Characterisation of function and regulation of the CD52 antigen on T and B lymphocytes

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To Mum and Dad,
Andrew,
Emma, Ross and Kerria

Abstract.

The CD52 antigen (CAMPATH-1) is a GPI-anchored protein, expressed on the surface of lymphocytes and monocytes. CAMPATH-1 antibodies are effective in *in vivo* lymphocyte depletion and have been used in the clinic for the treatment of a number of immune disorders (e.g. rheumatoid arthritis, T and B cell malignancies). The principal mechanism involved in lymphocyte depletion is thought to be by Antigen Dependent Cell mediated Cytotoxicity (ADCC). The studies described in this thesis suggest that CD52 could be involved in cell signalling and mediate apoptosis.

CAMPATH-1 antibodies were able to provide a costimulatory signal to induce CD45RA+ and CD45RO+ CD4+, and CD8+ T cell proliferation and cytokine production. Activation of CD4+ T cells was accompanied by down-regulation of CD52 surface expression. CAMPATH-1 induced proliferation of CD4+ T cells was inhibited by Cyclosporin A and Herbimycin A.

In Wien 133 (B cell line) and CD52 transfected Jurkat (T cell line) cells, CD52 ligation mediated growth inhibition and apoptosis. Induction of apoptosis was time and antigen density dependent, and in Jurkats required the presence of a functional T cell receptor.

Wien 133 cells surviving CAMPATH-1 treatment were shown to be deficient in CD52 expression as well as GPI-anchored CD55 and CD59. These cells remained sensitive to growth inhibition mediated via surface IgM. CD52 expression in these cell populations was restored following growth *in vivo* and expression of CD52 was maintained longterm in *in vitro* culture.

CD52 ligation induced increased tyrosine phosphorylation in peripheral CD4+ T cells, a T cell clone and the Wien 133 cell line. In CD4+ T cells, the phosphorylation was partially inhibited by Herbimycin A.

From the results obtained, it was concluded that CAMPATH-1 antibodies could induce lymphocyte depletion via discrete signals leading to apoptosis. In addition, results provide possible explanations for some of the side effects observed during and following CAMPATH-1 therapy. These are discussed in the final chapter.

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Table of Contents

TITLE 1

DEDICATION 2

ABSTRACT 3

ACKNOWLEDGEMENTS 4

TABLE OF CONTENTS 5

LIST OF FIGURES 11

LIST OF TABLES 14

ABBREVIATIONS 15

1. INTRODUCTION 18

1.1 THE IMMUNE SYSTEM 18

1.2 T CELL LYMPHOCYTE DEVELOPMENT AND THE LYMPHOID SYSTEM 19

1.2.1 Antigen presentation 21

1.2.2 The Major Histocompatibility Complex (MHC) 22

1.2.2.1 MHC class I processing 22

1.2.2.2 MHC class II processing 23

1.3 FUNCTIONS OF PERIPHERAL T CELLS 24

1.3.1 CD4+T cells 24

1.3.2 CD8+ T cells 25

1.4 T CELL SIGNALLING 26

1.4.1 The TCR signal (Signal 1). 27

1.4.2 T cell Costimulation (Signal 2). 30

1.5 T CELL ANERGY 33

1.5.1 Functional anergy. 33

1.5.2 Anergic signals. 34

1.6 T CELL APOPTOSIS. 35

1.7 B CELL LYMPHOCYTE DEVELOPMENT 36

1.7.2 B cell activation and signalling 37
1.8 GPI-ANCHORED PROTEINS 39
1.8.1 What is a GPI anchored protein? 39
1.8.2 The function of GPI anchors 42
1.9 THE CD52 ANTIGEN 44
1.10 CAMPATH-1 ANTIBODIES 46
1.10.1 The targets for CAMPATH-1 therapy 48
1.10.1.1 Rheumatoid arthritis 48
1.10.1.2 Multiple sclerosis 49
1.10.1.3 Lymphoproliferative disorders 50
1.10.1.4 Transplantation 50
1.10.2 Clinical trials with CAMPATH-1 antibodies 51
1.11 AIMS 53
2. MATERIALS AND METHODS. 54
2.1 MATERIALS. 54
2.1.1 Antibodies 54
2.1.2 Chemicals 55
2.1.3 Enzymes 55
2.1.4 Nucleic acids 55
2.1.5 Cell lines 56
2.2 PURIFICATION OF CD4+ T CELLS 56
2.3 PURIFICATION OF CD8+ T CELLS 57
2.4 PURIFICATION OF CD4+CD45RA+ AND CD4+ CD45RO+ T CELLS 58
2.5 T CELL PROLIFERATION ASSAY 58
2.6 T CELL CLONES 59
2.7 CYTOKINE PRODUCTION IN T CELLS 59
2.8 ELISA FOR THE DETECTION OF HUMAN TUMOUR NECROSIS FACTOR (TNF-α) 60
2.9 ELISA FOR THE DETECTION OF HUMAN INTERFERON-γ 60
2.10 ELISA FOR THE DETECTION OF IL-4 60
2.11 CELL ASSAY FOR MEASUREMENT OF IL-2 PRODUCTION 61
2.12 CELL GROWTH INHIBITION ASSAYS 61
2.13 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) 62
2.13.1 Autoradiography 63

2.14 General Method for Western blotting 63
2.15 Immunoprecipitation of Phosphotyrosine proteins 63
2.16 Phosphotyrosine blotting 64
2.17 Preparation and Purification of CAMPATH-1 (CD52) antigen 65
2.18 ELISA for the quantitation of CD52 antigen 65
2.19 Western blot detection of the CAMPATH-1 antigen 66
2.20 N-Glycanase digestion of the CD52 antigen 66
2.21 FACS Staining for analysis of CD52 expression 67
2.22 FACS analysis of other cell markers 67
2.23 PI-PLC treatment of cells 67
2.24 Preparation of RNA 68
2.25 RNA Dot blots 69
2.26 Agarose gel electrophoresis 69
2.27 Coupling antibodies to Sepharose 70
2.28 Detection of Apoptosis 70
2.28.1 Propidium Iodide staining of cells 71
2.28.2 Apoptosis: Giemsa stain 71
2.28.3 Apoptosis: DNA laddering 71
2.29 Transfections 72
2.30 Electron microscopy 73
2.31 Calcium flux measurements 73
2.32 In vivo experiments 74

3. THE ROLE OF CD52 ANTIGEN IN T CELL ACTIVATION 75

3.1 Introduction 75
3.2 Results 77
3.2.1 Induction of T cell proliferation by CAMPATH-1 antibodies. 77
3.2.2 CAMPATH-1 antibodies synergise with CD3-mediated signals to augment CD4+ T cell proliferation 78
3.2.3 CD52 is downregulated from the surface of CD4+ T cells following activation induced by anti-CD3 and CAMPATH-1H antibodies 78
3.2.4 Ability of cytokines to stimulate CD4+ T cell proliferation after crosslinking CD52 antigen 79

3.2.5 Anti-CD52 monoclonal antibodies induce cytokine release in CD4+ T cells79

3.2.6 Anti-CD52 antibodies induce proliferation in CD8+ and CD4+ CD45RA+ and CD4+ CD45RO+ T cells 81

3.2.7 The effect of cyclosporin A on the induction of T cell proliferation by anti-CD52 antibodies. 82

3.2.8 Down modulation of CD52 antigen following antigen specific activation. .. 83

3.2.9 CAMPATH-1H does not inhibit polyclonal or antigen-specific T cell activation 83

3.3 DISCUSSION 93

4. EFFECTS OF CROSSLINKING CD52 ON B AND T TUMOUR CELL LINES97

4.1 INTRODUCTION 97

4.2 RESULTS 99

4.2.1 Effect of crosslinking CAMPATH-1H antibody on the growth of different B and T tumour cell lines 99

4.2.2 The growth inhibition of the Wien 133 B cell lymphoma by CAMPATH-1H antibody is CD52 specific 100

4.2.3 Growth inhibition of B cell lymphoma is CD52 specific 100

4.2.4 The effect of isotype on the CD52 specific growth inhibition of Wien 133 cells ..... .102

4.2.5 Growth inhibition of B cell lymphoma induced via surface immunoglobulin 103

4.2.6 Surface IgM is not required for cell growth inhibition induced via the CD52 antigen 103

4.2.7 Does CAMPATH-1H synergise with anti-IgM antibody to mediate growth inhibition in Wien 133 cells? 104

4.2.8 The cell growth inhibition effect induced by CAMPATH-1H antibody in Wien 133 cells is time dependent 105

4.2.9 Effect of soluble cytokines on the inhibition of growth of Wien 133 cells induced by CAMPATH-1H antibody. 105

4.2.10 Effect of antibodies directed against CD23 and CD40 on growth inhibition in Wien 133 cells induced by crosslinking CD52 antigen 107

4.2.11 CAMPATH-1H antibody induces apoptosis in Wien 133 cells..... 107

4.2.12 CAMPATH-1H antibody induced apoptosis in Wien 133 cells does not depend on expression of CD95 (Fas) *110*
4.2.13 CAMPATH-1H induced apoptosis in Wien 133 cells is not due to TNF-α. *111*
4.2.14 N-Acetylcysteine can partially block CAMPATH-1H induced cell growth inhibition in Wien 133 cells *111*
4.2.15 Effect of Cyclosporin A on CAMPATH-1H induced growth inhibition of Wien 133 cells. *112*
4.2.16 The effect of other inhibitors on blocking CAMPATH-1H induced cell death in Wien 133 cells *112*
4.2.17 Signalling via the CD52 antigen in T cells requires the presence of a functional T cell receptor *113*
4.2.18 The effect of CAMPATH-1H antibody on the growth of CD52 Jurkat transfectants *114*
4.2.19 CAMPATH-1H and anti-CD3 synergise to inhibit the growth of CD52 transfected Jurkats *116*
4.2.20 The effect of CD52 ligation on release of IL-2 in Jurkats. *117*
4.3 DISCUSSION 147

5. CONTROL OF CD52 EXPRESSION IN WIEN 133 CELLS 152

5.1 INTRODUCTION 152
5.2 RESULTS 154
5.2.1 Selection of CAMPATH-1H resistant cells *154*
5.2.2 Quantitation and Western blot analysis of CD52 antigen in Wien 133 cells and CV lines *156*
5.2.3 CD52 antigen mRNA levels in Wien 133 cells and CV clones are comparable. *157*
5.2.4 Expression of other GPI-anchored proteins in Wien 133 cells and CV clones *157*
5.2.5 Western blot analysis of CD59 levels in Wien 133 cells and CV lines *158*
5.2.6 Surface IgD expression in Wien 133 cells is not sensitive to PI-PLC treatment. *158*
5.2.7 Restoration of CD52 expression in CV cells following in vivo growth in nude mice. *159*

5.2.8 Effect of cytokines on expression of CD52 in the CV clones 160

5.3 DISCUSSION 172

6. BIOCHEMISTRY OF SIGNALLING VIA THE CD52 ANTIGEN 176

6.1 INTRODUCTION 176

6.2 RESULTS 180

6.2.1 Effect of anti-CD52 antibodies on tyrosine phosphorylation in resting CD4+ T cells. 180

6.2.2 Coligation of CD52 and CD3 in resting CD4+ T cells leads to a phosphorylation pattern comparable to that seen when CD4 and CD3 are coligated.. 181

6.2.3 CD52 crosslinking induces tyrosine phosphorylation in a T cell clone 181

6.2.4 Immunoprecipitation of phosphorylated proteins in T Cells following CAMPATH-1H activation 182

6.2.5 Effect of herbimycin A on signalling in CD4+ T cells 183

6.2.6 Ligation of CD52 does not induce a calcium flux. 183

6.2.7 CD52 ligation induces phosphorylation in the Wien 133 B cell lymphoma cell line 184

6.2.8 Immunoprecipitation of CD52 antigen and associated proteins from Wien 133 cells 185

6.2.9 Western blot detection of lyn in Wien 133 cells 186

6.2.10 Protein sequence analysis of CD52 associated proteins 187

6.3 DISCU SSION 199

7. DISCUSSION 203

7.1 RELEVANCE OF STUDIES TO CLINICAL TRIAL DATA. 203

7.2 RELEVANCE OF STUDIES TO THE POSSIBLE FUNCTION OF THE CD52 ANTIGEN 205

7.2.1 The role of CD52 in T cell activation. 206

7.2.2 A role for CD52 in lymphocyte homing/ cell adhesion 209

7.2.3 A role for CD52 in protection from complement mediated attack. 210

7.3 CD52: THE FUTURE. 211

8. REFERENCES 213

List of Figures

FIGURE 1.1 T CELL SIGNALLING 31
FIGURE 1.2 T CELL/B CELL INTERACTIONS AND SIGNALS 38
FIGURE 1.3 GENERALISED STRUCTURE OF A GPI-ANCHOR 41
FIGURE 1.4 STRUCTURE OF THE CD52 ANTIGEN 45
FIGURE 3.1 CAMPATH-1 INDUCED PROLIFERATION OF CD4+ T CELLS 85
FIGURE 3.2 AUGMENTATION OF CD3 BUT NOT CD2, OR CD28-MEDIATED PROLIFERATION OF CD4+ T LYMPHOCYTES BY ANTI-CD52 ANTIBODY. 86
FIGURE 3.3 CD52 IS DOWNMODULATED FROM THE SURFACE OF CD4+ T CELLS FOLLOWING ACTIVATION IN THE PRESENCE OF ANTI-CD3 AND ANTI-CD52 ANTIBODIES 87
FIGURE 3.4 CD8+ T LYMPHOCYTES ARE INDUCED TO PROLIFERATE BY ANTI-CD52 ANTIBODIES 88
FIGURE 3.5 ANTI-CD52 ANTIBODIES CAN INDUCE PROLIFERATION IN BOTH NAIVE (CD45RA+) AND MEMORY (CD45RO+) CD4+ T CELLS. 89
FIGURE 3.6 CYCLOSPORIN A INHIBITION OF CD4+ T CELL PROLIFERATION INDUCED BY ANTI-CD52 ANTIBODIES. 90
FIGURE 3.7 CD52 SURFACE EXPRESSION IS DOWNMODULATED IN A T CELL CLONE FOLLOWING ANTIGEN ACTIVATION. 91
FIGURE 3.8 ANTI-CD52 ANTIBODIES DO NOT INHIBIT ANTIGEN SPECIFIC PROLIFERATION OF A T CELL CLONE. 92
FIGURE 4.1 EFFECT OF CAMPATH-1H ANTIBODY ON THE GROWTH OF B AND T CELL TUMOUR LINES. 118
FIGURE 4.2 CD52 ANTIGEN EXPRESSION IN WIEN 133, EBV, HUT 78, JURKAT AND CD52 TRANSFECTED JURKAT (CLONE 3B11) CELL LINES. 119
FIGURE 4.3 EXPRESSION OF SURFACE MARKERS ON WIEN 133 CELLS. 120
FIGURE 4.4 EFFECT OF CAMPATH-1H ANTIBODY CONCENTRATION ON THE GROWTH INHIBITION OF WIEN 133 CELLS. 121
FIGURE 4.5 EFFECT OF RAT CAMPATH-1G ANTIBODY ON THE GROWTH OF WIEN 133 CELLS. 122
FIGURE 4.6 GROWTH INHIBITION IN THE WIEN 133 CELL LINE VIA THE CD52 ANTIGEN REQUIRES CROSSLINKING. 123
FIGURE 4.7 GROWTH INHIBITION IN WIEN 133 CELLS INDUCED BY CAMPATH-1H ANTIBODY DOES NOT REQUIRE AN INTACT FC. 124
FIGURE 4.8 THE GROWTH INHIBITION INDUCED IN WIEN 133 CELLS BY CAMPATH-1H ANTIBODY IS NOT DUE TO INTERACTION VIA ANTIBODY FC REGIONS OR FORMATION OF NON SPECIFIC IMMUNE COMPLEXES. 125

FIGURE 4.9 EFFECT OF ANTIBODY ISOTYPE ON INDUCING CELL GROWTH INHIBITION IN WIEN 133 CELLS. 126
FIGURE 4.10 CROSSLINKING SURFACE IGG ENHANCES THE GROWTH INHIBITION IN WIEN 133 CELLS COMPARED TO THAT SEEN WHEN ANTI-HUMAN IGG ANTIBODY IS ADDED ALONE. 127
FIGURE 4.11 ANTI-IGM INDUCES GROWTH INHIBITION IN WIEN 133 CELLS. 128
FIGURE 4.12 WIEN 133 CELLS SURVIVING TREATMENT WITH ANTI-IGM ANTIBODY LOSE SURFACE IGM EXPRESSION BUT EXPRESS HIGH LEVELS OF SURFACE IGD..... 129
FIGURE 4.13 IGM NEGATIVE WIEN 133 CELLS REMAIN SENSITIVE TO GROWTH INHIBITION INDUCED BY CAMPATH-1H ANTIBODY..... 130
FIGURE 4.14 PARTIAL BLOCKING OF ANTI-IGM INDUCED CELL GROWTH INHIBITION IN WIEN 133 CELLS BY CAMPATH-1H ANTIBODY. 131
FIGURE 4.15 DIFFERENCES IN THE KINETICS OF CAMPATH-1H ANTIBODY INDUCED GROWTH INHIBITION IN WIEN 133 CELLS COMPARED TO ANTI-IGM TREATMENT..... 132
FIGURE 4.16 CAMPATH-1H TREATMENT OF WIEN 133 CELLS INCREASES THE NUMBER OF CELLS UNDERGOING APOPTOSIS..... 133
FIGURE 4.17 ELECTRON MICROGRAPHS OF CAMPATH-1H TREATED CELLS UNDERGOING APOPTOSIS (A) COMPARED TO UNTREATED CELLS (B). 134
FIGURE 4.18 DNA LADDERING IN CAMPATH-1H TREATED WIEN 133 CELLS..... 135
FIGURE 4.19 PROPIDIUM IODIDE STAINING OF CAMPATH-1H TREATED WIEN 133 CELLS SHOWS DNA FRAGMENTATION..... 136
FIGURE 4.20 N-ACETYL CYSTEINE PARTIALLY BLOCKS CAMPATH-1H INDUCED GROWTH INHIBITION IN WIEN 133 CELLS..... 137
FIGURE 4.21 CYCLOSPORIN A CAN PARTIALLY BLOCK CAMPATH-1H INDUCED GROWTH INHIBITION IN WIEN 133 CELLS..... 138
FIGURE 4.22 EXPRESSION OF CD52 IN TRANSFECTED JE6-1 JURKAT CLONES..... 139
FIGURE 4.23 EXPRESSION OF CD52 IN TRANSFECTED J.RT3-T3.5 JURKAT CLONES. 140
FIGURE 4.24 EXPRESSION OF CD52 IN TRANSFECTED J45.01 JURKAT CLONES..... 141
FIGURE 4.25 PHENOTYPE ANALYSIS OF CD52 TRANSFECTED JURKAT JE6-1 CLONES..... 142
FIGURE 4.26 PHENOTYPE ANALYSIS OF CD52 TRANSFECTED JURKAT J.RT3-T3.5 CLONES. 143
FIGURE 4.27 PHENOTYPE ANALYSIS OF CD52 TRANSFECTED JURKAT J45.01 CLONES..... 144
FIGURE 4.28 CAMPATH-1H INDUCED GROWTH INHIBITION IN JURKATS IS DEPENDENT ON THE DENSITY OF CD52 AND EXPRESSION OF A FUNCTIONAL TCR..... 145
FIGURE 4.29 CAMPATH-1H SYNERGISES WITH ANTI-CD3 TO INDUCE CELL GROWTH INHIBITION IN JURKATS..... 146
FIGURE 5.1 WIEN 133 CELLS RESISTANT TO GROWTH INHIBITION BY CAMPATH-1H ANTIBODY TREATMENT REMAIN SENSITIVE TO GROWTH INHIBITION BY IGM..... 161

FIGURE 5.2 DECREASED EXPRESSION OF CD52 IN CV CLONES COMPARED TO PARENTAL WIEN 133 C1 CLONE. 162
FIGURE 5.3 CELLS RESISTANT TO KILLING BY MONOCYTES IN THE PRESENCE OF CAMPATH-1H HAVE A SIMILAR PHENOTYPE OF CD52 EXPRESSION AS THE CV CLONES 163
FIGURE 5.4 PHENOTYPIC ANALYSIS OF WIEN 133 C1 CELLS FOLLOWING *IN VITRO* SELECTION BY CROSSLINKING WITH ANTI-CD52 ANTIBODIES. 164
FIGURE 5.5 THE LEVEL OF CD52 ANTIGEN IS DECREASED IN CELLS RESISTANT TO CAMPATH-1H TREATMENT. 165
FIGURE 5.6 THE MESSENGER RNA LEVELS FOR CD52 IN CAMPATH-1H SELECTED CELLS REMAIN COMPARABLE TO THE WIEN 133 PARENTAL CELL LINE. 166
FIGURE 5.7 CD55 AND CD59 SURFACE EXPRESSION IS REDUCED IN THE CAMPATH-1H SELECTED CELLS COMPARABLE TO REDUCED LEVELS OF SURFACE CD52. 167
FIGURE 5.8 WESTERN BLOT ANALYSIS OF CD59 ANTIGEN REVEALS COMPARABLE LEVELS IN THE WIEN 133 PARENTAL CELL LINE AND THE CV CLONES. 168
FIGURE 5.9 CV CLONES REVERT TO EXPRESSION OF SURFACE CD52 AT LEVELS COMPARABLE TO THE PARENTAL WIEN 133 CELLS FOLLOWING GROWTH *IN VIVO*. .. 169
FIGURE 5.10 *EX VIVO* CV CLONES ARE SENSITIVE TO GROWTH INHIBITION IN THE PRESENCE OF CAMPATH-1H. 170
FIGURE 5.11 SURFACE IGD EXPRESSION ON WIEN 133 CELLS IS NOT SENSITIVE TO PI-PLC TREATMENT. 171
FIGURE 6 1 CD52 LIGATION INDUCES TYROSINE PHOSPHORYLATION IN CD4+ T CELLS. . 188
FIGURE 6.2 THE PATTERN OF CD52 AND CD4 INDUCED TYROSINE PHOSPHORYLATION IS COMPARABLE. 189
FIGURE 6.3 CD52 LIGATION INDUCES TYROSINE PHOSPHORYLATION IN THE MF14 T CELL CLONE. 190
FIGURE 6.4 IMMUNOPRECIPITATION OF TYROSINE PHOSPHORYLATED PROTEINS FROM CD52 ACTIVATED CELLS DIFFERS FROM CD3 ACTIVATED CELLS. 191
FIGURE 6.5 HERBIMYCIN A PARTIALLY INHIBITS CD52 INDUCED CD4+T CELL PROLIFERATION. 192
FIGURE 6.6 HERBIMYCIN A PARTIALLY INHIBITS CD52 INDUCED TYROSINE PHOSPHORYLATION IN CD4 + T CELLS. 193
FIGURE 6.7 CAMPATH-1H DOES NOT SYNERGISE WITH ANTI-CD3 TO INDUCE A CALCIUM FLUX IN CD4+ T CELLS. 194
FIGURE 6.8 CD52 LIGATION INDUCES TYROSINE PHOSPHORYLATION IN WIEN 133 B CELLS: COMPARISON OF THE LEVELS OF TYROSINE PHOSPHORYLATION INDUCED BY CROSSLINKING THE CD52 ANTIGEN IN THE WIEN 133 B CELL LINE COMPARED TO THE CV5 CELL LINE. 195

FIGURE 6.9 CD52 COPRECIPITATES OTHER PROTEINS IN WIEN 133 B CELLS. 196
FIGURE 6.10 DETECTION OF CD52 IN CAMPATH-1H IMMUNOPRECIPITATES. 197
FIGURE 6.11 CD52 DOES NOT COPRECIPITATE LYN..... 198

List of tables

TABLE 3.1 CROSSLINKING CD52 IN THE PRESENCE OF IL-2, IL-4 AND IL-7 INDUCES CD4+ T CELL PROLIFERATION..... 80
TABLE 3.2 CAMPATH-1H INDUCES CYTOKINE RELEASE CD4+ T CELLS 80
TABLE 4.1 EFFECT OF CYTOKINES ON CAMPATH-1H INDUCED GROWTH INHIBITION IN WIEN 133 CELLS.....106
TABLE 4.2 ANTI-FAS ANTIBODY DOES NOT INDUCE GROWTH INHIBITION IN WIEN 133 CELLS.....110
TABLE 4.3 SUMMARY OF THE SURFACE PHENOTYPES OF CD52 TRANSFECTED JURKAT CELL CLONES.....115

Abbreviations

ADCC	Antigen dependent cell cytotoxicity
AP	Alkaline phosphatase
APC	Antigen presenting cell
ATP	Adenosine 5'-triphosphate
BCR	B cell antigen receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDR	Complementarity determining regions
CEA	Carcinoembryonic antigen
CHO	Chinese hamster ovary
C-1G	CAMPATH-1G
C-1H	CAMPATH-1H
cm	Centimetre
cpm	Counts per minute
CREB	cAMP response element binding protein
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte antigen
CV	CAMPATH variant
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanine 5'-triphosphate
dTTP	2'-deoxythymine 5'-trriphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
FACS	Fluorescence activated cell sorter

FADD	Fas associated death domain protein
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Gram
GM-CSF	Granulocyte monocyte colony stimulating factor
GPI	Glycosylphoshatidylinositol
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IFN-γ	Interferon-γ
IP_3	Inositol 3,4,5-triphosphate
IL	Interleukin
ITAM	Immunoreceptor tyrosine based activation motif
HRP	Horseradish peroxidase
JNK	c-Jun amino terminal kinase
kb	Kilobase pairs
KDa	Kilo Daltons
LFA	Lymphocyte function antigen
LPS	Lipopolysaccharide
m	Membrane
M	Mole
mA	Milli Amps
MAP	Mitogen-activated protein
mg	Milligram
MHC	Major histocompatability complex
MIgG	Mouse immunoglobulin
ml	Millilitre
mM	Millimolar
MR	Monocyte resistant
MS	Multiple sclerosis
NCAM	Neuronal cell adhesion molecule
NFAT	Nuclear factor of activated T cells
nm	Nanometre

PAGE	Polyacrylamide Gel Electrophoresis
PI3-K	Phosphatidylinositol 3-kinase
PI 4-P	Phosphatidylinositol 4-phosphate
PI 3,4-P_2	Phosphatidylinositol 3,4-biphosphate
PI 4,5-P_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PLC	Phospholipase C
pmol	Picomole
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SDS	Sodium dodecyl sulphate
SH	Src homology
SHP	SH2-domain containing phosphatase
SHIP	SH2-domain containing inositol polyphosphate 5-phosphatase
SSC	Saline sodium citrate
Tc	T cytotoxic
TCR	T cell receptor
Th	T helper
TNF	Tumour necrosis factor
TRADD	TNF receptor associated death domain protein
µg	Microgram
µl	Microlitre
µM	Micromolar
mRNA	Messenger ribonucleic acid
Tris	Tris (hydroxymethyl) aminomethane
PNH	Paroxysmal nocturnal haemoglobinuria
w/v	weight for volume
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
v/v	volume for volume
ZAP	Zeta associated protein

1. Introduction.

1.1 The immune system

The immune system has evolved to protect individuals against invading pathogens (eg. bacteria, viruses, protozoa, fungi, worms) or toxins (eg. snake venom, bacterial toxins).

The defense mechanisms available to the host can be subdivided into innate and acquired immunity (Reinisch and Litman, 1989).

Innate, or natural, immunity includes the use of physical barriers (eg skin), phagocytic cells which simply engulf foreign material (eg macrophages), granulocytes containing toxic molecules (eosinophils, basophils and neutrophils), natural killer cells and blood borne molecules (eg. complement). Natural immunity provides the first line of defence against foreign invasion, but is not antigen specific (Abbas et al., 1994).

In contrast, acquired, or adaptive, immunity is specific for a given antigen. This is achieved by a group of specialised cells called lymphocytes which are characterised by the possession of antigen specific surface receptors. Lymphocytes therefore, confer antigen specificity to an immune response. In addition, specific immunity enables the immune system to acquire memory. On future encounter to the same antigen, memory allows a rapid recognition and expansion of a cell population which can quickly deal with the invader (Gray, 1993).

The function of lymphocytes is dependent on antigen presenting and accessory cells which present antigen, provide the necessary costimulatory signals and secrete cytokines thereby initiating immune responses. Thus, innate and acquired immunity cooperate together to induce an immune response relevant to protecting against a specific antigen/organism (Janeway, 1992a).

Lymphocytes are subdivided into B cells and T cells. B cells recognise free antigen by means of surface immunoglobulin and respond by clonally expanding and differentiating into effector cells which produce soluble immunoglobulin (antibody). Serum antibodies

bear the same specificity as the parental cell and deal with antigen by neutralisation, activating the complement system or by opsonising antigens leading to enhanced phagocytosis by various cells such as macrophages. In addition, different antibody isotypes bind to specific Fc receptors on the surface of eosinophils, natural killer cells and mast cells and in response to antigen binding, trigger the functions of these cells (Burton and Woof, 1992). B cells thus confer humoral immunity.

T cells mediate cell immunity. Like B cells, T cells bear antigen specific receptors on their surface. In contrast to B cells, which recognise soluble antigen, T cells only see antigen in the form of short peptides bound to surface molecules of the Major Histocompatibility Complex (MHC) on other cells, including dendritic cells, B cells and macrophage antigen presenting cells, (APCs). T cell antigen recognition is followed by clonal expansion and differentiation into effector cells releasing distinct patterns of cytokines. The functions of effector T cells include help for B cell activation, macrophage activation or direct cytolytic activity (Griffiths, 1995; Mosmann and Coffmann, 1989).

The activation of B and T cells following specific antigen recognition can have functionally different outcomes. B and T cells, can respond to receptor ligation by undergoing clonal expansion and differentiation to produce effector cytokines, they can become anergised, or they die by apoptosis.

1.2 T cell lymphocyte development and the lymphoid system

Precursor T cells migrate from the bone marrow to the thymus via the bloodstream. In the cortex of the thymus, developing T cells start to express randomnly generated T cell receptors (TCRs) together with other proteins including CD3, CD4 and CD8. The expression of these proteins initiates the process of positive and negative selection. The end result is the generation of the peripheral T cell repertoire. Both foreign and self derived peptides are presented to the T cells by MHC class I and II proteins expressed on a variety of cells found in the thymus. These include thymic epithelium, macrophages

and dendritic cells. Failure of the TCR to recognise peptide bound to MHC molecules will trigger programmed cell death. Positive selection therefore, confers MHC restriction on a T cell - it will not respond to antigen unless the antigen is presented by a recognised MHC molecule. Recognition of peptide bound to MHC molecules by high affinity binding TCRs also results in programmed cell death. This ensures that abundant peptides which will largely represent self proteins, will not trigger inappropriate T cell responses on future encounters. Negative selection means that the mature T cell repertoire is self tolerant due to deletion of self reactive T cell clones in the thymus (Blackman et al., 1990; Kappler et al., 1987; van Boehmer and Kisielow, 1990).

When mature T cells leave the thymus, they enter the blood stream. At this stage, they are referred to as naive T cells because they have not encountered antigen. These cells are characterised by expression of CD45RA and by expression of high levels of L-selectin. L-selectin binds to specialised endothelium within lymph nodes and at sites of inflammation and mediates homing of naive T cells to lymph nodes (Bradley et al., 1994). If a naive T cell does not encounter its specific antigen within the lymph node, it leaves via efferent lymphatic vessels and eventually returns to the blood via the thoracic duct. There is a continuous recirculation of lymphocytes between the blood and the lymph organs and so there is a constant surveillance of the resident APC and their antigen cargoes (Goodnow, 1997). When a naive T cell does encounter its specific antigen in the lymph node, it becomes activated and enters into cell cycle thus clonally expanding a population of T cells specific for a given antigen. Triggering of a T cell via its specific antigen receptor increases expression of adhesion proteins, including LFA-1, VLA-4, VLA-5, VLA-6 and CD44 which help maintain the T cell:APC contact (Shimizu et al., 1990; Haynes et al., 1989). As the T cells differentiate, the affinity of the adhesion molecules decreases and enables the recently activated T cell to detach from the APC and return to the blood stream. Activated cells may recirculate and enter sites of inflammation. The majority of such cells die by apoptosis and this maintains homeostasis. A fraction survive and following the disappearance of antigen, return to a resting state. These T cells acquire a memory phenotype and are characterised by the expression of CD45RO (Young et al., 1997). Memory T cells also express low levels of L-selectin and as a consequence, no longer home efficiently to the lymph nodes. Instead by virtue of the new set of adhesion molecules they now express, migrate preferentially to

peripheral endothelium to sites of inflammation, which are often the sites of antigen entry (Cush et al., 1992; Tietz and Hamann, 1997). The selective migration of memory T cells to sites of antigen entry or of naive T cells to lymphoid organs is known as homing (Picker and Butcher, 1992; Bevilacqua, 1993).

1.2.1 Antigen presentation

Zinkernagel et al., 1997, proposed that antigen regulates the immune response which is dependent on antigen localisation, antigen dose and time. Antigen is concentrated within secondary lymphoid organs which means that the activation of lymphocytes is not dependent on chance encounters. This is important because the precursor frequency of lymphocytes for any given antigen is estimated to be less than 1/100,000 (Pape et al., 1997). Dendritic cells, which are found dispersed throughout the body in both solid tissue and the blood, take up antigen and transport it to secondary lymphoid organs, including the spleen, lymph nodes, mucosa associated lymphoid tissues and the cutaneous immune system. The structural organisation of lymphoid tissues optimises intimate contact and short range interactions between the cell populations that contribute to immune responses. Within the lymphoid tissues, different lymphocyte and non lymphocyte subpopulations concentrate in different areas of the lymph nodes which ensures that the right combinations of cells are making the necessary contacts (Mondino et al., 1996).

Antigen is also transported directly to lymph nodes via the lymphatic system. Lymph contains soluble and particulate self and foreign antigens. APCs residing in the lymph nodes trap antigen as it enters (Steinman et al., 1997).

The capture of antigen by APCs is achieved by different mechanisms in different cell types. Dendritic cells (DCs) are very effective APCs which take up particles and microbes by phagocytosis. They form large pinocytic vessicles in which extracellular solids and fluids are sampled (macropinocytosis) and they express receptors that mediate absorptive endocytosis, eg. C-type lectin, as well as the Fcγ and Fcε receptors. In the

presence of a number of different stimuli eg. IL-1, LPS, GM-CSF and TNF-α, DCs 'mature'. Mature DC are inefficient at capturing antigen, but very effective at antigen processing and presentation. These cells upregulate surface expression of MHC class II molecules and costimulatory molecules, including B7.1 and B7.2 (Reviewed by Banchereau and Steinman, 1998). DCs can stimulate both B and T cell activation (Tew et al., 1997).

Different types of APC may be involved at different stages of a T cell mediated immune response to protein antigens. For example, dendritic cells may be the primary cell involved in stimulating naive T cells. Subsequent exposure to the same antigen may lead to a differentiation phase of the immune response in which the activated T cells may develop into effector cells in response to stimulation by other types of APC such as B cells or macrophages (Abbas et al., 1994).

1.2.2 The Major Histocompatibility Complex (MHC)

MHC class I and class II gene products both display peptide/MHC complexes continuously as a normal function of the cell. The difference between the two MHC types is the way that antigen is acquired. MHC class I proteins associate with peptides generated proteolytically within the cytoplasm. In contrast, MHC class II molecules acquire peptides from proteins that have been taken up exogenously and endocytosed within the cell. Therefore, whether a protein peptide is presented to T cells via class I or class II, is dependent on the way in which the protein found its way into the cell.

1.2.2.1 MHC class I processing

Cytosolic proteolysis and delivery of peptides into the exocytotic pathway is achieved by the proteasome and the transporter in antigen processing (TAP) genes. Protein destined for degradation becomes covalently linked to ubiquitin polypeptides which targets the

protein to proteasomal enzymatic activity. The products of the TAP-1 and TAP-2 genes, pick up cytosolic peptides and transport them across the membrane, into the lumen of the endoplasmic reticulum (ER). Assembly and surface expression of stable class I MHC molecules requires the presence of peptides. Therefore, newly synthesised class I heavy chains and β2-microglobulin in the ER, bind peptide to form a stable MHC class I/peptide complex. The MHC class I/peptide protein complex is then transported across the ER, via the Golgi, to the cell surface (Elliot, 1997).

1.2.2.2 MHC class II processing

The first step in MHC class II processing is the uptake of the native protein by an APC. Antigen uptake by APC is achieved by various means in different cell types. Macrophages and dendritic cells bind many different antigens with little or no specificity, to surface molecules. Antibody coated (opsonised) antigens can be taken up by means of both Fc receptors on cells (CD16, CD32 and CD64) or alternatively, if CR3b complement protein is bound by the antibody, by complement receptors (CR3 and CR4). Uptake is by phagocytosis or by receptor mediated endocytosis in clathrin coated vesicles. Soluble antigens can be taken up by pinocytosis which does not require binding to the cell surface. Internalised antigens are localised in endosomes or lysosomes, both of which have an acidic pH, required to maintain enzymatic activity for proteolysis generating small peptides. MHC class II molecules consist of an αβ heterodimer and are kept in an unoccupied state by the invariant chain which effectively blocks the peptide binding cleft. The invariant chain also directs the movement of the MHC class II molecule out of the ER and through the Golgi complex to the membrane bound organelles of the endocytic pathway. Within the specialised MHC peptide loading compartment, the low pH and the chaperone protein, HLA-DM, cause the dissociation of the invariant chain and allow the MHC class II molecule to bind to peptides. MHC class II/peptide complexes are then transported to, and expressed at, the membrane surface (Kropshofer et al., 1997).

1.3 Functions of peripheral T cells

1.3.1 CD4+T cells

Helper T cells express the TCR co-receptor, CD4 molecule on their surface. CD4 recognises MHC class II molecules on the surface of APC. Depending on the context of the original signal, eg. peptide concentration, the type of APC and the cytokines secreted, the nature of costimulatory signals eg. B7.1 or B7.2 and CD40, CD4+ effector T cells can be further subdivided into Th1 and Th2 types based on the profile of cytokines they secrete (Constant and Bottomley, Rincon and Flavell, 1997).

In *in vitro* culture, CD4+ T cells cells stimulated in the presence of IL-12 and IFN-γ will differentiate to a Th1 cell phenotype, characterised by the production of inflammatory cytokines, including IFN-γ and TNF-α (Bradley et al., 1995). Dendritic cells and macrophages produce IL-12 and can direct the development of Th1 cells from naive CD4+ T cells (Macatonia, 1995; Heufler, 1996). TNF and IFN-γ upregulate MHC class I expression so increase the sensitivity of cells to CTL lysis. Therefore, Th1 cells predominate in response to viral infections and play a role in controlling the proliferation of tumour cells. Th1 cells enhance cellular immunity by activating cells such as macrophages and neutrophils (Belardelli, 1995).

In contrast, naive cells stimulated in the presence of IL-4, differentiate into a Th2 cell phenotype, characterised by the production of IL-4, IL-5, IL-6, IL-10 and IL-13 (Bradley et al., 1995; Abbas et al., 1994). The exact source of IL-4 required to initiate polarisation towards a Th2 phenotype is unclear, but recently, APC derived IL-6 has been shown to induce IL-4 in naive T cells (Rincon et al., 1997). Other possible sources include activated mast cells, basophils, NK cells and some CD8+ T cells, all of which are capable of producing IL-4 (Abbas et al., 1994). Th2 cells, via production of IL-4, are involved in the regulation of allergic reactions. IL-4 is the principal cytokine involved in stimulating the switch of B cells to producing IgE. IgE binds to receptors expressed on the surface of mast cells which induces release of histamines. Therefore, IgE is the principal mediator of immediate hypersensitivity reactions. In addition, IgE is important for controlling

helminthic infections. IL-4 inhibits macrophage activation, thereby inhibiting the production of cytokines, including IL-1, nitric oxide and prostaglandins. IL-4 is a growth factor for mast cells and synergises with IL-3 leading to mast cell proliferation. Another effect of IL-4 is to induce upregulation of the vascular cell adhesion molecule (VCAM) on endothelial cells which results in increased binding of lymphocytes, monocytes and eosinophils. Many of the functions of IL-4 are also induced by IL-13. IL-5, IL-6 and IL-10 all have roles in B cell activation and B cell differentiation. In addition, IL-5 activates eosinophils and IL-10 inhibits macrophage activation (Abbas et al., 1994).

1.3.2 CD8+ T cells

CD8+ T cells are commonly referred to as cytotoxic T lymphocytes (CTLs). Phenotypically, they can be distinguished from CD4+ T cells by the expression of the CD8 co-receptor molecule, which binds to cells expressing MHC class I molecules. Almost all cells express MHC class I, which ensures that any cell expressing foreign or mutant proteins can be targetted for killing by CTLs. Thus, CD8+ T cells act to protect the host from the threat of intracellular viral, protozoal or bacterial pathogens and potentially tumorigenic cells.

Like CD4+ T cells, they can be separated on the basis of cytokine profiles into two subsets, Tc1 and Tc2. This is dependent on the nature of the cytokines present during differentiation. Tc1 cells have been defined as CD8+ T cells secreting IFN-γ, but not IL-4 and IL-5, whereas Tc2 cells secrete IL-4 and IL-5, but not IFN-γ. IL-3, IL-6 and IL-10 are secreted by both Tc1 and Tc2 subsets but at much lower levels in Tc1 compared to Tc2 cells. Both Tc1 and Tc2 cells mediate cytotoxicity and can elicit DTH reactions. However, the expression of various cytokines suggests that CD8+ T cells can have other functions in addition to being cytotoxic and a role has been implicated in suppression of immune responses. The perforin knockout mouse has a higher incidence of tumour development suggesting a role for CD8+ T cells in immune surveilllance (Mosmann et al., 1997).

CD8+ T cell cytotoxicity is achieved by two main pathways (Henkart, 1997). The first, and the predominant cytokilling mechanism comprises the granule exocytosis pathway. TCR engagement on a naive CD8+ T cell, together with a second co-stimulatory signal, induces *de novo* synthesis of lytic granules containing perforin and serine proteases (granzymes). Granzymes are also found in NK and LAK cells. Cell/cell conjugation is followed by the migration and fusion of cytoplasmic granules with the plasma membrane and the subsequent release of perforin and granzymes into the intracellular space. Upon contact with a neutral environment and in the presence of calcium, perforin polymerises and forms pores on the target cell through which granzymes are able to pass. Cell death occurs via apoptosis, possibly by the direct effect of granzyme B by activating ICE proteases (caspases) within the target cell (Miller, 1997; reviewed by Pharm and Ley, 1997). The second pathway is by ligation of Fas on target cells. The role of Fas in signalling cell death is described in more detail later.

A single CTL can lyse multiple targets. Once primed, CTLs only require ligation via the TCR in order to lead to a very rapid degranulation (Reviewed by Griffiths, 1995).

1.4 T cell signalling

Activation of mature T cells by ligation of the T cell receptor can have functionally different outcomes - The cell can be induced to proliferate, it can respond by becoming functionally unreactive to further signals (anergised) or, alternatively, the cell can be induced to undergo apoptosis. In addition, T cells produce a range of cytokines and cell surface activation antigens.

The destiny of a naive T cell depends on the nature and the context in which the cell receives signals and how these signals are subsequently processed. For example, Valitutti et al., 1995, demonstrated that an activation signal by an agonist peptide, triggers TCRs serially, which as a consequence of ligation are down-regulated. In contrast, an antagonist peptide has been shown to interfere with the ongoing process of serial triggering (Viola et al., 1997).

1.4.1 The TCR signal (Signal 1).

The primary activation of naive T cells requires two signals after adhesion to APCs (Rudd et al., 1994). The first signal is via ligation of the T cell receptor (June et al., 1989).

The T cell receptor (TCR) complex comprises the variable α and β chains which together form the antigen recognition site. The invariant CD3 δ, ε and γ chains, together with the homodimer zeta chains are responsible for initiating signal transduction.

Following antigen recognition, the invariant chains of the TCR complex become tyrosine phosphorylated on conserved motifs (ITAMS or immunoreceptor tyrosine activation motifs) by src kinases, including lck and fyn (Reth, 1989; Weiss and Littman, 1994). Lck is non covalently associated with the cytoplasmic tails of both CD4 and CD8 (Veillette et al., 1988). Fyn has been shown to be constitutively associated with the TCR and is activated following TCR ligation (Cantrell, 1996). The phosphorylated ITAMS are able to bind SH2 domain containing proteins, including the tyrosine kinase, ZAP-70 (Chan et al., 1992). ZAP-70 is believed to play a pivotal role in T cell activation following the identification of defective ZAP-70 in a family group suffering from severe immunodeficiency (Elder et al., 1994; Chan et al., 1994). Adaptor proteins, including Grb-2, binding to ITAMS regulate the recruitment of downstream signalling pathways (Pawson and Scott, 1997).

Grb-2 is constitutively bound by the GTP/GDP exchange factor, Sos. Grb-2 binding to the ITAMS, thus brings Sos into close proximity with GTP binding Ras. The GTP binding proteins control a series of serine/threonine kinase cascades (MAPK cascades). The MAPK cascades, including the extracellularly regulated kinases (Erks), the stress activated protein kinases (SAPKs) and p38 kinase, constitute a group of signal transduction pathways characterised by the successive phosphorylation of coupled serine/threonine or dual specificity kinases. The conserved signalling module consists of a mitogen activated protein kinase (MAPK), a MAPK kinase (MAPKK), normally a dual specificity kinase that phopshorylates MAPK on threonine and tyrosine residues and a MAPKK kinase (MAPKKK) which phosphorylates MAPKK on serine and activates it (Marshall, 1995).

In T cells, the most important MAPK pathway linking the TCR to the cell interior leading to cell proliferation, is thought to be the Erk pathway. Ras-GTP binds to Raf-1 (MAPKKK) which becomes activated and phosphorylates Mek-1 and Mek-2 (MAPKK). These in turn phosphorylate and activate Erk-1 and Erk-2 which translocate to the nucleus and directly phosphorylate transcriptional regulatory proteins, including NFAT, Jun and Fos (Hardy and Chaudhri, 1997).

The SAPKs (also known as Jun NH2-terminal kinases or JNKs) are activated by inhibitors of protein synthesis, inflammatory cytokines eg. TNF-α and IL-1, and more generally by severe cellular stress. The small GTP binding proteins, Rac-1 and Cdc24, Pak-1 and Ras can activate the cascade leading to the activation of c-Jun and ATF-2 (Paul et al., 1997). SAPK activation is blocked by Cyclosporin (Su et al., 1994).

In addition to Sos, Grb-2 binds a 36 KDa protein, recently identified as LAT (Zhang et al., 1998). LAT becomes heavily phosphorylated following TCR ligation which coincides with the recruitment to the plasma membrane and the activation of PLC-γ. PLC-γ hydrolyses membrane phospholipids such as PI 4,5-P_2, generating the phosphoinositide second messengers, inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG)(Cantrell, 1998).

IP_3 binds to fyn phosphorylated receptors expressed on the ER membrane (Reviewed by Alberola-Ila et al., 1996) stimulating the release of calcium from intracellular stores. The best characterised downstream effector of the calcium signal is the calcium/calmodulin dependent serine phophatase, calcineurin. Calcineurin is the target of the immunosuppressive drug, Cyclosporin A. Substrates of calcineurin include NFAT transcription factors which activate IL-2 transcription and CaM-kinase II which inhibits IL-2 induction (Schreiber and Crabtree, 1992; Wiederrecht et al., 1993). The interactions between these two competing calcium dependent pathways is not yet understood and illustrates the complexity of the activation and regulation of signals emanating from the TCR.

DAG activates the serine kinase PKC family.

Other proteins which bind to Grb-2 include the adaptor protein SLP-76 and this in turn can bind to the adaptor protein Vav (Tuosto et al., 1996). A key role for Vav in TCR signalling is suggested by Vav knockout mouse models, in which B and T cell proliferation are impaired (Tarakhovsky et al., 1995). HS1, Lnk, Shb and TRIM have recently been identified as further adaptor proteins involved in proximal TCR signalling (Takaki et al., 1997; Welsh et al., 1998; Taniuchi et al., 1995; Bruyns et al., 1998). A wide range of adaptor proteins, therefore, provide the potential for a diverse range of effector mechanisms and regulation to be elicited by ligation of the TCR (Pawson and Scott, 1997; Peterson et al., 1998).

The diversification of signals probably initiates as a consequence of differences in phosphorylation at the level of the ITAMS on the TCR invariant chains (Shores and Love, 1997). The three ITAMS of the zeta chain are capable of binding different cytosolic effectors, including lck, fyn, ZAP-70 and syk which could lead to functionally different outcomes (Osman et al., 1995).

Phosphatases are involved in the regulation as well as the activation of kinase signals (Frearson and Alexander, 1996; Neel, 1997; Altin and Sloan, 1997). The most widely studied phosphatases known to influence the TCR signal are CD45 and SHP1. CD45 is a receptor-type phosphatase expressed at very high levels on the surface of haematopoietic cells and exists as numerous isoforms due to alternate splicing of exons encoding for the extracellular domain. Possible ligands for CD45 include CD4 (Dianzani et al., 1992). CD45 substrates include lck, fyn, ZAP-70 and possibly zeta (Furukawa et al., 1994; Mustelin et al., 1995). Both lck and fyn are negatively regulated by C terminal phosphorylation of a tyrosine residue and dephosphorylation by CD45 results in kinase activation (Mustelin and Burn, 1993). CD45 may also dephosphorylate tyrosine residues in fyn and lck and thereby reduce their activity (Ostergard and Trowbridge, 1990; Burns et al., 1994). SHP-1 suppresses TCR signals, associates with vav, Grb-2 and Sos and can bind and dephosphorylate ZAP-70 after T cell stimulation (Neel and Tonks, 1997).

Other examples of regulatory phosphatases include Pac-1, Mkp-1 and Mkp-2. These are induced by activation of the MAPK cascade and contribute to switching off the MAPK signal (Paul et al., 1997). The phosphatase SHP-2 associates with CTLA-4 which is a

negative regulator of TCR signalling (Reviewed by Scharenberg and Kinet, 1996; Unkeless and Jin, 1997). T cell signalling mediated via the TCR is represented schematically in figure 1.1.

1.4.2 T cell Costimulation (Signal 2).

Ligation of the TCR alone is insufficient to lead to T cell proliferation. A second signal is provided by one of several additional complexes on the T cell surface (Yi-Qun et al., 1996; Chambers and Allison, 1997). CD28 is regarded as one of the most important co-stimulatory molecules required for T cell activation (Bachmann et al., 1997; Reviewed by Janeway and Bottomley, 1994). However, studies with CD28 knock out mice suggest that CD28 is not indispensible for T cell activation (Shahinian et al., 1993).

In addition to CD28, other molecules which can provide costimulatory signals and induce T cell proliferation include CD2 (Clarke and Ledbetter, 1986), LFA-1 (Seventer et al.,1990) and 4-1BBL (DeBenedette et al., 1997). It is not clear whether these molecules induce second signals required for full T cell activation per se, or whether they act merely to provide a strong interaction between the T cell and the APC (Bachmann et al., 1997). All these proteins, in addition to extracellular domains, have cytoplasmic domains capable of linking up to intracellular signalling machinery. In contrast, there is a group of proteins which lack intracellular domains and are bound solely to the outer cell membrane via a glycophosphatidylinositol (GPI) anchor. Ligation of these proteins by antibodies can provide costimulatory signals to T cells necessary to induce cell proliferation. These include human CD55, CD59 and CD73 (Robinson, 1991; Deckert et al., 1995; Resta et al., 1994), murine Thy-1 and Ly-6, and rat RT-6 (Robinson, 1991) (see sections 1.8.1 and 1.8.2). CD28 is expressed on resting and activated T cells and its ligands, B7.1 (CD80) and B7.2 (CD86) are found on antigen presenting cells (APC) including B cells, macrophages and dendritic cells. Following a primary immune response, CD28 on the T cells and B7.1 and B7.2 on the APCs are both upregulated (Jenkins, 1994).

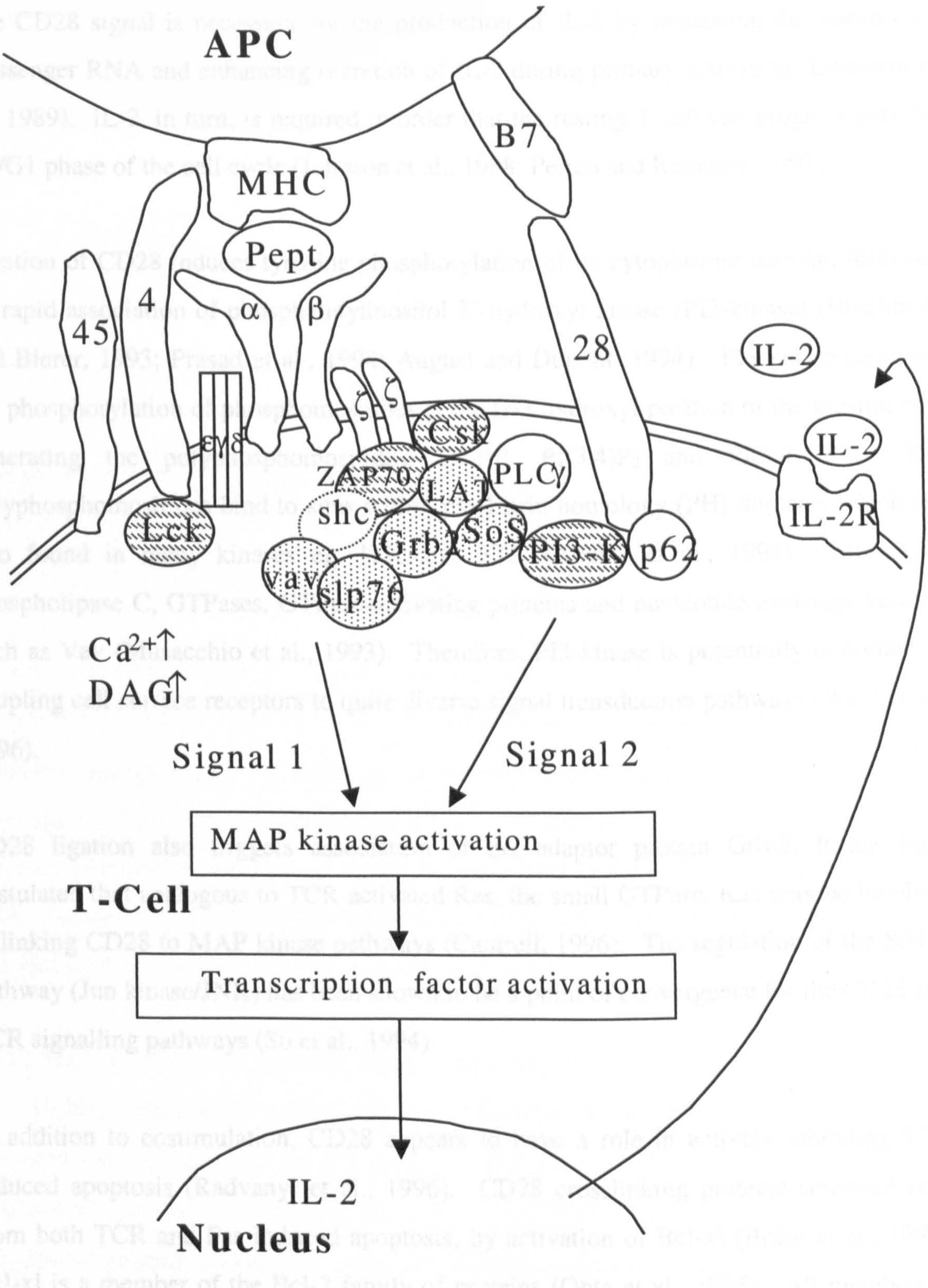

Figure 1.1 T cell signalling

Antigen bound in the context of MHC molecules is recognised by the TCR. ITAM motifs located on the TCR associated invariant chains (ε, δ, γ and zeta) are phosphorylated by src family kinases eg. lck. This enables recruitment of kinases (eg. ZAP-70) and adaptor proteins (eg. LAT) forming signalling complexes which mediate the release of secondary messengers (eg. DAG, calcium) and activate MAP kinase pathways. The convergence of signals from the TCR and CD28 leads to transcription factor activation and IL-2 transcription.

