



National Library  
of Canada

Bibliothèque nationale  
du Canada

Canadian Theses Service    Service des thèses canadiennes

Ottawa, Canada  
K1A 0N4

## NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

## AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

CHARACTERIZATION OF HLA CLASS I ANTIGENS ON PLATELETS AS  
INTEGRAL OR ADSORBED MEMBRANE PROTEINS

A THESIS SUBMITTED TO  
THE SCHOOL OF GRADUATE STUDIES AND RESEARCH  
THE UNIVERSITY OF OTTAWA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE  
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY  
SCHOOL OF MEDICINE

BY

VICTORIA HOGAN, R.T., B.Sc.



National Library  
of Canada

Bibliothèque nationale  
du Canada

Canadian Theses Service    Service des thèses canadiennes

Ottawa, Canada  
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-68107-1

Canada



UNIVERSITÉ D'OTTAWA  
UNIVERSITY OF OTTAWA

## ABSTRACT

HLA Class I antigens are present on platelets but the extent to which these antigens are intrinsic or adsorbed remains undefined. It has been suggested that HLA antigens are passively adsorbed from plasma and can be readily eluted using relatively mild treatments such as low pH or hypertonic salt solutions. To date, detailed biochemical analysis of platelet HLA antigens has failed to prove conclusively, the nature of their association with the platelet surface. Recent evidence that platelets possess specific mRNA sequences encoding integral membrane proteins such as GPIIIa argues in favour of the possibility that these antigens may also be integral in nature.

In order to determine whether HLA-A,B antigens on platelets are integral membrane constituents or simply represent adsorbed plasma proteins, the degree to which they are adsorbed, and the relative ease with which they elute off platelet membranes was studied using various treatments known to elute passively adsorbed membrane proteins. In addition, this question was investigated at the RNA level using phytohemagglutinin stimulation to determine whether platelets have the capacity to respond with de-novo synthesis of HLA antigens and by enzymatic amplification of platelet derived mRNA to attempt to demonstrate the presence of nascent message encoding for these antigens.

It was shown that HLA antigen present on platelet membranes did not elute when platelets were incubated in autologous plasma or in plasma from homologous, antigen negative donors. When HLA-A2 negative platelets were incubated in HLA-A2 positive plasma a small amount of HLA-A2 antigen was detectable indicating that platelets possess the ability, to a limited extent, to adsorb HLA antigen from the plasma, in vitro.

The ease with which these antigens could be eluted was assessed using hypertonic acid chloroquine (200 mg/ml, pH 5.0 for 1 hr. at 37°C), 3M KCl, 1M Urea and low pH (citric acid- $\text{Na}_2\text{HPO}_4$  buffer, pH 3.0). HLA antigen reactivity was considerably reduced after each treatment however, a residual level of HLA reactivity could be detected in all cases. Under the same conditions, the detectable level of  $\text{PL}^{\text{A1}}$  antigen, which is known to be associated with the integral GPIIIa molecules on platelet membranes, remained unchanged.

The results of these studies indicate that the majority of HLA antigen present on platelet membranes can be selectively eluted without a concomitant loss of known integral membrane proteins such as GPIIIa. These findings argue in favor of the existent hypothesis that HLA antigens are adsorbed platelet membrane proteins.

The results of the PHA stimulation and Polymerase Chain Reaction studies failed to indicate the presence of active mRNA sequences encoding HLA antigen within the platelet. The

possibility that this message is present but below the level of detection, even after amplification, or that a response was not seen with PHA because platelets lack specific membrane receptors for PHA, cannot be ruled out. The evidence presented does however support the conclusion that the majority of HLA antigen that resides on the platelet membrane can be easily eluted and that there is little or no de-novo synthesis and subsequent expression of these antigens carried out by the mature circulating platelet.

The residual HLA reactivity that remains after subjecting platelets to various elution treatments requires further study to determine if this subset of HLA antigens is in fact integral in nature. The possibility that these remaining antigens are characteristic of young platelets in the circulation that have recently been generated from megakaryocytes, which are known to possess nascent message for Class I HLA antigen synthesis and expression, is a newly formulated hypothesis which remains to be explored.

## ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude and thanks to Dr. Francine Decary for her patience, encouragement and guidance in this work. Words can scarce express my appreciation for her genuine interest and continuous support of my endeavors in life.

I would also like to acknowledge the guidance, assistance and technical support received from Dr. Pierre Chartrand, Director of Research at the Canadian Red Cross Society, Montreal Centre and his staff. Their sound advice, thoughtful suggestions and constructive criticisms were especially helpful and much appreciated.

I would like to thank Don Magee for his encouragement, graphic support and assistance provided in the preparation of this thesis.

Finally, I would like to express my sincere gratitude to my colleagues at the Canadian Red Cross Society, Ottawa Centre. Their collective encouragement and support throughout the course of my studies is largely responsible for enabling me to see this thesis through to completion.

## TABLE OF CONTENTS

ABSTRACT.....	i
ACKNOWLEDGEMENTS.....	iv
TABLE OF CONTENTS.....	v
LIST OF FIGURES.....	viii
LIST OF TABLES.....	ix
GLOSSARY OF ABBREVIATIONS.....	x
1. INTRODUCTION.....	1
1.1 General Introduction.....	1
1.2 Definition of Integral and Adsorbed Membrane Proteins.....	4
1.2.1 Properties of Integral Membrane Proteins..	4
1.2.2 Properties of Adsorbed Membrane Proteins..	5
1.3 Historical Review of Platelet Membrane Antigens..	6
1.4 Platelet Membrane Glycoproteins and Their Association With Platelet Specific Antigens.....	11
1.4.1 Platelet Membrane Glycoproteins.....	11
1.4.2 The Glycoprotein IIb-IIIa Complex.....	15
1.4.3 Association of The PL <sup>A1</sup> Antigen With Glycoprotein IIIa.....	19
1.5 HLA Antigens on Platelet Membranes.....	20
1.5.1 Class I HLA Antigens.....	21
1.5.2 Immunochemistry of Class I HLA Antigens..	22
1.5.3 Structure of Class I HLA Genes.....	26
1.6 Statement of Objective.....	27

2. MATERIALS AND METHODS.....	29
2.1 Materials.....	29
2.1.1 Reagents/Buffers.....	29
2.1.2 Equipment.....	37
2.2 Methods.....	39
2.2.1 Lymphocytotoxicity Test (LCT).....	39
2.2.2 Indirect Platelet Suspension Immunofluorescence Test (IPSIFT).....	39
2.2.3 Enzyme Linked Immunosorbant Assay (ELISA).....	40
2.2.4 Platelet Radioactive Antiglobulin Test (PRAGT).....	42
2.2.5 Western Blot Analysis.....	43
2.2.6 Immunoblot Assay.....	44
2.2.7 cDNA Synthesis From Total Cellular RNA...46	
2.2.8 Amplification of Specific DNA Sequences By Polymerase Chain Reaction.....	47
2.2.9 Agarose Gel Electrophoresis.....	48
2.3 Experimental Protocols.....	49
2.3.1 Passive Adsorption Experimental Protocol.49	
2.3.2 Passive Elution Experimental Protocol....50	
2.3.3 Chloroquine Elution Experimental Protocol.....	51
2.3.4 3M KCL/1M Urea Experimental Protocol....52	
2.3.5 Low pH Treatment Experimental Protocol...53	
2.3.6 PHA Stimulation Experimental Protocol....54	
2.3.7 PCR Experimental Protocol.....	56
3. RESULTS.....	58
3.1 Passive Adsorption and Elution of Class I HLA	

Antigens From Platelet Membranes.....	58
3.1.1 Rationale and Objectives.....	58
3.1.2 Results.....	58
3.2 Elution of Class I HLA Antigens From Platelet Membranes By Chloroquine.....	62
3.2.1 Rationale and Objectives.....	62
3.2.2 Results.....	63
3.3 Stability of Class I HLA and PL <sup>A1</sup> Antigens on Platelets After Treatment With a Hypertonic Salt Solution (3M KCl) And A Mild Denaturing Agent (1M Urea).....	68
3.3.1 Rationale abd Objective.....	68
3.3.2 Results.....	69
3.4 Effect of Low pH Treatment of Platelets on The Presence of Class I HLA and PL <sup>A1</sup> Antigens.....	74
3.4.1 Rationale and Objectives.....	74
3.4.2 Results.....	74
3.5 Effect of PHA Stimulation on The Expression of Class I HLA Antigens on Platelet Membranes.....	77
3.5.1 Rationale and Objectives.....	77
3.5.2 Results.....	77
3.6 Enzymatic Amplification of Platelet mRNA By The Polymerase Chain Reaction.....	81
3.6.1 Rationale and Objectives.....	81
3.6.2 Results.....	82
4. DISCUSSION.....	85
5. CONCLUSION.....	96
6. REFERENCES .....	98

## LIST OF FIGURES

FIGURE 1.	MODEL OF THE GPIIb-IIIa COMPLEX INSERTED INTO THE LIPID BILAYER.....	18
FIGURE 2.	STRUCTURE OF CLASS I HLA ANTIGEN.....	25
FIGURE 3.	SCHEMATIC DIAGRAM OF A CLASS I HLA GENE.....	28
FIGURE 4.	INTEGRITY OF PL <sup>A1</sup> ANTIGEN AFTER CHLOROQUINE TREATMENT OF PLATELETS.....	67
FIGURE 5.	EFFECT OF VARIOUS 3M KCL TREATMENT INTERVALS ON THE PRESENCE OF HLA-A,B ANTIGENS ON PLATELETS.....	70
FIGURE 6.	EFFECT OF VARIOUS 1M UREA TREATMENT INTERVALS ON THE PRESENCE OF HLA-A,B ANTIGENS ON PLATELETS.....	71
FIGURE 7.	STABILITY OF PL <sup>A1</sup> ANTIGEN ACTIVITY AFTER TREATMENT WITH HYPERTONIC SALT SOLUTION (3M KCL) AND A MILD DENATURING AGENT (1M UREA).....	73
FIGURE 8.	EFFECT OF VARIOUS TREATMENT INTERVALS WITH LOW PH CITRIC ACID ON THE LEVEL OF HLA-A,B ANTIGENS ON PLATELET MEMBRANES.....	75
FIGURE 9.	STABILITY OF PL <sup>A1</sup> ANTIGEN ON PLATELETS AFTER LOW PH TREATMENT WITH BUFFERED CITRIC ACID.....	76
FIGURE 10.	EFFECT OF INCREASING PHA CONCENTRATIONS ON THE EXPRESSION OF HLA-A,B ANTIGENS ON PLATELET MEMBRANES AFTER 48 HOURS OF INCUBATION.....	79
FIGURE 11.	HLA-A,B ANTIGEN EXPRESSION ON PLATELETS AFTER STIMULATION WITH A 25µg/ml CONCENTRATION OF PHA OVER VARIOUS INCUBATION INTERVALS.....	80
FIGURE 12.	ENZYMATIC AMPLIFICATION OF PLATELET AND LYMPHOCYTE DNA DERIVED FROM HLA-A2 POSITIVE CELLS USING SPECIFIC PRIMERS FOR HLA-A2 MESSAGE.....	84

## LIST OF TABLES

TABLE 1.	SUMMARY OF PLATELET SPECIFIC ANTIGEN SYSTEMS....	10
TABLE 2.	MAJOR PLATELET MEMBRANE GLYCOPROTEINS.....	13
TABLE 3.	PLATELET GLYCOPROTEIN COMPLEXES AND THEIR FUNCTIONAL RECEPTORS.....	14
TABLE 4.	PASSIVE ADSORPTION OF CLASS I HLA ANTIGENS ONTO PLATELET MEMBRANES.....	59
TABLE 5.	PASSIVE ELUTION OF CLASS I HLA ANTIGENS FROM PLATELET MEMBRANES.....	61
TABLE 6.	RESIDUAL HLA-A,B AND PL <sup>A1</sup> ACTIVITY AFTER TREATMENT WITH HYPERTONIC ACID CHLOROQUINE.....	64
TABLE 7.	PLATELET MEMBRANE FLUORESCENCE DUE TO HLA-A,B AND PL <sup>A1</sup> ANTIGEN EXPRESSION BEFORE AND AFTER CHLOROQUINE TREATMENT.....	65
TABLE 8.	STABILITY OF HLA AND PL <sup>A1</sup> ANTIGENS AFTER TREATMENT WITH 3M KCL AND 1M UREA.....	72

## GLOSSARY OF ABBREVIATIONS

aa	amino acid
A	adenine
ADP	adenosine diphosphate
B/T%	bound/total percent
$\beta 2\mu\text{g}$	beta-2-microglobulin
BSA	bovine serum albumin
bp	base pairs
$^{\circ}\text{C}$	degrees Celsius
C	cytosine
cdNA	complementary deoxyribonucleic acid
CPD-A1	citrate-phosphate-dextrose anticoagulant with adenine
cpm	count per minute
C-terminal	carboxy-terminal
ds	double stranded
DEPC	diethylpyrocarbonate
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
DNA	deoxyribonucleic acid
E1...8	exon 1 through 8 regions of mRNA encoding Class I HLA molecules
EM	electron microscopy
ELISA	enzyme linked immunosorbent assay
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
G	guanine
g	gram
GP	glycoprotein
HLA	human leukocyte antigen
HLA-A,B	human class I histocompatibility antigens
hr	hour
ICSH	International Committee For Standardization in Haematology
Ig	immunoglobulin
IgG	immunoglobulin G
IgGAM	immunoglobulin G, A and M
ISBT	International Society of Blood Transfusion
IPSIFT	Indirect Platelet Suspension Immunofluorescence Test
K	kilo ( $10^3$ )
Kb	kilo base
KDa	kilo dalton
Kg	kilogram
LCT	lymphocytotoxicity test
Mab	monoclonal antibody
Mab W6/32	specific monoclonal antibody directed against the heavy chain portion of Class I HLA molecules
MHC	major histocompatibility complex
min	minute
mM	milli molar ( $10^{-3}$ )
mmol	milli moles

MMLV	Moloney Murine Leukemia Virus
mosm	milli osmoles
mol. wt.	molecular weight
mRNA	messenger ribonucleic acid
NATP	neonatal alloimmune thrombocytopenia
nm	nanometres ( $10^{-9}$ metres)
N-terminal	amino-terminal
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PcR	polymerase chain reaction
PHA	phytohemagglutinin
pM	pico molar ( $10^{-12}$ )
PNPP	para-nitrophenyl phosphate
PPP	platelet poor plasma
PRAT	platelet radioactive antiglobulin test
PRP	platelet rich plasma
PTP	post transfusion purpura
RPMI 1640	Rockwell Park Memorial Institute nutrient medium 1640
RNA	ribonucleic acid
s	seconds
S.D.	one standard deviation
SDS	sodium dodecyl sulfate
ss	single stranded
T	thymine

T cell	thymus derived lymphocyte
Taq Polymerase	DNA polymerase enzyme from <u>Thermus aquaticus</u>
U	unit
$\mu\text{g}$	microgram ( $10^{-6}$ )
$\mu\text{M}$	micro molar ( $10^{-6}$ )
V	volts
vWF	von Willebrand Factor
WBC	white blood cell

## INTRODUCTION

### 1.1 General Introduction

Human blood platelets are cytoplasmic fragments of megakaryocytes (Brown et al. 1979; Kunicki 1988a; Levene et al. 1985) which range in size from less than  $5\mu^3$  to greater than  $12\mu^3$  and have an average life span in the peripheral circulation of 7 to 10 days. (Evatt and Stein 1988).

In-vivo, the resting platelet (ie. not activated) is a disc shaped cell with a porous membrane resembling a sponge. The invaginations of the surface membrane extend deep into the platelet cytoplasm and provide canals through which components within the granules of the platelet are released during activation. The platelet is covered by a plasma protein coat that potentiates the platelets' membrane adhesion function. When vascular endothelium is damaged, platelets adhere to exposed surfaces and release their granular constituents; thromboxane  $A_2$ , adenosine diphosphate (ADP), serotonin and epinephrine into the surrounding plasma. These mediators promote a number of important responses which serve to initiate blood coagulation and thus fulfill the platelets' biological function.

The need for platelets in the treatment of profoundly thrombocytopenic patients has increased significantly over the past 10 years. The Canadian Red Cross Society reports that production of platelet concentrate has been increasing at a rate of 10-12% each year in response to the increased demand

(The Canadian Red Cross Society Blood Services Statistical Report 1987/88). Many patients who receive repeated platelet transfusions develop an immunological refractory state due to the development of antibodies directed towards alloantigens present on platelet membranes. Platelet membranes bear several types of antigenic molecules including: HLA Class I gene products, ABO blood group antigens and a number of platelet specific antigens, all of which are capable of inducing an immune response during pregnancy, blood transfusion or transplantation (Decary 1982). Of these antigen systems, the HLA Class I antigens are by far the most significant in terms of their relative contribution to the overall refractory state in multi-transfused patients (Yankee et al. 1971; Duquesnoy 1979).

The Class I molecules present on the surface of platelet membranes are structurally and antigenically identical to those present on lymphocytes and other nucleated cells (Gockerman and Jacob 1979), however, there exists considerable controversy in current literature as to the nature of their expression. It has been reported by a number of investigators that HLA antigens can be passively adsorbed onto platelets from the surrounding plasma, thus suggesting that the majority, if not all, platelet HLA antigens are plasma derived (Blumberg et al. 1982, 1984; Lalezari and Driscoll 1982; Kao 1987). Conversely, there have been reports which conclude that HLA antigens on platelets are not readily adsorbed or eluted in vitro (Dunstan and Simpson 1985a; Warejcka et al.

1985; Santoso et al. 1986) and still others have convincingly demonstrated that the amount of HLA antigens adsorbed onto platelets after 18 hours of circulation in-vivo is insufficient to alter the specificity of the platelets (Santoso et al. 1986). Thus there exist two seemingly contradictory concepts with regard to the nature of expression of HLA Class I antigens on platelets. It is noteworthy to add that this same phenomenon has been observed for ABO antigens which are also detectable on platelet surfaces. Considerable work has focused on determining the integral or adsorbed status of these red cell antigens and the majority of evidence presented in the literature to date supports the view that only adsorbed ABO antigens are present on platelet surfaces (Lewis et al. 1960; Kelton et al. 1982; Dunstan et al. 1985a,b,c and Skogen et al. 1988).

Considering the important clinical role the HLA Class I antigens play in platelet transfusion therapy, such conflicting evidence merits further study. In order for the reader to understand the focus of this thesis and in turn, the experiments done, I will begin by presenting some background information on a number of relevant topics including; the criteria for classifying membrane proteins as integral or adsorbed, a brief historical review of the platelet antigens discovered to date, platelet membrane glycoproteins, the integral GPIIb-IIIa complex on platelet membranes and its association with the PL<sup>A1</sup> antigen and finally, a review of HLA Class I antigens and their immunochemistry.

## 1.2 Definition of Integral and Adsorbed Membrane Proteins

The criteria originally set forth by Singer and Nicolson (1972) to distinguish between an integral (native) versus an adsorbed (peripheral) membrane protein are generally considered to hold true today. The properties described below are those used to define integral and adsorbed proteins and are presented here as a basis for the experimental strategy employed in this research.

### 1.2.1 Properties of Integral Membrane Proteins

Integral proteins are by far the most abundant type of cellular membrane proteins and are characterized by the following properties:

- i) They are either completely or partially embedded in the lipid matrix of the cell membrane due to the existence of hydrophobic amino acid sequences.
- ii) They require relatively extreme treatments with reagents such as; detergents, bile acids, protein denaturants or organic solvents to dissociate them from the cell. Such treatments normally destroy the integrity of the cell membrane by dissolving or solubilizing it.
- iii) Once extracted from the cell they usually remain associated with the lipid fraction of the membrane and;
- iv) They are usually highly insoluble and often aggregated in neutral aqueous buffers.

### 1.2.2 Properties of Adsorbed Membrane Proteins

Adsorbed proteins may be characterized by the following properties:

- i) They are loosely associated with the cell membrane by weak, non-covalent, perhaps mainly electrostatic interactions, and do not possess structures which are embedded in the lipid bilayer of the membrane.
- ii) They require only mild treatments such as; changes in pH, increases in ionic strength of the medium, or the addition of a chelating agent, in order to dissociate them, molecularly intact, from the cell membrane. The integrity of the cell membrane is normally not affected by such treatments.
- iii) They dissociate from the membrane free of lipids and;
- iv) In their dissociated state they are relatively soluble in neutral aqueous buffers.

### 1.3 Historical Review of Platelet Membrane Antigens

The study of human platelet membrane antigens can be traced back to its beginning over 50 years ago when a group of German investigators (Schäfer and Gennerich 1939), first reported the presence of A,B blood group antigens on the surface of platelets. Despite this early report, the dawn of platelet research was not seen until the early 1950's when Harrington published the results of his classical experiments which proved that the etiological agent in idiopathic and neonatal alloimmune thrombocytopenic purpura (NATP) was a platelet agglutinin (Harrington et al. 1953).

These early studies evoked a good deal of interest in the field of platelet immunobiology and by the early 1960's, knowledge in the field had progressed considerably and numerous methodologies for detecting platelet agglutinins, thrombolysins, thromboprecipitins, thromboinhibitors and thromboallergins had been reported (Majsky 1969). A major focus in platelet research during this period was on the presence of blood group antigens and their significance (Ashurst et al. 1956; Coombs and Bedford 1955; Dausset and Malinvaud 1954; Dausset and Evelin 1958; Gurevitch and Nelken 1954; Jankovic and Arsenijevic 1959; Lewis et al. 1960; Moureau and André 1954; Ruffie et al 1959; Stefanini et al 1953).

The commonly accepted view among these investigators was that the blood group antigens were associated with the platelet as a fixed part of the platelet membrane. This

belief was first challenged by Lewis et al. (1960) who showed that bound A,B substance could be detected by the agglutination and mixed agglutination test on the surface of Group O platelets after incubation in saliva from a Group A,B individual. This bound A,B substance reportedly remained firmly fixed even after washing platelets twelve times in saline. A subsequent report by Majsky showed that repeated incubation of O platelets with fresh A or B plasma did not lead to binding of A,B substances on platelets and that the serological activity of A or B platelets did not change after repeated washings (Majsky 1962). The conclusion drawn from these conflicting reports was that blood group antigens on platelets were present in smaller quantities or in weaker forms than those of red cells and the question of their presence, as well as the nature of their expression, must await the introduction of more reliable and sensitive methods of detection (Majsky 1962).

More recent work has established the clinical significance of platelet antigens in patients receiving multiple platelet transfusions (Brand et al. 1986). In addition to ABO antigens, a number of other red cell antigens have been reported to be present on platelets and adsorbed in nature including; Ii (Dunstan et al. 1984), P (Dunstan et al. 1985d) and Le<sup>a</sup> (Dunstan et al. 1985e). The Rh antigen is reported to be absent from the platelet membrane, as have the antigens associated with the Duffy, Kell, Kidd and Lutheran blood group systems (Dunstan et al. 1983). The status of MNSs antigen

expression remains controversial at this time with earlier reports indicating the presence of these antigens (Moulinier 1958; Ducos et al. 1959; Majesky 1969; Urinson 1961) and more recently, a report presenting evidence to the contrary (Simpson et al. 1987).

