DEVELOPMENT AND STUDY OF A MONOCLONAL ANTIBODY TO THE HUMAN HLA-CLASS II INVARIANT CHAIN

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ABBREVIATIONS

BSA	bovine serum albumin
CLL	chronic lymphocytic leukemia
Con-A	concanavalin A
cpm	counts per minute
CSPG	chondroitin sulphate proteoglycan
DMSO	dimethyl sulphoxide
EBVL	Epstein-Barr virus transformed lymphocytes
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
HRP	horse radish peroxidase
hr(s)	hour(s)
HUCS	human umbilical cord serum
ICC	immunocytochemistry
IF	immunofluorescence
IFNγ	interferon γ
IgG	immunoglobulin G
Ii	invariant chain or γ chain
kDa	kilodaltons
MHC	major histocompatibility complex
min(s)	minute(s)
MLC	mixed lymphocyte culture
MoAb	monoclonal antibody
M_r	relative molecular mass
NP-40	Nonidet P-40

PAP	peroxidase-anti-peroxidase
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PVDF	polyvinylidenedifluoride membrane ("Immobilon")
RPMI-10	RPMI-1640 medium with 10% FCS and antibiotics
SA	Staphylococcus aureus
SDS	sodium dodecyl sulphate
SRBC	sheep red blood cells
TBS	tris-buffered saline
TRITC	tetramethylrhodamine isothiocyanate

INTRODUCTION AND SUMMARY

The work I present in this thesis was initiated by a request for a monoclonal antibody (MoAb) to the peripheral blood cells of a patient with chronic lymphocytic leukemia (CLL). It was initially hoped that such an antibody, if directed against an idiotypic determinant on the malignant B-cell surface, may prove to be useful therapeutically by virtue of its specificity and limited reactivity. At the time at which the request was made there had already been favourable reports of the use of MoAb's for the management of B-cell derived malignancies (1,2).

I fused immune splenocytes from a mouse that had received repeated injections of the patient's peripheral blood B cells with murine myeloma cells and obtained a MoAb that reacted strongly with a 35 kilodalton (kDa) antigen present in a detergent lysate of the patient's B cells. Preliminary studies with this antibody showed:

a) that it reacted with an antigen that was strongly represented in a lysate of leukemic B cells but not in similar lysates of normal peripheral blood mononuclear cells (PBMC). This suggested that the antibody might have useful discriminant value for distinguishing normal and malignant lymphocytes.

b) that the antibody did not react with an epitope present on the cell surface. Cells had to be fixed and permeabilized if the antigen was

to be demonstrated by immunofluorescence or immunohistochemistry in whole cell preparations. When normal PBMC were treated in this way approximately 20% reacted with the MoAb despite failure to detect the antigen in lysates of PBMC.

c) that expression of the antigen was not confined to peripheral blood B cells. The epitope was also strongly represented on Epstein-Barr virus transformed lymphocytes (EBVL), B lymphoblastoid cell lines, Reed-Sternberg cells of Hodgkins disease, monocytes, interdigitating reticulum cells and some melanoma cells.

The distribution of the antigen, its apparent molecular mass and its exposure by fixation and permeabilisation were similar to those described for HLA class II antigenic molecules by Cohen et al. (3). I accordingly sent Dr. Cohen, at the Clinical and Population Cytogenetics Unit at Western General Hospital in Edinburgh, samples of our MoAb and, in return, received samples of MoAbs that he had raised. The results of the experiments with these antibodies showed that the epitope with which our MoAb reacted was not present on the HLA class II alpha chain. I then wrote to Dr. V. Quaranta at the Research Institute of Scripps Clinic, La Jolla in California who had raised a MoAb against the HLA class II invariant chain (4) and I received a small amount of his antibody, VIC-Y1. Experiments with this antibody suggested that the molecule which reacted with our MoAb was the invariant (Ii) or γ-chain of the HLA class II antigen complex.

Having identified the epitope I performed a number of studies designed to characterize in quantitative, qualitative and kinetic terms, its expression on normal and malignant cells.

The antigen with which the antibody reacted was found to exist in three consistent cellular forms: a "free" form (M_r35kDa); a terminally glycosylated, "processed" form (M_r37kDa) and, in non-reduced samples, a "complexed" form (M_r72kDa) where it was associated with a second γ chain to form a dimeric complex.

The antibody precipitated a polydisperse set of chondroitin sulphate proteoglycan molecules (CSPG's) of M_r's varying between 55 and 97 kDa from ^{35}S-sulphate labelled lysates of B-lymphoblastoid Raji cells and melanoma cells but not of EBVL or CLL lymphocytes. Cell fractionation and Western blotting of the fractions with the antibody showed that the γ chain was localized intracellularly in the microsomal fraction.

Incubation of cells with the inhibitor of N-glycosylation, tunicamycin, followed by labelling with ^{35}S-methionine and immunoprecipitation with the antibody, showed that the γ chain was glycosylated.

When Raji cells and EBVL were labelled with ^{35}S-methionine followed by a pulse-chase and immunoprecipitation with the antibody, I found that in Raji cells the γ chain was rapidly processed to a higher M_r form whereas in EBVL it was "chased" out of the pool. The antibody co-precipitated molecules that were non-covalently associated with the γ chain, one being the class II α chain and the other a 25 kDa protein, which was weakly and less consistently demonstrated in ^{35}S-methionine-

labelled immunoprecipitates.

A 44 kDa molecule with the epitope was shown in low concentration in some Western blotting experiments. I could not demonstrate γ chain on the cell surface by immunoprecipitation, nor could I inhibit the mixed lymphocyte reaction by adding the antibody to the culture medium.

Having demonstrated differences in processing and sulphation of the invariant chain in Raji cells and EBVL and in view of the important functions of class II molecules in cell interactions, I studied the expression of γ chain in normal and pathological cells using an assay which enabled me to express γ chain content in terms of antibody binding sites per cell. With this assay, and with immunofluorescence and flow cytometry, I showed the following:

CLL peripheral blood lymphocytes expressed highly variable amounts of γ chain, varying between 99 and 355 x 10^3 sites per cell.

The distribution of γ chain in normal peripheral blood lymphocytes was bimodal with 10-20% of cells strongly positive and 25-50% weakly positive.

Four melanoma cell lines that I studied showed highly variable expression of invariant chain. The expression in one cell line was shown to be cell cycle-dependent and there was an inverse correlation between doubling time of different cell lines and Ii content. In two cell lines expression of γ chain was increased after incubation in serum-free medium.

CHAPTER 1

DEVELOPMENT OF VCD-1: A MONOCLONAL ANTIBODY TO AN EPITOPE ON THE HUMAN CLASS II INVARIANT CHAIN.

In an attempt to develop a therapeutic reagent for the management of a case of chronic lymphocytic leukemia, I raised a monoclonal antibody to the patient's peripheral blood lymphocytes. This antibody reacted with an epitope present on the human Class-II invariant chain and this chapter records the procedures that I used to develop and characterize the antibody and the results that I obtained.

METHODS

Separation of peripheral blood mononuclear cells (PBMC)

Freshly collected, heparinized venous blood was diluted with an equal volume of RPMI-1640 medium, and 8 ml of the diluted blood was layered on to 4 ml of Ficoll-Hypaque (density 1.077; Lymphoprep, Nycomed) in 12 ml centrifuge tubes. The tubes were centrifuged for 10 mins at 250g, the platelet-rich supernatant was removed to within 1 cm of the PBMC at the interface, and the tubes were centrifuged for a further 15 mins at 850g. The PBMC were removed from the interface, diluted with an approximately equal volume of RPMI-1640 medium, pelleted at 600g for 5 mins and washed once by resuspending and centrifuging.

B-cell enrichment of PBMC

A 1 ml tuberculin syringe was filled with teased nylon wool and this was flushed with 5% foetal calf serum (FCS) in RPMI medium (RPMI-5) at 37°C.

PBMC were separated from 50 ml heparinized blood and suspended in 0.5ml of RPMI-5. The cell suspension was pipetted into the syringe with nylon wool followed by 0.2ml RPMI-5 and the syringe was placed on its side in a 37°C incubator for 30-60 mins. T-cells were flushed out with 10ml of RPMI-5 at 37°C in a vertical position and B cells were dislodged by squeezing the wool with the plunger of the syringe and rinsing with medium.

Indirect immunofluorescence (IF) with cells attached to multi-well slides.

Teflon-coated, 8-well, microscope slides (Highveld Biological Cat. No. 806B) were incubated with a solution of poly-L-lysine (50μg/ml) in PBS for 30-60 mins and subsequently washed with PBS. Cells were washed twice and suspended in PBS at a final concentration of $1x10^6$ to $5x10^6$/ml (depending on the size of the cells) and 40μl aliquots of the cell suspension were applied per well. After 30-60 mins the slides were washed once with PBS, once with 2% FCS in PBS and then allowed to dry overnight in a dessicator.

For indirect immunofluorescence the cells were fixed with 1% formaldehyde in PBS for 30 mins or with acetone for 5 mins and washed

once in PBS. After blocking (40μl/well) for 10-15 mins with RPMI + 10% foetal calf serum (RPMI-10) the cells were incubated with undiluted hybridoma supernatant medium (MoAb harvest fluid), or ascites diluted 1/200-1/500 in RPMI-10, for 1 hr in a humidified box with ice. The slides were then washed twice in PBS in a Coplin jar and wells were incubated with fluorescein-conjugated goat anti-mouse IgG (FITC-GaM, Cappel 1211-0081) diluted 1/50 in RPMI-10 for 30 mins. Slides were washed twice with PBS, counterstained with ethidium bromide (2μg/ml in PBS) for 1 min, washed with PBS and mounted in 30% glycerol in PBS.

Indirect IF with cells in suspension:

Viable cells were suspended at $1x10^7$/ml in RPMI-10. Suspensions of fixed and permeabilized cells were prepared as follows:
Cells were washed twice with PBS and the pellets resuspended in 0.5ml of 1% formaldehyde in PBS containing 0.05% saponin. After 5 mins 10ml of 1% formaldehyde in PBS was added and the cells fixed for another 20 mins at room temperature. They were then washed once with PBS and suspended at $1x10^7$/ml in RPMI-10.

For the IF test 100μl cell suspension was mixed with 100μl MoAb (as for slide test) in 12 x 75mm tubes and left on ice for 1 hr. Cells were washed twice with PBS by centrifugation at 350g for 5 mins, incubated with 100μl FITC-GaM and washed twice with PBS.

The anti-HLA class I MoAb W6/32 was used as a positive control and an irrelevant mouse MoAb (to β-galactosidase or erythrina trypsin inhibitor) was used as a negative control.

Immunocytochemistry (ICC)

Cryostat sections were fixed in cold acetone for 10 mins; formaldehyde-fixed paraffin wax sections were de-waxed and hydrated using standard histological procedures.

Sections were pre-treated with non-immune rabbit serum (1/20 in PBS) for 10 mins, incubated with MoAb harvest fluid for 40 mins and washed with PBS. Bound murine antibody was detected according to Taylor (5). Briefly, slides were incubated for 30 mins with peroxidase-conjugated rabbit anti-mouse Ig (DAKO P161) that had been diluted 1/50 in PBS containing 4% normal human serum and washed again with PBS. When necessary this was followed by a further 30 mins incubation with peroxidase-conjugated swine anti-rabbit Ig (DAKO P217) (diluted as above) and a PBS wash. The substrate azinoethylcarbazole with hydrogen peroxide in acetate buffer was applied for 20-40 mins and the sections were counterstained with Mayer's haematoxylin.

Conjugation of VCD-1 antibody to fluorescein isothiocyanate (FITC)(6)

VCD-1 IgG was precipitated from ascites with 50% saturated ammonium sulphate and dialysed into PBS.

To 1.3ml of this solution, containing approximately 15mg of antibody, I added 150μl conjugation buffer (0.5M pH 9.5: 5.8ml of 5.3% Na_2CO_3 + 10ml of 4.2% $NaHCO_3$) and transferred the mixture to a tube containing 400-500μg FITC. The mixture was tumbled for 1 hr at room temperature and then passed through a Sephadex G-25M column, pre-equilibrated with

PBS, to separate free from bound FITC. The final solution contained ~6.5mg antibody/ml.

Double immunofluorescence(7)

To study the binding of two different murine monoclonal antibodies to the same cells I detected the first antibody by indirect immunofluorescence and the second by direct fluorescence of conjugated antibody. This was done as follows:

Cells were incubated with unconjugated first MoAb, washed twice with PBS, incubated with rhodamine conjugated sheep anti-mouse IgG 1/50 (TRITC-SaM, Cappel 22110084) and finally washed twice with PBS. To block remaining free binding sites of the TRITC-SaM I incubated the cells for 10 mins with irrelevant mouse ascites (1/200). The FITC-conjugated second murine antibody was then added and, after two washes, the cells were mounted in 30% glycerol.

Preparation of cell lysates

Cells were washed twice with PBS and suspended in lysis buffer (PBS containing 0.5% Nonidet P-40 and 1mM phenyl methyl sulphonyl fluoride (kept as a 0.1M stock solution in isopropanol at -20°C)) at a concentration of 10^8 PBMC/ml; 5 x 10^7 EBVL/ml; 5 x10^7 Raji cells/ml; or 2.5x10^7 melanoma cells/ml.

Cells in lysis buffer were kept on ice for 10-20 mins and then centrifuged at 1000g to remove nuclei.

Supernatants, if not used immediately, were stored frozen at -70°C without apparent loss of immunoreactivity.

Western blotting of cell lysates(8)

Proteins in cell lysates were separated by SDS-PAGE (see Appendix) in 11% polyacrylamide gels. The gels were placed in contact with polyvinylidene difluoride membrane (PVDF, "Immobilon", Millipore IPVH00010) which had been activated in methanol for a few seconds and soaked in water for 5 mins. The gels and PVDF membrane were wedged tightly between wetted Scotchbrite pads and double layers of 3MM filter paper in a perforated perspex holder which slotted into an electroblotting tank with the PVDF membrane on the positive side. The tank was filled with transfer buffer (see Appendix) and the gels were blotted at 7V/cm for 1.5 - 2 hrs or 3V/cm overnight. The track with the molecular weight markers was removed, stained for 10 mins in 0.1% PAGE blue (see Appendix), and de-stained.

The remaining membrane was immersed in blocking solution (5% FCS or 0.25% gelatin in Tris-buffered saline; TBS) for 30-60 mins with agitation. The membrane was then immersed and agitated in a solution of MoAb (ascites diluted 1/500 - 1/1000 in blocking solution) for 1 hr and washed twice with TBS. This was followed by sequential 30 min incubations in sheep anti-mouse IgG (SaM whole serum, produced in this laboratory; 1/200 in TBS) and peroxidase-anti-peroxidase (PAP, see Appendix, diluted 1/10 in TBS) with two 10 min washes in TBS after each incubation. The membrane was finally immersed in

diaminobenzidine (DAB) substrate with $CoCl_2$ (see Appendix) for 5 mins and washed with water.

Preparation of lymphocyte-conditioned medium.

PBMC from heparinized normal blood were separated by Ficoll-Hypaque flotation and suspended at 10^6/ml in RPMI-10 containing 20 μg/ml concanavalin A (Con A, Sigma C5275). The suspension was incubated for three days, after which the cultures were centrifuged to remove cells, methyl α-D-mannopyranoside was added to a final concentration of 10 mM and the medium was filtered through a 0.45μm Millipore filter.

Induction of VCD-1 antigen expression in K562 cells

K562 cells were suspended at 10^6/ml in RPMI-10 and mixed with an equal volume of lymphocyte-conditioned medium prepared as described above. The cells were incubated for several days during which time they were subdivided and supplemented with more medium when necessary. Control cells were incubated in RPMI-10 containing 20μg/ml Con A and 10 mM methyl α-D mannopyranoside. Cell samples were taken daily, dried on multi-well slides and examined by indirect IF, with MoAb harvest fluids VCD-1, W6/32 (anti-class I) and anti-class II α and β chain (3,9).

Antibodies and cells

Murine MoAb's of defined anti-human class II specificity were generously provided by Dr. B.B. Cohen of the Clinical and Population Cytogenetics Unit of the Western General Hospital in Edinburgh and by Dr. V. Quaranta of the Department of Immunology, Research Institute of

the Scripps Clinic, La Jolla, CA. These reagents are listed in Table 1.

Table 1: Antibodies used to characterize VCD-1

Designation	Specificity	Source	Ref.
4A5.1	Class II α chain	B.Cohen	(3)
2C4.4	Class II α chain	B.Cohen	(3)
2G1.4	Class II α chain	B.Cohen	(3)
DA6.147	Class II α chain	B.Cohen	(9)
CR3.43	Class II β chain	B.Cohen	(9)
VIC-Y1	Class II γ chain	V.Quaranta.	(4)
9.3F10	Class II β chain	ATCC Cat. No. HB180	(10)

Polyclonal and monoclonal antibodies used as reagents for detection of primary antibodies were obtained as follows:

FITC-GaM : fluorescein-conjugated goat anti-mouse IgG, Cappel Cat. No. 1211-0081

TRITC-SaM: rhodamine-conjugated sheep anti-mouse IgG, Cappel Cat. No. 2211-0084

SaM:[1] Sheep anti-mouse IgG link antibody, whole serum, produced in this laboratory, for use with "PAP" reagent.

MaHRP[1]: Murine monoclonal anti-horse radish peroxidase, produced in this laboratory,

HRP-RaM: Peroxidase-conjugated rabbit anti-mouse Ig, DAKO Cat. No. P161

HRP-SWaR: Peroxidase-conjugated swine anti-rabbit Ig, DAKO Cat. No. P217.

[1] The two antibodies were produced in this laboratory and were extensively used in a "PAP" detection system in which the ovine anti-mouse antibody was used to link primary monoclonal antibody to the MaHRP:HRP complex. The preparation of the PAP complexes is described in the Appendix.

Table 2: Target cells used to characterize VCD-I

Designation	Derivation	Ref
Raji	Burkitt's lymphoma	(11)
U-937	Histiocytic lymphoma	(12)
K562	Erythroleukemia	(13)
HL-60	Promyelocytic leukemia	(14)
UCT-GL1	Glioblastoma	No. 41 in (15)
UCT-GL2	Glioblastoma	No. 42 in (15)
UCT-BL1	Bladder ca.	No. 53 in (15)
F55	Foreskin fibroblasts	(*)
UCT-Br-1	Breast ca.	(16)
UCT-Mel 1	Melanoma	(17)
UCT-Mel 2	Melanoma	(17)
UCT-Mel 3	Melanoma	(17)
UCT-Mel 7	Melanoma	(17)
RPMI-7272	Melanoma (Bowes)	G. Moore, Denver, Colorado
EBVL	B lymphocytes	Appendix

(*) Foreskin fibroblasts were derived from primary cultures established in this laboratory.

Cell lines were maintained in RPMI-1640 medium with 10% foetal calf serum and antibiotics.

Adherent cells were removed from culture dishes with versene solution (see Appendix)

RESULTS

Mice were immunized with the patient's peripheral blood lymphocytes and immune splenocytes were fused with SP2 murine myeloma cells according to the standard procedure described in the Appendix. A clone that secreted antibody that reacted with the patient's cells was selected and designated VCD-1 (18). Isotyping of the antibody showed that it belonged to the IgG2a subclass. This isotype has remained stable over many passages.

VCD-1 did not react with viable cells.

To determine if VCD-1 reacted with a cell surface determinant I used an indirect immunofluorescence (IF) assay in which VCD-1 was the primary antibody and FITC-GaM the second antibody. Viable cells, or fixed and permeabilized cells were examined. The results (Fig. 1) showed that VCD-1 did not label viable cells. Fixation in the presence of detergent permeabilized the cell membranes and allowed antibody to penetrate. I concluded, therefore, that the epitope recognized by VCD-1 was intracellular.

Figure 1

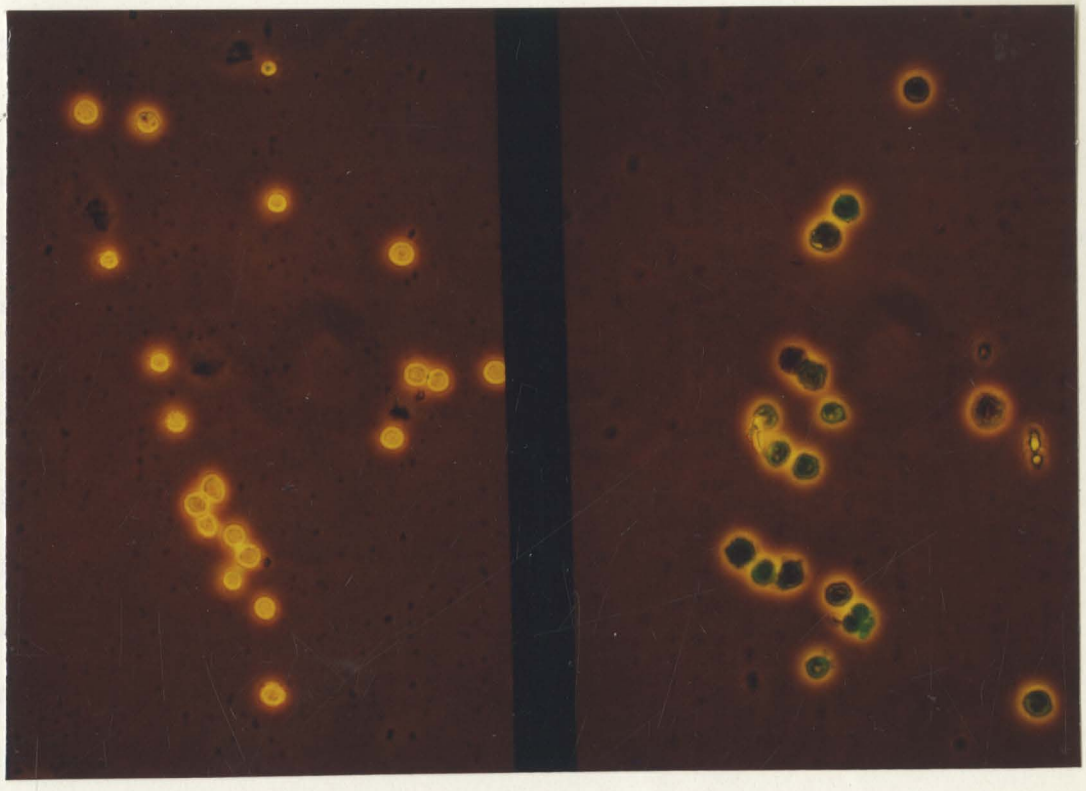


Fig. 1: CLL lymphocytes incubated with VCD-1 in the indirect IF test, photographed with phase contrast and fluorescence illumination combined.

A) viable cells incubated with VCD-1 and FITC-GaM

B) the same cells, fixed and permeabilized, incubated with VCD-1 and FITC-GaM

Distribution of VCD-1 antigen

To determine if expression of the VCD-1 antigen was restricted to CLL lymphocytes I examined the antibody for its ability to react with the cells listed in Table 3. Specific examples are shown in Fig. 2.

VCD-1 antigen was expressed by lymphoid cells, melanoma cells and interdigitating reticulum cells. It was not present on neutrophils or most epithelial lines. In these respects the distribution of the VCD-1 antigen closely resembled that of the HLA class II antigens.

VCD-1 bound to a 35-37kDa doublet in transblots of cell lysates separated by SDS-PAGE.

When SDS-PAGE gels of different cell lysates were transblotted on to Immobilon membrane and stained with VCD-1, the results shown in Fig. 3 were obtained. The epitope was strongly represented in CLL lymphocytes (lane 1), EBV-transformed B cells (lane 4) and Raji cells (lane 5). Normal peripheral blood mononuclear cells, enriched for B cells by adherence to nylon wool (lane 3) showed barely detectable levels of the antigen. T cell lysates contained no detectable antigen (lane 2). This technique failed to detect antigen in Ficoll-Hypaque separated PBMC without enrichment of B cells (data not shown).

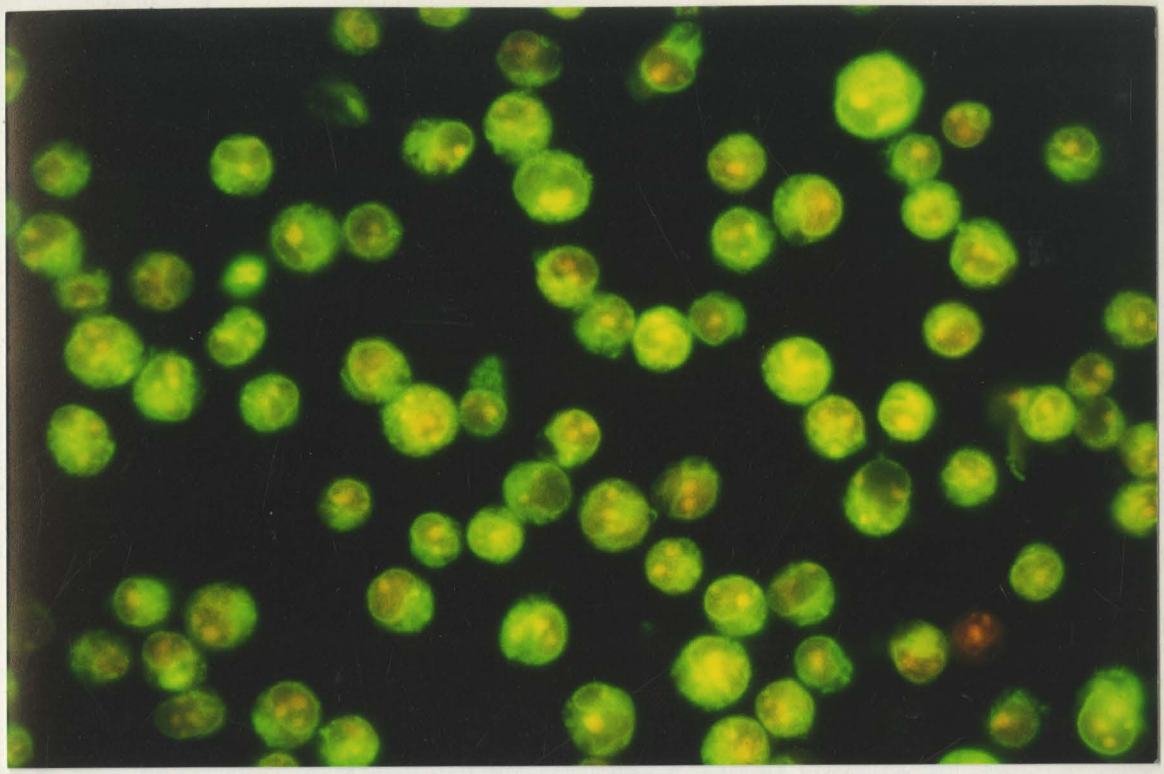
Table 3: Immunoreactivity of various cell types with VCD-1

Cell type	Results[1]		Approx. %+ve cells	Remarks
Normal cells:				
peripheral blood				
lymphocytes	+		20	see Fig. 4
monocytes	+		100	
neutrophils	-			
Interdigitating				
reticulum cells	+		100	see Fig. 2A
Lymph node follicle	+		100	see Fig. 2A
foreskin fibroblasts	-			
EBVL	+		>80	see Fig. 5A
Malignant cells:				
CLL lymphocytes	+	(17/17)[2]	50-100	
Reed-Sternberg cells	+	(1/1)	100	
Raji B lymphoblastoid	+		>95	see Fig. 2B
U-937 histiocytic				
lymphoma	+		>50	
K562, erythroleukemia	-			inducible(Fig.8)
HL-60,promyelocytic				
leukemia	-			
Breast carcinoma	+	(1/1)	90	
Melanoma	+	(4/5)	20-100	see Fig.27
Bladder carcinoma	-	(0/1)		
Glioblastoma	-	(0/2)		

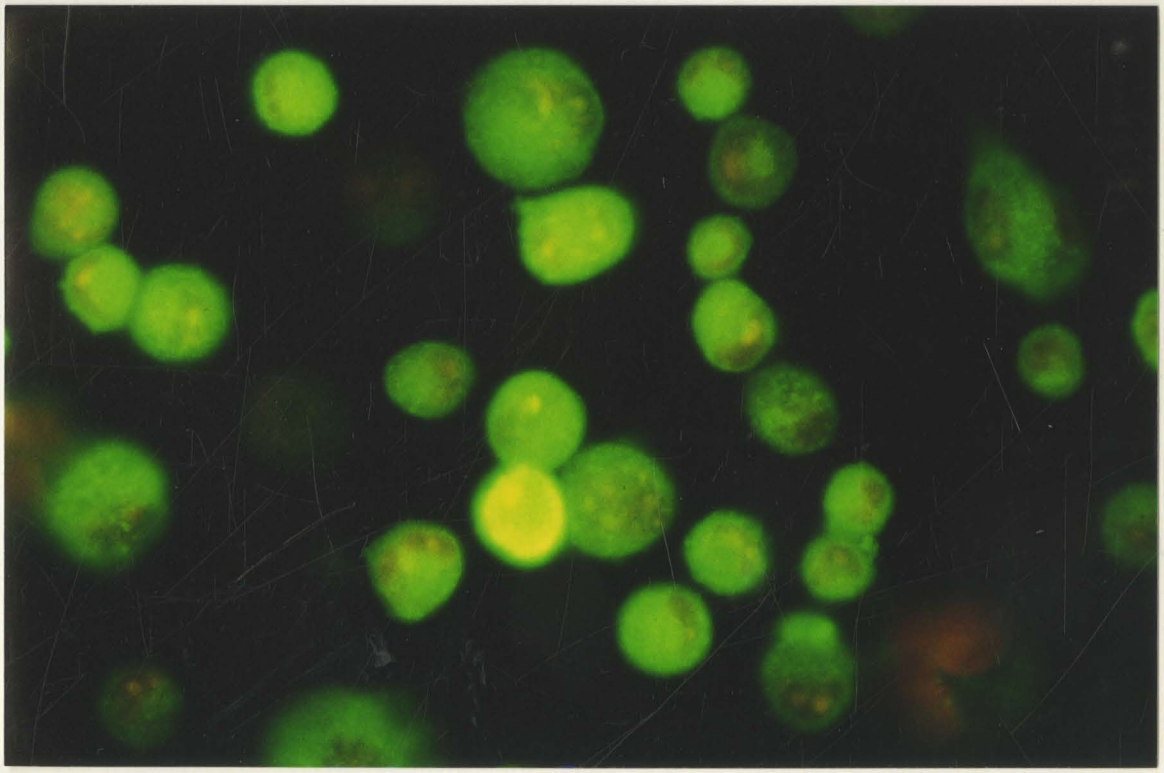
[1]Immunoreactivity of the various cell types was scored by immunofluorescence or immunohistochemical staining

[2] no. of cases +ve/no. tested.

