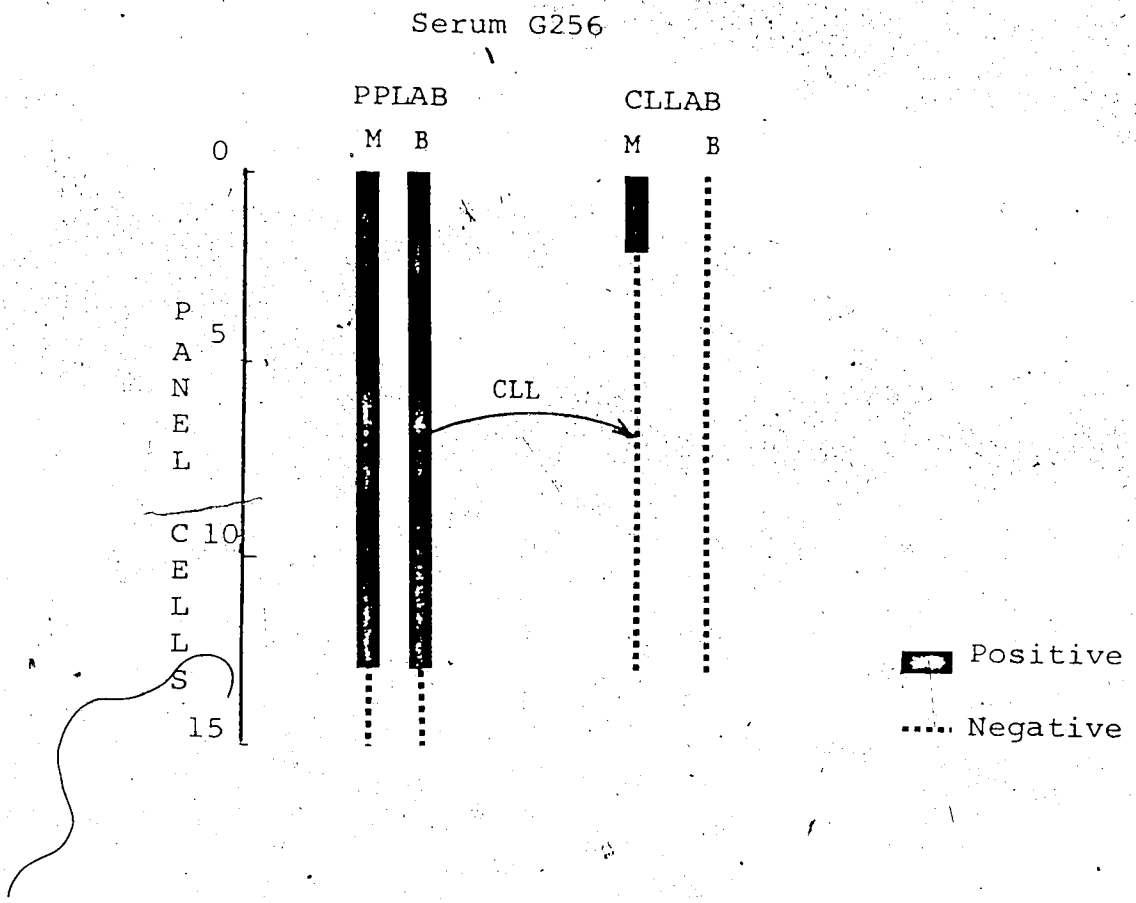


Figure 18 Histogram showing absortpion analysis of serum G256 (pooled platelet absorbed and CLL absorbed) by ADCC method. Pooled platelet absorbed serum G256 when tested with 15 panel members showed both M and B reactivity against 13 and 2 were negative with both M and B. CLL absorption removed B reactivity against all (13) whereas M reactivity persisted with two.

6) Serum 5018B.

This multiparous serum was known to have broad B reactivity. When the pooled platelet absorbed serum was tested with 19 panel members, B and M reactivity was present against 9 and no demonstrable B or M reactivity with the remaining 10 (Table 25 and Fig.19).

CLL cells from two patients which reacted with the serum was used for absorption. CLL absorbed serum was tested with 6 panel members all reacting with M and B. This showed (Table 26 and Fig.20) that CLL absorption removed both M and B reactivity against all panel members tested. Therefore this serum appeared to be a B cell specific serum without any monocyte specific reactivity.

Figure 21 illustrates the monocyte specific reactivity in 5 sera (2 transplant and 3 multiparous). Serum RCL shows broad monocyte specific reactivity compared to other sera. Monocyte specific and B cell specific reactions shown by the 6 broadly reactive sera are shown together in Fig. 22 along with the HLA-DRw antigens of the panel members.

Figure 23 illustrates the removal of B cell specific antibodies in these 6 broadly reactive sera by CLL absorption and the persistent monocyte specific activity

Table 25 ADCC reactions of serum 5018 B-PPLAB with panel cells*

Targets	specific ^{51}Cr release (%) M	B
1. SNAK	22	22
2. KJOH	21	22
3. VPAZ	16	13
4. MJOH	12	13
5. LMAR	17	19
6. NSHI	10	14
7. CFAL	23	10
8. MHIG	21	10
9. JPAZ	24	15
10. SSAI	1	1
11. JSCH	6	3
12. DDRE	5	4
13. KBET	5	3
14. JSLY	2	2
15. TKOV	2	1
16. JMUR	1	1
17. CJAC	-1	4
18. CMIL	0	1
19. SSHI	2	0

* Specific ^{51}Cr release of $\geq$10% was considered as positive.
1/8 dilution of serum was used in these experiments.
B and M reactivity was present against 9
No B or M reactivity with remianing 10

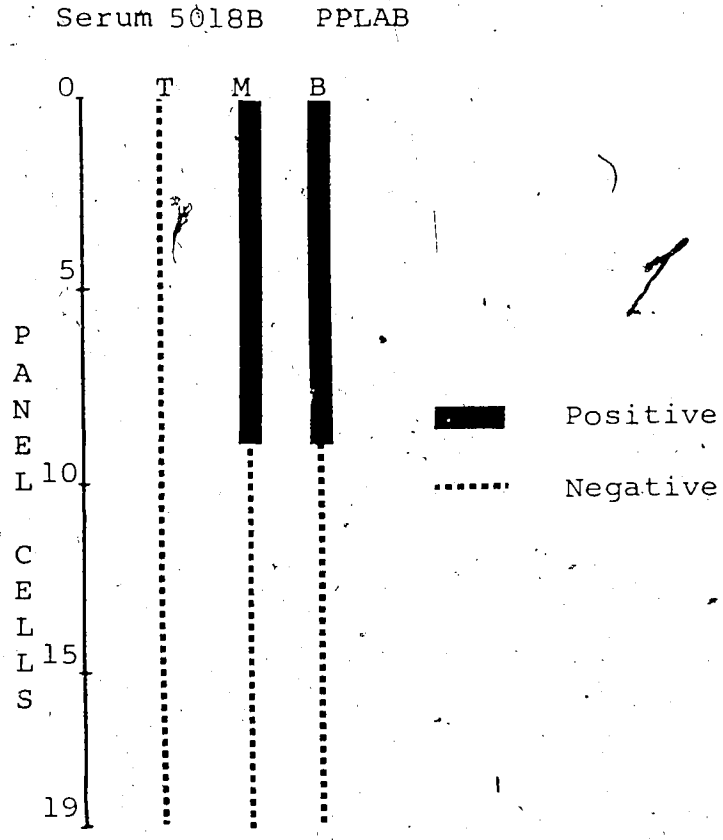


Figure 19 Histogram showing ADCC results of serum 5018B (platelet absorbed) with 19 panel cells - T,M and B. T reactivity was negative with all. Both M and B reactivity were present against 9 and both M and B were negative against 10.

Table 26 ADCC reactions of serum 5018 B-PPLAB[a] and CLLAB[b] with panel cells.