The presence of a common leucocyte and platelet antigen was first reported by Dausset (1959), who named the antigen Mac. This finding was later confirmed by other investigators (van Rood and Eernisse 1959; van der Wiel et al. 1961; Wilson 1963). During the 1960's, additional leucoplatelet antigens were discovered by different investigators who arranged them in systems with their own nomenclature. These leucoplatelet antigens were later proven to be HLA Class I antigens in experiments which demonstrated that volunteers injected with leucocytes of homologous donors became alloimmunized with antibodies reactive against both the lymphocytes and the platelets of the donors. (Wilson et al. 1968).

This same period saw considerable work done to define platelet specific antigen systems. The first platelet specific antigen was reported by Moulinier (1958) by means of an alloantibody detected in the serum of a woman (Duz) whose three previous infants had died of neonatal alloimmune thrombocytopenia (NATP). This antigen was named Duzo<sup>a</sup> and the corresponding antibody was named anti-Duzo. In 1959, a second platelet specific antigen named Zw<sup>a</sup> was detected using the platelet agglutination test (van Loghem et al. 1959). This

same antigen was later detected using the complement fixation test by an independent group of investigators who named it PL<sup>A1</sup> (Shulman et al. 1961). The corresponding allele in the PL<sup>A</sup> system (PL<sup>A2</sup> or Zw<sup>b</sup>) was reported four years later by van der Weerd et al. (1963).

Six other platelet specific antigen systems have been serologically defined since the early 1960's, including: Ko, PL<sup>E</sup>, Bak (Lek), Pen (Yuk), Br and PL<sup>t</sup>. Table 1 is a summary of these platelet specific antigen systems and the phenotypic frequencies of their corresponding alleles.

TABLE 1

SUMMARY OF PLATELET SPECIFIC ANTIGEN SYSTEMS\*

SYSTEM	ALLELE**	PHENOTYPIC FREQUENCY(%)	REFERENCE(YEAR)	GLYCOPROTEIN ASSOCIATION
Duzo	Duzo <sup>a</sup>	22.0	Moulinier (1958)	?
Zw	Zw <sup>a</sup>	97.6	van Loghem (1959)	IIIIa
(PL <sup>A</sup> )	(PL <sup>A1</sup> )	98.5	Shulman (1961)	
	Zw <sup>b</sup> (P1 <sup>A2</sup> )	26.2	van der Weerd (1963)	
Ko	Ko <sup>a</sup>	14.3	van der Weerd (1962)	?
	Ko <sup>b</sup>	99.4	van der Weerd (1965)	
PL <sup>E</sup>	PL <sup>E1</sup>	99.9	Shulman (1964)	Ib alpha
	PL <sup>E2</sup>	5.0	Shulman (1964)	
Bak	Bak <sup>a</sup> (Lek <sup>a</sup> )	85.0	von dem Borne (1980)	IIb alpha
(Lek)	Bak <sup>b</sup> (Lek <sup>b</sup> )	63.0	Kickler (1988)	
Pen	Pen <sup>a</sup> (Yuk <sup>b</sup> )	99.9	Friedman (1985)	IIIIa
(Yuk)	Pen <sup>b</sup> (Yuk <sup>a</sup> )	1.7	Shibata (1986)	
Br	Br <sup>a</sup>	98.9	Kiefel (1987)	Ia and/or IIa
	Br <sup>b</sup>	81.1	Kiefer (1987)	
PL <sup>t</sup>	PL <sup>t</sup>	---	Beardsley (1987)	V

\* A new nomenclature of Platelet Specific Antigens has recently been proposed by the ICSH/ISBT Working Party on Platelet Serology (von dem Borne and Decary 1990).

\*\* Alleles denoted in brackets represent alternate nomenclature for the same system.

#### 1.4 Platelet Membrane Glycoproteins and Their Association with Platelet Specific Antigens

The majority of clinically relevant, platelet specific antigens that have been identified to date are associated with and expressed on membrane glycoproteins (GP), (Table 1). Consequently, the understanding of platelet membrane antigens has evolved along with developments in the area of platelet glycoprotein biochemistry. In addition, platelet glycoproteins serve as important receptor sites for proteins which mediate such important biological functions as platelet adhesion and aggregation.

##### 1.4.1 Platelet Membrane Glycoproteins

In recent years, significant progress has been made in the identification and characterization of platelet membrane glycoproteins. Human platelets possess more than forty identifiable membrane proteins (Newman et al. 1988) although only nine major glycoproteins have been well described in the literature. (George et al. 1984; Solum 1985; Phillips and Fitzgerald 1987). At least six of the more prominent glycoproteins exhibit intrachain disulfide bonds in their native state (Table 2). Three of these, GPIb, GPIc and GPIIb, are composed of large subunits, or alpha chains, that are linked to smaller subunits called beta chains, through disulfide bonds. The alpha and beta chains dissociate upon reduction. The remaining three glycoproteins, GPIa, GPIIa and GPIIIa, consist of single polypeptide chains with several

intrachain disulfide bonds. The apparent molecular weight of these glycoproteins appears to increase slightly after reduction, an effect of the unfolding of the molecule during reduction, which causes it to migrate slower. The apparent molecular weights of the three additional glycoproteins, GPIIb (also known as GPIV), GPV and GPIX, do not change upon reduction. The major platelet membrane glycoproteins and their subunits are listed in Table 2.

Several of these glycoproteins exist in the form of complexes which are important to the biological function of the platelet (Table 3). GPIIb and IIIa exist in a calcium dependent heterodimer complex (Kunicki et al. 1981). The integrity of the complex is primarily important for the fibrinogen receptor activity of GPIIb-IIIa which mediates platelet cohesion, and to a lesser extent for the binding of fibronectin and von Willebrand Factor (vWF). Other platelet glycoproteins are reported to exist as noncovalently associated functional complexes, namely; GPIa-IIa, GPIb-IX and GPIc-IIa (Kunicki et al. 1988a; Nieuwenhuis et al. 1985; Santoso 1986).

A summary of these glycoprotein complexes and their associated functional receptors is given in Table 3.

TABLE 2  
MAJOR PLATELET MEMBRANE GLYCOPROTEINS

PLATELET GLYCOPROTEIN	MOLECULAR WEIGHT	
	INTACT MOLECULE	SUBUNIT(LARGE/SMALL)
Ia	155/170*	
Ib	170	143/22
Ic	150	134/27
IIa	130/145	
IIb	140	125/25
IIIa	105/115	
IIIb (IV)	95/95	
V	80/80	
IX	20/20	

\* non reduced/reduced molecular weights

**TABLE 3**  
**PLATELET GLYCOPROTEIN COMPLEXES AND THEIR FUNCTIONAL RECEPTORS**

<u>COMPLEX</u>	<u>RECEPTOR</u>	<u>BIOLOGICAL FUNCTION</u>
Ia-II	Collagen	adhesion
Ib-IX	vWF*	aggregation & adhesion
	Thrombin	coagulation
Ic-IIa	Fibronectin	adhesion
Ib-IIIa	Fibrinogen	coagulation
	Fibronectin	coagulation
	vWF	aggregation & adhesion

\* von Willebrand Factor

#### 1.4.2 The Glycoprotein IIb-IIIa Complex

The integral nature of GPIIb-IIIa molecules, the presence of the PL<sup>A1</sup> epitope on these complexes and the availability of high titre alloimmune sources of anti-PL<sup>A1</sup> with which to detect this antigen, is discussed here as it provides a model with which to study and compare the effect of various treatments of platelet membranes on the expression HLA Class I antigens.

The GPIIb-IIIa complex is the most abundant glycoprotein on the platelet surface. The average platelet has between 40,000 - 60,000 GPIIb-IIIa complexes (Pidard et al. 1983) which represents approximately 1% - 2% of the total platelet protein (Jennings and Phillips 1982). The majority of the GPIIb-IIIa complexes are randomly distributed on the surface of the platelet membrane while the remaining complexes (about 30% of total) are distributed between the membranes of the canalicular system. A detectable increase in the amount of GPIIb-IIIa is observed on the surface of the activated platelets, presumably because these "cryptic" complexes become accessible through an as yet unknown mechanism. (Hourdille et al. 1985; Woods et al. 1986).

GPIIb-IIIa is a heterodimeric complex composed of: one GPIIb molecule (mol. wt. = 140,000) and one GPIIIa molecule (mol. wt. = 105,000) associated in a calcium dependent conformation. The larger GPIIb molecule consists of 2 separate polypeptide units; an alpha subunit (mol. wt. = 125,000) and

a smaller  $\beta$  subunit (mol. wt. = 25,000) (Table 2) linked by one or more disulfide bonds to one GPIIIa molecule which exists as a single polypeptide chain (Phillips and Fitzgerald 1987). The functional role of this complex is evidenced by the fact that when platelets are suspended in  $\text{Ca}^{2+}$  chelating buffers, dissociation of the GPIIb-IIIa complex into monomeric subunits occurs resulting in a loss of the aggregation response of platelets (Jennings and Phillips 1982).

#### 1.4.2.1 Evidence That The GPIIb-IIIa Complex Exists As An Integral Platelet Membrane Protein

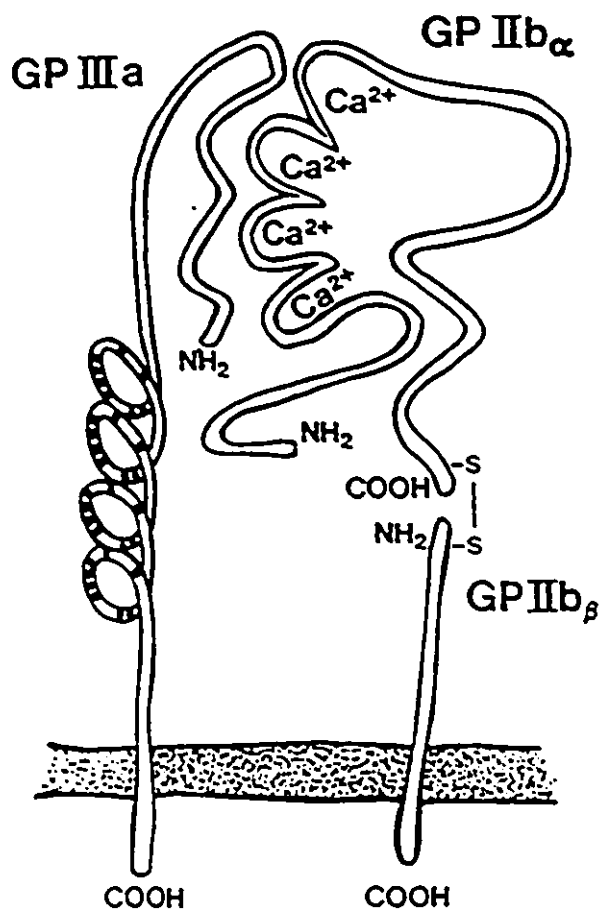
It has generally been accepted that the membrane glycoproteins of platelets represent true integral membrane proteins possessing hydrophobic amino acid sequences at their carboxy-terminal ends which correspond to their transmembrane binding regions (Lopez et al. 1988; Phillips et al. 1988). Several pieces of evidence exist that support the integral nature of the GPIIb-IIIa complex.

It has been reported by independent investigators that GPIIb-IIIa can be detected on immature megakaryocytes very early in their development process (Vinci et al. 1982; Koike et al. 1987) and that the assembly of the GPIIb-IIIa complex precedes its insertion into the platelet membrane (Rosa et al. 1986). From the results of monoclonal antibody (Mab) studies (Pidard et al. 1983), rotary shadowing EM studies (Carrell et al. 1985) and cDNA deduced protein sequences (Bray et al. 1988; Rosa et al. 1988), it is now possible to speculate about the

organization of the GPIIb-IIIa complex within the platelet membrane. It is presumed that GPIIb-IIIa is inserted into the lipid bilayer at two locations corresponding to the ends of the filamentous domains of GPIIb $\beta$  and GPIIIa (Figure 1).

Recently, Newman et al. (1988), convincingly demonstrated the integral nature of the GPIIIa protein using the polymerase chain reaction (PCR) to enzymatically amplify platelet derived mRNA encoding the GPIIIa molecule. Studies such as this one provide the investigator with new and novel approaches to understanding other antigenic structures on the platelet surface and the role they play in eliciting an alloimmune response.

**FIGURE 1. MODEL OF THE GPIIb-IIIa COMPLEX INSERTED INTO THE LIPID BILAYER.** GPIIb-IIIa is a heterodimeric complex composed of one 140,000 dalton GPIIb molecule and one 105,000 dalton GPIIIa molecule. The larger GPIIb molecule consists of 2 separate polypeptide units; an alpha subunit (125,000 daltons) and a smaller beta subunit (25,000 daltons). Each GPIIb molecule is linked by disulfide bonding to a single GPIIIa molecule. It is presumed that the GPIIb-IIIa complex is inserted into the lipid bilayer of the platelet membrane at two locations corresponding to separate hydrophobic aa sequences at the C-terminal ends of both GPIIb $\beta$  and GPIIIa. (Illustration taken from Phillips et al. 1988. Model is based on structural information obtained from primary amino acid sequencing of GPIIb-IIIa proteins)



1.4.3. Association of The PL<sup>A1</sup> Antigen With Glycoprotein IIIa

It has been established for some time that the PL<sup>A1</sup> and other platelet specific antigens (Table 1) are associated with the membrane glycoproteins of platelets. The PL<sup>A1</sup> antigen is associated with the GPIIb-IIIa complex (McMillan et al. 1982; Kunicki 1988a) and its precise location on this complex has just recently been reported (Newman et al. 1989).

It has been reported that roughly  $45,000 \pm 5,000$  molecules of anti-PL<sup>A1</sup> bind to platelets of persons homozygous for the PL<sup>A1</sup> antigen (PL<sup>A1</sup>/ PL<sup>A1</sup>), while approximately half that binds to persons who are heterozygous (PL<sup>A1</sup>/ PL<sup>A2</sup>), (Jansen et al. 1986). Since there are reportedly, about 40,000-50,000 GPIIb-IIIa complexes per platelet one can conclude that there is likely one PL<sup>A1</sup> epitope per GPIIb-IIIa molecule in the homozygous individual (Pidard et al. 1983).

The fact that anti-PL<sup>A1</sup> antibodies can inhibit aggregation suggest that PL<sup>A1</sup> antigen exists in close proximity or in association with the fibrinogen receptor site (Kunicki and Newman 1986). It has been reported that PL<sup>A1</sup> antigen can be adsorbed onto PL<sup>A1</sup> negative platelets when these platelets are incubated in plasma from PL<sup>A1</sup> positive individuals (Kickler et al. 1986; Warejcka et al. 1985). Such a phenomenon has been used to explain the mechanism of clearance of transfused platelets in cases of post transfusion

purpura (PTP) seen in PL<sup>A1</sup> negative individuals. A subsequent report by Ehmann et al. (1987), disputes the passive transfer of PL<sup>A1</sup> antigen suggesting that such findings represent an in-vitro artefact.

Despite the controversial nature of its expression on the platelet surface, PL<sup>A1</sup> antigen has considerable clinical significance compared to other platelet specific antigens defined thus far and is second only to that of the HLA Class I antigens (Decary 1982; Kao et al. 1986; Kunicki and Newman 1986). PL<sup>A1</sup> antigen has been widely studied and is the most common platelet antigen implicated in Neonatal Alloimmune Thrombocytopenia (NATP), a condition similar to Haemolytic Disease of the Newborn, where maternal alloimmunization to paternal alloantigens on fetal platelets, result in profound thrombocytopenia in the infant at birth (Blanchette et al. 1986; Decary 1982; Friedman and Aster 1985; Taaning et al. 1983).

#### 1.5 HLA Antigens on Platelets Membranes

The HLA Class I antigens are expressed on platelets but those of Class II are not. (Shulman et al. 1964; Santoso et al. 1986). The expression of Class I antigens is reported to vary considerably among individuals and from one HLA specificity to another (Szatkowski and Aster 1980; Kao et al. 1986; Kao 1987; Kao 1988 a,b; Pereira et al. 1988).

Despite their variable expression, it is currently widely accepted that only the gene products of the HLA-A and B loci are present in detectable quantities while those of the HLA-C locus are generally only weakly expressed and consequently often not detectable (Mueller-Eckhardt et al. 1980). Quantitative determinations of HLA antigens have only recently become technically possible and in one such report it was estimated that the average number of HLA Class I molecules per platelet varies between 50,000 and 120,000 among different individuals (Kao et al. 1988a).

The recent suggestion that variations in antigenic species present on platelet membranes may occur as a function of platelet age has been raised by Pereira et al. 1988 and is an interesting hypothesis which merits further attention and will be discussed subsequently in more detail.

As stated previously, the nature of the Class I HLA antigen species present on platelets has been the subject of unresolved controversy for a considerable period of time. Attempts to answer this question have been fraught with difficulties relating to the adsorptive nature of the platelet membrane itself as well as to limitations imposed by the available technology. A detailed discussion of this topic will be provided in the sections that follow.

#### 1.5.1 Class I HLA Antigens

Class I HLA antigens are found on all nucleated cells as well as platelets and function to restrict cytotoxic T-cell

activity, (Parham 1987). Recent evidence has established the Class I antigens as members of a large multigene family which exhibit highly conserved sequences (Strachan 1987). The Class I antigens are encoded within the Major Histocompatibility Complex (MHC) which is located on chromosome 6 while the invariant light chain ( $\beta 2\mu g$ ), found associated with all Class I molecules, is encoded at a single locus on chromosome 15 (Bodmer 1984).

Past attempts to isolate Class I molecules from lymphocytes in order to characterize them, have met with limited success due to the fact they represent such a small portion (<1%) of the total transmembrane protein present (Strachan 1987). More recent approaches, using cDNA clones of Class I mRNA have provided a substantial amount of information on the immunochemistry, molecular genetics and polymorphism of these molecules. The subject of HLA Class I molecules presents a most interesting and diverse topic on which much has been written. For the sake of brevity, the discussion of HLA Class I antigens will thus be limited to the areas of direct relevance to the question at hand.

#### 1.5.2 Immunochemistry of Class I HLA Antigens

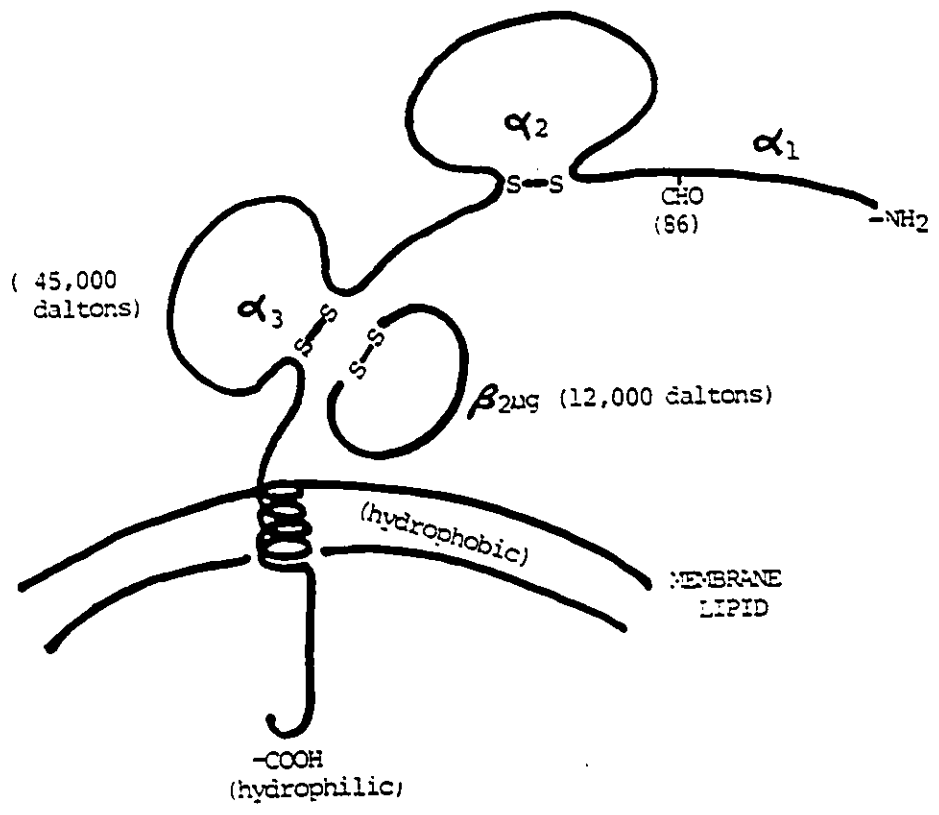
The HLA Class I antigens of lymphocytes are integral membrane glycoproteins which are firmly embedded in the lipid bilayer of the cell membrane (Walsh and Crumpton 1977; Krangel et al. 1979). After detergent solubilization of cell membranes these molecules can be shown to consist of two

polypeptide chains in a non-covalent association with one another. The large chain is referred to as the heavy chain and is a transmembrane glycoprotein of about 45,000 daltons. The smaller chain is a non-glycosylated invariant light chain, known as  $\beta$ -2-microglobulin ( $\beta$ 2 $\mu$ g) and has a molecular weight of about 12,000 daltons. (Snary et al. 1977; Orr and Strominger 1980; Ploegh et al. 1981a,b; Koller and Orr 1985; Strachan 1987).

The heavy chain is anchored in the lipid bilayer of the cell membrane by a short segment of hydrophobic amino acids (25 aa residues) followed by a longer hydrophilic tail region (30 aa residues) at its C-terminal end (COOH) which extends into the cytoplasm. The N-terminal end (NH<sub>2</sub>) of the heavy chain consists of three separate 90 aa residue domains known respectively as the alpha-1, alpha-2 and alpha-3 domains. The alpha-2 and 3 domains exhibit intrachain disulfide bonding through cysteine residues which cause the domains to fold in the disulfide loop pattern characteristic of immunoglobulin domain structure (Orr et al. 1979; Tragardh et al. 1980). The alpha-1 domain does not exhibit disulfide bonding but is unique in that it contains the glycosylated region (aa residue 86) of the molecule (Ploegh et al. 1981) as well as the variable regions which result in the considerable polymorphism seen within HLA Class I antigens. A small degree of sequence variation has been demonstrated in the alpha-2 domain however, the alpha-3 domain constitutes a highly conserved region (Tragardh et al. 1979a; Krangel et al. 1980).

The structure of Class I HLA molecules isolated from the surfaces of lymphocytes is shown in Figure 2. This same species is also present in soluble form in human plasma (Aster et al. 1973; Allison et al. 1977). Several groups have shown that HLA antigens isolated from papain solubilized platelets (Gockerman and Jacobs 1979) and detergent solubilized platelets (Tragardh et al. 1979b) are similar in all respects to those isolated from lymphocytes. Thus, the immunochemical structure given in Figure 2 can also be regarded as the species present on the platelet membrane.