Figure 2

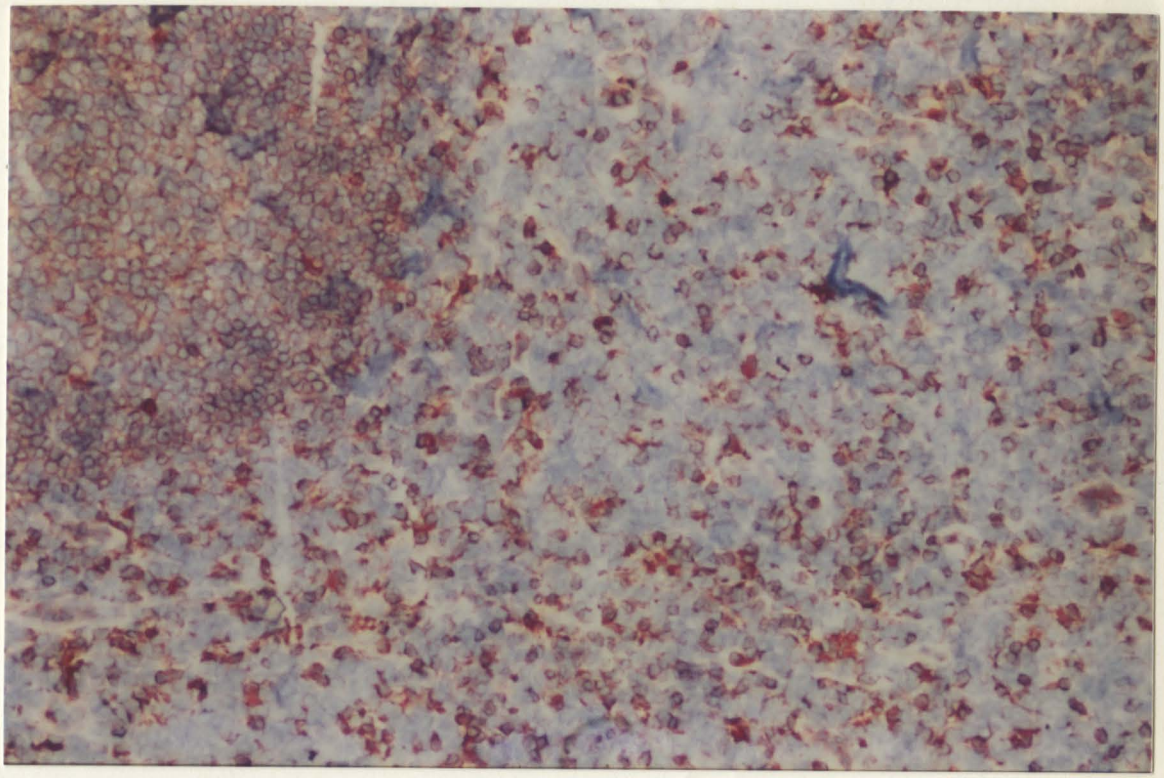


A



B

Figure 2



C

Fig. 2: Examples of cells expressing the VCD-1 antigen.

A) IF on B lymphoblastoid cells of the Raji cell line.

B) IF on melanoma cells of the UCT-Mel 7 cell line.

C) ICC on a frozen section of a normal lymph node. The red colour of the product of the immunoperoxidase reaction is seen on cells in the B cell rich follicle and on interdigitating reticulum cells in the inter-follicular area.

Figure 3

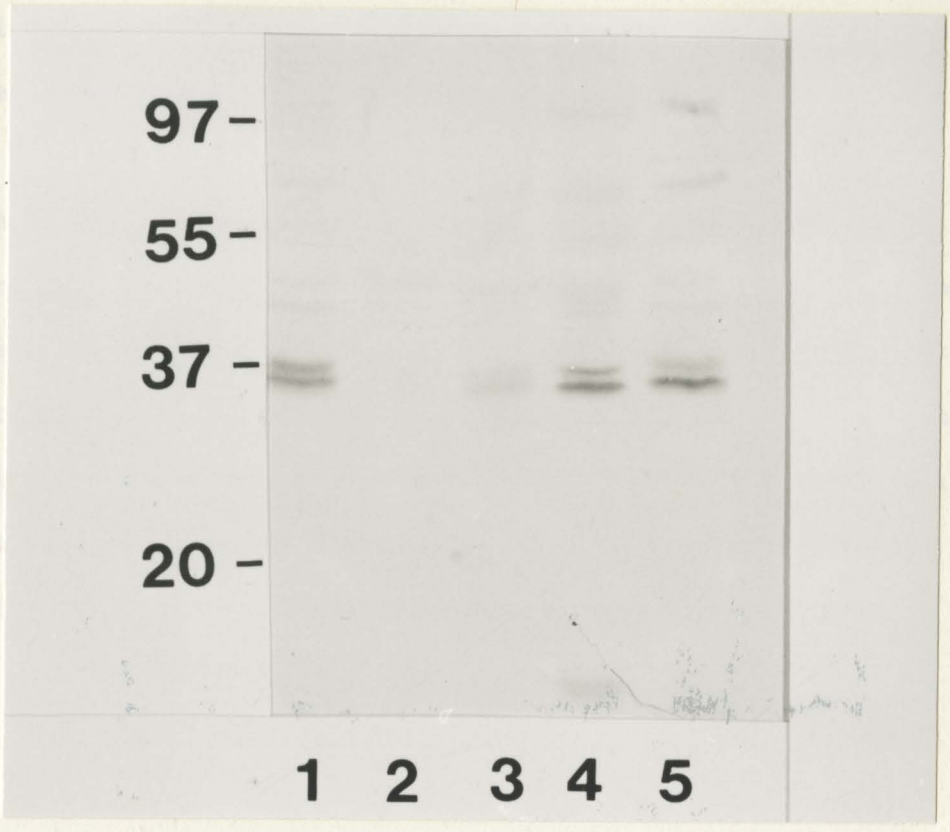


Fig. 3: Western blot of detergent lysates of cells with VCD-1.
CLL lymphocytes (10^8/ml): lane 1, T-lymphocytes (10^8/ml): lane 2, B-cell enriched PBMC (10^8/ml): lane 3, EBV-transformed lymphocytes ($5x10^7$/ml): lane 4, Raji cells ($5x10^7$/ml): lane 5, were treated with detergent and centrifuged to remove nuclei and the supernatant lysate electrophoresed in an 11% SDS-PAGE analytical gel under reducing conditions. Approximately 15μl of each lysate was applied per well.
Proteins were transblotted on to PVDF membrane probed with VCD-1.
Kilodaltons of molecular weight markers are indicated on the left.

Coexpression of the VCD-1 epitope and class II β chain

Normal PBMC were fixed, permeabilized and examined by double immunofluorescence, with the anti-class II β chain antibody CR3.43 (detected with TRITC-SaM), and FITC-conjugated VCD-1.

As shown in Fig. 4, many cells labelled intensely with both fluorescein and rhodamine to appear bright orange in the photograph. There were, in addition, cells that co-expressed both antigen, but less intensely, and yet others that expressed either class II-β-chain or VCD-1 antigen but not both together. The population more weakly reactive with VCD-1 could more readily be distinguished by flow cytometry and I discuss these cells more fully in Chapter 3.

VCD-1 did not bind to the epitope recognized by four anti-α chain MoAb's.

Anti-α chain antibodies (4A5.1, 2C4.4, 2G1.4 and DA6.147) obtained from Dr. Cohen were added in 8-fold excess over FITC-conjugated VCD-1 in a direct IF test with EBVL. The unlabelled antibodies did not compete with VCD-1 for binding to the cells whereas unlabelled VCD-1 did (Fig. 5).

Figure 4

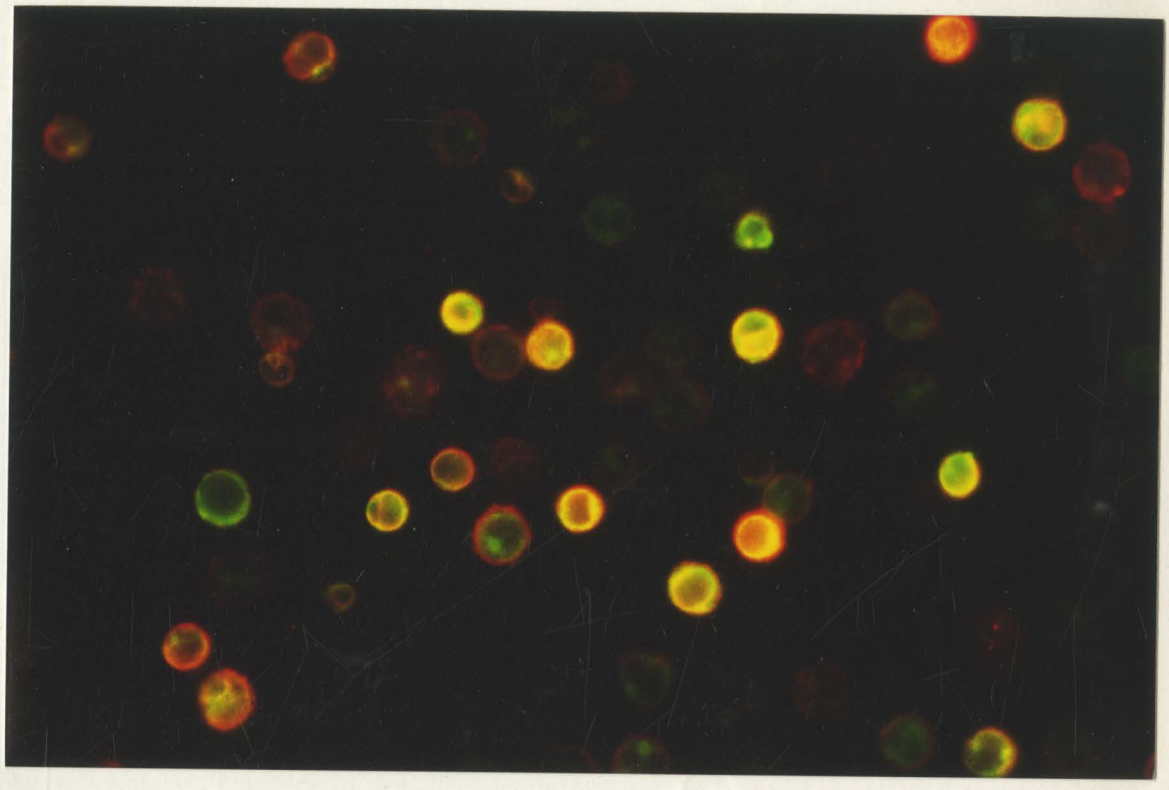


Fig. 4: Normal PBMC with double IF labelling.

PBMC were fixed, permabilized and analysed by double immunofluorescence as described in the Methods section. Antibodies to class II β-chain molecules were detected with rhodamine-labelled sheep anti-mouse antibody (red fluorescence); VCD-1 antigen was detected by direct analysis with FITC-labelled VCD-1 (green fluroescence).

The cells were not counterstained with ethidium bromide.

Figure 5

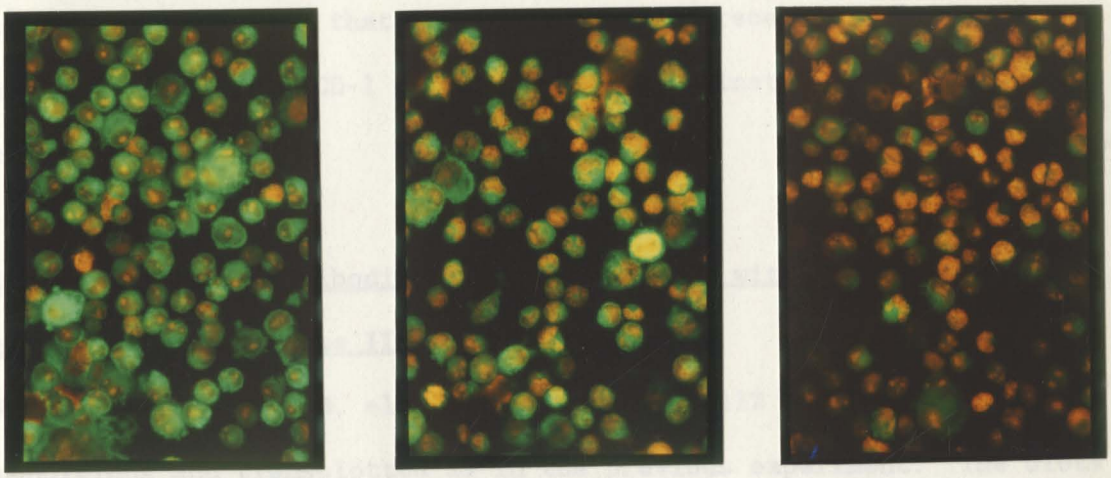


Fig. 5: Inhibition of binding of FITC-conjugated VCD-1 by unlabelled anti-class II α chain antibody on EBVL.

FITC-conjugated VCD-1 bound to cells when added alone (A) and in the presence of an approximately 8-fold excess of anti-α chain antibody 4A5.1 (B). Binding was inhibited by addition of a similar excess of unlabelled VCD-1 (C).

VCD-1 and anti-α chain antibody 4A5.1 identified different molecules. Raji cells were lysed, electrophoresed in an 11% gel under reducing conditions and transblotted as described in the methods section. The blots were then reacted with VCD-1 antibody, 4A5.1 anti-α chain antibody and a mixture of the two antibodies. It is quite clear from the results (Fig. 6) that the two antibodies recognized different molecules and that VCD-1 was not directed against an epitope on the α chain.

VCD-1 and VIC-Y1 antibodies appeared to react with the same molecule but not with the class II α chain.

Raji cells were lysed, electrophoresed in an 11% gel under reducing conditions and transblotted as in the previous experiment. The blots were reacted with anti-α chain antibody 4A5.1, VCD-1 and anti-γ chain antibody VIC-Y1. In Fig. 7 the patterns of immunoreactivity shown by the anti-γ chain antibody, VIC-Y1 (lane 3), and VCD-1 (lanes 2) were virtually identical whereas the anti-α chain antibody (lane 1) reacted with a molecule of slightly higher M_r than that identified by VCD-1.

Figure 6

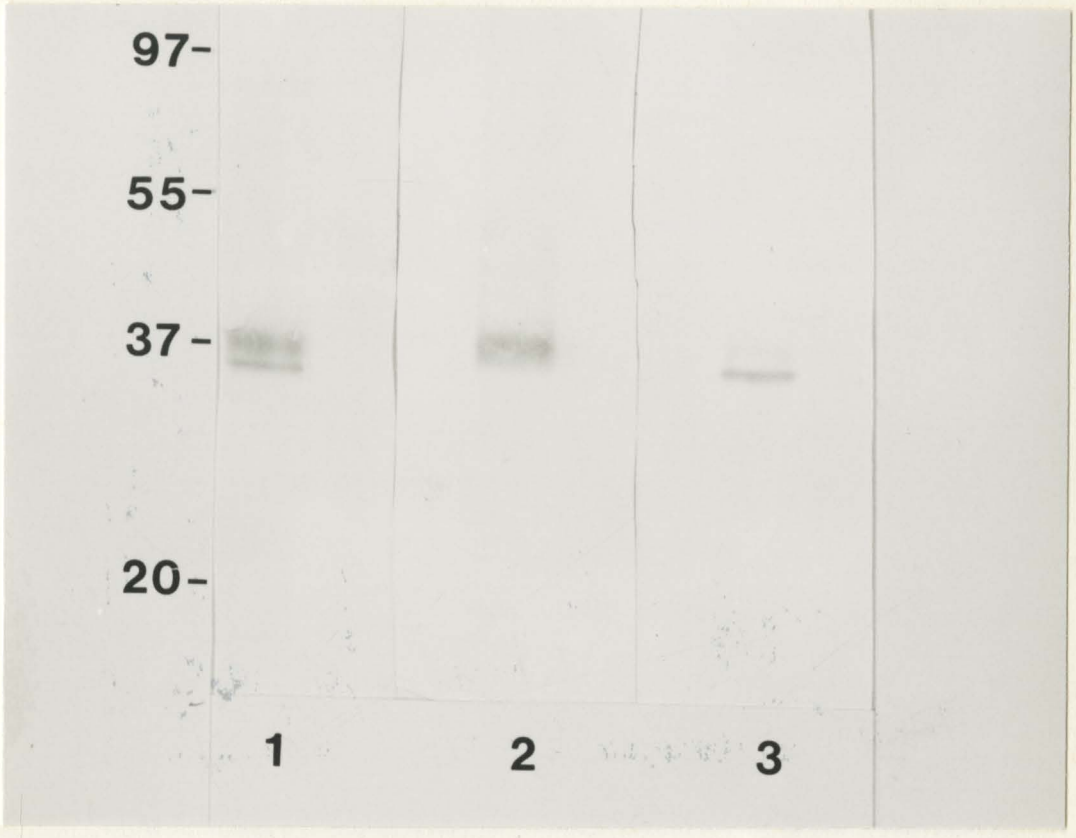


Fig. 6: Western blot of Raji cell lysate with VCD-1 and anti-α chain antibody.

Raji cells were lysed, electrophoresed in an 11% SDS gel under reducing conditions and transblotted on to PVDF membrane. The blots were reacted with VCD-1 antibody (lane 3), 4A5.1 anti-α chain antibody (lane 2) and a mixture of the two antibodies (lane 1).

Figure 7

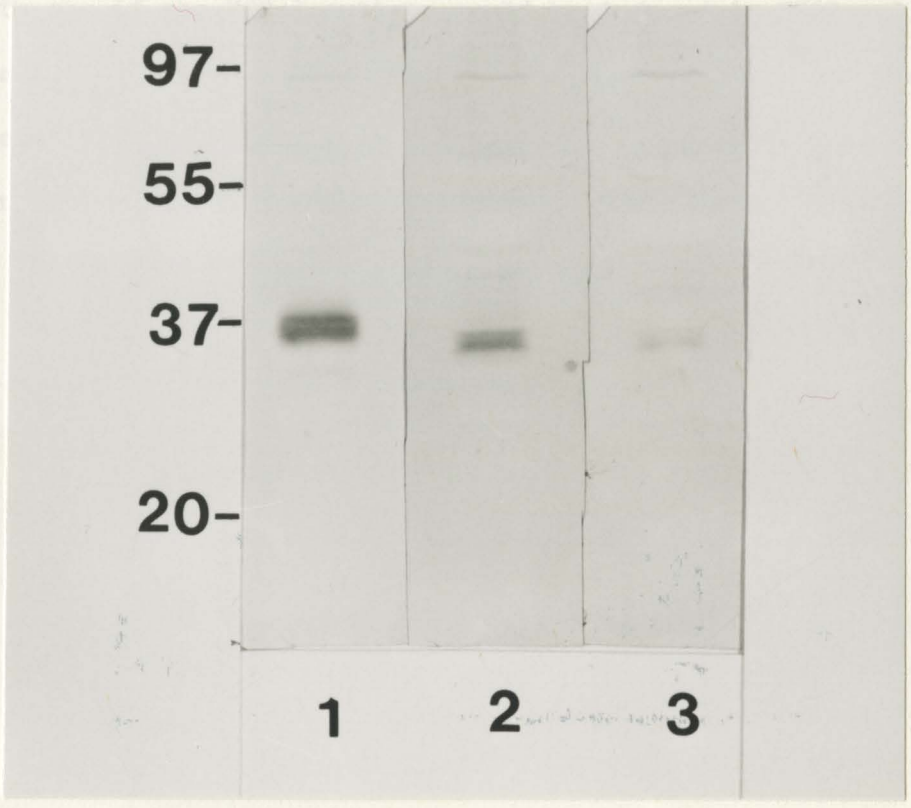


Fig. 7: Western blot of Raji cell lysate with VCD-1, anti-α chain and anti-γ chain antibody.

Raji cells were lysed, electrophoresed in an 11% SDS gel under reducing conditions and transblotted as for Fig. 6. Blots were reacted with anti-α chain antibody 4A5.1 (lane 1), VCD-1 (lane 2) and anti γ chain antibody VIC-Y1 (lane 3).

Cytokines induced expression of VCD-1 antigen in K562 cells

Since expression of HLA antigens in K562 cells is known to be modulatable by interferon γ (IFN γ)(19) I felt that it would be of interest to see if the VCD-1 antigen could be induced in K562 cells by the lymphokines present in the medium conditioned by Con-A stimulated lymphocytes. The results of this experiment (Fig. 8) showed that after four days of culture in the presence of the conditioned medium the cells quite clearly expressed VCD-1 antigen.

Figure 8

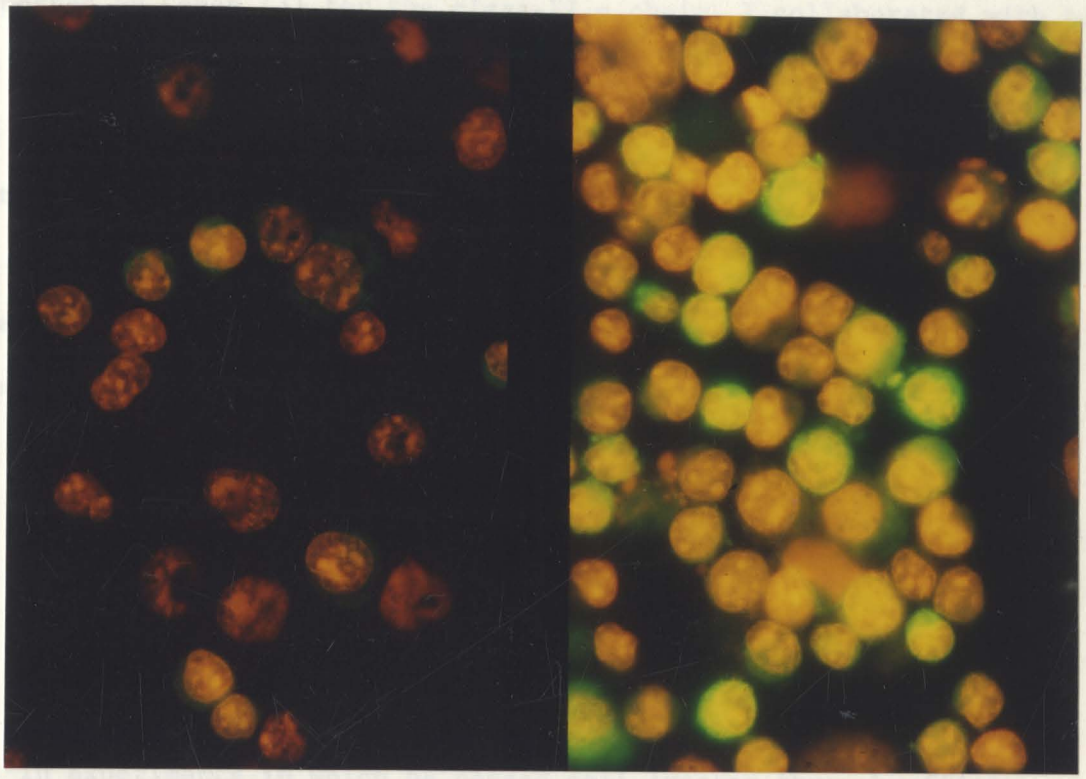


Fig. 8: Induction of VCD-1 antigen by lymphocyte-conditioned medium. K562 cells were grown in RPMI-10 mixed with an equal volume of Con-A lymphocyte-conditioned medium and tested for expression of the VCD-1 antigen with IF after (A) 2 days and (B) 4 days.

Experiments performed in collaboration with Dr. B. Cohen.

In view of the similarities between the VCD-1 antigen and that reported by Cohen (3) for the class-II α chain, I collaborated with him in a series of experiments in which I studied his antibodies (Table 1) in Cape Town and he compared VCD-1 with a panel of monoclonal antibodies that he had developed in Edinburgh.

The experiments performed by Dr. Cohen gave results that may be summarized as follows:

1. Although VCD-1 reacted with a protein of M_r similar to the α chain, it did not react with α/β dimers as might have been expected.
2. Western blots that he performed showed that VCD-1 reacted with a 34kDa component.
3. In experiments in which he passed cell lysates through affinity columns of immobilized VCD-1, anti-α- or anti-β-chain antibodies, and then tested the eluates in Western blots he found that:
   (a) VCD-1 did not remove class II α or β chain from the lysates.
   (b) an anti-α + anti-β affinity column removed the upper band of the 34kDa doublet and the 69kDa band recognized by VCD-1, but it did not remove the lower band of the 34kDa doublet
   (c) Eluates from a VCD-1 affinity column reacted with anti-α chain antibody.

DISCUSSION

In this chapter I have described the early work that led to the development of the hybridoma cell line, VCD-I, from the fusion of murine myeloma cells to splenocytes from a mouse that had been immunized with chronic lymphocytic leukemic cells. Initial hybridoma supernatant fluids were screened for antibodies that reacted with the CLL cells used to immunize the mice and VCD-I was selected on this basis.

Quite fortuitously, I screened the original hybridomas with cells that had been attached to microtitre plates with PLL and I added Tween 20 to wash fluids as a blocking agent. The effect of the detergent was to permeabilize the cells and expose the intracellular epitope recognized by the antibody. Had Tween 20 not been present, therefore, I should probably not have identified the VCD-I clone.