The CD28 signal is necessary for the production of IL-2 by increasing the stability of messenger RNA and enhancing secretion of IL-2 during primary activation (Lindstein et al., 1989). IL-2, in turn, is required in order that the resting T cell can progress past the G0/G1 phase of the cell cycle (Johnson et al., 1988; Perico and Remuzzi, 1997).

Ligation of CD28 induces tyrosine phosphorylation of its cytoplasmic domain, followed by rapid association of phosphatidylinositol 3'-hydroxyl kinase (PI3-kinase) (Hutchcroft and Bierer, 1993; Prasad et al., 1994; August and Dupont, 1994). PI3-kinase catalyses the phosphorylation of phosphoinositides at the D-3 hydroxyl position of the inositol ring generating the polyphosphoinositides, PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3. The polyphosphoinositides bind to sites within pleckstrin homology (PH) domains which are also found in many kinases eg. ITK and BTK (August et al., 1994); isoforms of phospholipase C, GTPases, GTPase-activating proteins and nucleotide exchange factors, such as Vav (Musacchio et al., 1993). Therefore, PI3-kinase is potentially important in coupling cell surface receptors to quite diverse signal transduction pathways (Ward et al., 1996).

CD28 ligation also triggers association of the adaptor protein Grb-2. It has been postulated that analogous to TCR activated Ras, the small GTPase, Rac may be involved in linking CD28 to MAP kinase pathways (Cantrell, 1996). The regulation of the SAPK pathway (Jun kinase/JNK) has been shown to be a point of convergence for the CD28 and TCR signalling pathways (Su et al., 1994).

In addition to costimulation, CD28 appears to have a role in actively inhibiting TCR induced apoptosis (Radvanyi et al., 1996). CD28 crosslinking protects activated cells from both TCR and Fas induced apoptosis, by activation of Bcl-xl (Boise et al., 1995). Bcl-xl is a member of the Bcl-2 family of proteins (Ohta et al., 1995). All members of the Bcl-2 family are able to bind Bcl-2, forming heterodimers and heteromultimers as demonstrated in a yeast two-hybrid system (Sato et al., 1994). Bcl-2 itself promotes cell survival as does Bcl-xl; in contrast, Bax induces cell death. The fate of cells is therefore determined by the ratio of positive versus negative protein interactions. Bcl-xl is also induced by ligation of the TCR alone, but declines rapidly unless CD28 is stimulated.

CD28 could therefore be important for either stabilising Bcl-xl mRNA levels or preventing Bcl-xl protein degradation (Mueller et al., 1996).

1.5 T cell anergy

1.5.1 Functional anergy.

Anergy is defined as a cellular state in which a lymphocyte remains viable but fails to respond to stimulation under optimal antigen specific and costimulation signals and does not produce IL-2 (Schwartz, 1996).

In vitro, signalling via the TCR, in the absence of costimulation, induces an anergic state (Harding et al., 1992). IL-4 and IFN-γ transcription are not affected by an anergic signal. However, anergy blocks the ability of the cells to become competent to proliferate to an IL-4 signal (Chiodetti and Schwartz, 1992). Becker et al., 1995, demonstrated the presence of increased amounts of a negative regulatory factor, Nil-2a, in anergic human T cells. Ligation of CD28 was shown to block production of Nil-2a. Therefore, both Th1 and Th2 cells can be anergised by lack of costimulation.

IL-10 has been shown to facilitate anergy induction and the mechanism proposed is that the cytokine functions by blocking the costimulatory pathway. IL-10 can convert activated macrophages to a resting monocyte-like state in which the production of pro-inflammatory cytokines, including IL-1, IL-12 and TNF-α and costimulatory molecules are downregulated. Dendritic cells are likewise affected (Schwartz, 1996). IL-10 downregulates class II and B7 molecules on DCs and APCs.

Altered peptide ligands (APL), as opposed to agonist peptide ligands, have been shown to induce a state of anergy (Madrenas et al., 1995).

One consequence of anergy in the T cell, is failure to express the CD40 ligand, therefore B cells are also anergised because they lack the appropriate second costimulatory signal

to become fully activated (Durie et al., 1994). CD40 crosslinking is also important for DC maturation and release of IL-12 for Th1 differentiation.

Studies with superantigens and adoptive transfer experiments with T cells from transgenic mice have demonstrated induction of anergy, or tolerance, *in vivo* (Schwartz, 1996).

1.5.2 Anergic signals.

A number of groups have provided evidence that there are clear changes in TCR proximal signals and downstream events leading to functional anergy. For example, studies performed comparing TCR peptide ligands to altered peptide ligands (APL), demonstrated a notable absence of ZAP-70 phosphorylation in response to TCR ligation by the APL, compared to the agonist ligand (Madrenas et al., 1995).

Boussiotis et al., 1996, showed that ligation of the TCR by alloantigen, in the absence of a second signal, resulting in functional anergy, activated tyrosine phosphorylation of TCR zeta and its association with fyn. In contrast, TCR ligation in the presence of B7 co-stimulation, activated tyrosine phosphorlyation of zeta and CD3 chains which associated with activated lck and ZAP-70. Similarly, studies by Salojin et al., 1994, found that hyporesponsive TCR signalling in cells from NOD mice appeared to correlate with elevated fyn kinase activity, at the expense of ZAP-70 activity, compared to normal mice.

Fyn phosphorylates the adaptor protein, Cbl. Phosphorylation of Cbl induces its dissociation from Grb-2 and its association with Crk, which binds the GTP/GDP exchange factor, C3G. The complexing of Crk with C3G activates Rap1. Activation of Rap1 coincides functionally with anergy induction (Boussiotis et al., 1997).

1.6 T cell apoptosis.

Antigen induced apoptosis in T cells is an important mechanism for maintaining T cell tolerance and homeostasis. Apoptosis is involved in the deletion of potentially self reactive T cells during thymic selection and in controlling an ongoing T cell response by limiting clonal expansion. CD8+ T cells, as mentioned in section 1.3.2, can induce target cell death by apoptosis.

T cell survival is controlled by expression of members of the tumour necrosis factor receptor (TNFR) family, including TNFR1 and TNFR2, Fas and TRAIL (Mariani and Krammer, 1998; Wong and Choi, 1997). The failure of apoptotic mechanisms to operate can lead to potentially deleterious effects with a breakdown in self tolerance leading to autoimmunity and lymphoproliferative disorders. Defects in apoptotic inductive genes have been shown to occur in the lpr and gld mouse models which have defects in the Fas protein (CD95) and Fas ligand repectively (Nagata and Golstein, 1995). A genetic defect in humans which results in mutations in Fas causes the autoimmune lymphoproliferative syndrome (ALPS) (Puck and Sneller, 1997).

Apoptosis occurs in two stages consisting of an inductive phase triggered by TCR stimulation and then an effector phase which involves the biochemical means necessary for cell death to occur. Fas ligand and TNF bind to their respective receptors, Fas and TNFR1. Fas and TNFR1 contain an aptly named 'death domain' within their cytoplasmic tails which mediates the induction of apoptosis in target cells. Following receptor ligation, FADD is recruited to the cytoplasmic domain of Fas and, likewise, TNFR recruits TRADD (Golstein, 1997). FADD and TRADD, in turn activate caspase cascades which culminate in DNA breakdown and cell apoptosis (Miller, 1997; Salvesen and Dixit, 1997). Recruitment of FLIP to the Fas receptor inhibits the binding of FADD and blocks the apoptosis pathway. Therefore, Fas expression is not sufficient to induce apoptosis and regulation of FLIP in T cells is critical to susceptibility to cell death (Irmler et al., 1997).

1.7 B cell lymphocyte development

B cells, like T cells originate in the bone marrow. During foetal development, B cell maturation proceeds in the liver and subsequently in the bone marrow and the periphery. Maturation of B cells can be followed through a series of distinct phenotypes. The precursor B cell does not possess surface Ig and so can not recognise or respond to antigen. Surface IgM is acquired by immature B cells. Immature B cells respond to antigen stimulation by undergoing apoptosis or functional unresponsiveness (tolerance). This property is important for maintaining tolerance to self antigens that the B cells encounter. Following migration from the bone marrow to the periphery and lymphoid tissues, B cells continue to mature even in the absence of antigenic stimulation. These cells are characterised by expression of both surface IgD and IgM (Abbas et al., 1994).

B cells concentrate in the germinal centres of secondary lymph organs where they detect soluble antigens or may recognise antigens that have been stored and concentrated on the surfaces of follicular dendritic cells (Tew et al., 1997). Depending on the nature of the antigen, B cells are regarded as T cell dependent and independent. Thus polysaccharide antigens which consist of multiple repeating epitopes are able to effectively aggregate surface receptors and do not require further signals from helper T cells. In contrast, protein antigens tend to contain single epitopes and these require T cell help in the form of secondary stimulation in order to get full B cell activation (Nishioka and Lipsky, 1994).

B cells take up antigen via surface immunoglobulin and process and present it to T cells in the context of MHC class II molecules. Stimulation via the T cell receptor, induces the T cell to upregulate CD40 ligand (CD154) expression, which binds to CD40 expressed on the surface of B cells, inducing the B cell to upregulate B7.1 and B7.2. B7.1 and B7.2 bind their cognate ligands, CD28 and CTLA-4, on the T cell. The T cell response is to produce IL-4 which acts as a growth factor for the B cell. IL-4 induces B cell clonal expansion and differentiation into either antibody secreting cells or memory B cells, which will respond rapidly in producing antibody on subsequent re-exposure to the antigen (Van Kooten and Banchereau, 1997). Blocking the CD40/CD40 ligand interaction results in a nonactivated B cell and an anergic T cell (Durie et al., 1994). In

naive B cells, ligation of the B cell receptor in the absence of a signal via CD40 results in cell death (Liu et al., 1991). The same occurs in the immature B cell line, WEHI 231 (Choi et al., 1995). Thus B and T cells are largely interdependent on each other for full activation. Protection from apoptosis coincides with upregulation of Bcl-xl expression. B cell/T cell interactions and signals are represented in figure 1.2.

1.7.2 B cell activation and signalling

Activation of B cells is initiated by ligation of surface immunoglobulin by soluble antigen which can be in the form of protein, polysaccharide or lipid. Like the TCR, the BCR has no intrinsic signalling capacity. The BCR is associated with the invariant chains, Igα (CD79α) and Igβ (CD79β) which exist as a transmembrane heterodimer. In the cytoplasmic domains of both CD79α and CD79β, is an ITAM. The ITAM is phosphorylated by members of the src family of tyrosine kinases including Lyn and Fyn, both of which are associated with the resting BCR via CD79α. Syk is also associated at low levels with the BCR prior to activation.

CD45 and csk are involved in regulating the src family kinase activity, as in T cells. Loss of CD45 results in hypoactive lyn in resting cells, as well as following BCR stimulation, and therefore, appears to be required for lyn activation. In contrast, the loss of csk results in a significant increase in the phosphorylation of substrates following BCR ligation. Therefore, BCR signalling is regulated by the balance of Csk and CD45 activities. Phosphorylation of the ITAMS leads to increased binding of src family kinases and Syk.

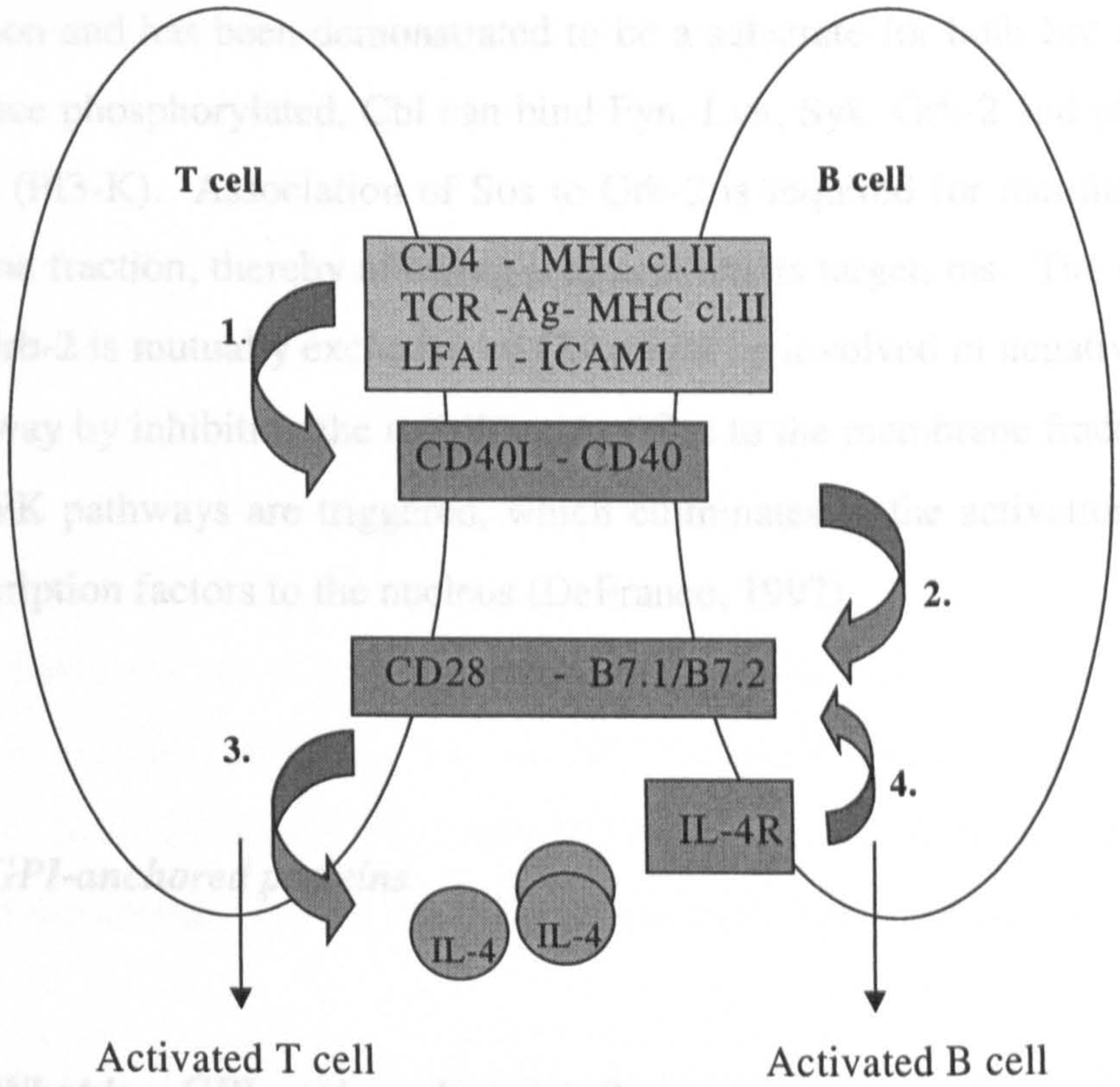

Figure 1.2 T cell/B cell interactions and signals.

Ligation of the TCR by antigen presented in the context of MHC class II molecules, leads to upregulated CD40 ligand expression (1.). CD40 ligand binds CD40 leading to upregulated B7.1 and B7.2 (2.). B7.1 and B7.2 upregulate IL-4 production by the T cell (3.) IL-4 binds to IL-4 receptors on B cells and T cells leading to cell differentiation (4.).

Increased activity of src family kinases, following BCR activation, correlates with increased activity of a Tec family kinase, Btk. Cbl is phosphorylated upon BCR stimulation and has been demonstrated to be a substrate for both Src family kinases and Syk. Once phosphorylated, Cbl can bind Fyn, Lyn, Syk, Grb-2 and phosphatidylinositol 3-kinase (PI3-K). Association of Sos to Grb-2 is required for recruitment of Sos to the membrane fraction, thereby allowing it to activate its target, ras. The binding of Sos and Cbl to Grb-2 is mutually exclusive so Cbl might be involved in negative regulation of the ras pathway by inhibiting the recruitment of Sos to the membrane fraction. As in T cells, the MAPK pathways are triggered, which culminates in the activation and translocation of transcription factors to the nucleus (DeFranco, 1997).

1.8 GPI-anchored proteins

1.8.1 What is a GPI anchored protein?

Membrane proteins can be broadly classified into two types; firstly, the integral membrane proteins, including the TCR, CD4, CD8 and CD28 in T cells, and the BCR, CD19 and CD40 in B cells. These proteins possess extracellular and intracellular protein domains.

The second group of membrane proteins are linked to the membrane via a covalent interaction of the protein with lipid and include glycosylphosphatidylinositol, or GPI-anchored proteins. GPI-anchored proteins have been found in organisms as diverse as protozoa, insects, yeasts, slime moulds, mammals and higher plants. The GPI-anchored proteins themselves are as diverse as the organsims in which they are found, both in terms of structure and function. Their molecular mass varies from 15 kDa to 300 kDa; most are additionally glycosylated but the extent and the nature of glycosylation varies considerably (Low and Finean, 1977; Ferguson and Williams, 1988).

The GPI precursor is transferred onto the polypeptide via a transamidase reaction following cleavage of the C terminal peptide. This allows the C terminus of the protein to be covalently linked to the GPI moiety (Yeh et al., 1994). Synthesis and assembly of GPI-anchored proteins is described in more detail in chapter 5.

The discovery of GPI anchored proteins followed observations made over forty years ago, that animals infected with *Bacillus anthracis* (Anthrax) had increased alkaline phosphatasemia (Smith et al., 1955). Later investigations showed that this was due to production of a soluble factor released by the bacteria which was identified as a phosphatidyl inositol specific phospholipase C (PI-PLC) (Low and Finean, 1977). PI-PLC mediated release of proteins from membranes is now recognised as an indicator of anchoring via GPI, although not all GPI anchored proteins are sensitive to PI-PLC treatment. In addition to PI-PLC, GPI-PLD is capable of releasing GPI-anchored proteins from cell membranes and the sites of action of both these proteins are shown in figure 1.3.

During the past 20 years, there has been an ever growing, diverse family of GPI-anchored proteins identified. These include hydrolytic enzymes such as alkaline phosphatase, ecto 5' nucleotidase (CD73) and acetyl choline esterase (Hooper, 1997). GPI-anchored decay accelerating factor (DAF) or CD55, and Protectin or CD59, regulate the activation of the complement cascade (Kirschfink, 1997). GPI-anchored proteins regulate cell adhesion and examples include NCAM, LFA-3, VCAM, Heparan sulphate proteoglycan and CEA. GPI anchors comprise the majority of protozoan parasite cell surface antigens eg. variant surface glycoprotein, VSG, in trypanosomes; major merozoite surface antigen in Plasmodium spp. (Cross, 1990). Other GPI-anchored proteins include CD14, the receptor for LPS; the Fc receptor, CD16 FcRIII; and the scrapie prion protein, Prp, the infectious agent for the disease, scrapie, which affects sheep and induces BSE in cattle. A number of GPI-anchored proteins have been implicated as having a role in T cell activation. These proteins include murine Thy-1, Ly6, J11d and Qa-2, rat RT-6, human DAF, CD59 and CD73 (Robinson, 1991).

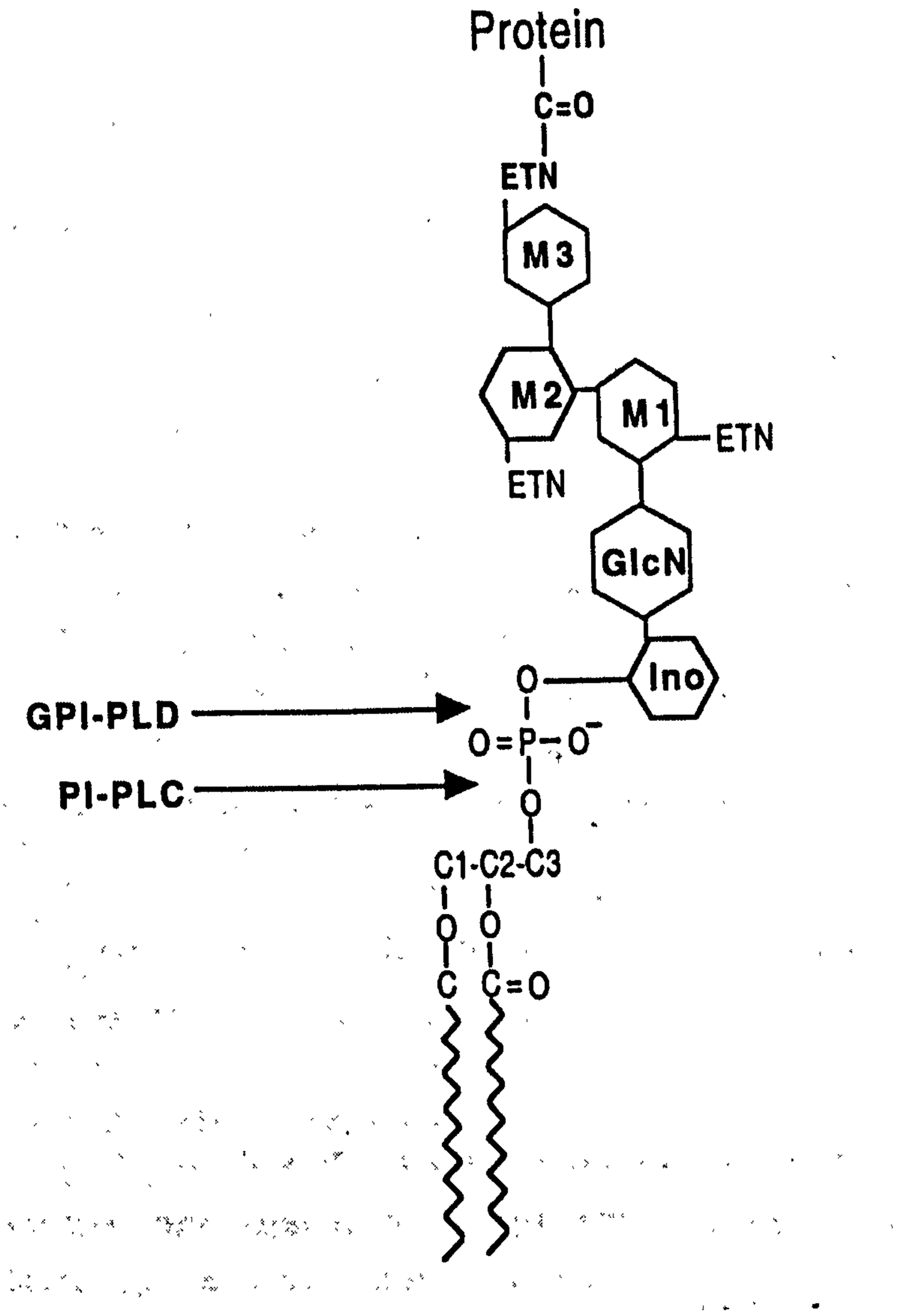

Figure 1.3 Generalised structure of a GPI-anchor

Ino, inositol; GlcN, glucosamine; M, mannose; ETN, ehanolamine phosphate. The conserved GPI core, which consists of ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6Inositol, is indicated by bold type lettering. The cleavage sites for PI-PLC and GPI-PLD are indicated by an arrow (Yeh et al., 1994).

1.8.2 The function of GPI anchors

The use of GPI tails for membrane attachment instead of a conventional transmembrane confers unique properties to a protein. A GPI tail gives increased lateral mobility to a protein in the membrane, a novel mechanism for shedding and or intercellular transfer of proteins (Reviewed by Censullo and Davitz, 1994; Anderson, 1994; Gaulton and Pratt, 1994; Ilangumaran et al., 1996).

Lateral mobility in the membrane is of obvious benefit to proteins like adhesion molecules such as LFA-3. LFA-3 is required for cell/cell interactions and freedom of movement in the membrane would enable the protein to localise and concentrate itself to the area of cell/cell contact maximising its effect.

Shedding one protein coat and expressing a new variant protein coat enables trypanosomes to effectively evade and delay recognition by the hosts immune system (Cross, 1990). Downmodulation of CD14 is achieved by shedding after activation of monocytes (Bazil and Strominger, 1991).

Intercellular transfer of proteins has been shown to occur in the epididymal tract where the complement regulatory proteins, CD55 and CD59 are secreted by epidermal cells and become incorporated into the outer spermatozoal membrane thus protecting the cells from the potential hostile environment which it is due to encounter in the female reproductive tract (Rooney et al., 1996).

A GPI-anchored mucin released from the membrane of *T. cruzi*, can alter macrophage function by binding to an epitope expressed by or proximal to CD62 L (L Selectin). As a consequence of this, TNF-α and IL-12 (Th1 type cytokines) were both inhibited which might contribute in the evasion of the immune response by the parasite (de Diego et al., 1997).

Anchorage by GPI would apparently preclude proteins from transmembrane signalling but this does not appear to be the case. Studies in T cells have used antibodies to mimic the effects of the putative ligands for proteins including Thy-1, Ly-6, Qa-2, CD55 and

CD59 and shown that resting T cells can be induced to proliferate (Robinson, 1991). Thus proteins with ascribed functions, for example, the complement regulatory proteins, CD55 and CD59 and the enzyme, ecto 5'nucleotidase (CD73) have also been shown to induce cell activation (Robinson, 1991; Resta et al., 1994). It is not clear how messages are transduced by such proteins and various models have been put forward and are discussed in more detail in chapter 6.

Studies in epithelial cells, have found that some GPI-anchored proteins become clustered together in small invaginations in the membrane termed caveolae (Lisanti et al., 1993). Caveolae are characterised by the association of a 22 kDa protein termed caveolin. In addition to caveolin, src family tyrosine kinases, small heterotrimeric G proteins, PKC isoforms and both an ATPase dependent Calcium pump as well as an IP_3 dependent calcium channel are found in these clusters (Fujimoto, 1993). Caveolae are involved in the uptake of small molecules via potocytosis.

Caveolae and caveolin are not found in T cells. In T cells, membrane domains have been identified and have been designated as DIGS (Detergent insoluble glycosphingolipid domains). DIGs consist of clusters of GPI anchored proteins eg. Thy-1, Ly-6A, CD48, CD52, CD55, CD59, together with numerous signalling proteins, including src family kinases, lck and fyn, CD45 phosphatase and the recently characterised adaptor protein, LAT (Brdicka et al., 1998). Immunoprecipitation studies have shown that GPI-anchored proteins can co-precipitate a kinase activity suggesting these might be functionally active (Stefanova et al., 1991). Both caveolae and DIGS are dependent on interactions with membrane lipids to maintain their integrity and cholesterol chelating agents such as nystatin and filipin cause the GPI-anchored proteins to disperse. Cells can then no longer be activated by ligation of GPI-anchored proteins (Stulnig et al., 1997).

1.9 The CD52 antigen

The CD52 antigen (CAMPATH-1 antigen) consists of a 12 amino acid mature protein which is linked to the cell membrane via a GPI-anchor. The peptide sequence is GQNDTSQTSSPS. The GPI-anchor is attached to the Ser-12 residue. The protein is very heavily glycosylated which accounts for a molecular weight of 21-28 kDa as determined by SDS PAGE followed by Western blotting (Hale et al., 1990; Xia et al., 1991). There are sites for both N and O linked glycosylation, but studies have shown that only the N linked site at Asn-3 is occupied by sugar side chains. The N-linked oligosaccharides consist of polylactosamine residues and these account for upwards of 50% by weight of the molecule (Treumann et al., 1995). The precise molecular weight of the N deglycosylated antigen was determined by electrospraymass spectrometry and found to be in exact agreement with the predicted molecular weight, suggesting that no other structures are present. The structure of the CD52 antigen is illustrated in figure 1.4.

The antigen in the form recognised by the CAMPATH-1 antibodies is found widely distributed on monocytes and lymphocytes but is not expressed on stem cells. Lymphocytes express the antigen at an estimated 5×10^5 copies per cell (Bindon et al., 1988). This is compared to the TCR which is expressed at 2.5×10^4 copies per cell (Male et al., 1987). Surface expression is lower in monocytes. Within granulocyte populations, it is expressed at significant levels on the surface of eosinophils but not on neutrophils and has been proposed as a marker to distinguish the two cell populations (Elsner et al. 1996).

In addition, CD52 has been termed the 'major maturation associated' sperm membrane antigen. CD52 is expressed at high levels within the epididymal epithelium, from where it appears that possession of a GPI-anchor allows the protein to be released and transferred onto the sperm cell (Perry et al., 1992; Kirchhoff, 1996).

Studies in monkeys have shown that lymphocytes of old world monkeys express the CD52 antigen whereas lymphocytes from new world species are negative. In baboons and macaques, erythrocytes are also positive for CD52 expression. In contrast, in

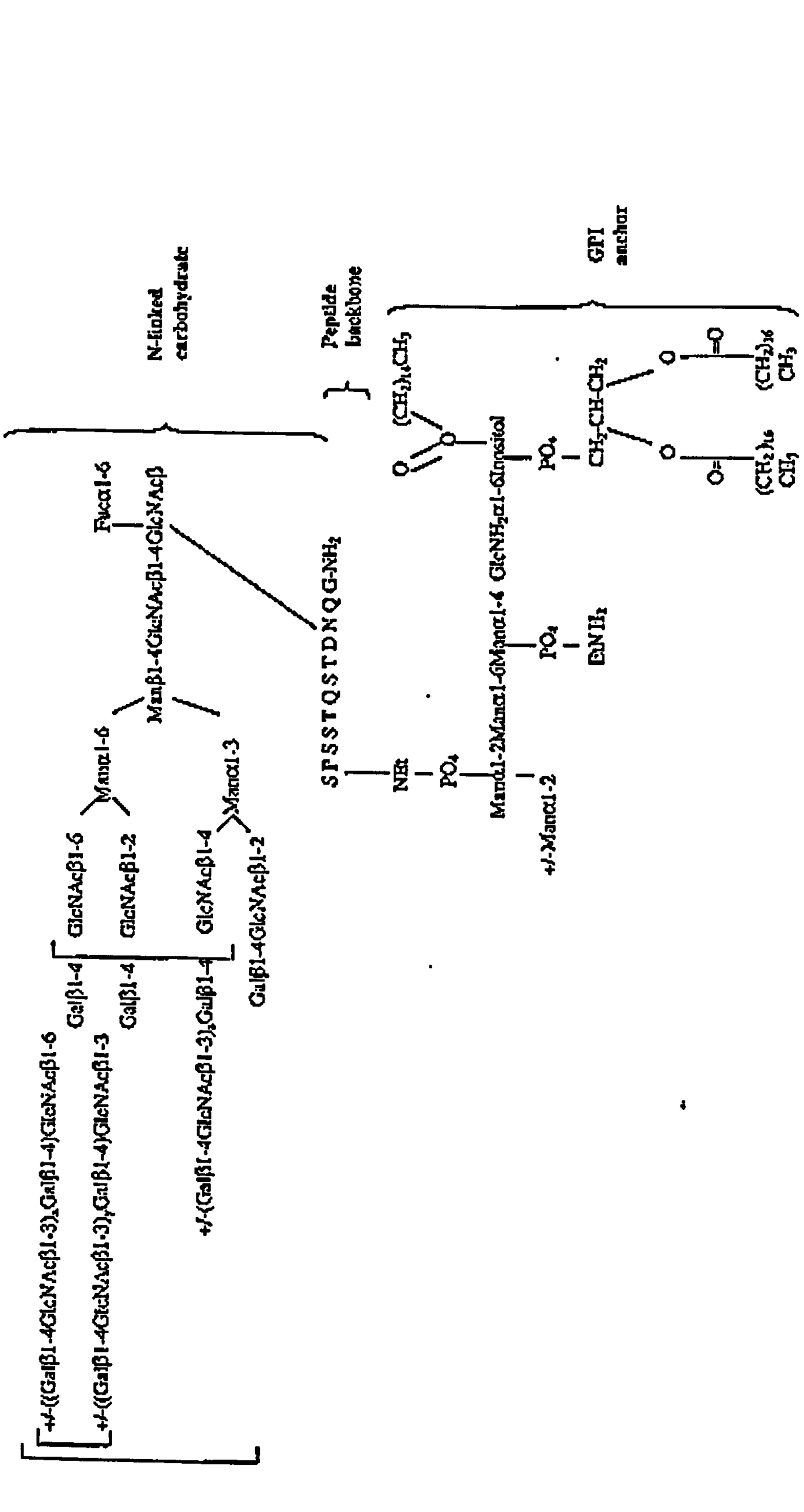

Figure 1.4 Structure of the CD52 antigen

(R. Lifely, personal communication; Treumann et al., 1995)

cynomolgous monkeys, erythrocytes can be negative or positive (Hale et al., 1990). This suggests that in monkeys, at least, the CD52 antigen has a redundant/non-essential role in cell function.

The CD52 antigen has been purified from human spleens by a chloroform/methanol extraction followed by antibody affinity chromatography (Xia et al., 1991). The CD52 antigen could be further purified by octyl sepharose chromatography, which is based on differences in hydrophobicity. This resulted in the separation of two distinct fractions differing in their susceptibility to phosphatidylinositol-specific phospholipase C. Approximately 50% of the CD52 antigen can be removed from lymphocytes by PI-PLC treatment (Hale et al., 1990). This might reflect the finding that there are two possible forms of the antigen, dependent on the site of GPI anchor attachment (Hale et al., 1996).

The physiological function of CD52 is still unknown.

1.10 CAMPATH-1 Antibodies

The CAMPATH-1 antibodies were generated in Professor Herman Waldmann's laboratory (Department of Pathology, University of Cambridge) during attempts to generate monoclonal antibodies which could be used as immunosuppressive agents to control transplant rejection and for the treatment of autoimmune disease. A series of monoclonal antibodies were produced by immunising rats with human T lymphocytes. CD52 specific antibodies, designated as CAMPATH-1 antibodies, were discovered due to their ability to induce T cell lysis in the presence of complement (Hale et al., 1983a; Hale et el., 1983b). These antibodies are amongst the most powerful and specific lymphocytic agents in humans (Hale, 1995).

The panel of monoclonal antibodies was notable for the fact that it consisted of nearly all IgG_{2c} or IgM antibodies. These isotypes are commonly made in response to carbohydrate epitopes and were thought to reflect the nature of the heavily glycosylated CD52 antigen. However, CAMPATH antibodies bind to the deglycosylated antigen and to a proteolytic

fragment, but not to synthetic peptide alone which suggests that the GPI anchor and the CD52 C-terminal amino acids, SPS, combine to form the epitope. The epitope recognised by CAMPATH-1 antibodies has been synthetically generated using peptide chemistry (Hale, 1995). The site of antibody recognition on the natural antigen would be located very close to the membrane which could account for the efficiency of the antibodies in mediating cell lysis (Xia et al., 1993).

One exception in the panel of monoclonal antibodies generated, was an IgG_{2a} isotype from which IgG_1 and IgG_{2b} (CAMPATH-1G) class switch variants were obtained (Hale et al., 1987). All IgM and IgG isotypes recognising the CD52 antigen are lytic in the presence of human complement. CAMPATH-1M antibody has been used to reduce the severity of Graft Versus Host Disease (GVDH) in transplantation studies; however, only the IgG_{2b} isotype proved to be effective during clinical studies to treat cases of lymphoid malignancies (Dyer et al., 1989). This is attributed to the ability of the IgG_{2b} isotype to invoke effector mechanisms including antibody dependent cell cytotoxicity (ADCC) by binding to Fc receptors on cells such as natural killer (NK) cells and macrophages (Wing et al., 1996).

A potential problem in using rat antibodies to treat human patients, was the development of an anti-immunoglobulin response. This would reduce the potential of the rat antibodies for therapeutic purposes especially in cases where the antibodies may require administration over long periods or for retreatment eg. in cases of reappearing lymphoid malignancies. In attempts to avoid this, the CAMPATH-1G antibody was the first antibody selected for 'humanisation'.

Humanisation of CAMPATH-1G utilised the 'CDR grafting technique', in which the CDRs (complementarity determining regions) from the rat antibody were 'transplanted' onto a suitable human immunoglobulin framework (Riechmann et al., 1988). The product of these manipulations, CAMPATH-1H, proved to be as effective as CAMPATH-1G, in both complement mediated and cell mediated cell lysis of human lymphocytes (Crowe et al., 1992).

1.10.1 The targets for CAMPATH-1 therapy

1.10.1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common autoimmune disease with a prevalence of 1% in the population. The cause of the disease is unknown. It is characterised by chronic inflammation of the synovial joints and infiltration of blood derived cells, particularly memory T cells, macrophages and plasma cells, all of which show signs of activation. This leads to progressive destruction of bone and cartilage, believed to be mediated by enzymes, including matrix metalloproteases, activated by cytokines. Familial genetic studies have demonstrated the involvement of certain MHC molecules (DR4 and DR1) and this has implicated a role for T cells at some stage of disease pathogenesis. CD4+ T cells may be involved in presentation of autoantigens, the nature of which is not clear. Collagen type II is one possible candidate for an autoantigen.

The role of T cells in established RA is not understood. T cell involvement has been shown by improved disease prognosis, following Cyclosporin A and anti-CD4 therapy. T cells do not appear to actively proliferate in RA joints and only very few cells producing IL-2 or INF-γ are found. T cells could therefore, be involved in disease induction and maintenance of disease. Thus reduced T cell function might be caused by the inhibitory cytokine environment with elevated IL-10 and TGF-β.

Most patients have IgM autoantibodies termed rheumatoid factors (Rfs) which recognise the Fc region of IgG. These are not present in all patients. Immune complexes of RF and IgG may contribute to disease activity by activating complement and stimulating cytokine synthesis. There is abnormal glycosylation of IgG in active RA.

TNF-α appears to head a proinflammatory cytokine cascade. The cytokines induced include both anti (IL-10, IL-1 rα, soluble TNF receptor) and pro-inflammatory cytokines (IL-6, IL-8, GM-CSF) and thus the balance of the two, towards proinflammatory decides the disease outcome (reviewed by Feldmann et al., 1996).

1.10.1.2 Multiple sclerosis

Multiple sclerosis (MS) is the most common autoimmune disease involving the nervous system. In common with RA, the cause of the disease is unknown. Studies looking at the prevalence of multiple sclerosis in monozygotic twins suggest that genetic factors are important. However, the concordance of the disease in monozygotic twins is 30% which means there are also environmental factors involved. MS is characterised by inflammation of venules surrounding and extending into the myelin sheath. Myelin reactive T cells can be detected in normal peripheral blood and one possibility is that these cells become activated by microbes. Therefore, similarities between an autoantigen and antigens expressed by infectious agents may lead to inadvertent autosensitisation during the course of the immune response to the foreign antigen. Myelin basic protein (MBP) shares extensive protein sequence homology with a number of common pathogens including measles, hepatitis B, influenza virus and adenovirus. MBP specific T cell clones derived from MS patients can be activated in response to contact with microbes (Wucherpfennig and Strominger, 1995). Once activated, T cells can cross the blood: brain barrier by virtue of the expression of integrins including VLA-4. VLA-4 binds to adhesion molecules on the surface of inflamed endothelium (Brocke et al., 1994). Th1 cytokines, such as IFN-γ, have been shown to worsen disease when given systemically to patients with MS and TNF-α is increased during exacerbations of MS. Th1 cytokines can increase the expression of MHC class II on endothelium and upregulate VLA-4. TNF-α kills myelinating cells in culture. Once immune cells access the white matter of the central nervous system, the immune response is targetted to several different antigens on myelin. There is an antibody response against myelin and the complement cascade is activated with membrane attack complexes appearing in the spinal fluid. T cells are targetted to myelin antigens and become activated producing cytokines. The cytokines in turn, induce macrophages to attack myelin and phagocytose large chunks of the myelin sheath. The consequence of the immune cell activity is demyelination of the myelin sheath surrounding nerve fibres. This results is impaired conduction of nerve impulses and produces pathological defects (Reviewed by Steinman, 1996).

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1.10.1.3 Lymphoproliferative disorders

Leukaemias are clonal expansions of lympho-haematopoietic cells. Generally, acute leukaemias present with high numbers of immature lympho-haematopoietic cells (blasts) in the blood and/or the bone marrow. In chronic leukaemias, the bulk of the neoplastic population is represented by mature looking cells. The incidence of acute and chronic myeloid leukaemias progressively increases with age. Acute lymphoblastic leukaemia, occurs more frequently in childhood and accounts for a sharp peak for leukaemia at 3- 4 years of age (Campana, 1998).

Lymphomas are tumours arising from lymphoid tissue. They are commonly multicentric, due to early spread. The spreading reflects the normal migratory behaviour of the cells of origin. The vast majority of human lymphomas of recognised lineage arise from T or B lymphoid cells and are usually confined to a restricted range of maturation. Lymphoma presents usually in a major lymphoid organ eg. bone marrow, thymus, spleen or mucosal tissue (Stevenson, 1998).

Dyer et al., 1990, looked at surface expression of CD52 on different lymphoid malignancies. 90% of B cell and 62 % of T cell malignancies were CD52 positive. Therefore, not all lymphoid malignancies are responsive to CAMPATH-1 therapy.

1.10.1.4 Transplantation.

Transplantation is the process of taking cells, tissue or organs (a graft) from one individual (the donor) and placing them into a recipient. A major setback in establishing successful transplants has been the natural immune response to foreign antigens (alloantigens) when grafts are transplanted between two genetically unidentical members of the same species (allografts). The immune response to alloantigens can be both cell mediated or humoral. The frequency of T cells in a recipient reactive with allogeneic MHC molecules can be as high as 2 per cent. This is because the foreign MHC molecule provides scope for presentation of many different determinants. Rejection of allografts is

generally as a result of recognition by both CD4+ and CD8+ T cells. The mixed lymphocyte reaction (MLR) has provided a model for understanding the cellular basis af alloantigen recognition by different T cell subpopulations. Mononuclear cells (T cells, B cells, NK cells, mononuclear phagocytes and dendritic cells) from two different individuals are cultured together. If differences exist between the MHC alleles of the two individuals, cell proliferation is induced. Allografts that differ from recipients at both class I and class II loci, leads to activation of both CD4+ and CD8+ T cells (Abbas et al., 1994).

Graft rejection can occur by different mechanisms. Alloreactive T cells can recruit and activate macrophages, initiating graft injury by delayed type hypersensitivity response. Alloreactive CTLs directly lyse graft endothelial and parenchymal cells. Alloantibodies bind endothelium, activate the complement system and injure graft blood vessels. If the recipient of an allograft has a fully functional immune system, transplantation almost invariably results in some form of rejection (Abbas et al., 1994). To overcome this, the graft recipients immune system can be suppressed or the graft can be made less immunogenic.

1.10.2 Clinical trials with CAMPATH-1 antibodies

CAMPATH-1H has been used in clinical trials to treat patients suffering from a number of immune disorders, for example, lymphoid malignancies (Dyer et al., 1997; Mehta et al., 1997) and autoimmune diseases (RA and MS)(Isaacs et al., 1992; Moreau et al., 1996), as well as for the prevention of GVDH in bone marrow transplantation (Hale et al., 1988a; Hale and Waldmann, 1994).

CAMPATH-1H treatment depletes peripheral B and T lymphocytes, but spares monocyte and NK cell populations. Patients are therefore, left severely immunosuppressed following treatment. B cell numbers return to pretreatment levels within a few months, but the number of peripheral blood CD4+ T cell lymphocytes do not fully recover, even after 2 years post-treatment (Brett et al., 1996a).

Treatment with CAMPATH-1 antibodies has been shown to benefit patients. For example, clinical trials in a group of patients suffering from rheumatoid arthritis resulted in 56% patients undergoing a remission period lasting between 3-6 months (Matteson et al., 1995).

In patients suffering with CD52 positive tumours, the results were variable depending on the tumour type. Patients suffering from CLL and PLL have responded well to CAMPATH-1 therapy. In these patients, the blood, the bone marrow and the spleen were cleared of lymphocytes. Patients responding less favourably, included those bearing bulky tumours (Dyer et al., 1989). The reason for this could be due to poor vascularisation of the tumour and the failure of the antibody and effector mechanisms to gain access to the target cells.

One consequence of treating patients affected with RA and B cell non-Hodgkin lymphoma has been the emergence of CD52 negative cells (Hertenstein et al., 1995; Brett et al., 1996b). These are thought to arise as the result of the expansion of a minor population of pre-existing CD52 negative cells. The phenotype of cells affected ressembles that of lymphocytes seen in people affected by paroxysmal nocturnal haemoglobulinaemia (PNH). PNH patients have deficient expression of GPI-linked molecules predominantly on red cells, neutrophils and some lymphocytes (Schubert et al., 1994). The common defects in all cases looked at are heterogeneous somatic mutations arising in the PIG A gene (Takeda et al., 1993). The product of the PIG A gene is involved in the assembly of the preformed GPI anchor and the nascent polypeptide into the mature GPI-anchored protein. The characteristic clinical manifestations of PNH include complement mediated haemolysis due to the absence of the complement regulatory proteins, DAF and CD59 (Holguin, 1989). Other symptoms of PNH include venous thrombosis, an increased incidence of leukaemia that arises from the affected cells and a tendency for association with aplastic anaemia (Rosse, 1989). Hertenstein, et al., 1995, showed that CD52 negative T cells arising following CAMPATH-1 treatment of a patient with B cell non-Hodgkin lymphoma had a defect in the PIG-A gene message. In addition to CD52, the cells affected were also shown to be negative for other GPI-anchored proteins, including CD55 and CD59.

A second problem that arose as a consequence of CAMPATH-1H therapy ha.was a first dose reaction characterised by the release of inflammatory cytokines, including TNF-α, IFN-γ and IL-6 (Wing et al., 1996). In some cases, in patients suffering from Multiple sclerosis, this had the effect of transiently exacerbating disease symptoms (Moreau et al., 1996).

In addition, studies by Brett at al., 1996a, found that T cell populations reemerging after CAMPATH-1 therapy, expressed the DR activation marker and the memory phenotypic marker, CD45RO. This suggested that during the course of antibody treatment these cells had become activated.

Despite these setbacks, CAMPATH-1H antibody provides a means for effective depletion of lymphoid populations with clinical benefit demonstrated in rheumatoid arthritis (Isaacs et al., 1992; Isaacs et al., 1996) and blood borne lymphoid malignancies (Dyer et al., 1988; Osterborg et al., 1997a; Pawson et al., 1997).

1.11 Aims

The aims of this study were to study the biological effects of CAMPATH-1 antibodies on T and B lymphocytes *in vitro,* in order to understand the mode of action and the possible mechanisms of the side effects seen *in vivo.*

This was studied by examining:

1. Cellular effects of CD52 ligation on normal peripheral human T cells.
2. Cellular effects of CD52 ligation on both human B and T cell tumour cell lines.
3. Biochemical effects of CD52 ligation on normal T cells and B cell tumour lines.

2. Materials and Methods.

2.1 Materials.

2.1.1 Antibodies

The CD52 specific rat monoclonal antibody CAMPATH-1G (IgG_{2b}) is a variant derived by loss of the Y3 myeloma light chain and class switching from the hybridoma clone YTH-34.5 (IgG_{2a}) (Hale et al., 1987). The reshaped humanised version of this antibody was joined to the human IgG_1 constant region (CAMPATH-1H) and expressed in Chinese Hamster Ovary cells (CHO) grown in serum free medium (Crowe et al., 1992; Riechmann et al., 1988). CHO culture supernatants were concentrated by ultrafiltration. Therapeutic grade CAMPATH-1H (>99% monomeric) was obtained following sequential Protein A affinity chromatography, cation exchange and gel filtration chromatography. The humanised anti-CD4 IgG1 monoclonal antibody was also produced in CHO cells (Gorman et al., 1991). Rat monoclonal antibodies IgG_{2b} anti-CD3 (YTH 12.5), IgG_{2c} anti-CD52 (YTH 361.10), IgG_{2b} anti-CD18 (YTH 51.1) and anti-CD28 (YTH 913.12) were kindly provided by Dr Herman Waldmann, University of Cambridge, UK. The human IgG_1 anti-tetanus toxoid antibody used as an isotype control, rabbit anti-TNF 214 and mouse monoclonal anti-TNF (clone 357/101) antibodies were kindly provided by Dr Nick Rapson (Wellcome, Beckenham, UK). Mouse monoclonal anti-CD3 (OKT3) antibody was obtained from the European Tissue Culture Collection (Porton Down, UK). Purified mouse monoclonal anti-CD2 (39C15) was purchased from Immunotech (Marseille-Luminy, France). Goat intact and $(Fab)_2$ anti-human IgG, goat anti-human IgM, goat anti-human IgD and anti-rat IgG were obtained from Sigma, UK. Purified anti-human DR (clone L243), anti-CD8 (clone Leu 2a), anti-CD20 (clone Leu 16), anti-CD4 (clone Leu 3a), anti-CD45 RA and anti-CD45 RO were obtained from Becton Dickinson, UK. Anti-human CD16 was obtained from Seralab, UK. Mouse monoclonal anti-phosphotyrosine (clone 4G10), rabbit polyclonal anti-lyn, anti-ZAP-70, anti-lck and anti-PI3-kinase, mouse IgM anti-Fas antisera were obtained from T.C.S., UK. Unpurified antibodies where required, were purified from culture supernatant or from ascites fluid using an FPLC Protein G Superose column (Pharmacia, Sweden). Horse radish peroxidase, or alkaline phosphatase conjugated anti-rat, anti-rabbit and anti-mouse antibodies were obtained from Sigma, UK. FITC labelled CAMPATH-1H $(Fab)_2$ and

FITC labelled anti-CD4 were kindly provided by Fiona Crisp (Wellcome, Beckenham, UK). FITC labelled anti-IgG, anti-IgD, anti-IgM, anti-DR, CD3, CD4, CD8, CD16, CD19, CD20, CD25, CD32 and CD64 antibodies were obtained from Becton Dickinson, UK, or Sigma, UK.

2.1.2 Chemicals

Herbimycin A was obtained from Calbiochem, UK; Lavendustin and Genistein were obtained from ; LNMMA was kindly provided by John Rhodes (Wellcome); Cyclosporin A was obtained from Sandoz, Switzerland; N-acetyl cysteine, Phytohaemagglutinin (PHA), Phorbol myristate acetate (PMA), Cycloheximide and Fibronectin were obtained from Sigma, UK; General laboratory chemicals were obtained from Sigma, UK, BDH, UK or Fluka, UK.

2.1.3 Enzymes

N-Glycanase, PI-PLC, RNAse, Proteinase K and Klenow were obtained from Boehringer Mannheim, UK.

2.1.4 Nucleic acids

BS Ag4 Plasmid containing the entire coding sequence of the CD52 antigen as a Hind III/ Eco RI insert and a plasmid containing the neomycin selection marker (p321 Neo) were provided by Chris Hale (Wellcome).

2.1.5 Cell lines

The Wien 133 B cell lymphoma cell line (Nacheva et al., 1987) was obtained from A. Karpas (University of Cambridge, UK), recloned by limiting dilution and maintained in Iscoves medium supplemented with 10% heat inactivated FCS/100 IU penicillin/ 100mg/ml streptomycin. Jurkat E6-1, J45.01 and J.RT3-T3.5 acute T cell leukaemia cell lines were obtained from ATCC, Maryland, USA, and maintained in RPMI 1640 medium supplemented with 10% heat inactivated autologous serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technogies, UK). CTLL cells were maintained with 10 ng/ml recombinant IL-2 in RPMI 1640 medium supplemented with 10% heat inactivated FCS, L-glutamine and penicillin/streptomycin.