**FIGURE 2. STRUCTURE OF CLASS I HLA ANTIGEN.** Class I HLA antigens consist of two polypeptide chains in a non-covalent association with one another. The heavy chain is a transmembrane glycoprotein with an approximate mol. wt. of 45,000, which is anchored in the lipid bilayer of the cell membrane by a short segment of hydrophobic aa residues (25 aa residues) at its C-terminal end (-COOH). The N-terminal end (-NH<sub>2</sub>) of the heavy chain consists of 3 separate 90 aa residue domains; the alpha-1, alpha-2 and alpha-3 domains. The alpha-2 and 3 domains exhibit intrachain disulfide bonding through cysteine residues, causing these domain structures to fold in loops characteristic of Ig domain structure. The alpha-1 domain contains the highly variable regions of the molecule which are responsible for the wide degree of polymorphism seen with Class I molecules. This domain also contains the glycosylated region of the molecule (aa residue 86). The alpha-2 domain exhibits a small degree of sequence variation however, the alpha-3 domain exists as a highly conserved region. The invariant  $\beta_2\mu$ g light chain has an approximate mol. wt. of 12 KDa, is non-covalently associated with the alpha-3 domain and is identical for all Class I HLA molecules.



### 1.5.3 Structure of Class I HLA Genes

The complete nucleotide sequences of several allelic genes at the HLA Class I region have been established using cDNA cloning techniques (Sodoyer et al. 1984; Strachan et al. 1984; Koller and Orr 1985). Comparison of these sequences with sequence data available at the protein level has established the organization of the functional gene encoding HLA Class I molecules as follows. The Class I heavy chain is encoded by eight distinct exons (E1 to E8) which are separated by introns of varying length. The entire gene spans a distance of about 3.5 kb of genomic DNA while that of the corresponding transcribed mRNA spans a distance of about 1.6 kb. Exon 1 (E1) encodes for a 24aa signal peptide, E2, E3 and E4 for the alpha-1, alpha-2 and alpha-3 domains respectively and E5 for the hydrophobic transmembrane region. From the sequence data available to date it appears that exons 6, 7 and 8 encode for the cytoplasmic tail regions in the case of HLA-A and C genes, however, only exons 6 and 7 encode for that section in the case of HLA-B genes. The last exon (E8) in these molecules appears to encode only the characteristic 3' untranslated sequences seen in the HLA-B genes (Koller et al. 1984; Strachan 1987). After translation, the Class I heavy chain consists of a signal peptide followed by a polypeptide chain consisting of between 338aa and 340aa residues. Post translational modifications which occur prior to insertion in the cell membrane include; glycosylation of the heavy chain, (at residue 86 in the alpha-1 domain), excision of the

signal peptide, associations with  $\beta 2\mu g$  and phosphorylation of the tail region, (Algranati et al. 1980). A schematic diagram of a Class I HLA gene is illustrated in Figure 3.

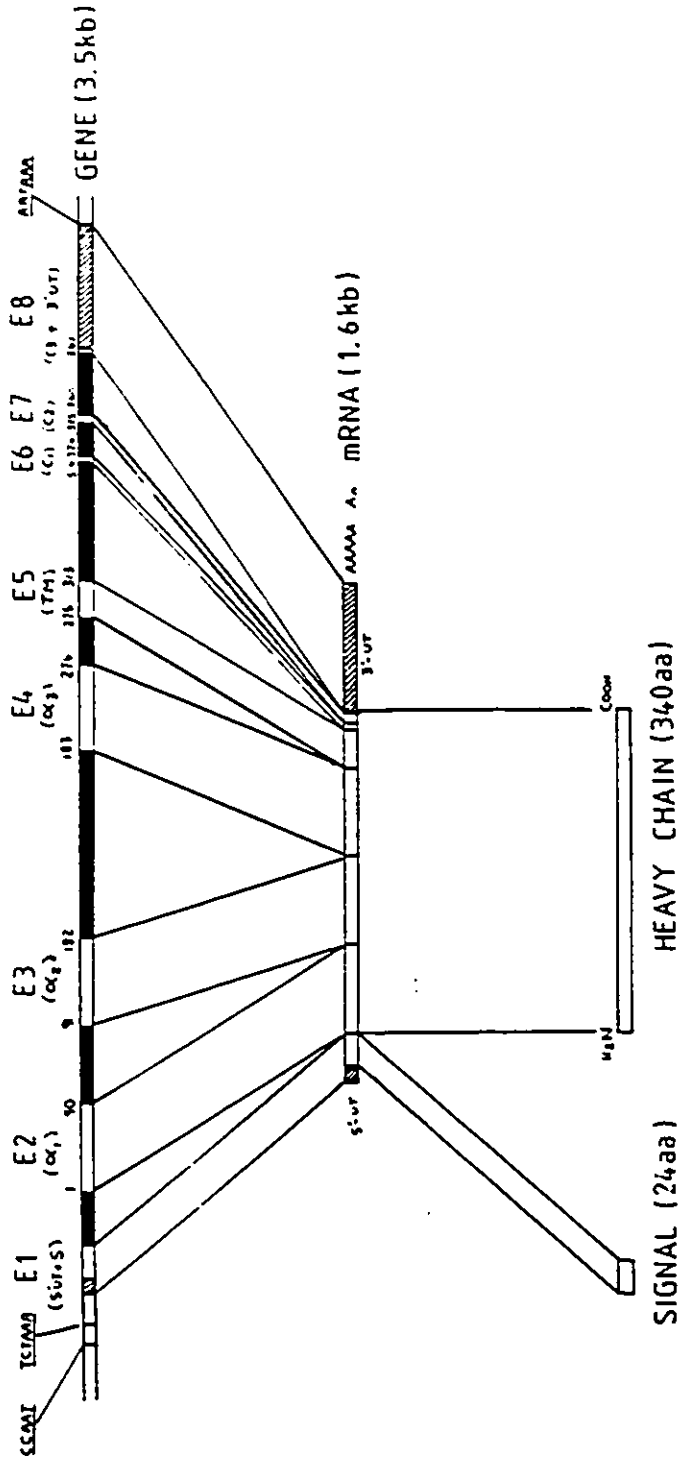
#### 1.6 Statement of Objective

One of the most perplexing issues regarding HLA antigens stems from the fact that, despite their being discovered on platelet surfaces over three decades ago, the means by which HLA antigen expression occurs continues to remain both an unresolved question and a controversial issue.

The objective of the work presented in this thesis is to further explore the nature of the binding of these antigens to platelet surfaces in an attempt to shed additional light towards resolving this issue. To further explore the question of integral vs. adsorbed membrane proteins at the cellular level, HLA Class I antigen binding on platelets was studied under a number of different conditions known to elute adsorbed proteins and compared to that of the platelet specific  $PL^{A1}$  antigen, which is known to be associated with the integral membrane GPIIIa.

In addition, this question was investigated at the RNA level for the first time, using enzymatic amplification of platelet derived RNA to attempt to demonstrate the presence of mRNA encoding for the Class I HLA antigens (specifically HLA-A2).

**FIGURE 3. SCHEMATIC DIAGRAM OF A CLASS I HLA GENE.** Class I heavy chains are encoded by eight distinct exons (E1 to E8) depicted as open boxes. The exons are separated by intervening sequences of varying lengths (solid boxes) and two small untranslated sequences at the 5' and 3' ends of mRNA (5'-UT; 3'-UT). The numbers over the beginning and end of each exon refer to equivalent aa positions in a typical A locus heavy chain. E1 encodes for a single 24aa signal peptide, E2, E3, and E4 for the alpha-1, alpha-2 and alpha-3 domains respectively and E5 encodes the hydrophobic transmembrane region (TM). E6, E7 and E8 encode for the 3 cytoplasmic regions (C1, C2 and C3) of Class I molecules. The entire genome spans a distance of approximately 3.5 kb of genomic DNA while that of the corresponding transcribed mRNA spans a distance of about 1.6 kb. (Diagram adapted from Strachan 1987).



**MATERIALS AND METHODS****2.1 Materials****2.1.1 Reagents/Buffers**

All chemicals used to prepare reagents and buffers were of laboratory grade with purities >99%. Chemicals used to prepare reagents for RNA isolation and PCR work were of the highest purity available and dedicated exclusively for use with DNA and RNA techniques. Materials, reagents and buffer formulations used are listed below in alphabetical order.

**Agarose** - purchased from Pharmacia Fine Chemicals, Piscataway, NJ. and used to prepare a 1% agarose/1% nuseive gel.

**Alkaline Phosphatase Conjugated Goat Anti-Human IgGAM** - purchased from Organon Teknika Cappel Laboratories, West Chester PA. Stored in 0.5 ml aliquots at -70°C.

**Alkaline Phosphatase Conjugated Goat Anti-Mouse IgGAM** - purchased from Organon Teknika Cappel Laboratories, West Chester PA. Stored in 0.5 ml aliquots at -70°C.

**Anti-HLA-A2** - alloantibody obtained as a gift from The Canadian Red Cross Society National HLA Reference Laboratory, Ottawa, Ont. Stored in 0.5 ml aliquots at -70°C.

**Anti-PL<sup>AI</sup> Antibody** - alloantibody obtained from a Group A woman whose infant suffered from NATP. This serum contained a high titre of anti-PL<sup>AI</sup> with no contaminating HLA antibodies. The anti-serum was

collected by plasmapheresis, defibrinated, adsorbed 3 times with washed Group B red cells and stored in 10 ml aliquots at  $-70^{\circ}\text{C}$ .

**Biotinylated Goat Anti-Human IgG** - purchased from Bethesda Research Laboratories (BRL) Gaithersburg, MD. and stored at  $4^{\circ}\text{C}$ . Used with Vectastain kit to detect bound antibody in immunoblot assay.

**Cesium Chloride Cushion** - 5.7M CsCl in 0.1M EDTA, pH 7.5. Stored at  $4^{\circ}\text{C}$ .

**Citric Acid (buffered)** - 10%  $\text{Na}_2\text{HPO}_4$  was added to concentrated citric acid drop wise until a pH of 3.0 was reached.

**Chloroform/Isoamyl** - 24 parts chloroform was added to 1 part isoamyl alcohol. Stored at  $4^{\circ}\text{C}$ .

**DEPC Treated Sterile Water** - 1 litre volumes of sterile double distilled water was treated with 1 ml (0.1%) diethylpyrocarbonate (DEPC) overnight then autoclaved to remove traces of DEPC. Stored at  $4^{\circ}\text{C}$ .

**DNA Amplification Kits (Gene-Amp Kits<sup>TM</sup>)** - purchased from Perkin Elmer Cetus, Norwalk CT. Stored at  $-40^{\circ}\text{C}$ . Used according to manufacturer's directions.

**Electrophoresis Buffer [20X]** - 20 mM Tris, 2 mM  $\text{Na}_2\text{EDTA-H}_2\text{O}$ , pH 8.4. Dilute [20X] stock buffer 1:20 with  $\text{H}_2\text{O}$  before use. Stored at  $20^{\circ}\text{C}$ .

**ELISA Stop Buffer** - 3.0M NaOH.

**Ethidium Bromide (EtBr)** - stock concentration of 10 mg/ml was added to agarose gels to a final concentration of 0.5 $\mu$ g/ml.

**Eosin Y** - purchased as a dry powder from Sigma Chemical Co. St. Louis, MI. and prepared as a 5% solution in distilled water. The reagent was stored at 4°C in a dark bottle and filtered before use.

**Fetal Calf Serum (FCS)** - sterile 500 ml bottles were obtained from Whittaker Bioproducts Inc., Walkersville, MD. FCS was inactivated at 56°C. for 30 minutes. They were then stored at -40°C in 50 ml aliquots.

**Ficoll-Hypaque** - sterile 100 ml. bottles were obtained from Pharmacia, Piscataway, NJ. and stored at 4°C. (ficoll 400 5.7 g, sodium diatrizoate 9.0 g in 100 ml.)

**FITC-Conjugated Goat Anti-Human IgGAM** - purchased from Behring, Montreal, Que. Obtained as a lyophilized reagent and stored at 4°C. Hydrated with 1.0 ml PBS-EDTA and stored in 0.5ml aliquots at -20°C.

**FITC-Conjugated Goat Anti-Mouse IgGAM** - purchased from Organon Teknika Cappel Laboratories, West Chester, PA. Lyophilized reagent stored at 4°C. Hydrated with 1.0 ml PBS-EDTA and stored in 0.5ml aliquots at -20°C.

**Formaldehyde (37% v/v)** - purchased from Sigma Chemical company St. Louis, MI. in 500ml bottles. Reagent prepared by adding 2 ml of 0.5% Phenol Red to 500 ml of formaldehyde and adjusting pH to 7.2 - 7.4 with concentrated NaOH.

Stored at 20°C in a dark bottle and filtered and pH adjusted immediately before each use.

**Guanidinium Isothiocyanate** - 4M guanidinium, 5mM Na Citrate, 0.1M 2-mercaptoethanol, 0.5% Sarkosyl, 0.1% Antifoam A. 40U/ $\mu$ l RNasin, pH 5.5. Stored at 4°C.

**HLA-A,B,C Typing Trays** - obtained from the Canadian Red Cross Society National HLA Reference Laboratory, Ottawa, Ont. Stored at -40°C.

**<sup>125</sup>I-Labelled Sheep Anti-Mouse Ig** - purchased from Amersham and stored at 4°C. Specific activity was 3.7 mBq/ml (100uCi/ $\mu$ g) at assay date.

**MMLV Reverse Transcriptase cDNA Synthesis Kit** - purchased from Bethesda Research Laboratories (BRL), Gaithersburg, MD. Stored at -20°C.

**Molecular Weight Standard** - purchased from Bio-Rad Laboratories, Richmond CA. Mol.wt. range: 40,000-200,000.

**Monoclonal Antibody W6/32** - purchased from Serotec, Toronto, Ont. Stored in 0.25 ml aliquots at -70°C.

**Monoclonal Antibody to HLA-A2** - purchased from Daymar Laboratories, Toronto, Ont. Stored in 0.25 ml aliquots at -70°C.

**96 Well Microplates** - Immulon<sup>TM</sup> 96 well flat bottom plates purchased from Dynatech Laboratories Inc. Chantilly, VA.

**Nitrocellulose paper** -0.45 $\mu$  pore nitrocellulose paper was purchased from Bio-Rad Laboratories, Richmond CA. and stored at 4°C.

**Normal Mouse Ascities Fluid** - purchased from Daymar Laboratories, Toronto, Ont. Stored in 0.5 ml aliquots at -70°C.

**Nuseive** - Purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Used to prepare a 1% agarose/1% nuseive gel. Gels were prepared in advance and stored at 4°C in 25ml aliquots in the case of mini gels or 100ml aliquots in the case of full size gels. EtBr was added to each gel to a final concentration of 0.5  $\mu$ g/ml at the time of preparation of the gels.

**Oligonucleotide Primers** - 1 set of oligonucleotide primers for Class I HLA message (HLA-A2) was custom synthesized by the Oligonucleotide Synthesis Laboratory at Queen's University, Kingston, Ont. The two primers (PCR-1 and PCR-2) were selected in the coding region for the alpha-2 domain of the HLA-A2 heavy chain (ie. Exon 3 region) and consisted of the following nucleotide sequences:

<u>Primer</u>	<u>Sense</u>	<u>5'-3' Nucleotide Sequence</u>	<u>Base No.</u>
PCR-1	RNA	CAAGAGTGTGGCAGGTCTCC	1240-1260
PCR-2	anti-sense	CCGTCATTCCTTCAGAGATT	1719-1740

The region bound by these two primers was a 500 base pair sequence of DNA in the Exon 3 coding region of an HLA-A2 gene. Primers were diluted to a stock concentration of 100  $\mu$ M and stored on desiccant at  $-40^{\circ}\text{C}$ .

**1 Kb DNA Ladder (Nucleotide Standard)** - purchased from Bethesda Research Laboratories (BRL) Gaithersburg, MD. and stored at  $4^{\circ}\text{C}$ . Nucleotide fragment range: 75-12,216 base pairs.

**PALL™ WBC Filters** - purchased from PALL Biomedical Products Corp. East Hills, NY. Used according to manufacturer's directions to remove contaminating WBC's from whole blood or Red Cell concentrates.

**Para-Nitrophenyl Phosphate (PNPP) Substrate** - 1 g vials were purchased from Sigma Chemical Co., St. Louis, MI. and stored in a desiccator at  $4^{\circ}\text{C}$ . PNPP was used at a concentration of 1mg/ml, pH 9.8.

**PBS-EDTA with Bovine Serum Albumin (PBS-EDTA-BSA)** - 0.0264M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 0.14M NaCl, 0.009M  $\text{Na}_2\text{EDTA}$ , 1% BSA, pH 6.8-7.0.

**PBS-EDTA with Tween (PBS-EDTA-Tween)** - 0.0264M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 0.14M NaCl, 0.009M  $\text{Na}_2\text{EDTA}$ , 0.05% Tween, pH 6.8-7.0.

**Phosphate Buffered Saline with EDTA (PBS-EDTA)** - 0.0264M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 0.14M NaCl, 0.009M  $\text{Na}_2\text{EDTA}$ , pH 6.8-7.0.

**Phytohemagglutinin** - PHA-P was purchased from Difco Laboratories, Inc. Detroit, MI. and stored at  $4^{\circ}\text{C}$ .

**Plasmalyte™** - 90 mmol/L NaCl, 23 mmol/L Sodium gluconate, 27 mmol/L Sodium acetate, 5 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, 294 mosm/L, pH=7.4. Purchased from Travenol Canada Inc. Mississauga, Ont.

**Platelet Wash Buffer (PBS-EDTA-Glucose)** - 0.0264M Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 0.14M NaCl, 0.009M Na<sub>2</sub>EDTA, 8.5mM glucose, pH 6.8-7.0.

**Prostaglandin-E<sub>1</sub>** - 1.0 g vial purchased from Sigma Chemicals and stored desiccated at -20°C. Stock concentration of 25mM was used to treat whole blood at a concentration of 50pM per ml.

**Protoblot™ Kit for Immunoblot Assay - Alkaline Phosphatase antibody detection kits** were purchased from Bio. Can. Science Laboratories, Mississauga, Ont. and stored at 4°C.

**Rabbit Complement** - purchased as lyophilized 1 ml aliquots from Cedarlane Laboratories, Mississauga, Ont. Stored at -40°C. Hydrated with 1 ml sterile H<sub>2</sub>O and used immediately in the lymphocytotoxicity test.

**RNA Resuspension Buffer** - 10mM Tris, 5mM EDTA, 1% SDS, pH 7.4. Stored at 4°C.

**RNasin** - placental RNA purchased from Promega Biotec, Madison, WI. Stock concentration = 40,000 U/ ml.

**RPMI 1640** - sterile 100 ml bottles were purchased from GIBCO Laboratories, Grand Island, N.Y. and stored at 4°C.

**Sample Buffer (10X)** - 60% saccharose, 1 mM EDTA, 1% SDS, 0.1% Bromophenol blue dye. Diluted 1:10 in sample to be run on agarose gel.

**Sodium Acetate** - 3M  $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$  in DEPC treated water, pH 5.2. Stored at 4°C.

**Trypan Blue Dye** - purchased as a 0.05% solution from Grand Island Biological Co. (GIBCO), Grand Island, N.Y. and stored at 20°C. Filtered before use.

### 2.1.2 Equipment

#### **Centrifuges -**

- i) Refrigerated bench top centrifuge - purchased from Beckman Instruments Inc. (Model TJ-6 Refrigerated Centrifuge), Fullerton, CA.
- ii) Microcentrifuge - Eppendorf (Model 5415) purchased from Fisher Scientific Co., Pittsburgh, PA.
- iii) Ultra Centrifuge - Beckman L8-M Ultra Centrifuge fitted with a Ti 70.1 Rotor. Purchased from Beckman Instruments, Fullerton, CA.

**CO<sub>2</sub> Incubator** - cell cultures were maintained in an automatic CO<sub>2</sub> incubator purchased from Precision Instruments Inc., Rockford, IL. This incubator was maintained at a temperature of 37°C. ± 0.5°C. and a % CO<sub>2</sub> of 5.0% ±1 throughout the cell incubation periods.

**DNA Thermal-Cycler** - purchased from Perkin Elmer Cetus, Norwalk, CT.

**Electroblot Chamber** - Transblot<sup>TM</sup> System purchased from Bio-Rad Laboratories, Richmond, CA.

#### **Electrophoresis Chambers -**

- i) PAGE - vertical gel apparatus (Protean II System), Bio-Rad Laboratories, Richmond, CA.
- ii) Agarose Gels - horizontal mini-gel electrophoresis system (Model H6), Bethesda Research Laboratories, Gaithersburg, MD.

**Gamma Counter** - purchased from LKB Instruments Inc.  
Piscataway, NJ.

**Microplate Reader** - purchased from DuPont, Wilmington, DE.

**Microscopes -**

- i) Inverted phase microscope fitted with an HLA tray holder. Zeiss, West Germany.
- ii) Epi-Fluorescence microscope fitted with a mercury vapour lamp, phase contrast optics and an FITC fluorescent filter set. Zeiss, West Germany.

**pH Meter** - Corning (Model 40) purchased from Fisher Scientific Co., Pittsburgh, PA.

**Pipettes -**

- i) Pipet'temen (P20, P100, P200 and P1000) and appropriate tips were purchased from Fisher Scientific Co., Pittsburgh, PA.)
- ii) Hamilton syringes (0.5 $\mu$ l, 1.0 $\mu$ l, 3.0 $\mu$ l and 5.0 $\mu$ l) were purchased from Hamilton Co., Reno, NV.

**UV Transilluminator** - purchased from Fisher Scientific Co., Pittsburgh, PA. and fitted with a Polaroid MP-4 Land camera.

**Vacuum Extractor** - Savant Speed Vac Concentrator purchased from Savant Instruments Inc. (Model No. SV0100H), Farmingdale, N.Y.

## 2.2 Methods

### 2.2.1 Lymphocytotoxicity Test (LCT)

HLA specificity of platelet and lymphocyte donors used in the various experiments was determined by the standard NIH microlymphocytotoxicity assay (Ray J.G. (ed.) NIAID Manual of Tissue Typing Techniques 1979) using HLA-A,B,C typing trays obtained from the Canadian Red Cross Society, National HLA Reference Laboratory.

### 2.2.2 Indirect Platelet Suspension Immunofluorescence Test (IPSIFT)

Platelets isolated from outdated 5 day platelet concentrates were washed in PBS-EDTA 3 times, subjected to various treatments and tested in this assay to determine the relative amounts of HLA Class I antigens and PL<sup>A1</sup> antigens detectable both before and after the various treatments. Platelets were tested according to the method described by von dem Borne et al. (1980) with the exception that the platelet paraformaldehyde fixation step was omitted. This step was omitted since, in our hands it did not contribute significantly to quenching non-specific fluorescence (unpublished results) and had the added drawback of causing cell lysis which resulted in a post treatment reduction in platelet concentration. Immunofluorescence intensity was assessed visually and graded as 0 (negative) or 1+ to 4+ (positive). HLA Class I antigen presence was assessed using the Mab W6/32 (which is directed against a common heavy chain

determinant of Class I molecules) at a dilution of 1:2000 (Barnstable et al. 1978; Parham et al. 1979). The presence of PL<sup>A1</sup> antigen was assessed using a 1:2 dilution of an alloimmune PL<sup>A1</sup> typing antiserum. This antiserum was harvested by plasmapheresis from a group A caucasian female whose infant had been diagnosed with NATP. There were no detectable HLA antibodies present in this antiserum and the weak anti-B present had been removed by multiple adsorptions with Group B defibrinated red blood cells harvested from a known PL<sup>A1</sup> negative donor. The antiglobulin reagent used was a FITC-conjugated goat anti-mouse IgGAM (ie IgG, IgA, IgM) used at a dilution of 1:50 in the case of HLA antigen detection and an FITC-conjugated goat anti-human IgGAM used at a concentration of 1:10 in the case of PL<sup>A1</sup> antigen detection. All antisera dilutions were previously determined as the optimal concentrations under the assay conditions described using a checkerboard titration against a pool of 10 PL<sup>A1</sup> positive platelet donors and appropriate negative controls which included; 2 PL<sup>A1</sup> negative platelet donors as the antigen negative control, mouse ascites fluid as the Mab negative control and serum from a group AB Rh-negative donor as the human serum (anti-PL<sup>A1</sup>) negative control.