While my early attempts to characterize VCD-I were in progress, I came across a paper by Cohen (3) in which he described a monoclonal antibody to class II α chain that seemed, in many respects, similar to mine: it reacted only with permeabilized cells; it recognized an epitope with similar tissue distribution to that recognized by VCD-I; and the M_r of the antigen (35kDa) as judged by SDS-PAGE and immunoblotting, was much the same.

Dr. Cohen generously agreed to an exchange of monoclonal antibodies so that I might undertake a series of comparative studies using his MoAb's in Cape Town while he compared VCD-1 with his panel in Edinburgh.

The upshot of these collaborative experiments was that VCD-I immunoprecipitated an antigen from cell lysates that reacted with Dr. Cohen's antibodies directed against the class II α chain. When used as a detecting antibody in Western blots, however, VCD-I identified a molecule that was clearly distinct from the α-chain recognized by MoAb 4A5.1. Furthermore, Dr. Cohen's anti-class II β-chain monoclonal antibody reacted, in a double immunofluorescence test, with the same subset of PBMC as did VCD-I. These close associations between the VCD-1 antigen and the class II α- and β-chains suggested that it might be the class II invariant (Ii) or γ-chain, particularly as this had an M_r (35kDa) similar to that reported by Quaranta (4) for the molecule.

Further circumstantial support for an association of the VCD-1 antigen and the class II complex came from observations that the expression of the antigen in K562 cells was induced by lymphocyte-conditioned medium as previously described by others (19).

I then corresponded with Dr. Quaranta, who supplied me with a small amount of his VIC-YI antibody. Western blots with this showed that VCD-I and VIC-YI identified epitopes on antigens with identical electrophoretic mobilities. Unfortunately I did not have sufficient VIC-YI to complete the competition studies that would have identified

the VCD-I antigen unequivocally as the class II γ-chain. I sent a sample of VCD-1 to Dr. Quaranta with the request that he compare it with VIC-YI. He acknowledged receipt of the antibody but I have since had no further report from him.

Claesson-Welsh et al.(20) subsequently reported on a rat monoclonal antibody to the invariant chain, and I wrote to her, requesting a sample of her antibody. Unfortunately, owing to the academic boycott of South Africa, she was unable to comply with my request.

The definitive identification of the antigenic molecule recognized by an antibody is generally achieved by a combination of several approaches.

It is common, in the first place, to use comparisons with reference antibodies to establish, by competition or by co-precipitation, that the antibody in question binds to the same molecule as the reference antibodies. This is the approach that I have described and, although the results of these immunochemical studies supported that VCD-1 bound to a determinant on the Ii chain, they were not entirely conclusive.

Secondly, one may use the antibody to purify a protein for subsequent amino acid sequencing or to identify a clone in an expression vector library for nucleic acid sequencing. Either of these two methods would provide rigorous biochemical identification. Unfortunately I had neither the background nor the resources to complete studies of this sort.

Finally, one may document the biochemical and cellular characteristics of the antigen in question and compare these with similar published descriptions in the literature. I report the results of the experiments using this approach in the next chapter.

CHAPTER 2

STRUCTURE, ASSOCIATIONS AND PROCESSING OF THE INVARIANT CHAIN.

The class II invariant chain (Ii) or γ chain was first discovered by Jones and her colleagues in 1979 (21) when they analysed immunoprecipitates of lysates of murine splenocytes that had been metabolically labelled with radioactive amino acid precursors. Monoclonal antibodies and alloantisera to the highly polymorphic I-A and I-E sub-region antigens co-precipitated an associated monomorphic molecule with an M_r of approximately 31kDa. Immunoprecipitates of cell lysates after surface-labelling with ^{125}I and lactoperoxidase did not show the Ii band and they concluded that the molecule was not expressed on the cell surface but was associated with intracellular I-A antigens.

Since that time the biosynthesis and processing of the class II α, β and γ chains have been extensively investigated in human and murine cells. For the most part these studies have involved metabolic labelling of class II positive cells with radioactive precursors, immunoprecipitation of labelled class II antigens from cell lysates and analysis of the radioactive precipitated antigen by one- or two-dimensional electrophoresis. Cell-free translation systems have

provided information regarding the assembly of class II antigens *in vitro* (22,23); cell fractionation procedures have defined their intracellular location (22,24,25,26); the use of appropriate inhibitors has established the extent to which they are glycosylated (23,24,25,26,27,28); and the genes that encode these antigens have been cloned and sequenced to give detailed information of their chromosomal location and chemical structure (reviewed in reference 29).

As a result, much is now known of the biochemistry of the class II associated Ii or γ chain. This knowledge and the major stages in its development may be summarized as follows:

Initially, as I have indicated above, the invariant chain was thought to occur only intracellularly because it could not be precipitated from lysates of cells which had been surface-labelled with ^{125}I.

This view was supported by the first report of a murine anti-human Ii MoAb, VIC-Y1 (4), which confirmed that the γ chain was not detectable on the cell surface. In 1982, however, a rat anti-murine invariant chain antibody was reported which reacted with cell-surface Ii that was not associated with I-A antigens (30).

The complete sequence of the mRNA for the γ chain (31) showed that it was a polypeptide that was unusual inasmuch as that there was a transmembrane region near the amino terminus and two glycosylation sites near the middle. This suggested that the molecule had a membrane polarity which was inverted relative to most transmembrane

proteins, the amino terminus being intracellular and the carboxy terminus extracellular. This is well shown in the hydrophilicity plot that I have constructed from the published sequence of the Ii chain (Fig. 9). In an elegant extension of these observations, Wraight et al (32) described MoAb's that reacted with epitopes that were mapped by mutant recombinant proteins. They established that the VIC-Y1 antibody, which binds only to an intracellular epitope, reacted with a determinant located between amino acids 1 and 73, whereas three other antibodies that recognized carboxy-terminal epitopes bound to an extracellular domain.

In the early 1980's, several papers were published that described the biosynthesis, intracellular transport and expression of the class II molecules (22,25,26,33). These showed that three polypeptides are involved in forming the oligomeric structure of the class II antigens. The α chain (M_r 35kDa), β chain (M_r 29 kDa) and γ chain (M_r 33 kDa) are co-translationally inserted into the membrane of the endoplasmic reticulum (ER). Oligomeric assembly of α, β and γ chains occurs shortly after their synthesis in the ER.

Gamma chains are synthesized in excess of α and β chains and they are thus found in the ER either in a complex with α and β or in a free form. Free γ chains remain in the ER, whereas γ chains that are complexed to α and β chains are transported to the Golgi apparatus and to the cell surface.

Figure 9

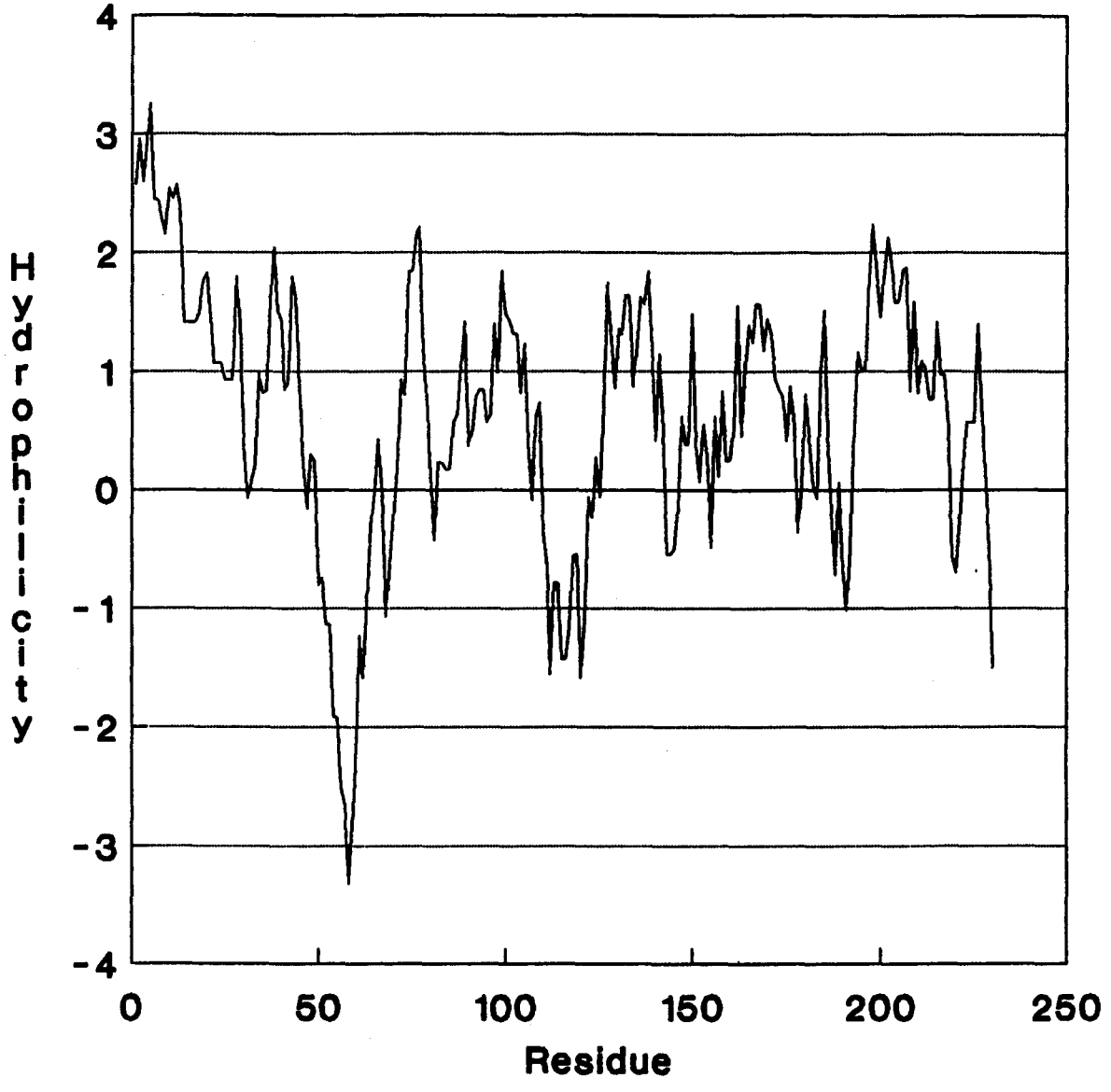


Figure 9: Hydrophilicity plot of the HLA class II invariant chain. Hydrophilicity was analysed by the method of Kyte and Doolittle (101) averaged over a window of seven residues. Note the hydrophobic transmembrane region extending from residue 50 to residue 70.

The molecular weight of the γ chains increases during transport as complex sugars are added in the Golgi complex. This is followed by the detachment of γ chains from the oligomeric complex and the appearance of α and β chains on the cell surface. Such Ii as is present on the surface does not appear to be associated with α or β chain.

The genes for both murine and human invariant chain have been mapped outside the MHC to chromosome 18 in mice and chromosome 5 in man. A c-DNA clone encoding the human invariant chain was isolated by Strubin et al. in 1984 (31) from which the unusual protein structure I have alluded to was deduced. They also demonstrated that it exists in two related forms p33 and p35, representing the products of translation from two in-phase AUG's in the message (34). The gene for the murine invariant chain was sequenced by Koch et al. in 1987 (35) who also identified an alternatively spliced exon which encodes a domain with high homology to a repetitive sequence in thyroglobulin. This exon is spliced into the mRNA coding for the Ii-related 41kDa protein that is frequently observed.

In addition to genetic mechanisms for the generation of multiple forms of γ chain, post-translational events also contribute to the heterodisperse species that are encountered in cellular lysates. The formation of covalent Ii chain dimers, different degrees of glycosylation and conversion to proteoglycans as well as limited proteolytic cleavage in the endosomal compartment (36,37,86) are known

to generate the additional bands identified by electrophoresis and immunoreactivity.

Sant et al. (38) discovered in 1985 that the murine invariant chain is the core protein of a 46-70kDa chondroitin sulphate proteoglycan (CSPG) which associates with the α/β heterodimer. Similar experiments with human cells were performed in 1986 by Giacoletto et al. (39). who found that CSPG labelled with ^{35}S-sulphate could be immunoprecipitated with antibodies against the invariant chain, indicating that it is an alternatively processed form of the invariant chain. The single attachment site of glycosaminoglycan was identified in the mouse by site-directed mutagenesis (40). The mutant protein lacking the glycosaminoglycan transported normally through the Golgi compartment and associated stably with I-A molecules. The CSPG has been shown to be associated with I-A at the plasma membrane as well as with an intracellular pool of these glycoproteins (39,41).

Rahmsdorf et al.(42,43) made the important observation that the γ chain is not constitutively expressed: its synthesis is modulatable by such compounds as IFNγ, mitomycin C, cycloheximide and lipopolysaccaride.

The close association of repression or stimulation of γ chain synthesis with corresponding changes in the expression of other class II antigens has been taken to indicate a regulatory role for the invariant chain in antigen presentation and the biological function of class II molecules.

In summary, therefore, the invariant chain may be defined as an inducible molecule that is closely associated, in all species so far studied, with the α and β chains of class II antigen in cells that express this oligomeric complex.

Physical association of Ii and class II antigens is readily demonstrable intracellularly where the major fraction of Ii appears to reside; Ii on the cell surface is not intimately associated with the α/β dimer.

The invariant chain has an unusual membrane polarity; it is glycosylated during the process of intracellular transport; and it forms the core protein of a cellular CSPG. Several isoforms of invariant chain are encoded by a single gene located on chromosome 5 in man and chromosome 18 in the mouse. These different forms of the invariant chain are the result of differential splicing and the product of translation from two in-phase start codons in the Ii mRNA.

In this chapter I present the results of the experiments that I undertook to characterize my antibody and to compare the behaviour of the antigen that it recognized with that of the invariant chain as described by others.

METHODS

Immunoprecipitation of antigen in cell lysates

VCD-1 antigen and the molecules with which it was associated were precipitated from cellular lysates by a minor modification of the method of Jones (44). Samples and reagents were kept on ice at 4°C throughout. Staphylococcus aureus (SA), purchased as a 10% formalin-fixed suspension (BRL 9321SA), was pelleted in a microfuge and resuspended to 10% in PBS containing 3% SDS w/v and 10% 2-mercaptoethanol. The suspended bacteria were heated to 95°C for 30 mins, washed twice with SA buffer (PBS containing 0.5% NP-40, 2mM methionine, 0.02% sodium azide), and resuspended at 10% in SA blocking solution (SA buffer containing 1mg/ml ovalbumin). The suspension was left on ice for 30 mins.

MoAb ascites was added at 5μl per 200μl SA suspension and the mixture left on ice for 1 hr with occasional mixing. Antibody-coated bacteria were then washed twice with SA buffer and resuspended in the original volume of SA blocking solution.

Cell lysates were first "cleared" by mixing with an equal volume of a suspension of SA coated with an irrelevant MoAb (anti-β-galactosidase). The mixture was left on ice for 1 hr with occasional mixing, centrifuged and the pellet discarded. I then added the suspension of SA coated with VCD-1 ascites (same volume as clearing SA) and incubated the mixture on ice for 1-16 hrs with occasional mixing. The

SA was then washed five times with SA buffer and pellets were resuspended in sample buffer and boiled for 2 mins. The mixture was centrifuged and the supernatant used for SDS-PAGE.

In the experiment shown in Fig. 11, immunoprecipitated, unlabelled antigen equivalent to 50μl lysate was loaded per track; in immunoprecipitations of ^{35}S- methionine-labelled lysate, the equivalent of 25μl lysate was loaded per track.

Sepharose beads covalently coated with rabbit- anti mouse IgG (RaM Sepharose) were generously provided by Pharmacia and were used in place of SA to precipitate immune complexes of antigens with antibodies (e.g. IgG1) whose binding to SA was uncertain or in circumstances when preliminary clearing of the lysate was not feasible. In these cases, 1.0 ml volumes of the suspension of RaM sepharose, as supplied, were centrifuged, resuspended in 100μl SA blocking solution and treated in the same way as SA.

Sub-cellular fractionation

The method used to prepare microsomal and cytosolic fractions was similar to that described by Graham (45). Cells were washed three times with PBS and resuspended at 10^8/ml in homogenization buffer (5mM Tris-HCl pH 7.6; 0.5mM $CaCl_2$; 0.5mM $MgCl_2$). The suspension was left on ice for 10 mins and then homogenized with 20 strokes of pestle A of a Dounce homogenizer. Cell disruption was checked by phase contrast microscopy and the suspension was made isotonic by adding 1/4 volume of 1.25M sucrose.

The homogenate was centrifuged at 1000g for 10mins to pellet nuclei. The supernatant was removed and centrifuged at 40 000g for 30mins to pellet microsomes. The supernatant cytosol was removed and mixed with an equal volume of sample buffer for SDS-PAGE. The crude microsomal pellet was first suspended in the original volume of cell lysis buffer (see Chapter 1), left on ice for 30mins and then mixed with an equal volume of sample buffer for SDS-PAGE.

Metabolic labelling with ^{35}S-methionine.

Cells were washed twice with PBS and suspended in methionine-free RPMI supplemented with 2% FCS which had been dialyzed against PBS. For continuous labelling, Raji cells and EBVL were suspended at $5x10^6$/ml, PBMC at $10x10^6$/ml, melanoma cells at $2.5x10^6$/ml and incubated at 37°C for 15-30mins. ^{35}S-methionine (NEN Cat. No. NEG-009A, 1111 Ci/mmol) was added at 50μCi/ml and the suspension incubated for 2 hrs with occasional mixing in a 12 ml centrifuge tube. The cells were washed twice with PBS and a cell lysate prepared as described in Chapter 1.

For the pulse chase experiment Raji cells and EBVL were suspended at $20x10^6$/ml in methionine-free and incubated for 15-30 mins at 37°C; ^{35}S-methionine was then added at 200μCi/ml. The cells were incubated at 37°C for 10 mins, diluted with 20 volumes of medium containing methionine, divided into aliquots and returned to the incubator for the chase. At the end of each chase time one tube was removed from the incubator and cooled in ice. The cells were washed twice with cold PBS, lysed and prepared for immunoprecipitation as described.

Tunicamycin treatment and metabolic labelling.

Cells were washed twice with PBS and suspended in methionine-free medium containing 2% dialysed FCS at $5x10^6$/ml. Tunicamycin (Sigma T-7765), 0.5mg/ml in dimethyl sulphoxide (DMSO), was added to a final concentration of 5μg/ml. DMSO was added to the control cells. I then incubated the cell suspensions at 37°C for 1 hr, added ^{35}S-methionine to a final concentration of 50μCi/ml and incubated them for a further 2 hours with occasional mixing. I then lysed the cells as described.

Immunoprecipitation of cell surface antigens.

One ml volumes of Pharmacia sepharose beads coated with RaM were spun in the microfuge and the pellets were resuspended in 200μl SA blocking solution. For the control immunoprecipitation of whole cell lysates I pre-incubated them with 5μl VCD-1 ascites and washed them twice with SA buffer.

EBVL were labelled with ^{35}S-methionine for 2 hrs as described and washed with PBS. I then divided the cells into two lots. One of these I lysed and immunoprecipitated the lysate with RaM sepharose coated with VCD-1 (100μl beads per 50μl cell lysate).

The other lot I resuspended in PBS in two parts of 0.5ml each; 5μl VCD-1 ascites was added to one and 5μl irrelevant ascites to the other. After 30 mins on ice the cells were washed twice with PBS and mixed with five times their number of unlabelled EBVL. These I lysed as described. The addition of unlabelled cells before lysis ensured that any free binding sites, on antibody bound to cell surface antigen or

on residual antibody that had not been removed by washing, were blocked with non-radioactive antigen and did not combine with radioactive intracellular antigen released by lysis.

Equal volumes of RaM sepharose were added to the lysates and the mixtures were left on ice for 1 hr. The sepharose pellets were washed five times with SA buffer before adding sample buffer for SDS-PAGE.

Immunoprecipitation of conditioned medium.

Raji cells were labelled for 2 hrs with ^{35}S-methionine as described and the medium was reserved. One ml volumes of conditioned medium were immunoprecipitated with RaM sepharose coated with VCD-1 or an irrelevant antibody. The immunoprecipitates were analysed by electrophoresis in an 11% SDS gel under reducing conditions followed by fluorography.

Mitogen stimulation and mixed lymphocyte culture in the presence of VCD-1

PBMC were separated from heparinized blood from normal donors and cultured with mitogens or mitomycin C-treated stimulator cells according to standard protocols (46). Purified VCD-1 (see Appendix) or normal mouse IgG was added to the cultures to a final concentration of 25μg/ml and the ^{3}H-thymidine incorporation of cultures compared.

Chondroitinase treatment of intact cells

Raji cells and EBVL were suspended at $5x10^{6}$/ml in PBS containing 0.02% sodium azide, 0.01% BSA and 1.5 u/ml chondroitinase ABC (Sigma

C-3509) Cells were incubated at 37°C in a water bath for 30 mins and washed twice with PBS. The viability of the cells was checked (>95%) and they were labelled in suspension with VCD-1 and FITC-GaM as described in Chapter 1.

Metabolic labelling of cells with ^{35}S-sulphate.

Sulphate-free medium was prepared by adding 100x MEM vitamin mixture (ISI cat. no. 268) and 50x MEM amino acid mixture (Flow Labs. 16-011-49) to the salts of MEM (Flow Laboratory catalogue. p. 46) with the 200mg/l $MgSO_4.7H_2O$ replaced by 170.8mg/l $MgCl_2.6H_2O$ as in BME Diploid (modified) medium i.e.

	mg/l
$CaCl_2.2H_2O$	265
$MgCl_2.6H_2O$	170.8
KCl	400
NaCl	6800
$NaHCO_3$	2000
$NaH_2PO4.H_2O$	340
Glucose	1000
L-glutamine	292.4

A 0.5% solution of phenol red was added at 2ml/l, the pH was adjusted to pH 7.2 and the medium sterilized by filtration.

Cells were washed twice with PBS and suspended in 4ml sulphate-free MEM containing 2% dialyzed FCS in a 60mm Petri dish containing $5x10^6$ Raji cells or EBVL, $10x10^6$ PBMC, or 2-3 x 10^6 melanoma cells (plated the previous day). ^{35}S-sulphate (Amersham SJS.1,1071 Ci/mmol) was added at 150μCi per dish and the cells were incubated overnight. Cells were washed twice with PBS and a lysate prepared as described.

RESULTS

<u>VCD-1 identified a prominent 35-37 kDa doublet in various cell lines that express class II antigens</u>

Since the class II α, β and γ chains are known to associate in the ER (4,20,22,23,25,26,27,33), I expected to find the VCD-1 epitope represented in a high M_r complex obtained from cell lysates. I therefore electrophoresed cell lysates in SDS-PAGE under non-reducing conditions and without boiling. After transfer onto PVDF membrane, proteins were reacted with VCD-1 and 4A5.1 antibodies (Fig.10). These Western blots showed that VCD-1 reacted with a 35-37kDa doublet and a 72-78kDa doublet. The higher M_r doublet represented the dimeric form of the 35-37kDa doublet described by others (47,48).

The anti-α chain antibody (lane 5) labelled a ~65kDa band identified by Cohen as the α/β complex. The anti-α chain antibody did not label any of the bands labelled by VCD-1. In two of the cell lines faint additional bands were observed at 12 and 44 kDa. It is worthy of note

Figure 10

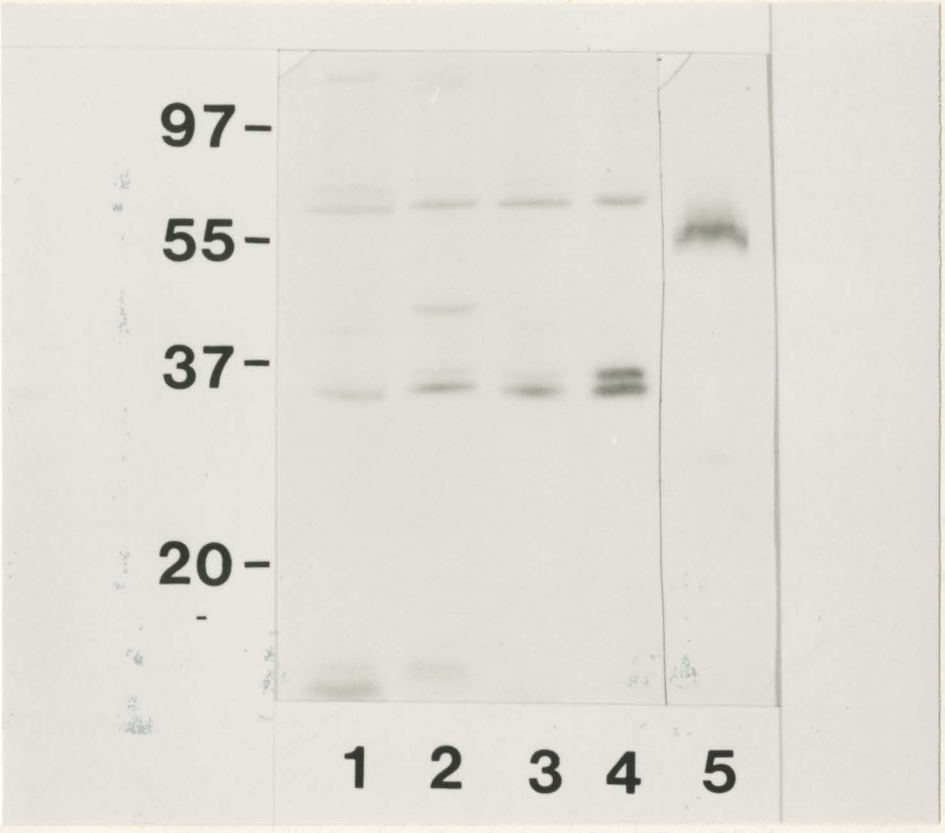


Fig. 10: Western blot of lysates of different cell types with VCD-1 and 4A5.1 antibody.

Detergent lysates of cells from the melanoma cell line UCT-Mel 7 at 2.5×10^7/ml (lanes 1 and 5), EBV-transformed lymphocytes at 5×10^7/ml (lane 2), Raji cells at 5×10^7/ml (lane 3) and CLL lymphocytes at 10×10^7/ml (lane 4) were electrophoresed in an 11% SDS polyacrylamide gel under non-reducing conditions and without boiling. After transfer onto PVDF membrane the proteins were reacted with VCD-1 (lanes 1-4) and 4A5.1 (lane 5) antibodies.

that CLL cells (lane 4) differed from the other cell types in that the higher M_r component of the 35-37kDa doublet was present in greater relative proportion.

Immunoprecipitation with VCD-1 precipitated a complex of VCD-1 antigen and class II α chain.

Kvist et al (22), in a series of studies designed to examine processing of class II antigens, showed that an antibody directed against the α chain co-precipitated γ chain from cell lysates, indicating that α/γ chain complexes exist. To confirm this observation with my antibody, I lysed Raji cells with detergent, immunoprecipitated VCD-1 reacting material with SA-bound VCD-1 and analysed the precipitate by electrophoresis and immunoblotting. The results (Fig. 11) showed that VCD-1 precipitated both a 35 kDa molecule (lane 1) and α chain, identified with 4A5.1 antibody (lane 2). These observations are concordant with those of Dr. Cohen (see Chapter 1) who showed, in experiments in which he passed cell lysates through an anti-α + anti-β chain immunoaffinity column, that the immobilized anti-class II antibody bound to a complex that included the 37 kDa component of the VCD-1 reacting doublet and a 72 kDa molecule that reacted with the VCD-1 antibody.