Targets		Specific ^{51}Cr release (%) PPLAB 1/8	PPLAB 1/16	CCLAB 1/8	CCLAB 1/16	PPLAB	CLLAB
1. SNAK	M	21	24	2	-5	+	-
	B	22	14	3	5	+	-
2. MJOH	M	11	1	-1	4	+	-
	B	13	10	1	1	+	-
3. KJOH	M	12	10	7	9	+	-
	B	12	12	7	3	+	-
4. LMAR	M	17	9	2	3	+	-
	B	19	12	4	3	+	-
5. NSHI	M	10	4	0	2	+	-
	B	14	13	3	3	+	-
6. CFAL	M	23	13	-5	2	+	-
	B	10	6	-1	1	+	-

[a] 5018 B-PPLAB - All 6 panel members showed M and B reactivity

[b] 5018 B-CLLAB - M and B reactivity removed against all

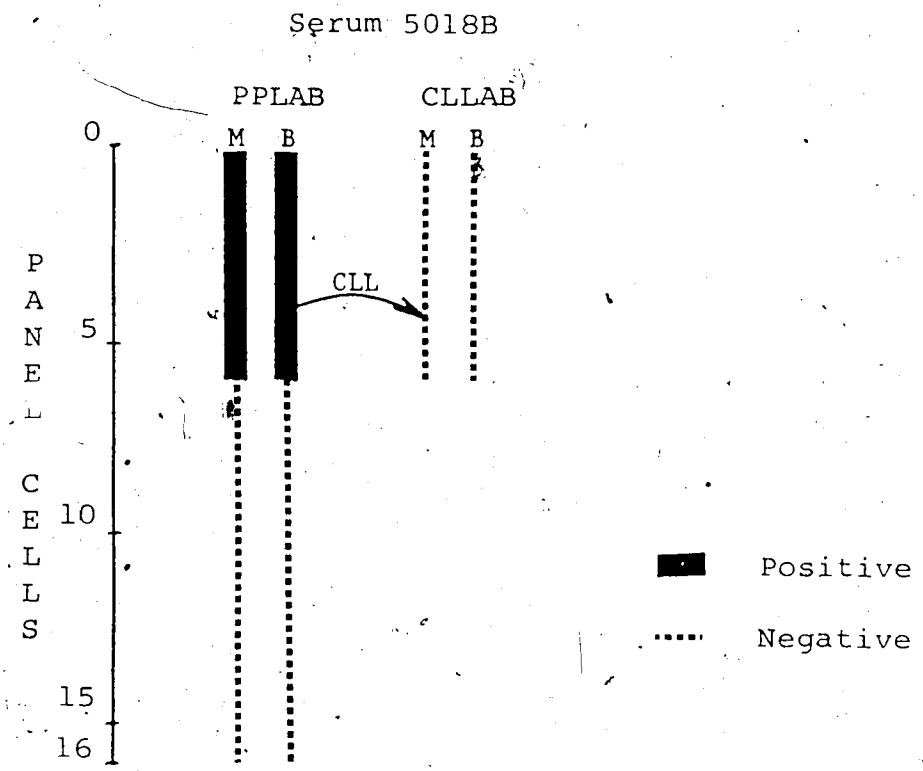


Figure 20 Histogram showing absorption analysis of serum 5018B (platelet absorbed and CLL absorbed) by ADCC method. Pooled platelet absorbed serum 5018B when tested with 16 panel members 6 showed both M and B reactivity, none with reactivity against M alone and 10 negative with both M and B. CLL absorption removed both M and B reactivity against all.

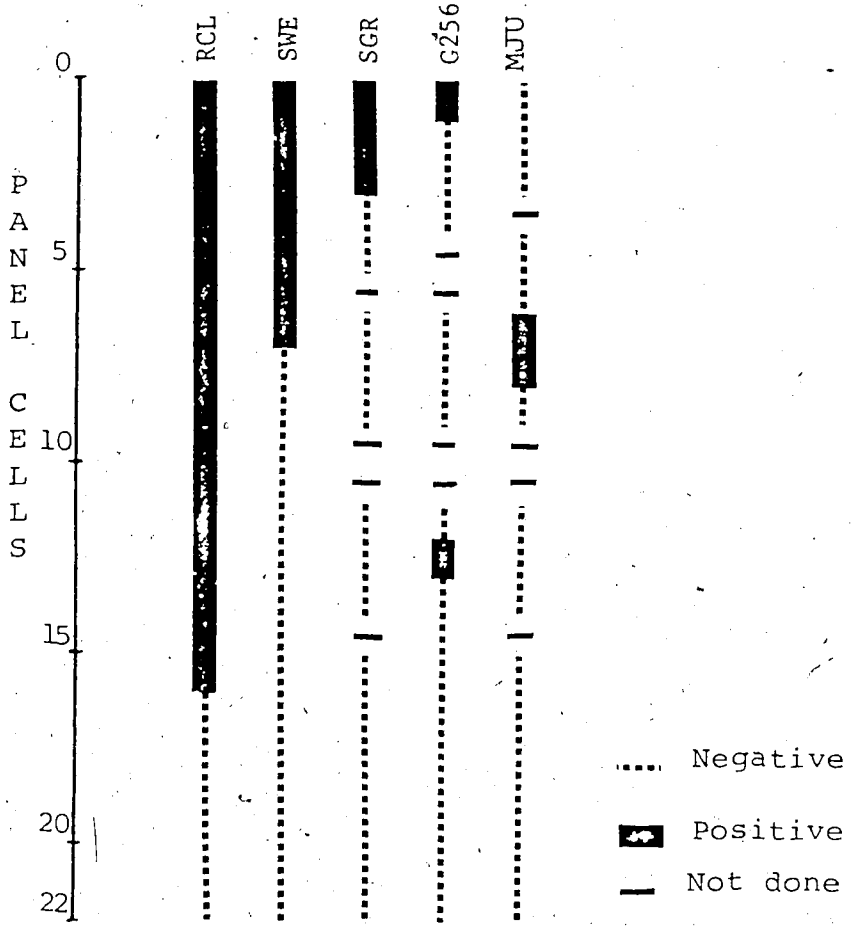
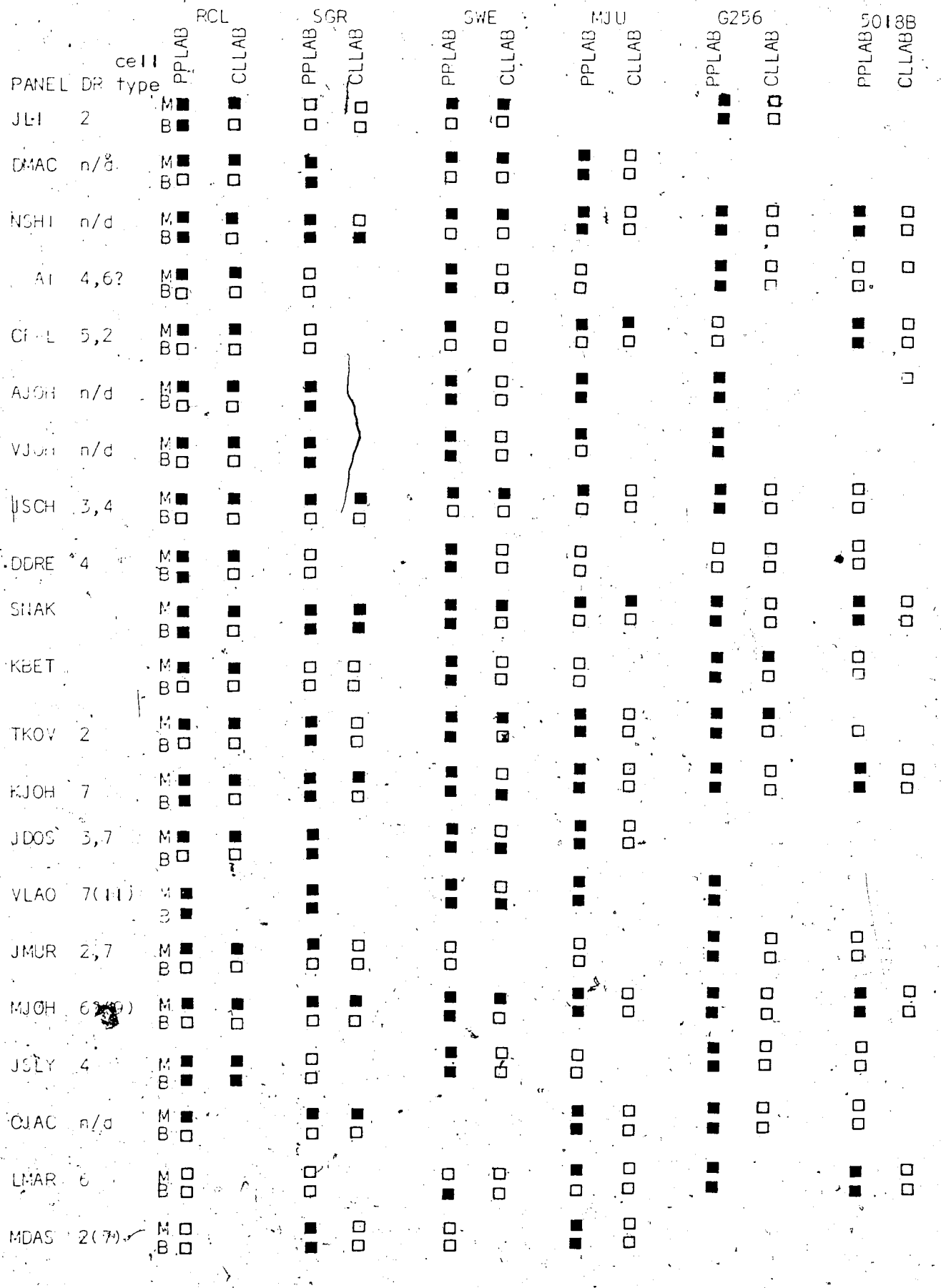