2.2 Purification of CD4 T cells

50 ml of defibrinated blood was collected as 2 x 25 ml aliquots in 50 ml centrifuge tubes. The blood was centrifuged at 2000 rpm in a bench top Sorvall Centrifuge for 10 minutes at 20°C. The serum was then removed and heat inactivated by heating in a waterbath at 56°C for 30 minutes. The remaining blood fraction in each tube was made up to a volume of 50 ml with PBS pH 7.2 and 4 x 25 ml layered onto 15 ml Lymphoprep (Flow Laboratories, UK) in 50 ml centrifuge tubes. Tubes were centrifuged at 2500 rpm for 20 minutes at 20°C. The buffy layers were carefully removed and transferred to 2 x 50 ml centrifuge tubes. The volume in each tube was made up to 50 ml with PBS pH 7.2 and the tubes were centrifuged at 2500 rpm for 10 minutes at 20°C. Cell pellets were resuspended in 20 ml RPMI containing 10% heat inactivated autologous serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technogies, UK), transferred to 75 cm^2 tissue culture flasks and incubated at 37°C, 5% CO_2 for 3 hours to allow monocytes to adhere. Non-adherent cells were carefully removed into a 50 ml centrifuge tube and centrifuged at 2000 rpm for 5 minutes at 20°C. The cell pellet was resuspended in 1 ml PBS pH 7.2. To the cell suspension, 100 µl anti-DR (clone L243), 100 µl anti-CD8 (clone Leu2a), 100 µl anti-CD20 (clone Leu 16) and 40 µl anti-CD16 antibody solutions were added and incubated at room temperature for 30 minutes. The

volume in the tube was made up to 50 ml with PBS pH 7.2 and the cells were then pelleted by centrifuging at 1600 rpm for 5 minutes at 20°C. The cells were resuspended in 1 ml PBS pH 7.2 and transferred to a 1.5 ml eppendorf tube containing 100 µl M-450 sheep anti-mouse IgG and 100 µl M-450 anti-human CD8 Dynabead suspensions (Dynal Ltd., UK). The tube contents were mixed by continuous inversion on a Rotator for 30 minutes at room temperature. The tube was placed in a Dynal magnet to remove beads and the depleted cell suspension was transferred to a second 1.5 ml eppendorf tube containing 50 µl M-450 sheep anti-mouse IgG and 50 µl M-450 anti-human CD8 Dynabead suspensions. The tube contents were mixed as previously for 10 minutes at room temperature. The beads were removed using the magnet to yield the purified CD4+ T cells. These were pelleted by microfuging the cells at 2000 rpm and then resuspending the cells in 1 ml PBS pH 7.2. A 10 µl aliquot of the cell suspension was mixed with 10 µl Trypan blue solution to check for cell viability (50 ml whole blood on average gives a final yield of 1 x 10^7 CD4+ T cells). The purity of the CD4+ T cells was determined by FACS analysis using FITC labelled non-competing antibodies (anti-CD3, CD4, CD8, CD16, CD19 and anti-DR antibodies were all obtained from Sigma, UK) and shown to be >95% pure. Efficient depletion of monocytes was determined by a lack of response to anti-CD3 antibody at a concentration of 0.1 µg/ml.

2.3 Purification of CD8+ T cells

Method as described above except that 100 µl anti-CD4 (clone Leu3a) (Becton Dickinson, UK) antibody solution was added in place of the anti-CD8 antibody and M-450 anti-human CD4 Dyna bead suspension was added in place of the M-450 anti-human CD8 Dynabead suspension. 50 ml whole blood on average yields 2-2.5 x 10^6 CD8+ T cells. FACS analysis showed the cells to be >95% pure

2.4 Purification of CD4+CD45RA+ and CD4+ CD45RO+ T cells

Method as described for CD4+ T cells, with the addition of either 100 µl anti-CD45 RA or anti-CD45 RO (Becton Dickinson, UK) to the above cocktail of monoclonal antibodies. FACS analysis showed the cells to be >95% pure

2.5 T cell proliferation assay

7×10^4-1×10^5 cells were cultured in 96 well flat bottomed tissue culture plates (Costar, UK) in RPMI-1640 supplemented with 10% heat inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies, UK.) in the presence or absence of the various activating agents described below, for 4 days at 37°C, 5% carbon dioxide. During the last 6 hours of the assay, cultures were pulsed with 1 µCi/well of [methyl ^{3}H]-thymidine (Amersham International, UK). Cells were harvested onto filter mats using a Skatron cell harvester (Wallac, UK) and counted using a Betaplate scintillation counter (Wallac, UK). Results were expressed as the arithmetic mean counts per minute (cpm) of triplicate cultures.

T cells were incubated in the presence or absence of CAMPATH-1H or CAMPATH-1G antibodies in the presence or absence of anti-human IgG or anti-rat IgG antibodies which were added in solution for the entire culture period, in the presence or absence of 10 ng/ml phorbol myristate acetate (PMA) (Sigma, UK). The optimum concentration of antibodies added in solution was found to be 20 µg/ml final concentration.

Alternatively, 96 well flat bottomed plates were prepared by first incubating with a suboptimal concentration of the mouse anti-CD3 (OKT3) antibody (0.1 µg/ml) 100 µl per well diluted in PBS, at 4°C. Wells were washed three times with PBS to remove unbound antibody. CAMPATH-1H antibody at concentrations of 0.1, 1 and 10 µg/ml in PBS, in the presence or absence of 20 µg/ml polyclonal anti-human IgG, monoclonal anti-CD2, anti-CD28 or anti-tetanus toxoid (all dilutions were carried out in PBS), were added in 100 µl volumes to wells and incubated at 37°C for 3 hours. In some experiments, anti-

CD3 coated plates were also incubated with Fibronectin (10 μg/ml) as described for antibodies. Unbound antibodies or fibronectin were removed by washing the plates 3 x with PBS.

2.6 T cell clones

The MF14 T cell clone was maintained in continuous culture by 7-14 day restimulation with flu antigen (X31) and irradiated PBMC (matched for DR) and Lymphocult T (Biotest, UK). Cells were harvested at least 7 days after the last activation and incubated in the presence or absence of different monoclonal antibodies as described above. 2×10^4 cells were added per well and cultured in the presence or absence of activating agents for 3 days at 37°C, 5% carbon dioxide. [^{3}H]-thymidine was added during the last 18 hours of culture to measure cell proliferation and harvested using a Skatron cell harvester. Counts were determined using a Beta plate scintillation counter.

2.7 Cytokine production in T cells

Cultures of CD4+ T cells were incubated with 20 μg/ml CAMPATH-1H antibody in the presence or absence of 20 μg/ml anti-human IgG for 1 hour at room temperature in 15 ml sterile tubes. Excess antibody was removed by washing the cells 2 x 10 ml PBS. Cells were resuspended in RPMI 1640/FCS/L-glutamine/penicillin/streptomycin at a concentration of 5×10^5 cells/ml. Cells were plated out 200 μl per well (1×10^5 cells per well) and incubated for 48 and 96 hours in the presence or absence of 10 ng/ml PMA. Supernatants from triplicate wells were then pooled and stored at -20°C until tested.

2.8 ELISA for the detection of Human tumour necrosis factor (TNF-α)

96 well EIA/RIA microtiter plates (Nunc, UK) were coated with 50 µl per well of rabbit anti-TNF 214 antibody diluted in PBS 0.002% sodium azide at a final concentration of 5 µg/ml. The plates were incubated overnight at 4°C. Wells were washed 5 x with 0.05% Tween 20 in PBS. Wells were blocked with 200 µl 1 % BSA in PBS, 0.002 % sodium azide, to prevent non-specific binding. The plates were incubated for 1 hour at room temperature. Wells were washed 5 x with 0.05% Tween 20 in PBS. 200 µl TNF standard or control samples, diluted in 1% BSA in PBS, was added per well and the plate was incubated for 2 hours at room temperature with gentle agitation. Wells were washed as previously. 50 µl per well mouse anti-TNF (clone 357/101) culture supernatant diluted 1/20 in 2% normal rabbit serum was added and incubated for 1 hour at room temperature. Wells were washed. 50 µl alkaline phosphatase conjugated anti-mouse IgG diluted 1/1000 in 2% normal rabbit serum was added to the wells and the plate was incubated for 1 hour at room temperature. The plate was washed as previously. 50 µl reconstituted substrate was added to each well and incubated for 20 minutes at room temperature. 100 µl reconstituted amplifier was added to each well and incubated for 10 minutes at room temperature. 50 µl /well 2M sulphuric acid was added per well to stop the reaction. The absorbance was read at 492 nm wavelength.

2.9 ELISA for the detection of human Interferon-γ

Interferon-γ was detected using a Genzyme kit (Cambridge, MA) following the manufacturer's instructions.

2.10 ELISA for the detection of IL-4

IL-4 was detected using a Genzyme kit, (Cambridge, MA) following the manufacturer's instructions.

2.11 Cell assay for measurement of IL-2 production

100 µl CTLL cells (IL-2 dependent mouse myeloma cell line) at 1 x 10^5 /ml were added/well to a 96 well flat bottomed tissue culture plate (Costar, UK). IL-2 (stock solution at 10,000 units/ml) was diluted to 200 units/ml in RPMI/FCS/L-glutamine/penicillin/ streptomycin. 100 µl/well IL-2 standard (dilutions added to wells to give final concentrations of 100, 50, 25, 12.5, 6.25, 3.25 and 0 units/ml) or 100 µl test culture supernatants were added to each well. Plates were incubated at 37°C, 5 % CO_2. 1 µCi per well [^{3}H]-thymidine was added during the last 5 hours of culture. Cells were harvested at 48 hours onto filter mats, using a Skatron cell harvester and then counted using a Beta scintillation counter (Wallac, UK).

2.12 Cell growth inhibition assays

Wien 133 (B cell lymphoma cell line) and Jurkat (T cell lymphoma cell line) cells were incubated in the presence or absence of antibodies added in solution at final concentration of 20 µg/ml, unless stated in the text, as described for CD4+ T cells. Antibodies added in solution, unless stated, were left in the cultures for the duration of the experiment. In some experiments, antibodies were immobilised onto plastic by coating the wells for 3 hours with antibodies in PBS. Unbound antibodies were removed by washing wells 3 x with PBS. The Wien 133 cell assays were performed in Iscoves medium supplemented with 10% heat inactivated FCS and penicillin/streptomycin (Life Technologies, UK). Jurkat cell assays were set up in RPMI medium supplemented with L-glutamine, 10% heat inactivated FCS and penicillin/streptomycin. 2 x 10^4 Wien 133 cells or 1 x 10^4 Jurkat cells were added/well. Samples were set up in triplicates. 1 µCi/well [^{3}H]-thymidine was added during the last 4 hours of a 5 day culture. Cells were transferred onto filter mats using a Skatron cell harvester and counted using a Beta scintillation counter (Wallac, UK).

2.13 SDS Polyacrylamide Gel Electrophoresis (PAGE)

Based on Laemmli's method (Laemmli, 1970).

Gels were prepared using the Mini Protean II apparatus set up according to the manufacturer's specifications (Biorad, UK) with 0.75 mm spacers and depending on the number of samples, either 10 well or 15 well combs. Separating gels were prepared using different final concentrations of acrylamide dependent on the size/sizes of the protein/proteins of interest. The final concentration of acrylamide (Biorad, UK) varied between 7.5% and 15% in 370 mM Tris pH 8.8, 0.01% SDS, 0.006% AMPS and Temed. The separating gels were poured to within 0.5 cm of the position of the base of the well-forming combs, overlaid with 0.5 ml 1:1 isopropanol:water mix and allowed to set. The isopropanol:water overlay was poured off and the top of the separating gel was rinsed with unpolymerised stacking gel consisting of 5% acrylamide, 125 mM Tris pH 6.8, 0.01% SDS, 0.006% AMPS and Temed. The stacking gel was then added, the combs inserted and the top gel allowed to set. After removing the combs, unpolymerised gel was rinsed out of the wells with SDS PAGE running buffer (25 mM Tris, 192 mM glycine, 1% SDS pH 8.3). Samples were prepared by adding an equal volume of sample buffer (125 mM Tris pH 6.8, 4% SDS, 4% glycerol, 0.01% bromophenol blue, 200 mM DTT). For analysis of proteins under non-reducing conditions, the DTT was omitted from the sample buffer. All samples were boiled in a heating block for 10 minutes at 100°C, microfuged briefly, then loaded into the wells using a Hamilton syringe. Gels were run under constant current (30-40 mA per gel) until the tracking dye was within 0.5 cm of the base of the separating gel.

2.13.1 Autoradiography

For radioactive protein samples, ^{14}C labelled Rainbow molecular weight standards (Amersham, UK) were used; these were treated in the same way as other samples as mentioned above. Proteins were fixed and stained by immersing the gels for 20-30 minutes in 1% Coomassie Brilliant Blue R-250 (Fluka, UK), 45% methanol, 10% acetic acid. Background staining was reduced by immersing gels in 2-3 changes of 20%

methanol, 7% acetic acid. Gels were then immersed in Amplify solution (Amersham, UK) for 30 minutes and dried under vacuum. Gels were exposed to X-Omat S film (Amersham, UK) in cassettes for varying lengths of time depending on the strength of the signal generated. Films were developed using an automatic Fuji X-Ray film processor RGII.

2.14 General Method for Western blotting

Western blotting was performed using a semi-dry blotting apparatus (Ancos, Denmark). Proteins were transferred from gels to nitrocellulose membrane (Schleicher and Schuell, UK), sandwiched between 3 sheets of 3MM Whatman filter paper. The membrane was soaked in distilled water; the filter papers were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol). The blotting was carried out for 15-20 minutes using a constant current of 200 mA per gel. Following transfer, membranes were immersed in blocking buffer, which varied in composition depending on the proteins being analysed (details below).

2.15 Immunoprecipitation of Phosphotyrosine proteins

All manipulations were carried out in 1.5 ml eppendorf tubes. Cells were activated as described for phosphotyrosine blotting (5×10^6 cells/immunoprecipitation reaction). The cells were lysed in 1 % n-octyl glucoside, 10 mM Tris pH 8.0, 50 mM NaCl, 10 mM iodacetamide, 10 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml chymostatin, 10 µg/ml leupeptin and 10 µg/ml pepstatin, on ice for 15 minutes and then microcentrifuged at 14,000 rpm for 15 minutes at 4°C. The lysates were transferred to fresh tubes and 20 µl 50% suspension anti-phosphotyrosine (clone 4G10)-agarose was added. The tube contents were incubated together, mixing continuously on a rotator for 30 minutes at room temperature. The immunoprecipitations were washed with 5 x 1 ml lysis buffer. 30 µl SDS PAGE sample loading buffer

containing 10 mM sodium orthovanadate was added and samples heated at 100°C in a heating block for 10 minutes. Samples were frozen at -20°C and reheated at 100°C for 10 minutes before loading onto SDS PAGE.

2.16 Phosphotyrosine blotting

Cells were resuspended on ice in RPMI/0.8% heat inactivated FCS/25 mM HEPES (1 x 10^6 cells/20 µl). Antibodies were added at a final concentration of 20 µg/ml and reactions incubated on ice for 30 minutes. Cells were washed with 1 ml PBS pH 7.2 and then resuspended in 20 µl RPMI/FCS/HEPES. The secondary antibodies were added at 20 µg/ml final concentration and the reactions incubated in a water bath at 37°C for the appropriate time. Reactions were stopped by adding 20 µl 2 x SDS PAGE sample loading buffer containing 10 mM sodium orthovanadate. Samples were heated at 100°C in a heating block for 10 minutes before either loading directly onto SDS PAGE or freezing at -20°C. If frozen, samples were reheated at 100°C for 10 minutes before loading onto SDS PAGE.

On a 10 well SDS PAGE minigel, 20 µl sample was loaded/track = 5 x 10^5 cells.

For proteins with a molecular weight < 40kDa, a 10% SDS PAGE was used.

For proteins with a molecular weight > 40kDa, a 7.5% SDS PAGE was used.

Following separation by SDS PAGE, the proteins were transferred to nitrocellulose. After transfer, the nitrocellulose was blocked with 10 ml 1% BSA in PBS pH 7.2 overnight at 4°C. Anti-phosphotyrosine 4G10 antibody was added to the blocking buffer at a final dilution of 1/2500 and incubated with the membrane for 2 hours at room temperature. The nitrocellulose was washed 3 x 1 minute and 3 x 5 minutes in PBS pH 7.2 containing 0.1% Tween 20. Anti-mouse IgG Fc specific HRP conjugate was added at a dilution of 1/10,000 in 10 ml blocking buffer and incubated for 1 hour at room temperature. The membrane was washed as previously and then incubated for 1 minute with 10 ml ECL detection reagent (Amersham, UK). The membrane was exposed to Hyperfilm film (Amersham, UK).

2.17 Preparation and Purification of CAMPATH-1 (CD52) antigen

Cells (10^8) were transferred to 30 ml glass corex tubes and centrifuged (Sorvall, SS-34 rotor at 5000 rpm for 5 minutes at 10°C) and then washed with PBS pH 7.2. The cell pellet was weighed and made up to 1 ml with water (assuming that 1g cells = 1 ml water). Methanol (2.67 ml) and chloroform (1.33 ml) were added to give a ratio of chloroform: methanol: water of 4:8:3. The mixture was vortexed and stirred for 30 minutes and then centrifuged (9000 rpm for 10 minutes at 10°C). The cell pellet was re-extracted with 5 ml chloroform: methanol: water (4:8:3) as previously and the supernatants bulked. The combined supernatant (10 ml) was mixed with 1.73 ml water to give a ratio of chloroform: methanol: water of 4:8:5.6. After stirring for 30 minutes at room temperature, the two phases were separated by centrifugation at 900 rpm for 10 minutes at 10°C. The upper phase was carefully removed from the lower phase and interface material. Both phases were rotary evaporated to dryness, resuspended in 2 ml water and then dialysed against water. Samples were stored at -20°C.

2.18 ELISA for the quantitation of CD52 antigen

96 well flat bottomed PVC flexible microtitre plates were coated with 50 µl/well two-fold dilutions of antigen (1:200 to 1:25,600) in 50% (v/v) methanol and left uncovered, allowing the methanol to evaporate to dryness. The plate was blocked with 50 µl/well of 2% BSA in PBS for 1 hour at room temperature. Wells were washed 5 x 0.05% Tween 20 in PBS and dried thoroughly. 50 µl/well CAMPATH-1H (2 µg/ml) in 2% BSA in PBS was added/well and incubated for 1.5 hours at room temperature. The plate was washed as previously. 50 µl/well alkaline phosphatse conjugated goat anti-human IgG diluted 1:500 in 2% BSA in PBS was added per well, and incubated for 1 hour at room temperature. The wells were washed 7 x with 0.05% Tween 20 in PBS and dried thoroughly. 50 µl/well substrate was added and incubated for 20 minutes at room temperature. 100 µl/well amplifier was added and incubated for 10 minutes at room temperature before stopping the reaction with the addition of 50 µl 2M sulphuric acid per

well. The absorbance was measured at a wavelength of 490nm in a Microwell plate reader.

2.19 Western blot detection of the CAMPATH-1 antigen

Samples were incubated with an equal volume of sample loading buffer containing DTT, heated at 100°C in a heating block and briefly centrifuged before loading onto a 15% SDS PAGE minigel (Biorad, UK). Rainbow molecular weight markers (Amersham, UK) from 2,340-46,000 kDa were treated in the same way. After electrophoresis, proteins were transferred to nitrocellulose membrane as previously described. The membrane was blocked overnight by immersion in 3% milk in PBS (Sigma) at 4°C. CAMPATH-1G antibody was added to the blocking buffer at a final concentration of 20 µg/ml and incubated with the membrane for 2 hours at room temperature. The membrane was washed 3 x 1 minutes and 2 x 10 minutes with PBS containing 0.1 % Tween 20. Horse radish peroxidase (HRP) anti-rat IgG antibody (Sigma) was added to a final concentration of 1/10,000 in blocking buffer and incubated with the membrane for 1 hour at room temperature. The blot was washed as previously and then immersed in ECL detection reagent (Amersham) for 1 minute. After air drying and wrapping in Saran wrap, the membrane was exposed to Hyperfilm (Amersham).

2.20 N-Glycanase digestion of the CD52 antigen

5 µl protein solution was incubated with 5 µl 1% w/v SDS/0.2 M mercaptoethanol and heated at 100°C for 5 minutes. This was made up to a final volume of 30 µl with 1M Tris pH 8.8 (200 mM final concentration), 0.5 M EDTA (10 mM final conc.), NP40 (1.25% final conc.) and 1 unit of N-glycanase and was incubated overnight at 37°C. The digest mix was mixed 1:1 with SDS PAGE sample loading buffer, heated 5 minutes at 100°C and loaded onto a 15% SDS PAGE gel.

2.21 FACS Staining for analysis of CD52 expression

Cells were washed in PBS and then resuspended in FACS staining buffer (5% heat inactivated FCS in PBS, 0.1% sodium azide). 100 µl cell suspension (1 x 10^6 /ml) was added to each well of a 96 round bottomed tissue culture plate (Costar, UK) and incubated with CAMPATH-1H $(Fab)_2$-FITC labelled antibody at a final concentration of 5 µg/ml. Control samples were incubated with 5 µl FACS staining buffer in the absence of antibodies. The plate was incubated for 30 minutes at 4°C, cells were then pelleted by centrifuging the plate for 2 minutes at 2000 rpm at room temperature (bench top Sorvall) and the antibody staining solution was then aspirated. Cell pellets were washed with 2 x 200 µl PBS by vortexing the plate to resuspend cells, adding the PBS and centrifuging between washes. The final cell pellet was resuspended and fixed by the addition of 100 µl 1% paraformaldehyde in PBS. Samples were stored for a minimum of one hour and for a maximum of 3 days at 4°C. FACS data was acquired using a FACScan and analysed using LYSIS II software (Becton Dickinson, Oxford, UK).

2.22 FACS analysis of other cell markers

Cells were stained with FITC labelled antibodies against a number of B and T cell markers using the method described above. Antibodies were purchased from Becton Dickinson or Sigma, UK, as described in section 2.1.1.

2.23 PI-PLC treatment of cells

100 µl cells (1 x 10^6 /ml) in complete medium were treated in the presence or absence of 5 units of phosphatidyl-inositol-specific phospholipase C (PI-PLC), (Boehringer Mannheim, UK) for 1 hour at 37°C. The cells were then washed in PBS and stained with

FITC labelled CAMPATH-1H $(Fab)_2$ antibody and analysed by FACScan using LYSIS II software (Becton Dickinson, UK).

2.24 Preparation of RNA

Stock Solution D (4M Guanidium thiocyanate/ 25mM trisodium citrate/ 0.5% Sarkosyl)

Preparation of Solution D - 250 g guanidium thiocyanate was dissolved in manufacturer's bottle with 293 ml water, 7.63 ml 0.75 M sodium citrate and 26.4 ml 10% Sarkosyl at 65°C. Solution D was prepared by adding 360 µl β mercaptoethanol to 50 ml stock solution. This can be stored in a dark bottle at room temperature for up to one month.

Caesium chloride stock solution - 47.5 g caesium chloride + 1.7g EDTA/100 ml was dissolved in distilled water and the pH adjusted to 7.3. This solution was then passed through a 0.22 µM filter and autoclaved [1 ml of solution should weigh 1.7 g +/- 0.3 g]. Cells were washed twice in PBS and the cell pellet lysed in 2.5 ml solution D. The lysate was sheared by passing it up and down in a syringe through a 23-25 gauge needle until the DNA had lost its stringiness. The lysates were then layered onto 2.5 ml caesium chloride/EDTA. Tubes were ultracentrifuged (Sorvall) in an SW50 rotor at 40,000 rpm at 15°C for 16 hours. The RNA precipitated to the base of the tubes, so for harvesting and to minimise DNA contamination, solution D and about half of the caesium chloride/EDTA was aspirated off. Using a clean pipette, the remaining caesium chloride/EDTA was removed and the tubes were inverted. After allowing any remaining fluid to drain away, the bottoms of the tubes were cut off using a sterile scalpel blade and placed on ice. The RNA pellet was rinsed 2-3 x with 70% ethanol and was then left on ice for 2-3 hours to allow the ethanol to evaporate. The RNA pellet was resuspended in 400 µl water and stored at -20°C.

The RNA was quantified by measuring the absorbance of a 1/100 diluted sample at a wavelength of 260 nm. The readings obtained were multiplied by 100 and then divided by a factor of 25 to get the RNA concentration in µg/ml.

2.25 RNA Dot blots

Doubling dilutions of RNA (20 µg/ml) were made in water in a 96 well round bottomed plate (Costar, UK). 60 µl 20 x SSC/ 37% formaldehyde (3:2) was added per well. The plate was incubated at 65°C for 15 minutes. 80 µl RNA was applied onto hybond-N membrane (Amersham, UK) using a manifold slot blot apparatus (Schleicher and Schuell, UK) and vacuum was applied. Samples were washed through with 2 x 100 µl water:10 x SSC: 37% formaldehyde (5:3:2). The RNA was fixed on the membrane using a Transilluminator and the membrane prehybridised in 1 % DNA, 2.5 x Denhardts, 0.5% SDS, 5 x SSC, 50% formamide at 42°C. The CD52 probe was added to the prehybridisation solution and incubated at 42°C for 48 hours. The filter was washed for 1 hour with SSC, 0.1 % SDS at 42°C; 1 x 20 minutes with 0.1% SDS at 42°C; 2 x 20 minutes with 0.5 x SSC, 0.1% SDS at 65°C; 2 x 10 minutes 0.1 x SSC, 0.1% SDS at 65°C. Filters were exposed to film at -70°C.

2.26 Agarose gel electrophoresis

The purity of the RNA was determined by running samples on a formaldehyde gel. 1g agarose was melted in 62 ml water in a microwave and cooled in a water bath before adding 20 ml 5 x RNA running buffer and 18 ml 12.3 M formaldehyde. The gel was cast in a 10 x 8 cm mini-gel tray (Pharmacia, Sweden) with 8 well gel-slot formers. 4.3 µg RNA in 4.5 µl water was mixed with 2 µl 5 x RNA running buffer, 3.5 µl 12.3 M formaldehyde and 10 µl formamide. Samples were heated in a water bath at 65°C for 15 minutes, chilled on ice and then 2 µl formaldehyde loading buffer was added. The gel was pre-run for 5 minutes at a constant voltage of 50 volts, the samples loaded and then a voltage of 10 volts applied. The gel was left running overnight and the voltage switched off when the tracking dye was approximately 2 cm from the end of the gel.

2.27 Coupling antibodies to Sepharose

CNBr-activated freeze-dried sepharose-4B powder (Pharmacia, Sweden) was rehydrated by washing on a sintered funnel with 1 mM hydrochloric acid. Antibodies, namely, CAMPATH-1H IgG1 or anti-CD4 IgG_1, were diluted in coupling buffer (0.1 M sodium bicarbonate pH 8.3, 0.5 M sodium chloride) at 2 mg/ml and added to the sepharose gel suspension in 15 ml tubes to give a final concentration of 2 mg antibody coupled per ml sepharose gel. The antibody solution and sepharose were incubated together for 5 hours at room temperature, mixing continously on a rotator. The antibody-coupled sepharose was precipitated by centrifugation in a bench top centrifuge at 2000 rpm for 5 minutes and then the coupling efficiency was determined by measuring the absorbance of the supernatant at 280 nm wavelength in a Spectrophotometer (Pharmacia). [1mg/ml antibody will have an absorbance of 1.32 at 280 nm wavelength and therefore, the % antibody coupled = total mg antibody remaining in the supernatant subtracted from the total mg antibody added initially, divided by the total mg antibody added initially, multiplied by 100]. Remaining active groups on the sepharose were blocked by overnight incubation with 1 M Tris pH 8.8. The antibody-coupled sepharose was washed with 10 volumes PBS and stored at 4°C in PBS 0.02% sodium azide.

2.28 Detection of Apoptosis

Wien 133 cells were incubated in the presence or absence of antibodies for 24, 72 and 96 hours. For the 24 hour time point, 1 x 10^6 cells were aliquoted into 24 well tissue culture plates (Costar, UK) in a final volume of 1 ml; for 72 and 96 hour time points, 5 x 10^5 cells were added to the wells. 20 µg CAMPATH-1H or 20 µg anti-human IgG or 20 µg CAMPATH-1H + 20 µg anti-human IgG or 4 µg anti-human IgM antibodies were added to the respective wells. Plates were incubated at 37°C, 5% CO_2. Harvested cells were washed with PBS.

2.28.1 Propidium Iodide staining of cells

Cells were transferred to 1.5 ml Eppendorf tubes and washed twice with 1ml PBS. Cell pellets were resuspended in 200 µl propidium iodide solution (50 mg/ml) (Becton Dickinson, UK) and incubated on ice for 10 minutes. Cells were washed twice with 1 ml PBS. The final cell pellet was resuspended, fixed in 1% paraformaldehyde and stored in the dark at 4°C prior to data aquisition using a FACScan and LYSIS II software (Becton Dickinson, UK).

2.28.2 Apoptosis: Giemsa stain

Cells were adhered to glass slides using a cytospin running at 500 rpm for 15 minutes. The slides were allowed to air dry and then cells were fixed with alcohol. Slides were placed in May-Grunwald stain (diluted 50:50 in buffered water) for 15 minutes, followed by 10 minutes in Giemsa (90:10 water:Giemsa stock). Slides were washed with three quick changes of buffered water and then with a final 5 minute wash in buffered water. Xylene was added and the slides mounted in DPX.

2.28.3 Apoptosis: DNA laddering

Cell pellets (1×10^6) were resuspended in 425 µl SSC (20 x stock solution made by dissolving 175.3 g NaCl sodium chloride and 88.2 g sodium citrate in 1 litre and adjusting pH to 7.0). 50 µl 5 mg/ml Proteinase K was added, followed by 25 µl 10% w/v SDS. DNA was extracted by adding 500 µl phenol saturated with SSC and 500 µl 24:1 chloroform:isoamyl alcohol; sample were vortexed and microfuged for 5 minutes at 12,000 rpm. The upper layer was re-extracted with 500 µl chloroform: isoamyl alcohol. 2 x volumes of ice cold 100% ethanol were added/tube and left at -70°C for 1 hour. After centrifugation at 14,000 rpm for 30 minutes, the pellets were washed with 1 ml 70% ethanol. Pellets were resuspended in 200 µl 0.1 x SSC and left overnight at 4°C to allow the DNA to fully dissolve. The sample volume was adjusted by adding 300 µl 0.1 x

SSC, 31.25 µl 20 x SSC and 31.25 µl 10 mg/ml RNase. Samples were incubated at 37°C for 30 minutes. 31.25 µl 5 mg/ml Proteinase K was added per tube and incubated at 37°C for a further 30 minutes. DNA was extracted as described above, precipitated and washed and dissolved in 100 µl 0.1 x TE. DNA was quantitated by measuring the absorbance of a 1/10 dilution at 260 nm wavelength, and then multiplying the reading by a factor of 50 and the dilution factor, giving the concentration in µg/µl. For labelling, 5 µl DNA was added to 1 µl ^{32}P ATP mix (3 µl 100 mM Tris pH 7.4, 50 mM magnesium chloride, 0.1 µl α^{32}P dATP, 1 µl dATP (3.3 µM), 1 µl (5 units) Klenow (Boehringer Mannheim, UK). Samples were incubated for 30 minutes at room temperature. Samples were mixed with 7 µl DNA loading buffer (1 µl 300 mM EDTA + 6 µl loading buffer). Samples were loaded onto a 1.8% agarose gel in TBE buffer (Molecular weight standard markers = 2 µl 1 Kb ^{32}P ladder + 3 µl water + 7 µl DNA loading buffer). After electrophoresis, the gel was fixed in 7.5% TCA and dried down by compression under filter paper.

2.29 Transfections

For each well, the following DNA mix was prepared: 5 µg plasmid containing CD52 insert, 1 µg plasmid 321 Neo, 25 µl 3 M sodium chloride in a final volume of 250 µl. 40 µl Transfectam (Sepracor, UK) was diluted with 210 µl water. The DNA mix and diluted Transfectam were mixed together 1:1. Cells (J45.01, Jurkat E6-1 and J. RT3-T3.5 cell lines) were washed in serum free medium (RPMI/L-glutamine/penicillin/streptomycin) and then resuspended in serum free medium at 1 x 10^6/ml. Cell suspensions (2 ml/well) were plated out in 6 well tissue culture plates (Costar, UK). 500 µl of DNA/transfectam mix was added dropwise to each well and the contents gently mixed and incubated at 37°C for 5 hours. 2 ml of RPMI/10% FCS/L-glutamine/penicillin/streptomycin was added to each well and the plates were left incubating at 37°C, 5% CO_2, for 2 days. Cell suspensions were transferred to tissue culture flasks (total volumes of 10 ml) and 100 µl Gentamicin G418 was added to give a final concentration of 500 µg/ml (Stock solution: 50 mg/ml in 100 mM HEPES). Non transfected cells were cultured in parallel to the transfected cells, in the presence of G418, in order to monitor selection. Transfected cells

surviving G418 selection were monitored for CD52 expression by FACS analysis. CD52 positive cells were dilution cloned in the presence of G418 and re-analysed for CD52 expression.

2.30 Electron microscopy

For TEM (Transmission Electron Microscopy), glutaraldehyde fixation was followed by a 5 minute distilled water wash and cells were post-fixed in 1% osmium tetroxide buffered with 0.1M sodium cacodylate pH 7.2 for 1 hour. The cells were rinsed for 5 minutes in distilled water and dehydrated in 50%, 70%, 90%, 100% and 100% ethanol, for 10 minutes in each. Two 5 minute rinses in propylene oxide were followed by infiltration in 1 : 1 propylene oxide : Araldite CY212 resin (Taab Laboratories, UK) for 30 minutes, Araldite CY212 for 2 hours and Araldite CY212 at 37°C for 1 hour. Polymerisation was performed at 60°C for 24 hours. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips TEM 420 at 80 kV.

2.31 Calcium flux measurements

Cells were resuspended in RPMI/30 mM HEPES and loaded with 3 mM Fluo-3 AM and 8 mM Fura red AM (Molecular Probes Europe, Netherlands) in the presence of pluronic F127 detergent (Sigma, UK) at a final concentration of 0.05 %, for 30 minutes at 37°C. Cells were then washed and resuspended at 5 x 10^5/ml and warmed to 37°C for 10 minutes before use.

Calcium flux was measured using a Coulter XL-MCL flow cytometer (Coulter, USA). Goat anti-mouse IgG (crossreactive with human IgG) was added for 1 minute, followed by the addition of the stimulating antibody. The mean ratio of Fluo-3/Fura-red fluorescence was measured during the acquisition time course and expressed graphically to indicate Calcium flux.

2.32 ***In vivo*** **experiments**

The parental Wien 133 cells or CD52 negative/low variants were washed in PBS and resuspended in PBS at a final concentration of 2 x 10^7/ml. The cells were mixed with an equal volume of 6 mg/ml matrigel. 0.1 ml was injected subcutaneously into nude mice. After approximately 1 month, tumours were excised from the animals, teased into single cell suspensions and analysed by FACS for CD52 expression.

3. The role of CD52 antigen in T cell activation

3.1 Introduction

The physiological activation of T cells, leading to proliferation, involves the interaction of the TCR-CD3 complex with processed antigen presented in the context of MHC gene encoded products, together with a second co-stimulatory signal provided by receptor-ligand contact between APC and T cells eg. B7-CD28 (Lenschow et al., 1996), ICAM-1-LFA-1 (Seventer et al., 1990), LFA-3-CD2 (Bierer et al., 1988). Ligation of the T cell receptor in naive T cells, either by antigen, or by antibodies directed against the invariable chains (CD3) of the T cell receptor complex, in the absence of a second co-stimulatory signal, results in the cells becoming unresponsive, or anergic (Jenkins et al., 1988; Schwartz, 1996).

The CD28-B7 interaction is commonly regarded as providing the main costimulatory signal to T cells. CD28 is expressed constitutively on naive T cells (June et al., 1990) and is upregulated following activation; its cognate ligands, B7.1 and B7.2, are expressed on the APCs, including activated B cells (Liu et al., 1992), macrophages (Liu and Janeway, 1991) and dendritic cells (Knight and Stagg, 1993). However, CD28 deficient mice are still able to mount adaptive immune responses (Shahinian et al., 1993) which infers that other surface molecules expressed by T cells are able to provide the costimulatory signals necessary to activate naive T cells.

In recent years, a number of GPI-anchored proteins have been shown to be capable of transducing signals resulting in T cell activation and proliferation. These include murine Thy-1, Ly-6 and Qa-2, human CD55, CD59, and CD73, and rat RT-6 T cell antigens (Reviewed by Robinson, 1991). Both polyclonal and monoclonal antibodies against these molecules can stimulate normal T cells in the presence of either Fc receptor bearing cells or anti-globulin antibodies. In some cases, for example CD59, activators of Protein Kinase C, such as PMA, are also required to stimulate proliferation (Korty et al., 1991). It has been suggested that T cell activation induced by antibodies to these GPI-anchored proteins may mimic the interaction of the proteins with their natural ligands *in vivo*.

The function of the CD52 antigen is as yet unknown but with the knowledge that ligation of GPI-anchored proteins can induce T cell activation, CAMPATH-1 antibodies were used in experiments to determine whether the CD52 antigen could also act as a co-stimulatory molecule and induce normal T cell proliferation.

3.2 Results

3.2.1 Induction of T cell proliferation by CAMPATH-1 antibodies.

In order to evaluate whether the anti-CD52 antigen monoclonal antibodies (CAMPATH-1 antibodies) were mitogenic for T cells, highly purified resting CD4+ T cells (purity > 95% as assessed by FACS analysis) were cultured with CAMPATH-1H in the presence of PMA and/or crosslinking anti-human IgG. CAMPATH-1H antibody could induce T cell proliferation, but only in the presence of a secondary crosslinking anti-globulin antibody and PMA. The optimum concentration of CAMPATH-1H for inducing T cell proliferation was 20 μg/ml (data not shown). This concentration was used for subsequent experiments. Using the same experimental conditions, the rat CAMPATH-1G antibody, crosslinked with anti-rat IgG, also induced T cell proliferation. In contrast, in the same experiment, crosslinking the CD4 molecule with humanised anti-CD4 antibody, of the same IgG isotype as CAMPATH-1H, together with anti-human IgG, in the presence of PMA, did not result in T cell proliferation. This showed that crosslinking of other surface markers such as CD4 did not result in T cell activation and implies that CD52 is linked up to different signalling pathways to CD4. Anti-CD3 antibody in the presence of PMA, without a second anti-globulin antibody, resulted in T cell proliferation. In addition to the CAMPATH-1 antibodies, the rat anti-CD52 IgG_{2c} antibody YTH 361.10, induced T cell proliferation in the total absence of other costimulatory signals. Addition of PMA could further enhance the YTH 361.10 antibody induced cell proliferation effect.

CAMPATH-1H did not induce T cell proliferation if the antibody was immobilised on plastic, even in the presence of PMA. Co-immobilisation of a secondary anti-globulin antibody had no effect. Results are shown in figure 3.1. YTH 361.10 antibody, immobilised onto plastic, also failed to induce T cell proliferation (data not shown).

3.2.2 CAMPATH-1 antibodies synergise with CD3-mediated signals to augment CD4+ T cell proliferation

A number of molecules on the T cell surface including CD2, CD18 and CD28 can synergise with signals delivered via the CD3/TCR complex to mediate T cell activation (Linsley et al., 1991; Van Seventer et al., 1991; Bierer et al., 1988). The possible role of the CD52 antigen being involved in delivering a co-stimulatory signal to cells was investigated by immobilising monoclonal anti-CD3 antibody at suboptimal doses onto plastic together with CAMPATH-1H antibody. Neither antibody alone was capable of driving the cells into proliferation, but in combination elicited a strong proliferative response in purified resting CD4+ T cells. In these experiments, there was no requirement for a secondary crosslinking anti-globulin antibody. However, the proliferative effect could be further enhanced by the addition of plate immobilised anti-human IgG.

The proliferative response was of the same order of magnitude as that observed when anti-CD2 6F10.3 or anti-CD28 (YTH 913.12.2) monoclonal antibodies were co-immobilised onto plastic with sub-optimal doses of anti-CD3 monoclonal antibody. In the presence of anti-CD3, rat anti-CD52 IgG_{2c} (YTH 361.10) antibody produced a significant T cell proliferative effect. The results are shown in figure 3.2[A].

The immobilised CAMPATH-1H antibody did not synergise with co-immobilised anti-CD2, or anti-CD28 monoclonal antibodies and addition of anti-human IgG had no effect (figure 3.2[B]).

3.2.3 CD52 is downregulated from the surface of CD4+ T cells following activation induced by anti-CD3 and CAMPATH-1H antibodies

T cell activation is accompanied by the downregulation of the TCR complex (Valitutti and Lanzavecchia, 1997). It was, therefore, of interest to determine the effect of activating CD4+ T cells in the presence of anti-CD3 and CAMPATH-1 antibodies, on the

surface expression of CD52. The results are shown in figure 3.3. FACS analysis of the surface expression of CD3, CD4, CD25, DR and CD52 indicated that at 24 hours post-stimulation, CD25 and DR were both upregulated, there was no significant change in the expression of CD3, whereas CD4 and CD52 were both downregulated.

3.2.4 Ability of cytokines to stimulate CD4+ T cell proliferation after crosslinking CD52 antigen

Proliferation of purified resting CD4+ T cells, induced by CAMPATH-1 antibody ligation of the CD52 antigen, together with a secondary anti-globulin antibody, could only be achieved by the addition of PMA. PMA acts by bypassing early signalling events induced by ligation of the TCR. The effect of various cytokines, including IL-1α, IL-2, IL-4, IL-6, IL-7 and IFN-γ, on T cell proliferation in response to anti-CD52, in the presence or absence of a secondary crosslinking anti-globulin antibody, was tested and compared with the effect of PMA. The cytokines were tested up to the maximum physiological concentrations suggested by the manufacturer. The results are shown in table 3.1.

IL-2, IL-4 and IL-7, all of which share a common γ chain (Kondo et al., 1993; Noguchi et al., 1993), are capable of replacing the requirement for addition of PMA and induced T cell proliferation; however, none of these was of the same magnitude of the T cell response induced by crosslinked CD52 antigen in the presence of PMA. IL-1α, IL-6 and IFN-γ did not show any effect on the response.

3.2.5 Anti-CD52 monoclonal antibodies induce cytokine release in CD4+ T cells

Activation of T cells is normally accompanied by release of cytokines, including IL-2 and IL-4, which drive the further proliferation of T cell populations, and IFN-γ and TNF-α, which play a role in inflammatory reactions in T cell mediated effects.

Table 3.1: Crosslinking CD52 in the presence of IL-2, IL-4 and IL-7 cytokines induces CD4+ T cell proliferation.

	Control	Anti-hulgG	C-1H	C-1H+anti-hulgG
Control	129 (46)	125 (15)	114 (23)	233 (68)
PMA	127 (20)	346 (80)	225 (52)	27281 (7052)
IL-2 (ng/ml)				
1	1598 (175)	2162 (105)	2166 (68)	6883 (70)
0.1	459 (54)	808 (88)	793 (88)	1902 (314)
0.01	91 (8)	79 (13)	155 (13)	474 (51)
IL-4 (IU/ml)				
100	1446 (268)	1566 (152)	1372 (77)	13341 (1574)
10	802 (22)	927 (111)	980 (135)	12452 (282)
1	545 (56)	587 (461)	724 (57)	5295 (548)
IL-7 (ng/ml)				
1	264 (32)	610 (33)	1216 (156)	4065 (248)
0.1	262 (54)	583 (31)	1247 (96)	2220 (421)
0.01	102 (12)	332 (62)	222 (88)	321 (76)

Purified CD4+ T cells (1 x 10^5 well) were cultured for 5 days in the presence of medium alone, CAMPATH-1H (C-1H) (20 μg/ml), anti-human IgG (20 μg/ml), C-1H + anti-human IgG in the presence or absence of IL-2 (0.01-1 ng/ml), IL-4 (1-100 IU/ml) or IL-7 (0.01-1 ng/ml). [^{3}H]-thymidine was added for the last 18 hours of the culture. Results are expressed as mean c.p.m. of triplicate cultures. The S.E.M. is given in brackets.

Table 3.2 Induction of cytokine release by CD4+ T cells in the presence of CAMPATH-1H antibodies.

	PMA	TNF-α (pg/ml)	IFN-γ (pg/ml)	IL-2 (IU/ml)	IL-4 (pg/ml)
Control		<40	<50	<0.005	<20
C-1H		<40	<50	<0.005	<20
anti-hulgG		<40	<50	<0.005	<20
C-1H+anti-hulgG		<40	<50	<0.005	<20
Control	+	<40	<50	<0.005	<20
C-1H	+	<40	<50	<0.005	<20
anti-hulgG	+	<40	<50	<0.005	<20
C-1H+anti-hulgG	+	507	116	5	60

CD4 + T cells (1 x 10^5/well) were preincubated for 1 hour with CAMPATH-1H (C-1H) (10 μg/ml) washed and further incubated in the presence or absence of anti-human IgG (10 μg/ml) for 1 hour at room temperature and washed to remove excess antibody. The cells were then incubated with or without PMA (10 ng/ml). Supernants from triplicate cultures were harvested at 48 hours for IL-2 and IL-4 assays and 96 hours for TNF-α and IFN-γ and stored at -20°C until tested.

Therefore, following crosslinking of the CD52 antigen on resting CD4+ T cells by CAMPATH-1 antibodies, culture supernatants were tested for the presence of cytokines (Table 3.2).

Supernatants from cells incubated with CAMPATH-1H in the presence of anti-human IgG and PMA contained high levels of IL-2 and lower, but significant, levels of IL-4, TNF-α and IFN-γ. In each case, there was no release of cytokine in the total absence of any stimulus, or with addition of antibodies or PMA alone.

To further evaluate the role of IL-2 and IL-4 cytokines in driving CD52 induced CD4+ T cell proliferation, anti-IL-2 and anti-IL4 neutralising antibodies were cultured with cells together with CAMPATH-1H antibody, anti-human IgG and PMA. The growth of cells cultured with anti-CD52, anti-human IgG and PMA, in the presence of anti-IL-2 antibody, was inhibited by up to 90%; under the same conditions, anti-IL-4 antibody only inhibited the cell growth by 15% (Sara Brett, personal communication).

In support of IL-2 playing a predominant role in the CD52 induced proliferation of CD4+ T cells, FACS analysis looking at surface expression of CD25 (IL-2 receptor), showed there was a significant increase, following coimmobilisation of CAMPATH-1H with anti-CD3. Neither anti-CD3 nor CAMPATH-1H antibody alone, was sufficient to induce CD25 expression (see figure 3.3).

3.2.6 Anti-CD52 antibodies induce proliferation in CD8+ and CD4+ CD45RA+ and CD4+ CD45RO+ T cells

The CD52 antigen is expressed at high levels on all T lymphocytes but this would not necessarily mean they would all be equally susceptible to the effects of anti-CD52 antibodies in inducing cell proliferation. Therefore, the ability of purified populations (purity > 95% as assessed by FACS analysis) of CD8+, CD4+ CD45RA+ and CD4+ CD45RO+ cells to respond to activation via the CD52 antigen was assessed.

In CD8+ T cells, as seen with the CD4+ T cells, crosslinking the CD52 antigen with CAMPATH-1H and anti-human IgG in the presence of PMA, induced a proliferative response. The CD8+ T cells also proliferated in response to treatment with the rat anti-CD52 IgG_{2c} YTH 361.10 antibody alone and this was enhanced further by the addition of PMA. Both CAMPATH-1H and the YTH 361.10 antibodies synergised with anti-CD3 antibody to induce CD8+ T cell proliferation, when co-immobilised onto plastic; fibronectin co-immobilised with anti-CD3 antibody was used as a positive control for driving CD8+ T cell proliferation. In the same assay, an anti-tetanus toxoid antibody included as a negative control, failed to synergise with anti-CD3 to activate the CD8+ T cells. The results are shown in figure 3.4.

Likewise, both the CD4+ CD45RA+ and the CD4+ CD45RO+ populations responded to CAMPATH-1H in the presence of anti-human IgG and PMA, or the YTH 361.10 antibody alone. Both cell populations also proliferated when CAMPATH-1H and anti-CD3 antibodies were coimmobilised onto plastic. The results are shown in figure 3.5.

3.2.7 The effect of cyclosporin A on the induction of T cell proliferation by anti-CD52 antibodies.

Cyclosporin A is a specific inhibitor of the calcium/calmodulin dependent phosphatase, calcineurin. Calcineurin is critical in the signalling pathway required for IL-2 production and hence T cell proliferation (Liu et al., 1991). Therefore, the effect of Cyclosporin A on CAMPATH-1 antibody dependent proliferation of CD4+ T cells was determined. The results are shown in figure 3.6. In order to compare the effect of the drug between the different treatments leading to cell activation, the results are expressed as a percentage of the proliferation obtained in the absence of Cyclosporin A.

Cyclosporin A inhibited the proliferation of CD4+ T cells induced under all activation conditions used ie. by crosslinking CD52 antigen with CAMPATH-1H antibody and anti-human IgG in the presence of PMA, or the rat anti-CD52 IgG2c YTH361.10 antibody alone, or coimmobilised CAMPATH-1H and anti-CD3, or fibronectin and anti-CD3

antibody. In every treatment, cell proliferation was inhibited in the presence of Cyclosporin A but there was variation between treatments and the degree to which the growth of the cells was inhibited. Thus, the stimulus inducing the strongest cell proliferation, namely, the fibronectin co-immobilised with the anti-CD3 was the hardest to inhibit. The difference in cell growth sensitivity to exposure to Cyclosporin A provides evidence that the drug is not grossly toxic; hence, concentrations of 30 and 100 ng/ml at which the fibronectin plus anti-CD3 response was only partly inhibited resulted in almost complete inhibition of the co-immobilised CAMPATH-1H and anti-CD3 antibodies, and the CAMPATH-1H and anti-human IgG plus PMA responses.

3.2.8 Down modulation of CD52 antigen following antigen specific activation.

Ligation of the T cell receptor by cognate ligand results in the down regulation of the TCR (Valitutti and Lanzavecchia, 1997) from the T cell surface. Having shown in previous experiments that CD52 can act as a costimulatory molecule, it was of interest to look at the effect of antigen specific activation using T cell clone, MF14, on expression of surface markers. The results are shown in figure 3.7. In the presence of antigen stimulation, CD3, CD4 and CD52 are all downregulated. In contrast, CD25 is upregulated. There is no change in the surface expression of CD18 or CD2.

3.2.9 CAMPATH-1H does not inhibit polyclonal or antigen-specific T cell activation

The data presented above indicates that ligation of the CD52 antigen on resting T cells purified from peripheral blood can provide a costimulatory signal leading to cell proliferation. Activation of peripheral CD4+ T cells in the presence of CAMPATH-1 antibodies was accompanied by downregulation of cell surface expression of CD3, CD4 and CD52. Likewise, in an antigen specific activation in a T cell clone, CD3, CD4 and CD52 were also downregulated.

Experiments were, therefore, carried out to determine whether, or not, CD52 plays an essential role in antigen specific T cell activation using CAMPATH-1 antibodies to block antigen mediated proliferation. Because CAMPATH-1H has been shown to be effective in ADCC, T cell activation was looked at using Fc negative antigen presenting cells, namely, DR1 transfected L cells, and a T cell clone, MF14, specific for an influenza A nucleoprotein. CAMPATH-1H intact antibody and a $(Fab)_2$ fragment did not block the antigen driven proliferative response, although a humanised anti-CD4 antibody, bearing the same isotype as CAMPATH-1H, totally blocked the proliferative effect. The results are shown in figure 3.8. These results indicate that CD52 is not required for antigen specific T cell activation, as antibodies against coreceptors or costimulatory molecules eg. CD4 or CD28, or adhesion molecules eg. LFA-1 will block T cell activation.

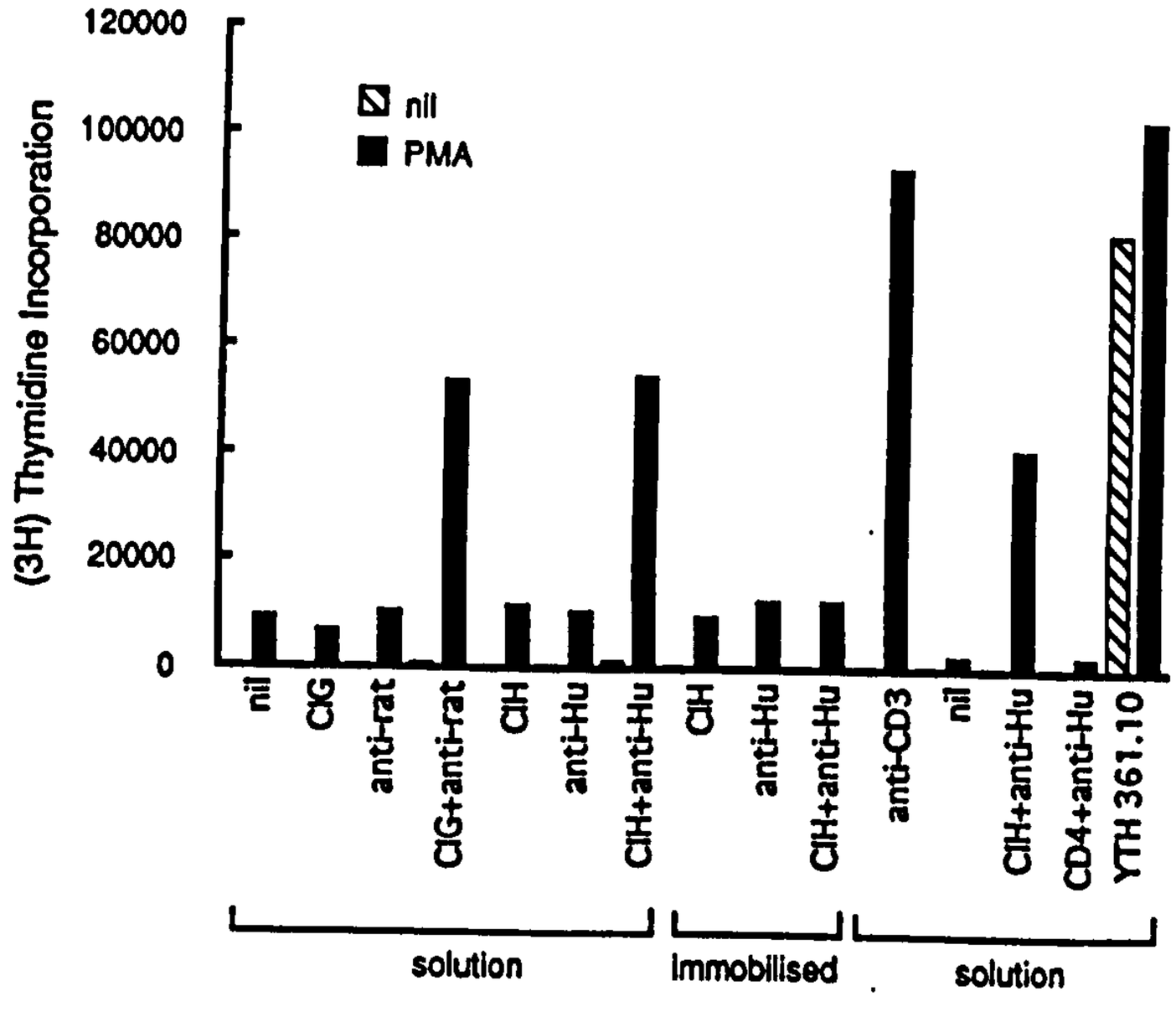


Figure 3.1 CAMPATH-1 induced proliferation of CD4+ T cells

Purified CD4+ T cells (1 x 10^5 well) were cultured for 5 days in the presence of medium alone, anti-CD3 (0.1 μg/ml), C-1H (20 μg/ml), anti-human IgG (20 μg/ml), C-1H + anti-human IgG, C-1G (20 μg/ml), anti-rat IgG (20 μg/ml), C-1G + anti-rat IgG or YTH 361.10 (20 μg/ml) added in solution, in the presence or absence of PMA (10 ng/ml). [^{3}H]-thymidine was added for the last 18 hours of the culture. Results are expressed as mean c.p.m. of triplicate cultures (SEM<15%). Background counts in the absence of PMA <500 c.p.m.