VCD-1 antigen was localized intracellularly in the microsomal fraction

Others have shown that class II γ chain is located, in cell homogenates, in the microsomal fraction (22,24,25,26). I accordingly analysed whole cells, cytosol and microsomal pellets from Raji cells that had been homogenized in hypotonic buffer as described in the

Figure 11

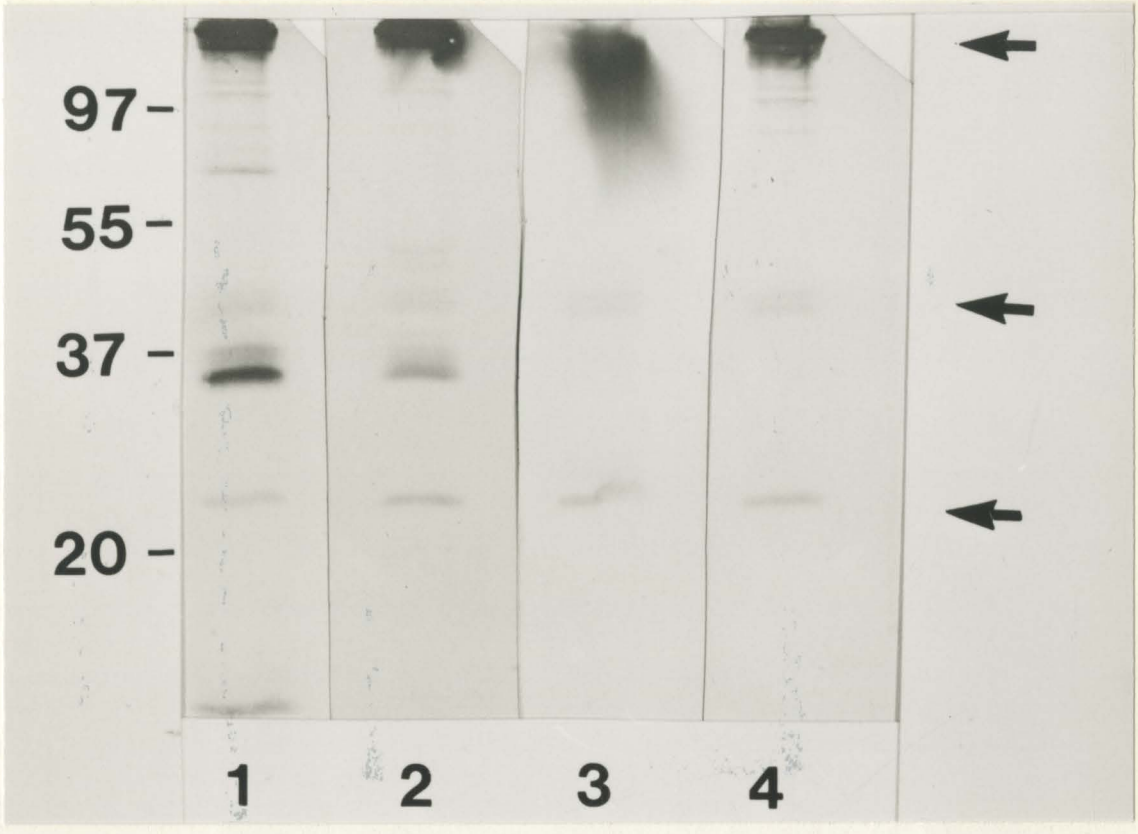


Fig. 11: Immunoprecipitation of Raji cell lysates with VCD-1 and identification of the precipitate by Western blot.
Raji cells were lysed with detergent at $5x10^7$/ml and the lysate was reacted with SA coated with VCD-1 antibody. The washed immunoprecipitant was treated with non-reducing sample buffer but not boiled to preserve the epitope of the anti-β chain antibody. The eluted proteins were electophoresed in an 11% SDS polyacrylamide gel, transferred onto PVDF membrane and reacted with VCD-1 (lane 1), 4A5.1 anti-α chain (lane 2), CR3. 43 anti-β chain (lane 3) and control antibody (lane 4).
Note that arrows mark the precipitating antibody which is transferred from the gel and is also labelled by the anti-mouse peroxidase detection system.

methods section and in the legend to Fig. 12. The Western blot (Fig. 12) showed that the VCD-1 antigen appeared to be confined to the microsomal fraction.

Immunoprecipitation of metabolically labelled CLL cells

Fluorographic analysis of SDS gels containing resolved VCD-1 immunoprecipitates from CLL cells metabolically labelled with ^{35}S-methionine showed two distinct ^{35}S-containing bands: a predominant 35 kDa band and a less obvious ~72 kDa band. Reduction of the immunoprecipitate by boiling with dithiothreitol caused the 72 kDa band to disappear and a 37 kDa band to appear. This is seen in lane 1 of Fig. 13 as a widening of the 35kDa band.

VCD-1 and VIC-Y1 precipitated an identical array of metabolically labelled molecules.

To obtain evidence for the identity of the VCD-1 antigen with the class II γ chain recognized by the reference antibody VIC-Y1, I prepared immunoabsorbents with these two antibodies and used them to precipitate antigens from ^{35}S-methionine-labelled Raji cells. As can be seen from Fig. 14 the patterns of radioactive bands precipitated by VCD-1 and VIC-Y1 were identical.

Kinetics of incorporation of ^{35}S methionine into VCD-1 antigen.

To study the synthesis and processing of the VCD-1 antigen as a function of time I performed a pulse-chase experiment in which Raji cells or EBV-transformed lymphocytes were incubated with ^{35}S-methionine for 10 mins followed by a "chase" with an excess of

Figure 12

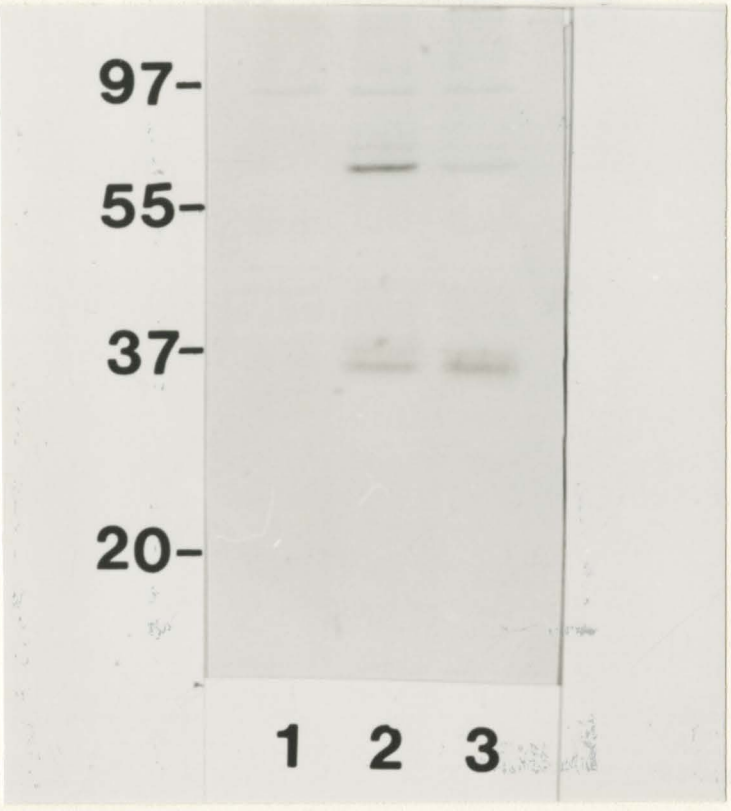


Fig. 12: Intracellular distribution of VCD-1 antigen.
Raji cells (10^8/ml) were homogenized in 5mM Tris-HCl pH 7.6 + 0.5mM $CaCl_2$ + 0.5mM $MgCl_2$. Nuclei were removed by centrifugation at 1000 g for 10 mins. and the microsomal pellet was obtained by centrifugation at 40 000 g for 30 mins. Whole cells (lane 3), cytosol (lane 1) and microsomal pellet (lane 2) were analyzed by SDS-PAGE under non-reducing conditions and immunoblotting with VCD-1 antibody

Figure 13

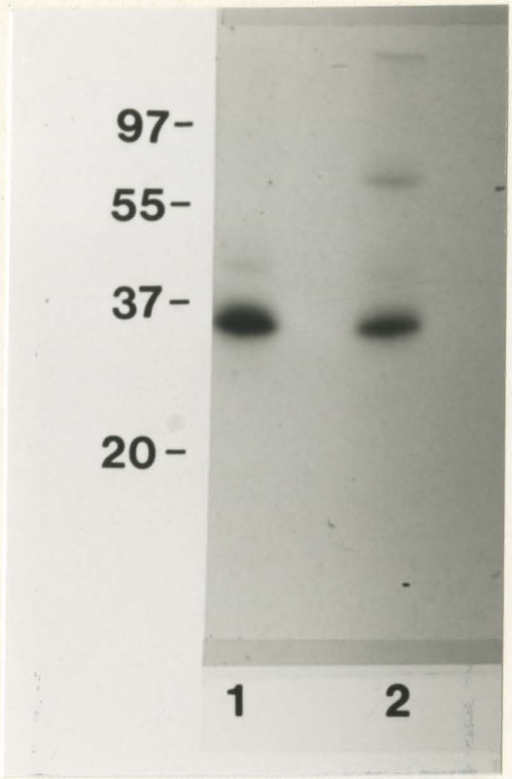


Fig. 13: Fluorographic analysis of VCD-1 immunoprecipitates from CLL cells .

CLL cells at 10^8/ml were metabolically labelled by incubating in methionine-free medium in the presence of 50 μCi/ml ^{35}S-methionine. After 2 hours the cells were lysed and immunoprecipitated with SA coated with VCD-1 antibody.

The immunoprecipitates were analyzed in an 11% SDS gel before (lane 2) and after (lane 1) boiling in the presence of 10 mM dithiothreitol. Note that the effect of reduction was to cause the disappearance of the ~ 72kDa band present in lane 2 and the appearance of a ~ 37kDa band in lane 1. The resolution of the fluorograph and photograph is such that it does not generate the appearance of a discrete doublet but rather a widening of the 35 kDa band.

Figure 14

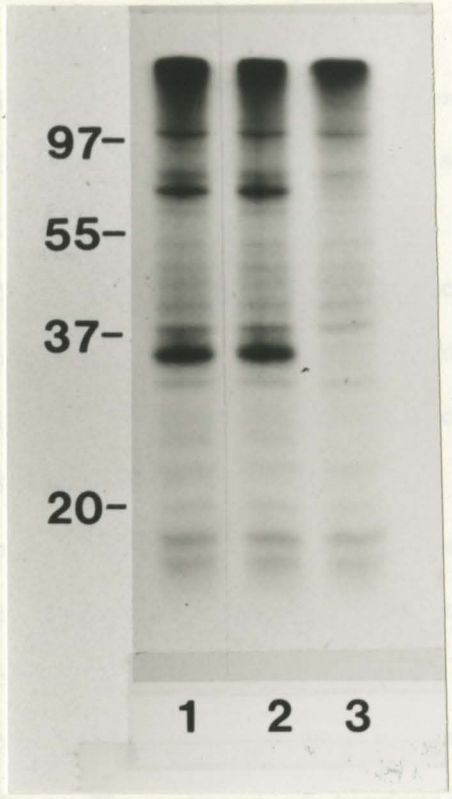


Fig. 14: Immunoprecipitation of Raji lysate with VCD-1 and VIC-Y1 antibody.

^{35}S-methionine-labelled Raji cells were lysed with detergent at 5 x 10^7/ml and the lysate immunoprecipitated with Sepharose coated with RaM IgG and VCD-1 (lane 1), VIC-Y1 (lane 2) or irrelevant antibody (lane 3). Immunoprecipitates were analyzed in an 11% SDS gel under non-reducing conditions and fluorographed. RaM Sepharose was chosen as immunoprecipitant in this case because VIC-Y1 antibody is of the IgG1 subclass which does not bind well to SA .

non-radioactive methionine. Cells were lysed at 0, 30, 60 and 120 minutes after addition of the chase and the lysates were analysed by immunoprecipitation, electrophoresis and fluorography. The results are shown in Fig. 15 and merit comment in the following respects:

VCD-1 antigen was rapidly labelled with the prominent appearance of three major clusters of bands within 10 mins. These clusters at ~35-37 kDa (arrowhead III), ~44 kDa (arrowhead II) and ~72-78 kDa (arrowhead I) were readily visible in the non-reduced lysates of both Raji and EBVL. When electrophoresed under reducing conditions the 72 kDa cluster became less obvious with accentuation of the 35-37 kDa and 44 kDa clusters. Furthermore, the 35 kDa band in the Raji lysates electrophoresed under non-reducing conditions (panel B) showed a noticeable disappearance with time with relative enrichment of the 37 kDa band. The bands in the gel run under reducing conditions (panel A), however, did not show this progressive increase in apparent M_r of the 35 kDa band with time. The pattern of temporal change in the EBV-transformed lymphocytes differed from that seen with Raji cells in that the 35 kDa band showed a steady diminution in radioactive intensity without change in apparent M_r.

A faint band with an M_r of 25 kDa was also precipitated; this disappeared after approximately 60 mins of chase.

Glycosylation of the VCD-1 antigen

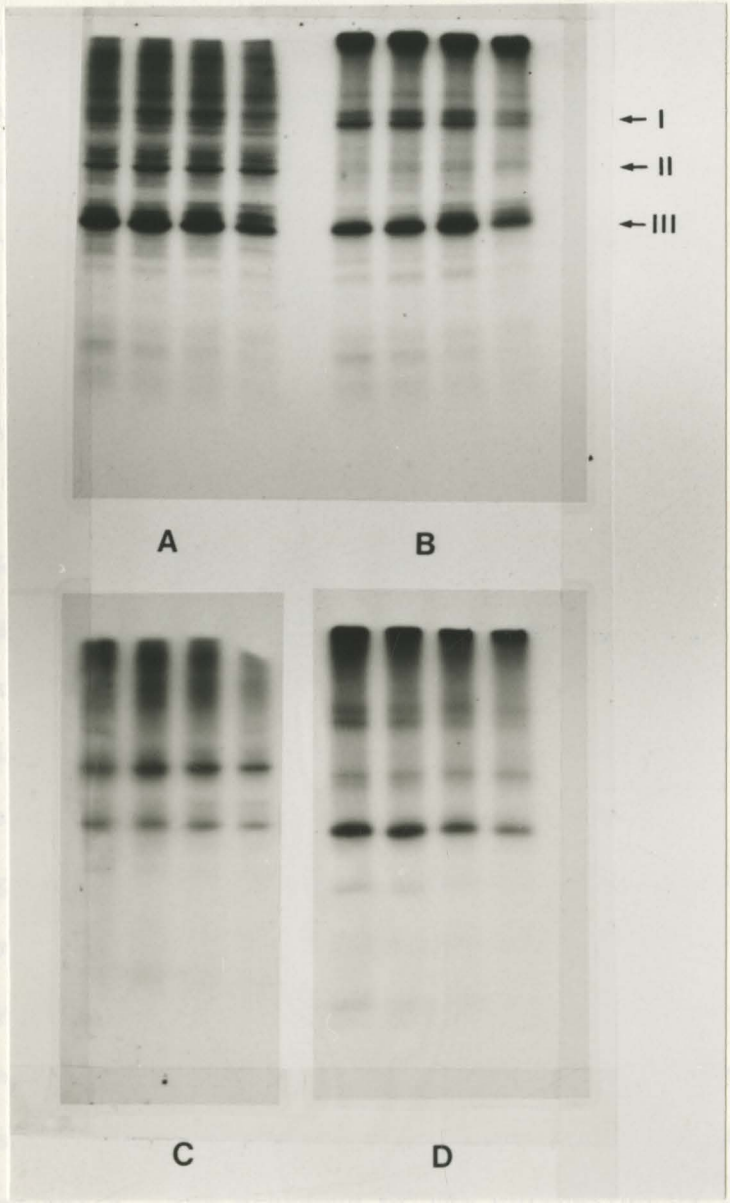
A number of reports have shown that the class II invariant chain is glycosylated during its transport to the Golgi apparatus

Figure 15

Fig. 15: "Pulse -chase" analysis of VCD-1 antigen synthesis and processing.

Raji cells (panels A and B) or EBV-transformed lymphocytes (panels C and D) were labelled for 10 mins. in methionine-free medium at a concentration of 20 x 10^6/ml with ^{35}S-methionine at 200 μCi/ml. The cells were then diluted with 20 volumes of regular medium, aliquots of cell suspension were withdrawn after chase times of 0, 30, 60, 120 minutes, the cells were lysed with detergent and immunoprecipitated with SA coated with VCD-1 antibody. The immunoprecipitates were analyzed by electrophoresis in 11% SDS gels under reducing (panel A and C) and non-reducing (panel B and D) conditions and fluorography (panel D is from a different gel).

Figure 15



(23,24,25,26,27,28). I thus performed the experiments to see whether this was true for the VCD-1 antigen.

Raji cells at 5 x 10^6/ml were metabolically labelled with ^{35}S-methionine in the presence or absence of 5μg/ml of the inhibitor of N-glycosylation, tunicamycin. At the end of the incubation the cells were harvested and lysed and immunoprecipitated with VCD-1 or control antibody. Electrophoretic and fluorographic analysis of the immunoprecipitates (Fig.16) showed that the VCD-1 antibody precipitated ~35 kDa and ~72 kDa molecules from lysates of control cells treated with the solvent DMSO alone (lane 3). The corresponding immunoprecipitate from the tunicamycin-treated cells showed the presence of ~27 kDa and ~64 kDa bands (lane 1)

<u>The VCD-1 epitope was located intracellularly, and was not represented on the cell surface or released into the medium</u>.

Despite the fact that the class II γ chain is known to be glycosylated and is found in the endoplasmic reticulum, most studies have failed to reveal its presence on the cell surface (4,22,24,25,33) or in the medium bathing cells (27) that express class II. To look for the cell surface expression of the VCD-1 determinant I labelled EBV-transformed lymphocytes by incubating them with ^{35}S-methionine. At the end of the incubation the cells were washed, treated with VCD-1 antibody, washed again, and lysed in the presence of a five-fold excess of unlabelled EBV-transformed cells. The presence of the unlabelled cells at the time of lysis ensured that free antibody sites would be occupied with non-radioactive antigen and hence unavailable for any radioactive

Figure 16

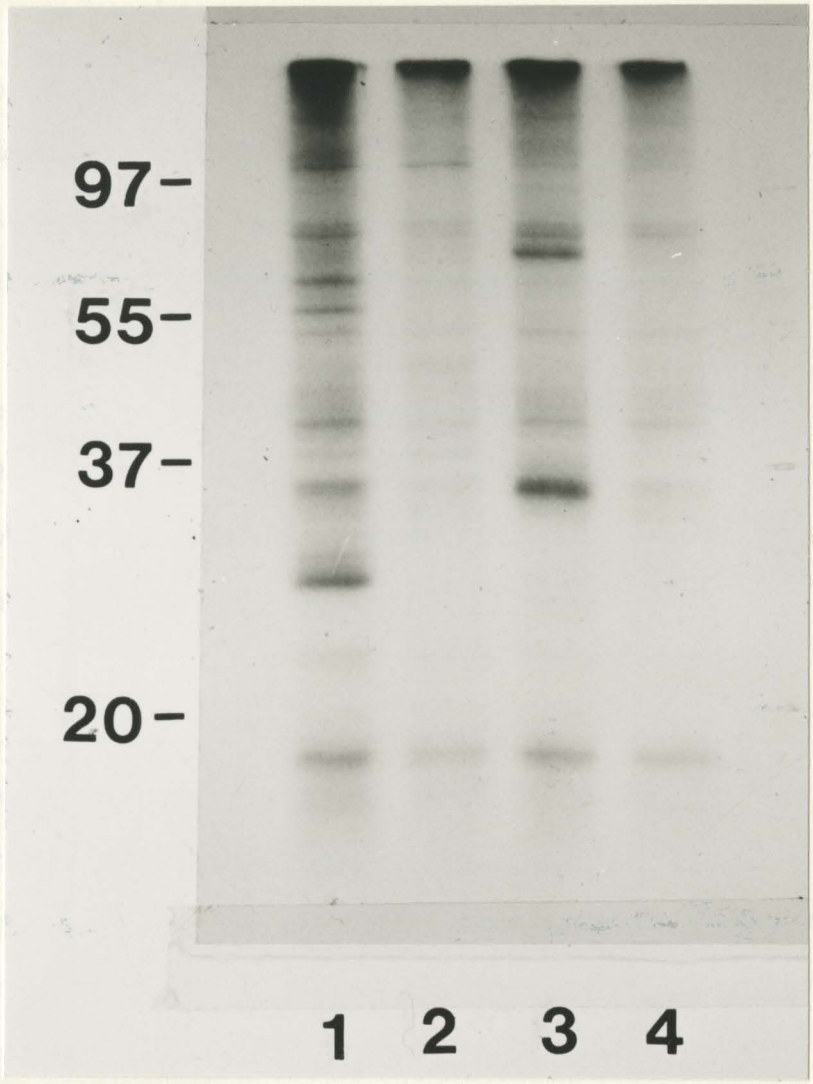


Fig. 16: Immunoprecipitation of tunicamycin-treated Raji cells. Tunicamycin dissolved in DMSO at 0.5 mg/ml (lanes 1 and 2) or DMSO alone (lanes 3 and 4) was added to Raji cells suspended in methionine-free medium at 5 x 10^6/ml to give a final concentration of 5 μg/ml tunicamycin. After 1 hour at 37°C ^{35}S-methionine was added at 50 μCi/ml and after a further incubation for 2 hours, the cells were lysed with detergent, immunoprecipitated with SA coated with VCD-1 antibody (lanes 1 and 3) or irrelevant antibody (lanes 2 and 4). Immunoprecipitates were electrophoresed in an 11% SDS gel under non-reducing conditions and fluorographed.

intracellular antigen that may be released (22,24). The VCD-1 antigen complexes were precipitated with solid phase immunoabsorbent and analysed by SDS-PAGE and fluorography. The results (Fig. 17A) showed quite clearly that the VCD-1 epitope was not present on the cell surface (lane 1); a simultaneous control experiment showed the abundant intracellular presence of antigen (lane 3).

Immunoprecipitation of the medium harvested from metabolically labelled Raji cells failed to reveal the presence of radioactively-labelled VCD-1 antigen (Fig. 17B, lane 1). Here again, intracellular antigen was readily demonstrated (lane 3)

VCD-1 had no effect on the Mixed Lymphocyte Reaction

To explore, by indirect means, the possibility that the VCD-1 epitope was present on the cell surface in functional association with class II antigens, I studied the effect of VCD-1 antibody on the mixed lymphocyte reaction (MLC). This experiment was based upon the assumption that an antibody to class II components might be expected to inhibit the MLC. Effects upon Pokeweed mitogen (PWM) and Phytohaemagglutinin (PHA) stimulation were studied as controls for the specificity of the effect. The results (Table 4) showed no effect with any of these stimuli.

Figure 17

Fig.17A: Immunoprecipitation of cell surface antigens of EBV-transformed lymphocytes.

EBV-transformed lymphocytes at 5 x 10^6/ml were labelled for 2 hours with 50 μCi/ml of ^{35}S-methionine in methionine-free medium. The cells were washed and incubated with VCD-1 (lane 1) or control antibody (lane 2)and washed again. A five-fold excess of unlabelled cells were added and the mixture lysed with detergent at 5 x 10^7/ml. Antigen/antibody complexes were precipitated with RaM sepharose and analysed by SDS-PAGE under reducing conditions and fluorography. Lysate from metabolically labelled control cells, not incubated with antibody and without unlabelled cells added, was immunoprecipitated with RaM Sepharose pre-incubated with VCD-1 (lane 3) or irrelevant antibody (lane 4).

Fig.17B: Immunoprecipitation of conditioned medium from metabolically labelled Raji cells.

5 x 10^6 Raji cells were incubated in 1 ml of methionine-free medium in the presence of 50 μCi ^{35}S-methionine for 2 hours. The labelling medium was reserved and immunoprecipitated with RaM Sepharose pre-incubated with VCD-1 (lane 1) or irrelevant antibody (lane 2). The immunoprecipitate was analysed by SDS-PAGE in an 11% gel under reducing conditions. The lysed labelled cells were similarly immunoprecipitated with VCD-1 (lane 3) and irrelevant antibody (lane 4) as a control for sufficient labelling of proteins.

Figure 17

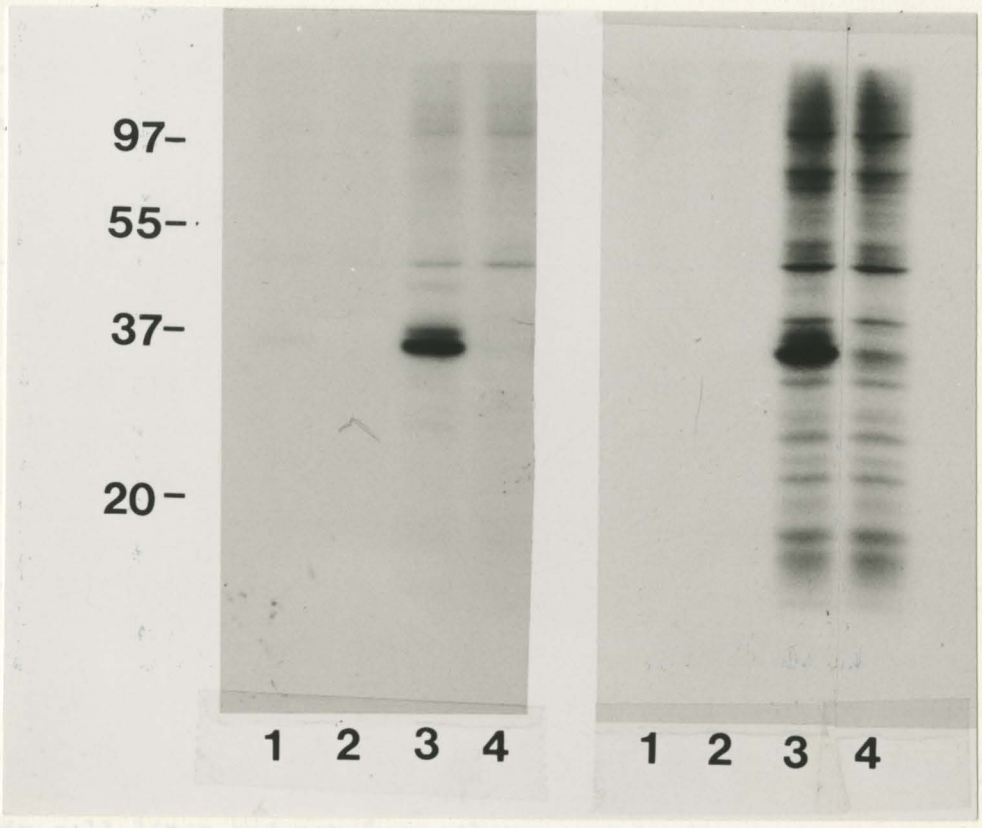


Table 4: ^{3}H-thymidine incorporation of stimulated PBMC in the presence of VCD-1

(Median cpm/$5x10^{4}$ cells)

Stimulus	VCD-1 (0.025mg/ml)	Normal IgG (0.025mg/ml)
MLC	9531	10425
PHA	16256	16760
PWM	7501	8549

VCD-1 antibody precipitated ^{35}S-sulphate-labelled macromolecules from certain cell lines and not from others

A number of authors (38,39,41) have produced evidence to indicate that the γ chain serves as the core protein for a cellular chondroitin sulphate proteoglycan (CSPG). To examine this observation I incubated Raji cells, EBV-transformed lymphocytes, melanoma cells and CLL lymphocytes for 16 hrs with ^{35}S-labelled sodium sulphate. I then lysed the cells and immunoprecipitated the lysate with VCD-1 antibody. SDS-PAGE and fluorographic analysis of the lysate and immunoprecipitates gave the striking results shown in Fig.18.