Figure 21 Histogram showing monocyte specific ADCC reactivity of 5 broadly reactive sera (2 transplant and 3 multiparous) after CLL absorption. Serum RCL showed broad monocyte specific reactivity (16/22). The other 4 sera, SWE (7/22), SGR (3/18), G256 (2/18) and MJU (2/18) showed more restricted monocyte specific reactivity.

Figure 22 (Right) Distribution of monocyte and B cell specific reactions of 6 broadly reactive sera (after pooled platelet absorption) with regard to the HLA-DRw antigens of panel members. Closed squares indicate positive and open squares negative.

Panel	DR	cell type	RCL PPLAB M	RCL PPLAB B	SGR PPLAB M	SGR PPLAB B	SWE PPLAB M	SWE PPLAB B	MJU PPLAB M	MJU PPLAB B	G256 PPLAB M	G256 PPLAB B	5018B PPLAB M	5018B PPLAB B
1.JLI	2		■	■	□	□	■	□			■	■		
2.DMAC	n/d		■	□	■	■	■	□	■	■				
3.NSHI	n/d		■	■	■	■	■	□	■	■	■	■	■	■
4.SSAI	4,6?		■	□	□	□	■	■	□	□	■	■	□	□
5.CFAL	5,(2)?		■	□	□	□	■	□	■	□	□	□	■	■
6.AJOH	n/d		■	□	■	■	■	■	■	■	■	■		
7.VJOH	n/d		■	□	■	■	■	■	■	□	■	■		
8.JSCH	3,4		■	□	■	□	■	□	■	□	■	■	□	□
9.DDRE	4		■	■	□	□	■	■	□	□	□	□	□	□
10.SNAK			■	■	■	■	■	■	■	□	■	■	■	■
11.KBET			■	□	□	□	■	■	□	□	■	■	□	□
12.TKOV	2		■	□	■	■	■	■	■	■	■	■	□	□
13.KJOH	7		■	■	■	■	■	■	■	■	■	■	■	■
14.JDOS	3,7		■	□	■	■	■	■	■	■				
15.VLAO	7(11)		■	■	■	■	■	■	■	■	■	■		
16.JMUR	2,7		■	□	■	■	□	□	□	□	■	■	□	□
17.MJOH	6?(9)		■	□	■	□	■	■	■	■	■	■	■	■
18.JSLY	4		■	□	□	□	■	■	□	□	■	■	□	□
19.CJAC	n/d		■	□	■	□			■	■	■	■	□	□
20.LMAR	6		□	□	□	□	□	■	■	□	■	■	■	■
21.MDAS	2(7)		□	□	■	■	□	□	■	■				
22.JPAZ	3		■	□	■	■	□	□	■	□	■		■	■
23.MLON	n/d				■	□	□	□	■	■				
24.CMIL	n/d		□	□	□	□	■	■	□	□	■	■		
25.MHIC	n/d		■	□	■	□	□	□	■	■			■	■
26.ELIE	'd		□	□	■	□	□	□	■	□				
27.AWIL	n/d		□	□			■	□	□	□				
28.LFAL	5				■	■								
29.SSHI	2,5		□	□	□	□	□	□	□	□				
30.VPAZ	3,6				■	■	□	□	■	■			■	■

Figure 23 (Right) This figure shows the distribu- of B and M specificities (after pooled platelet and CLL absorption) against panel members. DR typing of the panel members is also shown on the left hand side. Closed squares indicate positive and open squares negative. The pattern of reactivity with regard to known HLA-DRw antigens suggests that serum RCL contains DRw4 plus some other specificity. The broad nature of M specificity in this serum is evident. (? Public M specificity or multiple M specificities) Serum SWE contains DRw4 and probably additional weak DRw specificity(s). Monocyte specificity in this serum appears to be more restricted than that of serum RCL. Serum SGR, MJU and G256 contain undefined DR specificities and narrowly reacting M specificities. Serum 5018B shows only B specificity and no M specificity.


RCL
SGR
SWE
MJU
G256
5018B
PPLAB
CLLAB
PANEL
DR
cell type
JLI 2
DMAC n/d
NSHI n/d
AI 4,6?
CI L 5,2
AJOH n/d
VJOH n/d
JSCH 3,4
DDRE 4
SNAK
KBET
TKOV 2
KJOH 7
JDOS 3,7
VLAO 7(11)
JMUR 2,7
MJOH 6?(9)
JSLY 4
CJAC n/d
LMAR 6
MDAS 2(7)
M
B

in 5 of these sera. DRw antigens of the panel members are shown to demonstrate the pattern of reactivity. Distribution of the pattern of reactivity with regard to known HLA-DRw antigens suggests that serum RCL contains DRw4 plus some other DRw specificity(s). The broad nature of M specificity is evident (? public M specificity or multiple M specific antibodies). Serum SWE contains DRw4 and probably an additional weak DRw specificity. The monocyte specificity appears to be more restricted than that of serum RCL. The other three sera SGR, MJU and G256 contain undefined DR specificities in addition to the weaker M specificity. Serum 5108 shows no M specificity.

E. Prozone in ADCC against monocytes.

Table 27 illustrates prozone phenomenon observed with three sera (RCL, SGR and G256) in ADCC against monocyte. This phenomenon was more frequently observed when monocytes are used as targets compared with B cells.

F. Family segregation study.

Initial screening of 6 families with serum RCL known to have monocyte specific antibodies showed reactivity

Table 27 Prozone in ADCC with monocytes

PANEL CELLS	RCL (PPLAB) 1/5	1/10	1/20	1/40	SGR (PPLAB) 1/5	1/10	1/20	G256 (PPLAB) 1/10	1/20
1	-	25	34	-					
	-	6	17	-					
	4	9	18	-					
	6	10	17	-	15	21	-		
5	6	17	17	19	8	18	25		
					19	24	-		
7								31	44

Note the negative or lower readings with lower dilutions of sera compared to higher dilutions - prozone phenomenon.

with both parents, hence segregation study could not be informative with RCL serum.

Serum SWE was studied in the same six families; for 3 families both parents were positive and in one both parents were negative. Detailed studies were performed in the other two families, viz "FER" and "SWE".

1) "FER" family study.

As shown in Fig. 24, Terry, the father showed both M and B cell reactivity whereas Ruth, the mother, showed only M specificity. The father's HLA haplotypes were identified as A_2, B15 (a) and A_2Bw50 (b) and those of the mother, A_3Bw50 (c) and Aw32B_{14} (d). Children Brian and Laurie received "a" haplotype from the father and "c" haplotype from the mother. Both these children like the father, showed M and B reactivity. In contra- Bruce who received "b" haplotype from the father and "d" from the mother showed only monocyte specific reactivity. This indicated that the haplotype "d" of the mother carried M specific reactivity which was inherited by Bruce with identical reaction.