### 2.2.3 Enzyme Linked Immunosorbant Assay (ELISA)

Platelets isolated from outdated 5 day platelet concentrates were washed 3 times in PBS-EDTA , subjected to

various treatments and tested in this assay to determine the relative amount of both HLA Class I antigens and PL<sup>A1</sup> antigens remaining after each treatment. The method followed was essentially that described by Bishara et al. (1983) with the following modifications. Platelets isolated from PRP were washed 3 times in PBS-EDTA, adjusted to a concentration of  $50 \times 10^6$  cells in 50  $\mu$ l of PBS-EDTA and added to each well of 96 well flat-bottom microplates (Immulon, Dynatech Laboratories) in place of peripheral blood lymphocytes (PBL) described in the original method. Mab W6/32 (1:2000 dilution) was used in place of specific HLA alloantisera and the second incubation time was reduced from 16 hours to 2 hours.

Briefly, 50  $\mu$ l of PBS-EDTA-BSA platelets at a concentration of  $1 \times 10^6$  cells/ $\mu$ l was added to each well of a 96 well flat bottom microplate and the plates centrifuged for 10 min. at 1500 rpm. The PBS-EDTA-BSA was removed by aspiration and the platelets fixed with a 0.1% gluteraldehyde for 5 min. Following fixation, the platelets were washed 3 times with PBS-EDTA containing 0.05% Tween 20 and incubated with 50  $\mu$ l of the optimal dilution (determined previously) of appropriate antiserum (i.e. anti-PL<sup>A1</sup> at a dilution of 1:8 for detection of PL<sup>A1</sup> antigen and Mab W6/32 at a dilution of 1:2000 for detection of HLA Class I antigens) for 2 hours at room temperature.

After washing 3 times in PBS-EDTA-Tween, 50  $\mu$ l of a 1:300 dilution of alkaline phosphatase conjugated anti-mouse IgGAM

(to detect HLA antigens) or a 1:200 dilution of alkaline phosphatase conjugated anti-human IgGAM (to detect PL<sup>A1</sup> antigen) was added to the appropriate wells and incubated for 2 hours at room temperature. Excess conjugate was removed by washing the plates 3 times with PBS-EDTA-Tween followed by the addition of 100  $\mu$ l of P-nitrophenyl phosphate substrate (1 mg/ml, pH 9.8) to each well. The plates were further incubated for 30 min. at 37°C. then the colour reaction stopped by adding 100  $\mu$ l of 3M NaOH to each well. The optical density (O.D.) reading of each well was determined in a microplate reader at a wavelength of 405 nm. O.D. readings of the corresponding blanks run in parallel were subtracted from the O.D. readings of each test and the results expressed a relative net O.D. reading.

#### 2.2.4 Platelet Radioactive Antiglobulin Test (PRAT)

Platelets isolated from outdated 5 day platelet concentrates were washed 3 times in PBS-EDTA and subjected to various treatments. The quantity of Class I HLA antigens on platelets was assessed after each treatment using a sensitive platelet radioactive antiglobulin test as described below. Platelets were collected from PRP and washed three times in PBS-EDTA-BSA then adjusted to a concentration of  $5 \times 10^5$  cells/ $\mu$ l. 50  $\mu$ l aliquots of this platelet suspension was added to 12 x 100 mm polystyrene tubes and incubated with either 50  $\mu$ l of Mab to HLA-A2 (1:500), in the case of the

passive adsorption experiments or Mab W6/32 (1:2000 dilution), in the case of the passive elution experiments. In both instances, the incubation was carried out for 30 min. at 37°C. Following this step, the platelets were washed three times in PBS-EDTA-BSA then incubated with 50  $\mu$ l of  $^{125}$ I anti-mouse Ig (1:100 dilution, cpm = 10,000) for 30 min. at 20°C. The platelets were then subjected to a final 3 washes in PBS-EDTA-BSA and the amount of bound  $^{125}$ I was counted on a gammacounter. The results were expressed as a Bound/Total percent (B/T%) after subtracting the average background radioactivity of four blanks run in parallel.

#### 2.2.5 Western Blot Analysis

##### 2.2.5.1 Polyacrylamide Gel Electrophoresis (PAGE)

Platelets were harvested from fresh whole blood collected into EDTA vacutainers (Bectin Dickinson) and washed three times in PBS-EDTA containing the following inhibitors; 4 mM iodoacetamide, 4 mM n-ethylmaleimide and 500 U aprotinin. After the third wash, platelets were resuspended in PBS-EDTA without inhibitors, the count adjusted to  $4 \times 10^6$  cells/ $\mu$ l and placed at -40°C in 0.1 ml aliquots. Platelet samples were prepared for electrophoresis after thawing by adding 20  $\mu$ l of platelet sample (ie 5-10  $\mu$ g/ $\mu$ l of platelet protein) to an equal volume of SDS sample buffer (4% SDS, 0.125M Tris, 20% glycerol, 0.008% bromophenol blue) and boiling at 100°C. for

5 min. Electrophoresis was carried out under non-reducing conditions on 6% polyacrylamide gels using a 4% stacking gel and the Laemmli buffer system (Laemmli, 1970). Samples were electrophoresed in a vertical gel apparatus at 30V for 16-18 hours at 4°C. Molecular weight reference standards were run in parallel on each gel, (range, 40-200 KDa).

#### 2.2.5.2 Immunoblot Assay

The immunoblot assay used is that previously reported (Rock et al. 1987), with the exception that the protein banding patterns were detected using the Protoblot™ staining technique, (Bio Can Sci. Mississauga, Ontario) rather than the Vectastain™ method. In the Protoblot technique, the blotted nitrocellulose strips, after initial incubation with test serum, were incubated with alkaline phosphatase conjugated goat anti-human IgG, washed and transferred to a freshly prepared solution of colour development substrate. The blots were incubated with continuous agitation until bands were visible with the positive control serum tested in parallel.

#### 2.2.6 Extraction of Cellular RNA From Platelets and Lymphocytes

##### 2.2.6.1 Isolation of Platelets and Lymphocytes from Whole Blood

500 ml of fresh whole blood was collected from an HLA-A2 positive donor into a Fenwal blood bag (Baxter-Travenol Canada Inc. Mississauga, Ont) containing CPD-A1 anticoagulant

and PGE<sub>1</sub> was added to a final concentration of 50 nmol/litre. The unit of whole blood was then passed through a Pall™ WBC filter to remove contaminating leukocytes then spun at 1000 xg for 10 min. to prepare platelet rich plasma (PRP). Only the upper two thirds of the PRP was collected to further insure that contaminating leukocytes were removed from the platelet preparation. The platelets collected from this top fraction of PRP were then washed twice with Platelet Wash Buffer. The leukocyte contamination after these steps was determined to be less than 1 in 1,000,000 platelets by diluting the platelet suspension out to a concentration of  $1 \times 10^6$  cells in 4 $\mu$ l, loading this entire volume of platelet suspension onto a haemocytometer and counting the number of WBC's present. A total of  $1 \times 10^{10}$  platelets were used to isolate platelet RNA for subsequent amplification in the PCR.

Lymphocyte RNA was extracted from the PBL of this same HLA-A2 positive donor for use as a positive control in the PCR experiments. PBL were isolated from 30 ml of fresh whole blood drawn into heparin anticoagulant (Vacutainer™ Becton Dickinson) using a Ficoll-Hypaque gradient. The cells were harvested from the gradient interface and washed twice in RPMI 1640 with 10% FCS. A total of  $1 \times 10^4$  PBL's were used to isolate lymphocyte RNA for amplification in the PCR.

#### 2.2.6.2 Guanidinium-Isothiocyanate Extraction of Cellular RNA.

After isolation, platelets ( $1 \times 10^{10}$ ) and PBL ( $1 \times 10^4$ ) were pelleted and solubilized in 3.3 ml of guanidinium-isothiocyanate reagent then layered over a 1.2 ml cushion of 5.7 M CsCl and centrifuged at 20°C in a Ti-70i rotor at 35,000 rpm for 16 hours. (Beckman Ultracentrifuge, Fullerton, CA.). The RNA pellets were resuspended individually in 100  $\mu$ l of buffer (10 mM Tris, 5 mM EDTA and 1% SDS, pH 7.4) and extracted three times with equal volumes of chloroform isoamyl alcohol (24:1) essentially as described by Maniatis et al. (1982). The resulting platelet and lymphocyte total RNA extracts were used in the subsequent cDNA synthesis and DNA amplifications steps.

#### 2.2.7 cDNA Synthesis From Total Cellular RNA

Total RNA isolated from each aliquot of platelets ( $1 \times 10^{10}$ ) and lymphocytes ( $1 \times 10^4$ ) were resuspended in 10  $\mu$ l of 0.1% DEPC treated water containing 1U of RNasin in sterile DEPC treated 1.5 ml polypropylene tubes. cDNA synthesis was performed according to the manufacturer's directions using MMLV reverse transcriptase as the DNA polymerizing enzyme and a cDNA synthesis kit (Bethesda Research Laboratories) containing the necessary dNTP reagents and an oligo (dT) primer. The primer supplied with the kit was used to synthesize cDNA from both platelet and lymphocyte total RNA preparations in the case of the respective DNA controls. In

the case of test DNA for amplification, the PCR-2 primer for HLA-A2 message was used in place of the oligo (dT) primer to direct first strand synthesis of specific message (i.e. HLA-A2 message). A  $1.0\mu\text{M}$  concentration of PCR-2 was added to the reaction mixture and cDNA synthesis was carried out at  $37^{\circ}\text{C}$  for 60 min. then stopped by placing the tubes at  $0^{\circ}\text{C}$ .

#### 2.2.8 Amplification of Specific DNA Sequences By Polymerase Chain Reaction

After cDNA synthesis was complete, the first strand reaction mixture from both test DNA samples and control DNA samples was subjected to DNA amplification in a Thermal-Cycler, according to the method described by Newman et al. (1988). PCR reaction buffer was added to the negative control DNA samples for both platelets and lymphocytes in place of specific primers and these samples were subjected to the same amplification steps as the test DNA. All PCR reactions were carried out in a total volume of 0.1 ml. In the case of the test DNA, the first strand reaction buffer was diluted with an equal volume of PCR buffer containing; 25mM KCl,  $1\mu\text{M}$  PCR-2 oligonucleotide ( $2\mu\text{M}$  PCR-2 in the case of the DNA controls),  $2\mu\text{M}$  PCR-1 oligonucleotide and 0.02% gelatin then heated to  $94^{\circ}\text{C}$  for 2 min. in a DNA Thermal-Cycler (Perkin Elmer Cetus Corp. Norwalk, CT.) to denature and separate the ds DNA. Samples were allowed to cool to  $37^{\circ}\text{C}$ . for 10 min. before adding 5U of Taq polymerase to initiate the PCR. Following addition of the DNA polymerase enzyme, tubes were overlaid

with 50  $\mu$ l of oil to prevent evaporation and subject to 30 amplification cycles according to the method of Newman et al. (1988), as follows: denaturation for 1 min, 20s at 94°C, primer annealing for 3 min. 20s at 37°C and primer extensions for 10 min. at 72°C. An additional 2.5U of Taq Polymerase was added after cycles 10 and 20. Following this amplification step, samples were stored at -20°C until analyzed by agarose gel electrophoresis.

The 2 oligonucleotide primers used were selected in the coding region for the alpha-2 domain of the HLA-A2 heavy chain (i.e Exon 3 coding region). PCR-1 corresponded to a 20 nucleotide DNA strand spanning nucleotides 1240 - 1260 of the published sequence for an HLA-A2 gene (Koller and Orr, 1985). PCR-2 corresponded to a 21 nucleotide anti-sense strand spanning nucleotides 1740 - 1719. The region bound by these 2 oligonucleotides was a 500 base pair sequence of DNA in the Exon 3 coding region of the HLA-A2 gene.

#### 2.2.9 Agarose Gel Electrophoresis

Electrophoresis of PCR reaction products was carried out on 1% agarose/ 1% Nuseive gels containing 0.5  $\mu$ g/ml of ethidium bromide (EtBr) according to the technique described by Maniatis et al. (1982). Samples (15 $\mu$ l) were prepared for loading by adding 10X sample buffer to a final dilution of 1:10 and spinning the tubes for 2 minutes in an eppendorf centrifuge. Gels were run at 30V for 2 hours at 20°C in the

case of minigels and overnight at 20°C in the case of large gels. Resulting DNA bands were visualized and photographed using a UV-transilluminator fitted with a Polaroid camera and compared to a 1 Kb DNA ladder standard. (range = 75-12,216 bp).

## 2.3 Experimental Protocols

### 2.3.1 Passive Adsorption Experimental Protocol

500 ml units of fresh whole blood was collected from 6 Group O, HLA-A2 negative donors, and one Group C, HLA-A2 positive donor, into CPD-A1 anticoagulant and centrifuged at 1000xg for 10 minutes. PRP was collected from each donor and further centrifuged at 1800g for 20 minutes to collect both the platelets and PPP. Each of the 6 HLA-A2 negative platelet samples were washed 3 times in PBS-EDTA then pelleted and resuspended to a concentration of  $4 \times 10^5$  cells/ $\mu$ l in 25 ml of homologous plasma from the HLA-A2 positive donor and in 25 ml of autologous plasma as a control. All platelet samples were incubated for 24 hours at 37°C with continuous end over end rotation then pelleted and resuspended in fresh 25 ml aliquots of homologous HLA-A2 positive plasma, autologous plasma (ie. HLA-A2 negative) and plasmalyte™. The resuspending medium was replaced with aliquots of the appropriate fresh plasma or plasmalyte™ each 24 hr. period over 5 days of incubation. Following this, platelets were collected from each sample,

washed 3 times in PBS-EDTA and tested in the IPSIFT, PRAT and ELISA assays using a Mab to HLA-A2. Controls included platelets from these same 6 donors tested fresh (on the day of collection) and after 48 hours incubation in autologous plasma and plasmalyte™ (a non-plasma buffer which sustains platelet viability).

### 2.3.2 Passive Elution Experimental Protocol

500 ml units of fresh whole blood was collected from 6 Group O donors into CPD-A1 anticoagulant and processed into platelet PRP by centrifugation at 1000g for 10 minutes. The PRP was centrifuged at 1800g for 20 minutes to collect both the pelleted platelets and the PPP. The platelet pellet was divided into three equal parts and treated in the following manner: one third of the platelet pellet was aseptically resuspended in one third the original volume of autologous PPP, one third of the platelets were resuspended in one third of the plasma from a different donor (ie. homologous plasma negative for all HLA antigens present on the platelets) and the final one third of the platelets were resuspended in the same volume of sterile plasmalyte™. Platelets were incubated for 5 days at 37°C with continuous end over end rotation, then washed 3 times in PBS-EDTA and tested in the IPSIFT, PRAT and ELISA assays to determine the quantity of Class I HLA antigen present. As a control, platelets were tested fresh (on day

of collection) to determine the baseline levels of Class I antigen detection.

### 2.3.3 Chloroquine Elution Experimental Protocol

Platelets were isolated from 5 day old platelet concentrates collected from 6 Group O PL<sup>A1</sup> positive donors and washed as described previously. The platelets were then treated with hypertonic acid chloroquine according to the method described by Blumberg et al. (1984). As a control, platelets incubated in PBS-EDTA (i.e. without chloroquine) were run in parallel. Following chloroquine treatment, test and control platelets were washed 3 times in PBS-EDTA-BSA and tested for HLA-A,B and PL<sup>A1</sup> activity in both the ELISA assay and the IPSIFT. In addition, the protein banding pattern and integrity of HLA and PL<sup>A1</sup> antigen (associated with GPIIIa) were assessed before and after chloroquine stripping. This was accomplished by PAGE of solubilized platelet membranes followed by electroblotting onto nitrocellulose paper. The protein banding pattern was demonstrated on both treated and untreated platelets from each of the 6 donors using the following technique. Platelets were electrophoresed under non-reducing conditions on a 6% polyacrylamide gel and electroblotted onto nitrocellulose paper according to the method of Rock et al. (1987). Protein banding patterns were visualized by staining the blots with amido black dye.

To assess the integrity of PL<sup>A1</sup> antigen after chloroquine stripping (ie. it's ability to resist elution), treated and untreated platelets from each of the 6 donors were electrophoresed under non-reducing conditions on a 6% polyacrylamide gel, electroblotted onto nitrocellulose paper and incubated with a serum containing anti-PL<sup>A1</sup> according to the method of Rock et al. (1987). The resulting blots were developed with Protoblot<sup>TM</sup> kits according to the manufacturer's directions.

#### 2.3.4 3M KCl/1M Urea Experimental Protocol

Platelets were isolated from 5 day old platelet concentrates from 6 different donors, washed in PBS (pH=6.0) and resuspended to a concentration of  $5 \times 10^6$  cells/ $\mu$ l. Platelets from 100  $\mu$ l aliquots of the suspension ( $5 \times 10^8$  platelets) were pelleted and resuspended in 1ml of 3M KCl or 1M Urea for various incubation times from 0 to 30 hrs at 4°C in the case of 3M KCl and 0 to 6 hrs at 4°C in the case of 1M Urea, in order to determine the effect of each treatment over time.

Following each incubation interval, the treated platelets were washed 3 times with PBS-EDTA (pH=6.0) and tested in the ELISA assay using Mab W6/32 antibody to detect the presence of residual HLA antigen activity. Untreated platelets (ie. platelets treated with PBS-EDTA over the various incubation periods) were run in the ELISA assay as a control.

In a separate experiment, platelets from the same 6 donors were harvested, washed in PBS and  $5 \times 10^8$  platelets were treated with both 3M KCl and 1M Urea for 20 hours and 2 hours respectively. Following incubation, the treated platelets were washed 3 times in PBS-EDTA and tested in the ELISA assay according to the above protocol using secondary antibodies to detect the presence of HLA Class I antigens (W6/32) and PL<sup>A1</sup> antigen. To further investigate the stability of PL<sup>A1</sup> antigen after these treatments, platelets from each of the 6 donors (post-treatment) were subjected to 6% PAGE, Western Blotted onto nitrocellulose paper and incubated with an anti-PL<sup>A1</sup> antibody to detect the presence of intact PL<sup>A1</sup> antigen.

#### 2.3.5 Low pH Treatment Experimental Protocol

$5 \times 10^8$  platelets were isolated from 5 day old platelet concentrates collected from 6 different Group O PL<sup>A1</sup> positive donors. The platelets were washed as previously described, resuspended in 0.5 ml of buffered citric acid solution (citric acid -  $\text{Na}_2\text{HPO}_4$  pH=3.0) and incubated for various times from 0 to 30 minutes at 4°C. The platelet suspensions were then neutralized with excess citrate buffer (pH=6.0) and washed 3 times in PBS-EDTA (pH=6.0). The treated platelets were then tested in the ELISA assay using a previously determined optimal concentration of Mab W6/32 (1:2000 dilution) to detect HLA antigen activity. Untreated control platelets (ie.

platelets incubated in PBS-EDTA over the various incubation periods) were run in the ELISA assay as a control.

In a separate series of experiments, platelets from the same 6 donors were treated with buffered citric acid for 10 minutes according to the above protocol and then tested in the ELISA assay using secondary antibodies to detect the presence of HLA antigens (Mab W6/32) and PL<sup>A1</sup> antigens (anti-PL<sup>A1</sup>).

To further investigate the stability of PL<sup>A1</sup> antigen after this treatment, platelets were incubated for 10 minutes in buffered citric acid, solubilized in SDS and subjected to 6% PAGE. Following this step, the gels were electroblotted on to nitrocellulose paper and the blots were incubated with anti-PL<sup>A1</sup> and developed with the Protoblot<sup>TM</sup> staining kit.

#### 2.3.6 PHA Stimulation Experimental Protocol

Platelets and lymphocytes were collected from 3 random donors in the following manner. 50 ml of fresh whole blood was collected into heparin anticoagulant (Vacutainer<sup>TM</sup> Becton Dickenson) and spun at 1,000g for 10 mins. The PRP was collected into one 10 ml tube and the buffy coat layer collected into a separate 10 ml tube. Platelets were washed 3 times as in PBS-EDTA as previously described and resuspended in Plasmalyte<sup>TM</sup> to a concentration of 500,000 cells/ $\mu$ l. The lymphocytes were separated on a Ficoll-Hypaque gradient by diluting the buffy coat 1:2 with RPMI 1640, layering it over

the gradient and centrifuging at 1,500g for 30 min. Following this step, lymphocytes were washed 2 times in RPMI 1640 containing 5% FCS and resuspended to a concentration of 5,000 cells/ $\mu$ l. PHA was added to 1 ml aliquots of each platelet and lymphocyte preparation to the following final concentrations: 1, 5, 10, 25 and 50  $\mu$ g/ml and incubated for 48 hours at 20°C in the case of platelets and 37°C in the case of lymphocytes. Following incubation, the cell suspensions were washed 2 times in either PBS-EDTA or RPMI 1640 with 5% FCS and resuspended once again to a concentration of 500,000 cells/ $\mu$ l for platelets and 5,000 cells/ $\mu$ l for lymphocytes. Negative controls consisted of cells incubated for 48 hours at the appropriate incubation temperatures without PHA added. All cells were tested in the PRAT using Mab W6/32 to detect HLA antigen and an  $^{125}$ I anti-mouse Ig second antibody to detect bound Mab W6/32. Blank controls for the PRAT consisted of the untreated (i.e. not stimulated with PHA) platelet and lymphocyte preparations incubated with normal mouse ascites fluid in place of the Mab W6/32. The results, expressed as a Bound/Total %, were determined by subtracting the average background radioactivity of the corresponding blanks from each test then dividing by the total amount of radioactivity added to each test and multiplying by 100 to obtain a percent value. Platelet and lymphocyte viability was assessed using Trypan Blue dye, before and after incubation in PHA to insure that

the cells remained viable under the various conditions studied.

A second experiment was run using the same experimental protocol with the exception that the concentration of PHA used for stimulation was held constant (25 $\mu$ g/ml) and the incubation times increased over a period of 3 days in order to determine the optimal stimulation time for lymphocytes and to investigate if a longer stimulation time was needed for platelets to show a detectable response to PHA.