Figure 18

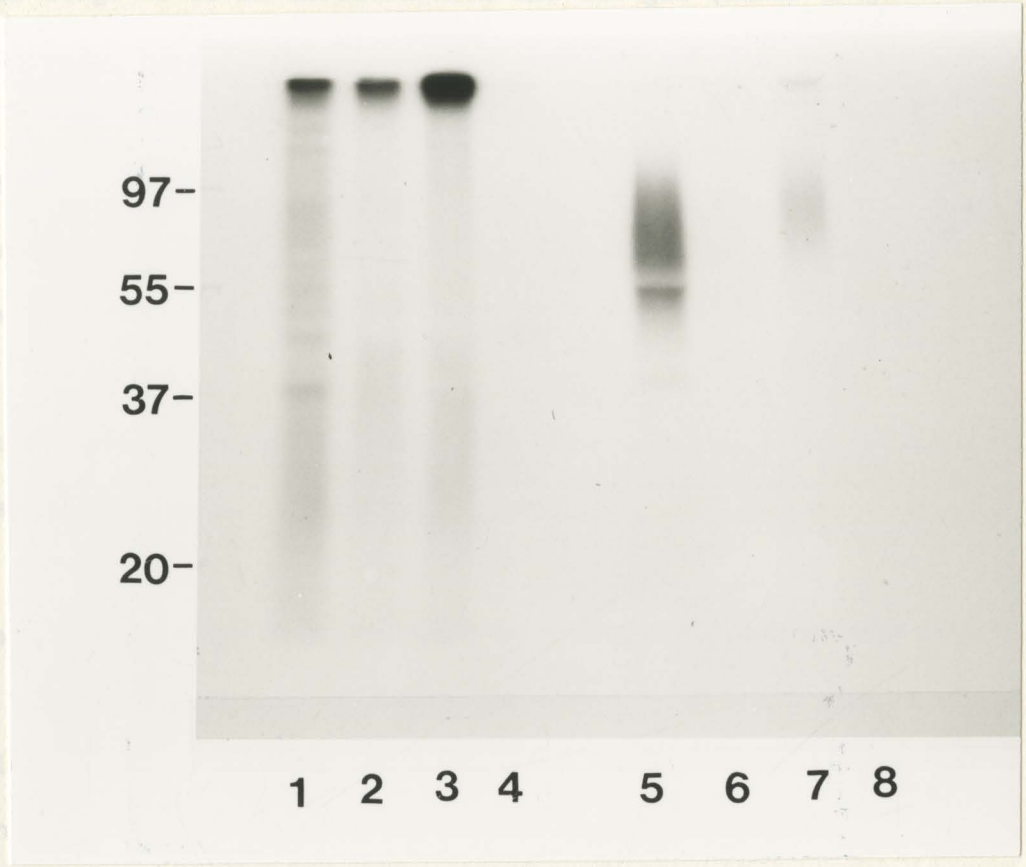


Figure 18: Immunoprecipitation of ^{35}S-sulphate-labelled cell lysates with VCD-1

Raji cells (lanes 1 and 5), EBV-transformed lymphocytes (lanes 2 and 6) UCT-Mel 7 melanoma cells (lanes 3 and 7) and CLL cells (lanes 4 and 8) were labelled overnight in sulphate-free medium containing 40μCi/ml of ^{35}S-sodium sulphate. The cells were lysed, immunoprecipitated with SA coated with VCD-1, electrophoresed in an 11% SDS gel under reducing conditions and fluorographed. The unprecipitated lysates were electrophoresed in lanes 1 to 4 and the VCD-1 immunoprecipitates in lanes 5 to 8.

All of the cell types, with the exception of the CLL lymphocytes, synthesized sulphate-labelled macromolecules, the majority of which were so large as to be excluded from the 11% gel (lanes 1-4). The immunoprecipitates of the Raji cells (lane 5) and the melanoma cells (lane 7) contained $^{35}SO_4$-labelled macromolecules that appeared as a polydisperse "smudge" of radioactive species with M_r's ranging from ~ 63 kDa to ~ 97 kDa. A faint ~ 38 kDa band and a more prominent 55 kDa band were evident in the Raji cell immunoprecipitate.

EBV-transformed lymphocytes and CLL lymphocytes contained no such sulphated molecules.

To examine the relationship between the sulphated species and the VCD-1 antigen I performed a further experiment in which separate identical cultures of Raji cells were incubated simultaneously and continuously for 16 hrs with $Na_2{}^{35}SO_4$ or with ^{35}S-methionine. Electrophoretic analysis of immunoprecipitates of lysates of these cells (Fig. 19) showed, as before, that VCD-1 precipitated a polydisperse set of sulphate-labelled molecules with M_r's ranging from ~63 kDa to ~97 kDa and a well-defined band at ~55 kDa (lane 2). The immunoprecipitate of the ^{35}S-methionine-labelled cell lysate contained 3 prominent, well-defined VCD-1 reacting species with M_r's of ~ 35 kDa, ~ 55 kDa and ~ 80 kDa (Fig. 19, lane 4).

The immunoprecipitated 55kDa M_r bands labelled with $^{35}SO_4$ or ^{35}S-methionine were monodisperse. The finely resolved, 80kDa molecule in the ^{35}S-methionine-labelled lysate did not appear to be sulphated

Figure 19

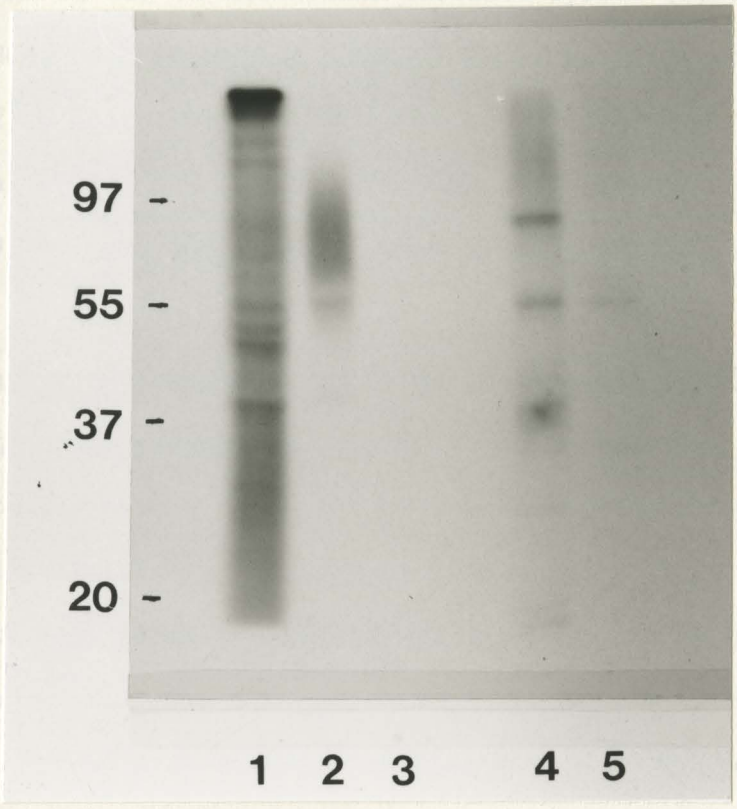


Figure 19: Immunoprecipitation of Raji cells labelled in parallel with ^{35}S-sulphate and ^{35}S-methionine.

Raji cells were labelled overnight, in separate cultures, with ^{35}S-sulphate and ^{35}S-methionine at 40μCi/ml. Detergent lysates of ^{35}S-sulphate labelled cells (lanes 2-3) and ^{35}S methionine-labelled cells (lanes 4-5) were immunoprecipitated with SA coated with VCD-1 (lanes 2 and 4) or irrelevant antibody (lanes 3 and 5), electrophoresed in an 11% SDS gel under reducing conditions and fluorographed. Lane 1 shows unprecipitated ^{35}S-sulphate labelled lysate.

since it was not represented as a distinct band in the heterogeneous $^{35}SO_4$-labelled species.

The 35 kDa band in the ^{35}S-methionine labelled lysate (lane 4) showed no corresponding band in lane 2. The 35 kDa γ chain is thus poorly sulphated, if at all; the fact that VCD-1 precipitated a polydisperse sulphate-labelled species is consistent with the observations of others (38,39,41) that the γ chain forms the core protein of a CSPG.

Failure to detect cell-surface VCD-1 antigen was not attributable to masking by attached glycosaminoglycans.

I was consistently unable to demonstrate VCD-1 antigen on the surface of living cells (Chapter 1, Fig. 1; Chapter 2, Fig. 17A). Since recent reports have produced unequivocal evidence to show that γ chain is present on the cell surface (32), it occurred to me that the VCD-1 epitope, while readily detectable intracellularly, might be masked on the surface by attachment of glycosaminoglycans. This seemed possible in view of the studies showing that the γ chain forms the core protein for a cell surface CSPG (39).

To investigate this possibility I treated Raji cells and EBVL with chondroitinase ABC by the method of Quaranta et al. (39). Immunofluorescence studies of the viable cells both before and after removal of the surface glycosaminoglycan failed to reveal staining by VCD-1 (results not shown).

DISCUSSION

In most respects the results that I have obtained with my VCD-1 antibody are consistent with those obtained by others who have used antibodies to study the class II invariant chain.

VCD-1 identified, by Western blotting or immunoprecipitation, several electrophoretic bands that were consistently observed.

The first group of these comprised molecules that expressed the VCD-1 epitope:

- a 35kDa, intense band that represented the free γ chain in the intracellular pool
- a 37kDa, weaker band which represented the processed γ chain after addition of complex sugars in the Golgi compartment. The M_r of this band was the same as that of class II α chains in one-dimensional SDS-PAGE
- a 44 kDa band as shown in the Western blot in Fig. 10 and the pulse chase experiment in Fig. 15 (arrowhead II) and which cannot be identified with certainty. The presence of this band has been reported in immunoprecipitates of murine (35,47,49) and human (32,34,50) cell lysates with anti-class II antibodies but its characterization has not as yet been completed. As a prominent and fairly consistent co-precipitant with γ chain it merits further study.

Yamamoto et al. (49), working with murine cells, identified a 41 kDa protein that was serologically related to the γ chain and that represented the product of the same gene that encoded the 31 kDa protein.

Strubin et al. (34) found that human lymphocytes contained two forms of the invariant chain that resulted from alternative initiations at two in-phase AUGs. They, too, mention a 41 kDa serologically related band whose origin was not fully understood and that they elected not to discuss further.

- a 72-78 kDa doublet which I have taken to be the dimeric form of the 35-37 kDa species that has been reported by others (47,48,85).

The second group consisted of molecules that co-precipitated with Ii chain:

- class II α-chain was seen as a 37 kDa band with the same M_r as processed γ chain. Its identification as α chain was confirmed by probing transblots with specific anti-α chain antibody after electrophoresis of VCD-1 immunoprecipitates (Fig. 11).

The intracellular association as a non-covalent dimeric complex of α chain and γ chain has been consistently reported by many authors (22,85) and, historically, formed the basis for associating Ii chain with class II antigen (21).

- A faint band, with an M_r of 25 kDa, was occasionally seen in ^{35}S-methionine-labelled immunoprecipitates and disappeared after

60 min of chase in the experiment depicted in Fig. 15. It was not seen in any of the Western blots that I examined.

The nature of this band is somewhat controversial. Thomas et al. (36) have attributed it to the 25 kDa C-terminus fragment of the Ii chain that is released by limited proteolysis in the endosomal compartment. They were unable to precipitate it with a monoclonal antibody directed to the N-terminal, intracellular domain of the molecule. VCD-1, which reacts with an intracellular epitope, would, therefore, not be expected to react with the 25kDa band in Western blots; my finding is in accord with this.

On the other hand, my antibody and the similar VIC-Y1 antibody of Quaranta (see Fig. 1 of ref. 32) do precipitate the p25 band despite the fact that they bind exclusively to intracellular epitopes. These findings would, therefore, suggest that the p25 band co-precipitates with the Ii chain and is not necessarily derived from it. This view is shared by Sachs (51) who feels that it may represent co-precipitated tumour necrosis factor. Finally, it is of interest to note that the non-immune rabbit serum used by Sorli et al. (Fig. 1C of ref. 85) as a control non-specifically precipitated a prominent band in the 25kDa region. I have on occasion observed similar faint bands in non-specific precipitates.

- an 80kDa protein similar to that described by Sorli et al. (85) that was only seen after prolonged labelling (Fig. 19). This

non-sulphated molecule was finally resolved by ^{35}S-methionine labelling but was not evident in the heterogenous 63-97 kDa species that were labelled with ^{35}S-sulphate. The elegant experiments performed by Sorli et al. using two dimensional electrophoresis under reducing and non-reducing conditions with or without prior linking with the heterobifunctional reagent sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (SASD), showed that the 80kDa protein was unaffected by incubation with chondroitinase ABC and was non-covalently associated with Ii chain in detergent lysates of Raji cell microsomal preparations. The function of this protein is uncertain.

- a 55 kDa sulphated molecule was also observed after prolonged metabolic labelling as a VCD-1 co-precipitant. The nature of this component is uncertain; it was not evident in the Western blots that I performed and I have found no specific reference to it in the relevant literature.
  A similar band can be discerned, however, in Fig. 4 of reference 38 after labelling murine spleen cells for 9-10 hrs. The authors make no mention of this band in their paper.

Analysis of immunoprecipitates of lysates of cells that had been treated with tunicamycin and metabolically labelled showed a reduction in M_r of the Ii chain from 35kDa to 27kDa. The glycosylation of the Ii chain that can be inferred from this experiment has been reported by others (23,24,25,26,27,28).

Lastly, VCD-1 immunoprecipitates of Raji cell lysates after labelling with ^{35}S-sulphate identified the characteristic polydisperse "smudge" of sulphated molecules of M_r ~63 to ~97 kDa. Quaranta et al.(39) with their VIC-Y1 antibody found a similar heterogeneous proteoglycan population, the only difference being that in their case the CSPGs migrated with electrophoretic mobilities corresponding to a mass range of 40-70 kDa. The M_r differences between their findings and mine may be due to the fact that they labelled for only 5 hrs whereas I labelled for 16 hrs. They used a DR homozygous cell line, Swei, whereas I used Raji cells.

In some respects my experimental results differed from those of others. I have, for example, been consistently unable to co-precipitate class II β chain with the VCD-1 antibody. The nature of the association between class II α, β and γ chains is not well understood and experimental conditions such as freezing and thawing of cell lysates and ionic strength of solvents, which were shown to be the cause of variable results in other laboratories (52), may explain my findings in this respect.

Other workers have not described the differences that I observed in the processing of γ chain in Raji cells and EBV-transformed lymphocytes. In my pulse chase experiments the 35kDa band in the Raji cells showed an increase in M_r with time whereas the same band in EBV-transformed lymphocytes was chased out of the pool. I also found that a 37 kDa processed form of the γ chain was more prominent in CLL lymphocytes than in Raji cells, EBVL or melanoma cells.

Finally, in the experiments shown in Fig. 18, VCD-1 immunoprecipitates of ^{35}S- sulphate-labelled molecules showed striking differences between the four class II positive cells tested. No CSPG molecules with the VCD-1 epitope could be precipitated from EBVL and CLL lymphocytes whereas Raji cells contained large quantities and melanoma cells a small amount. This too, has not been described by others.

The function of the Ii chain is as yet more a question of speculation than of fact. Its close intracellular association with class II α and β chains, its localization in intracellular membrane-bound vesicles and its presence as the core protein for a cell surface proteoglycan (indicating that it has passed through the Golgi apparatus) have been interpreted as evidence for Ii chain involvement in the intracellular traffic of class II α and β chains into the lysosomal compartment of antigen-presenting cells where they become associated with processed exogenous antigens before appearing on the cell surface.

The function of the Ii chain in this context has been variously suggested as being:

(a) to associate with the α/β chain and so to protect the peptide-binding groove of class II polypeptides from self peptides until peptides from the endocytic processing pathway are available (48,53,54).

(b) to keep class II molecules in a conformation with a low affinity for peptides until, in the endosomes, the γ chain is removed and the α/β complex acquires a high affinity conformation (55).

(c) to route class II molecules into the endosomes where exogenous antigen is met (56).

Plausible as these suggestions for γ chain function in antigen-presentation may be, they have not consistently fulfilled the prediction that interference with γ chain synthesis or expression should compromise class II-mediated interactions with responding T cells. On the one hand it was found that the absence of Ii does not affect the ability of class II transfected fibroblasts to present intact antigen but increases the ability to present antigenic peptides (57).

A human fibroblast line transfected with DRα and β chain but not γ chain genes and infected with measles virus was lysed effectively by class II-restricted measles virus-specific T-cells, showing that γ chain is not required for antigen presentation (87).

Contradicting this are the findings of Stockinger et al (58) that only fibroblasts supertransfected with the invariant chain gene were able to present native antigen whereas those transfected only with class II genes could only present cyanogen bromide-fragmented antigen. It has also been shown that selective interference with Ii conversion to its

CSPG form renders class II positive cells less effective in presenting antigen to T cells (59).

It is possible, in the light of the experimental results that I report in Chapter 3, that the association of Ii chain with α/β chain function has been over-emphasized and that the γ-chain may have a more general intracellular role to play - a role that is only partially related to class II MHC function and whose elucidation will require further experimental data.

CHAPTER 3

EXPRESSION OF THE CLASS II INVARIANT CHAIN IN NORMAL AND PATHOLOGICAL CELL TYPES

Once a new biological molecule has been discovered, and some account has been given of the circumstances that govern its synthesis and expression, clinical interest naturally leads us to look for abnormalities that we may associate with disease. There are good reasons for subjecting the expression and processing of class II invariant chain to such scrutiny.

In the first place, the molecule is known to be closely associated with the α and β chains of the class II antigens that form the antigen presenting structures on cell surfaces that are critical for the induction of responses in $CD4^+$ MHC-restricted T cells. This association strongly suggests a regulatory role for the Ii molecule that might be deranged in various immunopathological states.

Secondly, the synthesis and processing of Ii chain are subject to modulation. For the most part, changes in class II α and β gene expression correlate with that of the Ii chain and isolated reports of dissociation between these components of the class II complex have appeared (60,61,62,63). There is reason to believe, therefore, that the Ii chain might be abnormally regulated or processed in disease.

Thirdly, although there have been reports of Ii chain expression being inversely related to proliferation in human and murine B lymphocytes

and myeloma cells (42,64), and murine pre-B cells (64), these observations have not been extended to other cells that are known to express class II antigens. Furthermore, to the best of my knowledge, there have been only three reports of Ii chain expression in B cell neoplasia (60,65,66).

Finally, the Ii chain is interesting for the fact that it is subject to glycosylation and further processing to form a cell surface chondroitin sulphate proteoglycan. Since the addition of glycosaminoglycans to core proteins is a terminal event in the synthesis of a number of biologically important molecules, the Ii chain provides a useful model for the study of intracellular post-translational modification and processing. It is distinctly possible that disturbed intracellular protein traffic will prove to be a feature of disease.

For the most part studies of Ii chain expression in disease have been descriptive and limited in their extent. Table 5 summarizes the most important observations that have been made.

Table 5: Ii chain expression in disease.

Cell Type	Observation	Reference
B cell lymphoma of high and low grade malignancy	DR not expressed in the absence of Ii; 2 of 30 highly malignant tumours expressed Ii only; lack of DR correlated with high-grade malignancy	60
Keratinocytes in diseased skin	Ii negative but DR positive	30
Colorectal adenoma	in 15 of 20 cases Ii positive cells outnumber DR positive cells	61
Colorectal carcinoma	in 51 of 70 cases Ii positive cells outnumber DR positive cells	61
Hairy cell leukemia spleen cells	Accumulation of Ii but absence of p25 proteolytic cleavage fragment	50,67
CLL lymphocytes (blood and bone marrow)	Increased expression of Ii mRNA compared to normal and other leukemic lymphocytes	65
B cell haematological malignancies	Ii mRNA expression varied with B lymphocyte differentiation - highest at intermediate maturation	66
Lymphoblastoid B-cell lines from patients with severe combined immunodeficiency	No expression of α, β or γ chain protein but mRNA for γ chain only	62
Acute lymphocytic leukemia	in 4 cases cells were class II positive but Ii negative	4

In this chapter I present the results of studies that I have done to document Ii chain expression in various normal and abnormal cell types. I have, in particular, addressed the question of relative expression of Ii chain in normal and leukemic peripheral blood mononuclear cells and Ii chain expression in melanoma cells.

METHODS

Iodination of VCD-1 antibody

Antibody was purified from ascites by ammonium sulphate and caprylic acid precipitation as described in the Appendix. The purity of the antibody was established by SDS-PAGE and the concentration determined spectrophotometrically using the extinction coefficient.

$A_{280nm}(1\%) = 13.8$ (68)

I labelled the antibody with ^{125}I with Iodogen (1,3,4,6 - tetrachloro-3α, 6α diphenyl glycoluril, Pierce No. 289600) using equimolar amounts of IgG and iodide(69).

Briefly, a 1.5 ml plastic centrifuge tube was coated with 2μg Iodogen by evaporating 50μl of a solution of 1 mg Iodogen in 25 ml dichloromethane while rotating the tube. The following were then added to the tube in the sequential order given:

6 μl PBS

8 μl of 50 μM NaI

2 μl (200 μCi) of ^{125}I (as 50μM NaI, Amersham 1MS.30)

34 μl Ab (75 μg)

The mixture was incubated at room temperature for 10 mins with frequent mixing. Free iodide was then separated from radio-iodinated antibody by centrifuging the mixture through a 1 ml Sephadex G50 column that was packed in a tuberculin syringe (70). The labelled antibody was collected into 200μl of 1% BSA in PBS. This yielded 75μg of ^{125}I-labelled antibody in 250μl BSA solution; 99.3% of the radioactivity was TCA-precipitable.

The immunoreactivity of the radiolabelled protein was measured as the maximal amount of antibody that bound to permeabilized cells that expressed the antigen. This was done using a minor modification of the method described by Trucca and de Petris(71). Briefly, serial 1.5 fold dilutions of fixed and permeabilized EBVL (from 5x10^6/ml to 0.44x10^6/ml) were prepared in PBS. I then mixed 200μl of each suspension with 250μl of 0.2% BSA in PBS and 50μl of a 1/500 dilution of the radiolabelled antibody solution. The tubes were incubated at 4°C for 2 hrs and the cell suspensions underlayered with 100μl FCS and centrifuged. The supernatant was removed and the pellets counted. Maximum cellular uptake was determined graphically by plotting the reciprocal of the bound radioactivity as a function of the reciprocal of the cell concentration in the incubation mixture (Fig. 20). The

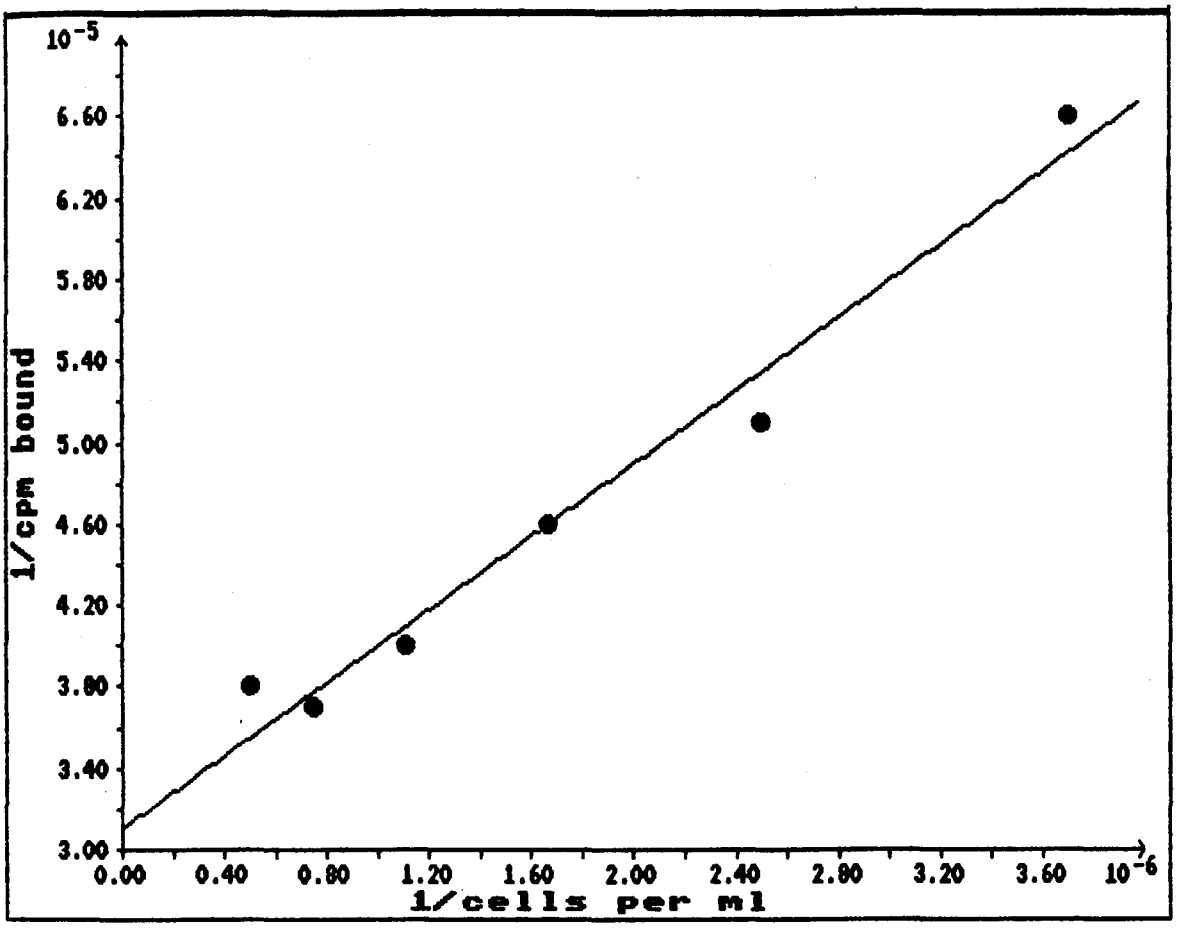


Fig. 20. Double recipocal plot of radiolabelled VCD-1 binding to fixed and permabilized EBVL.

Total radioactivity added was 39513 cpm. The results were plotted using a non-linear regression data analysis programme (84). The reciprocal of the Y axis intercept (32 200 cpm) was equivalent to 81% of radioactivity added; this was taken as the percentage of the labelled antibody that was immunoreactive.

extrapolated intercept on the ordinate axis was used to calculate antibody bound at infinite cell concentration.

Preparation of cells for quantitative antibody binding studies

PBMC were separated from heparinized blood as described in Chapter 1 and stored frozen in RPMI supplemented with 10% FCS and 10% DMSO. On the day of the study they were thawed rapidly at 37°C, diluted with approximately 10ml RPMI-10, pelleted and resuspended in 2ml of RPMI-10 in a centrifuge tube. To avoid lysis of the cells during subsequent fixing and permeabilization I incubated the suspension for 2 hr at 37°C with occasional mixing. Thawed normal PBMC were incubated for 2 hrs in glass Petri dishes to remove most of the monocytes. After incubation any clumps of cells were removed with a Pasteur pipette; the cells were then fixed with formaldehyde and saponin as described in Chapter 1 and suspended in PBS at $5x10^6$/ml.

Binding analysis

Antibody binding studies for subsequent Scatchard analysis were performed as follows:

A series of 1.5ml plastic centrifuge tubes was prepared, each of which contained:

50 μl of a serially diluted solution of unlabelled VCD-1 in 0.2% BSA. The starting and final dilution are given in the results section; typically these provided final MoAb concentrations ranging from approximately 0.05 to 1μg/ml.

200 μl 0.2% BSA

50 μl of a solution of ^{125}I-VCD-1 containing 0.03μg (39500 cpm) of labelled antibody.

200 μl cell suspension containing 10^6 cells

Tubes containing unlabelled antibody at a concentration 10-fold higher than the highest specific assay tube were included to correct for non-specific binding. Non-specific cpm were subtracted from specific counts to obtain "bound radioactivity".

PBS was used to prepare solutions and cell suspensions.

The tubes were incubated at 4°C for 2 hrs (or overnight with similar results).