2) Family SWE.

Serum SWE was studied in the family of the serum donor,

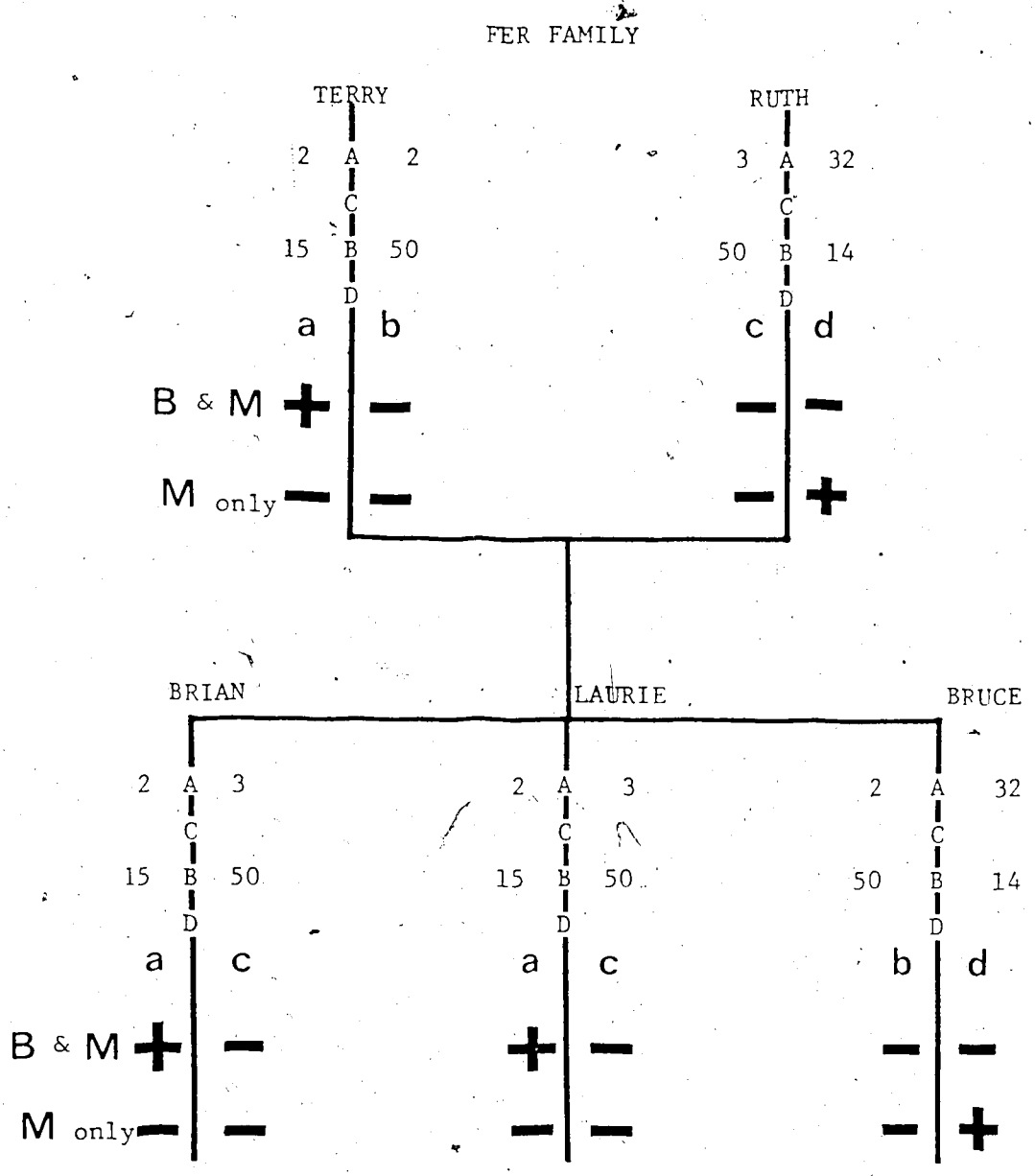


Figure 24 Segregation of M specificity of serum SWE (pooled platelet absorbed) by ADCC in family "FER". See text for interpretation.

Table 28 ADCC reactions of serum SWE-PPLAB with members of family "SWE"**

Targets		specific 51_{Cr} release (%)* 1/16	1/12	
1. Clarence(father)	M	38	43	+
	B	43	40	+
2. Ann	M	11	8	+
	B	3	1	-
3. Edward	M	22	19	+
	B	1	0	-
4. Fred	M	19	15	+
	B	5	3	-
5. Silvia	M	34	35	+
	B	9	8	-
6. John	M	10	6	+
	B	56	51	+
7. Adrian	M	23	24	+
	B	36	26	+

* Specific 51_{Cr} release of ≥10% was considered positive.

** Clarence(father) and two children, John and Adrian showed B and M reactivity
The other 4 children, Ann, Edward, Fred and Silvia showed M alone

See Fig.25 for HLA-A,B,C and DRw antigens of SWE family members

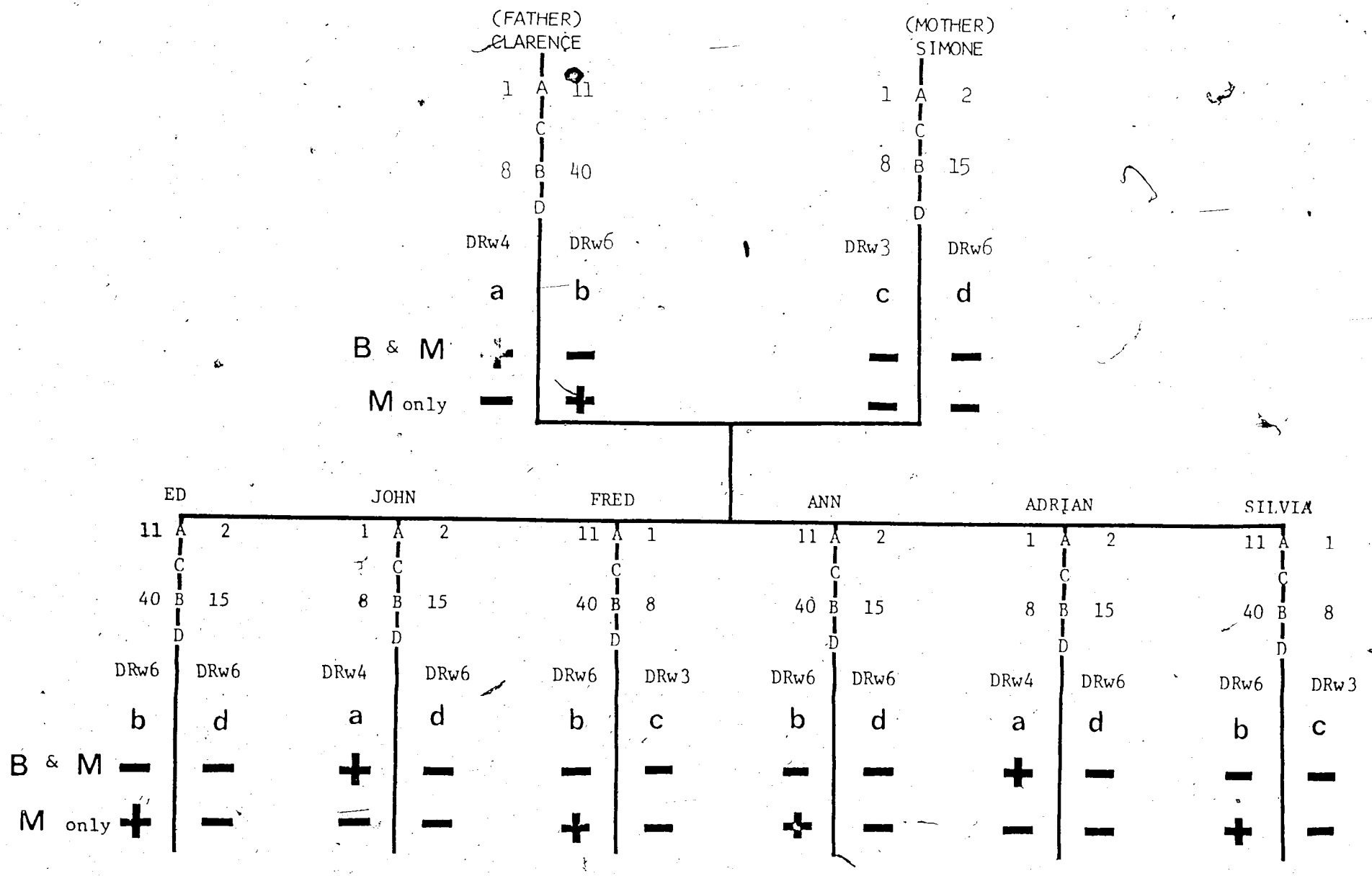


Figure 25 Segregation of M specificity of serum SWE (platelet absorbed) by ADCC in family "SWE". See text for interpretation.