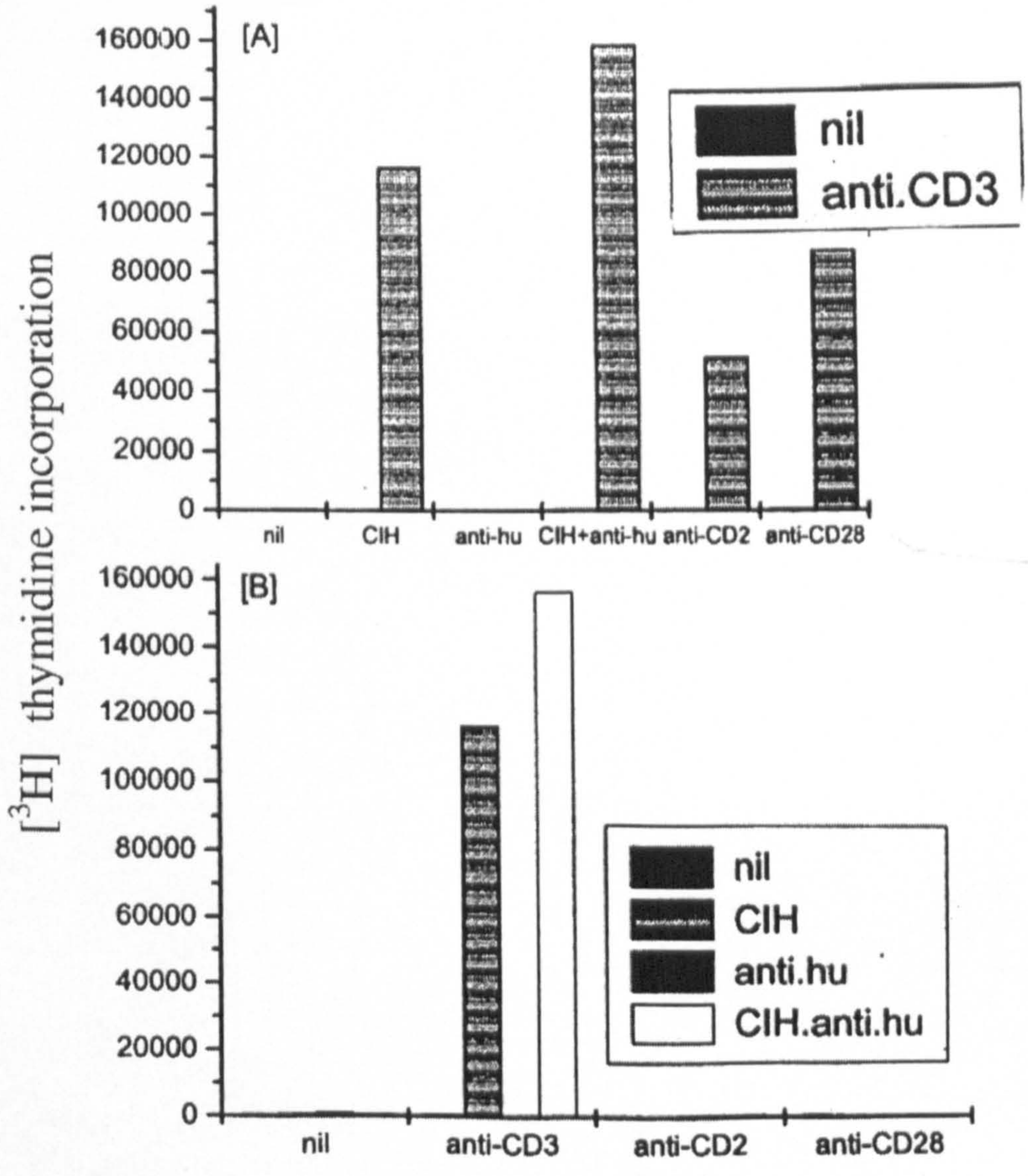


Figure 3.2 Augmentation of CD3 but not CD2, or CD28-mediated proliferation of CD4+ T lymphocytes by anti-CD52 antibody.

[A] Anti-CD3 mAb (0.1 μg/ml) was co-immobilised onto plastic microtitre plates in the presence or absence of CAMPATH-1H (C-1H), anti-human IgG, C-1H + anti-human IgG, anti-CD2 (39C15) or anti-CD28 mAb, all coated at 20 μg/ml. [B] C-1H in the presence or absence of anti-human IgG was coimmobilised together with anti-CD3, anti-CD2 or anti-CD28 mAbs coated at a concentration of 20 μg/ml. Purified CD4+ T cells (1 x 10^5/well) were cultured with immobilised antibodies for 5 days. [^{3}H]-thymidine was added for the last 18 hours of the culture. Results are expressed as c.p.m. of triplicate cultures (SEM<15%). [^{3}H]-thymidine incorporation of cultures in the presence of medium alone or with individual antibodies was <1000 cpm.

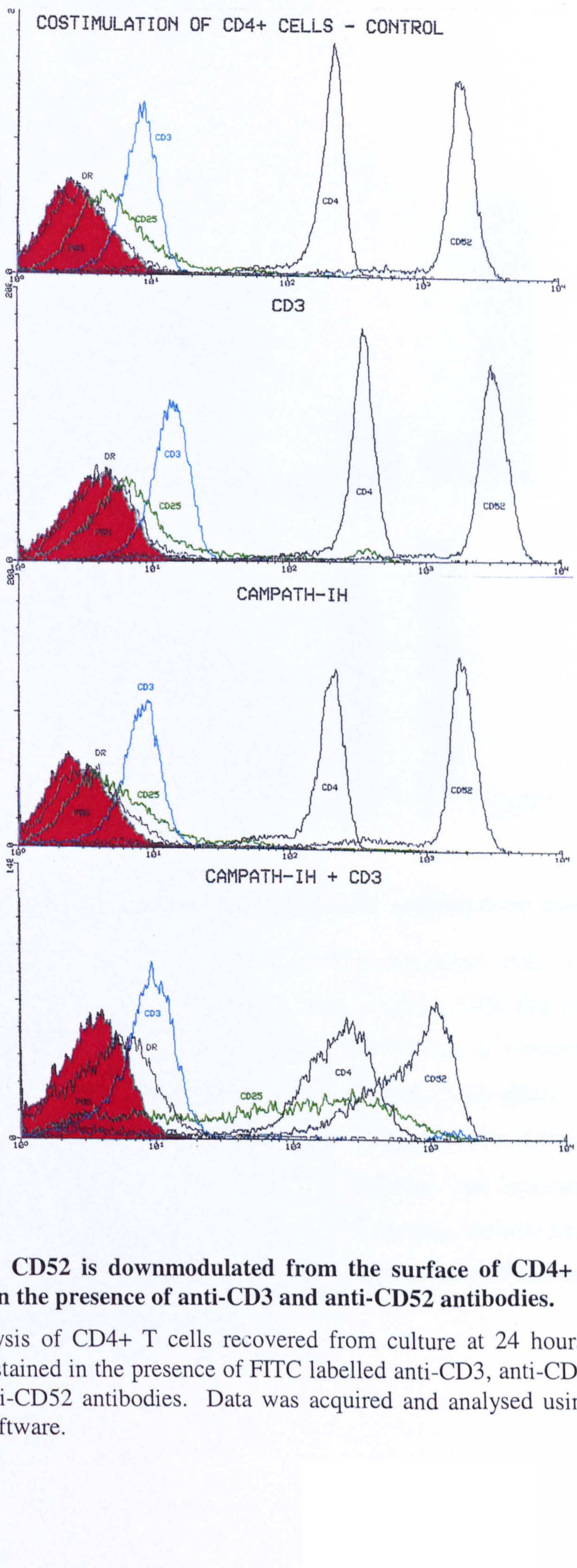


Figure 3.3 CD52 is downmodulated from the surface of CD4+ T cells following activation in the presence of anti-CD3 and anti-CD52 antibodies.

FACS analysis of CD4+ T cells recovered from culture at 24 hours post stimulation. Cells were stained in the presence of FITC labelled anti-CD3, anti-CD4, anti-CD25, anti-DR and anti-CD52 antibodies. Data was acquired and analysed using a FACScan and LYSIS II software.

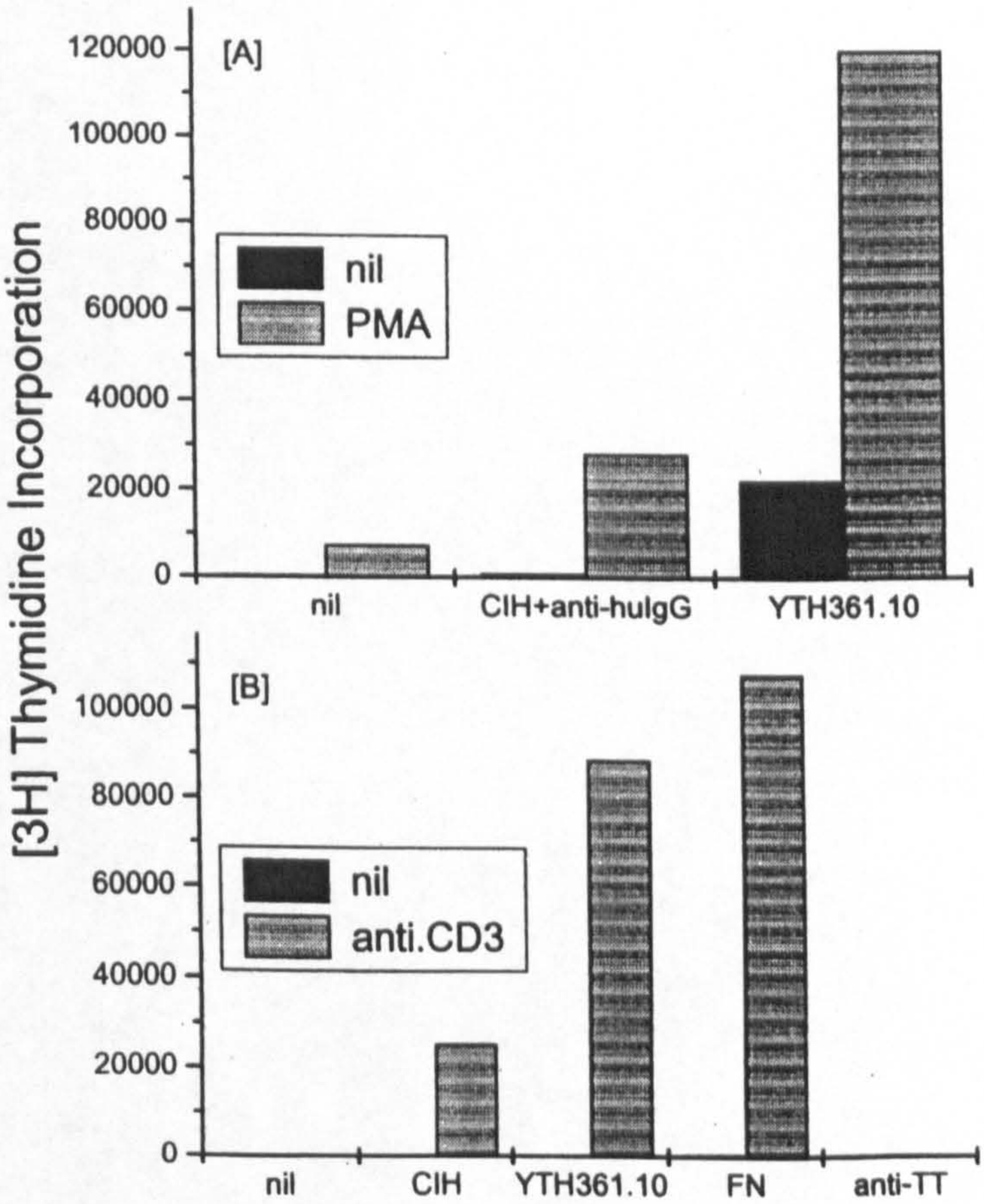


Figure 3.4 CD8+ T lymphocytes are induced to proliferate by anti-CD52 antibodies.

Purified CD8+ T cells (1 x 10^5/well) were incubated with, [A] medium alone, CAMPATH-1H (C-1H) + anti-human IgG or rat IgG_{2c} YTH 361.10 in the presence or absence of PMA or, [B] medium alone, C-1H, YTH 361.10, fibronectin (FN) or human anti-tetanus toxoid (anti-TT) IgG_1 mAb immobilised onto plastic, in the presence or absence of sub-optimal anti-CD3 antibody (0.1 μg/ml). Other antibodies were used at 20 μg/ml. Fibronectin was used at 10 μg/ml. Cultures were incubated for 5 days. [^{3}H]-thymidine was added during the last 18 hours of culture. Results are expressed as c.p.m. of triplicate cultures (SEM<15%). Counts in the absence of PMA or with each antibody alone were <500 c.p.m.

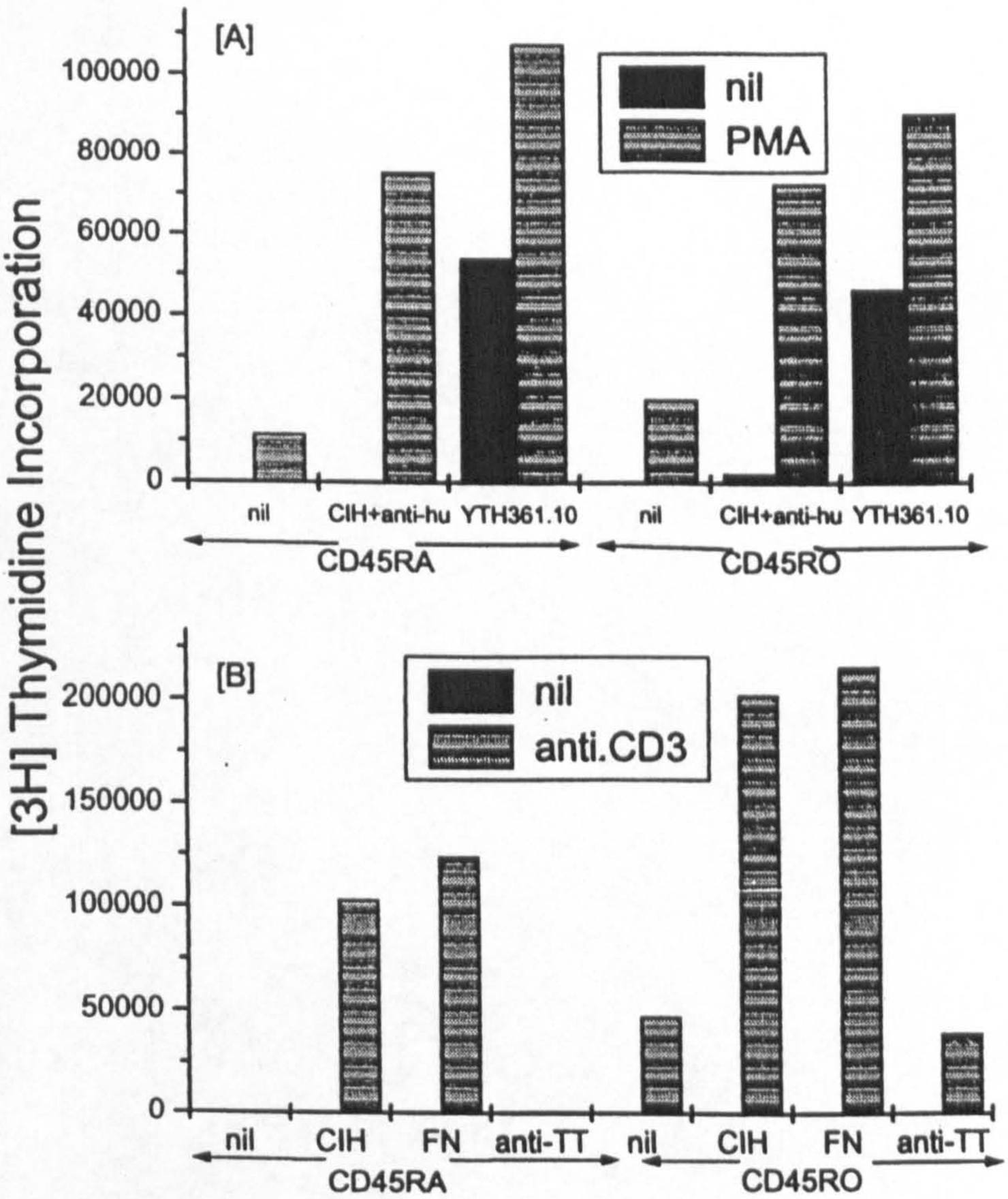


Figure 3.5 Anti-CD52 antibodies can induce proliferation in both naive (CD45RA+) and memory (CD45RO+) CD4+ T cells.

Purified CD45RA+ and CD45RO+ CD4+ T cells (1 x 10^5/well) were incubated with, [A] medium alone, CAMPATH-1H (C-1H) + anti-human IgG or rat IgG_{2c} YTH 361.10 in the presence or absence of PMA or, [B] medium alone, C-1H, fibronectin (FN) or anti-tetanus toxoid (anti-TT) mAb immobilised onto plastic in the presence or absence of sub-optimal anti-CD3 (0.1 μg/ml). All other antibodies were used at 20 μg/ml. Fibronectin was added at 10 μg/ml. Cultures were incubated for 5 days. During the last 18 hours of culture, [^{3}H]-thymidine was added. Results are expressed as mean c.p.m. and SEM, 15%. Background cultures in the absence of PMA or with each antibody alone were <800 c.p.m.

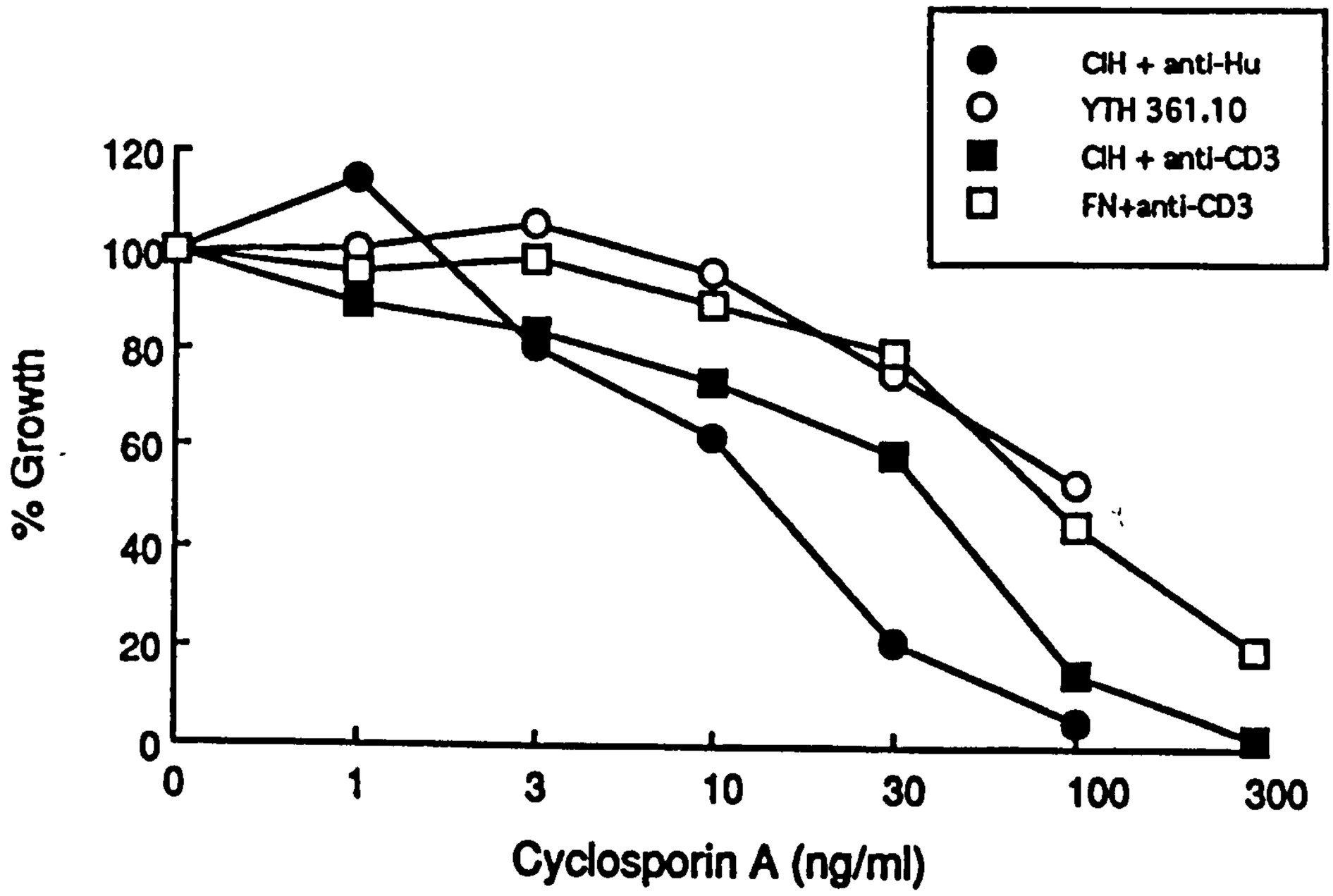


Figure 3.6 Cyclosporin A inhibition of CD4+ T cell proliferation induced by anti-CD52 antibodies.

Purified CD4+ T lymphocytes were incubated with concentrations of Cyclosporin A ranging from 0 - 300 ng/ml and stimulated with CAMPATH-1H (C-1H) + anti-human IgG + PMA (10 ng/ml), or rat IgG_{2c} YTH 361.10 added in solution, or alternatively, C-1H + sub-optimal anti-CD3 (0.1 μg/ml) and anti-CD3 + fibronectin (FN) coimmobilised onto plastic. All antibodies except anti-CD3 were used at 20 μg/ml. Fibronectin was used at 10 μg/ml. Cultures were incubated for 5 days. [^{3}H]-thymidine was added during the last 18 hours of culture. Results are shown as percentage growth of cultures without cyclosporin A. Proliferation in the absence of cyclosporin in terms of c.p.m. were as follows: C-1H + anti-human IgG + PMA, 772,629; YTH 361.10, 147,089; C-1H + anti-CD3, 147,089; fibronectin + anti-CD3, 354,154. Proliferation in the presence of each antibody alone was <1000 c.p.m.

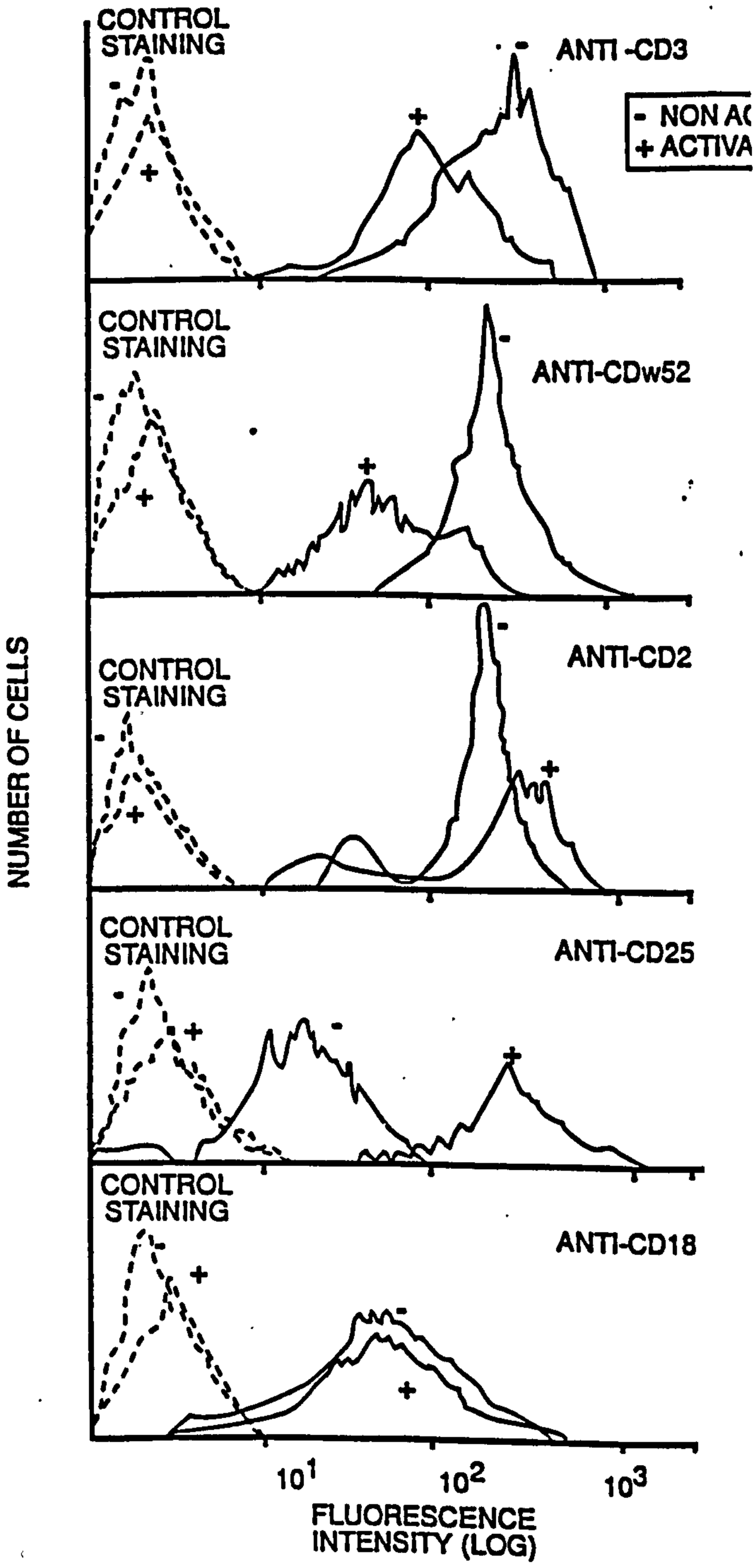


Figure 3.7 CD52 surface expression is downmodulated in a T cell clone following antigen activation.

MF14 T cells were activated by culturing together with DR1 transfected L cells, in the presence (+) or absence (-) of flu peptide. After 24 hours, cells were recovered and stained with FITC labelled antibodies against CD3, CD52, CD2, CD25 and CD18. Control staining with a mouse antibody isotype control is shown by the broken line profiles. Cells were analysed using a FACScan and LYSIS II software.

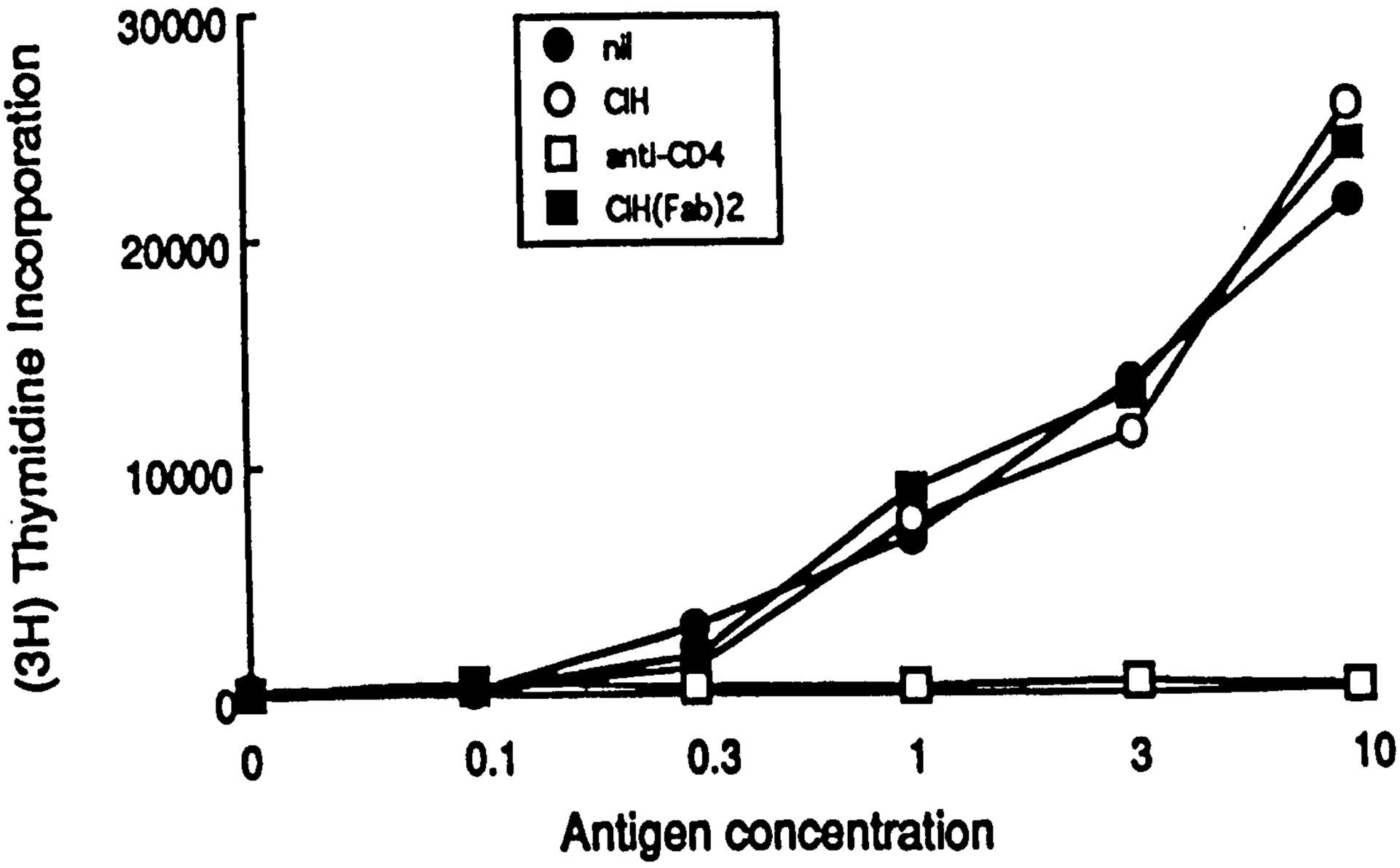


Figure 3.8 Anti-CD52 antibodies do not inhibit antigen specific proliferation of a T cell clone.

T cell clone MF14 cells (2 x 10^4/well) were stimulated with DR1 transfected fibroblasts and concentrations of peptide 206-229 derived from influenza nucleoprotein ranging from 0 - 10 μg/ml together with either medium alone, humanised anti-CD4 mAb, C-1H or C-1H $F(ab)_2$. All antibodies were used at a final concentration of 10 μg/ml. Cultures were incubated for 3 days. [^{3}H]-thymidine was added during the last 18 hours of culture. Results are expressed as mean c.p.m. and SEM<15%.

3.3 Discussion

The results above showed that as with Thy-1, Ly-6, Qa-2, CD55, CD59 and CD73 (reviewed by Malek et al., 1994; Robinson, 1991), antibodies directed against the GPI-anchored CD52 antigen can lead to T cell activation.

CAMPATH-1 antibodies have been shown to induce activation in both naive (CD45RA+) and memory (CD45RO+) CD4+ T cells and CD8+ T cells. Effector CD4+ T cells can be subdivided into two populations based on the profile of cytokines they secrete. Thus, Th1 cells secrete proinflammatory cytokines, including IFN-γ and TNF, whereas Th2 cells secrete cytokines including IL-4, IL-9, IL-10 and IL-13 and are responsible for controlling humoral immune responses (Reviewed by Constant and Bottomley, 1997). Analysis of cytokine release following CAMPATH-1 induced activation in CD4+ T cells indicated that both Th1 and Th2 type cytokines were released, those measured being IL-2, IL-4, IFN-γ and TNF. CD52 ligation does not, therefore, appear to be involved in polarising T cells towards a particular phenotype.

Activation of CD4+ T cells by CAMPATH-1H and CAMPATH-1G antibodies (human IgG_1 and rat IgG_{2b} antibodies, respectively), required secondary crosslinking and the presence of either PMA, or cytokines. Interestingly, the receptors of the cytokines found to be effective, namely, IL-2, IL-4 and IL-7, all share a common functional component, described previously as the IL-2 receptor gamma chain (Kondo et al., 1993; Noguchi et al., 1993).

In contrast, activation of CD4+ T cells by the rat YTH 361.10 anti-CD52 antibody (IgG_{2c}), added in solution, did not require secondary crosslinking, or addition of PMA. Addition of PMA could augment the proliferation induced by the anti-CD52 IgG_{2c} antibody alone, suggesting that activation was not optimal. Experiments performed in murine T cells with a panel of antibodies directed against the Thy-1 antigen found likewise (Kummer et al., 1993). Generally, anti-Thy-1 antibodies could activate only when crosslinked with a secondary antibody together with PMA; however antibodies bearing the IgG_{2c} isotype did not require secondary crosslinking or addition of PMA. The

mitogenicity of these antibodies could be explained by the ability of the IgG_{2c} isotype Fc regions to self associate (Kummer et al., 1993). In addition, the YTH 361.10 antibody has an approximately 10 fold higher affinity for the CD52 antigen compared to CAMPATH-1H and CAMPATH-1G (R. Lifely, personal communication).

In agreement with several other studies on T cell activation by ligation of GPI-anchored proteins, it was found that immobilisation of anti-CD52 antibodies onto plastic did not stimulate activation in the presence of PMA (Robinson, 1991). This could be because the activation process is dependent on the lateral mobility of the GPI-anchored proteins in the membrane and/or the requirement for the GPI-anchored proteins to be internalised to activate signalling mechanisms (Bamezai, 1989). The GPI-anchor appears to play an important role for these proteins to function in activation. This has been demonstrated by studies in transgenic mice in which the Qa-2 molecule was expressed as either a GPI-anchored or a transmembrane protein bearing the transmembrane domain of class I molecules. Only T cells from mice bearing GPI-anchored Qa-2 could be activated with Qa-2 specific antibodies (Robinson et al., 1989).

In contrast to the above, when anti-CD52 antibodies were coimmobilised onto the same surface as anti-CD3 antibodies, cells were stimulated to proliferate. The reason for this difference is not clear. Expression of a functional T cell receptor appears to be essential for activation by GPI-anchored proteins (Korty et al., 1991, Gunter et al., 1987). Therefore, by bringing the CD52 and CD3 molecules into close proximity, the appropriate interactions could take place. It also implies that the reasom why plastic immobilised CAMPATH-1 antibodies in the presence of PMA, do not stimulate proliferation, is not due to the inability of the cells to internalise the GPI-anchored CD52. In addition, activation of T cells is accompanied by downregulation of the TCR complex (Valitutti and Lanzavecchia, 1997) and the fact that proliferation is achieved with immobilised anti-CD3 suggests that immobilisation of the activating antibodies is not preventing the internalisation of the target receptors.

The involvement of the calcium dependent calmodulin-calcineurin pathway in activation following CD52 ligation is suggested by the observation that these responses are blocked by cyclosporin A, which is a specific inhibitor of this pathway (Liu et al., 1991). This

pathway is critical for full induction of IL-2 production following ligation of the T cell receptor. This supports the observations made that the proliferation induced following crosslinking by CD52 is predominantly driven by IL-2 and indicates that similar or complementary pathways may be used by both the TCR and CD52.

The relevance of T cell activation in response to CAMPATH-1H antibody *in vivo*, is unclear. The most dramatic effect of the antibody was to completely deplete lymphocytes from peripheral blood (Brett et al., 1996a). However, the extent of depletion in lymphoid organs is less clear. A noticeable finding following CAMPATH-1 therapy, was that lymphocytes repopulating the blood, nearly all showed evidence of recent activation, by expression of HLA-DR, CD45RO (Brett et al., 1996a; Jendro et al., 1995) and CD25 (Sara Brett, personal communication). One explanation for these observations, is that in certain lymphoid areas, CAMPATH-1H coated T cells, could interact with Fc receptors on dendritic cells. A possible consequence of this could be that these cells were then activated, rather than depleted. DCs are not lytic, are Fc receptor positive and express many costimulatory molecules (Banchereau and Steinman, 1998). Also DCs will cluster T cells, which may be releasing cytokines, including IL-2 and IL-4. This may then have a bystander effect. The results in this chapter have shown that these cytokines will synergise with crosslinked CAMPATH-1H antibody to induce T cell proliferation. Such T cells may then subsequently enter recirculation and be detected as recently activated cells in peripheral blood.

An alternative explanation to the above, is that activated surviving T cells occur in response to opportunistic infections which were characteristic of many of the patients treated with CAMPATH-1 antibodies. It has been shown that there is a dramatic clonal expansion of activated T cells following certain acute viral infections eg. EBV (Borthwick et al., 1997). In addition, it was shown that following antigen specific activation, CD52 was downmodulated from the T cell surface. These T cells would then no longer be susceptible to CAMPATH-1 therapy. Therefore, an ongoing T cell response at the time of CAMPATH-1 therapy could provide a source of T cells which would not be depleted during treatment. After the disappearance of CAMPATH-1 antibody from the circulation, expansion of these cells could account for the appearance of T cells bearing a recently activated phenotype. In support of this, Jendro et al., 1995, showed that the T

cell populations which emerged following CAMPATH-1H treatment in RA patients, were oligoclonal. These cells could represent a small number of T cells activated by antigen during CAMPATH-1 therapy.

In summary, it has been shown *in vitro* that CAMPATH-1 antibodies together with a second signal (provided by PMA, or suboptimal doeses of anti-CD3, or IL-2, IL-4 or IL-7 cytokines) can induce T cell proliferation. CAMPATH-1 antibody could not block an antigen specific activation of a T cell clone, suggesting that under normal physiological conditions, CD52 is not involved in T cell activation. However, the data suggests that in the 'right' environment, CAMPATH-1 antibodies could be responsible for the presence of recently activated T cells observed *in vivo,* following CAMPATH-1 treatment.

4. Effects of crosslinking CD52 on B and T tumour cell lines

4.1 Introduction

The CD52 antigen is expressed at high levels on all B lymphocytes and on the majority of B cell lineage malignancies (Dyer et al., 1990). Therefore, antibodies directed against the CD52 antigen have been investigated for their potential as therapeutic agents in a number of lymphoproliferative diseases including non-Hodgkin's lymphoma (NHL) (Hale et al., 1988b). A panel of rat monoclonal CD52 specific antibodies, including both IgM and IgG isotypes have been shown in *in vitro* assays to be very efficient at cell lysis in the presence of human complement (Bindon et al., 1988). *In vivo,* however, only the rat IgG_{2b} antibody, namely CAMPATH-1G, has proved to be effective in the depletion of lymphoid cells (Dyer et al., 1989; Dyer et al., 1990). The reason for this has been attributed to the fact that in addition to complement mediated lysis, the rat IgG_{2b} isotype and the humanised version, CAMPATH-1H (IgG1) bind to FcIII receptors on effector cells and thereby bring about cell mediated killing. The mechanism proposed for the cell mediated killing is ADCC, or antibody dependent cell-mediated cytotoxicity (Hale et al., 1985; Riechmann et al., 1988; Crowe et al., 1992).

Whether the mechanism of action of CAMPATH-1 antibodies *in vivo* in depletion of lymphocytes is entirely due to these mechanisms is unknown. An additional mechanism by which antibodies could have a direct effect on lymphocyte depletion is by inducing Programmed Cell Death (PCD), also referred to as apoptosis.

There are precedents in the literature for antibodies directed against various lymphocyte surface antigens initiating signalling pathways and driving cells into apoptosis. These include antibodies to the B cell receptor (Benhamou et al., 1990; Hasbold and Klaus, 1990), CD3/T cell receptor complex (Odaka et al., 1990; Smith et al., 1995) and the Fas/apo-1 (CD95) antigen (Alderson et al., 1995; Mapara et al., 1993). *In vivo* studies in mice have shown that antibodies directed against CD95 have a lethal effect (Ogasawara et al., 1993). Many B and T tumour cell lines have been shown to undergo apoptosis

following ligation of the antigen specific receptor (Ishigami et al.,1992; Ucker et al., 1989).

Apoptosis is a controlled form of cell death first described by Kerr et al. in 1972. It is characterised morphologically by a shrinkage in cell volume, the organelles become tightly packed, the chromatin clumps and the cell exhibits zeiosis. The chromatin subsequently collapses into crescents along the nuclear envelop. The collapsed nucleus breaks up into spheres following endonuclease activation and the cell fragments into apoptotic bodies. During this whole series of processes, the cell maintains membrane permeability. The apoptotic bodies are rapidly ingested by phagocytes and so there is no localised inflammatory reaction (Savill et al., 1993). This is in contrast to necrosis or accidental cell death in which the cell loses membrane permeability and is unable to regulate its osmotic pressure; the cell then swells and lyses releasing cell contents into the environs and this in turn causes localised inflammatory responses (Cohen, 1991).

Evidence has been provided in the previous chapter that crosslinking CD52 on normal human T lymphocytes, as observed with other GPI-linked molecules eg.Thy-1 (Robinson et al., 1991), triggered clonal expansion. It was therefore, of interest to determine the effects of crosslinking CD52 on transformed lymphoid cells as these are a clinical target for the CAMPATH-1 antibodies. The following studies suggest that signals via the CD52 antigen are capable of inducing apoptosis in both B and T tumour cell lines *in vitro*, in the absence of complement and effector cells and that this could, therefore, present an alternative mechanism whereby CAMPATH-1 antibodies effectively deplete lymphoid cells *in vivo*.

4.2 Results

4.2.1 Effect of crosslinking CAMPATH-1H antibody on the growth of different B and T tumour cell lines

Cell lines available to determine the effects of CAMPATH-1 antibody on the growth of B and T tumour cells included the B cell lines, Wien 133 and EBV, and the T cell lines, HUT 78 and Jurkats, including the parental line and a CD52 transfected line, designated 3B11 (provided by C. Hale, Wellcome, Beckenham, UK). Antibodies were added in solution at a final concentration of 20 μg/ml and left in the culture for the duration of the experiment. After 5 days culture, the growth of the cells was assessed by their ability to incorporate [^{3}H]-thymidine. Results are shown in figure 4.1. In both the Wien 133 and the CD52 transfected Jurkat 3B11 cell lines, CAMPATH-1H antibody alone had no effect; anti-human IgG alone did have a slight effect on inhibiting the growth of the cells in some experiments in the Wien 133 cell line; the combination of the two antibodies resulted in a significant reduction in cell growth. In the HUT 78 and the parental Jurkat cell lines, crosslinking CAMPATH-1H had very little effect. The growth of the EBV cell line was partially inhibited by crosslinking CAMPATH-1H. The differences in the effects of CAMPATH-1H on the growth of the different cell lines could be explained by the expression levels of the CD52 antigen, shown in figure 4.2. Both EBV and parental Jurkat cells have minimal levels of CD52 expressed on the cell surface with Mean Fluorescence Indexes (M.F.I.) of 10 and 6.5 respectively; the HUT 78 cell line expresses moderate levels of CD52 with M.F.I. of 72; the Wien 133 cell line and the CD52 transfected Jurkat 3B11 cell lines express high levels of CD52 antigen with M.F.I. of 873 and 400 respectively. In comparison, the M.F.I.s for CD52 staining of T and B cells taken from normal human peripheral blood were 617 and 510 respectively (Gill Baxter, personal communication).

4.2.2 The growth inhibition of the Wien 133 B cell lymphoma by CAMPATH-1H antibody is CD52 specific

The Wien 133 cell line was selected for further study because it was the only naturally occurring cell line expressing high levels of CD52 antigen as shown in figure 4.2. In addition to CD52, the line is positive for surface IgG, IgD, IgM, CD19, CD20, HLA DR and FcγII receptor, CD32 (Figure 4.3). The line is negative for the other two Fcγ receptors, namely, CD16 and CD64. The dose response effect of CAMPATH-1H antibody crosslinked with secondary anti-human IgG on the growth of Wien 133 cells is shown in figure 4.4. From these results, an optimal concentration of 20 μg/ml of each antibody was selected for further experiments.

Growth inhibition of Wien 133 cells was also obtained, if the experiment was performed coating the CAMPATH-1H and anti-human IgG antibodies to the plates. Antibodies were diluted in PBS at 20 μg/ml and coated to the plates by incubation at 37°C for 3 hours. Unbound antibody was removed by washing the plates three times in PBS prior to the addition of cells. Within 1 hour, in the presence of CAMPATH-1H and anti-human IgG, the cells' morphological appearance had altered; in the absence of antibodies the cells were spherical in shape and tended to form large clusters, but in the presence of antibodies the cells assumed spindly shapes, becoming spread with numerous appendages and did not form clusters. Under these culture conditions, using toluidine blue, the cells were shown to have become adherent and therefore, following ^{3}H-thymidine incorporation, the plates were frozen at -20°C and thawed before harvesting, to ensure that all the viable cells were counted.

4.2.3 Growth inhibition of B cell lymphoma is CD52 specific

In some experiments, anti-human IgG alone had a partial effect on inhibiting cell growth and so to show that the growth inhibition of Wien 133 cells in the presence of a humanised antibody directed against the CD52 antigen is not solely due to formation of immune complexes or signalling via the surface IgG, the experiment above was repeated

using the rat CD52 specific IgG2b antibody, CAMPATH-1G and crosslinking with a secondary anti-rat IgG. The results are shown in figure 4.5. CAMPATH-1G and anti-rat IgG antibodies alone had no effect on inhibiting cell growth. However, when CAMPATH-1G was incubated with the cells in the presence of anti-rat IgG (not cross reactive with human IgG), there was significant inhibition in cell proliferation. This would suggest that the growth inhibition observed was a specific effect induced via crosslinking the CD52 antigen and not due solely to a synergistic effect crosslinking CD52 and surface IgG.

To further investigate the interaction between CD52 and surface IgG, cells were preincubated in the presence of anti-human IgG and then excess antibody was removed by washing the cells before adding the CAMPATH-1H antibody. Under these conditions, the cells were not susceptible to growth inhibition. If the reverse experiment was performed ie. cells were incubated with CAMPATH-1H antibody first, excess antibody removed by washing and then culturing the cells in the presence of anti-human IgG, there was growth inhibition. Results are shown in figure 4.6. This indicates that secondary crosslinking of the CAMPATH-1H antibody is required to induce growth inhibition.

In addition to surface immunoglobulin, CD32 Fc receptors are also present as markers on B cells. The inhibition of Wien 133 cell growth could be due to the CAMPATH-1 antibodies binding to and crosslinking Fc receptors. Experiments were carried out using $F(ab)_2$ fragments of CAMPATH-1H and anti-human IgG antibodies. As with the intact antibodies, CAMPATH-1H $F(ab)_2$ had no effect on cell growth, anti-human IgG $F(ab)_2$ partially inhibited the growth of Wien 133 cells in some experiments. In combination, the CAMPATH-1H and anti-human IgG $F(ab)_2$ fragments resulted in growth inhibition, indicating that there is no requirement for Fc interactions. Results are shown in figure 4.7.

For additional confirmation that the growth inhibitory effects observed by CAMPATH-1H antibody were not due to immune complex formation, the effect of humanised anti-CD4 antibody, bearing the same isotype as CAMPATH-1H antibody, was studied in combination with anti-human IgG. B cells do not have CD4 as a surface marker and so any interaction with the B cells would have to be via the Fc portion of the antibody. Anti-

CD4 antibody had no effect; as observed previously, the anti-human IgG antibody partially inhibited the growth of cells, but the effect was not enhanced when used in combination with the anti-CD4 antibody. Results are shown in figure 4.8. Therefore, the inhibition of cell growth is not due to the formation of non-specific immune complexes formed via Fc interactions.

4.2.4 The effect of isotype on the CD52 specific growth inhibition of Wien 133 cells

In vivo studies with CD52 specific antibodies suggest that the successful depletion of lymphocytes by unknown mechanisms is dependent on isotype. Therefore, in addition to the human CAMPATH-1H IgG_1 and the rat CAMPATH-1G IgG_{2b} antibodies, it was of interest to compare the effect of a number of CD52 specific antibodies available bearing different isotypes, including, a rat IgG2c, rat IgM and a human IgG_4 on the growth of Wien 133 cells. Cells were incubated with the different anti-CD52 antibodies in the absence of a secondary crosslinking antibody or with anti-human IgG or with anti-rat IgG. Results are shown in figure 4.9. As in previous experiments, human CAMPATH-1H (IgG_1) and rat CAMPATH-1G (IgG_{2b}) had no effect on cell growth unless they were crosslinked with the relevant second antibody. Rat IgM had no effect. Human IgG4 had a partial effect on inhibiting cell growth alone; this was augmented in the presence of anti-human IgG. Rat IgG_{2c} partially inhibited the growth of Wien 133 cells alone, this was augmented by the presence of anti-rat IgG. Biocore data looking at the affinity of the different CAMPATH-1 isotypes for CD52 antigen showed rat IgG_{2c} >rat IgG_{2b} = human IgG_1 = human IgG_4 > rat IgM (Personal communication R. Lifely); this could in part explain differences in the extent of growth inhibition in Wien 133 cells induced by the different isotypes. In addition, IgG_{2c} isotypes have the ability to self aggregate via Fc:Fc interactions (Kummer et al., 1993) and can directly activate T cells (Chapter 3) which could account for the ability of this antibody to partially inhibit the growth of Wien 133 cells without secondary crosslinking. The human IgG_4 antibody provided contained aggregates (N. Rapson, personal communication) and this again could explain why this particular antibody partially inhibited the growth of the Wien 133 cells without secondary crosslinking with anti-human IgG.

4.2.5 Growth inhibition of B cell lymphoma induced via surface immunoglobulin

Surface immunoglobulin is the antigen specific B cell receptor and depending on the activation state of the cell, crosslinking the receptor can push the cells towards proliferation (Cooke et al., 1994) or alternatively result in cell death by apoptosis (Benhamou et al., 1990). In Wien 133 cells, FACS analysis (Figure 4.3) using FITC-labelled anti-IgG, anti-IgD, anti-IgM and CAMPATH-1H showed that whereas the levels of expression of the CD52 antigen and surface IgM and IgD were high, the expression of IgG was lower (Figure 4.3). As indicated above, the growth of Wien 133 cells could be partially inhibited by anti-human IgG alone; this could be enhanced by adding a second crosslinking antibody (Figure 4.10). In contrast, anti-IgM alone was sufficient to cause significant cell growth inhibition and the effect was apparent at comparatively low antibody concentrations of 0.8 μg/ml final concentration (Figure 4.11).

4.2.6 Surface IgM is not required for cell growth inhibition induced via the CD52 antigen

Evidence from T cell tumours indicates that growth inhibition induced by crosslinking GPI linked surface molecules, such as CD59 (Korty et al., 1991) requires the presence of a functional antigen receptor. To determine whether the B cell surface immunoglobulin and hence the signalling apparatus linked to the receptor is required to obtain the growth inhibition effect of crosslinking the CD52 antigen, attempts were made to generate surface Ig negative cell lines. Repeated efforts of culturing the Wien 133 cell lines with a cocktail of antibodies against IgG, IgD and IgM failed to produce any surviving cells. However, using anti-IgM alone at 3 μg/ml, a population of cells was obtained which by FACS analysis were shown to be negative for surface IgM, but expressed surface IgD at high levels (Figure 4.12); CD52 expression remained high; surface IgG expression remained low. The IgM-, IgD+ cells were cloned by limiting dilution. Resulting clones were tested to see the effect of crosslinking CD52 on the cell surface with CAMPATH-1H and anti-human IgG and as in experiments with the parental cell line described above, there was growth inhibition. There was no growth inhibition effect when the cells were

treated with anti-IgM as one would expect; however, the cells were sensitive to treatment with anti-IgD without the requirement for further crosslinking and cell proliferation was inhibited (Figure 4.13). This is in contrast to the effect of anti-IgD on the parental Wien 133 cell line which, although positive for IgD expresssion, was not sensitive to anti-IgD induced growth inhibition. The reason for this could be attributed to the Wien 133 C1 cells expressing a variable level of IgD, compared to the IgM selected cells. This suggests that growth inhibition by IgD is dependent on antigen density.

These results indicate that surface IgM is not required for cell growth inhibition via the CD52 antigen. Other surface immunoglobulin molecules, namely IgG or IgD, may compensate for this as they can also mediate the growth inhibitory effect.

4.2.7 Does CAMPATH-1H synergise with anti-IgM antibody to mediate growth inhibition in Wien 133 cells?

CAMPATH-1H together with anti-human IgG and anti-IgM alone have been shown to inhibit the growth of Wien 133 cells. It was of interest to see whether anti-IgM at suboptimal levels is able to synergise with CAMPATH-1H to inhibit cell growth in the absence of crosslinking antibody. For this experiment, tissue culture plates were coated with anti-IgM at a range of antibody concentrations from 0 - 20 μg/ml, with and without CAMPATH-1H antibody at a constant concentration of 20 μg/ml. After 5 days culture, there was no apparent synergy between CAMPATH-1H and anti-human IgM leading to growth inhibition. However, at low doses of anti-IgM (0.8 μg/ml), the presence of CAMPATH-1H appeared to partially reduce the cell growth inhibition effects induced by anti-IgM (Figure 4.14).