#### 2.3.7 PCR Experimental Protocol

RNA extracts were prepared from platelets and lymphocytes collected from a known HLA-A2 donor according to the procedures already described. The total platelet and lymphocyte RNA recovered from each aliquot of cells was used in all subsequent cDNA synthesis and amplification steps according to the methods previously described.

Six separate RNA extractions (3 platelet and 3 lymphocyte) were performed on aliquots of cells, each containing  $1 \times 10^{10}$  platelets or  $1 \times 10^4$  lymphocytes and treated as follows; one RNA preparation for each cell type was treated as described below for the negative control, one preparation of each was subjected to cDNA synthesis and DNA amplification as described below for the test samples and the third RNA extract for each cell type was subjected to 2 serial 1:10 dilutions with 0.1% DEPC treated sterile water before cDNA synthesis and DNA amplification steps were

performed. These diluted total RNA samples were treated in an identical manner to that of the undiluted test DNA samples and were included to provide a means of assessing the relative efficiency of the PCR in its ability to amplify specific HLA-A2 message to a detectable level.

cDNA synthesis was performed on the lymphocyte and platelet DNA controls using the oligo (dT) primer supplied with the cDNA kit which directs first strand synthesis of all message present in the respective extracts. These negative control samples were then subjected to amplification by the PCR in the absence of specific primers for HLA-A2 message. In the case of the test DNA samples run in the PCR, cDNA synthesis was performed on both the lymphocyte and platelet RNA extracts using a 1.0 $\mu$ M concentration of PCR-2 to direct first strand synthesis of specific HLA-A2 message only. In subsequent amplification steps, both oligonucleotide primers for HLA-A2 message were added to the PCR reaction mixture such that amplification of this specific message could occur if the appropriate complementary DNA sequences were present in the original RNA extract. A "no-DNA" control was run in parallel with the above control and test samples to insure that the PCR reaction mixture (containing both primers) did not contribute to the formation of any amplified sequences in the absence of appropriate message.

## RESULTS

### 3.1 Passive Adsorption and Elution of Class I HLA Antigens from Platelet Membranes

#### 3.1.1 Rationale and Objectives

The degree to which platelets are reported to passively adsorb or elute Class I HLA antigens varies considerably among different groups of investigators (Lalezari and Driscoll 1982; Blumberg et al. 1984; Warejcka et al. 1985; Santoso et al. 1986; Kao et al. 1987). Thus, as a starting point in the investigation of the origin of Class I HLA antigens on platelet membranes, it was deemed appropriate to begin by determining the degree to which passive adsorption and elution of Class I antigens from platelets takes place in vitro.

#### 3.1.2 Results

The results of the adsorption and elution experiments described in the experimental protocol sections 2.3.1 and 2.3.2 are given in tables 4 and 5 respectively. By comparison to the negative controls in the adsorption experiments, a small amount of HLA-A2 antigen was detected by all three assays performed on A2-negative platelets after 5 days of incubation in plasma containing soluble HLA-A2 substance. This result supports the findings of other investigators (Lalezari and Driscoll 1982; Santoso et al. 1986) that at least some of the HLA antigen present on platelets is likely adsorbed.

TABLE 4

PASSIVE ADSORPTION OF CLASS I ANTIGENS ONTO PLATELET MEMBRANES<sup>1</sup>Assay Using Mab to HLA-A2

<u>Incubation Conditions</u>	<u>IPSIFT<sup>*</sup> (1+-4+)</u>	<u>PRAT<sup>*</sup> (B/T%)</u>	<u>ELISA<sup>*</sup> (O.D.)</u>
FRESH	neg	1.4 ± .022	.254 ± .026
5 days in HLA-A2 Positive Plasma	1+	4.2 ± .036	.352 ± .018
5 days in Autologous Plasma	neg	1.5 ± .020	.259 ± .023
5 days in Plasmalyte	neg	1.3 ± .018	.267 ± .021

1. All Platelet donors selected were HLA-A2 negative.

\* Mean ± S.D. n=6

The results of the passive elution experiments (Table 5) show no significant change in the total amount of Class I antigen present before and after 5 days incubation in either; homologous plasma containing soluble HLA antigens other than those present on the test platelets, autologous plasma containing the same soluble HLA antigens as present on the platelets or plasmalyte<sup>TM</sup>, which was devoid of soluble HLA antigens. These results support the belief that platelet HLA antigens, whatever their source of origin, do not readily elute once bound.

TABLE 5

PASSIVE ELUTION OF CLASS I HLA ANTIGENS FROM PLATELET MEMBRANES

Incubation Conditions	Assay Using W6/32 Mab		
	IPSIFR* (1-4+)	PRAT* (B/T%)	ELISA* (O.D.)
FRESH	3+	16.4 ± .130	.962 ± .039
5 days in Homologous Plasma	3+	18.1 ± .142	.985 ± .042
5 days in Autologous Plasma	3+	17.1 ± .126	.940 ± .04 0
5 days in Plasmalyte	3+	17.5 ± .129	.972 ± .045

\* Mean ± S.D. n=6

### 3.2 Elution of Class I HLA Antigens from Platelet Membranes by Chloroquine

#### 3.2.1 Rationale and Objectives

It has been previously reported that hypertonic acid chloroquine (200 mg/ml, pH 5.0) is effective in eluting HLA antigen from platelet membranes but not lymphocyte membranes (Blumberg et al. 1984; Nordhagen and Flaathen 1985; Kao 1987) as well as in eluting passively adsorbed IgG antibodies from the platelet surface (Masel et al. 1988), and red cell membranes (Holtz et al. 1973; Mantel and Holtz 1976; Edwards et al. 1982). Furthermore, it has been suggested by several authors that this phenomenon is consistent with the hypothesis that HLA-A,B antigens are adsorbed onto platelet surfaces (Blumberg et al. 1984; Nordhagen and Flaathen 1985) and that if these antigens are in fact adsorbed membrane proteins, then chloroquine would not be expected to elute those membrane proteins which are integral in nature (Kao 1988). To test the latter hypothesis, platelets were treated with a solution of hypertonic acid chloroquine and tested in the ELISA assay and IPSIFT to determine the residual HLA-A,B reactivity as well as that of the PL<sup>A1</sup> antigen, which is associated with the integral membrane GPIIIa. Protein banding patterns before and after chloroquine stripping were analyzed and the integrity of the PL<sup>A1</sup> antigen was assessed by immunoblot analysis.

### 3.2.2 Results

The results of the chloroquine stripping experiments described in section 2.3.3 are given in Table 6, Table 7, Figure 4. Table 6 shows the HLA-A,B and PL<sup>A1</sup> serological activity detected before and after chloroquine treatment as assessed by the ELISA assay. HLA antigen reactivity on platelets was detected by using Mab W6/32 and PL<sup>A1</sup> antigen reactivity was detected using an alloimmune source of anti-PL<sup>A1</sup>. The amount of HLA antigen detected on the platelets decreased markedly after chloroquine treatment from an initial O.D. reading of 0.924 to 0.302. The amount of PL<sup>A1</sup> antigen reactivity detected after treatment with chloroquine was comparable to the pre-treatment values obtained.

Table 7 shows the relative fluorescent staining intensity of platelets incubated with either the Mab W6/32 or anti-PL<sup>A1</sup>, before and after chloroquine stripping. The fluorescent staining intensity obtained after incubation with Mab W6/32 was significantly reduced after chloroquine treatment in all 6 platelet donors while that of platelets incubated with anti-PL<sup>A1</sup> remained unchanged. Weak fluorescence was detected after chloroquine stripping using the Class I framework Mab in 2 of the 6 platelet donors.

TABLE 6  
RESIDUAL HLA-A,B AND PL<sup>A1</sup> ACTIVITY AFTER  
TREATMENT WITH HYPERTONIC ACID CHLOROQUINE

MEMBRANE PROTEIN	DETECTING ANTIBODY	TREATMENT	
		PBS-EDTA <sup>1</sup> (before)	CHLOROQUINE <sup>1</sup> (after)
HLA	W6/32	.924 ± .039	.302 ± .065
PL <sup>A1</sup>	Anti-PL <sup>A1</sup>	.683 ± .039	.697 ± .071

1. Values represent O.D. readings at 405 nm (mean ± S.D.) n=6

TABLE 7

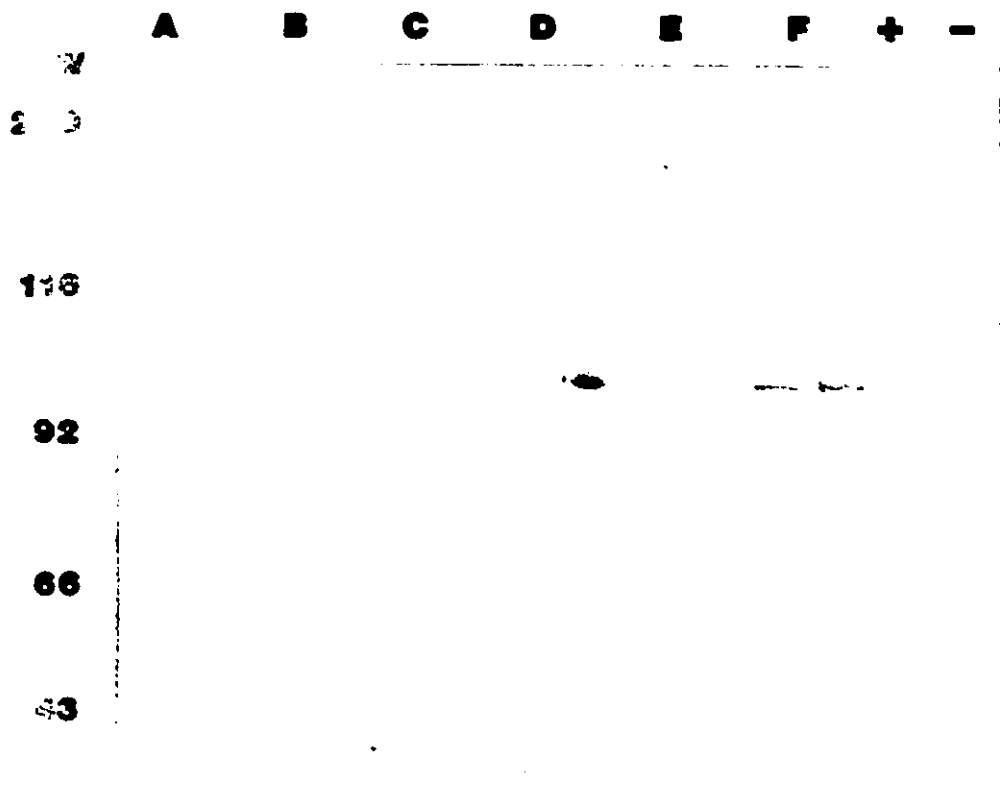
PLATELET MEMBRANE FLUORESCENCE DUE TO HLA-A, B  
AND PL<sup>A1</sup> ANTIGEN EXPRESSION BEFORE AND AFTER  
CHLOROQUINE TREATMENT

PLATELET DONOR	CHLOROQUINE TREATMENT			
	BEFORE		AFTER	
	HLA	PL <sup>A1</sup>	HLA	PL <sup>A1</sup>
1	4+	4+	+/- <sup>1</sup>	4+
2	4+	3+	-	3+
3	4+	3+	-	3+
4	3+	4+	-	4+
5	4+	4+	+/-	4+
6	4+	3+	-	3+

1. Membrane fluorescence weakly detected.

The integrity of PL<sup>A1</sup> antigen after Chloroquine treatment of platelets is shown in Figure 4. The bands appearing at an approximate mol. wt. of 105,000 correspond to the binding of anti-PL<sup>A1</sup> to the PL<sup>A1</sup> epitope present on GPIIIa molecules. The ability to demonstrate PL<sup>A1</sup> antigen by immunoblot indicates that hypertonic acid chloroquine treatment neither elutes integral membrane proteins such as GPIIIa nor does it affect the integrity of PL<sup>A1</sup> antigen.

FIGURE 4. INTEGRITY OF PL<sup>A1</sup> ANTIGEN AFTER CHLOROQUINE TREATMENT OF PLATELETS. SDS solubilized platelets from 6 PL<sup>A1</sup> positive donors (A through F) were electrophoresed on a 6% PAGE before treatment (1st Lane) and after treatment (2nd Lane) with hypertonic acid chloroquine under non-reduced conditions. The gel was electroblotted onto nitrocellulose paper, incubated with an alloimmune serum containing anti-PL<sup>A1</sup> then developed using the Protoblot<sup>TM</sup> system. Untreated platelets from a known PL<sup>A1</sup> positive donor (+) and a known PL<sup>A1</sup> negative donor (-) were run as controls. The bands migrating at an apparent mol. wt. of 105 KDa, correspond to the binding of antibody to the PL<sup>A1</sup> epitope present on GPIIIa. Note the relative staining intensities of the PL<sup>A1</sup> bands are comparable for both treated and untreated platelets. Note also the disappearance of the high mol. wt. bands migrating at an approximate mol. wt. of 200,000 in the chloroquine treated platelets (2nd Lane for each donor). These high mol. wt. bands correspond to the loosely bound IgG immune complexes which are known to be passively adsorbed onto platelet membranes.



3.3 Stability of Class I HLA and PL<sup>A1</sup> Antigens on Platelets After Treatment With a Hypertonic Salt Solution (3M KCl) And A Mild Denaturing Agent (1M Urea)

3.3.1 Rationale and Objectives

According to the definition of Singer and Nicolson (1972), integral membrane proteins can be distinguished from adsorbed membrane proteins by the ease with which they dissociate from the cell membrane. As such, mild treatments such as increasing ionic strength of the surrounding medium and mild denaturation should be effective in eluting only loosely adsorbed proteins and should not affect the presence or expression of integral proteins. To further investigate the binding stability of platelet HLA-A,B antigens, platelets were subjected to treatment with 3M KCl and 1M Urea in separate experiments. If the HLA antigens on platelets are indeed adsorbed in nature then it was rationalized that such mild treatments should be capable of eluting HLA antigen (to some extent) without adversely affecting other known integral membrane proteins such as platelet GPIIb-IIIa.

Lymphocytes were run in parallel to compare the stability of the HLA-A,B antigens on lymphocytes under the same conditions however these cells did not remain intact (osmotically) during the course of treatment with these agents.

In a further set of experiments, the stability of PL<sup>A1</sup> antigen was assessed after both 3M KCl and 1M Urea treatment by Western Blot analysis (see experimental protocol section 2.3.4).

### 3.3.2 Results

The results of these experiments are shown in Figures 5, 6 and 7 and Table 8. Figures 5 and 6 show respectively, the effect of 3M KCl and 1M Urea treatment of platelets on the detectable quantity of HLA-A,B over various incubation intervals. Both these treatments were effective in significantly reducing the the amount of detectable HLA-A,B over the time periods studied however, residual HLA remained in both cases after the optimal treatment interval had been reached. This residual HLA antigen could not be eluted by extending the treatment intervals or by increasing the molar concentration of the KCl to 4M and that of the Urea to 2M (Results not shown).

Table 8 shows the effect of 3M KCl and 1M Urea on the stability of both HLA- A,B and PL<sup>A1</sup> antigens after treatment for 15 hours and 2 hours at 4°C respectively.

Figure 7 visually demonstrates the stability of PL<sup>A1</sup> antigen after PL<sup>A1</sup> positive donor platelets were subjected to treatment with 3M KCl for 15 hours at 4°C and 1M Urea for 2 hours at 4°C. The staining intensity of the PL<sup>A1</sup> bands did not differ from the untreated control platelets indicating that neither 3M KCL nor 1M Urea were effective in eluting integral GPIIIa molecules from platelet membranes.

FIGURE 5. EFFECT OF VARIOUS 3M KCL TREATMENT INTERVALS ON THE PRESENCE OF HLA-A,B ANTIGENS ON PLATELETS. Platelets treated with 3M KCl over a period of 0-30 hours (□) and untreated (i.e. incubated with PBS-EDTA) platelets (■) were tested in the ELISA assay to determine the level of HLA-A,B antigen expression. Maximal elution of HLA-A,B was seen after 15 hours of incubation however residual HLA could still be detected after 30 hours of treatment with this hypertonic salt solution.

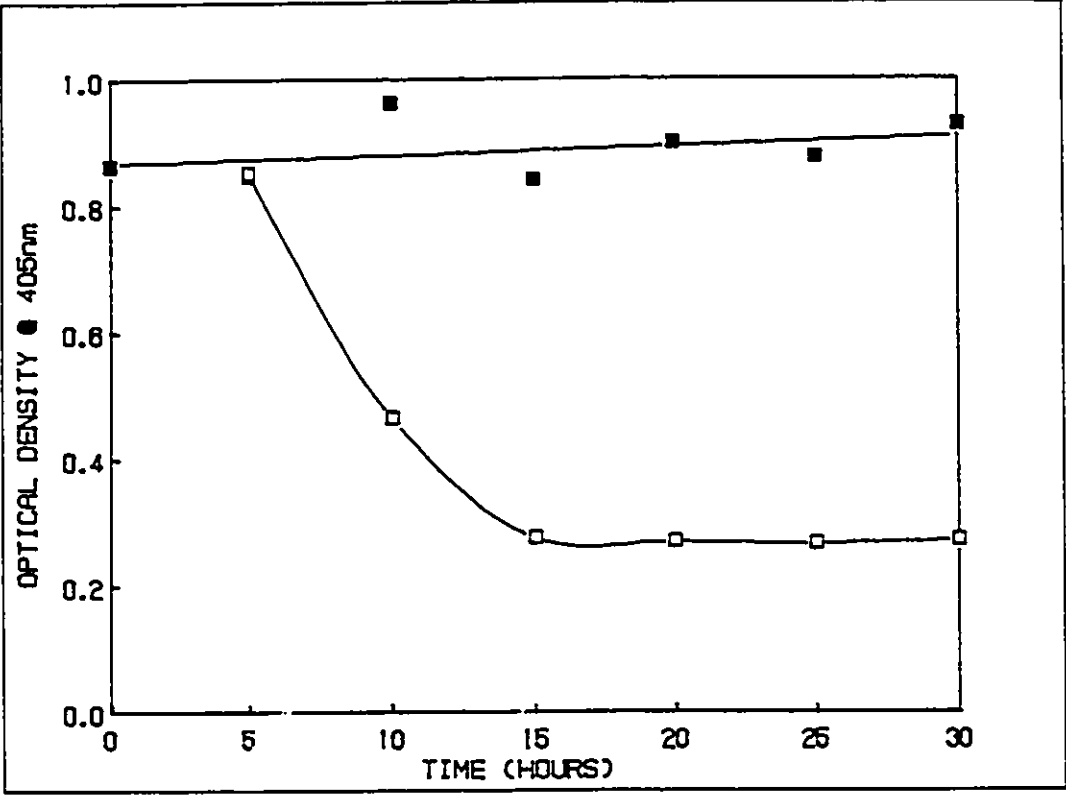
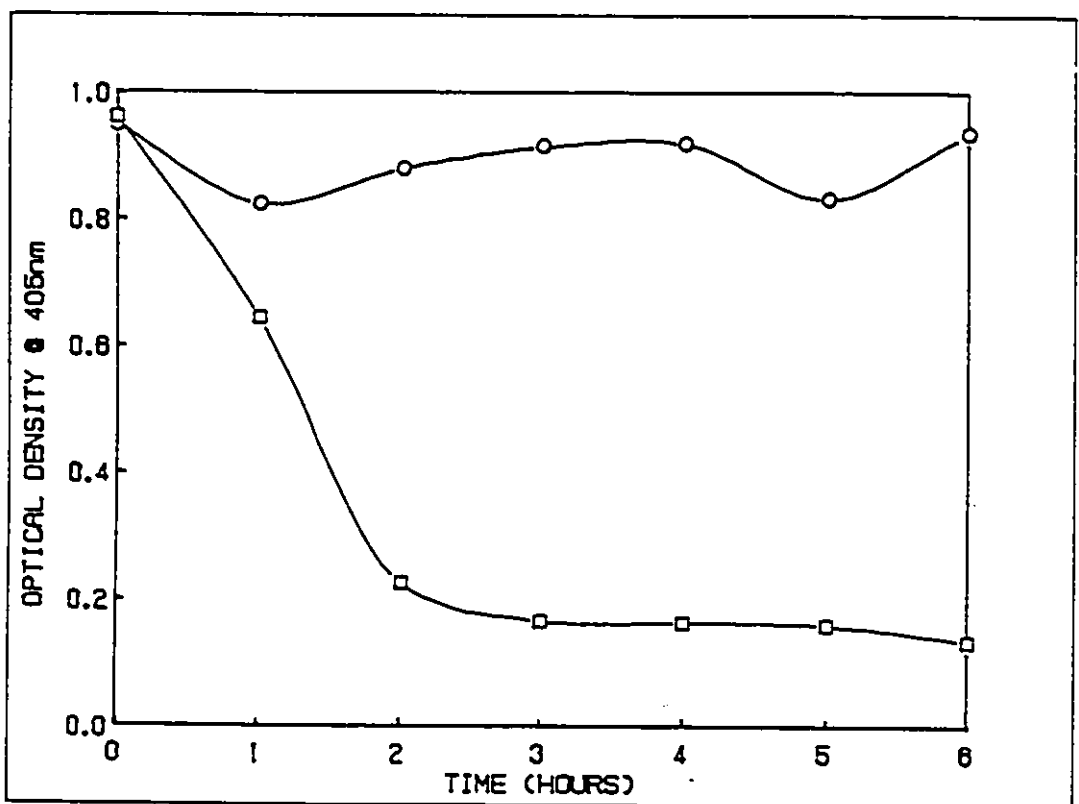


FIGURE 6. EFFECT OF VARIOUS 1M UREA TREATMENT INTERVALS ON THE PRESENCE OF HLA-A,B ANTIGENS ON PLATELETS. Platelets treated with 1M Urea (◻) over a period of 0-6 hours at 4°C and untreated (i.e. PBS-EDTA treated) platelets (○) were tested in the ELISA assay to determine the detectable level of HLA-A,B antigen. Maximal elution of these antigens was seen after 2 hours of treatment with 1M Urea. Residual HLA (i.e. a non-elutable HLA component) could be detected at all remaining treatment intervals and was present on platelets that had been incubated in 1M Urea for up to 24 hours (results not shown).