The contents were then mixed, the cell suspensions were underlayered with 100 μl FCS and the tubes were spun at 600g for 5 mins. The supernatant including most of the FCS, were removed by careful suction and the pellets were counted in a γ-counter. Total radioactivity added was determined by counting duplicate 50 μl aliquots of radioactive antibody. Bound antibody in μg/ml was calculated from the formula:

$$\text{Bound Ab } (\mu g/ml) = \frac{\text{bound radioactivity(cpm)} \times \text{total Ab concentration } (\mu g/ml)}{\text{immunoreactive Ab added (cpm)}}$$

Binding of VCD-1 to CLL lymphocytes at saturating Ab concentration

Cells were prepared and antibody binding was measured as above. In this case, however, binding was determined in duplicate tubes that contained 0.44μg/ml unlabelled VCD-1 and 0.06μg/ml radiolabelled VCD-1. These concentrations exceeded those, deduced from the Scatchard analysis and confirmed experimentally, at which saturation of antibody-binding sites was observed. Duplicate tubes to control for non-specific binding contained a 20-fold excess of unlabelled VCD-1.

Flow cytometry of normal PBMC.

One million frozen normal PBMC, depleted of monocytes and fixed and permeabilized for the ^{125}I antibody binding as described, were incubated overnight at 4°C in 0.1% BSA in PBS with VCD-1, 9.3F10 anti-cell surface class II MoAb (ATCC Cat. No. HB180) or control Ab harvest fluid 1/20. The suspensions were diluted 10-fold with PBS, allowed to stand for 15 mins on ice and centrifuged. After decanting the supernatants the cells were incubated with FITG-GaM (see Chapter 1) diluted 1/400 in PBS containing 2% human AB serum for half an hour and again diluted 10-fold with PBS, allowed to stand for 15 mins and centrifuged.

The labelled cells were then analyzed in the Coulter cell sorter (Model TPS 1TC) with the light scatter settings so adjusted that the forward light scatter peak was clearly resolved from debris in the lower channels while retaining the cell population within the analytical range of the instrument. An electronic gate selected cells from the forward light scatter spectrum for fluorescence analysis.

The data were captured on a Hewlett-Packard desk-top computer (Model 85) and analyzed using a simple program that displayed the fluorescent cell spectrum as a distribution relative to the number of cells in the maximal channel. The gated fluorescent counts for control runs in which an irrelevant mouse Ab was used were subtracted from the test runs. Integrated counts were collected over channels that were selected visually.

The flow cytometric analysis of Ii distribution in normal cell populations showed a bimodal distribution. The way in which this was analyzed is presented in the Results section.

Mitomycin C treatment of melanoma cells.

UCT-Mel 3 cells were suspended at 10^6/ml in RPMI-10 and Mitomycin C (Sigma M-0503) was added to a final concentration of 100μg/ml. The cells were incubated at 37°C for 1 hr, washed three times with RPMI medium and resuspended in RPMI-10 at ±2 x 10^5/ml. The viability of these cells was 90% (trypan blue exclusion); they did not incorporate ^{3}H-thymidine. After overnight culture the cells were dried on multiwell slides and reacted with VCD-1 and 9.3F10 antibody for indirect IF as in Chapter 1.

Depletion of class II positive melanoma cells of the cell line UCT-Mel 3.

UCT-Mel 3 cells that expressed surface class II antigen were removed from a cell suspension by forming rosettes with antibody-coated erythrocytes and centrifuging these through a Ficoll-Hypaque layer (72). The melanoma cells, suspended in RPMI-10 at 2 x 10^6/ml were reacted with anti-class II MoAb, 9.3F10, by adding an equal volume of a 1/200 dilution of mouse ascites and incubating the suspension on ice for 30 mins. They were then washed twice with PBS and suspended, at 2 x 10^6/ml, in PBS containing 10% FCS.

Sheep red blood cells (SRBC) coated with goat anti-mouse IgG were prepared as follows: After 4 washes with 0.9% NaCl, 60μl packed SRBC were suspended in 1 ml of 0.9% NaCl and 18μl (0.4mg) goat anti-mouse IgG (IgG fraction,Cappel Cat. No. 0211-0231) in 0.9% NaCl was added, followed by 100μl of 0.1%(w/v) $CrCl_3$ in water. The mixture was incubated at room temperature for 5 min after which I added 2 ml PBS, washed the cells once with PBS and suspended them in 3ml PBS containing 10% FCS.

This suspension was mixed with an equal volume of the antibody-coated UCT-Mel 3 cells, centrifuged (200g; 5 min; room temperature) and the pellet gently resuspended. I confirmed the presence of rosettes by microscopy, layered the suspension on to an equal volume of Ficoll-Hypaque and centrifuged it (850g; 15 min.; room temperature). Unrosetted melanoma cells were collected from the interface, washed and cultured in RPMI-10.

Depletion of class II positive cells of normal PBMC for flow cytometry

Thawed normal PBMC were depleted of adherent cells as for the saturation binding assay, treated with 9.3F10 antibody, rosetted with GaM-coated SRBC and centrifuged on Ficoll-Hypaque as described above for the depletion of class II positive melanoma cells. Unrosetted cells were collected from the interface, fixed, permeabilized and labelled with 9.3F10 or VCD-1 MoAb and FITC-GaM as described.

RESULTS

Scatchard analysis

To establish the conditions for saturation of antigenic sites on CLL cells, binding of radiolabelled VCD-1 was determined at varying antibody concentrations as shown in Table 6.

TABLE 6: Scatchard analysis of radio-labelled VCD-1 binding to CLL cells

Total Ab mg/ml	Bound cpm[1]	Bound Ab µg/ml	Free Ab µg/ml
0.929	6816	0.197	0.732
0.489	12400	0.188	0.301
0.269	17438	0.146	0.123
0.159	20142	0.099	0.060
0.104	22651	0.073	0.031
0.077	23694	0.057	0.020
0.049	23519	0.036	0.013

[1]cpm bound after subtracting non-specific control cpm (=1678) bound in the presence of 8.8 µg/ml unlabelled VCD.

total cpm added per tube = 39513

total cpm corresponding to immunoreactive Ab = 32200 (Fig. 20)

Fixed and permeabilized PBMC from CLL patient ST were used for the analysis.

Figure 21

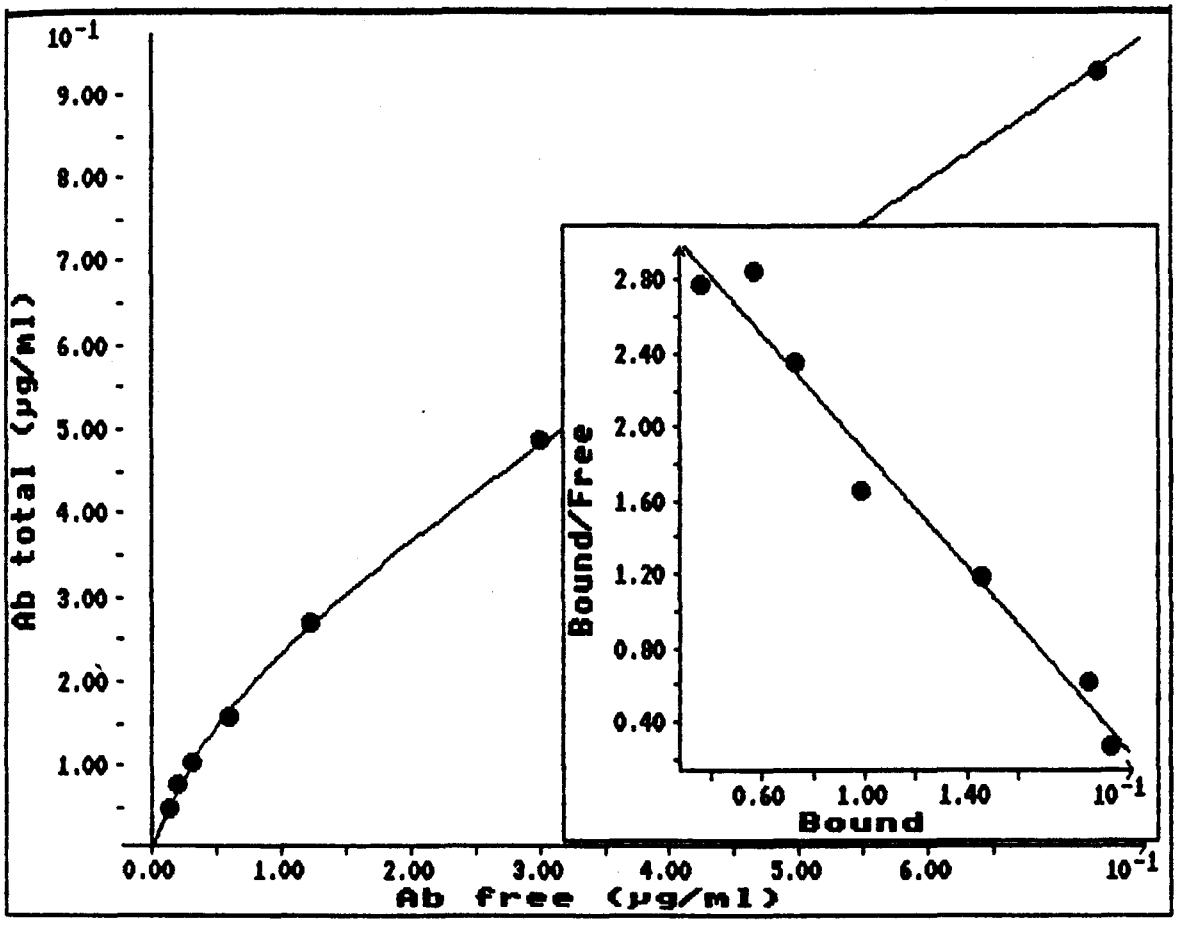


Fig. 21: Scatchard analysis of VCD-1 binding to CLL lymphocytes.

The results summarized in Table 6 were plotted by non-linear regression data analysis (84).

The X-axis intercept representing the number of sites = 0.219µg/ml which corresponds to 4.4 x 10^5 molecules per cell. The slope representing the affinity constant = 15.75ml/µg = 2.36x10^9l/mole.

The results, plotted by non-linear regression data analysis (84) are shown in Fig. 21.

Antibody bound at saturation was calculated to be 0.219 μg/ml which corresponds to 4.4×10^5 molecules per cell, assuming that at saturation only one binding site per antibody molecule is occupied.

The dissociation constant was calculated to be 0.0635μg/ml

$$\text{Therefore the affinity constant} = \frac{1}{0.0635} = 15.75 \text{ ml}/\mu\text{g}$$

$$= 2.36 \times 10^9 \text{ litres/mole}$$

Invariant chain expression, in normal, transformed and malignant PBMC.

Having shown that class II γ chain was readily demonstrable in the lymphocytes of patient ST, I examined other CLL cells for its presence. I obtained blood from patients with CLL, other leukemias or lymphomas and from these samples I separated the PBMC and stored them in liquid nitrogen.

Some of the cells from each patient were used to prepare NP-40 lysates (see Chapter 1 Methods) and these I examined for γ chain in a simple dot-blot test in which 2μl of lysate was spotted onto an Immobilon membrane and probed with VCD-1 as in Western blotting described in Chapter 1. The γ chain could be demonstrated by this technique in all of the 22 CLL lysates tested. This method did not detect γ chain in lysates of mixed PBMC isolated from normal peripheral blood.

A representative sample of the CLL lysates was then examined by electrophoresis and immunoblotting (Fig. 22). In this experiment, γ chain antigen was demonstrable in all of the CLL lysates tested. As previously seen with the lysate of cells from patient ST (Chapter 2, Fig. 10), the relative amounts of the upper and lower components of the ~ 36kDa doublet were similar in the CLL lysates, in contradistinction to the situation in EBV-transformed normal lymphocytes, Raji cells and melanoma cells, where the lower band predominated.

I measured cellular Ii-chain content of various cell types by assaying ^{125}I-labelled VCD-1 binding to a suspension of fixed, permeabilized cells at saturating antibody concentrations.

In comparing the VCD-1 expression on resident normal peripheral blood lymphocytes and lymphocytes obtained from leukemic subjects or from EBV-transformed cultures I was confronted with the need to correct for differences in the proportion of B cells present in the samples being analyzed. CLL samples, for example, could be expected to consist of up to 100% of mature B cells whereas adherent cell-depleted PBMC isolated by Ficoll-Hypaque density gradient centrifugation from normal venous blood would be expected to contain 5-15% B cells (73).

Figure 22

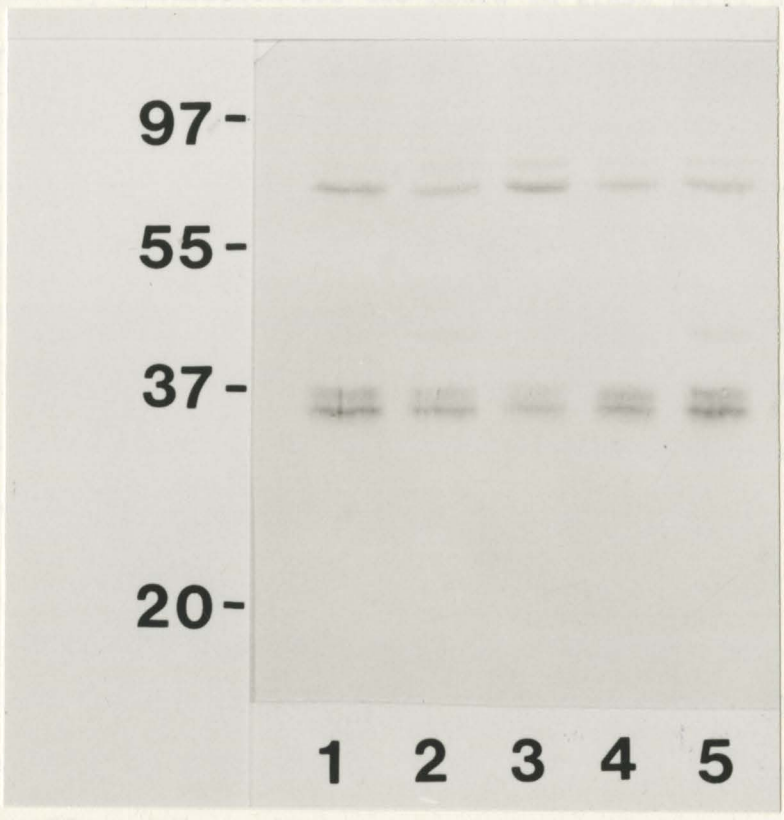


Fig. 22: Western blot of CLL cell lysates with VCD-1.

PBMC of CLL patients were lysed with detergent and electrophoresed in an 11% SDS gel under non-reducing conditions. The separated proteins were transblotted on to PVDF membrane and reacted with VCD-1 antibody as described in Chapter 1.

One way of addressing this difficulty would have been to isolate, for the analysis, pure populations of B cells from normal peripheral blood. To do this I developed a procedure based upon the removal of adherent cells by incubation for two hours in glass petri dishes followed by treatment with OKT-3 anti T-cell MoAb and complement and finally by removal of dead cells by passage through cotton wool columns (74). Unfortunately, however, this was a time-consuming and expensive procedure and recoveries were poor. I would thus have required large volumes (ca 100ml) of blood in order to obtain sufficient B cells for confident final estimates of VCD-1 antibody binding and I was reluctant to ask normal volunteers to donate samples of this size.

I therefore chose to express my results for the normal PBMC population in terms of VCD-1 antibody binding per class II positive cell. I felt justified in doing so since, in the first place, the great majority of surface class II positive cells in a normal, adherent cell-depleted PBMC population are B cells (73). Similarly, CLL cells and EBV- transformed cells are known to express class II α and β (75). This approach had the technical advantage that it enabled me to measure, in the same cell population and in the same experiment, the number of class II positive cells and the number of cells that expressed Ii chain.

Scatchard analysis of the results obtained with the CLL cells from patient ST provided an estimate of the antibody concentration that would be required for the saturation of all cellular binding sites.

Radio-labelled antibody at this concentration was then used to measure total cellular binding sites in 17 other CLL lymphocyte samples by saturation binding analysis. Cells from three patients with chronic myeloid leukemia (CML), one patient with acute lymphocytic leukemia (ALL), two patients with lymphomas (LYM) one patient with a myeloproliferative disorder (MYEL), one sample of EBV-transformed lymphocytes and adherent cell-depleted PBMC from 14 normal subjects were also studied. The results are presented in Tables 8, 9 and 10 in the Appendix and are shown graphically in Fig 23.

The results obtained with mixed populations of normal PBMC are presented in Fig. 23 as two sets of graphic data labelled Norm(1) and Norm(2). I have done this to accommodate a problem that I had not anticipated in my experimental design and that requires brief mention at this point:

Flow cytometry showed that VCD-1 positive cells in normal PBMC were bimodally distributed with a large, prominent peak of surface class II-negative cells that showed homogeneous weak fluorescence, and a much smaller, disperse population of more strongly fluorescent cells. The data showing this are given below. This meant that the results of the VCD-1 binding measurements for normal peripheral blood lymphocytes could be expressed on the basis of Ii chain binding sites per class II positive cell or per Ii positive cell. These results are given in the histograms labelled Norm(1) and Norm(2) respectively. A typical calculation to normalize binding sites in these two ways would have been as follows.

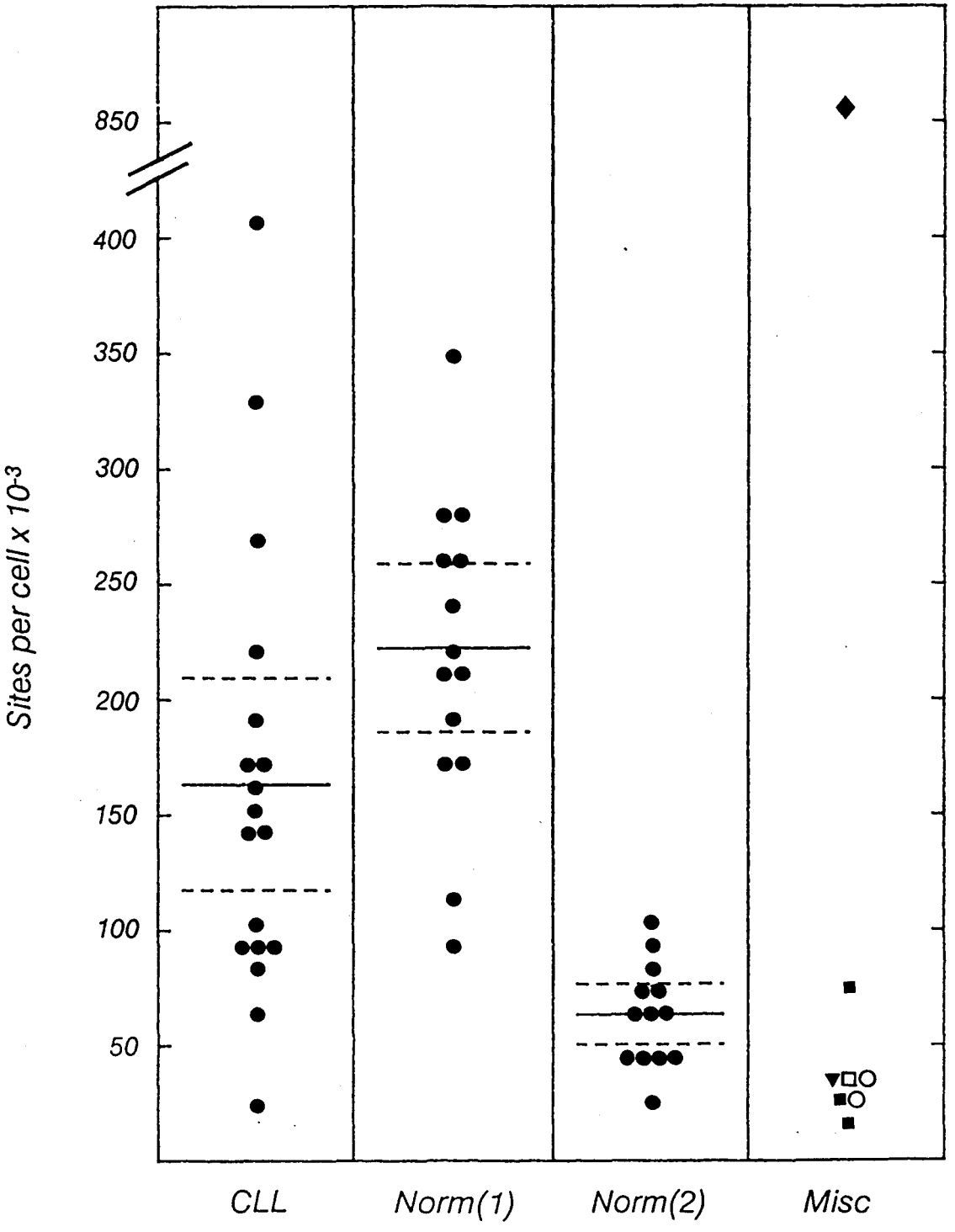
Figure 23

Fig. 23: Ii sites per cell in malignant, transformed and normal PBMC. Ii sites were determined by saturation binding of ^{125}I-labelled VCD-1 to fixed and permeabilized cells in suspension. The average value obtained for normal PBMC was corrected by dividing by the fraction of class II positive cells (NORM 1) or by the fraction of Ii positive cells (NORM 2). Means and standard errors are indicated by solid and dotted lines respectively. Sites per peripheral blood lymphocyte in miscellaneous lymphoid disorders (MISC) are shown by the symbols:

■ CML, □ ALL, ▼ myeloproliferative disorder, ○ lymphoma

◆ EBV-transformed normal lymphocytes

Figure 23


850
400
350
300
250
200
150
100
50
Sites per cell x 10-3
CLL
Norm(1)
Norm(2)
Misc

For donor FI:

average sites per cell = 29×10^3

% class II positive cells = 17%

sites per class II positive cell = $\frac{29 \times 10^3}{0,17}$

= 172×10^3

% VCD-1-positive cells = 43%

sites per VCD-1 positive cell = $\frac{29 \times 10^3}{0.43}$

= 68×10^3

CLL cells showed a wide variation in Ii chain content, ranging from 26 to 413×10^3 molecules/cell with a mean (±SEM) of $163(\pm 23) \times 10^3$. Surface class II positive normal PBMC contained a mean of $222(\pm 18) \times 10^3$ sites per cell with a scatter ranging from 99 to 355×10^3. Taking all of the Ii positive cells into account, the mean number of Ii molecules/cell was $64(\pm 7) \times 10^3$ varying from 23 to 106×10^3. The one sample of EBVL analysed contained 883×10^3 sites/cell. Peripheral blood mononuclear cells from patients with other forms of leukemia, myeloproliferative disorder or lymphoma contained numbers of Ii molecules per cell that varied from 14 to 74×10^3.

The results of the experiments that identified the two populations of Ii-chain positive cells are summarized in Figs. 24 to 26. In this experiment I depleted PBMC of surface class II-positive cells by the "rosetting" technique described in the Methods section, in which cells were coated with class II-specific 9.3F10 antibody, complexed with erythrocytes coated with goat anti-mouse IgG Ab, and centrifuged through a Ficoll-Hypaque cushion. The class II-depleted cell

population was collected from the interface and processed for flow cytometry.

Flow cytometric analysis of normal peripheral blood lymphocytes with 9.3F10 MoAb showed a prominent peak of non-specific fluorescence in channel 5 and a clearly visible population, that showed specific fluorescence, in the higher channels (Fig. 24A and B). This latter population was removed by rosetting with 9.3F10 antibody while the non-specific population was not affected by this procedure (compare Figs. 24B and C). I was thus able to remove, by rosetting, all surface class II positive cells.

Flow cytometry of normal peripheral blood lymphocytes stained with VCD-1 antibody clearly resolved, in the lower channels, a weakly fluorescent cell population that overlapped with the non-specific fluorescence spectrum given by cells treated with irrelevant murine antibody (Fig. 25A). This "non-specific" peak was subtracted from the test cell population to leave a specific cell population histogram that peaked in channel 10 (Fig. 25B). It is just possible to discern in Fig. 25A a second, smaller population of cells whose fluorescence intensities ranged from channel 20 through to the higher channels, with a mode in the vicinity of channel 40. The frequency distribution of this population is better shown as a continuous tracing in the expanded diagram in Fig. 26C. Analysis of normal peripheral blood lymphocytes after removal of cells that expressed surface class II β chain showed no appreciable effect on the weakly fluorescent population (Fig. 26A and B) whereas the cells constituting the

relatively scant strongly fluorescent population were removed (Fig. 26C).

Integrated counts of fluorescent cells that accumulated in the specific channels showed that the VCD-1 positive cells belonging to the weakly fluorescent population represented approximately 25-50% of the total light scatter gate count whereas those in the disperse, strongly fluorescent population represented approximately 10-20% (Table 11, Appendix). The unimodal class II positive population that was removed by rosetting also comprised approximately 10-20% of cells that passed the light scatter gate.

These results showed that Ii +ve lymphocytes in the peripheral blood of normal individuals could be divided into a major fraction that did not express surface class II and a smaller fraction that did.

The results for the CLL cells are expressed on the basis of binding sites per cell, on the assumption that they were all class II +ve (75). Since I have not analysed CLL cells by flow cytometry, I am unable to say whether they have a unimodal frequency distribution of γ chain content or whether, like normal cells, a weakly fluorescent population is also present. This clearly requires further studies for definitive conclusions to be drawn.

The EBV-transformed lymphocytes contained a surprisingly high number of 883 x 10^3 Ii molecules per cell. Cells from patients with AML, CML, lymphoma or myeloproliferative disorder contained low levels of

Figure 24

Fig. 24: Flow cytometry of normal and class II depleted normal lymphocytes. Adherent cell-depleted normal PBMC were coated with 9.3F10 anti-class II Ab and mixed with SRBC coated with GaM Ab. The rosette-forming cells were removed by centrifugation through Ficoll-Hypaque and the remaining cells were fixed and permeabilized and labelled with MoAb for flow cytometry.

Cells labelled with 9.3F10 Ab before removal of class II positive cells are shown as a continuous line in Fig. 24A and the control population labelled with irrelevant MoAb as a dotted line. In Fig. 24B these two cell populations are shown on an expanded scale. Fig. 24C shows the class II depleted cells labelled with 9.3F10 Ab as a continuous line and those labelled with irrelevant Ab as a dotted line.