4.2.8 The cell growth inhibition effect induced by CAMPATH-1H antibody in Wien 133 cells is time dependent

A comparison of the time course of growth inhibition induced by crosslinking CD52 or IgM on the surface of Wien 133 cells is shown in Figure 4.15. Wien 133 cells were cultured in the presence of CAMPATH-1H and anti-human IgG (20 μg/ml) or anti-IgM (4 μg/ml) and cells harvested at 4, 24, 48, 72 and 96 hours. The effect on cell growth with anti-IgM was apparent from as early as 4 hours post-treatment. Anti-human IgG transiently inhibited the growth of cells at 24 hours, but then the cells showed some recovery and there was no further effect with time. CAMPATH-1H antibody alone had no effect. CAMPATH-1H and anti-human IgG antibody together, resulted in growth inhibition at the 48 hour time point which, unlike the effect of anti-human IgG alone, was sustained with time over 72 and 96 hours.

4.2.9 Effect of soluble cytokines on the inhibition of growth of Wien 133 cells induced by CAMPATH-1H antibody.

Numerous reports in the literature have shown that growth inhibition of both normal B cells and some B cell lines, can, in response to various external stimuli, be reversed in the presence of certain cytokines (Dancescu et al., 1992; Buschle et al., 1993). The effect on the growth inhibition of Wien 133 cells induced by crosslinking surface CD52, cultured in the presence of a number of cytokines known to affect B cells, was investigated. The experiment was set up as previously described, adding the antibodies in solution, together with the cytokines for the duration of the assay at a range of physiological concentrations (The results are presented in table 4.1). Interleukin-1α did not show consistent results. IL-2, IL-6 and IFN-γ had no effect. IL-4 appeared to synergise with CAMPATH-1H and IgG to inhibit cell growth. However, at 0.3 ng/ml, TNF-α was toxic to cell growth, anti-human IgG antibodies to enhance the growth inhibition of cells. IL-7 synergised with anti-human IgG alone to increase the growth inhibition of cells. TNF-α at 0.3 ng/ml synergised with anti-human IgG alone and CAMPATH-1H in the presence of anti-human

Table 4.1. Effect of cytokines on CAMPATH-1H induced growth inhibition in Wien 133 cells.

Interleukin-1 (pg/ml)						
	0	1	3	10	30	100
Control	100	103	80	87	81	83
C-1H	81	81	78	81	82	78
Anti-hulgG	97	91	93	88	86	86
C-1H+anti-hulgG	17	14	25	25	23	21

Interleukin-2 (ng/ml)						
	0	0.01	0.03	0.1	0.3	1
Control	100	100	105	102	107	108
C-1H	98	96	103	93	103	100
Anti-hulgG	71	66	77	67	69	71
C-1H+anti-hulgG	17	14	17	17	16	17

Interleukin-4 (ng/ml)						
	0	0.3	1	3	10	30
Control	100	103	104	99	96	93
C-1H	89	96	92	96	95	102
Anti-hulgG	71	70	65	62	63	62
C-1H+anti-hulgG	29	14	11	10	10	11

Interleukin-6 (ng/ml)						
	0	0.1	0.3	1	3	10
Control	100	102	104	101	107	105
C-1H	95	98	100	102	109	109
Anti-hulgG	66	65	56	62	65	67
C-1H+anti-hulgG	19	17	18	19	17	17

Interleukin-7 (ng/ml)						
	0	0.1	0.3	1	3	10
Control	100	86	89	88	82	81
C-1H	93	94	88	79	77	77
Anti-hulgG	60	53	47	38	34	30
C-1H+anti-hulgG	14	13	15	13	9.5	7

Interferon-Gamma (U/ml)						
	0	0.3	1	3	10	30
Control	100	90	95	95	96	70
C-1H	92	89	89	81	87	91
Anti-hulgG	59	59	54	60	60	63
C-1H+anti-hulgG	16	14	17	18	16	17

TNF (ng/ml)		
	0	0.3
Control	100	44
C-1H	90	49
Anti-hulgG	58	7.4
C-1H+anti-hulgG	12	0.002

Wien 133 cells (2 x 10^4/well) were incubated for 5 days in the presence of medium alone, anti-human IgG alone, C-1H, or C-1H + anti-human IgG in the presence of cytokines, added at a range of concentrations as indicated. All antibodies were added at 20 µg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as percentage inhibition of control cell growth.

Ig to inhibit the growth of cells. At this concentration, TNF-α in the absence of antibody, inhibited cell growth to less than 50% of the final concentration. Overall, the addition of any of the recombinant cytokines tested did not consistently rescue cells from anti-CD52 induced growth inhibition.

4.2.10 Effect of antibodies directed against CD23 and CD40 on growth inhibition in Wien 133 cells induced by crosslinking CD52 antigen

Reports in the literature have shown that antibodies directed against CD23 in the presence of interleukin-1α and antibodies to CD40 can prevent cell growth inhibition of certain B cell tumours eg. WEHI-231 (Valentine and Licciardi, 1992). Monoclonal antibodies to CD23 or CD40 at a range of concentrations were cultured with Wien 133 cells in the presence of CAMPATH-1H and anti-human IgG as described above. Anti-CD23 was also tested in the presence of interleukin-1α (range 0 - 100 pg/ml. Neither anti-CD23 nor anti-CD40 appeared to have any effect on preventing the CAMPATH-1H induced cell growth inhibition; In fact anti-CD23 alone, at 1/50 dilution, caused partial growth inhibition. Anti-CD23 did not synergise with interleukin-1α to prevent growth inhibition.

4.2.11 CAMPATH-1H antibody induces apoptosis in Wien 133 cells

Growth inhibition in Wien 133 cells follows different time courses dependent on the stimulus. Thus, the effect on cell growth inhibition mediated by anti-IgM is very rapid and long lasting, whereas anti-human IgG has a transient effect on cell growth inhibition. The growth inhibition mediated by CAMPATH-1 antibodies crosslinked by secondary antibody has a much slower induction phase compared to IgM. However, in contrast to the effect of anti-human IgG alone, and akin with the effect of anti-IgM, the effect is long lasting. Numerous reports in the literature have shown that ligation of IgM on the surface of B cell lines induces apoptosis (Benhamou et al., 1990). A number of characteristic features are associated with the onset of apoptosis, including their morphological

appearance and the breakdown of cellular DNA (McCloskey et al., 1994). Experiments were, therefore, performed to investigate whether the long lasting effects of CAMPATH-1 antibodies in the presence of secondary crosslinking antibody on the growth of Wien 133 cells is due to induction of apoptosis.

Light microscopy of cells treated with Giemsa/May-Grunwald stain, enabled identification of apoptotic cells. Apoptotic cells were identified as having condensed nuclei, blebbed membranes and being smaller than normal cells due to shrinkage of the cell cytoplasm. Cells were cultured in the presence of either no antibody, CAMPATH-1H alone, anti-human IgG alone, CAMPATH-1H and anti-human IgG together, or anti-IgM. Samples were taken at 4, 24, 48, 72 and 96 hours. Cells were cyto-centrifuged and slides were stained. Examination of the slides revealed that at 4 and 24 hours, there were very few apoptotic cells following treatment of the cells with either no antibody, CAMPATH-1H alone, anti-human IgG alone or CAMPATH-1H together with anti-human IgG; in contrast, anti-IgM resulted in a significant number of apoptotic cells as early as 4 hours post-treatment. At 72 hours, there was an increase in the percentage of apoptotic cells following treatment with CAMPATH-1H and anti-human IgG antibodies; there was still very little evidence of apoptosis in cells cultured in the absence of antibody or with CAMPATH-1H or anti-human IgG antibody alone. The number of apoptotic cells was counted and expressed as a percentage of the total number of cells looked at. The results are shown in figure 4.16. The 96 hour time point gave very similar figures to the 72 hour time point.

In addition to light microscopy, electron microscopy enabled higher magnification of cells for examination. Wien 133 cells were cultured in the absence of antibody, with CAMPATH-1H alone, with anti-human IgG alone or with CAMPATH-1H antibody together with anti-human IgG as previously. Cells were harvested at 24, 48, 72 and 96 hours, fixed using glutaraldehyde and then samples were prepared for electron microscopy. Apoptotic cells were identified as those with classical morphological features as described in the literature, including a blebbed, but relatively intact membrane, condensed chromatin, sparse cytoplasm but intact organelles (Figure 4.17).

Apoptosis in some cells has been shown to involve the cleavage of DNA by endonucleases into 200 bp fragments which when separated by electrophoresis on agarose gels produce a characteristic laddering effect (Nakashima et al., 1993). Cells were treated with antibodies as described previously and samples taken at 24, 48, 72 and 96 hours post-treatment. Incorporation of α^{32}dATP into DNA extracted from the cells enabled visualisation of fragmented DNA following electrophoresis (Figure 4.18). After 24 hours, cells treated with anti-IgM produced a distinct laddering pattern; at the 96 hour time point, cells treated with CAMPATH-1H and anti-human IgG produced a smear rather than a distinct laddering pattern. A possible explanation for this difference could be a reflection of the asynchronous apoptosis following CD52 crosslinking over a period of 5 days as compared to the very rapid cell death induced by treatment with anti-IgM which takes effect within 24 hours. With no phagocytes to take up the apoptotic bodies, cells would eventually become necrotic and so in CAMPATH-1H treated cells there is likely to be an accumulation of apoptotic cells becoming necrotic.

Changes in DNA composition due to cells undergoing apoptosis can also be demonstrated by fixing cells in ethanol, staining cellular DNA with propidium iodide and analysing the cells by FACS (Figure 4.19)(McCloskey et al., 1994). Cells treated with antibodies as previously described were cultured for 24, 48, 72 or 96 hours. Results are given for the 96 hour time point. In the control cells (untreated), there is a small sub G0/G1 peak representing apoptotic cells, this is followed by a peak representing the cells in G1 phase of the cell cycle, followed by a percentage of cells in S phase and finally, the cells staining strongest with the propidium iodide, representing the cells in G2/M phase. The same pattern is seen in when cells are treated with CAMPATH-1H alone. With anti-human IgG alone, there is an increase in the number of cells in the sub G0/G1 peak. There was a further increase when CAMPATH-1H was crosslinked with anti-human IgG. By 96 hours, the majority of the anti-IgM treated cells are dead, demonstrated by Trypan blue staining. This is reflected in the propidium iodide staining where these cells appear as small staining debris.

4.2.12 CAMPATH-1H antibody induced apoptosis in Wien 133 cells does not depend on expression of CD95 (Fas)

Studies in the literature have indicated that the induction of Fas and Fas ligand in T and B cells is an important pathway for apoptosis (reviewed by Van Parijs and Abbas, 1996). Therefore, experiments were performed to investigate if Fas was involved in anti-IgM or CAMPATH-1H induced cell death in Wien 133 cells.

Wien 133 cells were treated with no antibody, CAMPATH-1H alone, anti-human IgG alone, CAMPATH-1H and anti-human IgG together, and anti-IgM and then analysed by FACS at 6, 24, 72 and 96 hours for expression of Fas. Wien 133 cells prior to treatment were negative for Fas expression and there was no increase in Fas cell surface expression following antibody treatment when compared to the negative control.

Further confirmation for Fas not being involved in mediating cell death in the Wien 133 cells was obtained by incubating the cells in the presence of anti-Fas antibody, capable of inducing apoptosis in Fas expressing cells. The results are shown in table 4.2. Addition of anti-Fas did not increase the number of apoptotic cells present, suggesting that Fas is not involved in CAMPATH-1 mediated cell death. Anti-Fas did not synergise with CAMPATH-1H and anti-human IgG to induce cell death.

Table 4.2. Anti-Fas antibody does not induce growth inhibition in Wien 133 cells.

	Control	C-1H	anti-huIgG	C-1H+anti-hu	anti-IgM
Control	311,167 (16)	288,280 (19)	290,591 (7)	127,464 (15)	8156 (13)
Anti-Fas	298,422 (30)	257,161 (22)	337,743 (22)	121,458 (20)	2931 (11)

Wien 133 cells were cultured in the presence of medium alone, CAMPATH-1H, anti-human IgG, CAMPATH-1H together with anti-human IgG, or anti-IgM. CAMPATH-1H and anti-human IgG antibodies were added at a final concentration of 20 μg/ml; anti-IgM was added at 4 μg/ml final concentration. Anti-Fas antibody was added at a final concentration of 100 ng/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures (% observed error is given in brackets).

4.2.13 CAMPATH-1H induced apoptosis in Wien 133 cells is not due to TNF-α

The Wien 133 cell line was shown to be very sensitive to growth inhibition in the presence of TNF-α (See table 4.2). An ELISA set up to measure TNF-α suggested that following CD52 antigen crosslinking, low levels of TNF-α were secreted (N. Rapson, personal communication). This could contribute to, or represent part of the mechanism of CAMPATH-1H induced apoptosis in the Wien 133 cells. Therefore, the effect of neutralising anti-TNF-α antibodies on the CAMPATH-1H induced growth inhibition of Wien 133 cells was investigated. Antibodies were added in solution for the duration of the experiment. Anti-TNF-α antibodies did not inhibit the CAMPATH-1H induced cell death (data not shown).

Adding back culture supernatant from cells which were growth inhibited in the presence of CAMPATH-1H and anti-human IgG, to untreated cells, had no effect on cell growth. This suggests that cell death induced by CAMPATH-1 antibodies in Wien 133 cells, is not due to the presence of soluble factors.

4.2.14 N-Acetylcysteine can partially block CAMPATH-1H induced cell growth inhibition in Wien 133 cells

Hydroxyl radicals are reactive oxygen free radical species capable of direct oxidative damage to macromolecules including DNA, protein and lipid membranes (Halliwell and Gutteridge, 1989). The excess production of free oxygen radicals leads to some of the features indicative of apoptosis, including DNA strand breaks and membrane blebbing. A role of free radicals in apoptosis has been demonstrated following other stimuli eg. removal of IL-3 in IL-3 dependent cell lines (Hockenbery et al., 1993) and therefore, the involvement of free radicals in driving Wien 133 cells to apoptose following treatment with CAMPATH-1H was investigated using N-acetyl cysteine as an anti-oxidant.

The effects of culturing Wien 133 cells with medium alone, CAMPATH-1H alone, anti-human IgG, or CAMPATH-1H together with anti-human IgG, in the presence of N-

acetylcysteine at a range of concentrations are shown in figure 4.20. The results indicate that N-acetylcysteine can partially block CAMPATH-1H induced cell growth inhibition, suggesting that free radicals are involved in the signalling pathway downstream of CD52.

The same experiments were performed in the presence of LNMMA, an analogue of arginine and an inhibitor of nitric oxide synthase. Addition of LNMMA had no effect, suggesting that nitric oxide does not have a role in CAMPATH-1H induced growth inhibition in the Wien 133 cell line.

4.2.15 Effect of Cyclosporin A on CAMPATH-1H induced growth inhibition of Wien 133 cells.

Cyclosporin A inhibits pathways downstream of calcium dependent calcineurin. Cyclosporin A was used at a range of concentrations from 0 - 3000 ng/ml. The results are shown in figure 4.21. At high concentrations, Cyclosporin A alone caused growth inhibition. Between 3 and 100 ng/ml, Cyclosporin A partially reduced the growth inhibition effect seen with anti-human IgG alone. The growth inhibition induced by CAMPATH-1H in the presence of anti-human was also inhibited, but to the same extent as with anti-human IgG alone. This suggests that Cyclosporin A inhibits the effect which anti-human IgG contributes to growth inhibition of Wien 133 cells, but does not effect the CAMPATH-1H mediated growth inhibition.

4.2.16 The effect of other inhibitors on blocking CAMPATH-1H induced cell death in Wien 133 cells

The effect of a range of other inhibitors on CAMPATH-1 antibody induced growth inhibition of Wien 133 cells was investigated. Herbimycin A, Genistein and Lavendustin (tyrosine kinase inhibitors), Staurosporine (Protein kinase C inhibitor), and cycloheximide (inhibitor of novel protein synthesis) all proved to be extremely toxic to

cell growth in medium alone. Therefore, it was not possible to draw any conclusions about other signalling pathways operating downstream of CD52 using these inhibitors.

4.2.17 Signalling via the CD52 antigen in T cells requires the presence of a functional T cell receptor

Previous studies have shown that GPI-anchored proteins require the presence of a functional antigen specific receptor for signalling (Gunter et al., 1987; Sussman et al., 1988). Surface Ig negative Wien 133 cells were not obtained and it was not possible to study the requirements for a functional surface antigen receptor for CD52 mediated growth inhibition. The growth of a CD52 transfected Jurkat T cell line was shown previously to be inhibited in the presence of CAMPATH-1H and anti-human IgG (see section 4.2.1). Jurkat T cell line variants were available commercially which were deficient in TCR expression and CD45. Therefore, Jurkat cells were chosen to look at the effect of TCR expression on CAMPATH-1H mediated growth inhibition. In addition, Jurkat cells do not express Fc receptors or surface Ig.

The three Jurkat cell lines chosen for further experimentation were the Jurkat parental clone E6-1, J45.01 (CD45 deficient clone derived from the E6-1 clone) and J.RT3-T3.5 (derivative mutant of the E6-1 clone lacking the beta chain of the T cell antigen receptor). These were transfected with the Blue Script plasmid containing the entire coding sequence for the CD52 antigen and p321 Neo plasmid as a selection marker. Cells were cultured in the presence of Gentamicin G418. After 3 weeks, cells surviving under selection pressure, were screened using FACS to look for expression of the CD52 antigen. By FACS analysis, prior to transfection, all three parental cell lines expressed very low levels of the CD52 antigen; the E6-1 and J.RT3-T3.5 cell lines were positive for CD45 expression whereas the J45.01 cell line expressed CD45 at very low levels; the E6-1 and the J45.01 cell lines were positive for CD3 expression whereas the J.RT3-T3.5 cell line was negative for CD3 expression. Following transfection, FACS analysis revealed that in all three cell lines, there were cells expressing the CD52 antigen at comparatively high levels. The cells in each case, were dilution cloned: the CD52 transfected Jurkat E6-1 cell line produced 30 viable clones; the

J.45.01 cell line produced 22 clones; the J.RT3-T3.5 cell line produced 20 clones. The clones were checked for expression of the CD52 antigen by FACS analysis and the results are shown in figure 4.22, 4.23, 4.24.

Clones from each set of transfectants were selected for further study and in addition to CD52, the expression of CD3, TCR and CD45 was looked at by FACS analysis. Results are illustrated in figures 4.25, 4.26, 4.27.

For the Jurkat parental E6-1 CD52 transfectants (CD3 positive, CD45 positive), clones selected included #9, #10, #11, #12, #15, #16, #21and #25. Clones #1, #9, #12 and #16 had low/negative levels of CD3 and TCRαβ expression, despite the fact that the parental cell line did express these markers, reflecting variability in uncloned cell lines.

The J.RT3-T3.5 (TCR negative) CD52 transfected clones all expressed CD52 at comparable levels with one exception. Clone #10 expressed the CD52 antigen at relatively high levels and in addition, unlike the other transfected clones in this series, was also positive for CD3 and the TCR. Additional clones selected for further study included #3,#4, #7, #14,#17 and #18.

In the J45.01 (CD45 negative) CD52 transfectants, clones #3, #4, #5, #11, #12, #14, #16 and #22 had relatively similar FACS profiles for the expression of CD3, TCR and CD52.

4.2.18 The effect of CAMPATH-1H antibody on the growth of CD52 Jurkat transfectants

From the panel of transfected clones available, cells were chosen for further study on the basis of having comparable levels of CD52 expression; namely, J E6-1CD52 +ve clones #11 and #16, J.RT3-T3.5 CD52 +ve clones #4 and #18 and CD45.01 CD52+ve clones #4 and #11. In addition, J.RT3-T3.5 CD52+ve clone #10 was selected for further study because it expressed CD52 antigen at a higher, approximately one log greater mean fluorescence compared to the clones above and because by FACS analysis it was positive for CD3

expression (despite the fact that the clone was derived from a parental cell line negative for CD3 expression); J E6-1 CD52 +ve clone #21 had a similar phenotype as measured by FACS to J.RT3-T3.5 CD+ve clone #10. The phenotypes of the clones selected for further study are summarised in table 4.3.

Table 4.3. Phenotypic analysis of the expression of selected surface markers

Identification	CD3	TCR	CD45	CD52
JE6 Parental	+	+	+	-
JE6 CD52+ #11	+	+	+	+
JE6 CD52+ #16	-	-	+	+
JE6 CD52+ #21	+	+	+	++
J45.01 Parental	+	+	+/-	-
J45.01 CD52+ #4	+	+	+/-	+
J45.01 CD52+ #11	+	+	+/-	+
J.RT3-T3.5 Parental	-	-	+	+/-
J.RT3-T3.5 CD52+ #4	-	-	+	+
J.RT3-T3.5 CD52+ #10	+	+	+	++
J.RT3-T3.5 CD52+ #18	-	-	+	+

Cells were stained with FITC labelled anti-CD3, anti-TCRαβ, anti-CD45 or CAMPATH-1H antibodies and analysed by FACScan using LYSIS II software. + indicates cells positive for surface marker expression, - denotes negative expression, +/- denotes negligible expression, ++ denotes high expression

Cells were cultured in the absence of Gentamicin G418 for two weeks prior to setting up assays. FACS analysis following this period showed that the expression of CD52 was the same as in cells maintained in the presence of selection medium ie. CD52 expression appeared to be stable in the transfected cell lines.

In order to determine the effect of crosslinking CD52 on the surface of the Jurkat transfectants, cells were cultured for 5 days in the presence of medium alone, CAMPATH-1H antibody alone, anti-human IgG, or CAMPATH-1H together with anti-human IgG. The only cell line affected by CAMPATH-1H treatment, was the JE6-1 CD52+clone #21. Thus, the growth of cell lines expressing low levels of CD52 (<100 MFI) were not inhibited in the presence of CAMPATH-1H antibody and secondary crosslinking antibody. This suggests that the levels of CD52 expression are important in determining the functional effect of the antibody. The JE6-1 CD52+ clone #21 expresses high levels of CD52 comparable to the JRT3-T3.5 CD52+ clone #10. However, the growth of the latter clone was not affected by crosslinking CAMPATH-1 antibodies (Figure 4.28). Interestingly, although the JRT3-T3.5

CD52+ clone #10 expresses CD3, it was derived from a CD3 negative cell line which might suggest there is some other underlying defect in the TCR signalling complex in this clone. This is supported by experiments in which this clone failed to respond to anti-CD3 treatment (see section below). This suggests that CD52 density and expression of a functional TCR are important for signalling via the CD52 antigen.

4.2.19 CAMPATH-1H and anti-CD3 synergise to inhibit the growth of CD52 transfected Jurkats

In normal resting T cells, anti-CD3 at suboptimal doses (ie. doses at which anti-CD3 alone has no effect on the growth of the cells) synergised with CAMPATH-1 antibodies to drive the cells into proliferation (Chapter 3). The experiment to look at the effect of anti-CD3 in combination with CAMPATH-1H antibody was therefore repeated with a titration of anti-CD3 (0.1, 1 and 10 μg/ml) to determine whether or not at suboptimal concentrations of anti-CD3, there was synergy between the two antibodies leading to cell growth inhibition.

In the J E6-1 CD52+ve #21 cell line, anti-CD3 antibody alone at 0.1 μg/ml had a small effect on inhibiting the growth of the cells; however, in the presence of CAMPATH-1H antibody (10 μg/ml), the growth of the cells was inhibited to approximately 30% of the control cell growth (figure 4.29).

As one would predict, in the absence of CD3 expression, the growth of the J RT3-T3.5 untransfected cell line and J RT3-T3.5 CD52 +ve clones #4 and #18 was not affected by either anti-CD3 antibody alone, CAMPATH-1H antibody alone or the combination of anti-CD3 and CAMPATH-1H antibodies.

The growth of JRT3-T3.5 CD52+ clone #10 which expressed surface CD3, was not inhibited in the presence of anti-CD3 antibody. This suggested that although CD3 was expressed in this clone, there was a defect in the TCR signalling machinery.

Inhibition by anti-CD3 appeared to require the presence of CD45 indicated by the fact that the growth of the J45.01 untransfected parental cell line and J45.01 CD52+ve clones #4 and #11 was not affected by the presence of anti-CD3 and CAMPATH-1H antibodies.

4.2.20 The effect of CD52 ligation on release of IL-2 in Jurkats.

48 hours after setting up the assays for measuring the effects of CAMPATH-1 antibodies on Jurkat cell growth, 50 µl supernatant was removed from each well to look for IL-2 production in a CTLL assay. No IL-2 was detected and, therefore, the growth inhibition effect observed did not involve IL-2 production. These Jurkats did not produce IL-2 in response to anti-CD3.

Addition of PMA had no effect on the observations made above. The results of the CTLL assay, indicated that under the conditions used, IL-2 was not produced in response to anti-CD3 and CAMPATH-1H antibodies.

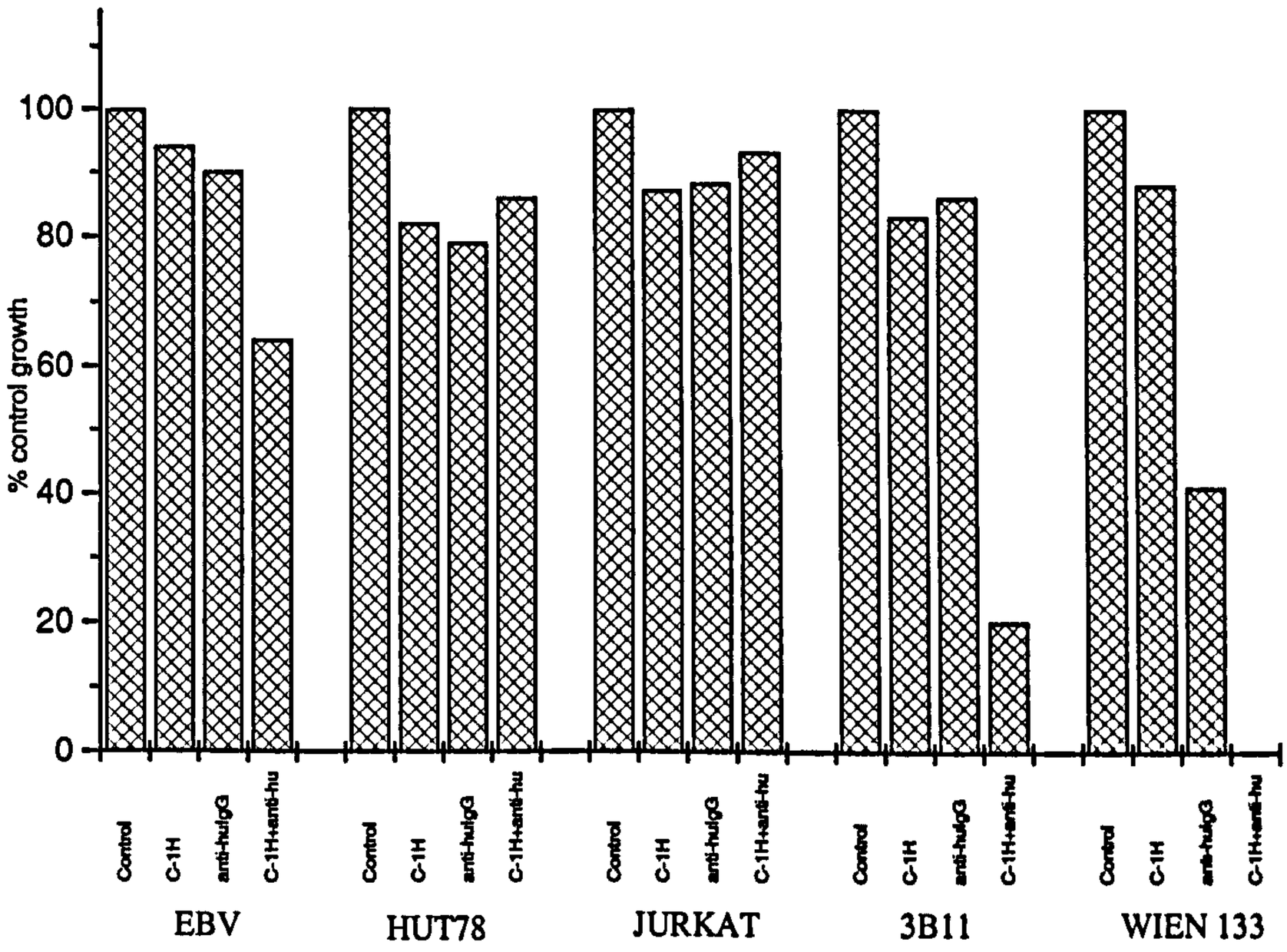
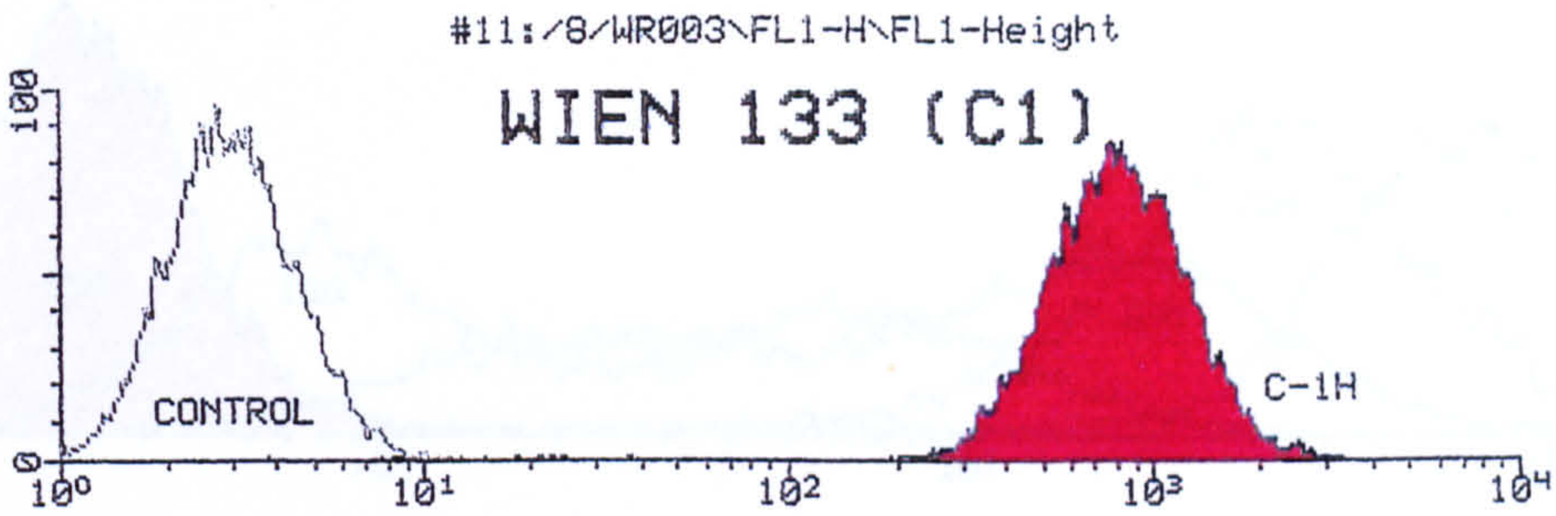
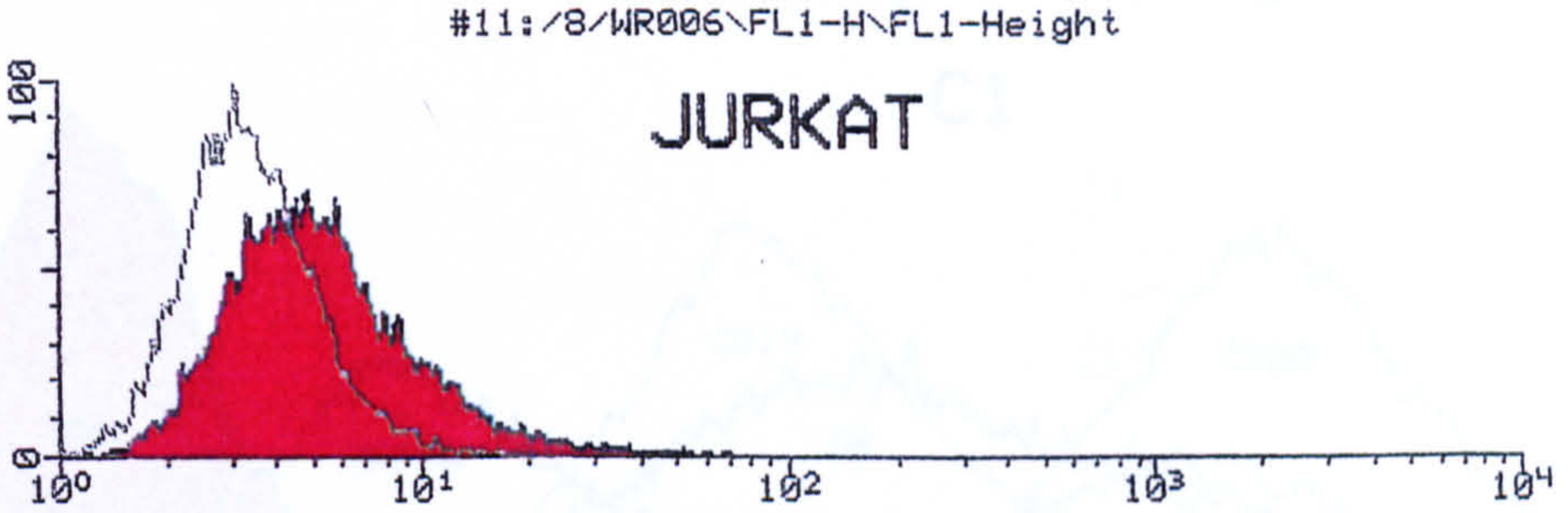
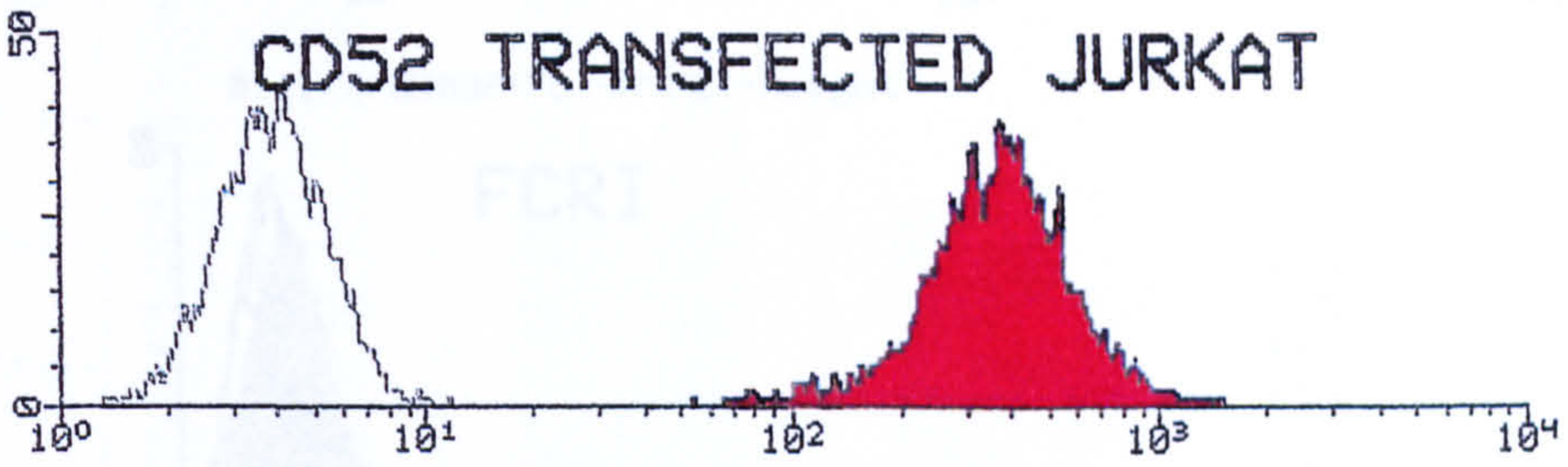


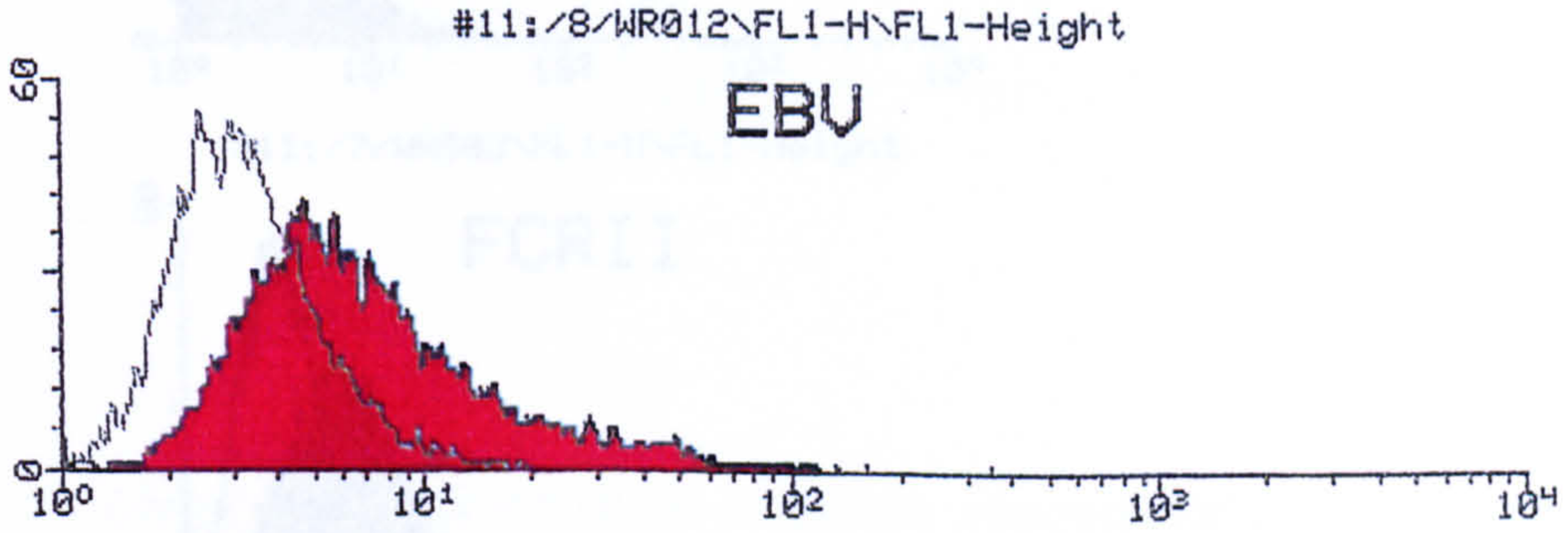
Figure 4.1 Effect of CAMPATH-1H antibody on the growth of B and T cell tumour lines.

EBV, HUT 78, Jurkat and CD52 transfected Jurkat 3B11 T cell lines and Wien 133 cells were cultured for 5 days in the presence of medium alone (control), C-1H (CAMPATH-1H)(20 μg/ml), anti-human IgG (20 μg/ml) or C-1H + anti-human IgG. Antibodies were added in solution. [^{3}H]-thymidine was added for the last 4 hours of culture. Results are expressed as percentage inhibition of control growth which in terms of c.p.m. were as follows: EBV (44,744); HUT78 (275,082); Jurkats (330,343); 3B11 (285,394); Wien 133 (150,423).









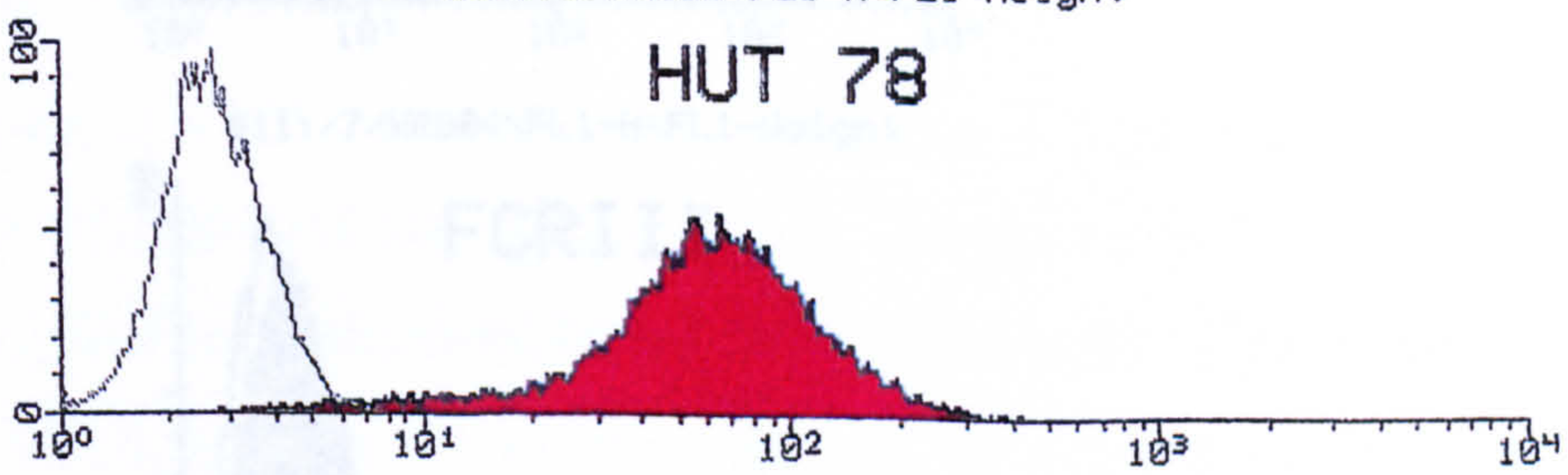


Figure 4.2 CD52 antigen expression in Wien 133, EBV, HUT78, Jurkat and CD52 transfected Jurkat (Clone 3B11) cell lines.

Cells were stained for CD52 expression using FITC labelled CAMPATH-1H (Fab)2 antibody and then analysed using a FACScan and LYSIS II software. The cell number is shown on the y axis; fluorescence intensity is shown on the x axis.

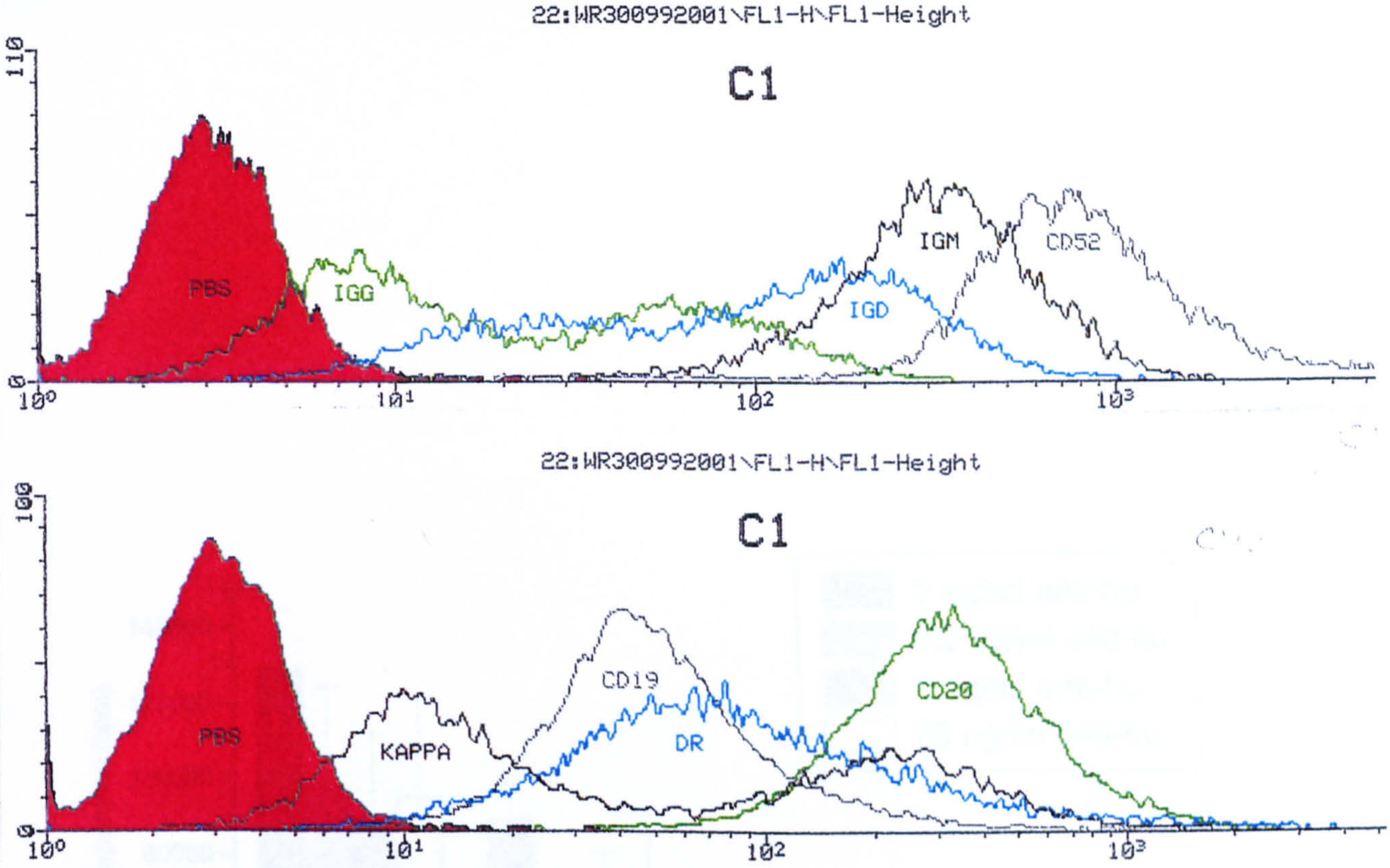


Figure 4.3 Expression of surface markers on Wien 133 cells.

Wien 133 cells were stained for surface expression of IgG, IgD, IgM, CD19, CD20, DR, CD16, CD32 and CD64 FITC labelled antibodies. Cells were analysed by FACScan using LYSIS II software. Cell number is represented by the y axis and the fluoresecence intenisty is shown on the x axis.

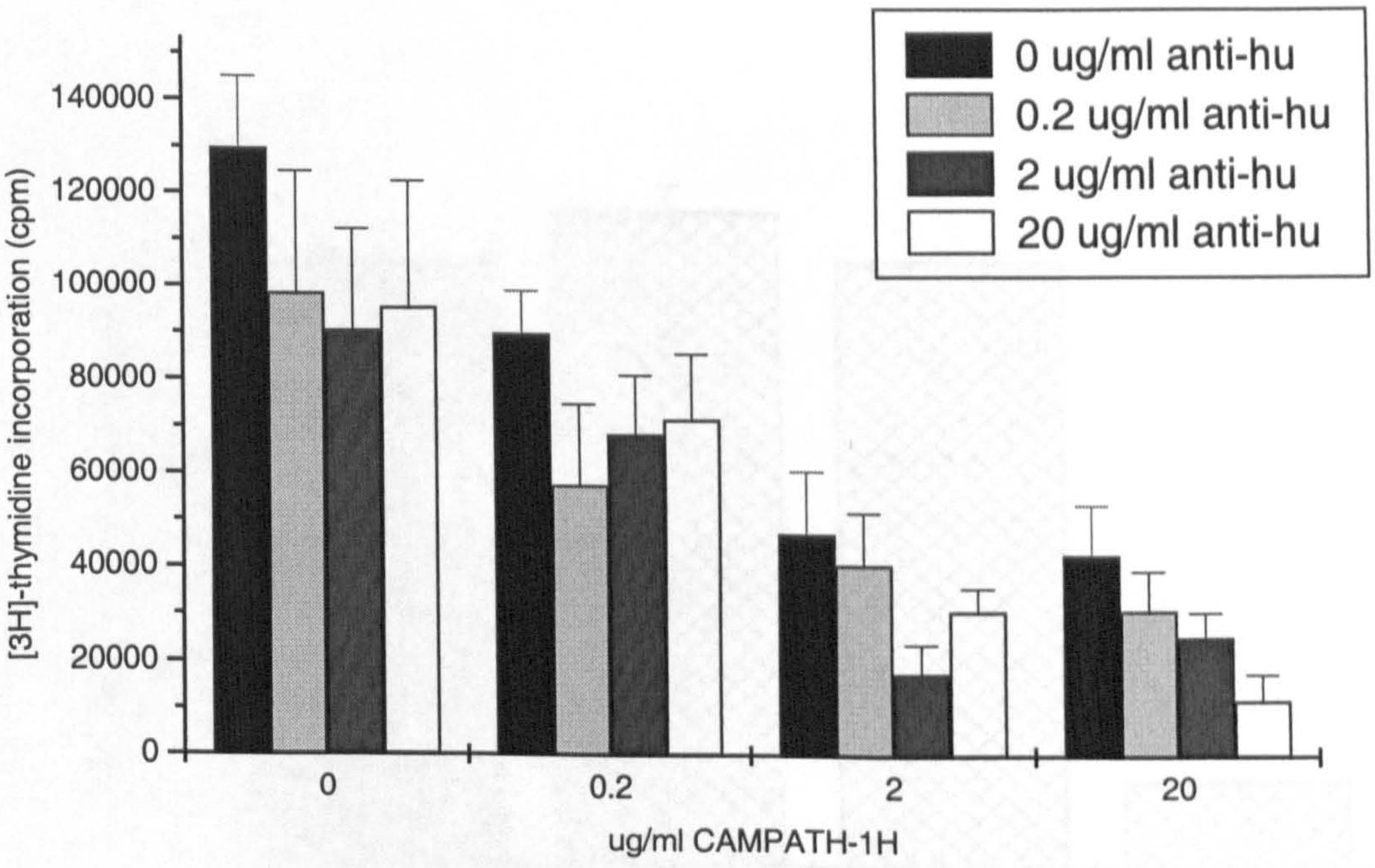


Figure 4.4 Effect of CAMPATH-1H antibody concentration on the growth inhibition of Wien 133 cells.

Wien 133 cells (2 x 10^4 cells/well) were cultured for 5 days in the presence of medium alone, C-1H (CAMPATH-1H) antibody alone, anti-human IgG or C-1H together with anti-human IgG at the concentrations indicated on the graph. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the standard error of the mean (SEM).

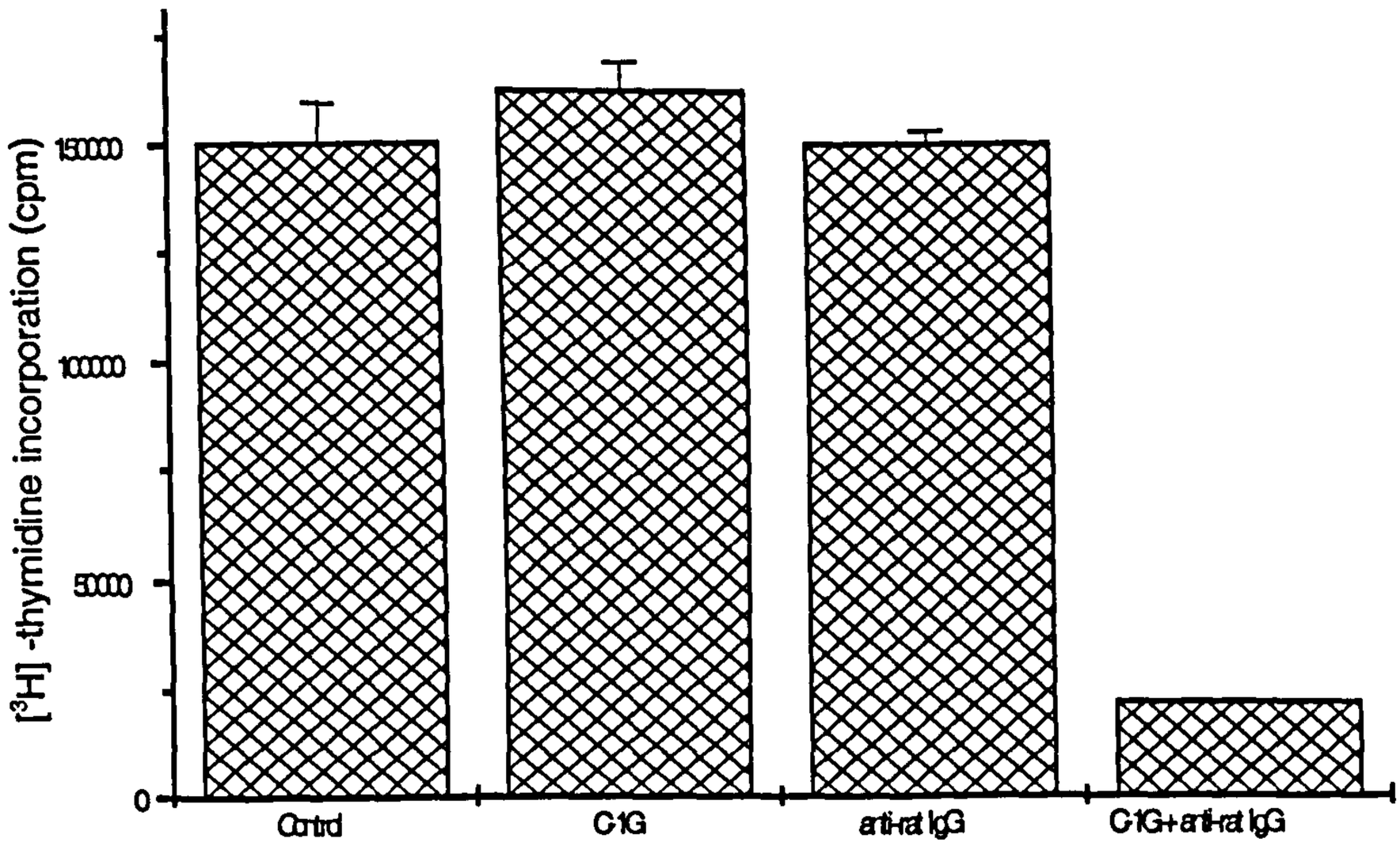


Figure 4.5 Effect of rat CAMPATH-1G antibody on the growth of Wien 133 cells.

Wien 133 cell were cultured for 5 days in the presence of medium alone, CAMPATH-1G (C-1G), anti-rat IgG or C-1G together with anti-rat IgG. Antibodies were all added at a final concentration of 20 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the standard error of the mean.