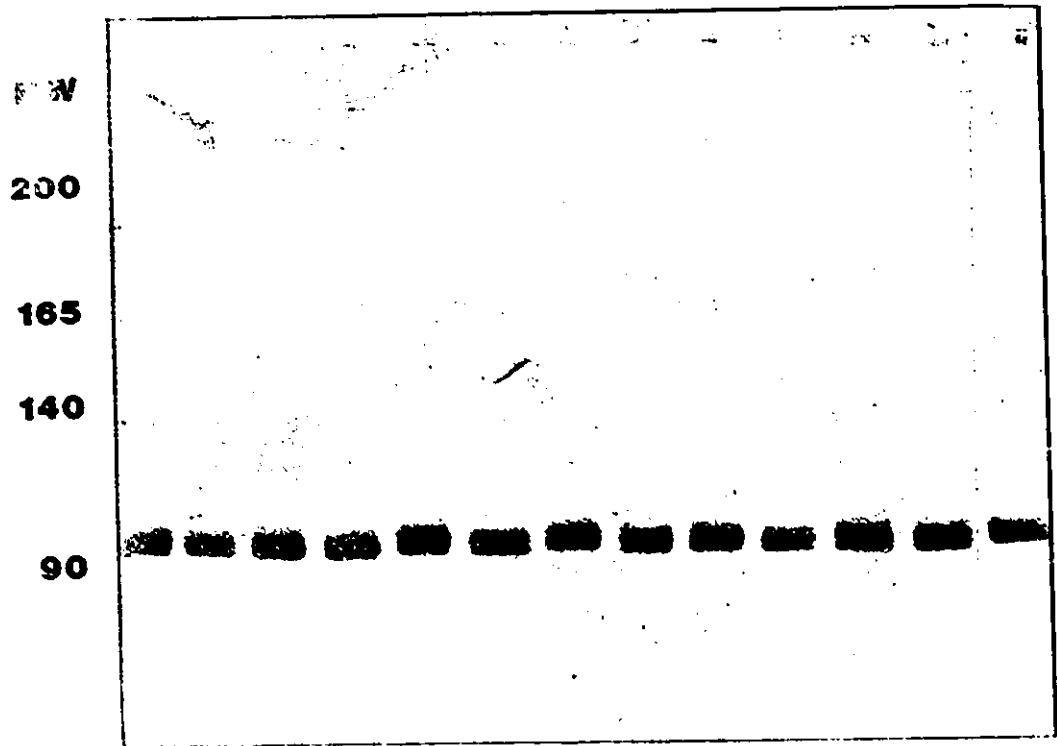
**TABLE 8**  
**STABILITY OF HLA AND PL<sup>A1</sup> ANTIGENS AFTER TREATMENT**  
**WITH 3M KCL AND 1M UREA**

TREATMENT	MEMBRANE PROTEIN DETECTED <sup>1</sup>	
	HLA (W6/32) <sup>2</sup>	PL <sup>A1</sup> (ANTI-PL <sup>A1</sup> ) <sup>3</sup>
3M KCl	.323 ± .064	.611 ± .068
PBS-EDTA (control)	.862 ± .044	.579 ± .037
1M Urea	.156 ± .083	.577 ± .076
PBS-EDTA (control)	.785 ± .048	.622 ± .036

1. Figures represent O.D. readings at 405nm. Mean ± S.D. n=6.
2. HLA activity detected using McAb W6/32. Negative control = PBS-EDTA treated platelets + normal mouse ascites.
3. PL<sup>A1</sup> activity detected using an alloimmune source of anti- PL<sup>A1</sup>. Negative control = PBS-EDTA treated platelets + normal AB Rh neg human serum.

FIGURE 7. STABILITY OF PL<sup>A1</sup> ANTIGEN ACTIVITY AFTER TREATMENT WITH HYPERTONIC SALT SOLUTION (3M KCL) AND A MILD DENATURING AGENT (1M UREA). SDS solubilized platelets from 6 PL<sup>A1</sup> positive donors (a through f) were electrophoresed on a 6% PAGE under non-reducing conditions after treatment with 3M KCl for 15 hours at 4°C. (donor group A) or with 1M Urea for 2 hours at 4°C. (donor group B). Platelets from an untreated PL<sup>A1</sup> positive donor were run in parallel as a control (+). The resulting gel was electroblotted onto nitrocellulose paper, incubated with an alloimmune source of anti-PL<sup>A1</sup>, then developed using the Protoblot<sup>TM</sup> system. The presence of bands migrating at an apparent mol. wt. of 105 KDa, corresponds to the binding of antibody to the PL<sup>A1</sup> epitope present on GPIIIa. The staining intensity of the bands on the treated platelets does not differ from that of the control platelets indicating that neither 3M KCl and 1M Urea are effective in eluting GPIIIa molecules off platelet membranes.

A B  
: a b c d e f : a b c d e f : +



### 3.4 Effect of Low PH Treatment of Platelets on The Presence of Class I HLA and PL<sup>A1</sup> Antigens

#### 3.4.1 Rationale and Objectives

Recently it has been reported that the ability to serologically detect surface Class I HLA can be eliminated without significant cell destruction by brief exposures to low pH (Sugawara et al.

1987, Kurata et al. 1989). The presence of HLA-A,B antigens was thus further investigated after treatment with citric acid at a pH of 3.0 over various treatment intervals and compared to that of PL<sup>A1</sup> antigen expression on platelets after identical treatment in citric acid for 10 min at 4°C.

#### 3.4.2 Results

The results of these experiments are shown in Figures 8 and 9. Figure 8 shows the effect of low pH treatment over time on the expression of HLA-A,B on platelet membranes. A significant reduction in HLA-A,B activity was seen after subjecting platelets to low pH for only 10 minutes. Figure 9 visually demonstrates the stability of PL<sup>A1</sup> antigen on platelet membranes after low pH treatment for 10 min at 4°C. The presence of intact PL<sup>A1</sup> bands on the immuoblot indicates that low pH citric acid treatment has no effect on integral proteins such as GPIIIa.

FIGURE 8. EFFECT OF VARIOUS TREATMENT INTERVALS WITH LOW PH CITRIC ACID ON THE LEVEL OF HLA-A,B ANTIGEN DETECTED ON PLATELET MEMBRANES. Platelets treated (□) with buffered citric acid (pH=3.0) over a period of 0-30 minutes at 4°C then tested in the Elisa assay to determine the relative amounts of HLA-A,B that remained over time. Untreated platelets (■) were run in parallel as a negative control. A significant reduction in the quantity of membrane bound HLA-A,B was seen after treatment for only 10 min in buffered citric acid. This treatment was very effective in eluting HLA antigen from the platelet membrane however, a residual amount of antigen activity could still be detected after 30 min of exposure to citric acid.

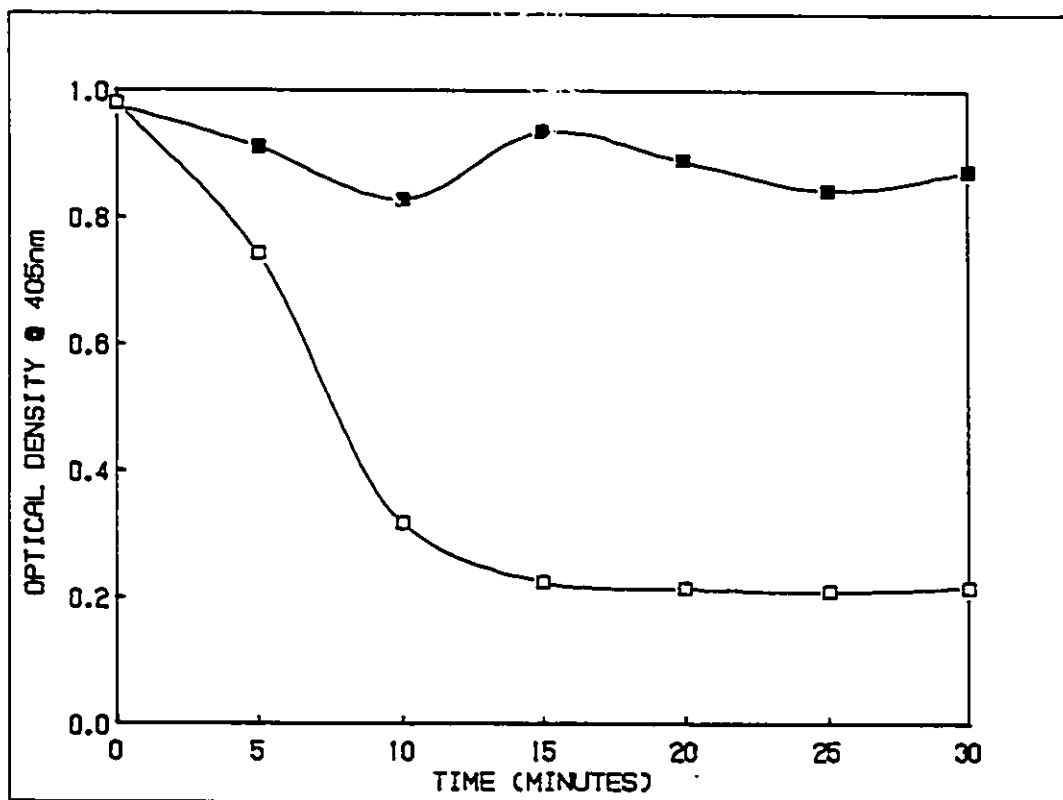


FIGURE 9. STABILITY OF PL<sup>A1</sup> ANTIGEN ON PLATELETS AFTER LOW PH TREATMENT WITH BUFFERED CITRIC ACID. SDS solubilized platelets from 6 PL<sup>A1</sup> positive donors (A through F) were electrophoresed on a 6% PAGE under non-reducing conditions after low pH treatment for 10 min at 4°C. The resulting gel was electroblotted onto nitocellulose paper then incubated with an alloimmune source of anti-PL<sup>A1</sup> and developed using the Protoblot<sup>TM</sup> system. The band migrating at an apparent mol. wt. of 105, corresponds to the binding of antibody to the PL<sup>A1</sup> epitope present on GPIIIa. The presence of this band indicates that low pH treatment of platelets does not elute the integral bound GPIIIa molecules present on platelet membranes.

**A B C D E F**

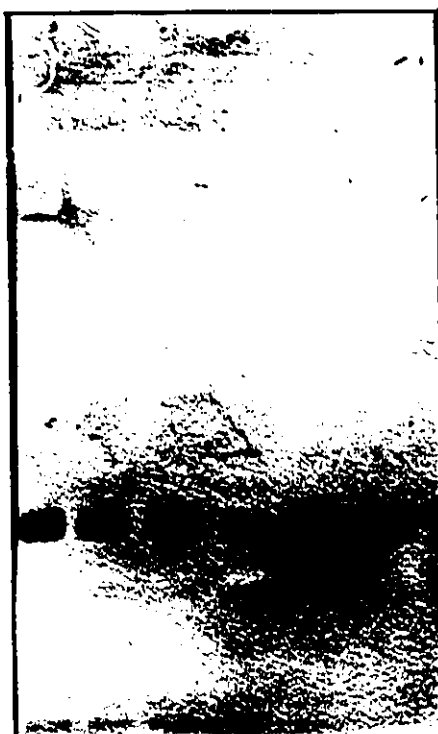
**NW**

**200**

**165**

**140**

**90**



### 3.5 Effect of PHA Stimulation on The Expression of Class I HLA Antigens on Platelet Membranes

#### 3.5.1 Rationale and Objectives

Phytohemagglutinin (PHA) has previously been shown to stimulate an increase in the expression of Class I HLA antigens on the surface of lymphocytes (McCune et al. 1975). These investigators showed that a significant increase in HLA antigen density could be observed within 12 hours of incubation and that the increased expression induced by PHA was dose-dependent. Lymphocyte blast transformations of this nature require the presence of both a carbohydrate receptor for PHA on the cell surface and nascent message (ie. mRNA for Class I antigen synthesis) for Class I HLA synthesis and expression. In order to investigate the possibility that platelets may possess the necessary membrane receptors for PHA, mRNA and appropriate protein synthesis machinery to respond to PHA stimulation in a similar manner to lymphocytes, viable platelets were exposed to various concentrations of PHA over time and the relative amounts of HLA antigen detected before and after stimulation was compared to that of lymphocytes collected from the same donors.

#### 3.5.2 Results

The results given in Figure 10 show that platelets did not respond to PHA stimulation after 48 hours incubation since the amount of Mab W6/32 bound by the platelets did not differ

significantly over the range of concentrations studied, from the unstimulated, baseline value of 17.2 B/T%.

Lymphocytes, on the other hand, showed a maximal stimulation over the 48 hours of incubation in the presence of 25 $\mu$ g/ml of PHA. Figure 11 shows the effect of holding the concentration of PHA used to stimulate the cells constant (25 $\mu$ g/ml) and incubating the cells over a period ranging from 2 to 72 hours. Again, the platelets did not show a change in the detectable level of Class I HLA antigen, even after 72 hours of incubation in the presence of PHA. The control lymphocytes run in parallel, showed a greater than 2 fold increase in the amount of detectable HLA Class I antigen after exposure to PHA over the same 72 hour incubation period.

FIGURE 10. EFFECT OF INCREASING PHA CONCENTRATIONS ON THE EXPRESSION OF HLA-A,B ANTIGENS ON PLATELET MEMBRANES AFTER 48 HOURS OF INCUBATION. Platelets (□) and lymphocytes (○) from the same donors (n=6) were incubated with increasing concentrations of PHA (1, 5, 10, 25, 50 $\mu$ g/ml) over a period of 48 hours. Negative controls run in parallel consisted of platelets (■) and lymphocytes (●) from the same 6 donors incubated in the absence of PHA under the same conditions. HLA antigen expression on lymphocytes showed a marked increase (24.3 B/T% to 38.4 B/T%) over the range of PHA concentrations studied however the expression of these antigens on platelets remained constant under the same conditions (17.2 B/T% to 18.1 B/T%). Maximal HLA antigen expression on lymphocytes after 48 hours of PHA stimulation was seen at a PHA concentration between 20 and 30  $\mu$ g/ml.

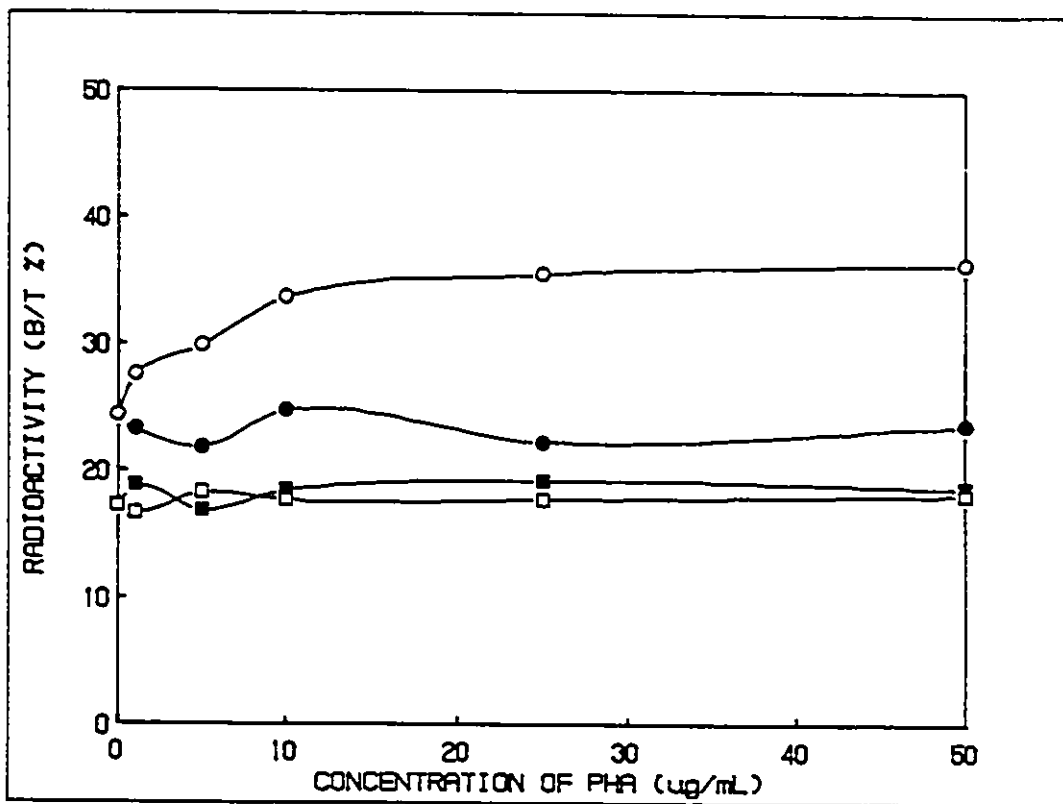
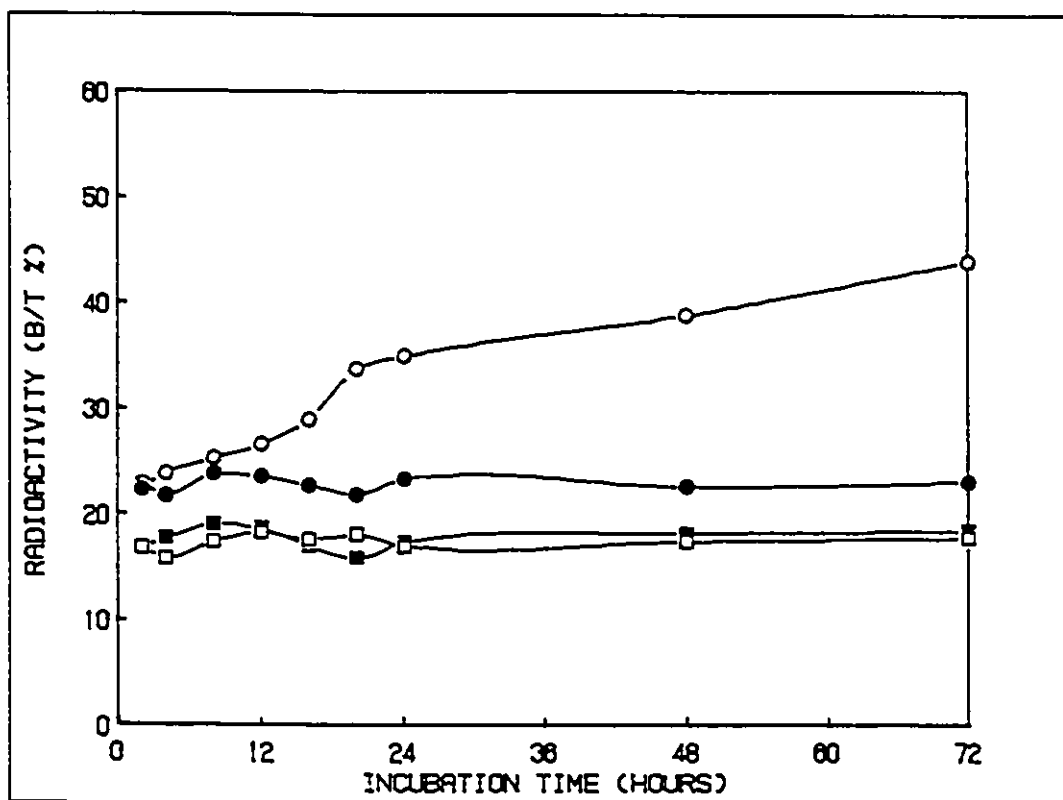


FIGURE 11. HLA-A,B ANTIGEN EXPRESSION ON PLATELETS AFTER STIMULATION WITH A 25 $\mu$ g/ml CONCENTRATION OF PHA OVER VARIOUS INCUBATION INTERVALS. Platelets (□) and lymphocytes (○) from the same donors (n=6) were incubated in 25 $\mu$ g/ml of PHA over various incubation intervals from 0 to 72 hours. Negative controls run in parallel consisted of platelets (■) and lymphocytes (●) from the same 6 donors incubated in the absence of PHA under the same conditions. HLA antigen expression on platelets remained constant (16.8 B/T% to 17.4 B/T%) over the course of the incubation intervals studied while that of lymphocytes increased greater than 2 fold (22.3 B/T% to 48.4 B/T%) over the same 72 hour exposure to PHA.



### 3.6 Enzymatic Amplification of Platelet mRNA by The Polymerase Chain Reaction

#### 3.6.1 Rationale and Objectives

A method to enzymatically amplify short segments of platelet derived DNA was recently reported by Newman et al. 1988. These authors reported the direct amplification of GPIIIa message contained in RNA extracts prepared from platelets isolated from the upper half of the PRP derived from 50 ml of whole blood (i.e. about  $5 \times 10^9$  platelets). A major deterrent to investigating the primary structure of platelet specific proteins has been the inability to obtain sufficient mRNA to produce informative DNA libraries. This report represents the first time that investigators have been able to demonstrate the presence of nascent message in the vestigial RNA that exists within platelets.

It was rationalized that if it is possible to demonstrate with this technique, that GPIIIa message is present in platelets, then it should also be possible to demonstrate the presence of nascent message encoding for HLA Class I antigens, if in fact, such message actually exists within platelets. Obviously, if message for HLA Class I antigen could be demonstrated to be present in platelet derived RNA, it would provide considerable evidence in favor of the existence of an integral HLA component.

To investigate the possibility that HLA Class I message exists in platelets, RNA extracts were prepared from both

platelets and lymphocytes (positive control) isolated from a known HLA-A2 donor, These total RNA extracts were then subjected to cDNA synthesis and subsequent amplification by the polymerase chain reaction using specific oligonucleotide primers for HLA-A2 message.

### 3.6.2 Results

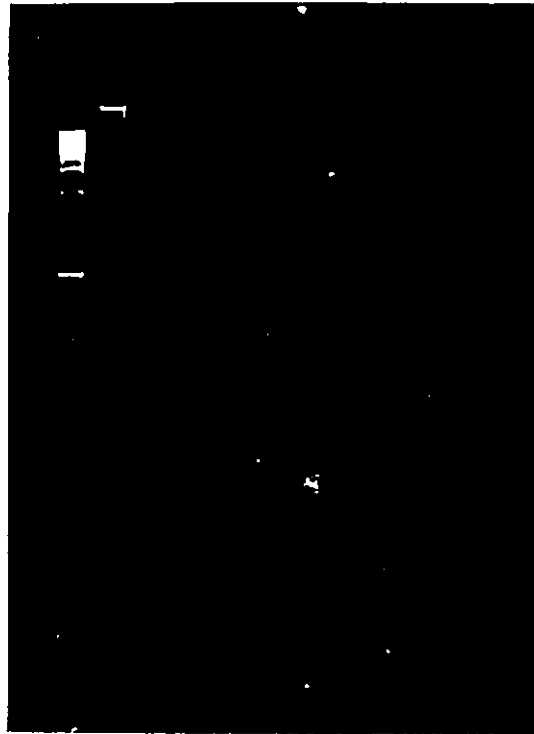
The results of the PCR experiments conducted are shown in Figure 12. The lymphocyte control DNA samples (Lanes D, E and F) show a 500 kb fragment of amplified DNA. This band was shown to be specific for HLA-A2 message by hybridization of a  $^{32}\text{P}$ -labeled probe prepared from PCR-1 (results not shown). The intensity of the EtBr staining of this fragment diminished as the amount of lymphocyte DNA used in the PCR was decreased (Lane D= $10^4$ , Lane E= $10^3$  and Lane F= $10^2$  lymphocytes used for extraction). This amplification strategy failed to detect the presence of HLA-A2 message in the platelet derived DNA samples (Lanes G,H and I). In an attempt to determine if the message was present but still below the level of detection, the 3 platelet samples were placed back in the PCR thermal-cycler and run for a further 30 amplification cycles (see methods section for cycle parameters) after replenishing the reaction mixture with a  $2\mu\text{M}$  concentration of each primer and 5U of Taq Polymerase. The 500 kb sequence could not be detected even after a further 30 amplification cycles (results not shown). This same

experiment was repeated several times, allowing the PCR to cycle 30, 60 and 90 times without interruption, with the same negative results. A further experiment using  $2 \times 10^{10}$  platelets in the RNA extraction and the same conditions for cDNA synthesis and DNA amplification (i.e. 30, 60 and 90 cycles) also failed to detect the presence of HLA-A2 message. Further platelet extractions using more cells were not contemplated as the volume of cells used in the extraction exceeded the solubilizing capability of the guanidinium isothiocyanate at numbers much greater than  $1 \times 10^{10}$ .