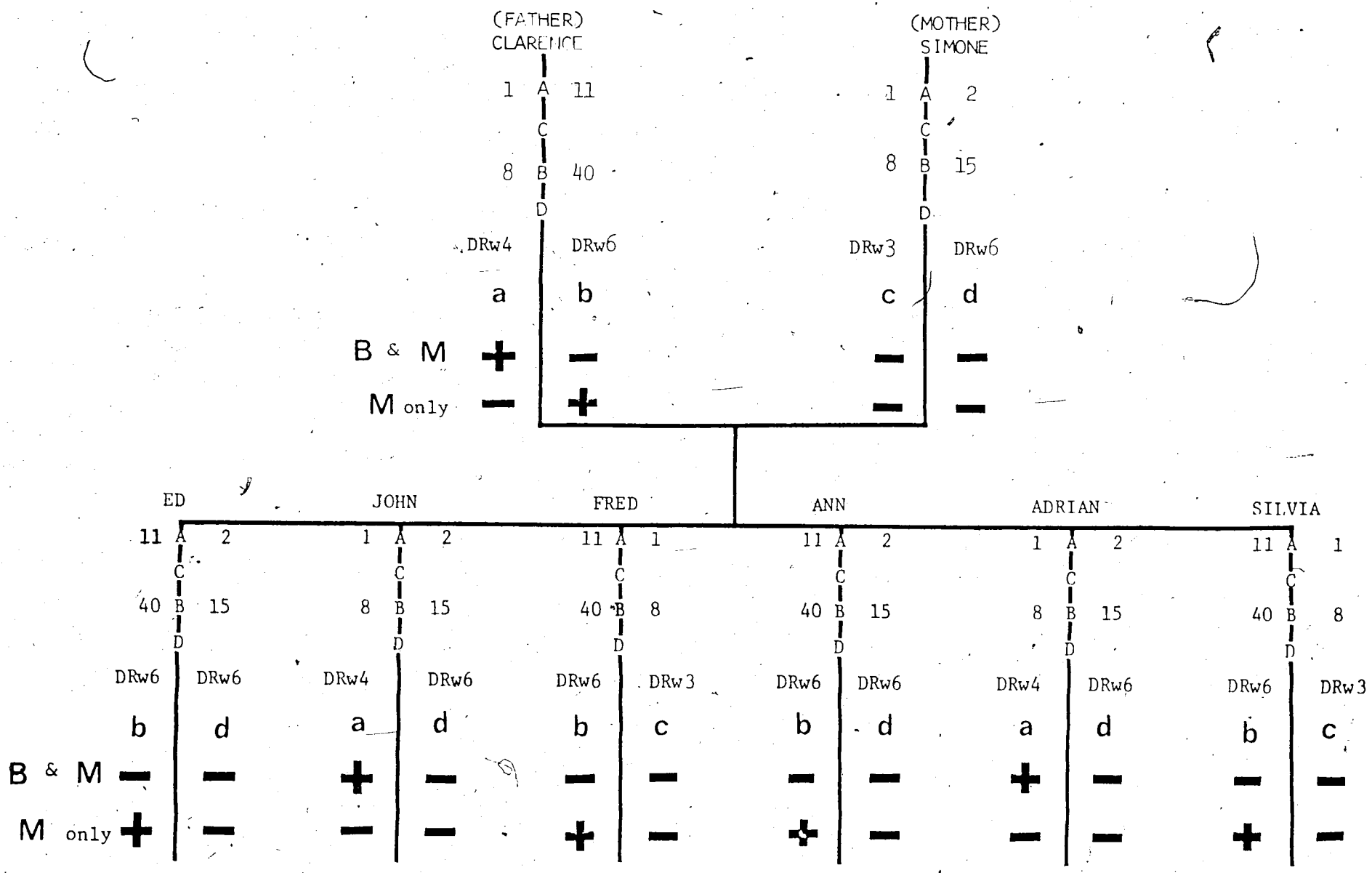


Figure 25 Segregation of M specificity of serum SWE (platelet absorbed) by ADCC in family "SWE". See text for interpretation.

a multiparous mother of 10 pregnancies. The serum had been found to have both B specific and M specific antibodies as described earlier (see page 80). Her HLA haplotypes were identified as $A_1B_8DRw_3$ (c) and $A_2B_{15}DRw_6$ (d), and those of her husband Clarence as $A_1B_8DRw_4$ (a) and $A_{11}B_{40}DRw_6$ (b) (see Fig.25 and Table 28). The husband showed strong monocyte and B reactivity when tested with his wife Simone's serum (SWE).

Two children, John and Adrian, who received the "a" haplotype from the father, showed both M and B reactivity though the former was weaker. The other four children Ed, Fred, Ann and Sylvia, who received the "b" haplotype from the father, expressed only monocyte specific reactivity but no B cell reactivity. These results indicated that the M specific reactivity was linked to the "b" haplotype of the father.

Simone's serum (SWE) is also known to have DRw_4 antibodies based on the observation at the 3rd North American Histocompatibility Workshop. Since monocytes also bear DR antigens Clarence, John and Adrian, all sharing the "a" haplotype bearing DRw_4 specificity showed M and B cell reactivity with the mother Simone's

serum. It could be inferred from these observations that M specificity was linked to the "b" haplotype of the father and the B specificity (DRw_4) to the "a" haplotype.

IV. Discussion and Conclusions

A. Discussion.

Our study of monocyte specific antigens was stimulated by two observations.

First, a group of antigens expressed specifically on endothelial cells and monocytes but not detectable on T or B lymphocytes aroused considerable interest since recent reports from Stastny and associates (Moraes and Stastny 1977a,b). The antibodies could not be absorbed with platelets or B lymphocytes but were readily removed by absorption with monocytes or endothelial cells. Pre-treatment of these cells with anti-human B2-microglobulin blocked subsequent lysis by endothelial-monocyte (E-M) antibodies suggesting that these were associated with B2-microglobulin and were distinct from HLA-DR. It was further suggested on the basis of family studies that most but not all monocyte specific reactions segregated in linkage with HLA.

Since vascular endothelium appears to be an important target in renal allograft rejection, those newly defined endothelial antigens also present on monocytes have aroused special interest in view of their possible effect on transplant survival. Further discussion of possible biological significance of monocyte specific

antigens will follow later.

The second reason that prompted our interest in monocyte specific antigens was the observation made by many investigators (van Rood et al, 1975a,b; Bodmer et al, 1976 and 1978) that, as a rule the reactivity of anti-DR sera is broader than corresponding Dw specificity. Since most routinely prepared B cell enriched preparations often contain variable number of monocytes, the extra-reactions in anti-DR sera could be due to the presence of monocyte specific antibodies.

Our search for monocyte specific antibodies was started by screening 28 serum samples from post transplant patients collected during episodes of rejection, 7 multiparous sera with known broad reactivity and 5 SLE sera (Table 2).

The presence of monocyte specific activity was detected in 32% of post transplant sera (9/28). However, only 7% (2 patients) had strong reactivity whereas the other 25% (7 patients) showed weak reactivity. The incidence of monocyte specific reactivity in multi-parous sera could not be assessed from this study as these sera were selected on the basis of known broad reactivity. All SLE sera were negative.