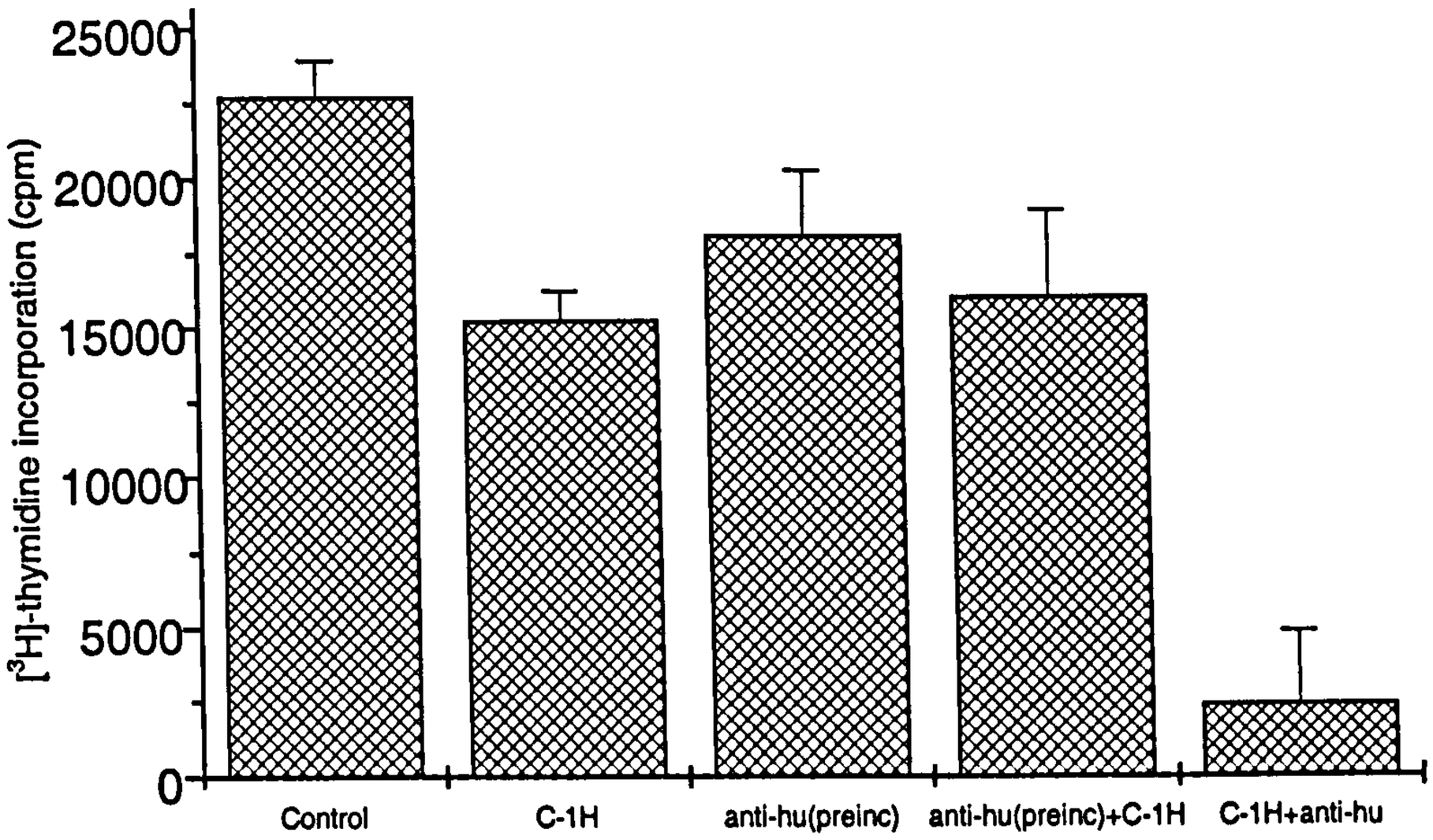


Figure 4.6 Growth inhibition in the Wien 133 cell line via the CD52 antigen requires crosslinking.

Wien 133 cells were preincubated +/- anti-human IgG and excess antibody removed by washing cells in PBS. Cells (2 x 10^4/well) were cultured for 5 days in the presence of medium alone, CAMPATH-1H antibody (C-1H), anti-human IgG, or CAMPATH-1H together with anti-human IgG. All antibodies were added at a final concentration of 20 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the standard error of the mean.

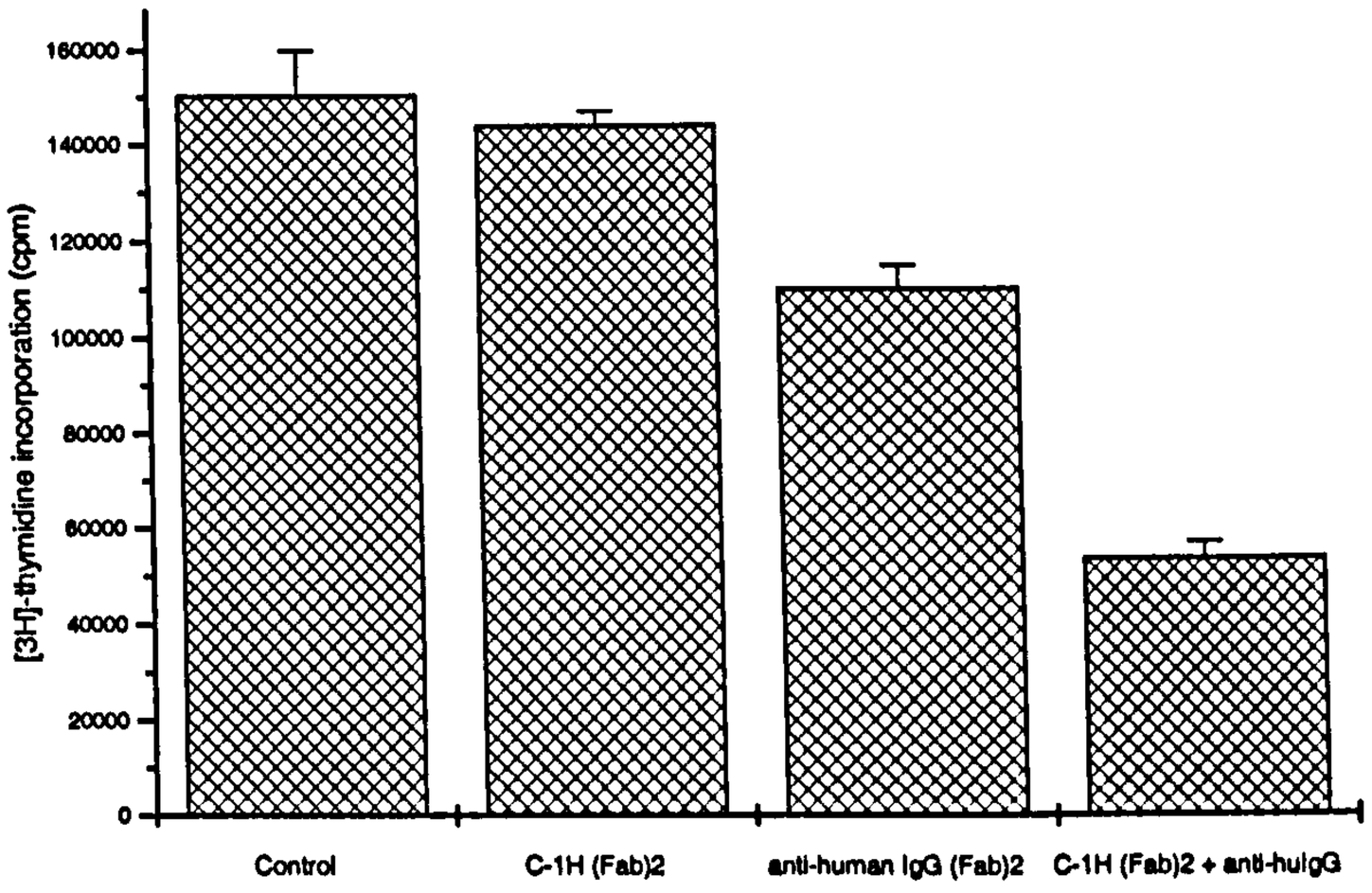


Figure 4.7 Growth inhibition in Wien 133 cells induced by CAMPATH-1H antibody does not require an intact Fc.

Wien 133 cells (2 x 10^4/well) were cultured for 5 days in the presence of medium alone, CAMPATH-1H $(Fab)_2$ [C-1H $(Fab)_2$], anti-human IgG $(Fab)_2$, or CAMPATH-1H $(Fab)_2$ together with anti-human IgG $(Fab)_2$. All antibodies were added at a final concentration of 20 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the standard error of the mean.

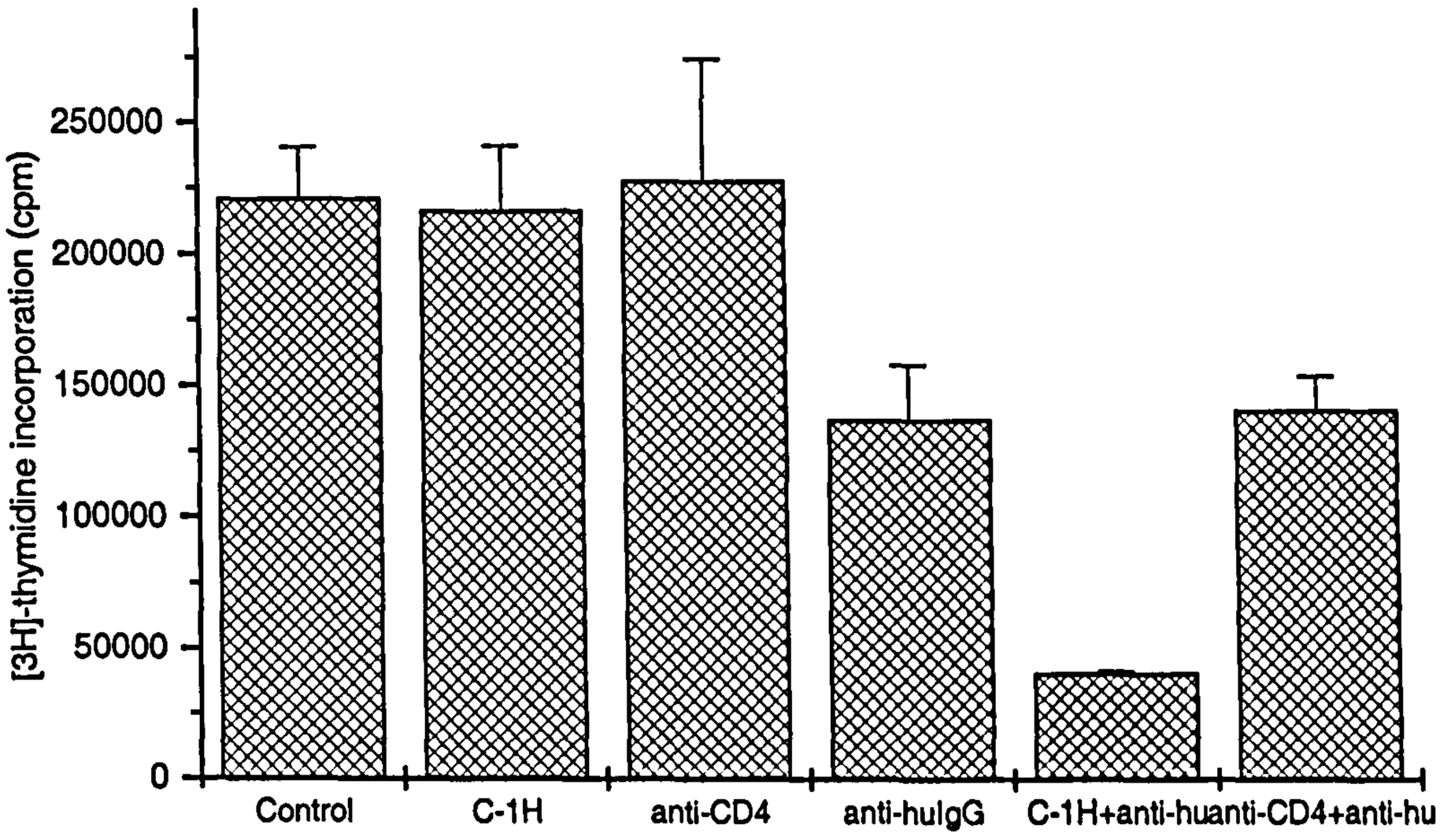


Figure 4.8 The growth inhibition induced in Wien 133 cells by CAMPATH-1H antibody is not due to interaction via antibody Fc regions or formation of non specific immune complexes.

Wien 133 cells (2 x 10^4/well) were cultured for 5 days in the presence of medium alone, CAMPATH-1H (C-1H), anti-CD4, anti-human IgG, CAMPATH-1H together with anti-human IgG, or anti-CD4 together with anti-human IgG. All antibodies were added at a final concentration of 20 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the standard error of the mean.

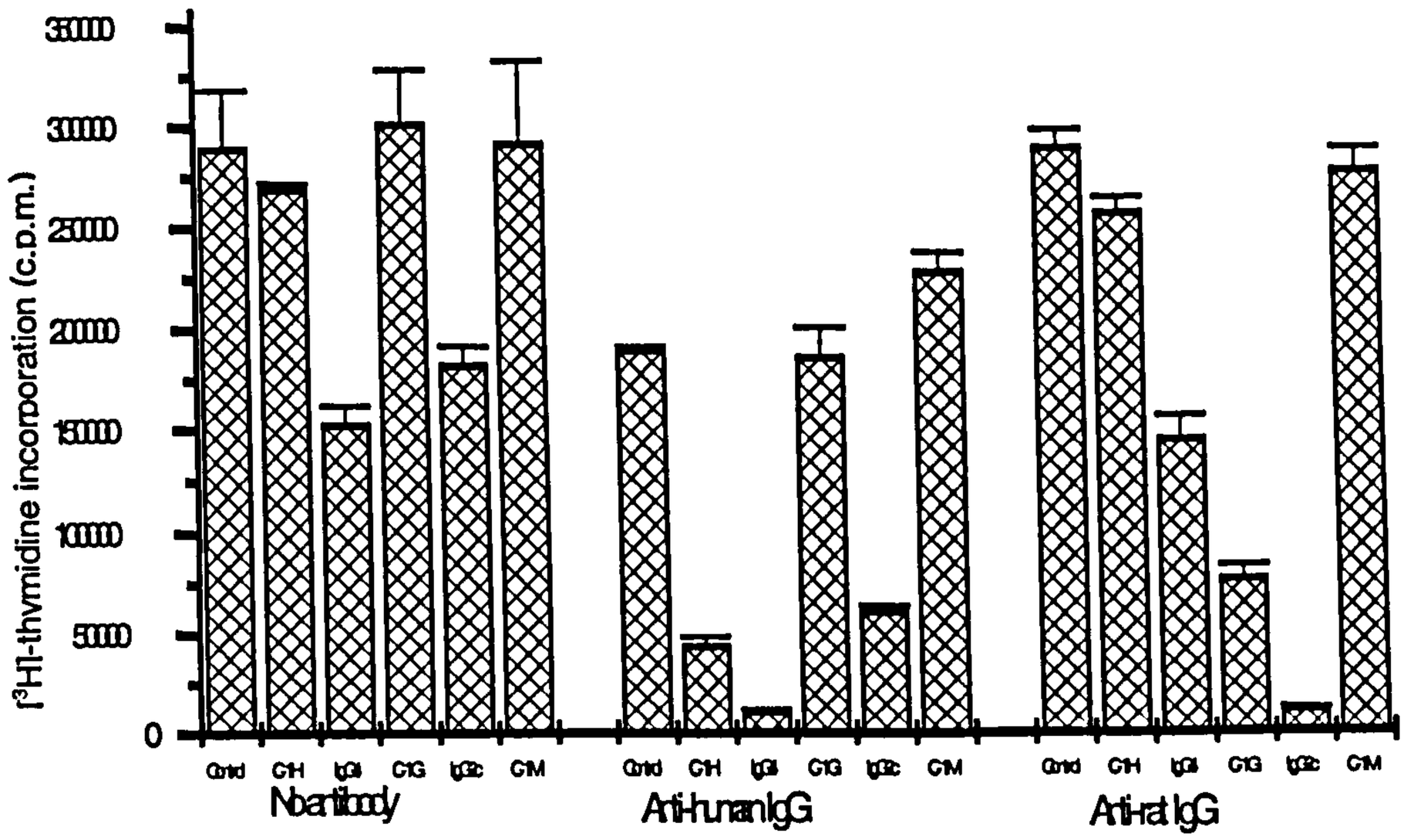


Figure 4.9 Effect of antibody isotype on inducing cell growth inhibition in Wien 133 cells.

Wien 133 cells (2 x 10^4/ well)were cultured for 5 days in the presence of medium alone, human IgG_1 CAMPATH-1H (C-1H), human IgG_4, rat IgG_{2b} CAMPATH-1G (C-1G), rat IgG2c or rat IgM CAMPATH-1M (C-1M) either alone or in the presence or absence of anti-human IgG or anti-rat IgG. All antibodies were added at a final concentration of 20 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the standard error of the mean.

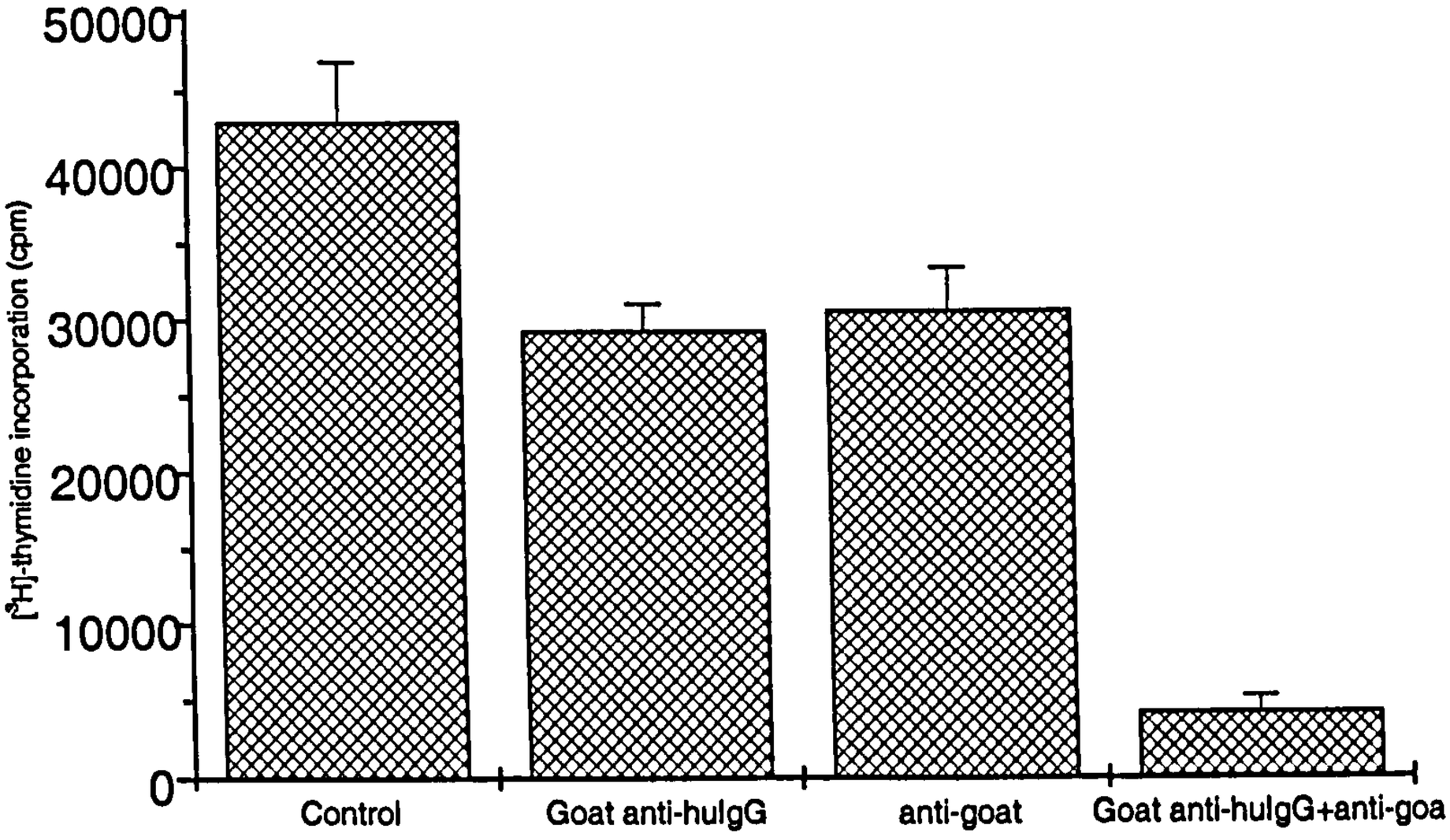


Figure 4.10 Crosslinking surface IgG enhances the growth inhibition in Wien 133 cells compared to that seen when anti-human IgG antibody is added alone.

Wien 133 cells (2 x 10^4/well) were cultured for 5 days in the presence of medium alone, goat anti-human IgG, anti-goat IgG, or goat anti-human IgG together with anti-goat IgG. All antibodies were added at a final concentration of 20 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the standard error of the mean.

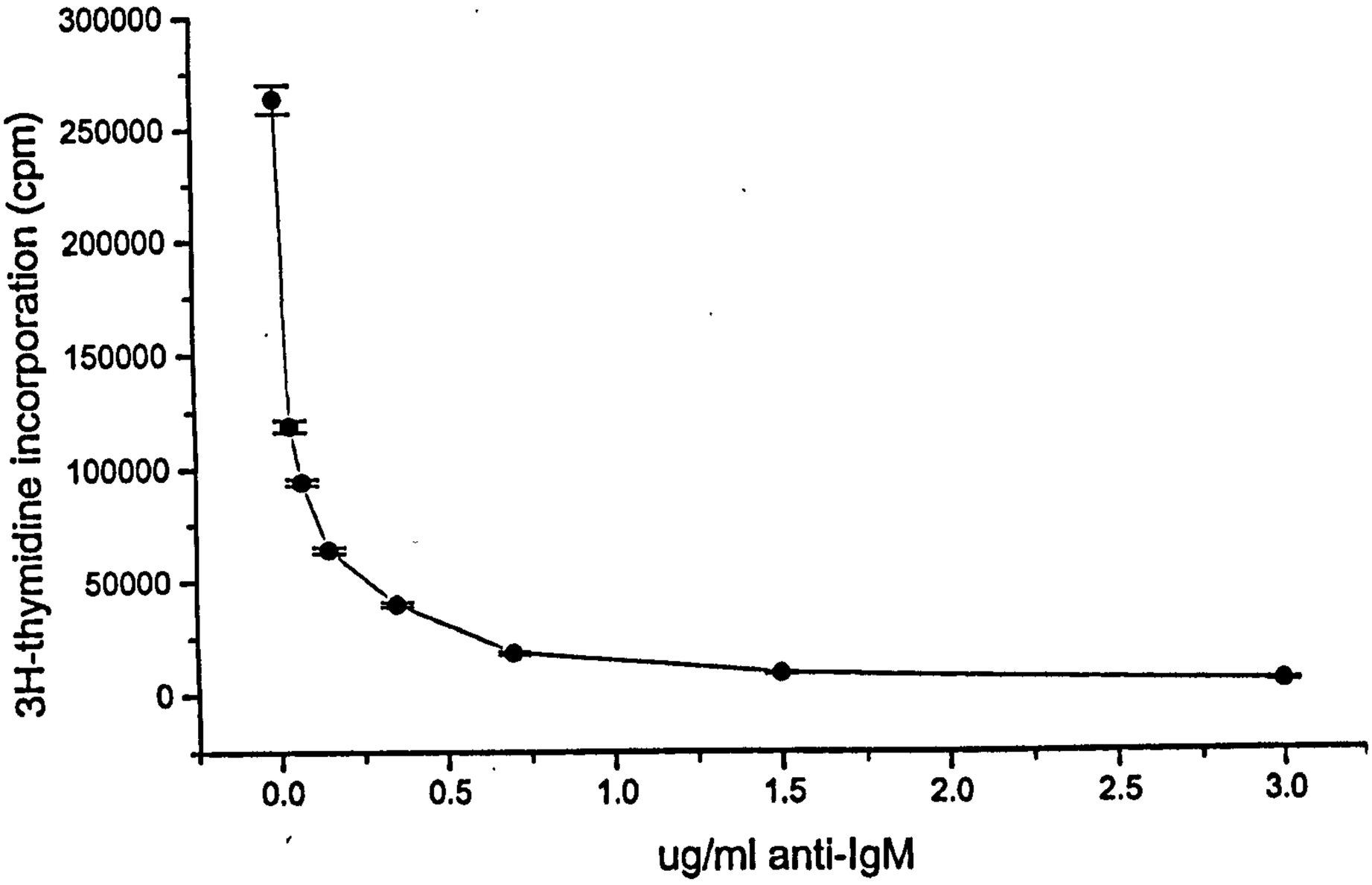


Figure 4.11 Anti-IgM induces growth inhibition in Wien 133 cells.

Wien 133 cells (2 x 10^4/well) were cultured for 5 days in the presence of medium alone or anti-IgM at a range of concentrations from 0 - 20 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the mean SEM.

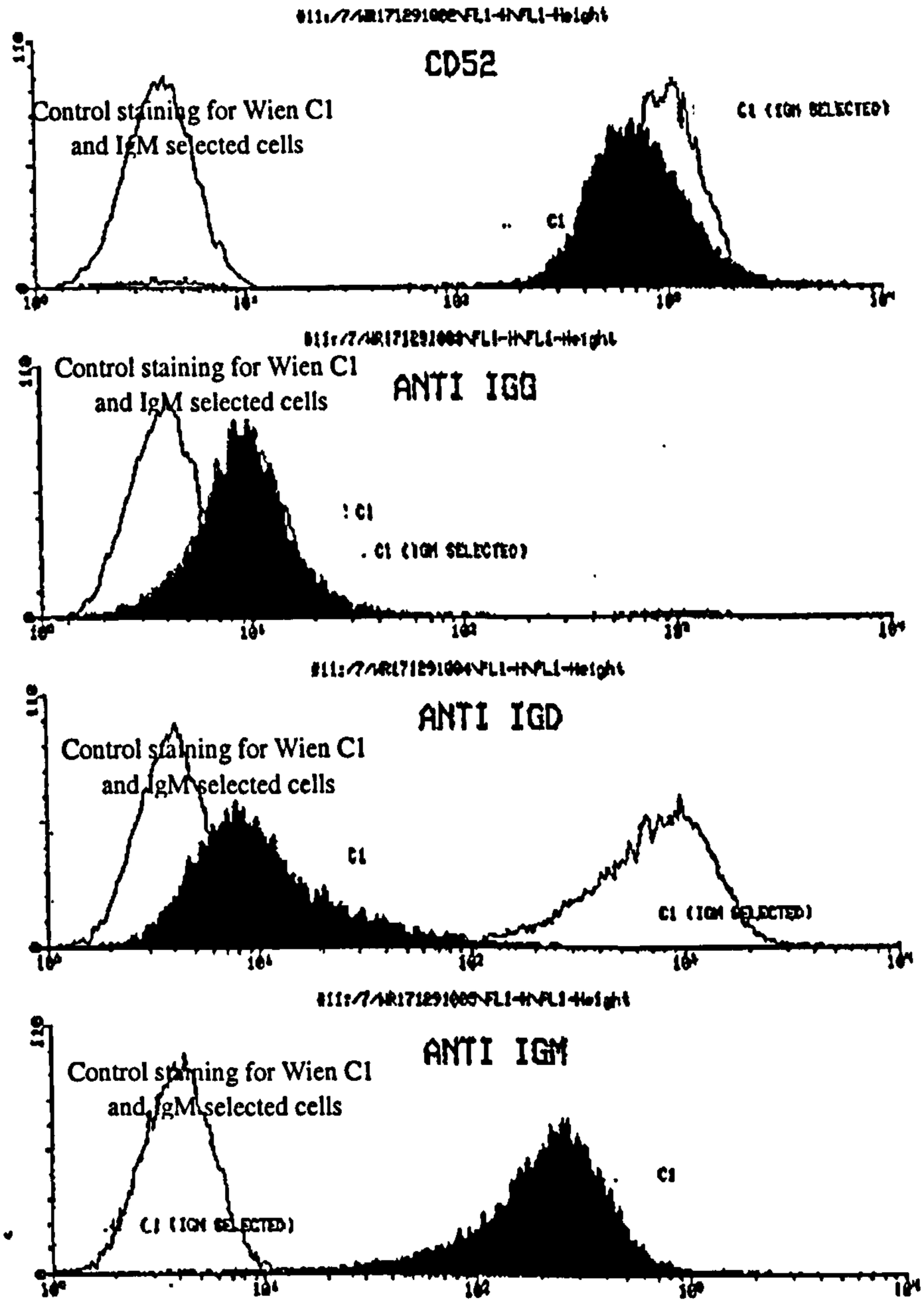


Figure 4.12 Wien 133 cells surviving treatment with anti-IgM antibody lose surface IgM expression but express high levels of surface IgD.

Wien 133 C1 cells and cells surviving treatment with anti-IgM antibody were stained with FITC labelled antibodies against CD52, IgG, IgD and IgM. Cells were analysed by FACScan using LYSIS II software. The negative control is represented by cells stained by a nonspecific FITC labelled anti-mouse IgG1. The x axis represents fluorescence intensity whilst cell number is shown on the y axis.

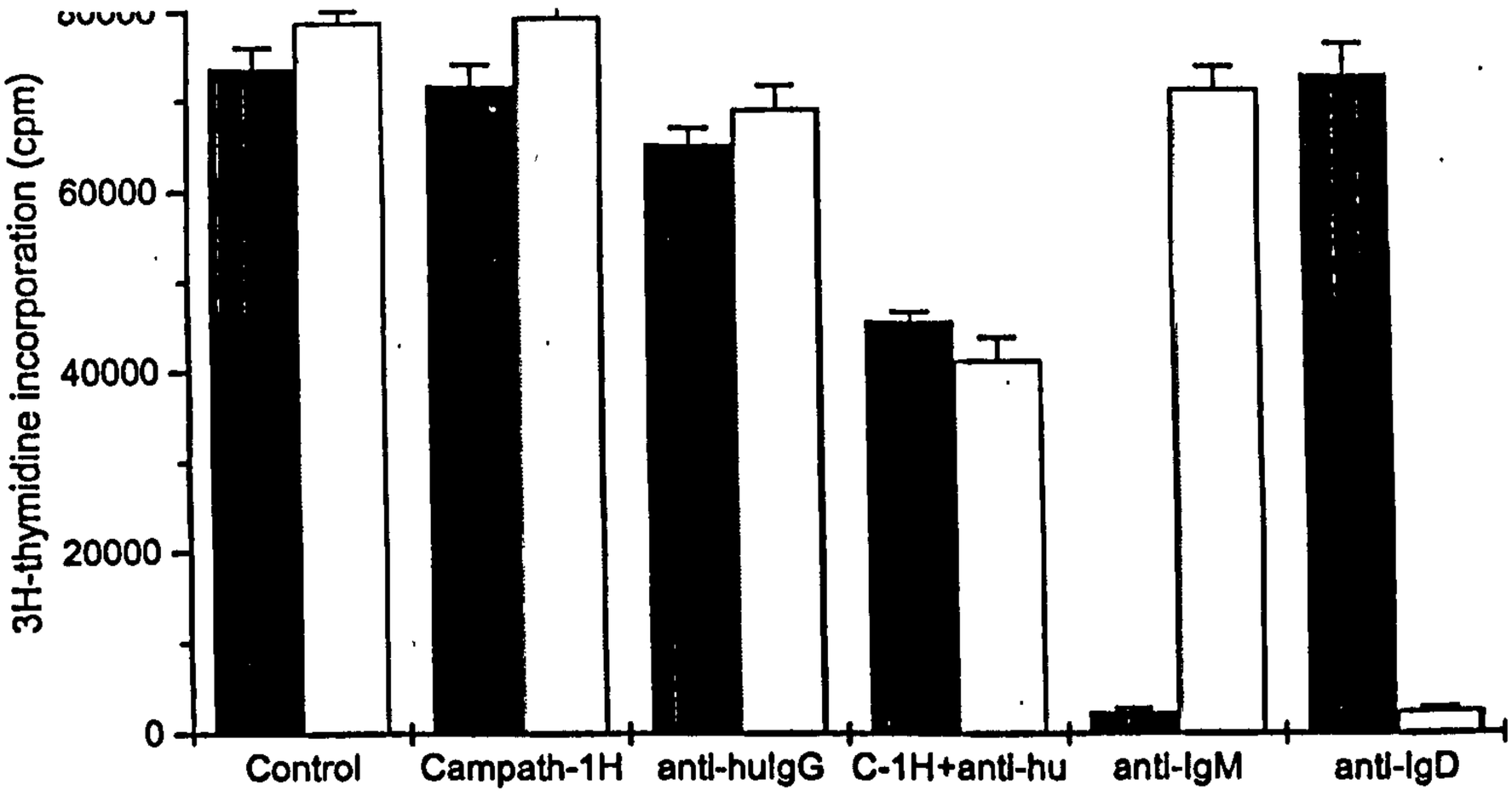


Figure 4.13 IgM negative Wien 133 cells remain sensitive to growth inhibition induced by CAMPATH-1H antibody.

Parental Wien 133 cells (Black bars) or sIgM- sIgD+Wien 133 cells (White bars) (2 x 10^4/well) were cultured for 5 days in the presence of medium alone, CAMPATH-1H (C-1H), anti-human IgG, CAMPATH-1H together with anti-human IgG, anti-IgM, or anti-IgD antibodies. CAMPATH-1H and anti-human IgG were each added at a final concentration of 20 μg/ml, anti-IgM was added at a final concentration of 4 μg/ml and anti-IgD was added at a final concentration of 4 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the mean c.p.m. of triplicate cultures and error bars give the mean SEM.

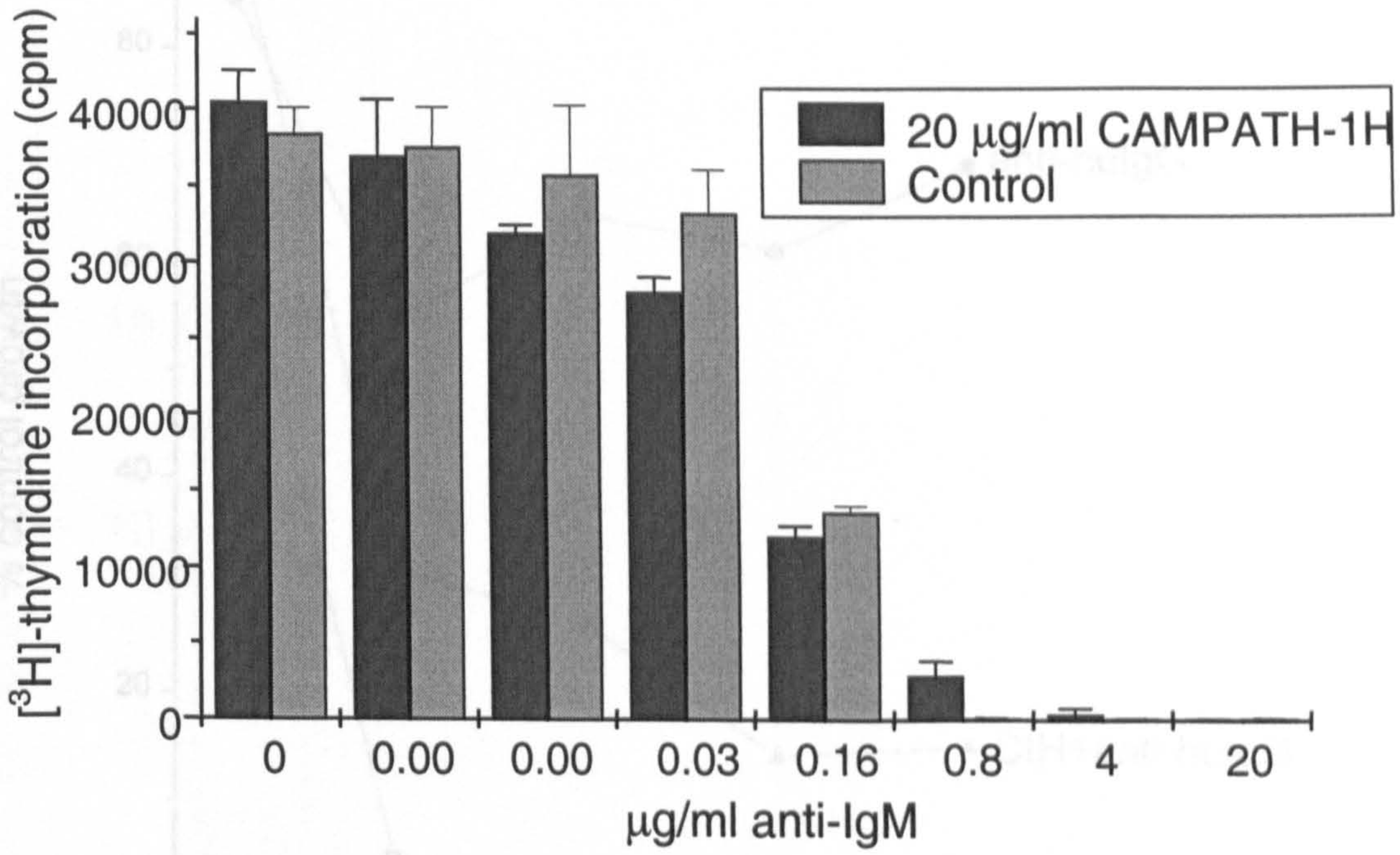


Figure 4.14 Partial blocking of anti-IgM induced cell growth inhibition in Wien 133 cells by CAMPATH-1H antibody.

Wien 133 cells (2 x 10^4/well) were cultured for 5 days in the presence of anti-IgM at concentrations ranging from 0 - 20 μg/ml in the presence or absence of CAMPATH-1H antibody added at a final concentration of 20 μg/ml. [^{3}H]-thymidine was added during the last 4 hours of the culture. The results are expressed as the mean cpm of triplicate cultures and error bars give the standard error of the mean.

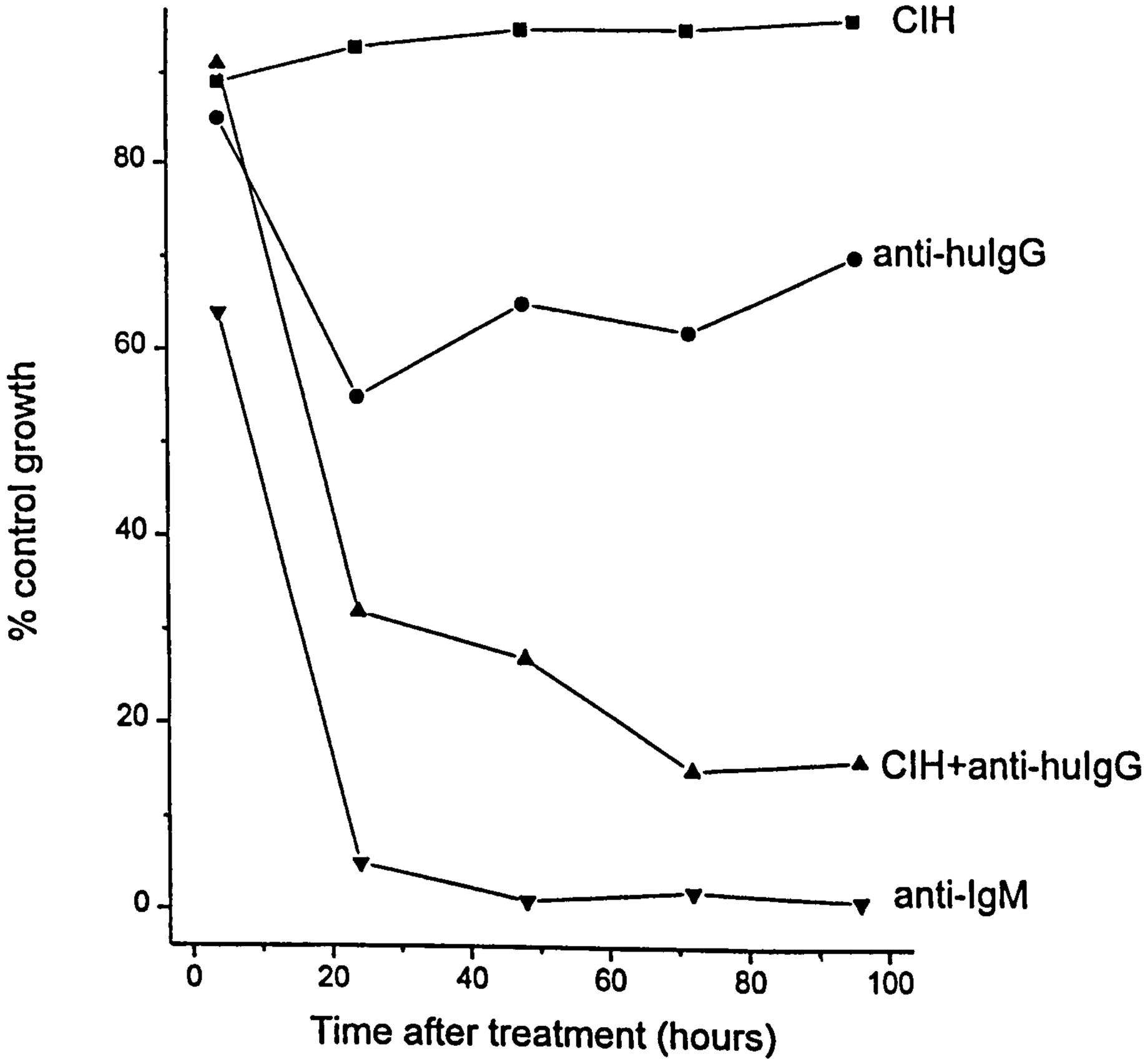


Figure 4.15 Differences in the kinetics of CAMPATH-1H antibody induced growth inhibition in Wien 133 cells compared to anti-IgM treatment.

Wien 133 cells (2 x 10^4/well) were cultured in the presence of medium alone, CAMPATH-1H, anti-human IgG, CAMPATH-1H together with anti-human IgG, or anti-IgM. All antibodies were added at a final concentration of 20 μg/ml except anti-IgM which was added at a final concnetration of 4 μg/ml. Cells were harvested at 6, 24, 48, 72 and 96 hours. [^{3}H]-thymidine was added for the last 4 hours of culture. Results are expressed as the percentage of control cell growth in the absence of antibodies. Proliferation in the absence of antibodies in terms of c.p.m. were as follows: 24 hours, 27612; 48 hours, 55491, 72 hours, 124,869; 96 hours, 161,848.

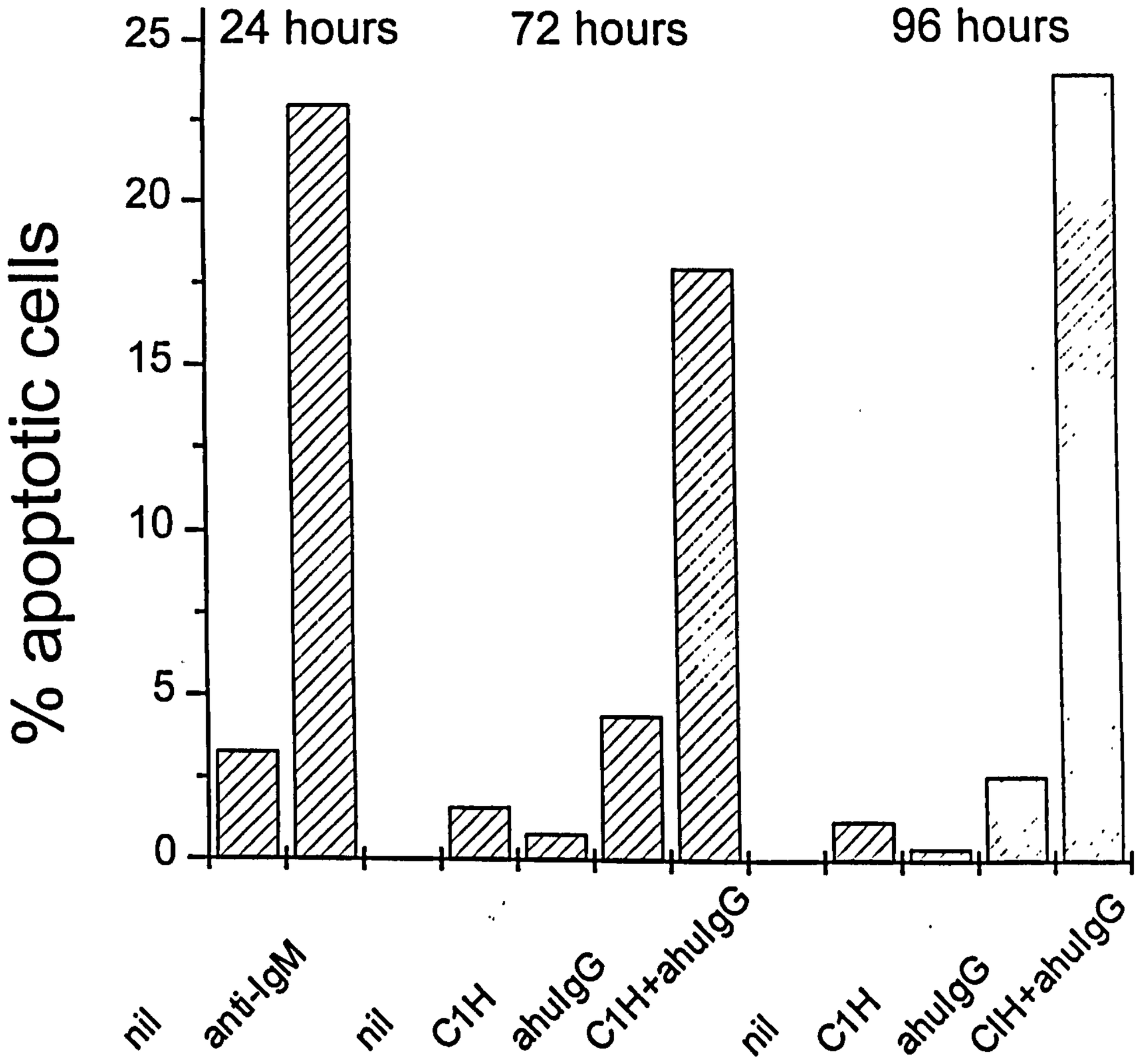


Figure 4.16 CAMPATH-1H treatment of Wien 133 cells increases the number of cells undergoing apoptosis.

Wien 133 cells were cultured in the presence of medium alone, CAMPATH-1H, anti-human IgG, CAMPATH-1H together with anti-human IgG, or anti-IgM. CAMPATH-1H and anti-human IgG antibodies were added at a final concentration of 20 μg/ml; anti-IgM was added at 4 μg/ml final concentration. Apoptotic cells were visualised by light microscopy after treatment with Giemsa/May Grunwald stain. The results are expressed as the percentage of apoptotic cells of the total cell number count (A total of 500 cells were counted for each treatment).

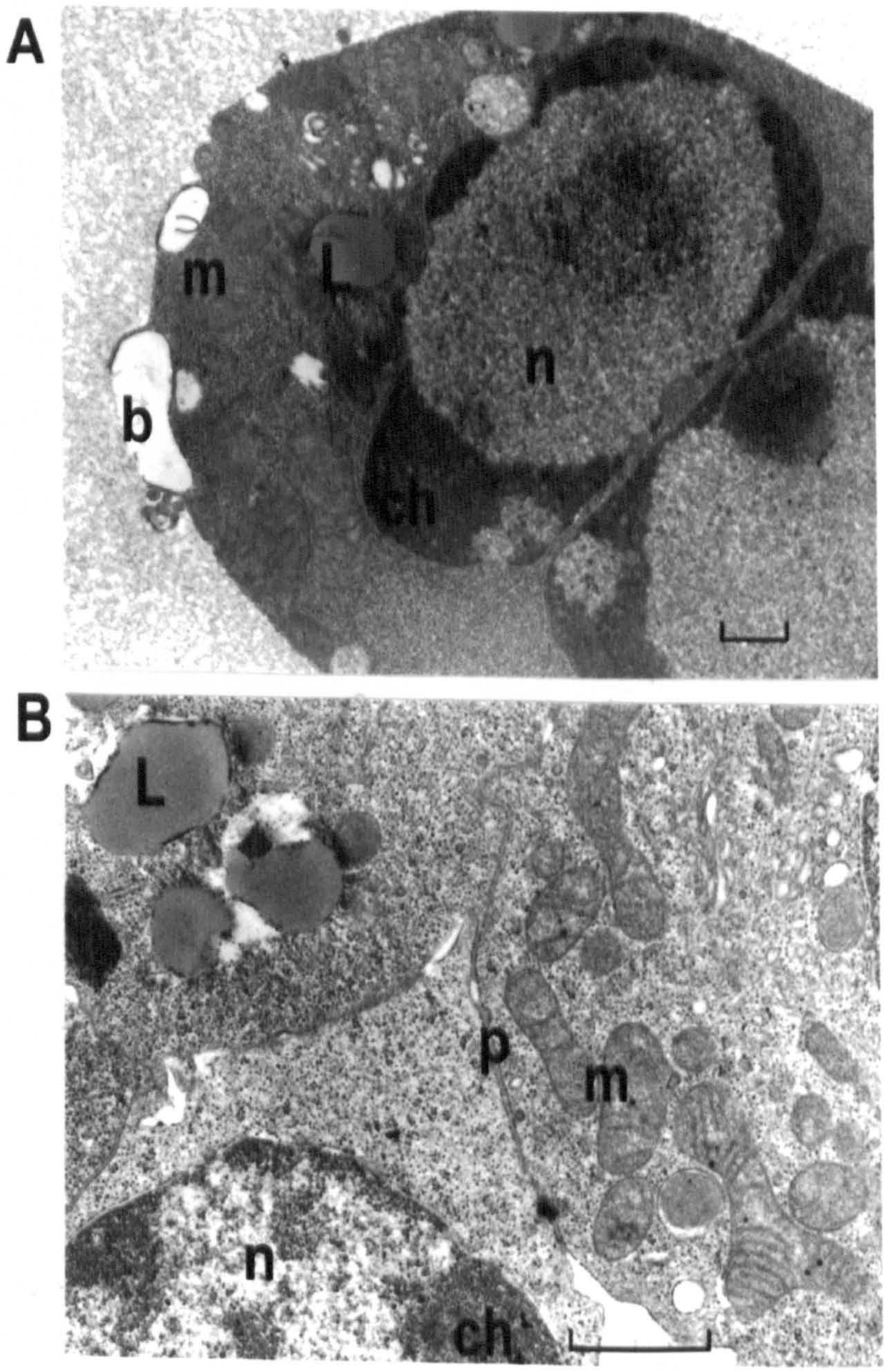


Figure 4.17 Electron micrographs of CAMPATH-1H treated cells undergoing apoptosis (A) compared to untreated cells (B).

In figure A), the nucleus (n) has condensed and marginated chromatin (ch), the cytoplasm is dense (c) with numerous intact mitochondria (m) and occasional lipid droplets (L). A cytoplasmic bleb (b) can also be seen. Scale bar = 500nm. In figure B), the nucleus (n) is intact with well dispersed chromatin (ch). The plasma membrane (p) is intact and there are numerous mitochondria (m) and occasional lipid droplets (L). Scale bar = 500nm.

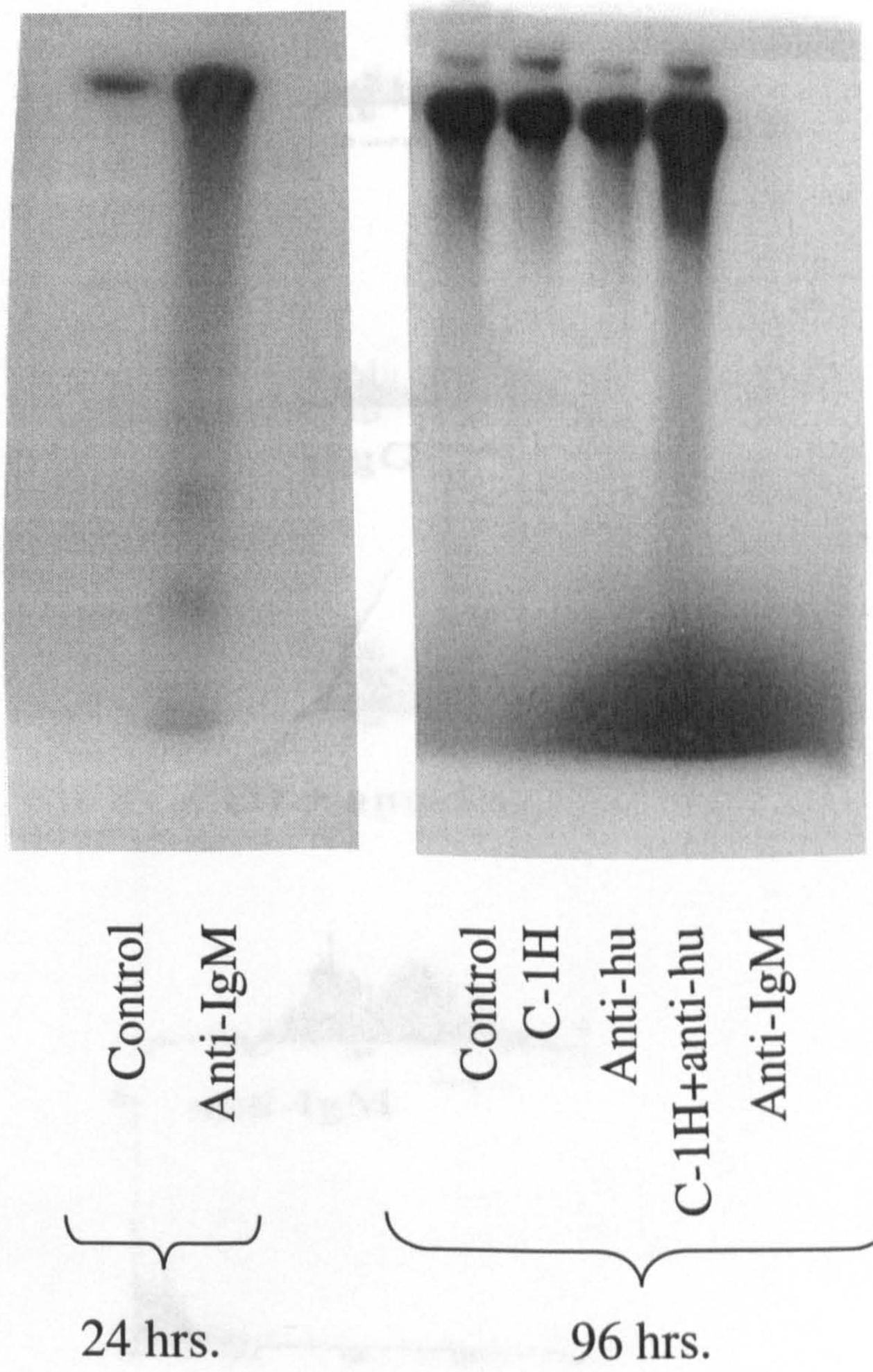


Figure 4.18 DNA laddering in CAMPATH-1H treated Wien 133 cells

Wien 133 cells were cultured in the presence of medium alone, CAMPATH-1H, anti-human IgG, CAMPATH-1H together with anti-human IgG, or anti-IgM. All antibodies were added at 20 μg/ml final concentration, with the exception of anti-IgM which was added at 4 μg/ml final concentration. DNA extracted from cells at the times indicated was labelled in the presence of α^{32}dATP and then separated on a 1.8% agarose gel.