FIGURE 12. ENZYMATIC AMPLIFICATION OF PLATELET AND LYMPHOCYTE DNA DERIVED FROM HLA-A2 POSITIVE CELLS USING SPECIFIC PRIMERS FOR HLA-A2 MESSAGE. Total platelet and lymphocyte RNA was transcribed to cDNA and enzymatically amplified in the presence of specific primers for HLA-A2 message (test DNA samples) or in the absence of these primers (control DNA samples). After amplification by PCR was completed, 15 $\mu$ l samples of each reaction mixture was analyzed on a 1% agarose/1% nuseive gel and the resulting bands visualized by EtBr staining. Lanes A, B and C correspond to the negative control samples as follows; lymphocyte DNA control (lane A), platelet DNA control (lane B) and no DNA control (lane C). Lanes D, E and F correspond to the PCR positive control samples which consisted of DNA extracts prepared from lymphocytes as follows; 1 X 10<sup>4</sup> lymphocytes (lane D), 1 X 10<sup>3</sup> lymphocytes (lane E) and 1 X 10<sup>2</sup> lymphocytes (lane F). lanes G, H and I correspond to platelet DNA isolated from; 1 X 10<sup>10</sup> platelets (lane G), 1 X 10<sup>9</sup> platelets (lane H) and 1 X 10<sup>8</sup> platelets (lane I). The resulting DNA bands are compared to a 1 Kb DNA standard run in parallel (STD). Note the presence of a 500 bp amplified DNA sequence in lanes D and E (lymphocyte DNA), which is not detected in the platelet DNA samples run in lanes G, H and I.

**STD**  
**A B C D E F G H I**

**Kb**  
**3054**  
**2036**  
**1635**  
**1018**  
**520**



## DISCUSSION

The availability of effective platelet products has dramatically improved the survival of patients suffering from a variety of neoplastic and hematological disorders. As the treatment for these disorders has advanced, the demand for platelets has increased proportionally. This increased platelet utilization has, in turn, been paralleled by the need for a better understanding of platelet biochemistry, function, optimal storage parameters and post-transfusion efficacy. This thesis presents the studies done in an attempt to further our knowledge with regards to the presence of HLA-A,B antigens on platelets, with new information that will, hopefully, promote future interest in investigating new and novel approaches to providing compatible platelets to alloimmunized patients.

The primary objective of this investigation was to explore the nature of the association of HLA Class I antigens with platelet membranes (ie. as integral or adsorbed membrane proteins) in hopes of discovering new information about their origin. In an attempt to further this objective, the expression of these antigens was studied at both the cellular and the RNA level. At the cellular level, HLA antigen expression was studied under a number of different conditions known to elute adsorbed membrane proteins. The results of such treatments were compared to that of the platelet specific PL<sup>A1</sup> antigen which is known to be associated with the integral

membrane protein GPIIIa. The studies done at the RNA level represent a new approach to this question, made possible by the development of the PCR technique which enables one to enzymatically amplify specific DNA sequences from  $10^5$  to  $10^9$  times (Saiki et al. 1988). The results of the studies conducted and presented in Section 3.0 (Results) are discussed below.

The controversy which exists in the literature, stems from conflicting reports as to the nature of platelet HLA specificity (i.e. antigens). Depending on the studies conducted and the conclusions drawn, it has been reported both that; HLA specificity is adsorbed readily from plasma (Blumberg et al. 1982; Lalezari and Driscoll 1982) and, that HLA specificity cannot be acquired from plasma (Warejcka et al. 1985). It has also been reported that various amounts of HLA antigen activity on platelets can be removed without a concomitant loss of known integral membrane proteins under certain relatively mild conditions which include; treatment with hypertonic acid chloroquine (Blumberg et al. 1984; Nordhagen and Flaathen 1985; Kao 1987) and low acid pH treatment (Sugawana et al. 1986; Kurata et al. 1988). Finally, there exists contradicting reports as to the relative amounts each species (i.e adsorbed and integral) which range from reports that; (i) the majority of HLA antigens on platelets are acquired from plasma (Lalezari and Driscoll 1982) to, (ii) >50% of these antigens are acquired (ie. adsorbed) in nature (Kao 1987) to (iii) the major portion of

HLA antigens present on platelets appear to be integral in nature (Santoso et al. 1986).

As a starting point, studies were conducted to determine the relative amount of passive adsorption and elution of Class I antigens. The results of the adsorption experiments showed that HLA-A2 specificity could be weakly detected on HLA-A2 negative platelets after 5 days of incubation in plasma from an HLA-A2 positive individual. These results support the findings of other investigators (Lalezari and Driscoll 1982; Santoso et al. 1986) that platelets are capable of passively adsorbing HLA antigens and that at least some of the HLA activity on platelet membranes is adsorbed. The results of the passive elution experiments showed no appreciable change in the amount of HLA-A,B expression detected after 5 days of platelet incubation in either; homologous plasma, autologous plasma or Plasmalyte™, a synthetic plasma-free nutrient medium for platelets which is devoid of any source of protein, including soluble HLA antigen. The fact that HLA expression did not diminish during prolonged incubation in this plasma-free medium suggests that all HLA antigens on platelets are relatively stable in their association with the platelet membrane and therefore, do not passively elute. In addition, these results suggest that the adsorptive properties of platelets are not gradient or concentration dependent, and that the concentration of HLA antigens on platelets is independent of the concentration of soluble HLA antigen in plasma. These findings support previous reports that there

is no correlation between adsorbed HLA concentration and plasma HLA concentration (Kao et al. 1988) and confirm the findings of Dunstan and Simpson (1985) that Class I HLA antigens are stable under a variety of storage conditions.

The results of the chloroquine elution studies confirm previous reports that HLA antigens are elutable with this treatment. The extent to which these antigens could be eluted was assessed by several semi-quantitative assay techniques and compared to the elution of a known integral membrane protein, GPIIIa, from platelets subjected to identical treatments. The ELISA assay results showed a 70-75% reduction in the amount of detectable HLA after platelets were subjected to chloroquine elution (Table 6). The results obtained in the IPISFT showed that the HLA antigen activity had been reduced, after chloroquine stripping, to an undetectable level in 4 of the 6 platelet donors tested. In the remaining 2 donors, a weak fluorescence could still be detected (Table 7). The intensity of the integral GPIIIa bands present did not diminish after chloroquine treatment (Figure 4) thus indicating that chloroquine was effective in eluting the majority of HLA antigens but had no effect on integral GPIIIa proteins.

The results of the 3M KCl, 1M Urea and low pH experiments are consistent with the concept that mild treatments such as increasing the ionic strength of the surrounding medium and mild denaturation of proteins, would be capable of eluting proteins which are only loosely associated with the cell

membrane, but not those which are integral in nature. The results of the experiments conducted to evaluate the binding stability of HLA antigens indicate that all 3 treatments were effective in eluting HLA antigen, but not PL<sup>A1</sup> (GPIIa) antigen. A residual HLA activity which remained constant could be detected after 15 hours of treatment with 3M KCl, after 2 hours of treatment with 1M Urea and after only 10 minutes of treatment in buffered citric acid (i.e. low pH). The incubation time needed to achieve maximal elution of HLA activity varied widely with the 3 treatments however, the residual activity that remained was remarkably constant once maximal elution was achieved (O.D. range in ELISA = 0.195 - 0.323). These results argue strongly in favor of the majority of HLA activity (70-75% in the experiments conducted) being adsorbed in nature. An interesting finding which is unexplained is that only 20-30% of the total HLA activity could be detected after maximal elution had occurred for each treatment. This finding is consistent with the findings of Kao (1987) who conducted quantitative studies on the concentration of HLA antigens on platelets, before and after chloroquine elution and reported that >50% of HLA antigens on platelets are acquired. There is however, a considerable disparity between these two percentages which is larger than expected and hence in need of an explanation. One possible explanation is that the platelets used to conduct these experiments were collected from 5 day platelet concentrates which had just reached their outdating period instead of from

fresh PRP, as was the case in the experiments conducted by Kao. It could be argued that this difference is a result of the difference in mean age of the platelet populations used in the experiments and thus, that the relative concentration of integral HLA species on platelets is a function of platelet age.

The PHA stimulation experiments conducted were not informative from the stand point of furthering the aim of this study since PHA failed to stimulate HLA Class I antigen expression in platelets. The fact that PHA is not capable of stimulating platelet HLA antigen expression is however, new information and therefore noteworthy. Possible explanations for this negative response include; (i) platelets may not have membrane receptors for PHA which would enable them to bind with this mitogen, (ii) platelets do not possess nascent message for the *de-novo* synthesis of HLA antigen or, (iii) that PHA is capable of stimulating synthesis of HLA antigens but that the rate of synthesis is too slow to be detectable over the time period studied. The first two explanations would seem plausible, the latter unlikely. The negative findings in the PCR experiments are not surprising in view of the fact that platelets are known to possess only vestigial amounts of mRNA and no organized nucleus (Evatt and Stein 1988). The inability to demonstrate nascent message in platelets by this technique could be due to one or more of several possible explanations including; (i) HLA Class I message exists within platelets but the amount of specific

mRNA is so minute that it cannot be amplified to a detectable level, (ii) the experimental conditions used in the cDNA synthesis steps and subsequent amplification strategy may not have been optimal for amplification of HLA Class I message, (iii) platelet mRNA is highly unstable and subject to rapid degradation by endogenous RNases such that any HLA-A,B message present in platelet RNA extract would not remain intact long enough to be transcribed into cDNA or, (iv) nascent message for HLA antigen synthesis does not exist within platelets and thus these findings represent the true situation that exists with respect to the presence of specific HLA Class I message in platelets.

The first explanation is unlikely since the PCR is reported to be capable of amplifying even a single mRNA sequence to the point of detection if conducted under optimal conditions (Saiki et al. 1985). The fact that these studies employed identical experimental conditions and amplification strategy to that described by Newman et al. (1988) to demonstrate the presence of mRNA encoding for GPIIIa, also argues against the explanation that the amount of message present exists at a sub-detectable level. The final evidence that argues against this explanation is the fact that HLA message could not be detected even when the total RNA extract used in the PCR step was prepared from  $2 \times 10^{10}$  platelets (ie four times the number used by Newman and his co-workers).

The second explanation is again unlikely since the 500 kb amplified segment was seen in RNA isolates prepared from

as few as  $10^3$  lymphocytes. The presence of this amplified sequence in the positive control DNA indicates that both the cDNA synthesis step, which used PCR-2 to direct the first strand synthesis of specific HLA-A2 message only and the amplification conditions and strategy employed in the PCR step were sufficiently optimal to produce amplification of the message. The fact that amplified message was not detected after allowing the PCR to continue for 90 amplification cycles, further argues against the suggestion that sub-optimal assay conditions were responsible for the outcome. This is the case since if sub-optimal conditions existed during the PCR then it is likely that by doubling or tripling the number of amplification cycles, the amount of message generated will eventually reach a detectable level. The third explanation presented represents a possibility that can not be ruled out as very little is currently known about the existence, stability, half-life or steady state of mRNA present in platelets. The fourth possibility (ie. nascent message for HLA antigen synthesis does not exist in the mature platelet) would thus appear to provide the best explanation for the negative results obtained in these experiments.

The findings of this work suggest that adsorbed HLA-A,B antigen is the predominant species present on platelet membranes however, a detectable amount of the integral species is also present. A recent report by Pereria et al. (1988) introduces the concept that variations in Class I HLA antigen expression seen between high density (older, more picnotic

platelets) and low density (younger, larger platelets) platelet cohorts, may provide a useful marker of platelet age. It can be hypothesized from the findings presented in this thesis that this variable expression is seen as a direct result of the amount of integral vs. adsorbed antigens which in turn is a function of the platelets' age. Thus, young platelets would possess predominately vestigial HLA antigens originating from megakaryocyte synthesis which would be integral in nature and older platelets would possess predominately adsorbed HLA antigen which would be acquired over the course of their circulation in the peripheral blood.

It is further hypothesized that these vestigial integral HLA antigens are shed into the plasma as platelets age and are replaced over the course of the platelets' 10 day life span, with the adsorbed species. Such a hypothesis would be consistent with the reported findings of a 50%/50% ratio of elutable vs. non-elutable HLA species in an unfractionated platelet population such as that found in fresh whole blood and a 75%/25% adsorbed (elutable) vs. integral (non-elutable) ratio seen in a 5 day old population of viable platelets.

The fact that mRNA encoding for HLA-A2 could not be demonstrated in platelet RNA derived from an HLA-A2 positive donor can be explained by the fact that mRNA in all mammalian cells is constantly being degraded at a rate that is equal to the specific half-life of that particular mRNA (Darnell et al. 1986). Platelets are anucleated cells and as such, do not possess DNA from which new mRNA can be generated. It is

unlikely then, that platelet mRNA exists in any form of steady state concentration and likely that the presence of any given mRNA in circulating platelets would be a function of; the relative concentration of the message at the point of megakaryocyte fragmentation, the specific half-life of that particular mRNA and the mean age of the platelet population being studied. Thus it seems reasonable to conclude that once platelets are generated from their megakaryocyte precursors and are released into the circulation, any mRNA which may have been present immediately after megakaryocyte fragmentation will be degraded at a rate determined by its specific half-life. Once degraded, the mRNA is not regenerated and the eventual outcome is a complete loss of ability to direct HLA Class I antigen synthesis.

In summary, the data presented in this thesis are consistent with previous reports that the majority of HLA-A,B antigens on platelets are associated with the platelet surface in a manner different from that of lymphocytes (i.e. adsorbed in nature). In addition, they indicate that an integral component does in fact exist but that this species is not likely generated by de-novo protein synthesis in the mature circulating platelet. Finally, the findings presented here support the hypothesis that the integral HLA component present on platelets may exist as a vestigial remain of endogenous synthesis which occurs in the megakaryocyte during thrombopoiesis and diminishes over the life span of the platelet. If such a hypothesis were true, integral HLA

antigens would be the predominate species early in the platelet life-cycle but their production and subsequent expression would diminish as specific mRNA is degraded and existing antigens are shed into the plasma during the aging process.

**CONCLUSION**

The main objective of this study was to further characterize the nature of the expression of HLA Class I antigens on platelet membranes in an attempt to shed additional light on the controversy which presently exists with regards to their origin. This question is of relevance since HLA antigens on lymphocytes play an important role in mediating cell - cell recognition in the immune response and thus in determining the survival of various transplanted organs and tissues. The biological significance of their presence on platelet membranes is as yet uncertain however in the clinical setting, it has been known for some time that these antigens are capable of inducing immune responses which can lead to subsequent febrile transfusion reactions in individuals receiving multiple platelet transfusions. More recently it has been reported that platelets and plasma account for >90% of HLA antigens in blood (Kao et al. 1988). It follows then, that further studies on the origin and binding mechanism of these platelet antigens might lead to the development of ways to manipulate their expression, either quantitatively and/or qualitatively, such that their immunogenicity and hence clinical significance could be diminished or even abolished in the future.

The results of the studies reported here have not allowed one to conclude with absolute certainty, the nature of HLA-A,B expressed on platelets. They do however, add considerably

to the growing evidence that exists in support of the hypothesis that both integral and adsorbed forms of these antigens exist on platelet membranes. In addition, they support the hypothesis that the relative degree of expression of each species may in fact be a function of platelet age.

Similar studies to those reported here, performed on platelets harvested from a patient in an acute thrombolytic state (i.e. a neo-platelet population) along with DNA amplification studies performed on mRNA isolated from megakaryocytes, might hold the key to resolving the present controversy to everyone's satisfaction. The knowledge gained in such studies could potentially open the door to future research aimed at exploring such possibilities as genetic regulation of HLA antigen expression in megakaryocytes or characterization of the binding sites involved in binding the adsorbed antigen species. Such information will determine the feasibility of pursuing the ultimate goal of developing novel approaches to platelet concentrate manufacturing processes which would optimize the removal of elutable HLA antigens and/or block the adsorption of these antigens without affecting platelet viability or function.

## REFERENCES

- Adams, G.A., Swenson S.D., and G. Rock. 1985. Platelet storage in a plasma-free medium. *Transfusion* 25: 551-556.
- Adams, G.A., Swenson S.D., and G. Rock. 1986. Survival and recovery of human platelets stored for 5 days in a non-plasma medium. *Blood* 67: 672-675.
- Adams, G.A. and G. Rock. 1987. Storage of human platelet concentrates in an artificial medium without dextrose. *Transfusion* 28: 217-220.
- Algranati, I.D., Milstein, C., and A. Ziegler. 1980. Studies on biosynthesis assembly and expression of human major transplantation antigens. *Eur. J. Biochem.* 103: 197-207.
- Allison, J.P., Pellegrino, M.A., Ferrone, S., Callahan, G.N. and R.H. Reisfeld. 1977. Biologic and Chemical characterization of HLA antigens in human serum. *J. of Immunol.* 118: 1004-1009.
- Ashurst, D.E., Bedford, D., and R.R.A. Coombs. 1956. Examination of human platelets for the ABO, MN, Rh, Tj<sup>a</sup>, Lutheran and Lewis system of antigens by means of mixed erythrocyte-platelet agglutination. *Vox Sang.* 1: 235-240.
- Aster, R.H., Mikovich, B.H., and G.E. Rodey. 1973. Histocompatibility antigens of human plasma. *Transplantation* 16: 205-210.
- Barnstable, C.J., Bodmer, W.F., Brown, G., Milstein, C., Williams, A.F., and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens - new tools for genetic analysis. *Cell* 14: 9-20.
- Beardsley, D.S., Ho, J.S., and T. Moulton. 1987. PL<sup>t</sup>: A new platelet specific antigen on glycoprotein V. *Blood* 70: 347-352.
- Bishara, A., Nelken, D., Bonavida, B. and C. Brautbar. 1983. Enzyme-linked immunosorbent assay for determination of HLA: gene dose effect. *Tissue Antigens* 23: 284-289.
- Blanchette, V.S., Peters, M.A. and K. Pegg-Feige. 1986. Alloimmune Thrombocytopenia. Review from a neonatal intensive care unit. In "First Canadian Workshop and Conference on Platelet Serology, Ottawa, 1985. Current

- Studies in Hematology and Blood Transfusion". F. Decary and G. Rock (eds.), Karger, Basel 52: 87-96.
- Blumberg, N., Masel, D., Mayer, T., and J. Heal. 1982. Acquisition of new HLA-A,B, phenotypes by human platelets. *Blood* 60: 276.
- Blumberg, N., Masel, D., Mayer, T., Horan, P. and J. Heal. 1984. Removal of HLA-A,B antigens from platelets. *Blood* 63: 48-450.
- Bodmer, W.F. 1984. The HLA System, 1984. p.11-22. In "Histocompatibility Testing, 1984", E.D. Albert (ed.), Springer-Verlag, Berlin, Heidelberg. pp. 11-22.
- Brand, A., Sintrnicolaas, K., Claas, F.H.J., and J.G. Eernisse. 1986. ABH antibodies causing platelet transfusion refractoriness. *Transfusion* 26: 463-466.
- Bray, P.F., Barsh, G., Rosa, J.F., Luo, X.Y., and E. Magenis. 1988. Physical linkage of the genes for platelet membrane glycoproteins IIb and IIIa. *Proc. Natl. Acad. Sci.* 85: 8683-8687.
- Brown, G., Biberfeld, P., Christensson, B. and D.Y. Mason. 1979. The distribution of HLA on human lymphoid, bone marrow and peripheral blood cells. *Eur.J.Immunol.* 9: 272-275.
- Canadian Red Cross Society (ed.) In "Blood Services Statistical Report 1987/88". The Canadian Red Cross Society, Ottawa, Ontario. pp. 18.
- Coombs, R.R.A. and D. Bedford. 1955. The A and B antigens on human platelets demonstrated by means of mixed erythrocyte-platelet agglutination. *Vox Sang.* 5: 111-115.
- Darnell, J.E., Lodish, H. and D. Baltimore. 1986. In "Molecular Cell Biology". Scientific American Books, Inc. New York, New York. p 363-365.
- Dausset, J. and G. Malinvaud. 1954. Normal and pathological platelet agglutinins investigated by means of the shaking method. Confirmation of the presence of A and B antigens in platelets. *Vox Sang.* 4: 204-213.
- Dausset, J. and J. Evelin. 1958. Présence de l'antigène Rh(D) dans les leucocytes et les plaquettes humaines. *Vox Sang.* 3: 266-276.

- Dausset, J. 1959. Iso-anticorps et antigènes des leucocytes et de plaquettes independants de ceux des érythrocytes. *Blood* 30: 634-642.
- Decary, F. 1982. Platelet antigens. *Plasma Ther. Transfus. Technol.* 3: 251-258.
- Doherty, P.J., Huesca-Contreras, M., Dosch, H.M. and S. Pan. 1989. Rapid amplification of Complementary DNA from small amounts of unfractionated RNA. *Anal. Biochem.* 177: 7-10.
- Dunstan, R.A., Simpson, M.B. and W.F. Rosse. 1983. Erythrocyte antigens on human platelets: absence of Rhesus, Duffy, Kell, Kidd and Lutheran antigens. *Transfusion* 24: 243-246.
- Dunstan, R.A., Simpson, M.B., and W.F. Rosse. 1984. The Presence of Ii blood group system on human platelets. *Amer. J. Clin. Path.* 82: 74-77.
- Dunstan, R.A. and M.B. Simpson. 1985a. Stability of platelet surface antigens during storage. *Transfusion* 25: 563-566.
- Dunstan, R.A. and C.M. Mansbach. 1985b. Biochemical characterization of A blood group activities on human platelets. *Vox Sang.* 49 : 149-153.
- Dunstan, R.A., Simpson, M.B., Knowles, R.W. and W.F. Rosse. 1985c. The origin of ABH antigens on human platelets. *Blood* 65: 615-619.
- Dunstan, R.A., Simpson, M.B., and W.F. Rosse. 1985d. Presence of P blood group antigens on human platelets. *Amer. J. Clin. Path.* 83: 731-735.
- Dunstan, R.A., Simpson, M.B., and W.F. Rosse. 1985e. Le<sup>a</sup> blood group antigen on human platelets. *Amer. J. Clin. Path.* 83: 90-94.
- Duquesnoy, R.J. 1979. Role of HLA and ABO blood group matching in platelet transfusion therapy of alloimmunized thrombocytopenic patients. In *Proceedings of Haemonetics Research Institute Advanced Component Seminar*. Haemonetics Research Institute, Boston.
- Edwards, J.M., Moulds, J.J. and W.J. Judd. 1982. Chloroquine dissociation of antigen-antibody complexes. A new technique for typing red blood cells with a positive direct antiglobulin test. *Transfusion* 22: 59-61.