Higher frequency of occurrence of monocyte specific antibodies was reported by Stastny and associates (Moraes et al,1977) in 70 multiparous and 40 post transplant sera (38% and 70% respectively). However, much lower frequency (1%) was observed by Cicciarelli et al,(1978) in a study of 35 sera (13 post transplant and 22 multiparous(?)). CDC technique by dye exclusion was used by Cicciarelli et al,whereas both CDC and PDA methods were employed by Stastny and associates. Our studies indicated that the ADCC technique was significantly more sensitive than complement dependent methods (Fig,4). This technique was hence used for the analysis of serum reactivity and detection of antigenic specificities on separated subpopulations of PBL (T,B and M) throughout our study. High sensitivity of ADCC technique became particularly important in view of multiple absorptions that were necessary in preparation of sera for detection of monocyte specific antibodies (Fig.2). Repeated absorptions usually resulted in a significant dilution of the serum sample contributing to low sensitivity of complement dependent methods.

The removal of anti HLA-A,B,C antibodies was achieved using pooled platelet absorption. Though

absorption with platelets of the required type would be more economical as to the number of platelets needed, use of pooled platelets (from 70-100 donors) was very efficient and convenient since large stocks of outdated platelets could be kept at 4°C and used as required. In our experiments sera had to be absorbed 3-5 times to ensure complete removal of HLA-A,B,C antibodies.

Since monocytes also bear DR antigens on their surface (Wernet,1976; Stastny,1978a; Drew et al,1978) removal of DR antibodies from the serum is important for detection of monocyte specific antigens. This could be achieved by absorption with chronic lymphatic leukaemia (CLL) cells or pooled B lymphocytes. CLL cells form a convenient source of B-enriched population since they have high lymphocyte counts of which 90% may have the Ia bearing characteristics of B lymphocytes. Thus an almost pure preparation of Ia bearing cells can be obtained without complicated separation needed for B lymphocytes (Bodmer,1978; Gosset et al,1977). In our experiments two absorptions with 150 x 10^6 CLL cells were required to remove the B specificity when specific CLL cells were used.

Several technical modifications were introduced in the process of our study which contributed to the purity of cell preparations and sensitivity and reproducibility of the assay.

E rosetting of PBL followed by sedimentation on F-H was used to separate the rosetted T cells from non-rosetted cells. Pre-treatment of SRBC with neuraminidase (Bach and Dardene, 1972; Weiner et al,1973) was found to be essential for stabilization of rosettes, thereby facilita ng easy handling and increasing purity in preparation. Our results of E rosetting of 67-77% in 21 panel members is comparable to other reports (Kaplan et al,1976). The purity of T cells so separated was over 90% (Table 4). Non-rosetted population showed only ~ 5-10% T cell contamination on re-rosetting.

Non-rosetted cells were adhered on plastic surfaces for separation of monocytes (adherent) and B lymphocytes (non-adherent). The B-enriched population (11±3% of PBL) showed S.Ig positivity by immunofluorescence in 76% (Table 4). Monocyte enriched preparations in this study (8±3% of PBL) gave 86±6% positivity by acridine orange staining (Table 4).

The problem of clumping of monocytes (when used as targets in ADCC) was largely overcome with the use of EDTA

in the suspending medium. With this modification the background readings were often less than 20%.

As in other ADCC systems, pre-selection of donors with high effector cell activity was critical as otherwise false negatives resulted from poor killing of target cells. Adult males were preferred as effector cell donors since females and young children were reported to have low effector cell activity (Kovithavongs et al,1974a).

Difference in uptake of ^{51}Cr (Kovithavongs et al, 1977) by monocytes, B cells and T cells (Table 7) did not interfere with the results of the assay since purified subpopulations of PBL were used as targets. It could be speculated that the ^{51}Cr uptake was a function of the size of the cell itself, to explain the high uptake by monocytes over B and T lymphocytes.

Twenty eight sera from transplant patients were obtained from frozen samples collected during periods of rejection. Of these, five patients were known to have broad HLA reactivity in CDC using dye exclusion method. Further analysis of the broad reactivity in these sera was possible with the use of ADCC as a sensitive method for detection of monocyte specificity. Two of these 5 broadly reactive transplant sera showed monocyte specific

reactivity in addition to B specificity. The other three sera had only B specificity.

Serum RCL was one of the sera that showed strong monocyte specific reactivity. This serum was obtained from a patient who had two previous transplants in the preceding 6 years and received several blood transfusions leading to broad tissue sensitization. Monocyte reactivity was examined one month after a third transplant from a pre-transplant CDC cross match negative cadaver donor. Post transplant period was not complicated by any clinical rejection episodes. This serum showed the presence of M and B specific reactivity against 14/27 and 7/27 panel members respectively (Fig.5 and Table 13). Absorption with pooled B cells or CLL cells removed B cell specific reactivity sparing M specificity (Table 16 and 17 and Fig.8) whereas absorption with pooled monocytes removed the M and B specificities confirming the presence of M specificity in this serum.

Serum SGR from another transplant patient also showed strong monocyte specific reactivity. This serum was from a female patient two months after transplantation. Post transplant period was threatened by several episodes of rejection leading to graft loss subsequently. Serum SGR showed more predominant B cell specific

reactivity (13/29) than monocyte specific reactivity (6/29) (Table 14 and Fig.7). Monocyte specific reactivity appeared to be more restricted than that of RCL (4/29, serum after CLL absorption) (Table 18 and Fig.9).

Seven serum samples were obtained from multiparous women. They all showed broad reactivity when examined by ADCC against B and M subpopulations. However, monocyte specific activity was demonstrated in only 3 of them (Table 2). B specificity was more dominant in all sera except serum SWE which showed persistent M reactivity in 7/18 panel members after CLL absorption (Fig.12 and Table 20).

CLL absorption removed both M and B reactivity from serum 5108 indicating the B cell specificity of the serum 5108 without M specificity (Fig.20 and Table 26).

The pattern of reactivity of 6 sera (2 transplant and 4 multiparous) after pooled platelet absorption and CLL absorption, against separated M and B cells of 21 panel members is represented in Fig.23. Sera SGR, SWE, MJU and G256 showed relatively narrow range of M reactivity whereas RCL appeared to be considerably broader (Fig.21). This broad reactivity of the serum might indicate either the presence of multiple monocyte

specific antibodies or a public monocyte specificity. An attempt was made to clarify this question using absorption with monocytes of a single positive panel member. Complete removal of reactivity against the panel member as well as the rest of the panel members would then indicate a public specificity. In contrast persisting reactivity with some of the panel members would suggest multiple private monocyte specific antibodies. Results of this study was inconclusive since complete removal of the reactivity to the absorbing panel members' monocytes could not be achieved. This could have been due to the low yield of monocytes obtained from peripheral blood (8±3% of PBL). In this experiment the amount of monocytes obtained from 200 ml. of peripheral blood was not sufficient for complete absorption of 0.2 ml. of ½ diluted RCL serum. According to our calculations more than 500 ml. of whole blood would be necessary to absorb this amount of serum. Because of the difficulty in performing absorption analysis of monocyte specific antisera, the question of the number of alleles controlling monocyte specific antigens can not be answered at the moment. None of the antisera so far analyzed, showed mutually exclusive (allelic) relationship. Further monocyte specific antisera should

be searched for and analysed before the answer to this question could be provided.

The number of genetic loci involved in the control of monocyte specific antigens is also so far unknown. Stastny,(1977) indicated, that most but not all monocyte specific reactions segregated in linkage with HLA. In his recent report (Stastny,1978) he suggested that antibodies to monocyte specific antigens could be directed against two kinds of surface products, one containing B2-microglobulin and the other without it. He also reported that reactions that are blocked by anti-B2-microglobulin can be shown to be inherited in linkage with HLA.

We have attempted to analyze segregation of monocyte specific antigens in two informative families. Family studies with serum SWE (multiparous) were clearly indicative of M specificty linked to HLA haplotype. Linkage was demonstrated when serum SWE was studied within her family (Fig.25) as well as in another family ("FER" family). The relatively narrow but strong specificity helped in clearly isolating this linkage to HLA haplotype. In contrast the HLA segregation of M specificity in RCL could not be defined since both parents' monocytes reacted with serum in all the six

families studied. This was probably related to the broad nature of M specific reactivity in serum RCL.