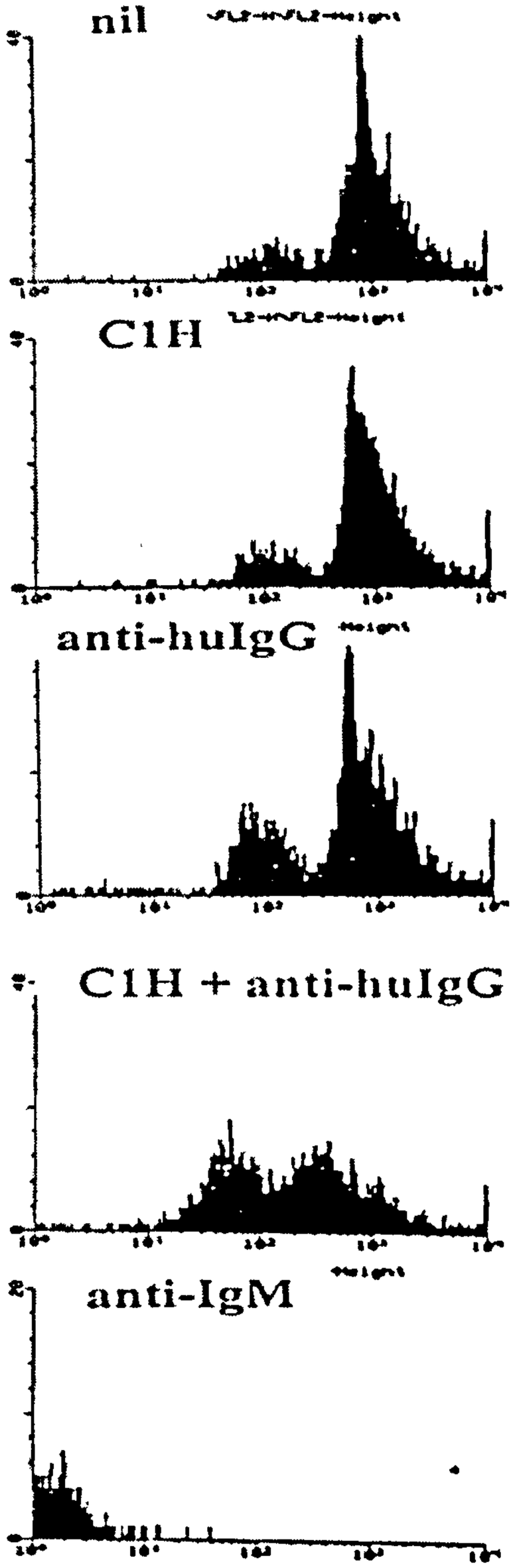
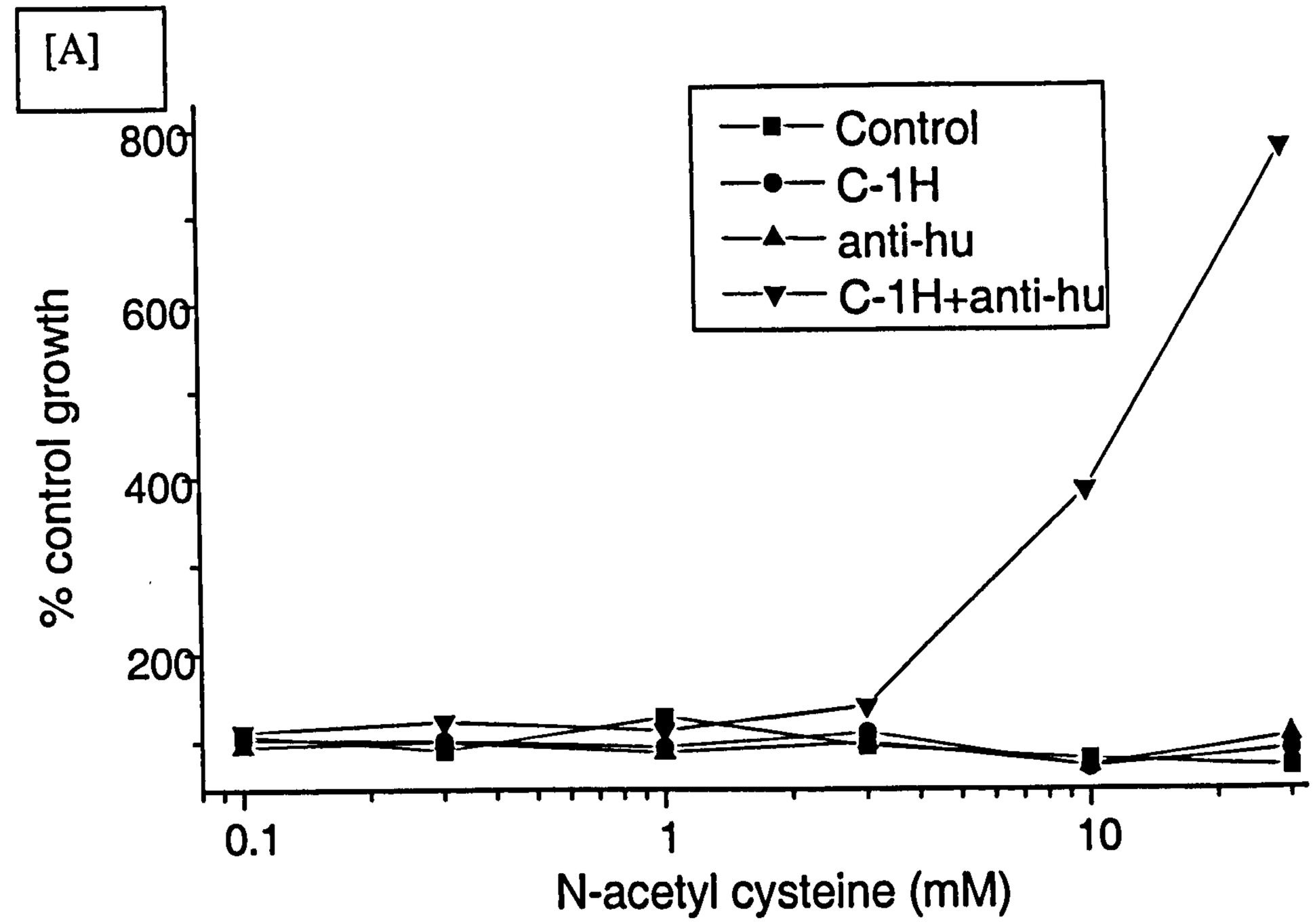


Figure 4.19 Propidium iodide staining of CAMPATH-1H treated Wien 133 cells shows DNA fragmentation.

Wien 133 cells were cultured in the presence of medium alone, CAMPATH-1H, anti-human IgG, CAMPATH-1H together with anti-human IgG, or anti-IgM. All antibodies were added at 20 μg/ml final concentration, with the exception of anti-IgM which was added at 4 μg/ml final concentration. After 72 hours, cells were stained with propidium iodide and analysed by FACScan using LYSIS II software.



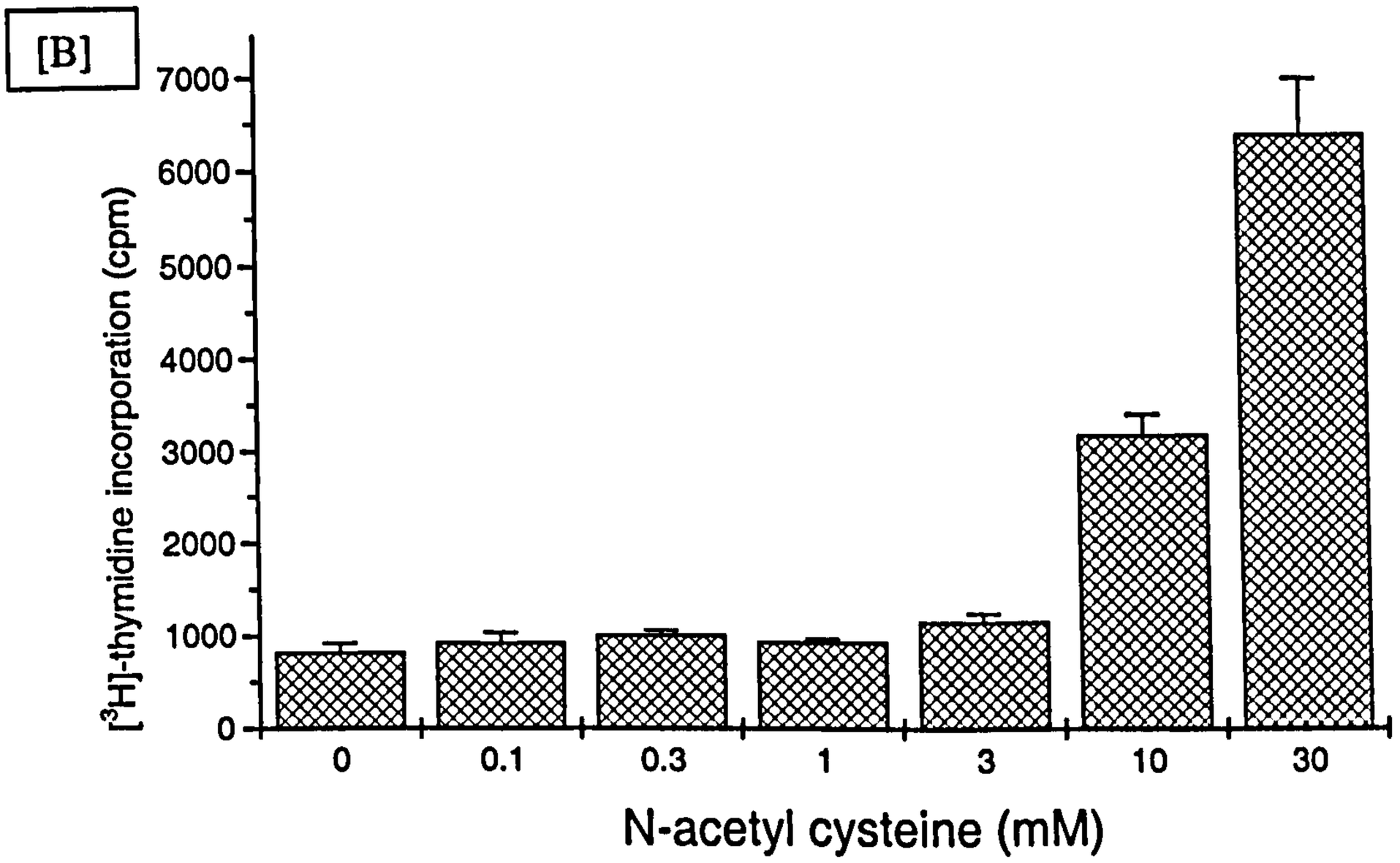


Figure 4.20 N-acetylcysteine partially blocks CAMPATH-1H induced growth inhibition in Wien 133 cells.

Wien 133 cells (2 x 10^4/well) were cultured for 5 days in the presence of medium alone, CAMPATH-1H (C-1H), anti-human IgG (anti-hu), or CAMPATH-1H together with anti-human IgG. All antibodies were added at a final concentration of 20 μg/ml. N-acetyl cysteine was added at a range of concentrations from 0 - 30 mM. [^{3}H]-thymidine was added during the last 4 hours of the culture. [A] Results are expressed as the percentage control growth for medium alone (109452), C-1H (106981), anti-human IgG (84386) and C-1H + anti-human IgG (819). Figure [B] shows the [^{3}H]-thymidine incorporation.

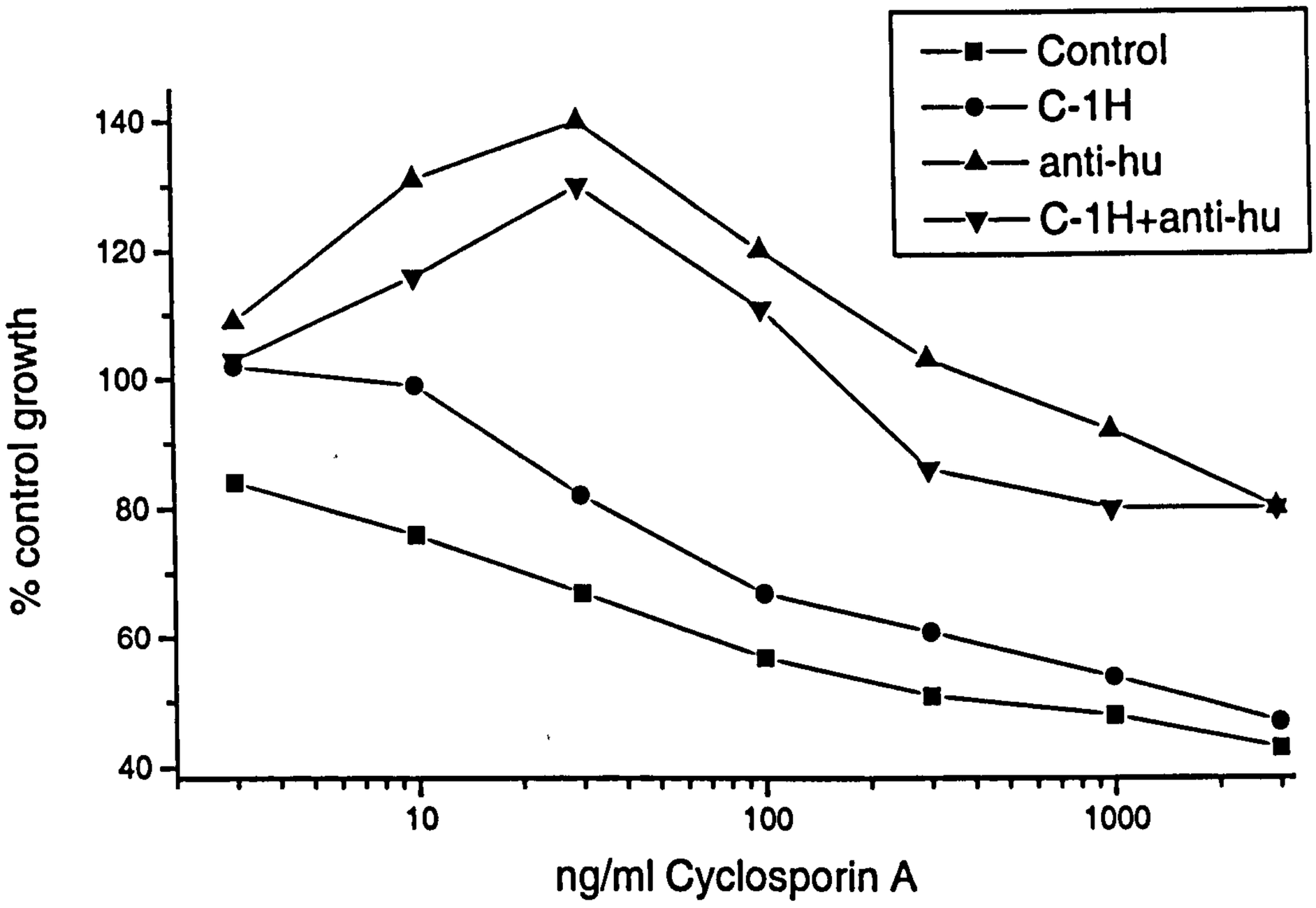


Figure 4.21 Cyclosporin A can partially block CAMPATH-1H induced growth inhibition in Wien 133 cells

Wien 133 cells (2 x 10^4/well) were incubated for 5 days in the presence of medium alone (control), anti-human IgG (anti-hu) alone, CAMPATH-1H (C-1H), or C-1H + anti-human IgG in the presence of Cyclosporin A, added at a range of concentrations from 0 - 3000 ng/ml. All antibodies were added at 20 μg/ml final concentration. [^{3}H]-thymidine was added during the last 4 hours of the culture. Results are expressed as the percentage of control growth for medium alone (252368), C-1H (264265), anti-hu (189397), or C-1H + anti-hu (28589).

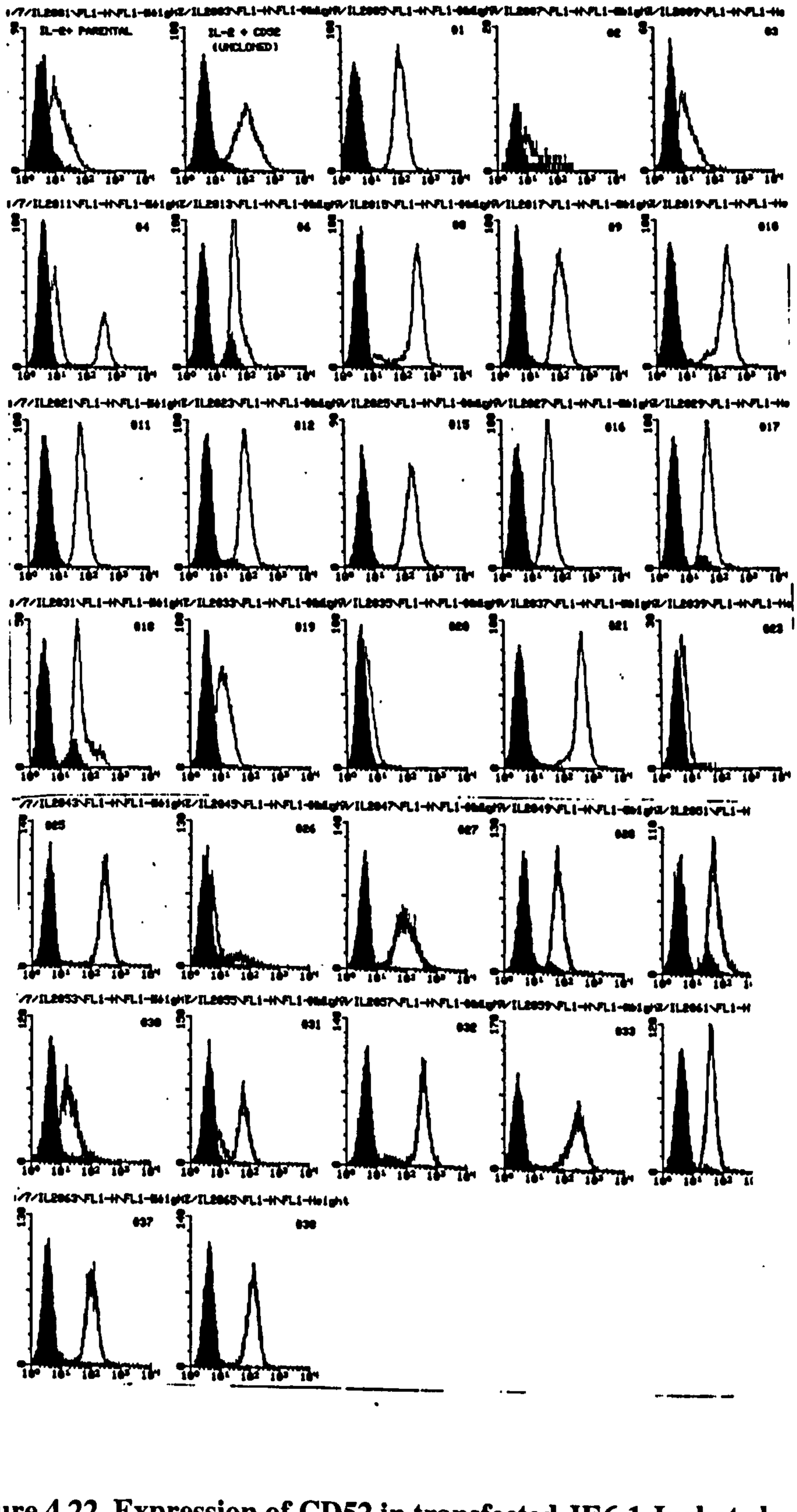


Figure 4.22 Expression of CD52 in transfected JE6-1 Jurkat clones.

CD52 transfected JE6-1 (Parental) Jurkat cells were stained with FITC labelled anti-mouse IgG1(Control)(Black profile) or C-1H $(Fab)_2$ (White profile) antibodies and analysed by FACScan using LYSIS II software. Cell number is shown on the y axis and the fluorescence intensity is shown on the x axis.

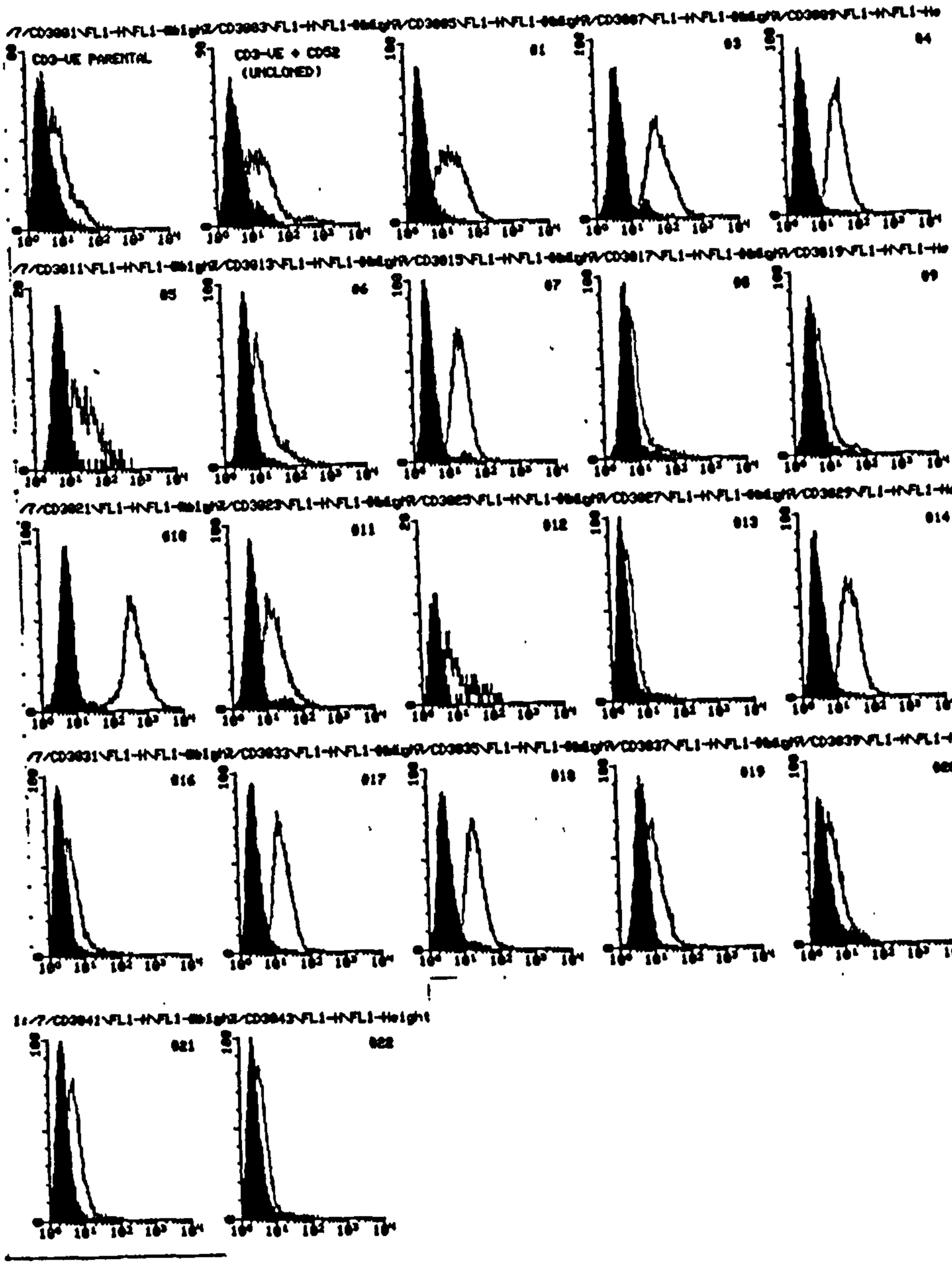


Figure 4.23 Expression of CD52 in transfected J.RT3-T3.5 Jurkat clones.

CD52 transfected J.RT3-T3.5 (TCR negative) cells were stained with FITC labelled anti-mouse IgG1(Control) (Black profile) or C-1H $(Fab)_2$ (White profile) antibodies and analysed by FACScan using LYSIS II software. Cell number is shown on the y axis and the fluorescence intensity is shown on the x axis.

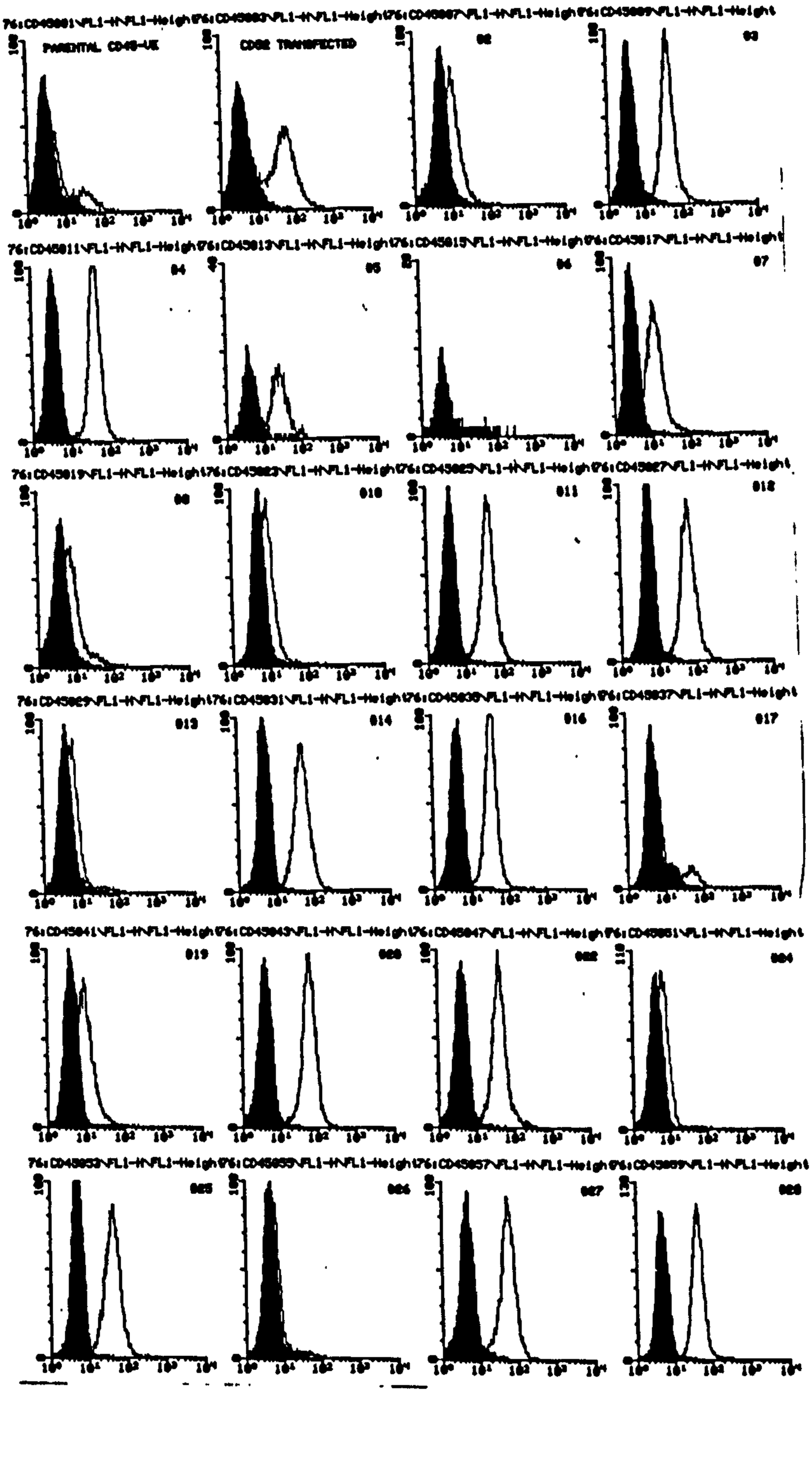


Figure 4.24 Expression of CD52 in transfected J45.01 Jurkat clones.

CD52 transfected J45.01 (CD45 negative) Jurkat cells were stained with FITC labelled anti-mouse IgG1 (Control) (Black profile) or C-1H $(Fab)_2$ (White profile) antibodies and analysed by FACScan using LYSIS II software. Cell number is shown on the y axis and the fluorescence intensity is shown on the x axis.

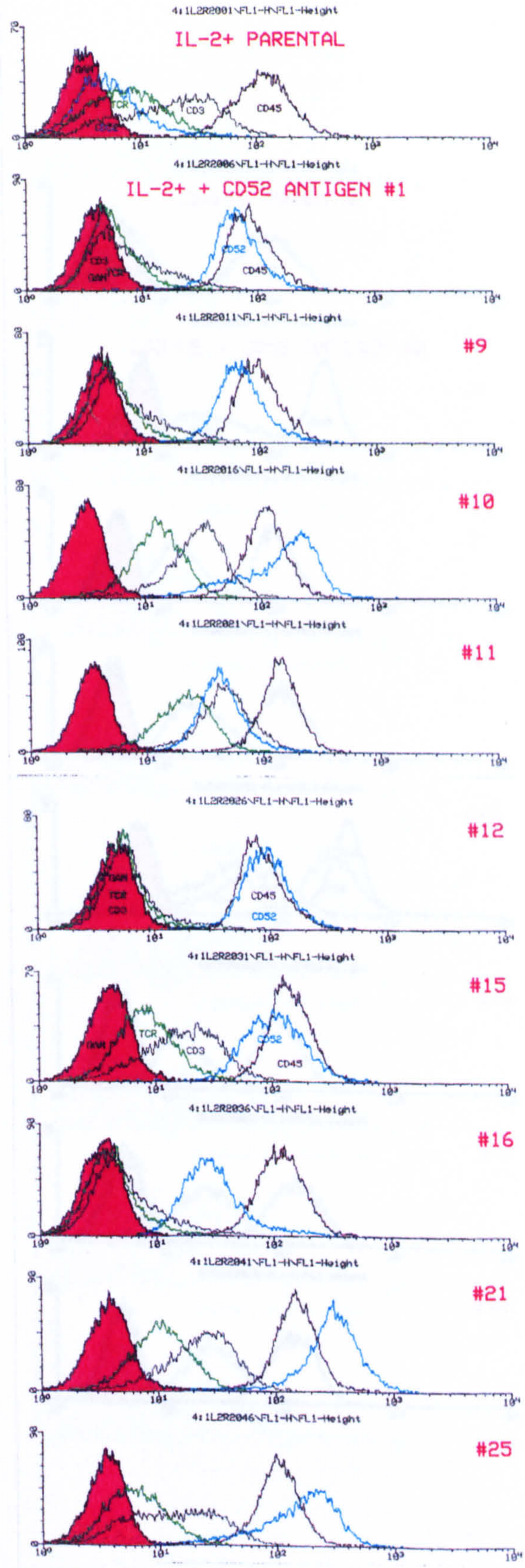


Figure 4.25 Phenotype analysis of CD52 transfected Jurkat JE6-1 clones.

CD52 transfected Jurkat JE6-1 cells were stained with FITC labelled anti-mouse IgG1 (Control), anti-CD3, anti-CD45 and anti-TCRαβ antibodies and analysed by FACScan using LYSIS II software. Cell number is shown on the y axis and the fluorescence intensity is shown on the x axis.

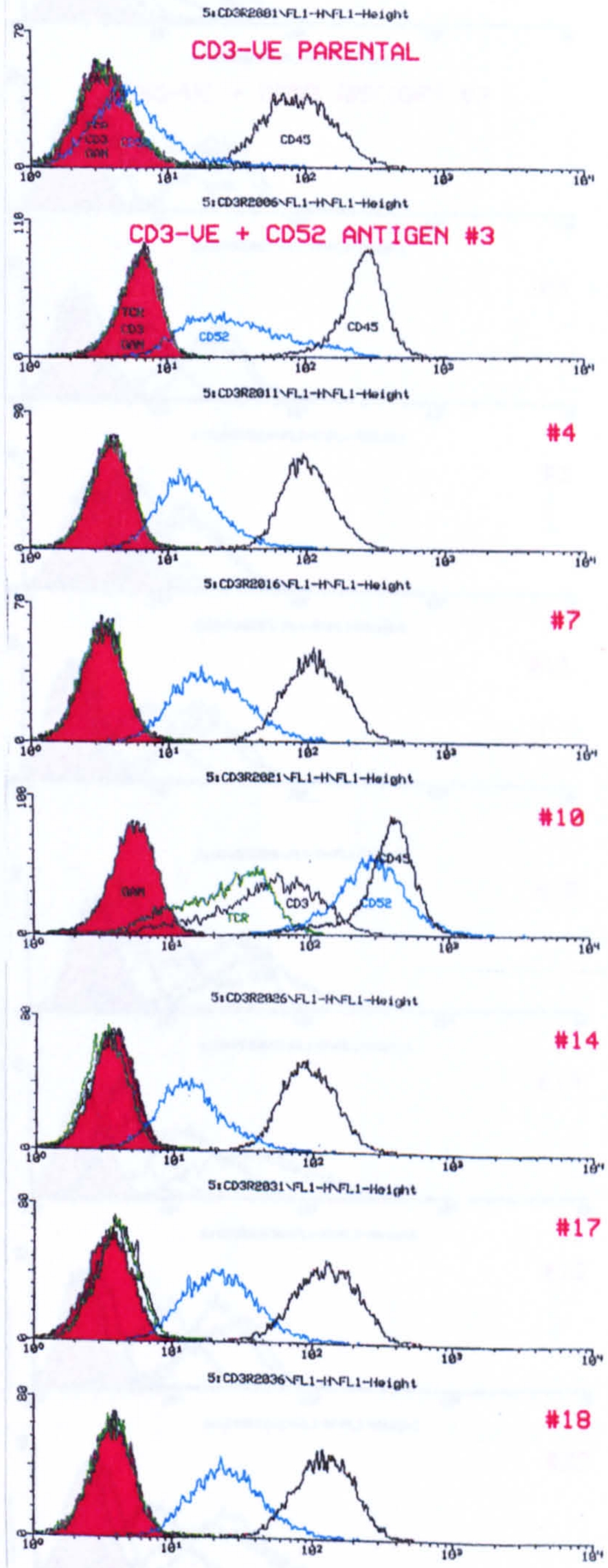


Figure 4.26 Phenotype analysis of CD52 transfected Jurkat J.RT3-T3.5 clones.

CD52 transfected Jurkat J.RT3-T3.5 cells were stained with FITC labelled anti-mouse IgG1 (Control), anti-CD3, anti-CD45 and anti-TCR αβ antibodies and analysed by FACScan using LYSIS II software. Cell number is shown on the y axis and the fluorescence intensity is shown on the x axis.

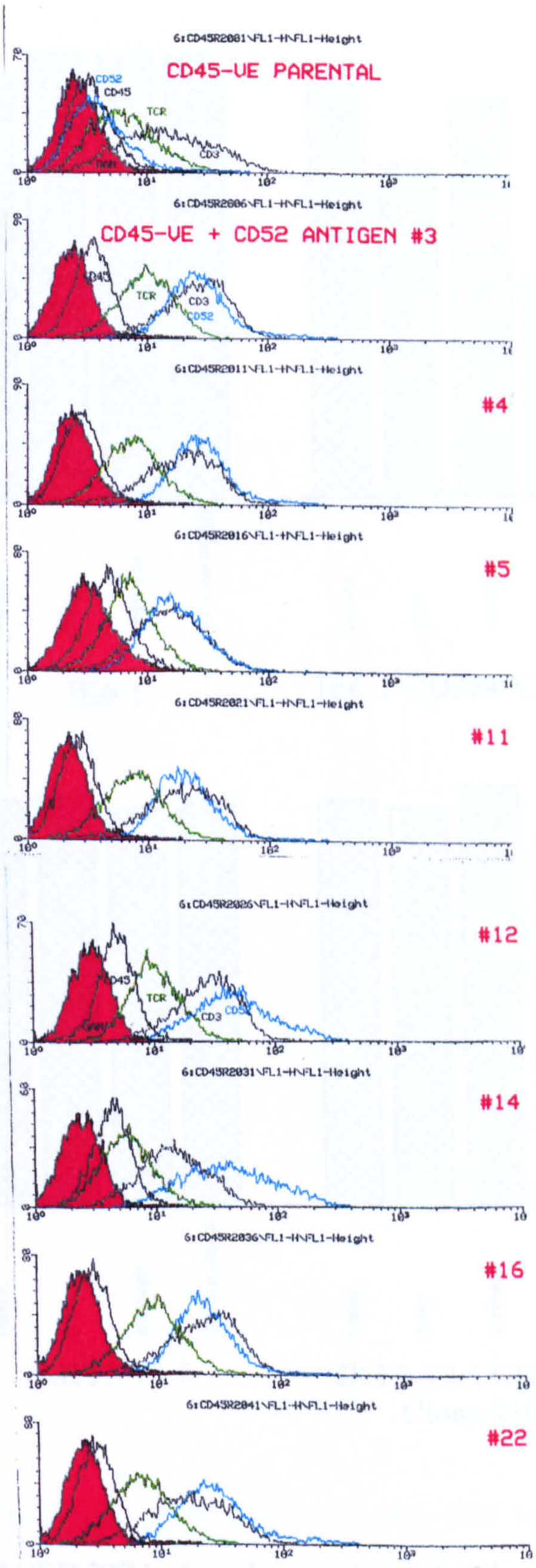


Figure 4.27 Phenotype analysis of CD52 transfected Jurkat J45.01 clones.

CD52 transfected Jurkat J45.01 cells were stained with FITC labelled anti-mouse IgG1 (Control), anti-CD3, anti-CD45 and anti-TCRαβ antibodies and analysed by FACScan using LYSIS II software. Cell number is shown on the y axis and the fluorescence intensity is shown on the x axis.

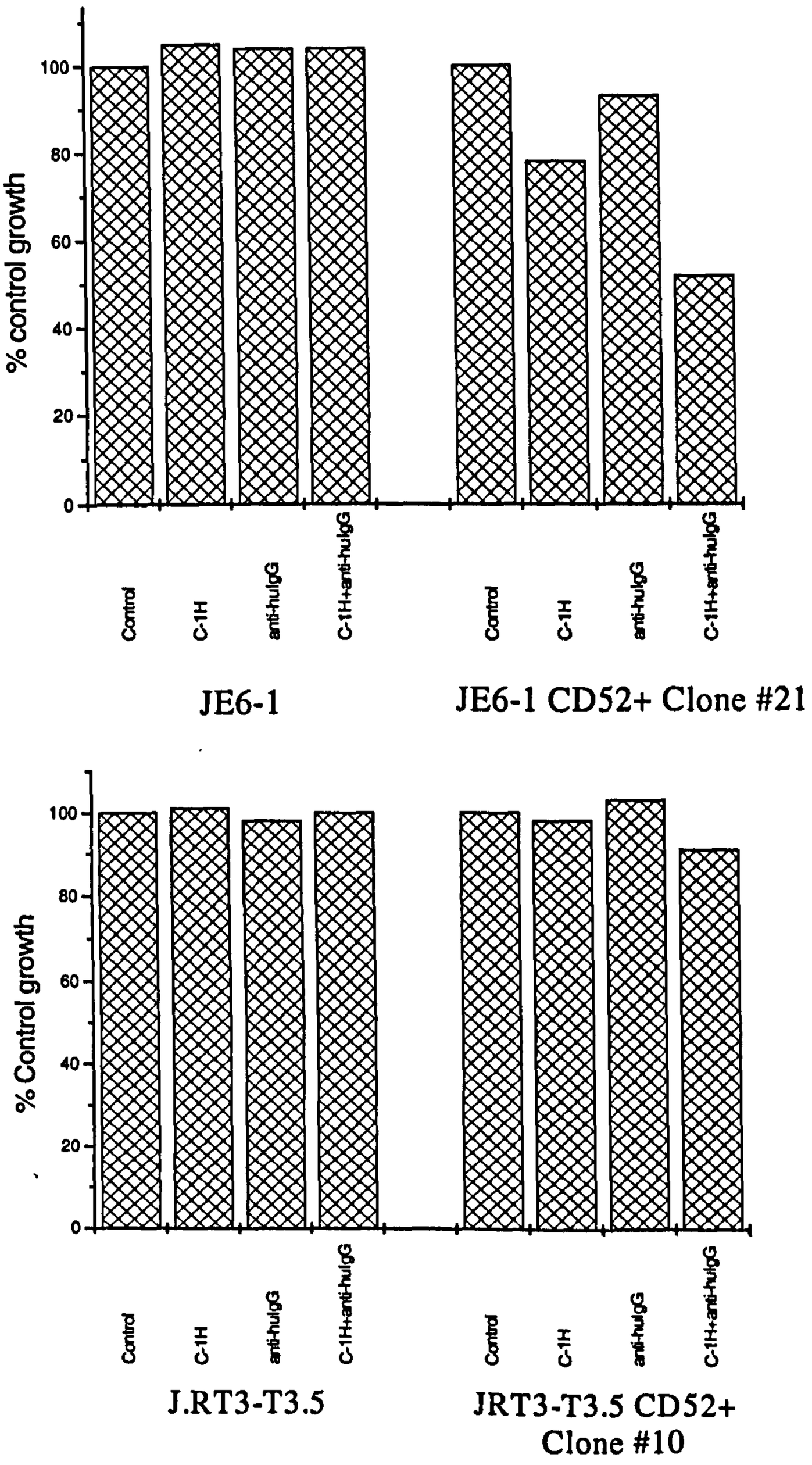


Figure 4.28 CAMPATH-1H induced growth inhibition in Jurkats is dependent on the density of CD52 and expression of a functional TCR.

Cells (1 x 10^4/well) were cultured for 7 days with medium alone, CAMPATH-1H (C-1H) antibody, anti-human IgG (anti-huIgG), or CAMPATH-1H and anti-human IgG together. Antibodies were all added in solution at a final concentration of 20 μg/ml and left in for the duration of the assay. Wells were set up in replicates of six. [^{3}H]-thymidine was added to the wells during the last 4 hours of culture. Results are expressed as percentage inhibition of control growth.

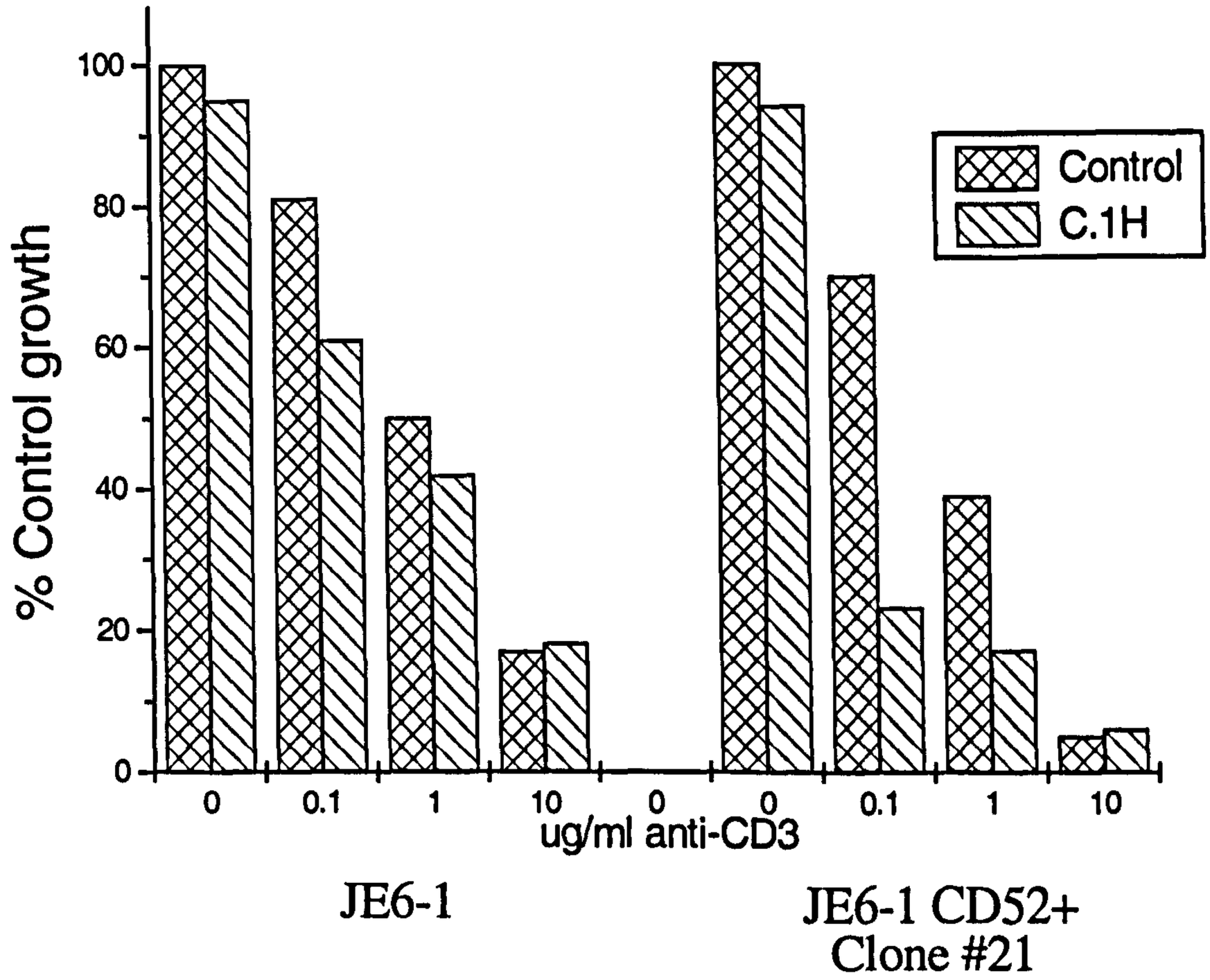


Figure 4.29 CAMPATH-1H synergises with anti-CD3 to induce cell growth inhibition in Jurkats.

Plates were coated with either no antibody, anti-CD3 antibody alone, CAMPATH-1H antibody alone, or anti-CD3 together with CAMPATH-1H antibody. All antibodies were used at 10 μg/ml. The assay was set up in the presence and absence of PMA (10 ng/ml). Cells were added at 1 x 10^4 per well and cultured for 7 days prior to harvesting. [^{3}H]-thymidine was added to the wells during the last 4 hours of culture. Results are expressed as percentage inhibition of control growth.

4.3 Discussion

This chapter shows that crosslinking the CD52 antigen on the surface of both B and T cell tumour cell lines can induce growth inhibition and subsequent apoptosis and this is achieved in the absence of effector cells or complement.

Growth inhibition/apoptosis induced by crosslinking the CD52 antigen is dependent on antigen density and initially, the only tumour cell line available expressing high enough levels of CD52 to observe these effects was the Wien 133 B cell lymphoma line.

In addition to high levels of CD52, the Wien 133 cell line also expresses surface immunoglobulin and Fc receptors. Both of these have been shown to also induce negative effects in terms of cell growth in B cell tumour cell lines (Hasbold and Klaus, 1990; Oberbarnscheidt and Kolsch, 1978) and therefore, various control experiments were carried out in order to determine whether the growth inhibition/apoptosis induced in Wien 133 cells following the crosslinking of the CD52 antigen was CD52 specific.

The use of $F(ab)_2$ fragments of antibodies showed that growth inhibition could still be achieved in the absence of Fc binding. In addition, the use of an antibody isotype control against an antigen not expressed on the surface of the Wien 133 cells had no effect on cell growth again, concluding that growth inhibition was not due to Fc interactions. Surface Ig may contribute to the CD52 induced growth inhibition as the growth of cells could be partly inhibited by the secondary anti-human IgG crosslinking antibody alone. However, experiments using a rat primary CD52 specific antibody followed by a secondary crosslinking anti-rat antibody (absorbed against human serum proteins to prevent crossreactivity), suggest that the negative signal on cell growth, induced via ligation of CD52, does not require coligation of surface immunoglobulin. In addition, the growth of the Wien 133 cells was partially inhibited in the presence of a rat anti-CD52 antibody classified as an IgG_{2c}, without the requirement for secondary crosslinking. This was attributed to the fact that the Fc portion of antibodies bearing this particular isotype can self aggregate (Kummer et al., 1993; Dhein et al., 1992); addition of anti-human IgG had no further effects on growth arrest which was in contrast to addition of anti-rat IgG which

resulted in complete growth arrest. These results therefore suggest that crosslinking CD52 alone is sufficient to cause growth arrest in Wien 133 cells.

Surface immunoglobulin represents the antigen specific receptor on B cells and dependent on the state of activation of the cell, ligation can have various functional outcomes including proliferation, anergy and apoptosis as a consequence of the triggering of signalling pathways linked to the receptor via transmembrane and cytoplasmic associations. Studies with other GPI-anchored proteins, including Thy-1 and Ly-6 in murine T cells (Sussman et al., 1988) and CD59 in human Jurkat T cells (Korty et al., 1991) have demonstrated that signalling is dependent on the coexpression of CD3, the antigen specific receptor of T cells. Repeated attempts to generate surface immunoglobulin (IgD, IgG and IgM) negative Wien 133 cells with a cocktail of anti-human isotype specific antibodies failed and so it was not possible to determine whether CD52 signalling in the Wien 133 cell line is dependent on the presence of surface immunoglobulin and its associated signalling machinery. However, culturing the Wien 133 parental cell line in the presence of anti-IgM alone resulted in cloned surface IgM negative, surface IgD positive cell lines and it was possible to conclude that signalling via CD52 does not require the presence of surface IgM but surface IgD may compensate. The inability to generate a totally negative surface immunoglobulin cell line may be explained by observations made by Paramithiotis et al., 1995, who reported that the loss of surface immunoglobulin in the Bursa of Fabricus triggered programmed cell death at certain stages in B cell development.

In order to totally eliminate the possible contribution of Fc interactions and crossreaction of the secondary crosslinking antibody with surface immunoglobulin, the Jurkat T cell leukaemia cell line was chosen. The CD52 antigen was transfected into Jurkat cells in the presence of a functional T cell receptor, in the absence of a functional T cell receptor and in the presence of a T cell receptor but in the absence of the CD45 phosphatase. A number of cloned cell lines were obtained with varying levels of CD52 expression. Experiments carried out using these, made it possible to conclude that growth inhibition via CD52 is dependent on the CD52 antigen density and expression of a functional antigen specific receptor. The relevance of these studies for CD52 signalling is discussed in more detail in chapter 7.

More detailed studies of the functional outcome of CD52 ligation were carried out in the Wien 133 cell line as it was deemed less artificial than the transfected Jurkat cells. Growth inhibition was shown to precede apoptosis. Apoptosis was demonstrated by a number of diagnostic techniques, including FACS analysis following propidium iodide staining of DNA, electron and light microscopy enabling visualisation of morphological features characteristic of apoptosis and gel electrophoresis to demonstrate DNA laddering. It is unclear how ligation of CD52 mediates growth arrest and apoptosis. Many previous studies with B and T cell tumour cell lines have shown that signalling via the antigen receptor is associated with growth arrest resulting in abortive cell cycle often accompanied by apoptosis (Scott et al., 1986; Fischer et al., 1994). Several studies have shown that cells are sensitive to growth inhibitory effects at the G1/S stage of the cell cycle (Ezhevsky et al., 1996). In the experiments described above, signalling via anti-CD3 in the Jurkats or anti-IgM in the Wien 133 cells, resulted in a complete growth inhibition and this was followed by rapid apoptosis. In contrast, the effects of ligating the CD52 antigen were much slower and complete growth inhibition was not always observed. This might suggest that the growth inhibition/apoptosis that occurs in response to ligation of the CD52 antigen requires additional protein expression/protein interactions eg. Fas and the Fas ligand; TNF-α receptor and TNF-α. Both TNF-α receptor and Fas are members of a family which when ligated with their cognate ligand are coupled to signalling pathways which can bring about apoptosis of the cell on which they are expressed (Reviewed by Wallach et al., 1997). The Wien 133 cell line was shown to be extremely sensitive to the presence of TNF-α but antibodies capable of neutralising TNF-α failed to prevent CD52 induced cell death. This does not necessarily preclude that membrane bound TNF-α which is not recognised by the neutralising antibody, is responsible for the cell death observed. The Fas pathway did not appear to have a role in CD52 induced cell death in the Wien 133 cells as they were shown not to express Fas and it was not induced following CD52 crosslinking. This is in agreement with the literature that in contrast to T cell tumour cell lines, apoptosis in many B cell lines is not induced by Fas (Peter et al., 1995). Since these experiments were performed, other potential candidates for inducing apoptosis in the Wien 133 cell line have been described, including wsl (Kitson et al., 1996), DR4 (Pan et al., 1997) and TRAIL (Mariani and Krammer, 1998). The growth arrest/apoptosis effect induced by crosslinking CD52 on the surface of Wien 133 cells could not be transferred by culturing cells with culture

supernatants from CAMPATH-1H treated cells. This suggests that the effects could well be mediated by some kind of membrane bound factor; alternatively, the factor is extremely labile and therefore not transferrable. Attempts to rescue the Wien 133 cell line from growth arrest/apoptosis with numerous cytokines, including IL-1, IL-2, IL-4, IL-6, IL-7, IFN-α and IFN-γ were unsuccessful. IL-4 and IFN-γ have been shown in the literature to rescue CLL B cells from apoptosis (Dancescu et al., 1992; Buschle et al., 1993). Antibodies against CD40 and CD23, both of which have been reported to be capable of reducing growth inhibition/apoptosis induced by ligation of surface IgM in B cell lymphoma cell lines or germinal centre B cells (Valentine and Licciardi, 1992), also had no effect. This could be due to the activation state of the cells, the Wien 133 cell line bearing the phenotype of a relatively mature B cell expressing high levels of surface IgM and also secreting IgG. Similar results have been described with the B104 B cell tumour line, which is phenotypically similar to the Wien 133 cell line (Ishigami et al., 1992). Cyclosporin A was able to partially block CAMPATH-1 induced growth inhibition in the Wien 133 cell line. Recent studies looking at TRAIL (member of the TNF family) have shown that its expression is Cyclosporin A sensitive (Mariani and Krammer, 1998).