- Ehmann, W.C. Dancis, A., Ferzinger, R., and S. Karpatkin. 1987. Post-transfusion purpura: Evidence that conversion of PL<sup>A1</sup> negative platelets to PL<sup>A1</sup> positive phenotype is an in-vitro artifact. *Blood* 70: 339a.
- Evatt, B.L. and S.F. Stein. 1988. Platelet Structure and function. In Smith, D.M. and Summers, S.H. (eds) "Platelets". Amer. Assoc. Blood Banks, Arlington VA. pp. 1-14.
- Friedman, J.M. and R.H. Aster. 1985. Neonatal alloimmune thrombocytopenic purpura and congenital porenciphaly in two sibilings associated with a new maternal anti-platelet antibody. *Blood* 65: 1412-1415.
- George J.N., Nurden, A.T. and D.R. Phillips. 1984. Molecular defects in interactions of platelets with the vessel wall. *New Eng. J. Med.* 311: 1084-1098.
- Gockerman, J.P. and W. Jacobs. 1979. Purification and Characterization of papain-solubilized HLA antigens from human platelets. *Blood* 53: 838-850.
- Guindo, M. and W.V. Miller. 1981. Application of the enzyme linked immunospecific assay (ELISA) for the detection of platelet antibodies. *Blood* 57: 32-37.
- Gurevitch, J. and D. Nelken. 1954. ABO groups in blood platelets. *J. Lab. Clin. Med.* 44: 562-570.
- Gyllensten, U.B. and H.A. Erlich. 1988. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. *Proc. Natl. Acad. Sci.* 85: 7652-7656.
- Harrington, W.J., Sprague, C., Minnich, V., Moore C.V., Aulvin, R.C. and R. Dubach. 1953. Immunological mechanism in idiopathic and neonatal thrombocytopenic purpura. *Ann. Int. Med.* 38: 433-469.
- Holtz, G. Mantel, W. and W. Buck. 1973. The inhibition of antigen-antibody reactions by chloroquine and its mechanism of action. *Z. Immun.-Forsch. Bd.* 146: 145-157.
- Hourdille, P., Benabdallah, S., Belloc, F. and A.T. Nurden. 1985. Distribution of glycoprotein IIb-IIIa complexes in the surface membranes of human platelets and megakaryocytes. *Br. J. of Haematol.* 59: 171-182
- Jankovic, B.D. and K. Arsenijevic. 1959. Histochemical demonstration of A and B antigens in platelets. *Nature* 183: 695-696.

- Janson, M., McFarland, J. and R.H. Aster. 1986. Quantitative determination of platelet surface alloantigens using a monoclonal probe. *Human Immunol.* 15: 251-262.
- Jennings, L.K. and D.R. Phillips. 1982. Purification of glycoproteins IIb and IIIa from human platelet membranes and characterization of a calcium-dependent glycoprotein IIb-IIIa complex. *J. Biol. Chem.* 257: 10458-10464.
- Kao, K.J., Cook, D.J., and J. Scornik. 1986. Quantitative analysis of platelet surface HLA by W6/32 anti-HLA monoclonal antibody. *Blood* 68: 627-632.
- Kao, K.J. 1987. Plasma and platelet HLA in normal individuals: Quantitation by competitive enzyme-linked immunoassay. *Blood* 70: 282-286.
- Kao, K.J., Scornik, J.C., Riley, W.J. and C.F. McQueen. 1988a. Association between HLA phenotype and HLA concentration in plasma or platelets. *Human Immunol.* 21: 115-124.
- Kao, K.J. 1988b. Selective elution of HLA antigens and  $\beta_2$ -microglobulin from human platelets by chloroquine diphosphate. *Transfusion* 28: 14-17.
- Kelton, J.G., Hamid, C., Aker, S. and M.A. Blajchman. 1982. The amount of blood group A substance on platelets is proportional to the amount in the plasma. *Blood* 59: 980-985.
- Kickler, T.S., Ness, P., Herman, J.H. and W.R. Bell., 1986. Studies on the pathophysiology of posttransfusion purpura. *Blood* 68: 347-350.
- Kickler, T.S., Herman, J.H. Furihata, K., Kunicki, T.J. and R.H. Aster. 1988. Identification of Bak<sup>D</sup>, a new platelet specific antigen associated with post-transfusion purpura. *Blood* 70: 894-898.
- Kiefel, V., Santoso, S., Katzman, B., and C. Mueller-Eckhardt. 1987. Neonatal Thrombocytopenia caused by a new platelet specific alloantibody Br<sup>a</sup>. *Blood* 70: 340.
- Kieffer, N., Boizard, B., Didry, D., and J. Wautier. 1984. Immunochemical characterization of the platelet specific alloantigen Lek<sup>a</sup>. A comparative study with the PL<sup>A1</sup> alloantigen. *Blood* 64: 1212-1219.
- Koike, T., Aoki, S., Maruyama, S., Narita, M., Ishizuka, T., Imanaka, H., Adachi, T., Maeda, H. and A. Shibata. 1987. Cell surface phenotyping of megakaryoblasts. *Blood* 69: 957-960.

- Koller, B.H., Sidwell, B. Demars, R. and H.T. Orr. 1984. Isolation of HLA locus-specific DNA probes from the 3'-untranslated region. *Proc. Natl. Sci.* 81: 5175-5178.
- Koller, B.H. and H.T. Orr. 1985. Cloning and Complete sequence of an HLA-A2 Gene: Analysis of two HLA-A alleles at the nucleotide level. *J. Immunol.* 134: 2727-2733.
- Krangel, M.S., Orr, H.T. and J.L. Strominger. 1979. Assembly and maturation of HLA-A and HLA-B antigens in-vivo. *Cell* 18: 979-991.
- Krangel, M.S., Orr, H.T. and J.L. Strominger. 1980. Structure, function, and biosynthesis of the major histocompatibility antigens (HLA-A and HLA-B). *Scand. J. Immunol.* 11: 561-571.
- Kunicki, T.J., Pidard, D., Rosa, J.P. and A.T. Nurden. 1981. The formation of calcium dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. *Blood* 58: 268-278.
- Kunicki, T. and P.J. Newman. 1986. The biochemistry of platelet-specific alloantigens. *Curr. Stud. Haematol. Blood Transf.* 52: 18-32.
- Kunicki, T.J. 1988a. Human platelet antigen systems. In "Platelets". Smith, D.M. and Summers, S.H. (eds.) Amer. Assoc. Blood Banks, Arlington, VA. pp. 15-53.
- Kunicki, T.J. Nugent, D.J., Staats, S., Orckowski, R.P., Wayer, E.A. and W.G. Carter. 1988b. The human fibroblast class II extracellular matrix receptor (ECMR II) mediates platelet adhesion to collagen and is identical to the platelet glycoprotein Ia-IIa complex. *J. Biol. Chem.* 263: 4516-4519.
- Kurata, Y., Oshida, M., Take, H., Furubayashi, T., Nakao, H., Tomiyama, Y., Kanayama, Y., Yonezawa, T. and S. Tarui. 1988. Elimination of antigenicity of surface HLA Class I antigens from platelet membrane by acid treatment at pH 3.0. In Press. *Vox Sang.*
- Laemmlli, V.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature* 227: 685-688.
- Lalezari, P. and A.M. Driscoll. 1982. Ability of thrombocytes to acquire HLA specificity from plasma. *Blood* 59: 167-170.

- Levene, R.B., Lamaziere, J.D., Broxmeyer, H.E. and E.M. Rabellino. 1985. Human megakaryocytes. *J. Exp. Med.* 161: 457-474.
- Lewis, J.H., Draude, J. and W.J. Kuhns. 1960. Coating of "O" platelets with A and B group substances. *Vox Sang.* 5: 434-440.
- Lopez, J.A., Chung, D.W., Fujikawa, K., Hagen, F.S., Davies, E.W. and G.J. Roth. 1988. The alpha and beta chains of human platelet glycoprotein Ib are both transmembrane proteins containing a leucine-rich amino acid sequence. *Proc. Nat. Acad. Sci.* 85: 2135-2139.
- Majsky, A. 1962. AB blood group substance on platelets. *Z. Ges. Inn. Med.* 17: 220-224.
- Majsky, A. 1969. Antigenicity of blood platelets. *Current Top. Microbiol. Immunol.* 58: 138-173.
- Mantel, W. and G. Holtz. 1976. Characterization of autoantibodies to erythrocytes in autoimmune haemolytic anemias by chloroquine. *Vox Sang.* 30: 453-463.
- Maniatis, T., Fritsch, E.F., and J. Sambrook. 1982. In "Molecular Cloning. A Laboratory Manual". Cold Spring Harbor Laboratory, Cold Spring Harbor NY. pp. 196.
- Masel, D.S., Blumberg, N. and J.M. Heal. 1988. A chloroquine elution technique for platelet serology. *Transfusion* 28: 132-136.
- McCune, J.M., Humphreys, R.E., Yocum, R.R., and J. Strominger. 1975. Enhanced representation of HLA antigens on human lymphocytes after mitogenesis induced by phytohemagglutinin or Epstein-Barr virus. *Proc. Nat. Acad. Sci.* 72: 3206-3209.
- McMillan, R. Mason, D., Tani, P. and G.M. Schmidt. 1982. Evaluation of platelet surface antigens: localization of the PL<sup>A1</sup> alloantigen. *Br. J. Haematol.* 51: 297-304.
- Metcalf, P., Minchinton, R.M., Murphy, M.F. and A.H. Waters. 1985. Use of chloroquine-treated granulocytes and platelets in the diagnosis of immune cytopenias. *Vox Sang.* 49: 340-345.
- Mezzano, D., Hwang, K., Catalano, P., and R.H. Aster. 1981. Evidence that platelet buoyant density, but not size, correlates with platelet age in man. *Amer. J. Hematol.* 11: 61-76.

- Minchinton, R.M. and A.H. Waters. 1984. Chloroquine stripping of HLA antigens from neutrophils without removal of neutrophil specific antigens. *Br. J. Haematol.* 57: 703-706.
- Morgan, R., and F. Anderson. 1989. DNA technology continues to improve. *Transfusion* 29: 189.
- Moulinier, J. 1958. Iso-immunization maternelle anti-plaquettaire et purpura néo-natal. Le système de groupe plaquettaire "DUZO". In "Proc. 6th Cong. European Society of Haematology". S. Karger, New York.
- Moureau, P. and A. André. 1954. Présence des antigènes A et B dans les plaquettes. *Vox Sang.* 4:46-51.
- Mueller-Eckhardt, G., Hauck, M., Kayser, W. and C. Mueller-Eckhardt. 1980. HLA-C antigens on platelets. *Tissue Antigens.* 16: 91-94.
- Newman, P.J., Gorski, J., White, G.C., Gidwitz, S., Cretney, C.J. and R.H. Aster. 1988. Enzymatic amplification of platelet-specific messenger RNA using the polymerase chain reaction. *J of Clin. Invest.* 82: 739-743.
- Newman, P.J., Derbes, R.S. and R.H. Aster. 1989. Human platelet alloantigens PL<sup>A1</sup>/PL<sup>A2</sup> are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa and are distinguishable by DNA typing. *J of Clin. Invest.* 83: 1778-1781.
- Nieuwenhuis, H.K., Akkerman, J.W.N., Houdjik, W.P.M., and J.J. Sixma. 1985. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* 318: 470-472.
- Nordhagen, R. and S.T. Flaathen. 1985. Chloroquine removal of HLA antigens for the platelet immunofluorescence test. *Vox Sang.* 48: 156-159.
- Orr, H.T., Lopez de Castro, J.A., Lancet, D., and J.L. Strominger. 1979. Complete amino acid sequence of a papain-solubilized human histocompatibility antigen, HLA-B7. Sequence determination and search for homologies. *Biochemistry* 18: 5711-5719.
- Orr, H.T. and J.L. Strominger. 1980. Towards a structural definition of serological specificity: The HLA system. In "The Biology and Function of The Major Histocompatibility Complex". J.J. McCullough and J.H. Hansen (eds.) Amer. Assoc. Blood Banks, Arlington, Va. pp. 9-22.

- Parham, P., Barnstable, C.J., and W. Bodmer. 1979. Use of a monoclonal antibody (W6/32) in structural studies of HLA-A,B,C antigens. *J. Immunol.* 123: 342-349.
- Parham, P. 1987. Functional sites of human class I MHC molecules: Paradigms a dozen? *Immunol. Res.* 6: 153-178.
- Pereira, J., Cretney, C. and R.H. Aster. 1988. Variation of class I HLA antigen expression among platelet density cohorts: a possible index of platelet age? *Blood* 71: 516-519.
- Phillips, D.R. and L.A. Fitzgerald. 1987. Platelet membrane glycoproteins. In "Hemostasis and Thrombosis: Basic Principles and Clinical Practice". Colman, R.W., Hirsh, J., Marder, V.J. and E.W. Salzman (eds.) Philadelphia, Lippincott, pp. 572-593.
- Phillips, D.R., Charo, I.F., Parise, L.V. and L.A. Fitzgerald. 1988. The platelet membrane glycoprotein IIb-IIIa complex. *Blood* 71: 831-843.
- Pidard, D. Montgomery, R.R., Bennett, J.S. and T.J. Kunicki. 1983. Interactions of AP-2, a monoclonal antibody specific for the human platelet glycoprotein IIb-IIIa complex, with intact platelets. *J. Biol. Chem.* 258: 12582-12586.
- Pincus, J.H., Kahan, B.D., and K.K. Mittal. 1976. A role for cAMP in the preparation of human platelets for the extraction of histocompatibility antigens. *Immunochemistry* 13: 565-570.
- Ploegh, H.L., Orr, H.T., and J.L. Strominger. 1981a. Biosynthesis and cell surface localization of nonglycosylated human histocompatibility antigens. *J. Immunol.* 126: 270-275.
- Ploegh, H.L., Orr, H.T. and J.L. Strominger. 1981b. Major histocompatibility antigens: The human (HLA-A,-B,-C) and Murine (H-2K, H-2D) class I molecules. *Cell* 24: 287-299.
- Ray, J.G. 1979. (ed.) In "NIAID Manual of Tissue Typing Techniques". Rockville, Md. DEHW publication (NIH) no. 80-545: 39-41.
- Rock, G., Decary, F., Tittley, P. and V. Fuller. 1987. Electroblotting and immunohistochemical staining for identification of platelet antibodies. *Br. J. Hematol.* 67: 437-441.

- Rosa, J.P., Cevallos, M. and R.P. McEver. 1986. Fibrinogen receptor assembly in human erythroleukemia (HEL) cells. *Blood* 68: 325a.
- Rosa, J.P. Bray, P.F., Gayet, O., Johnston, G.I., Cook, R.G., Jackson, K.W., Shuman, M.A. and R.P. McEver. 1988. Cloning of glycoprotein IIIa cDNA from human erythroleukemia cells and localization of the gene to chromosome 17. *Blood* 72: 593-600.
- Ruffie, J., Broussy, J. and J. Ducos. 1959. Mise en évidence de l'antigène P dans les plaquettes sanguines humaines. *Sangre* 4: 156-162.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Millis, K.B., and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.
- Santoro, S.A. 1986. Identification of a 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependant adhesion of platelets to collagen. *Cell* 46: 913-920.
- Santoso, S., Mueller-Eckhardt, G., Santoso, S., Kiefel, V. and C. Mueller-Eckhardt. 1986. HLA antigens on platelet membranes. In vitro and in-vivo studies. *Vox Sang* 51: 327-333.
- Schäfer, K. and H. Gennerich. 1939. Die Blutgruppen Spezifität der Thrombozyten. *Klin. Wschr.* 18: 491-492.
- Shibata, Y.T., Miyai, T., Ichikawa, Y. and I. Matsuda. 1986. A new platelet antigen system, Yuk<sup>a</sup>/Yuk<sup>b</sup>. *Vox Sang.* 51: 334-336.
- Shulman, N.R., Aster, R.H., Leitner, A. and M.C. Hiller. 1961. Immunoreaction involving platelets. Posttransfusion purpura due to a complement-fixing antibody against a genetically controlled platelet antigen. A proposed mechanism for thrombocytopenia and its relevance in "autoimmunity". *J. Clin. Invest.* 40: 1597-1620.
- Shulman, N.R., Hiller, M.C. and E.M. Collier. 1964. (eds.) Platelet and leucocyte isoantigens and their antibodies. Serologic, physiologic and clinical studies. In "Progress in Hematology". Grune and Stratton, New York and London, Vol 4. pp 42.
- Simpson, M.B., Dunstan, R.A., Rosse, W.F., Munro, A.C., Fraser, F., and M.E. Nichols. 1987. Status of the MNSs antigens on human platelets. *Transfusion* 27: 15-18.

- Singer, S.J. and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175: 720-731.
- Sintnicolaas, K., van der Steuijt, K.J.B., Putten, W.L.J. and R.L.H. Bolhuis. 1987. A microplate elisa for the detection of platelet alloantibodies: comparison with the platelet immunofluorescence test. *Br. J. Hematol.* 66: 363-367.
- Skogen, B., Rossebo Hansen, B., Husebekk, A., Havnes, T. and K. Hannestad. 1988. Minimal expression of blood group A antigen on thrombocytes from A<sub>2</sub> individuals. *Transfusion* 28: 456-459.
- Snary, D., Barnstable, C.J. Bodmer, W.F. and M.J. Crumpton. 1977. Molecular structure of human histocompatibility antigens: the HLA-C series. *Eur. J. Immunol.* 8: 580-585.
- Sodoyer, R., Damotte, M., Delovitch, T.L., Trucy, J., Jordan, B.R. and T. Strachan. 1984. Complete nucleotide sequence of a gene encoding a functional human class I histocompatibility antigen (HLA-CW3). *EMBO J.* 3: 879-885.
- Solum, N.O. 1985. Platelet membrane proteins. *Seminars In Hematol.* 22: 289-302.
- Stefanini, M., Plitman, G.I., Dameshek, W. Chatterjea, J.B., and S.B. Mednicoff. 1953. Studies on platelets. XI antigenicity of platelets and evidence of platelet groups and types in man. *J. Lab. Clin. Med.* 42: 723-734.
- Strachan, T., Sodoyer, R., Damotte, M. and B.R. Jordan. 1984. Complete nucleotide sequence of a functional class I HLA gene, HLA-A3: implication for the evolution of HLA genes. *EMBO J.* 3: 887-894.
- Strachan, T. 1987. Molecular genetics and polymorphism of class I HLA antigens. In "HLA in Medicine". M.J. Crumpton (ed.) Churchill Livingstone, New York.
- Sugawara, S., Abo, T. and K. Kumagai. 1987. A simple method to eliminate the antigenicity of surface class I MHC molecules from the membrane of viable cells by acid treatment at pH 3.0. *J. Immunol. Methods* 100: 83-90.
- Szatkowski, N.S. and R.H. Aster. 1980. HLA antigens of platelets. IV. Influence of "Private" HLA-B locus specificities on the expression of BW4 and BW6 on human platelets. *Tissue Antigens* 15: 361-368.

- Taaning, E., Antonsen, H., Peterson, S., Svejgaard, A., and M. Thomsen. 1983. HLA antigens and maternal antibodies in alloimmune neonatal thrombocytopenia. *Tissue Antigens*. 21: 351-359.
- Tragardh, L., Rask, L., Wiman, K. Fohlman, J. and P.A. Peterson. 1979a. Amino acid sequence of an immunoglobulin-like HLA antigen heavy chain domain. *Proc. Natl. Acad. Sci.* 76: 5839-5842.
- Tragardh, L. Klareskog, L., Curman, B., Rask, L. and P.L. Peterson. 1979b. Isolation and properties of detergent-solubilized HLA antigens obtained from platelets. *Scand. J. Immunol.* 9: 303-314.
- Tragardh, L., Rask, L., Wiman, K. Fohlman, J. and P.A. Peterson. 1980. Complete amino acid sequence of pooled papain-solubilized HLA-A, -B, and -C antigens: relatedness to immunoglobulins and internal homologies. *Proc. Natl. Acad. Sci.* 77: 1129-1133.
- van der Weerd, CH.M., van der Wiel-Dorfmeijer, H., Engelfriet, C.P. and J.J. van Loghem. 1962. A new platelet antigen. In "Proc. 8th Congress European Society Haematology". S. Karger, New York. p. 379.
- van der Weerd, CH.M., Veenhoven-von Riesz, L.E. Nijenhuis, L.E. and J.J. van Loghem. 1963. The Zw blood group system in platelets. *Vox Sang.* 8: 513-530.
- van der Weerd, CH.M. 1965. Iso-immunization against blood platelets. *Ref. Belg. Path.* 31: 118-122.
- van der Wiel, W.M., van der Wiel-Dorfmeijer, H. and J.J. van Loghem. 1961. Studies on platelet antibodies in man. *Vox Sang.* 6: 641-668.
- van Loghem, J.J., Dorfmeijer, H., Van der Hart, M. and F. Schreuder. 1959. Serological and genetical studies on a platelet antigen (Zw). *Vox Sang.* 4: 161-169.
- van Rood, J. and J.G. Eernisse. 1959. Leucocyte antibodies in sera of pregnant women. *Vox Sang.* 4: 427-444.
- Vinci, G., Tabilio, A., Dechamps, J.F., Van Haeke, D., Henri, A., Guichard, J., Tetteroo, P., Landsorp, P.M., Hercend, T., Vainchenker, W., and J. Breton-Gorius. 1982. Immunological study of in vitro maturation of human megakaryocytes. *Br.J. Haematol.* 58: 589-xxxx.
- von dem Borne, A.E.G.Kr., Verheugt, F.W.A., Oosterhol, F., von Riesz, E., Brutel de la Rivière, A. and C.P. Engelfriet. 1978. A simple immunofluorescence test for

- the detection of platelet antibodies. Br. J. Haematol. 39: 195-207.
- von dem Borne, A.E.G.Kr., von Riesz, E., Verheugt, F.W.A., Cate, J.W., Koppe, J.G., Engelfriet, C.P. and L.E. Nijehuis. 1980. Bak<sup>a</sup>, a new platelet specific antigen involved in neonatal allo-immune thrombocytopenia. Vox Sang.: 113-120.
- von dem Borne, A.E.G.Kr. and Decary. 1990. ICSH/ISBT working party on platelet serology. Nomenclature of platelet specific antigens. Vox Sang. 58: 176.
- Walsh, F.S. and M.J. Crumpton. 1977. Orientation of cell-surface antigens in the lipid bilayer of lymphocyte plasma membrane. Nature 269: 307-311.
- Warejcka, D., Janson, M. and R.H. Aster. 1985. The PL<sup>A1</sup> antigen, but not HLA antigen, can be acquired by platelets from plasma. Abstract. Amer. Soc. Hematol. 321: 109a.
- Wilson, D.B. 1963. Blood platelets and transplantation antigens. Transplantation 1: 318-326.
- Wilson, H.E., Johnson, H.M. and M.C. Dodd. 1968. Induction of isoantibodies to human leucocytes. Transplantation 6: 374-381.
- Woods, V.L.Jr., Wolff, L.E. and D.M. Keller. 1986. Resting platelets contain a substantial centrally located pool of glycoprotein IIb/IIIa complex which may be accessible to some but not other extracellular proteins. J. Biol. Chem. 261: 15242-15248.
- Yankee, R.A., Grumet, F.C. and G.N. Rogentine. 1971. Importance of histocompatibility in platelet transfusion therapy. Vox Sang. 20: 419-426.