Our results of family studies should be considered rather preliminary. These have demonstrated linkage between HLA and the locus controlling monocyte specific antigens (M-locus). To make any definite conclusion as to the position of this locus on chromosome 6 (inside or outside the HLA complex) it is essential to perform extensive studies in numerous families. It is especially important to include into this analysis the families with known HLA recombinants and perform studies of other 6 chromosomal markers in order to map M locus on chromosome 6.

Although the biological role of monocyte specific antigens is not as yet clear it is conceivable that they could play an important role in transplant survival. This assumption is based on the observation by Stastny, (1977) that monocyte specific antigens are expressed on endothelial cells and on the well known fact that endothelial lining of blood vessels in a transplant is often a primary target for the attack by recipient's lymphocytes (Hirschberg and Thorsby, 1975). Using endothelial cells from umbilical cord veins and monocytes from cord blood Moraes and Stastny, (1977a,b) demonstrated that these cells

share an antigenic moiety which is distinct from HLA-A,B,C and DR.

The problem of biological significance of monocyte specific antigens in transplant survival could be approached in two ways. First, the typing for monocyte specific antigens could be done on donors and recipients and the degree of compatibility could be correlated with post transplant course. The pre-requisite for this approach is to have a battery of anti-monocyte sera detecting as many different specificities as possible. Also, the genetic control of monocyte specific antigens (number of loci, extend of polymorphism, extend of linkage with HLA) must be clarified first.

The second approach would involve the analysis of anti-monocyte activity in pre-transplant (possible pre-sensitization) and post transplant sera (sequential study). The correlation between occurrence of anti-monocyte activity and graft function, if observed, might provide evidence as to the role of monocyte antigens in histocompatibility. However, a clear delineation of the role of monocyte antibodies to transplant rejection is possible only when it could be isolated from the deleterious effect of the other better defined antigens of the HLA complex. Antibodies to HLA-A,B,C loci has

been widely accepted as deleterious to transplant survival whereas the critical role of DR antibodies is not clearly defined (Ettenger et al 1977; Silberman et al 1978; Myburg and Smith, 1978; Suthanthiran et al, 1978). The role of cell mediated tissue damage as examined by the lymphocyte mediated cytolysis (LMC) test has been shown to be associated with poor graft survival (Garovoy et al 1973; Grunnet et al 1975; Stiller et al 1976; Kovithavongs et al, 1978a). Application of a battery of cell mediated and serological tests is important in deciding the outcome of the graft.

In this study of 28 patients examined for monocyte specific antibodies during rejections, 9 patients showed their presence, 2 of which were strongly reactive. Of these 9 patients with M specificity two also had both cytotoxic HLA antibodies and LMC reactivity. Four patients had either cytotoxic HLA antibodies or positive LMC. Thus in all these 6 instances, the critical role of monocyte specific antibodies to graft rejection could not be separated from the deleterious role of cytotoxic HLA antibodies and cell mediated immunity (LMC). Another 3 patients with monocyte specific reactivity had neither LMC nor HLA antibodies demonstrable. However, the M reactivity in all of them was only weak, and also one of

these patients subsequently had an excellent graft function at three years follow-up.

Stastny (1977) and Cerilli (1977) have both shown that the presence of endothelial antibodies following transplantation are associated with poor graft outcome. The former group has further demonstrated that monocyte and endothelial antigens are identical thus attributing a critical role to M specific antibodies in transplant rejections. While the detection of endothelial antibodies in the absence of HLA-A,B,C antibodies was shown to be deletrious, they had not excluded the pathogenetic role of cell mediated immunity (by LMC test) in those patients. Thus the critical contribution of monocyte specific antibodies to graft outcome needs further definition.

B. Conclusions.

Following conclusions can be drawn from the studies in this thesis:

1. Monocyte specific antibodies may be present in transplant and multiparous serum. This may probably reflect the repeated immunogenic challenge due to blood transfusions, organ transplantation and pregnancies.

2. Compared to complement dependent methods ADCC is a more sensitive method in detecting monocyte specific antibodies.

3. Extra reactions in B reactive sera may partly be due to the presence of monocyte specific antibodies, as most B-enriched preparations often contain variable numbers of monocytes.

4. The biological significance of monocyte specific antibodies needs further clarification since the sera with strong monocyte specific reactivity also had cytotoxic HLA antibodies.

5. Monocyte specificity may segregate with HLA haplotypes in families.

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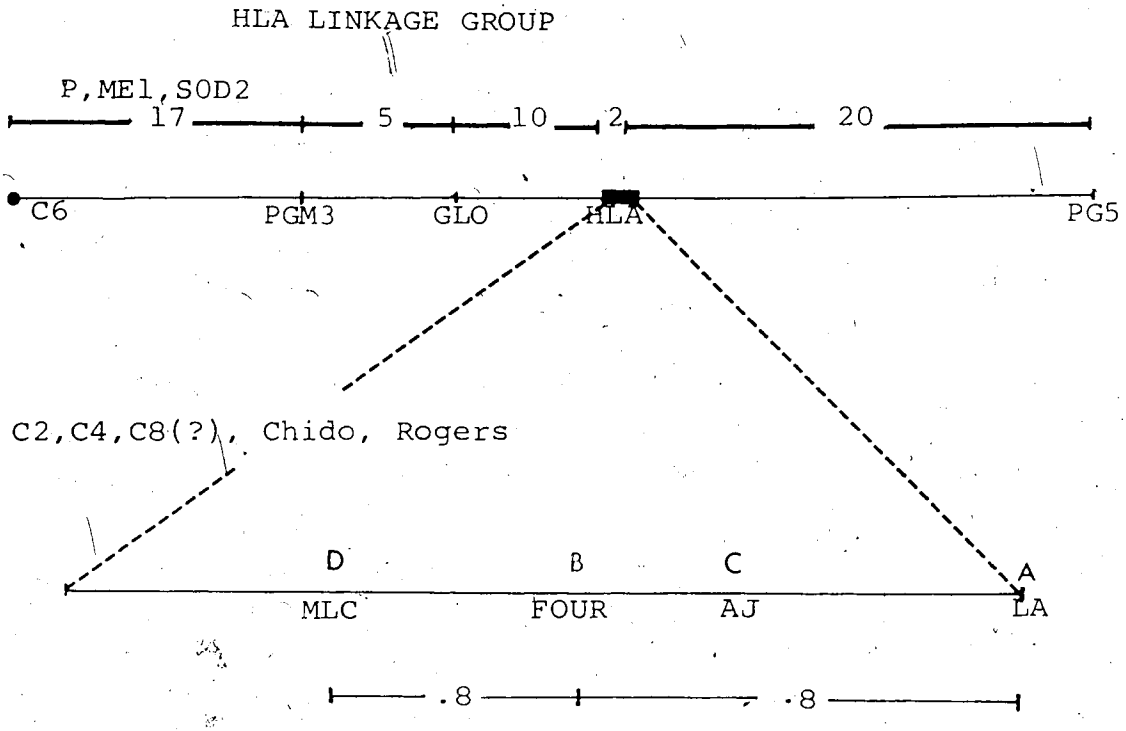


Appendix A. Schematic representation of the HLA supergene

From: van Rood, J.J., van Leeuwen, A., Termijtelen and Keuning, J.J.. B cell antibodies, Ia-like determinants and their relation to MLC determinants in man. Transplant. Rev. 30,122,1976.