Figure 24

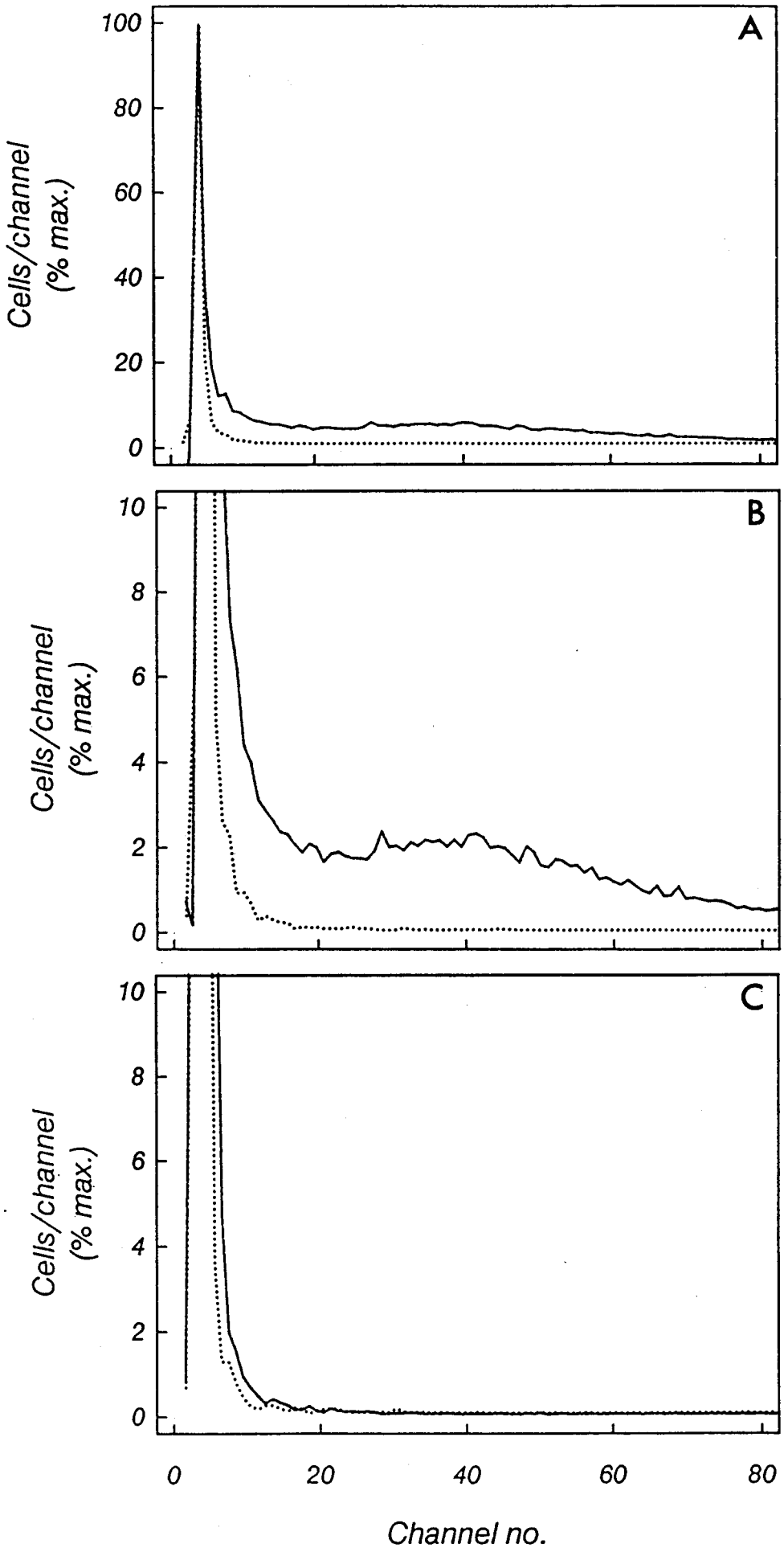

A
B
C
Cells/channel
(% max.)
100
80
60
40
20
0
10
8
6
4
2
0
0
20
40
60
80
Channel no.

Figure 25

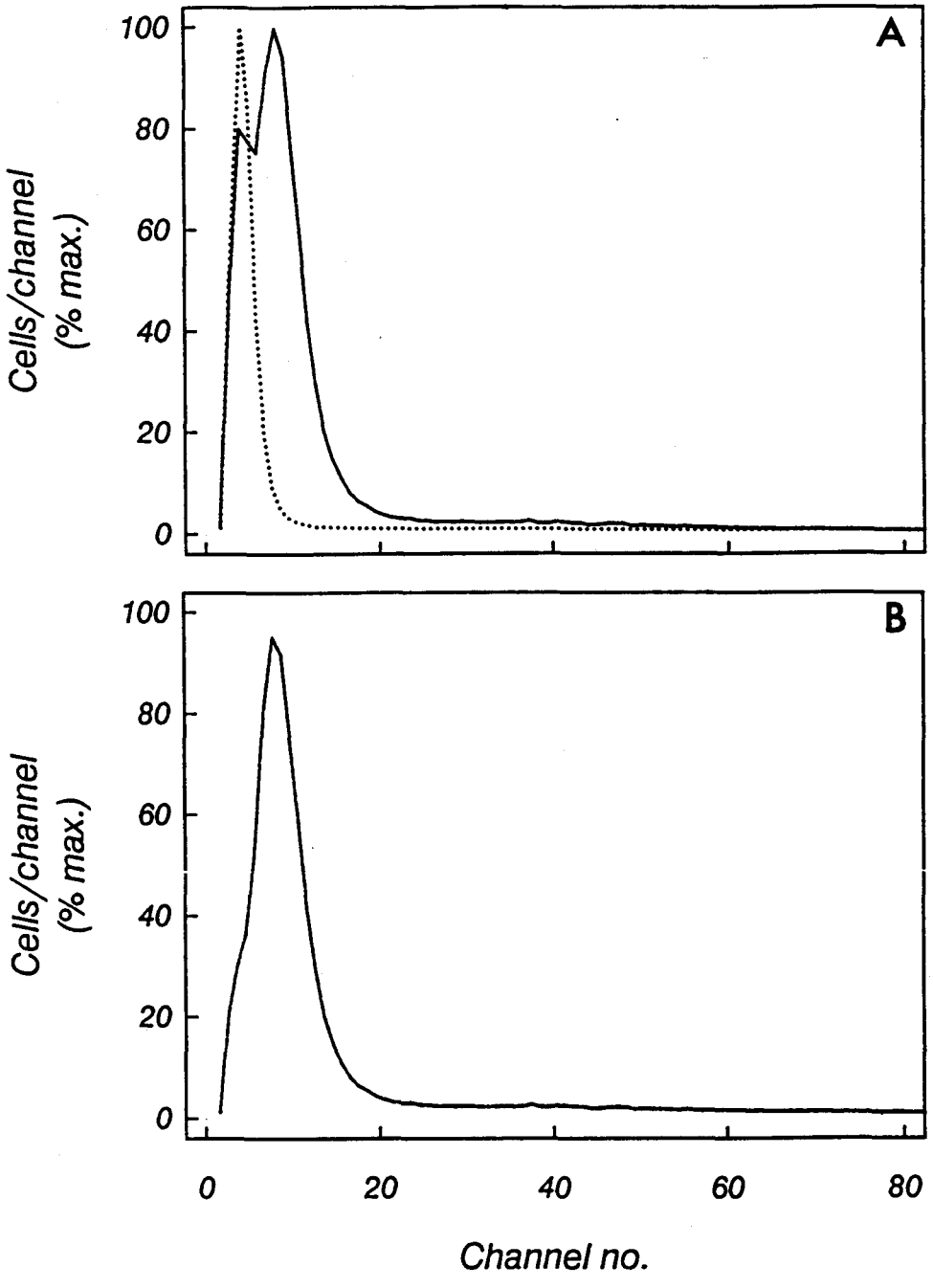


Fig. 25: Flow cytometry of normal lymphocytes.

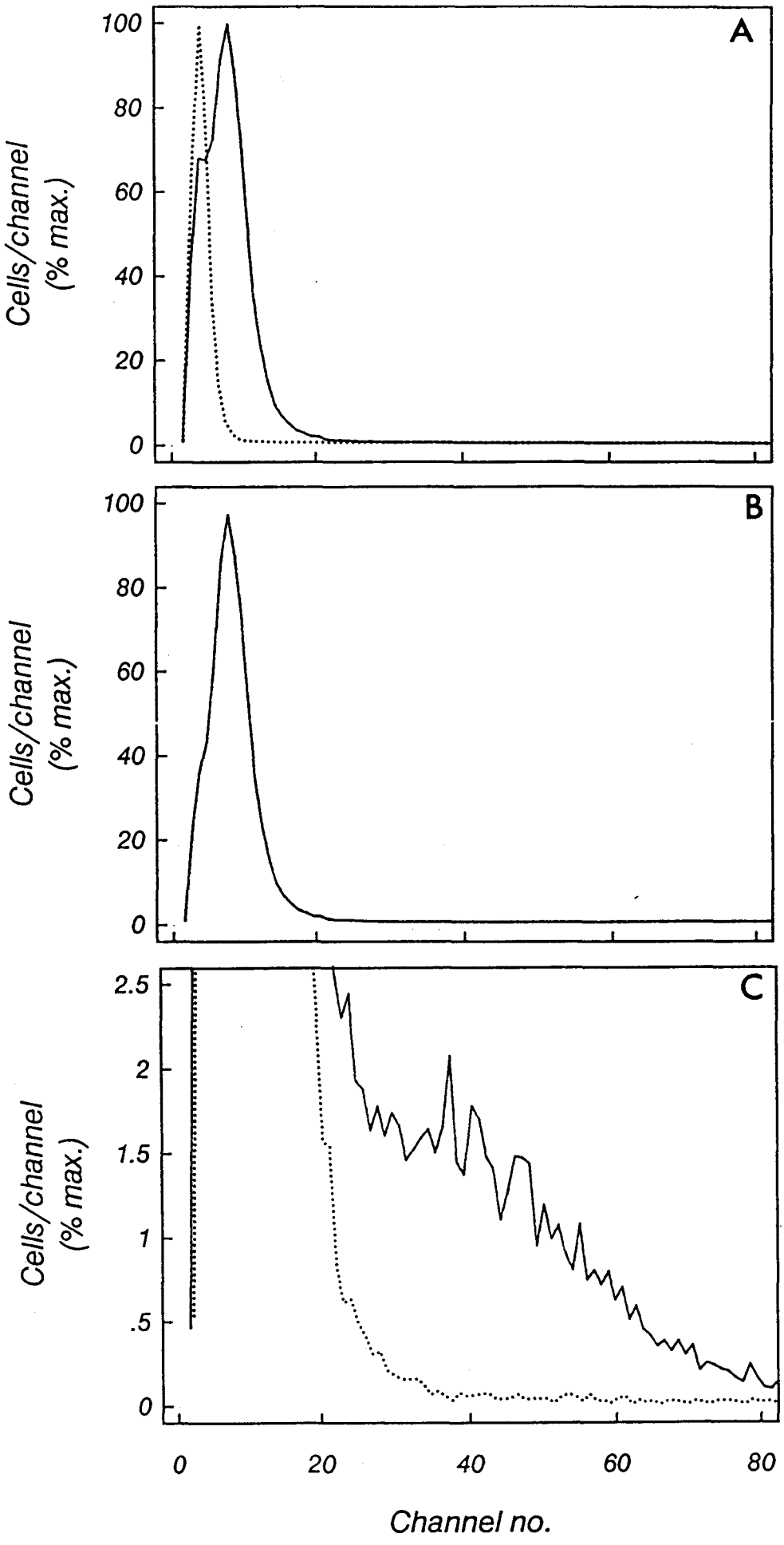
Fixed and permeabilized adherent cell depleted normal PBMC were labelled with irrelevant MoAb (A, dotted line) and VCD-1 (A, solid line). VCD-1 positive cells after subtracting the control cell population are shown in B.

Figure 26

Fig. 26

Flow cytometry of normal and class II depleted normal lymphocytes. Normal adherent cell depleted and class II depleted PBMC were prepared as for Fig. 24 and were labelled with irrelevant Ab (A, dotted line) and VCD-1 (A, solid line). VCD-1 positive cells after subtracting the control cell population are shown in Fig. 26B. This population is shown on an expanded scale as a dotted line in Fig. 26C. The VCD-1 positive population before removal of class II positive cells (as shown in Fig. 25B) is also shown, as a continuous line, on the expanded scale in Fig 26C.

Figure 26


A
B
C
100
80
60
40
20
0
2.5
2
1.5
1
.5
0
Cells/channel
(% max.)
0
20
40
60
80
Channel no.

Ii chain. The cell populations analyzed, however, were not pure and the number of specimens studied was too small to warrant any confident opinion regarding Ii chain expression in these cells types.

Invariant chain expression in human melanoma cells cultured *in vitro*. Although it is well known that class II antigens are present on melanoma cells in modulatable and significant amounts, most studies have been confined to the measurement of α and β chains (33,76,77,78) with few references (33) to the co-expression of invariant chain. Since expression of the Ii chain in murine and human lymphoid cell lines can be induced by culture conditions that inhibit proliferation (42,43,64) and since dissociation of class II γ and α/β expression has been described in other cell types (60,61), I felt it would be of interest to study Ii chain expression in the human melanoma cell lines I had at my disposal.

Melanoma cell lines differed widely in the extent to which they expressed Ii chain (Fig.27 and Table 7). At one extreme confluent or semi-confluent cultures of Bowes melanoma cells showed no evidence of γ chain while, at the other extreme, UCT-Mel 7 cells consistently expressed large amounts of the antigen. The other three cell lines showed intermediate levels with UCT-Mel 2 > UCT-Mel 3 > UCT-Mel 1.

These results are confirmed by saturation binding studies with ^{125}I-labelled VCD-1 (Table 7).

Figure 27

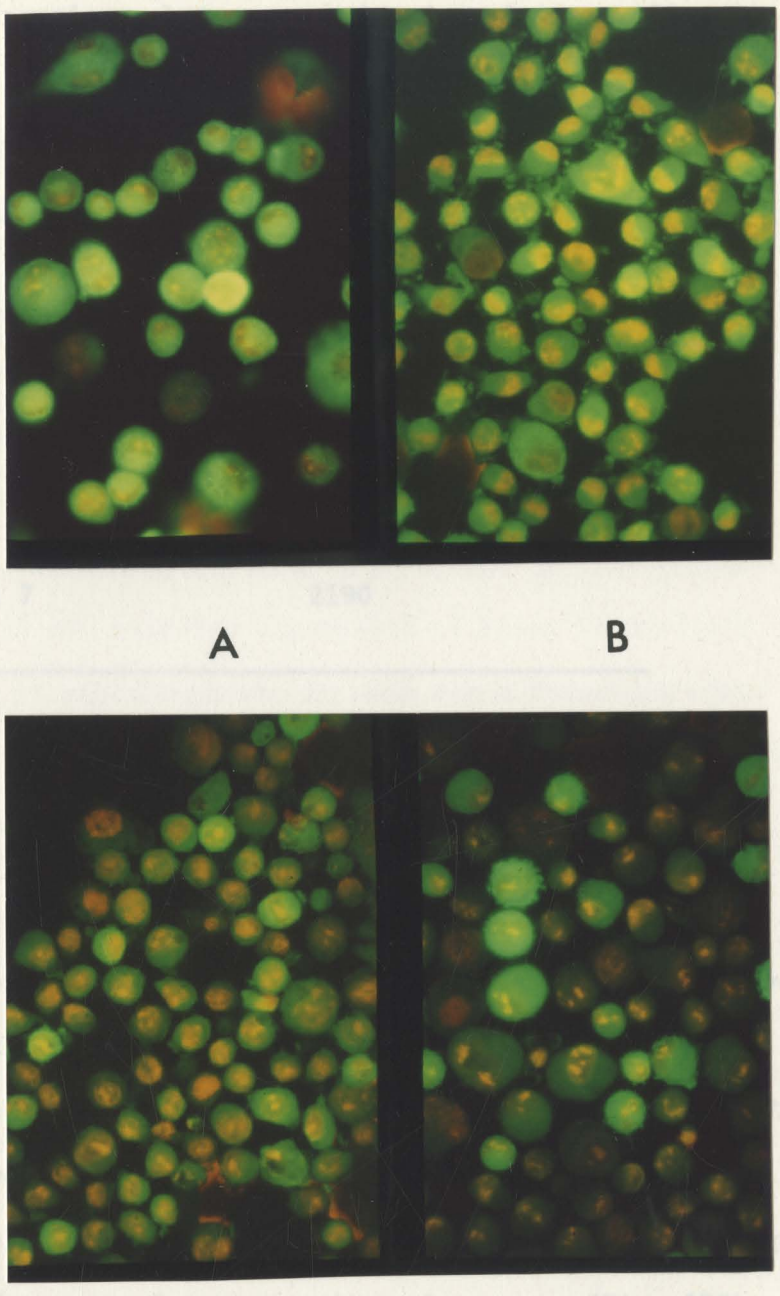


Fig. 27: Expression of γ chain in 4 different melanoma cell lines. UCT-Mel 7 (A); UCT-Mel 2 (B); UCT-Mel 1 (C) and UCT-Mel 3 (D). Cells from culture were dried on multi-well slides, fixed with acetone and reacted with VCD-1 and FITC-GaM. UCT-Mel 7 cells were photographed with a shorter exposure due to the extremely bright fluorescence.

Table 7: Invariant chain expression in melanoma cells.

Cell line	Ii molecules/cell ($x10^{-3}$)
Bowes	8
UCT-Mel 1	84
UCT-Mel 3	98
UCT-Mel 2	159
UCT-mel 7	2190

These between-cell line differences were attributable both to the varying proportions of cells that expressed γ chain and to the intensity of this expression in the cells that were positive. This is best illustrated in the comparisons shown in Fig. 27
UCT-Mel 7 cells were all positive and intensely so (Fig. 27A).
UCT-Mel 2 were all positive but weakly so (Fig. 27B).
Approximately 15% of UCT-Mel 1 cells were positive, varying in intensity from very faint to moderately strong (Fig. 27C).
Approximately 20% of UCT-Mel 3 cells were strongly positive, in sharp contrast to the 80% that were negative (Fig. 27D).

The Ii chain, in UCT-Mel 3 cells, was localized to a small, concentrated area against a weak, diffusely fluorescent background

(Fig. 27D). The other positive cell lines showed some evidence of discrete or "granular" accumulations of the γ chain but generally it was more diffusely distributed.

Parallel immunofluorescence studies with a MoAb against class II β chain in these melanoma cell lines showed similar reactivities (results not shown).

The fact that clonally derived cell populations, that were phenotypically stable for many generations in cell culture showed variation in the cellular expression of class II antigens suggested that class II expression was in some way a transient event that was related to a phase of the cell cycle or to some other state of differentiation of a sub-population of the culture.

I accordingly performed a number of experiments with melanoma cells to explore the notion that γ chain expression was inversely related to cellular proliferation and to document the rate of re-appearance of α/β positive cells after depletion of the population.

In the first experiment I exposed melanoma cells to concentrations of Mitomycin C that were sufficient to inhibit DNA synthesis. The cell line UCT-Mel 3 was chosen for this experiment for the fact that, by virtue of the proportion of positive cells and the intensity of cellular expression of Ii chain, it would have been relatively easy for me to detect the induction of expression. I would have expected a

greater proportion of cells to express Ii chain after Mitomycin C treatment.

The result of this experiment was negative in the sense that I was unable to demonstrate any effect of Mitomycin C on synthesis or accumulation of Ii chain (results not shown).

Since cells in culture are dependent upon growth factors in foetal calf serum for their continued growth, I felt it would be of interest to study the effect of serum deprivation on the expression of γ chain. Removal of serum from UCT-Mel 1 cells had no effect on expression of γ chain (results not shown) during the ensuing three days. In the case of UCT-Mel 2 cells, however, a consistent and pronounced increase in cellular γ chain expression was detectable after three days of serum deprivation. In UCT-Mel 3 cells, the proportion of Ii positive cells increased after serum deprivation. These effects are demonstrated in Fig. 28.

Indirect evidence for a relationship between proliferative potential *in vitro* and γ chain expression is evident from the significant inverse correlation seen between doubling time of the cells and Ii chain content (Fig 29). All of the indications were that Ii chain expression in melanoma cells was maximal during the resting phase of the cell cycle.

To establish the rate of reappearance of class II antigen positive cells in a depleted population of UCT-Mel 3 cells, I removed those

Figure 28

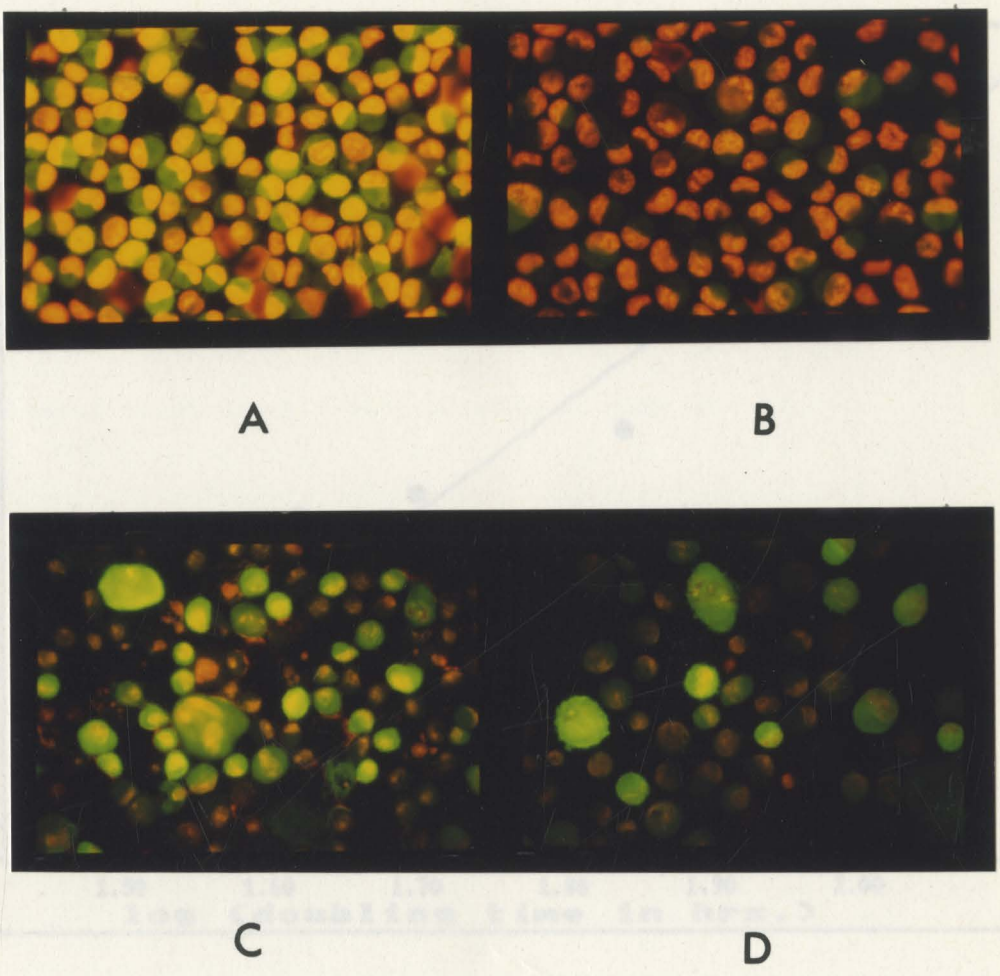


Fig. 28: The effect of serum deprivation on γ chain expression in two melanoma cell lines.

UCT-Mel 2 (A and B) and UCT-Mel 3 (C and D) were grown in medium without serum for 3 days (A and C) or in complete medium (B and D), dried on multiwell slides, fixed with acetone and labelled with VCD-1 and FITC-GaM. Cells in (A) and (B) were from a different experiment to those in (C) and (D).

Note that in the photograph strong green fluorescence seems to enhance the orange fluorescence of the ethidium bromide counter stain.

Figure 29

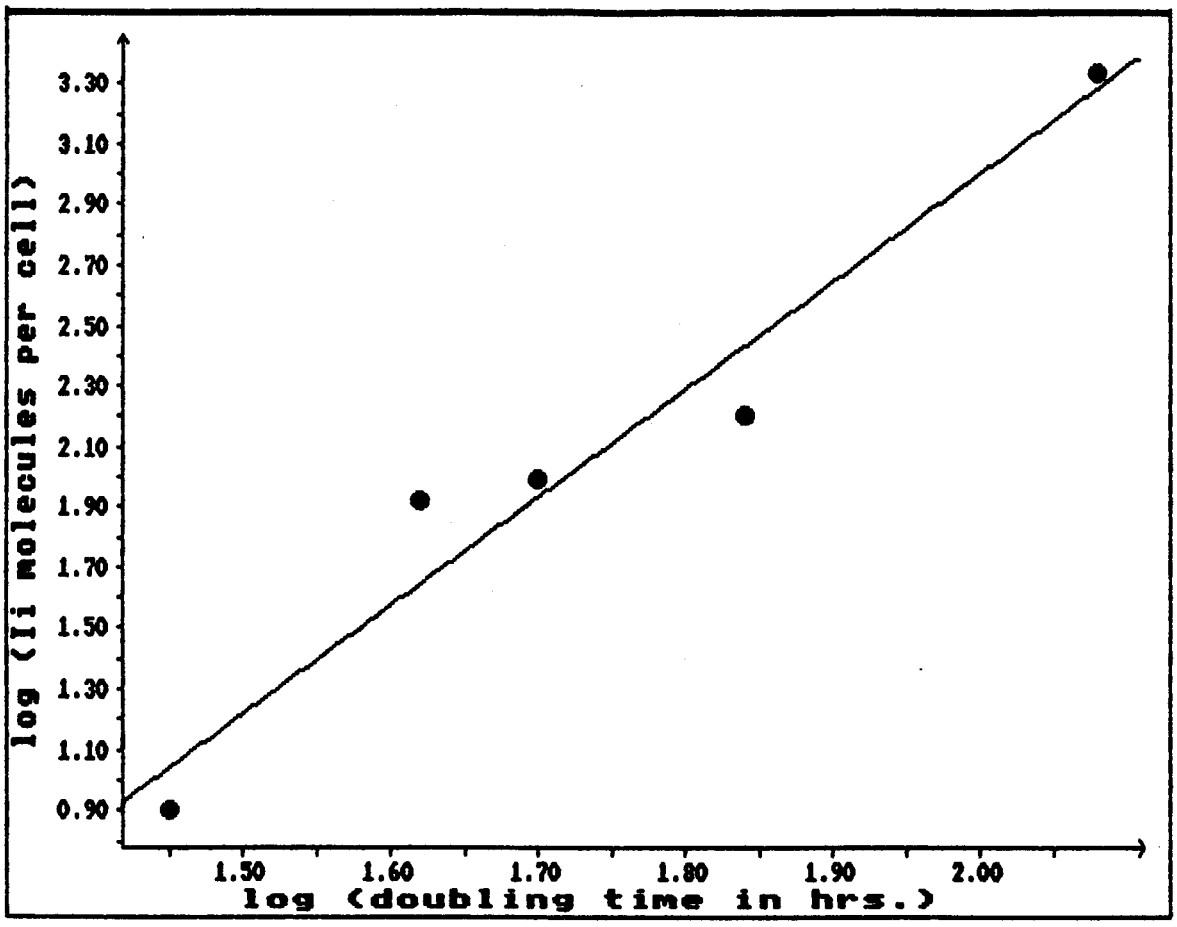


Fig. 29: The relationship between doubling time and Ii expression in melanoma cells.

Doubling time was plotted against Ii sites per cell, determined by saturation binding of ^{125}I-labelled VCD-1, in 5 different melanoma cell lines: Bowes, UCT-Mel 1, UCT-Mel 3, UCT-Mel 2 and UCT-Mel 7 (in order of magnitude; see Table 12, Appendix).

that expressed surface class II by density gradient centrifugation after rosetting with specific antibody-coated erythrocytes. Fig. 30 shows that cells from the top of the Ficoll-Hypaque were devoid of cells that expressed class II β chain. A small number of cells that expressed Ii chain faintly could, however, be detected in this population. When these cells were returned to culture and examined again after three days and six days, Ii positive cells reappeared in numbers and intensity that approximated those present before depletion. Class II β chain expression on the cell surface similarly re-appeared spontaneously with passage of time in culture.

DISCUSSION

Most authors who have studied Ii chain expression have emphasised the co-ordinate cellular expression of γ chain and class II α and β molecules. Although there have been reports to indicate that γ chain may be synthesized in the absence of class II α and β chains (60,61) and, conversely, that class II may be expressed without concomitant expression of Ii chain (4,63), these dissociations have generally been regarded as unusual phenomena. To the best of my knowledge the data I present give the first quantitative account of γ chain expression without class II α/β expression as a frequent phenomenon in both normal and abnormal cell types.

Figure 30

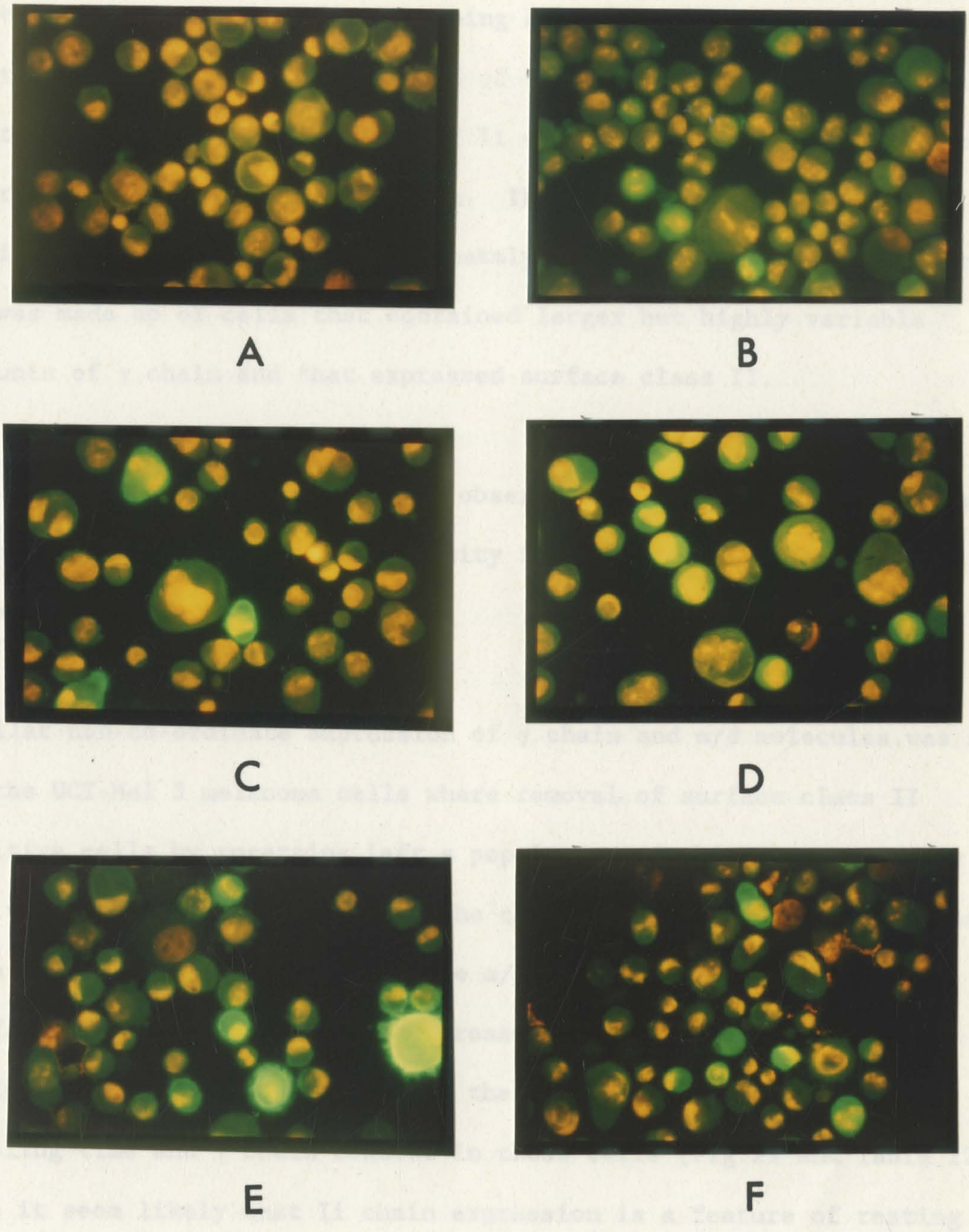


Fig. 30: Expression of γ chain during cell cycle of melanoma cells. Cells of the melanoma cell line UCT-Mel 3 depleted of class II positive cells (A,B), incubated for 3 days (C,D) and 6 days (E,F) and labelled with 9.3F10 antibody (A,C,E) and VCD-1 (B,D,F) by indirect IF of acetone-fixed cells.

The flow cytometry results were unequivocal in demonstrating two distinct populations of Ii-containing PBMC: the first a population that constituted approximately 40% of the cells analyzed and that contained uniform small amounts of Ii chain; this population did not express surface class II α/β chain. The second population of Ii positive PBMC constituted approximately 10% of the total population: it was made up of cells that contained larger but highly variable amounts of γ chain and that expressed surface class II.

The practical implication of this observation was that it is necessary to consider cellular Ii chain density in the two populations separately.

Similar non-co-ordinate expression of γ chain and α/β molecules was seen in the UCT-Mel 3 melanoma cells where removal of surface class II positive cells by rosetting left a population of which approximately 10% were weakly Ii positive. In the case of the melanoma cells I was able to show that, by returning the α/β depleted cells to culture, surface class II came to be re-expressed. Furthermore, the serum depletion experiments (Fig.28) and the direct relationship between doubling time and γ chain content in these cells (Fig.29 and Table 12) make it seem likely that Ii chain expression is a feature of resting cells whose proliferation has been arrested in the resting phase of cell cycle.

One might propose, therefore, a situation in melanoma cells in which Ii chain is present for the major part of G1 and surface class II only

appears in a subpopulation that progresses to further differentiation or at some other stage of the cell cycle. Since melanoma cells may be considered a clonally derived malignant cell population, one presumes that those with the Ii chain positive, surface class II negative phenotype and those with the Ii positive and class II positive phenotype are derived one from the other and are members of the same parent population. This being so, it is reasonable to assume that Ii chain synthesis and intracellular metabolism are functionally related to α/β chain synthesis and expression - a view that has been proposed by others (48,53,55,56,58,59).

In the case of the lymphocytes, however, similar assumptions are not so easy to make. Surface class II positive lymphocytes are predominantly cells of the B cell lineage and, in normal resting PBMC, constitute no more than 15% of the cells present. I was able to show that Ii chain may be expressed in as many as 50% of the PBMC analyzed; of these only a fraction were surface class II positive and in numbers that one would expect for the normal B cell population (Table 11; Appendix). I conclude, therefore, that a population of PBMC exists in which Ii chain expression is unrelated to subsequent class II expression. Although Ii chain is associated with intracellular α/β chains in immunoprecipitation experiments my conclusion would imply that this physical association seen experimentally is of questionable functional significance. Reports are now appearing that give weight to this argument (57,63,79).

Other reports of non-coordinate expression or induction of α/β and Ii chain, and observations to the effect that the γ chain is not required for antigen presentation, add further to the doubt concerning the role of Ii chain in the economy of cellular class II expression (57).

It has been suggested, on the basis of cellular mRNA content, that excessive Ii chain synthesis is a feature of the chronic lymphocytic leukemic cell (65). This suggestion, and the results of initial studies that showed that γ chain was readily detectable in PBMC from CLL patients but not from normal patients, led me to undertake a survey of cellular Ii chain content in leukemic blood samples. My findings, Fig. 23, warrant comment in several respects.

Firstly, leukemic PBMC's varied widely in the amounts of Ii chain they contained. This may have been due to the fact that the different leukemic cell populations were derived from different malignant clones that expressed, phenotypically, widely varying amounts of Ii chain. If, as Narni et al. have shown (65), all CLL cells contain large amounts of Ii chain mRNA we must assume that malignant lymphocytes show clonal differences in translational control without strict quantitative concomitance between transcription and γ chain synthesis. This assumption is consistent with the results obtained by Schneider et al. (63) who treated Ia negative murine bone marrow macrophages with γ interferon. The induced cells contained transcripts of α, β and γ chain, but α and β chains were the only final translated products detected.

It is also possible that the CLL cells that I analyzed were not all derived from the leukemic B cell clone and may have been diluted with other PBMC's of non-B cell lineage. It is recognised, for example, that peripheral blood lymphocytes of patients with CLL may contain as many as 30% T cells (80).

Secondly, having shown that normal Ii chain positive PBMC comprised two distinct populations, I now have the problem which of these two populations should be used to compare with the leukemic cells. Using, as the basis for comparison, data from the normal PBMC that were corrected to consider only surface class II positive cells (Fig. 23 NORM(1)) it seemed that CLL cells and normal cells express very similar amounts of Ii chain. If, on the other hand, one takes as the basis for comparison the average normal cellular Ii content for both populations (Fig. 23 NORM (2)) CLL cells contained significantly more Ii chain than normal cells.

Since I do not have all of the data required for valid comparisons, further work needs to be done to resolve the question of whether or not CLL cells do contain more Ii chain. It is necessary, for example, to compare leukemic and normal cell populations that are comparable in such other respects as surface class II expression, surface immunoglobulin expression and the presence or absence of other B cell markers. It is also of importance to know whether Ii expression in leukemic cells is unimodally or bimodally distributed. I intend to complete the experiments that will provide this information and I should like to explore further γ chain expression in EBVL and CLL cells.

EBV-transformed B cells, in the one sample assayed for Ii chain, showed an exceptionally high content of Ii chain and, if confirmed in more EBV-transformed cell lines, may have implications for the understanding of the mechanism by which EBV immortalizes B cells.

I should also like to extend the survey of patients with lymphocytic malignancies to consolidate my view that lymphocytic leukemia is associated with defects in the processing and sulphation of the Ii chain. The striking difference between the degree of sulphation of the Ii chain that I observed in the Raji cells, which are derived from Burkitt's lymphoma (an EBV-associated tumour), and EBV-transformed normal peripheral blood lymphocytes, suggests the possibility of a link between the terminal addition of glycosaminoglycan to core protein and the expression of the neoplastic as opposed to the immortal phenotype.

It is also important, I believe, to relate transcription of γ chain message to the time of appearance of translated products in cell populations that have been depleted of class II. The observations of Narni et al. (65), that showed so clearly that increased γ chain message was a characteristic of CLL, and my findings that there was no difference in γ chain content between class II +ve normal and CLL lymphocytes would suggest that, in CLL cells, there is a defect in translation of Ii chain.

Finally, I intend to clarify the relationship between Ii expression and the phase of the cell cycle in melanoma cells. This could be

approached in three ways: Firstly, I could deplete an asynchronously dividing cell population of surface class II +ve cells by the rosetting technique that I described in Chapter 3. By pulsing the depleted cells with ^{3}H-thymidine it should be possible to discern a temporal pattern of incorporation of radioactivity consistent with the depletion of a G1 population. Secondly, by performing dual fluorescent cytometry with ethidium bromide and FITC-labelled VCD-1, cells that contain a single complement of DNA should express more Ii chain than cells in S phase, G2 or M. Thirdly, the simultaneous use of VCD-1 and a MoAb to bromodeoxyuridine, after a pulse with this analog, should enable me to identify that phase of the cell cycle in which Ii chain is predominantly expressed.

APPENDIX

Phosphate-buffered saline (PBS) pH 7.2

(0.009M Phosphate; 0.145M Na; 0.004M K; 0.140M Cl; pH 7.2)

NaCl	8g	0.137M
KCl	0.2g	0.003M
$Na_2HPO_4.2H_2O$	1.44g	0.008M
KH_2PO_4	0.2g	0.001M

dissolved and made up to 1 litre with distilled water.

Tris-buffered saline (TBS) pH 7.6

(0.15M NaCl; 0.05M Tris-HCl, pH 7.6 at room temperature)

NaCl	8.77g
Tris base	6.05g

(Tris (hydroxymethyl) aminomethane)

dissolved in approximately 900 ml distilled water, pH adjusted to 7.6 with conc. HCl and made up to 1 litre

Transfer buffer for Western blots pH 8.3

Tris base	12.1g
Glycine	28.8g
Methanol	400ml

made up to 2 litres with distilled water.

Diaminobenzidine (DAB) substrate for Western blots

A stock solution of DAB at 25mg/ml in TBS was stored frozen in aliquots at -20°C. Just before use the stock solution was diluted 1/50 in TBS, 30% H_2O_2 was added to 1/1000, and a 3% stock solution of $CoCl_2$ to 1/100 dropwise with stirring.

Blots were left in this solution for 5 minutes and then rinsed well with distilled water. (Caution: DAB is carcinogenic).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

(essentially as in ref. 44)

Running gel buffer:

18.17g Tris base was dissolved in approximately 80ml distilled water and adjusted to pH 8.8 with concentrated HCl. After adding 4.0ml of 10% SDS, the volume was made up to 100ml.

Stacking gel buffer:

6.057g Tris base was dissolved in approximately 80ml distilled water and adjusted to pH 6.8 with conc. HCl. After adding 4.0ml of 10% SDS the volume was made up to 100ml.

Reservoir buffer (pH 8.5):

Tris base	3.03g
Glycine	14.41g
10% SDS	10ml

made up to 1 litre with distilled water.

2x sample buffer (total volume 10ml):

stacking gel buffer	2.5ml
sucrose or glycerol	2.0g
10% SDS	4.6ml
0.04% bromophenol blue	1.0ml
H_2O	0.5ml

For reducing sample buffer, 0.2ml of 1M dithiothreitol (DTT) was added to the above mixture.

Samples for SDS-PAGE were mixed with an equal volume of 2x sample buffer and boiled for 2 mins.

Acrylamide-bis acrylamide (A-bis-A):

acrylamide	30g
N,N-methylene-bis-acrylamide	0.8g

dissolved in distilled water, made up to 100ml and filtered (caution : acrylamide is toxic)

To prepare 11% gels of 120 x 80 x 1mm:

Running gel mixture

A-bis-A	3.3ml
running gel buffer	2.25ml
distilled water	3.45ml
N,N,N,N'-tetramethylene diamine (TEMED)	5μl
10% ammonium persulphate	45μl

Stacking gel mixture:

A-bis-A	0.5ml
stacking gel buffer	1.25ml
H_2O	3.25ml
TEMED	10μl
10% ammonium persulphate	100μl

Staining solution:

0.1g PAGE blue 83 (BDH 44246) in 100ml of 30% methanol, 10% acetic acid.

De-staining solution

30% methanol, 10% acetic acid

For staining and de-staining molecular weight markers blotted onto Immobilon membrane (Millipore Cat No. IPVH00010) 50% methanol, 10% acetic acid was used to make up 0.1% PAGE blue and to de-stain.

Molecular weight markers (Boehringer "Combithek")

	Daltons
alpha macroglobulin (reduced)	170 000
phosphorylase b	97 000
glutamate dehydrogenase	55 400
lactate dehydrogenase	36 500
trypsin inhibitor	20 100

Fluorography of polyacrylamide gels:

The lane containing the resolved molecular weight markers was sliced from the gel, stained for 30-60 mins and de-stained overnight. The remaining gel was fixed overnight in 30% methanol, 10% acetic acid, immersed in "Amplify" (Amersham NANP.100) for 15 mins. with agitation and then for another 10-15 mins. in approximately 3% glycerol in water. After drying between filter paper and cellophane under vacuum, the gel was kept in a casette between intensifying screens with Cronex 4 X-ray film (Du Pont) or Hyperfilm-HP autoradiography film (Amersham) for several days at -70°C.

Molecular weights of unknown proteins were determined by reference to the electrophoretic mobility of the molecular weight markers. A linear relationship was observed between mobility and log M_r.

Epstein-Barr virus transformation of lymphocytes

PBMC were separated from heparinized blood of a normal donor by Ficoll-Hypaque flotation (see Chapter 1).

The cells were suspended at 2 x 10^6/ml in RPMI supplemented with 15% FCS, antibiotics and 5μg/ml phytohaemagglutinin, and mixed with an equal volume of tissue culture supernatant from the cell line B95-8. The cells were cultured for 2-3 weeks until transformed cells proliferated; the cultures were subdivided and supplemented with 15% FCS in RPMI when necessary.

Production of monoclonal antibody VCD-1

(essentially as in ref 81)

Media (sterilized by filtration):

RPMI 1640 powdered tissue culture medium (GIBCO 074-01800)

Foetal calf serum (FCS) from the State Vaccine Institute, Cape Town, heated at 56°C for 30 mins.

Pooled human umbilical cord serum (HUCS), heated at 56°C for 30 mins.

Penicillin and streptomycin (PS) 100xstock: 10 000 U/ml penicillin and 10mg/ml streptomycin sulphate in PBS

Freezing medium: 10% dimethyl sulphoxide (DMSO), and 10% FCS in RPMI 1640.

Polyethylene glycol (PEG), 50% in RPMI: 2g PEG MW 4000 (Merck 9727) was autoclaved and 2ml warm RPMI added while the PEG was still liquid.

Versene Solution, 5x stock:

Na_2EDTA	0.65g	(0.003M)
KCl	0.5g	(0.013M)
NaCl	20g	(0.684M)
$Na_2HPO_4.2H_2O$	2.85g	(0.032M)
KH_2PO_4	0.5g	(0.007M)

in 500ml distilled water. Adjust pH to 7,2-7,4.

Aminopterin (A), 100x stock:

1.76mg aminopterin was dissolved in 25ml distilled water + 0.5ml of 5N NaOH, then neutralized with 5N HCl, made up to 100ml and stored at -20 C.

Hypoxanthine and thymidine (HT), 100x stock

hypoxanthine	0.34g
thymidine	0.0968g
glycine	0.0055g
sodium pyruvate	2.7515g

Approximately 125ml distilled water was added to the hypoxanthine powder and then 10M NaOH dropwise with stirring until dissolved. The remaining ingredients were added, the solution made up to 250ml and frozen at -20 C in aliquots.

Cell fusion and cloning:

The spleen of a Balb/c mouse, that had been immunized with PBMC from the CLL patient, was removed and disrupted by forcing through a stainless steel mesh with a syringe plunger into RPMI medium. The cells were washed once in RPMI and mixed with SP2 myeloma cells at a ratio of 10 spleen cells:1 SP2 cell.

The mixed cells were pelleted at 100g and the supernatant removed. Warm 50% PEG (1.5 ml over 1 min) was added to the pellet dropwise with mixing and mixing was continued for another minute. Warm RPMI was added slowly with constant mixing, at first at a rate of 2 ml over 2 mins and then 8 ml over 2-3 minutes. Approximately 35 ml of warm RPMI was then added by pouring down the side of the tube.

The cells were then pelleted (100g; 3min), the supernatant was removed and the pellet was gently resuspended in 100ml (per 10^8 spleen cells) of 10% HUCS in HAT medium. After culturing the cells in 24-well plates for 2 weeks, the culture medium was sampled and tested for MoAb against CLL lymphocytes in ELISA using the KPL Hybri-clonal EIA screening kit (KPL Cat No. 54-18-09) as per instruction. Cells were attached to PVC ELISA plates (Flow Labs. 77-173-05) by pre-incubating the plates for 30 mins with a solution of poly-L-lysine (1mg/ml in 0.15M NaCl) removing the PLL solution and adding 25μl of a suspension of $5x10^6$ cells/ml of PBS. The plates were incubated for 10 min at 4°C and centrifuged at 250g for 2 mins.

Hybridomas producing antibody were cloned twice by limiting dilution in 10% HUCS in HT medium in 96 well plates. The final clone was expanded in 10% FCS in RPMI and the cells were injected into pristane-primed mice to produce ascites.

Purification of MoAb from mouse ascites (82)

Ascites was diluted with two volumes of 0.06M acetate buffer pH4 and the pH was adjusted to 4.8 with HCl. Caprylic acid (Sigma C-2875), 1.1 ml per 100ml diluted ascites, was added dropwise with stirring and mixing was continued for 30 mins at room temperature. The mixture was centrifuged (10 000g; 30 mins, at room temperature) and the supernatant containing the MoAb was dialyzed against PBS. The immunoglobulin was precipitated by stirring with an equal volume of saturated ammonium sulphate for 30 mins and collected by centrifuging (10 000g; 20 min). The precipitate was resuspended in PBS and dialyzed against PBS.

Preparation of peroxidase-anti-peroxidase (PAP) complexes.

Anti-horse radish peroxidase (HRP) MoAb produced in this laboratory was purified by precipitation with caprylic acid. Horse radish peroxidase (Seravac 161451) was dissolved at 100μg/ml in PBS containing 0.5% BSA and 0.01% thiomersal. Purified anti HRP(2.2mg/ml) was added to 1/100 (relative concentrations were determined in ELISA). This stock solution was diluted 1/10 in TBS for use in Western blots.

Sub-isotyping of MoAb by a dot-blot test.

Rabbit anti-mouse isotyping sera (Miles) were diluted 1/20 in PBS and 2μl droplets were spotted onto nitrocellulose membrane which was left to dry. The membrane was blocked by shaking for 1 hr in TBS containing 5% FCS. The blocking solution was removed, 5μl samples of MoAb harvest fluid were applied to the spots and incubated for 5 mins. The membrane was washed twice with TBS and 5μl volumes of peroxidase-conjugated goat anti-mouse Ig (Cappel 3211-0231 diluted 1/200 in blocking solution), were applied to the spots for 5 mins. The membrane was washed three times in TBS and immersed in DAB substrate for 5 mins.

Table 8: Ii molecules per cell on PBMC of CLL patients

Patient	WBC/l ($\times 10^{-9}$)	% Lymphs	Ii molecules per cell ($\times 10^{-3}$)
ST	320	-	413
JO	12.0	54	223
VA	20.5	89	162
DE	7.6	58	140
PE	136	97	333
PW	8.4	30	178
MA	21.2	51	140
HA	19.3	71	170
KH	4.08	78	197
GU	26.2	88	95
GI	63.7	88	94
CA	23.3	64	68
BO	24.2	-	154
DU	7.4	54	94
MT	3.8	16	26
QU	15.2	47	81
PI	15.2	66	271
MAK	9.5	81	101

Table 9: Ii molecules per cell on malignant and transformed cells other than CLL

Patient	Diagnosis	Ii molecules/cell(x 10^{-3})
BA	CML	14
HA	CML	74
FO	CML	28
GA	ALL	37
BAR	LYM	32
SE	LYM	27
MO	MYEL	34
MI	EBVL	883

Table 10: Ii molecules per cell on normal lymphocytes ($x10^{-3}$)

Cell donor	Ii molecules per cell	% class II +ve cells	% VCD-1 + ve cells	Ii molecules per class II + ve cell	Ii molecules per VCD-1 + ve cell
FI	29	17	43	172	68
SC	31	11	52	285	60
VE	21	8	44	261	47
BU	12	12	51	99	23
PO	17	15	40	116	44
RO	45	16	43	283	106
TW	59	28	-	213	-
AD	41	23	55	179	75
FR	29	15	39	194	75
KE	35	14	53	249	66
FL	26	12	62	219	42
JA	25	7	58	355	43
ME	55	21	58	263	96
PH	43	19	53	225	81

Table 11: Flow cytometry results of adherent cell-depleted normal PBMC (fluorescent cells as % of total light scatter gate count)

Cell donor	Positive with 9.3F10 Ab	Strongly positive with VCD-1	Weakly positive with VCD-1
FI	17	17	26
SC	11	10	42
VE	8	8	36
BU	12	10	41
PO	15	11	29
RO	16	12	31
AD	23	18	37
FR	15	12	27
KE	14	10	43
FL	12	12	50
JA	7	11	47
ME	21	19	39
PH	19	13	40

Table 12: Ii expression and doubling time of melanoma cells

Cell line	Ii molecules/cell ($x10^{-3}$)	Doubling time (hrs)
Bowes	8	28
UCT-Mel 1	84	42
UCT-Mel 3	98	50
UCT-Mel 2	159	70
UCT-Mel 7	2190	119

CORRESPONDENCE FROM DR. B.B. COHEN

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Crewe Road, Edinburgh
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telephone 031-332 2471

Your reference

Our reference

11-3-88

Dear Professor Doudle,

I'm sorry for the delay but enclosed is the results I got with your MoAbs. As you can see it is apparently not as "good" as our own.

I confirm it corresponds to the κ chain and that it does not appear to react with κ/β dimers.

However by our arguments I am puzzled and ask why?:—

1) Since you immunised with intact cells most anti Class II MoAbs should have been derived against β chains and not κ chains as we and others found with our early studies in the DA6-series raised against Daudi cells.

2) For the above reason your anti κ should react with intact κ/β dimers as did the one anti κ we raised in the DA6 series (DA6-147)
However all of the new anti κ raised against affinity purified and lemic acid-eluted class II do not react with intact cells and poorly with intact κ/β dimers.

Should I send you cell lines for you to try in case your MoAbs didn't travel well.
Also do you want any of our MoAbs?

I hope this result is of use and does not fade with time!

Yours Sincerely
Brian Cohen

May 26th 1988

Dear Professor Dowdle,

Enclosed are some more assays. May I revise my opinion and suggest that VCD1 is probably not an anti α or else it is a very specific one since:

1) The bands although very similar to the authentic α bands are not just exactly coincident (in two experiments)

2) VCD appears to react with several components approx 69K, a doublet at 34K and 12K

3) Analysis of a panel of cell lines showed that in most, a high [α] coincides with high VCD epitope but there are a couple of exceptions viz. 4, 5 and 6

If I have time I will try to do a more definitive experiment by taking lysate through an anti Class II affinity column and checking the reactivity of the bound and unbound in blots. Similarly lysate through a VCD column.

I enclose these results to let you know I have not given up on your mAbs

I have asked Keith Guy our Chief Res. Officer to check the VCD, (in the FACS) binding pattern X to a panel of fixed cells with known sites of DR, DP and D

I wonder whether it could be anti Class I?

Yours sincerely

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10 June 1988

Professor E B Dowdle
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Dear Professor Dowdle

Further to my last letter - I actually went ahead and did the affinity coloumns and enclose the results (VCD[1] is sometimes labelled SA for SouthAfrica!)

Cell Lines: BSMC and JAB lysed in NP40 Buffer and spun to clarify

Sepharose CNBr. Affinity columns: a) VCD1
b) Mixture of DA6-147 (anti α) and CR3/43 (anti β)

Each lysate put through each column and unbound lysate collected. Then columns eluted with successive aliquots of glycine HCL pH 2.5 so that for each cell lysate there are -

Original
Unbound to VCD1 Column
Unbound to anti-Class II Column
Eluted from VCD1 Column
Eluted from anti-Class II Column

All the samples were then added with Laemmli sample buffer without mercaptoethanol and without boiling. Note that the only acidified samples are those eluted from the affinity columns.

In the first experiment all of the samples were blotted with CR3/147 Mixture or VCD1 and developed with alkaline phosphatose anti-mouse conjugate.

Exp 1A Samples
1 Original JAB
2 Original BSMC
3 JAB Unbound to VCD1 Column
4 JAB Unbound to anti Class II Column
5 BSMC Unbound to VCD1 Column
6 BSMC Unbound to anti Class II Column

LHS Blotted with CR3/147 RHS with VCD1

Conclusions: a) Anti Class II removes practically all Class II. VCD1 does not remove Class II. (From LHS)
b) VCD1 removes all VCD1 epitopes and Anti Class II seems not to remove VCD1 epitopes although the upper bands seen to disappear(?) Again as stated earlier the doublet recognised by VCD-1 seems to differ from the α chain pattern with DA6-147.

2

10 June 1988

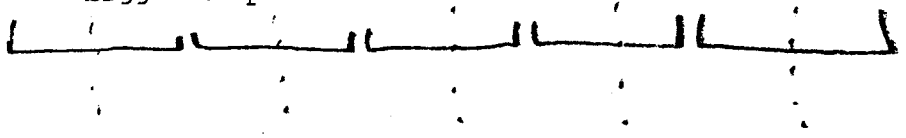
Professor EB Dowdle

Exp 1B Reading Left to Right

Elutions 1-4 from BSMC lysate bound to VCD1 column
Elutions 1-4 from BSMC lysate bound to 147/CR3 column
Elutions 1-4 from JAB lysate bound to 147/CR3 colimn
Elutions 1-4 from JAB lysate bound to VCD1
Original JAB
Original BSMC

Conclusions
1) Anti Class II pulled out Class II (Lower Picture)
2) AVCD1 pulled out VCD1 epitopes (Upper Picture)
3) Anti Class II pulled out small amounts of VCD1 epitopes! (Upper Picture)
4) VCD1 did not pull out Class II epitopes (Lower Picture)

Exp II I then took the residual fractions and re-ran them using 5F2.3 (in our hands the best anti α blotter) and CR3/43 using larger wells to load bigger samples.



Then cutting as shown and blotting as on the cellulose nitrate.

Well 1 Pooled elutions from VCD column BSMC
2 Pooled elutions from anti Class II Column BSMC
3 Pooled elutions from anti Class II Column JAB
4 Original mixture of JAB and BSMC
5 Pooled elutions from VCD Column JAB

Confirms conclusions above and also shown that elutions from the VCD columns react with 5F2.3

The overall conclusions at this stage I would say are that VCD1 either does not react with MHC Class II α chains or else if it does, they are a minority group or else the VCD1 molecules and the Class II molecules are loosely linked together on the membrane and co-ppt? What next?

With best regards

Brian Cohen

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