Appendix B Nomenclature for factors of the HLA system - 1977

Locus A	Previous
A1	
A2	
A3	
A9	
A10	
A11	
A25(10)	AW25
A26(10)	AW26
A28	
A29	
AW19	
AW23(9)	
AW24(9)	
AW30	
AW31	
AW32	
AW33	
AW34	
AW36	
AW43	

Locus B	Previous
BW4	W4,4a
BW6	W6,4b

Locus B	Previous
B5	
B7	
B8	
B12	
B13	
B14	
B15	BW15
B17	BW17
B18	
B27	
B37	BW37
B40	BW40
BW16	
BW21	
BW22	
BW35	
BW38(16)	
BW39(16)	
BW41	
BW42	
BW44(12)	B12(notTT*)
BW45(12)	TT*
BW46	HS,SIN2
BW47	407*,M066, CAS,BW40C
BW48	KSO,JA, BW40.3
BW49(21)	BW21.1, SL-ET
BW50(21)	BW21.2, ET*
BW51(5)	B5.1
BW52(5)	B5.2
BW53	HR
BW54(22)	BW22j,SAP1 SN1,J1

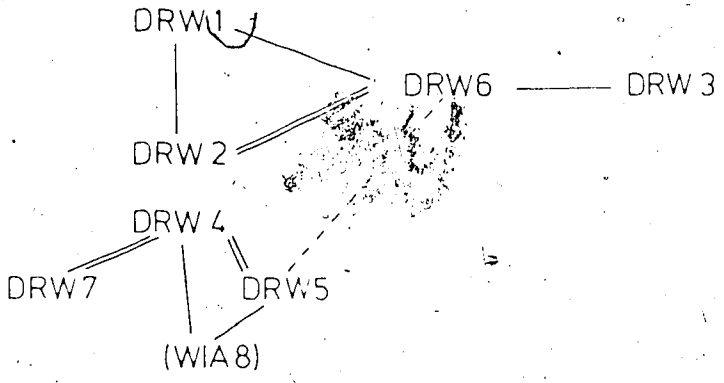
Locus C	Previous
CW1	
CW2	
CW3	
CW4	
CW5	
CW6	T7

Locus D	Previous
DW1	
DW2	
DW3	
DW4	
DW5	
DW6	
DW7	LD107
DW8	LD108
DW9	TB9,OH
DW10	LD16
DW11	LD17

Locus DR	Previous (workshop)
DRW1	WIA1, Te6
DRW2	WIA2, Te4
DRW3	WIA3, Te5
DRW4	WIA4, Te1.1
DRW5	WIA5, Te5.2
DRW6	WIA6, Te10
DRW7	WIA7, Te3

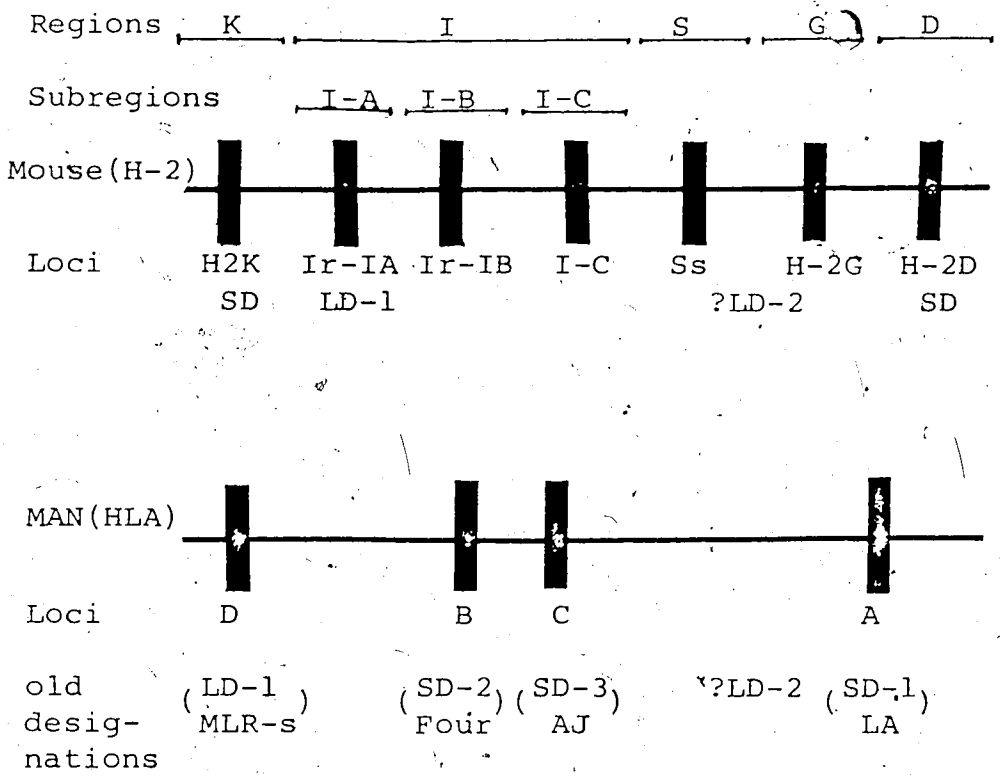
By W.H.O. Committee on leukocyte nomenclature.
The broad specificities are shown in parentheses following particular splits. Based on Histocompatibility Testing 1977. Bodmer W.F. (ed) Munksgaard, Copenhagen.

Appendix C Patterns of cross-reaction among DR antigens



═══ ——— ---- indicates, respectively decreasing extents of cross-reaction

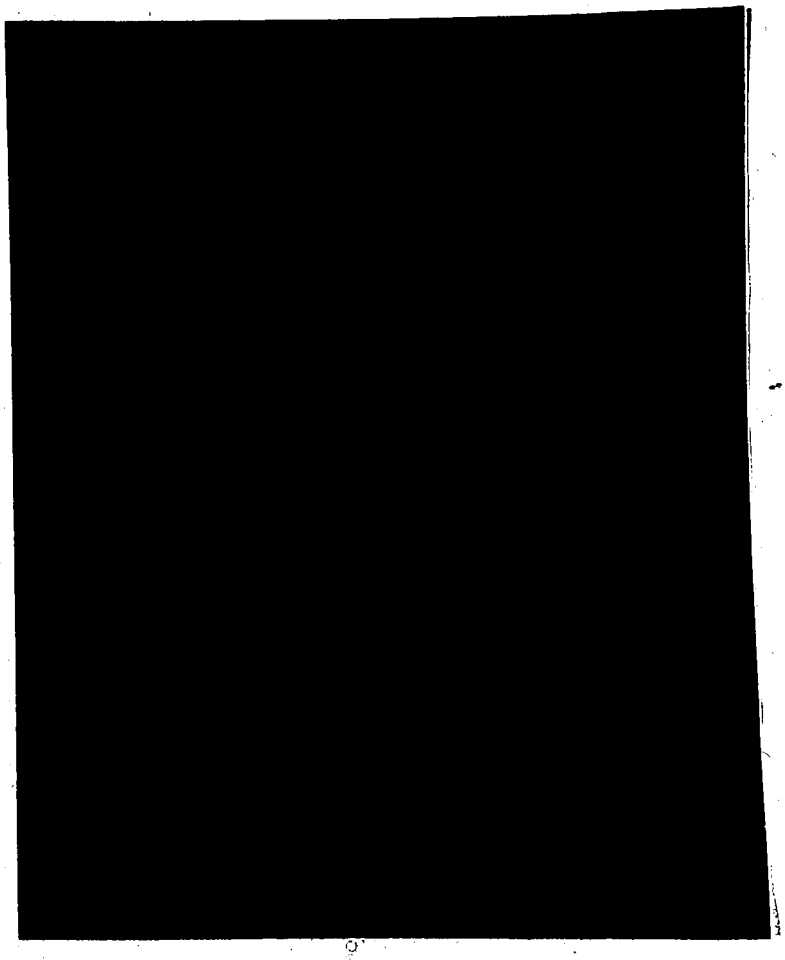
From: Bodmer (1978) Ia serology. 10. Summary and Conclusions. Histocompatibility Testing 1977, p.351 Bodmer W.F. (ed) Munksgaard, Copenhagen.



Appendix D Comparison of the major histocompatibility complex in mouse and man.

From: Bach, F.F., and van Rood, J.J. (1976) The Major Histocompatibility Complex - Genetics and Biology. New Eng. J. Med. 295,807.

Appendix E Demonstration of surface Ig on B lymphocyte using fluorescent antisera directed against Ig molecules on the cell surface (see page 37).



Appendix F Acridine orange staining of monocytes. Note the bright orange staining granules in cytoplasm and pale green coloured characteristic large nucleus (see page 37).