In conclusion, the above studies have demonstrated clearly that crosslinking the CD52 antigen on B and T tumour cell lines can induce growth arrest and apoptosis. These effects occur in the absence of effector cells such as NK cells or macrophages or active serum complement. The relative importance of these mechanisms as a means of depleting cells *in vivo* remains unclear. *In vivo* studies with a number of anti-CD52 antibodies looking at lymphocyte depletion have demonstrated that the extent of depletion is dependent on antibody isotype. The reasons have been attributed to the abilities of different antibodies to effect different mechanisms such as ADCC and complement fixation (Hale et al., 1983b; Hale et al., 1985; Riechmann et al., 1988). The involvement of Fc binding is suggested by the fact that following administration of CAMPATH-1H antibody, patients suffer a strong first dose reaction, associated with the release of inflammatory cytokines including TNF-α, IL-6 and IL-1 release. This phenomenon involves CD16 crosslinking, which supports a role for ADCC in tumour clearance, at least in peripheral blood (Wing et al., 1996). In addition, the time course for tumour depletion in peripheral blood is extremely rapid and more closely follows the time course for ADCC of 4-6 hours than that seen for direct growth inhibition which occurs over

several days. However, this does not preclude a role for growth inhibition or apoptosis of tumour cells in different anatomical localities eg. in lymph nodes. In addition, some tumour types eg. PLL or CLL, are more sensitive to treatment with CAMPATH-1H than other tumours (Dyer, personal communication).

5. Control of CD52 Expression in Wien 133 Cells

5.1 Introduction

CAMPATH-1 antibodies have been used in clinical trials for the treatment of rheumatoid arthritis and lymphoid malignancies (Hale et al., 1988b; Dyer et al., 1989; Isaacs et al., 1992). During the treatment of both diseases, in a minority of cases, patients have developed populations of lymphoid cells which have lost expression of CD52 and other GPI-anchored proteins, including CD55 and CD59 (Brett et al., 1996b; Hertenstein et al., 1995). The absence of surface GPI-anchored proteins on cells in these patients appeared to have no serious consequences. Those patients affected, recovered from antibody therapy with a better prognosis for disease symptoms than those retaining normal levels of CD52 expression. However, loss of CD52 expression, especially on tumour cells, means that these patients can no longer be treated with CAMPATH-1 specific antibodies.

In addition, there is the concern that the longterm loss of GPI-anchored proteins from the surface of lymphocytes could lead to adverse side effects as can be illustrated by people who suffer from paroxysmal nocturnal hemoglobinuria (PNH) (Takeda et al. 1993; Schubert et al., 1994). PNH is an acquired disease which affects hematopoietic stem cells and is characterised by recurrent intravascular haemolysis which can lead to life threatening thrombosis. Abnormal cells in PNH patients have been shown to lack expression of GPI-anchored proteins, including acetylcholine esterase, alkaline phosphatase, 5' nucleotidase, CD55, CD59 and LFA-3. In all PNH patients looked at to date, the defect has been attributed to mutations arising in the PIG-A gene. The PIG-A gene is involved in the early steps of GPI-anchor biosynthesis. Mutations range from those in the coding region (missense, nonsense, frameshift) to splicing abnormalities and unstable transcripts (Miyata et al., 1994; Yamada et al., 1995).

The biosynthetic pathway of the GPI-anchor has been identified in mammalian cells using a panel of T cell mutants which lack expression of GPI-anchored proteins including Thy-1 and Ly-6A, and the use of complementation mutants (reviewed by Yeh et al., 1994). The

first step in anchor biosynthesis, involves the transfer of N-acetylglucosamine to a phosphoinositol acceptor to form N-acetylglucosamine-phosphatidyl inositol and this appears to require a functional PIG-A gene. N-acetylglucosamine-phosphatidyl inositol is then deacetylated to form N-glucosamine phosphatidyl inositol. Fatty acid is added to the inositol ring and three mannose residues are transferred to the elongating GPI core. The final step in the synthesis of a GPI-anchor is the transfer of ethanolamine phosphates to the mannose residues. The entire synthesis of the GPI is thought to take place in the cytoplasm. The implication is, therefore, that the mature GPI anchor precursor flips into the ER lumen (Vidugiriene and Menon, 1994). Proteins destined to be GPI anchored have two signal sequences; the carboxyl terminus signal peptide gets cleaved off enabling the transfer of the preformed GPI anchor onto the nascent polypeptide by a transamidase reaction. The amino terminus signal peptide allows the fully formed GPI-anchored protein to be translocated across the ER membrane. If proteins do not become GPI-anchored, they do not get expressed on the cell surface but instead are retained, degraded or secreted (Field et al., 1994; Takeda and Kinoshita, 1995).

The emergence of CD52 negative cells in CAMPATH-1H treated patients suggests that antibody therapy is placing cells under selection pressure and therefore, it is of interest to determine at what level, the loss of CD52 expression is occurring (ie. transcription, translation or failure to assemble a fully formed GPI-anchored CD52 protein) and whether the phenotype is irreversible.

Studies were therefore initiated in the Wien 133 cell line which was described in the previous chapter (Chapter 4). The Wien 133 cell line was shown to be very sensitive to growth inhibition and cell death following the crosslinking of surface CD52 with CAMPATH-1 antibodies. Cells resistant to cell death expressed negligible levels of CD52 and so provided a system for looking to see what stage/stages in the synthesis and expression of GPI-anchored CD52, were being affected by CAMPATH-1H treatment and what the implications might be for patients treated with the antibody who develop populations of CD52 negative cells.

5.2 Results

5.2.1 Selection of CAMPATH-1H resistant cells

The Wien 133 B cell lymphoma cell line was selected for study because it maintains a high level of CD52 antigen expression *in vitro* and has been demonstrated to be sensitive to growth inhibition and cell death in the presence of CAMPATH-1H antibody (Chapter 4). Wien 133 cells (1 x 10^6/ml) were cultured in the presence of 20 μg/ml CAMPATH-1H and 20 μg/ml anti-human IgG. After 14 days, a small population of cells which remained viable after antibody treatment, were expanded in culture to generate the CV series. These cells were resistant to growth inhibition and cell death induced in the presence of CAMPATH-1H in contrast to the parental cell line; however, in common with the parental cell line, were sensitive to killing by an anti-IgM antibody (Figure 5.1).

In addition to cell growth inhibition induced by CAMPATH-1H in the presence of a second crosslinking antibody, Wien 133 cells treated with CAMPATH-1H alone are sensitive to killing in the presence of monocytes. A second set of CAMPATH-1H resistant cell lines, was generated by selection with human monocytes and anti-CD52. The MR cell series were completely resistant to killing by monocytes plus CAMPATH-1H antibody, but still sensitive to killing in the presence of anti-CD38 antibody (J. Tite, personal communication).

Phenotypic analysis showed that both selection procedures generated populations in which the majority of cells expressed negligible levels of CD52, with a small subpopulation of cells expressing levels of CD52 were comparable to the parental Wien 133 cell line. The levels of selected B cell markers, CD19, CD20, DR and IgM expression in both variant cell line series compared to the parental cell line. There were differences in the expression of surface IgD; both the MR and CV cell series expressed comparable levels of IgD, with a more heterogeneous expression pattern of IgD than in the parental Wien 133 cell line.

The CV (CAMPATH variant) cells were dilution cloned generating CV1, CV2, CV3 etc. The level of CD52 expression in the clones was assessed by FACS and three clones were selected for further analysis, namely CV3, CV4 and CV5. The FACS data indicated that in all three cloned cell lines, there was a percentage of cells which expressed low to moderate levels of CD52 antigen (Figure 5.2). In the CV3 clone, 10% of the cells expressed 'high' levels of CD52; in the CV4 clone, the number was 42% and in the CV5 clone, the number was 6%. Cells resistant to killing by monocytes were dilution cloned as above, generating the MR (monocyte resistant) cell clones and showed a similar pattern of expression to the CV clones (figure 5.3).

The expression of other selected B cell surface markers, namely surface IgG, IgM, CD19, CD20 and DR was the same in the parental Wien 133 cell line and in the CV clones. IgD levels were lower in the CV3 and CV5 clones compared to the C1 parental cell line (Figure 5.4).

FACS analysis showed that despite cloning, the bimodal expression pattern of CD52 was still apparent, suggesting that the CAMPATH-1H resistant cell lines still posessed a functional gene for CD52 but the regulation of CD52 expression in these populations was distorted. The level of expression was not related to stages in the cell cycle as cells sorted for high, medium and low expression of CD52 contained identical percentages of cells in G1, S and G2/M phases of the cell cycle (J. Tite, personal communication).

Further evidence for a regulatory defect in the selected variants came from experiments in which cells sorted into two separate populations of cells for high and low expression of CD52, reverted back to a bimodal pattern of CD52 expression after several days in culture. In addition, a further round of dilution cloning still produced clones maintaining a bimodal pattern of CD52 expression.

5.2.2 Quantitation and Western blot analysis of CD52 antigen in Wien 133 cells and CV lines

The CD52 antigen was extracted from cell pellets ($3x10^8$ cells/pellet) and the total amount of CD52 antigen in each preparation was determined by ELISA. A standard curve was set up using affinity purified antigen from human spleens. Dilutions were made of the Wien 133 C1 and CV antigen extracts to enable readings to be taken from the standard curve and thus quantitate the amount of CD52 antigen present. The Wien 133 parental line contained 4000 pmol CD52 antigen/10^8 cells, CV3 = 288 pmol antigen/10^8 cells, CV4 = 2500 pmol antigen/10^8 cells and CV5 = 76 pmol antigen/10^8 cells. These figures would appear to correlate with the level of expression of the CD52 antigen determined by FACS (Figure 5.2).

After quantitation, for each antigen preparation, 5 µl was treated in the presence or absence of N-Glycanase to remove N-linked sugars. Following SDS PAGE, proteins were transferred to nitrocellulose and antigen detected using the rat CAMPATH-1G antibody (Figure 5.5). The results obtained from the Western blot confirm the presence of CD52 antigen in the preparations ie. the molecular weights of the proteins treated in the presence (6,000 KDa) or absence (20,000-26,000 kDa) of N-Glycanase are characterisic of the CD52 antigen (Xia et al., 1991). The results confirm the ELISA and FACS data that there are differences in the levels of CD52 expression in the Wien 133 parental cell line and the CV clones. The results obtained following treatment with N-Glycanase suggest that the differences are not due to changes in glycosylation ie. the amount of CD52 antigen in the CV5 cell line appears comparable with and without N-Glycanase treatment as is the case in the Wien 133 parental cell line. There is however, a marked difference between the amount of CD52 antigen present, if the CV5 cell line is compared to the Wien 133 parental cell line.

The results obtained from the ELISA and Western blotting indicate that the total amount of CD52 antigen as detected by the CAMPATH-1 antibodies, varies between the Wien 133 parental cell line and the CV clones. The CD52 antigen in the form recognisable by CAMPATH antibody is not being withheld intracellularly.

5.2.3 CD52 antigen mRNA levels in Wien 133 cells and CV clones are comparable.

Total RNA was extracted from Wien 133 cells and the CV clones and quantitated using a spectrophotometer. The quality of the preparations was assessed by electrophoresing the RNA on agarose gels and staining with ethidium bromide to visualise the 30s and 70s ribosomal subunits. After standardising the initial starting amounts of total RNA, the RNA was titrated by doubling dilution, applied to Hybond-N membrane and CD52 specific message detected using a labelled linearised plasmid DNA insert corresponding to the entire CD52 coding sequence. The results are shown in figure 5.6. There was no marked difference between the level of message RNA in the CV clone and the Wien 133 parental cell line. Filters were then stripped and reprobed with a DNA insert specific for β-Actin to show that the initial loadings were the same. The results indicate that the differences in levels of expression of CD52 antigen on the surface of Wien 133 and CV cells is not due to a failure in transcription.

5.2.4 Expression of other GPI-anchored proteins in Wien 133 cells and CV clones

Using FACS analysis, the expression of two other GPI-anchored proteins, CD55 (DAF) and CD59 (protectin), was examined in the Wien 133 parental cell line and the CV3, CV4 and CV5 clones (Figure 5.7). The results indicate that in contrast to the expression of typical B cell transmembrane markers which were comparable in the parental cell line and the CV clones (See figure 5.4), the expression of other GPI-anchored proteins in addition to CD52 were also reduced. The parental Wien 133 cell line expressed both CD55 and CD59 although these markers appeared to be present at a far lower density on the cell surface compared to CD52. The CV3 and CV5 cell lines expressed low/negative levels of CD55 and CD59. The CV4 cell line appeared to have two populations of cells present; the first, expressing CD52 antigen, CD55 and CD59 at levels comparable to the parental Wien 133 cells; the second, expressing low levels of CD52, low CD59 and negative for CD55.

These results point to a general defect in the expression of GPI-anchored proteins.

5.2.5 Western blot analysis of CD59 levels in Wien 133 cells and CV lines

The anti-CD59 antibody was used in a Western blot detection system to look at the total amount of CD59 antigen present in the cells. This antibody detects the extracellular region of the CD59 protein and not the GPI anchor (Figure 5.8). The levels of CD59 were comparable in the parental Wien 133 cell line, the CV3, CV4 and CV5 clones. The molecular weight of the protein detected on the blot (20 kDa) corresponded to that documented for the CD59 antigen. There was no suitable negative control cell line available; in the absence of primary antibody, the second antibody used for the detection of the anti-CD59 antibody did not produce any bands, indicating that the results were specific to the first antibody.

This data suggests that there is a defect in the assembly of GPI-anchored proteins. The failure to detect intracellular CD52 antigen can be explained by CAMPATH-1 antibodies either recognising an epitope on the antigen involving the GPI anchor or that the presence of the GPI anchor contributes to the formation of the epitope recognised by the CAMPATH-1 antibodies.

5.2.6 Surface IgD expression in Wien 133 cells is not sensitive to PI-PLC treatment

The CV clones have been shown to have reduced surface expression of GPI-anchored CD52, CD55 and CD59. The expression of other B cell markers, including CD19, CD20, DR, sIgG and sIgM did not change. The exception was sIgD which was expressed at lower levels in the variants compared to the parental cells. Reports in the literature have shown that sIgD can be expressed as both a transmembrane and a GPI-anchored protein (Wienands and Reth, 1991). Lankester et al., 1993, found no evidence for GPI-anchored sIgD in primary B cells, but did not exclude the possibility of a GPI-anchored form in transformed cells. The variable expression of sIgD could be explained by the existence of both forms in Wien 133 cells. Therefore, when GPI-anchored sIgD was lost under selection pressure, the transmembrane form could still expressed. In order to address this, Wien 133 cells were treated in the presence and absence of PI-PLC which cleaves GPI-

anchored proteins. Results are shown in figure 5.9. Only a proportion of CD52 is affected by treatment with PI-PLC and there is no effect on the expression of sIgD. It is therefore not possible to draw any conclusions as the possibility remains that a proportion, or all, of sIgD is GPI-anchored but it is not sensitive to removal by PI-PLC treatment.

5.2.7 Restoration of CD52 expression in CV cells following *in vivo* growth in nude mice

The CD52 antigen is expressed at high levels on normal T and B lymphocytes *in vivo*. The majority of T and B tumour cell lines cultured *in vitro*, in contrast to Wien 133 cells, express very low levels of CD52 antigen (Hale et al., 1990; figure 4.1). To investigate the effect of the *in vivo* environment on CD52 expression, parental and variant clones (CV and MR lines) of Wien 133 cells were injected subcutaneously into nude mice.

After several weeks, mice injected with both parental and variant lines developed tumours. At day 38, the incidence of tumours was higher and tumour size was bigger in mice injected with Wien 133 parental C1 cells compared to those injected with variant derived cells (Peter Topley, personal communication).

Tumours were recovered from the mice and FACS analysis showed that irrespective of the phenotype of the cells with which the mice had originally been injected, all cells now expressed higher levels of CD52 than their counterparts maintained *in vitro* (Figure 5.10). *Ex vivo* cells were maintained in culture for several months. Increased CD52 expression once re-established was apparently quite stable.

To determine whether increased CD52 expression following *in vivo* growth was associated with selection of a few pre-existing CD52 high expressing cells, or due to the upregulation of enzymes controlling GPI-anchor production, the CV5 cells were treated with CAMPATH-1H in the presence of complement to remove CD52 positive cells; alternatively, CD52 negative cells were selected by removing CAMPATH-1 positive cells using Dynal beads or by use of the FACS Sort. These were then injected into mice as

described above or returned to *in vitro* culture. Again cells recovered from mice which had received CD52 negative cells had increased levels of CD52 expression suggesting that the re-expression of CD52 is due to upregulation of expression rather than selection.

Cells which had increased expression of CD52 after *in vivo* growth, were once again sensitive to growth inhibition by crosslinking CD52 antigen with CAMPATH-1H and anti-human IgG (Figure 5.11).

5.2.8 Effect of cytokines on expression of CD52 in the CV clones

The data showing that *ex vivo* CV clones expressed high levels of surface CD52, suggests that the loss of antigen expression following treatment with CAMPATH-1 antibodies is due to a regulation of GPI-anchored protein expression rather than a permanent defect. Cytokines known to effect B cells were therefore added to CV clones over a range of physiological concentrations to see whether the expression of CD52 could be induced. 5 x 10^5 cells per well were seeded in 2 ml volume in 24 well plates. Cells were incubated with cytokines as detailed below and then harvested at 24 and 48 hours and analysed by FACS for expression of CD52. IL-1α (1, 10 and 100 pg/ml), IL-2 (0.01,0.1 and 1 ng/ml), IL-4 (0.1, 1 and 10 ng/ml), IL-5 (0.01, 0.1 and 1 ng/ml), IL-6 (0.001, 0.01 and 0.1 ng/ml) and IL-7 (0.01, 0.1 and 1 ng/ml) did not induce upregulation of CD52 expression.

In addition, culture supernatants from the parental Wien 133 C1 cells had no effect on upregulating the expression of CD52 in the CV clones.

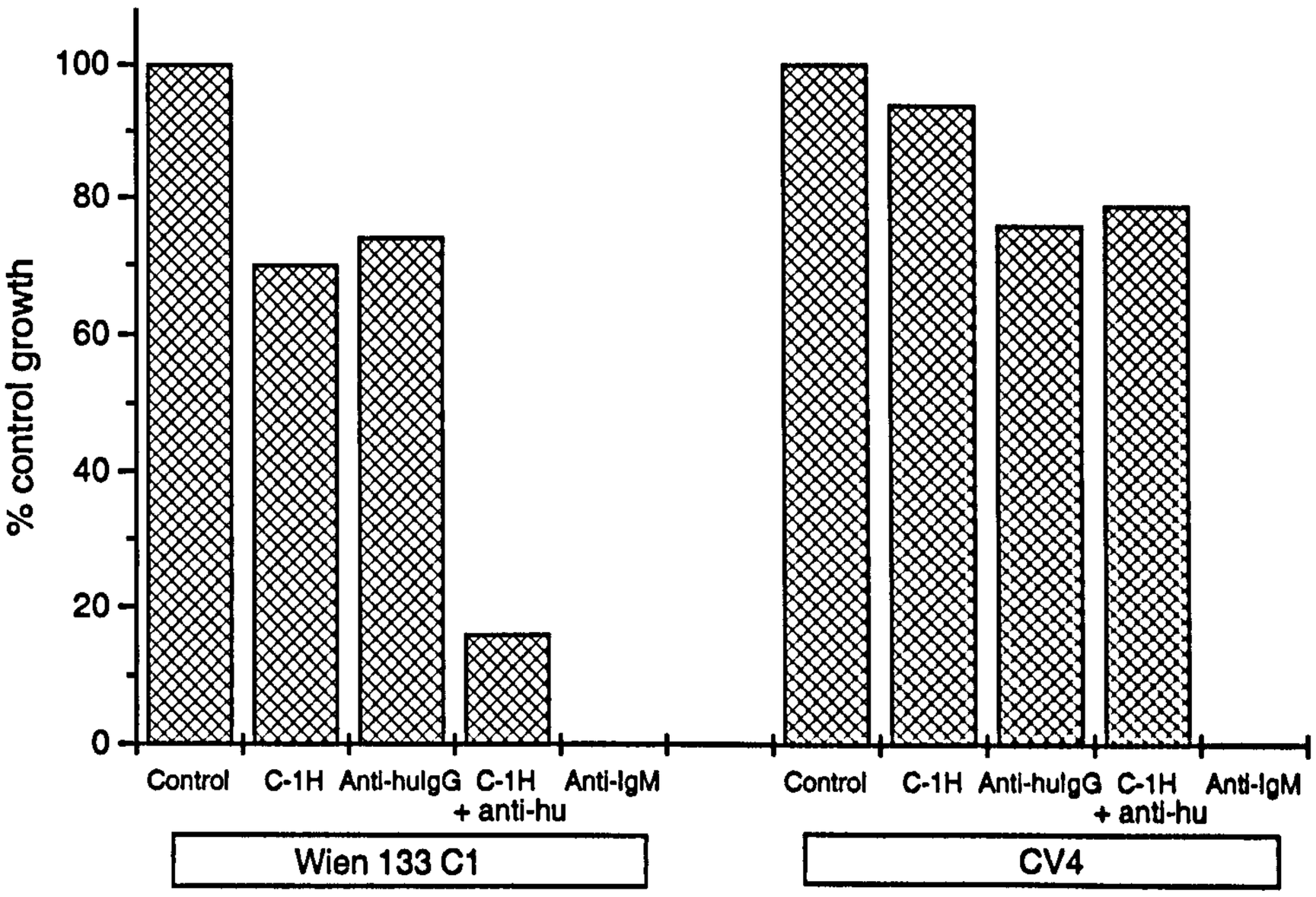


Figure 5.1 Wien 133 cells resistant to growth inhibition by CAMPATH-1H antibody treatment remain sensitive to growth inhibition by IgM.

Cells (2 x 10^4/ well) were cultured for 5 days in the presence of medium alone, C-1H alone, anti-human IgG alone, C-1H + anti-human IgG or anti-human IgM alone. Antibodies were all added at a final concentration of 20 μg/ml with the exception of anti-human IgM which was added at a final concentration of 4 μg/ml. [^{3}H]-thymidine was added for the last 6 hours of the culture. Results are expressed as mean +/- SEM of triplicate cultures.

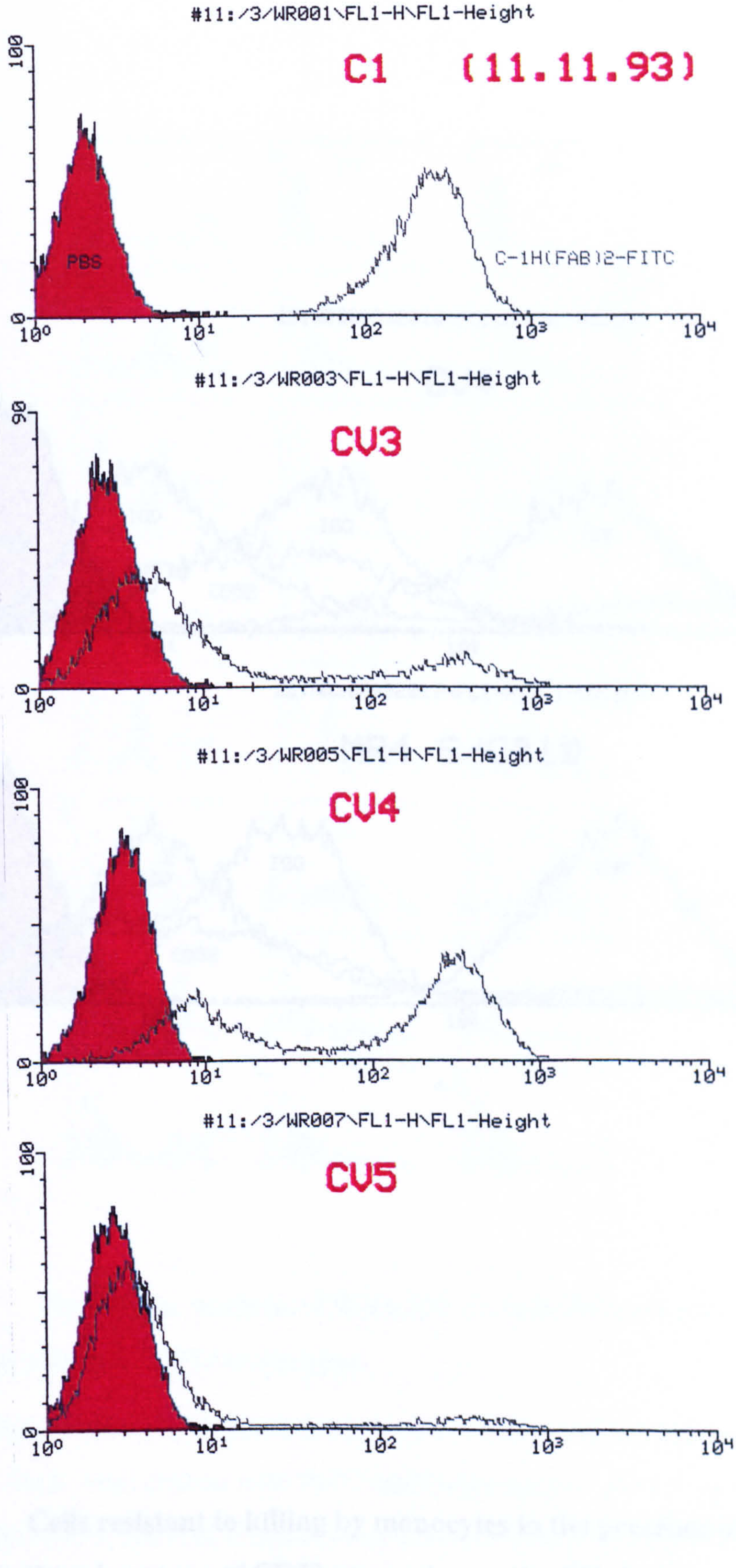


Figure 5.2 Decreased expression of CD52 in CV clones compared to parental Wien 133 C1 clone.

Cells were stained for surface expression of CD52 using FITC-labelled CAMPATH-1H (Fab)2 and analysed by FACScan using LYSIS II software.

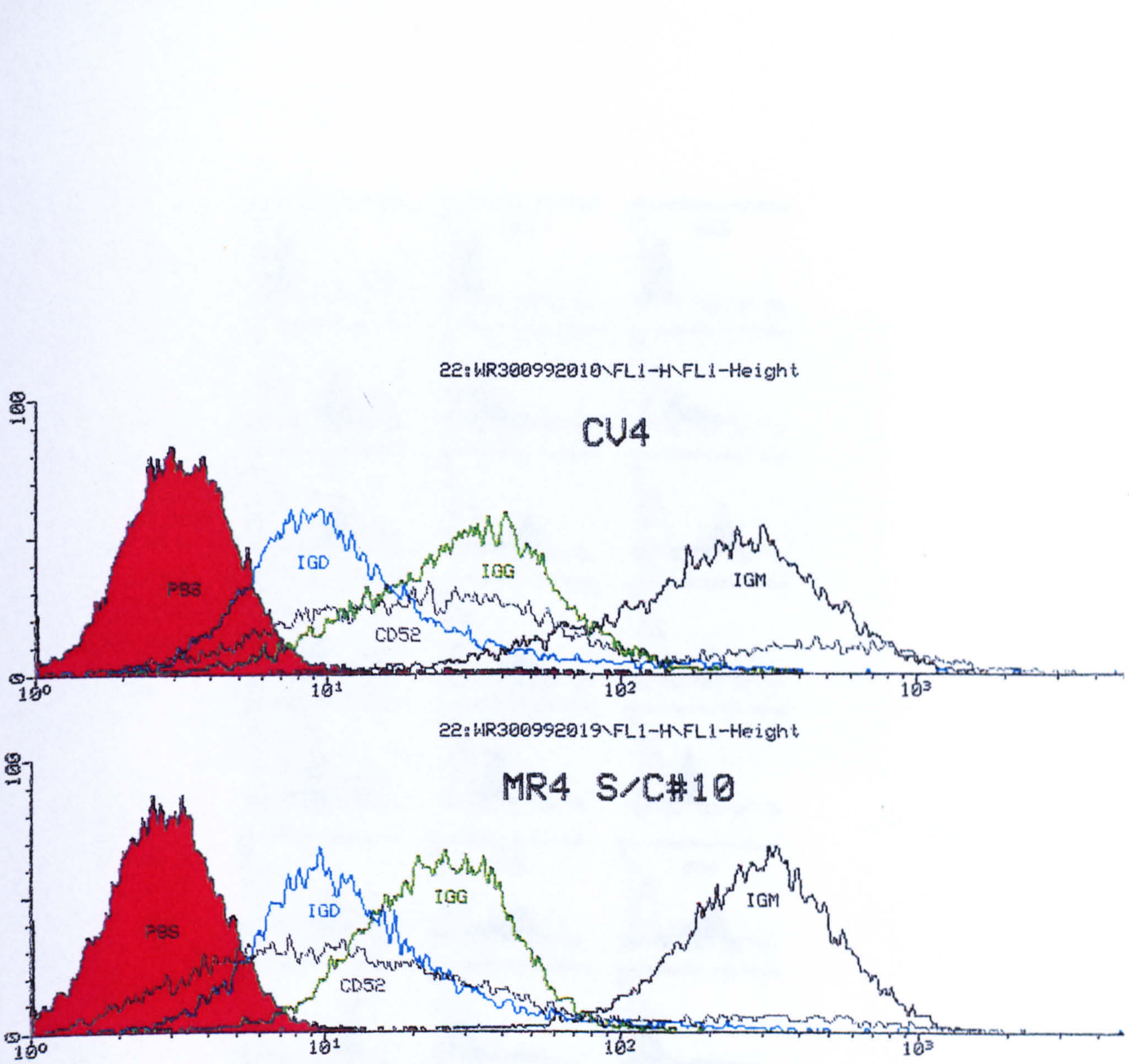


Figure 5.3 Cells resistant to killing by monocytes in the presence of CAMPATH-1H have a similar phenotype of CD52 expression as the CV clones.

Cells were stained using FITC-labelled CAMPATH-1H, IgG, IgD and IgM antibodies and analysed by FACScan using LYSIS II software.

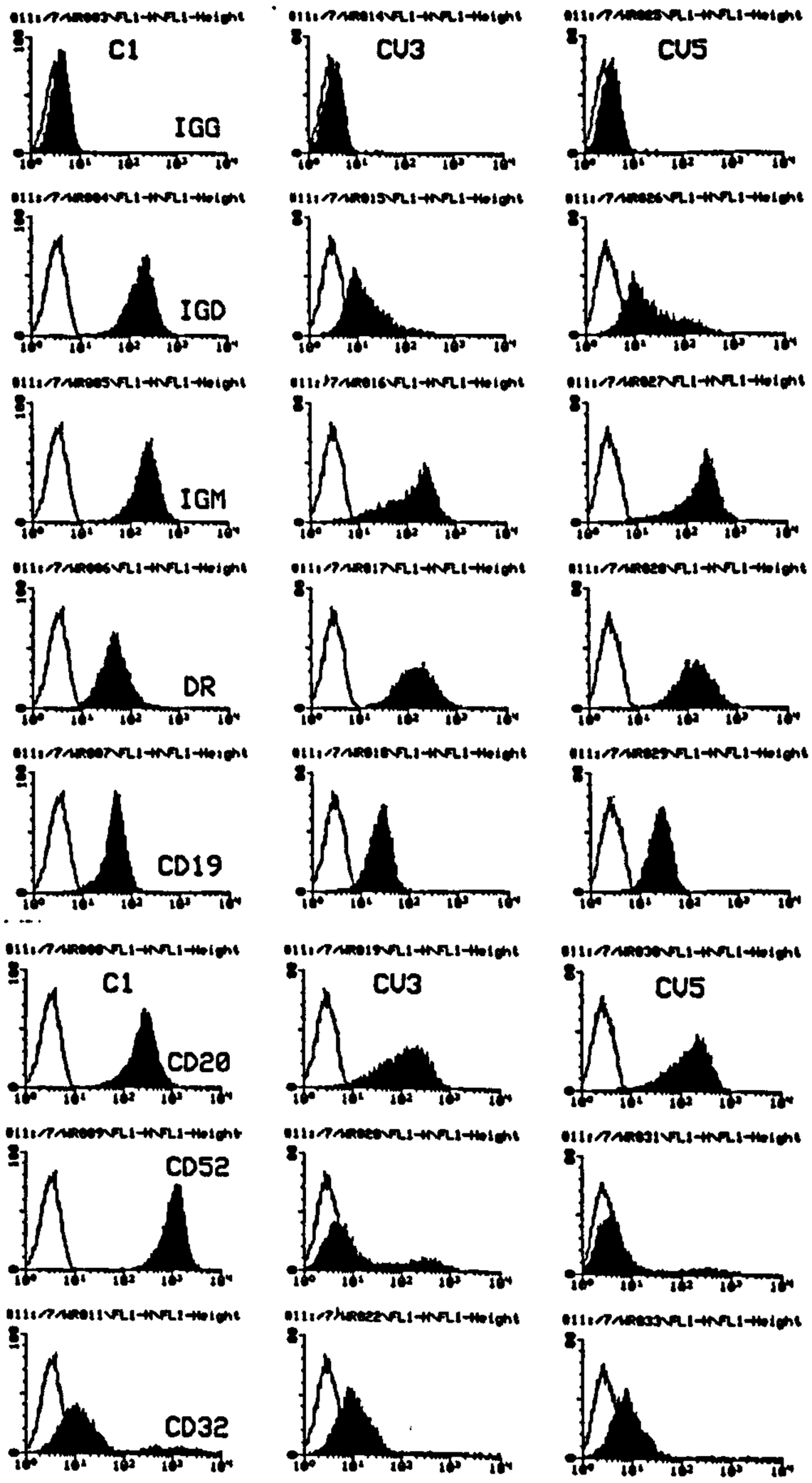


Figure 5.4 Phenotypic analysis of Wien 133 C1 cells following *in vitro* selection by crosslinking with anti-CD52 antibodies.

FACS analysis of parental (C1) and 2 clones derived from CD52 mediated selection (CV3 and CV5). Cells were stained with FITC-antibodies against surface IgG, IgD, IgM, DR, CD19, CD20, CD52, and CD32 (solid shaded area) and analysed by FACScan and LYSIS II software. Control staining with FITC labelled anti-mouse IgG1 is shown as unshaded area.

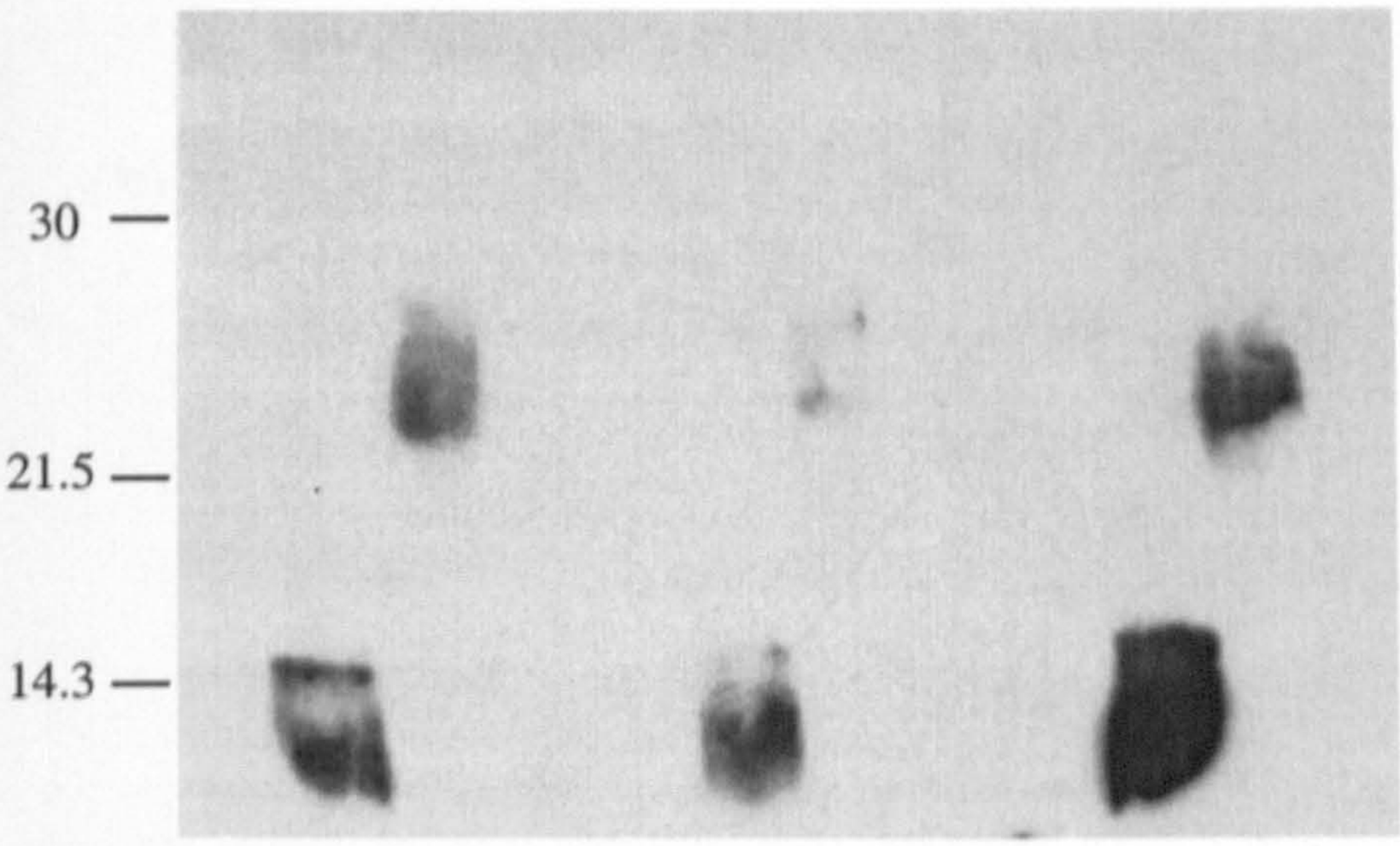


Figure 5.5 The level of CD52 antigen is decreased in cells resistant to CAMPATH-1H treatment.

CD52 antigen extracted from equivalent cell numbers of Wien 133 C1, CV3, CV4 and CV5 clones, was separated by 15% SDS PAGE, transferred to nitrocellulose and then visualised using the rat anti-CD52 specific CAMPATH-1G antibody and ECL detection reagents. Samples of antigen were treated in the presence and absence of N-Glycanase.

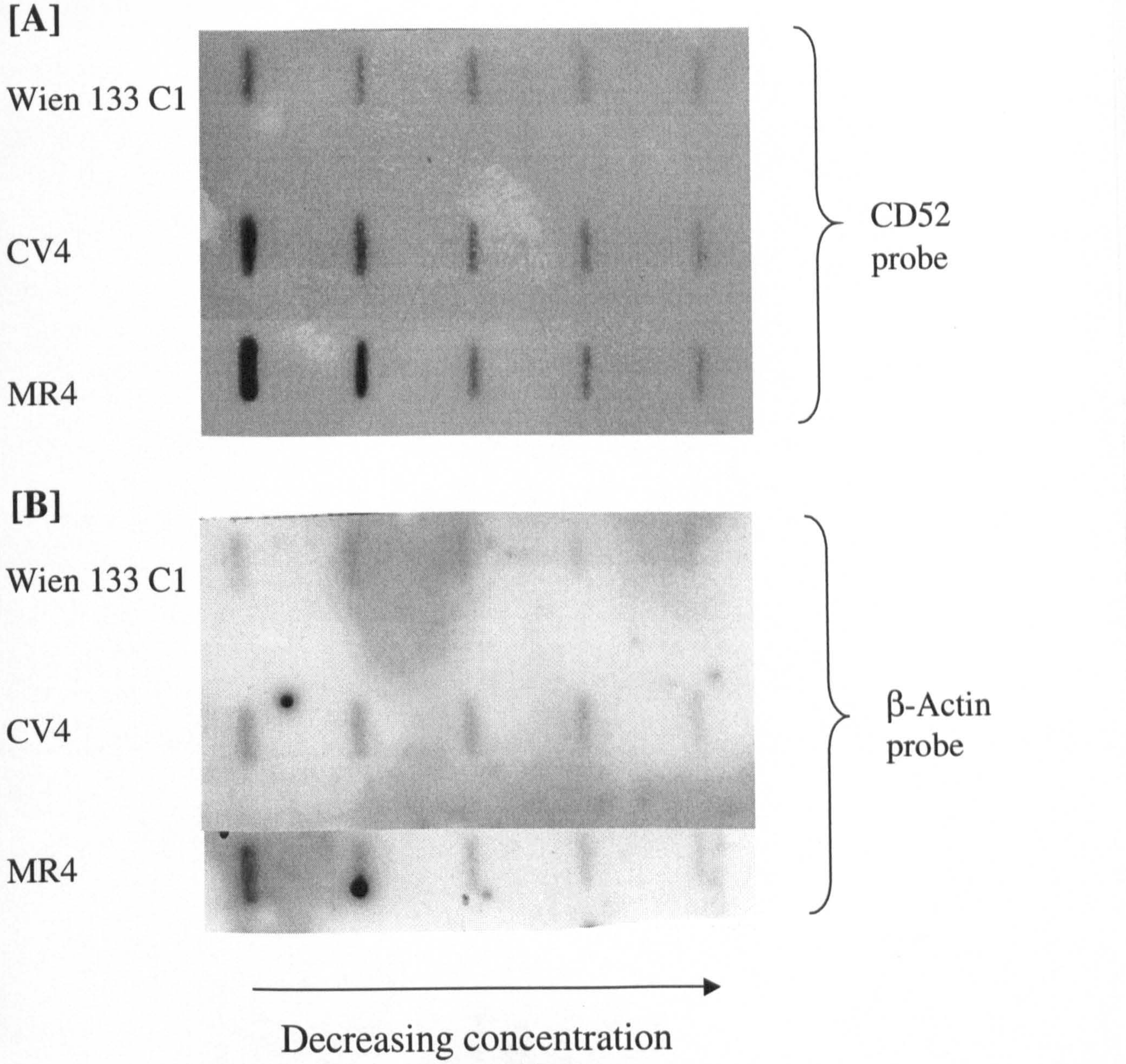


Figure 5.6 The messenger RNA levels for CD52 in CAMPATH-1H selected cells remain comparable to the Wien 133 parental cell line.

Total RNA was applied at doubling dilutions starting from 5 μg/ml to the wells in a slot blot manifold. Specific CD52 mRNA was detected using a ^{32}P labelled cDNA probe corresponding to the entire coding sequence for CD52 [A.]. To show that there was equal loading of RNA in each sample, filters were stripped and then reprobed with a ^{32}P labelled probe for β-actin [B].

Figure 5.7 CD55 and CD59 surface expression is reduced in the CAMPATH-1H selected cells comparable to reduced levels of surface CD52.

The Wien 133 parental, CV3, CV4 and CV5 cell lines were stained using FITC labelled antibodies for the expression of CD52, CD55 and CD59 and analysed by FACScan using LYSIS II software.

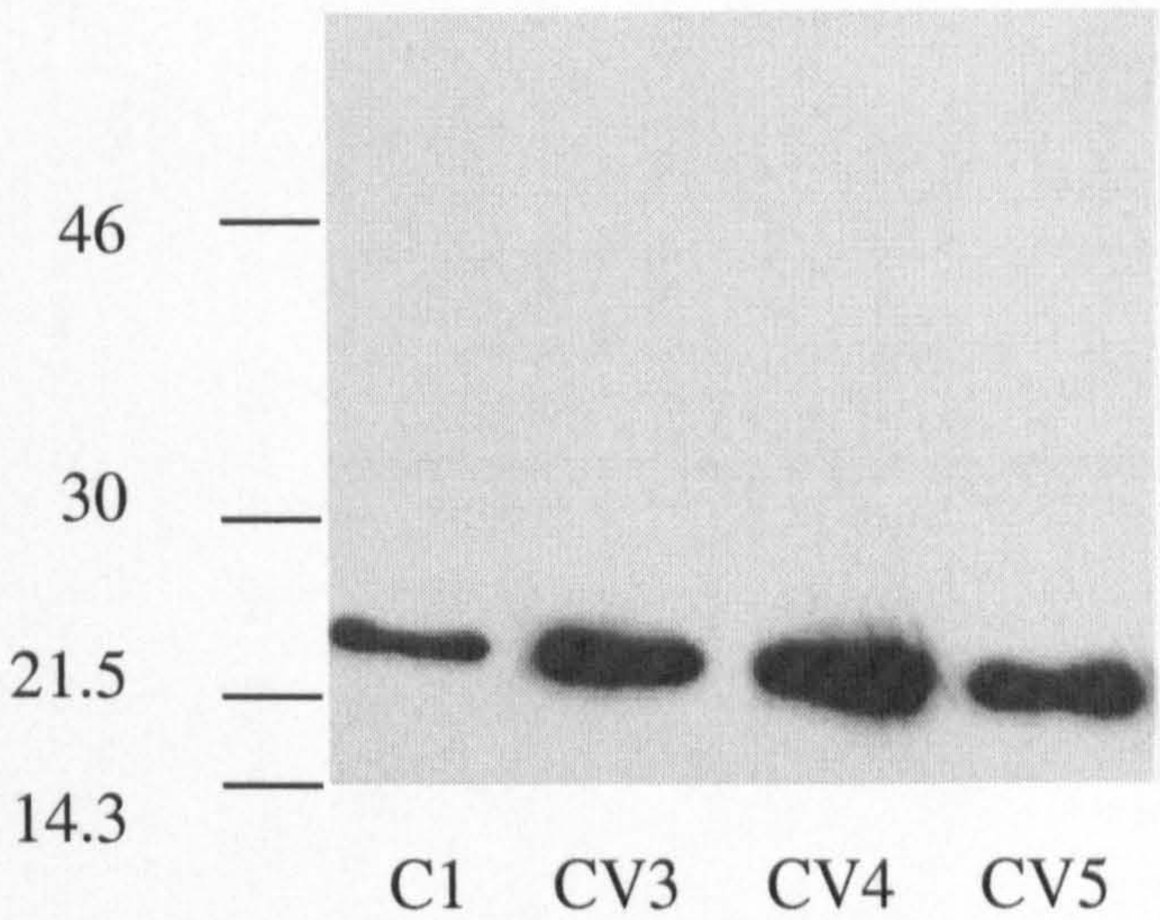


Figure 5.8 Western blot analysis of CD59 antigen reveals comparable levels in the Wien 133 parental cell line and the CV clones.

Total cell lysates were separated by 15% SDS PAGE, blotted onto nitrocellulose membrane and then probed with anti-CD59 antibody. CD59 antigen was visualised using the ECL detection system.

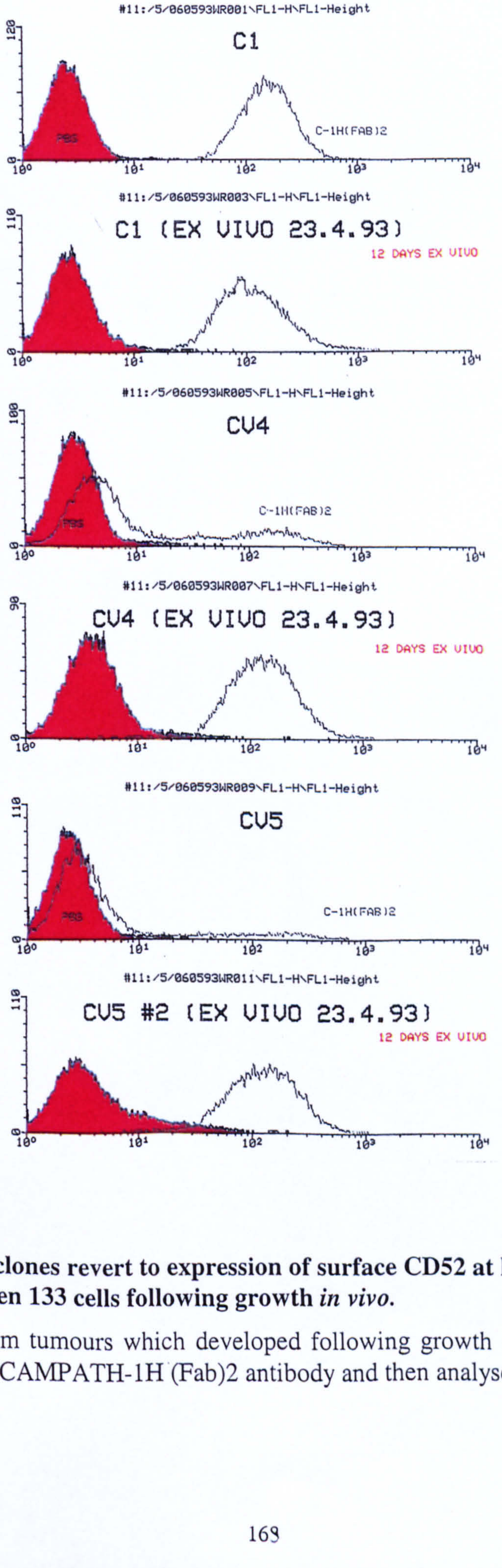


Figure 5.9 CV clones revert to expression of surface CD52 at levels comparable to the parental Wien 133 cells following growth *in vivo*.

Cells recovered from tumours which developed following growth in mice were stained with FITC labelled CAMPATH-1H (Fab)2 antibody and then analysed by FACScan using LYSIS II software.

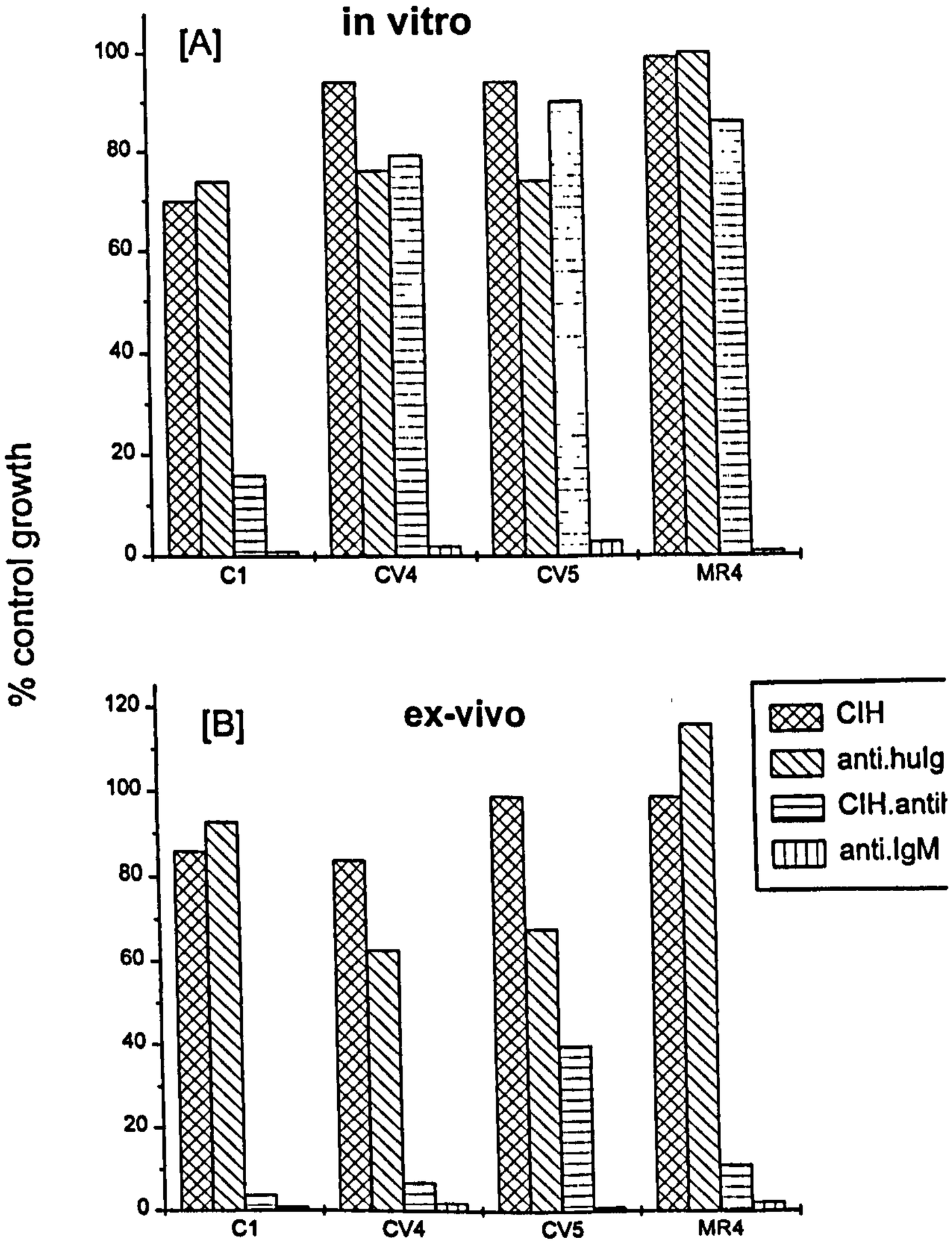


Figure 5.10 ***Ex vivo*** **CV clones are sensitive to growth inhibition in the presence of CAMPATH-1H.**

Cells (2 x 10^4/ well) were cultured for 5 days in the presence of medium alone, C-1H alone, anti-human IgG alone and C-1H + anti-human IgG. Antibodies were all added at a final concentration of 20 μg/ml. [^{3}H]-thymidine was added for the last 6 hours of the culture. Results are expressed as % control cell growth in the absence of antibodies.

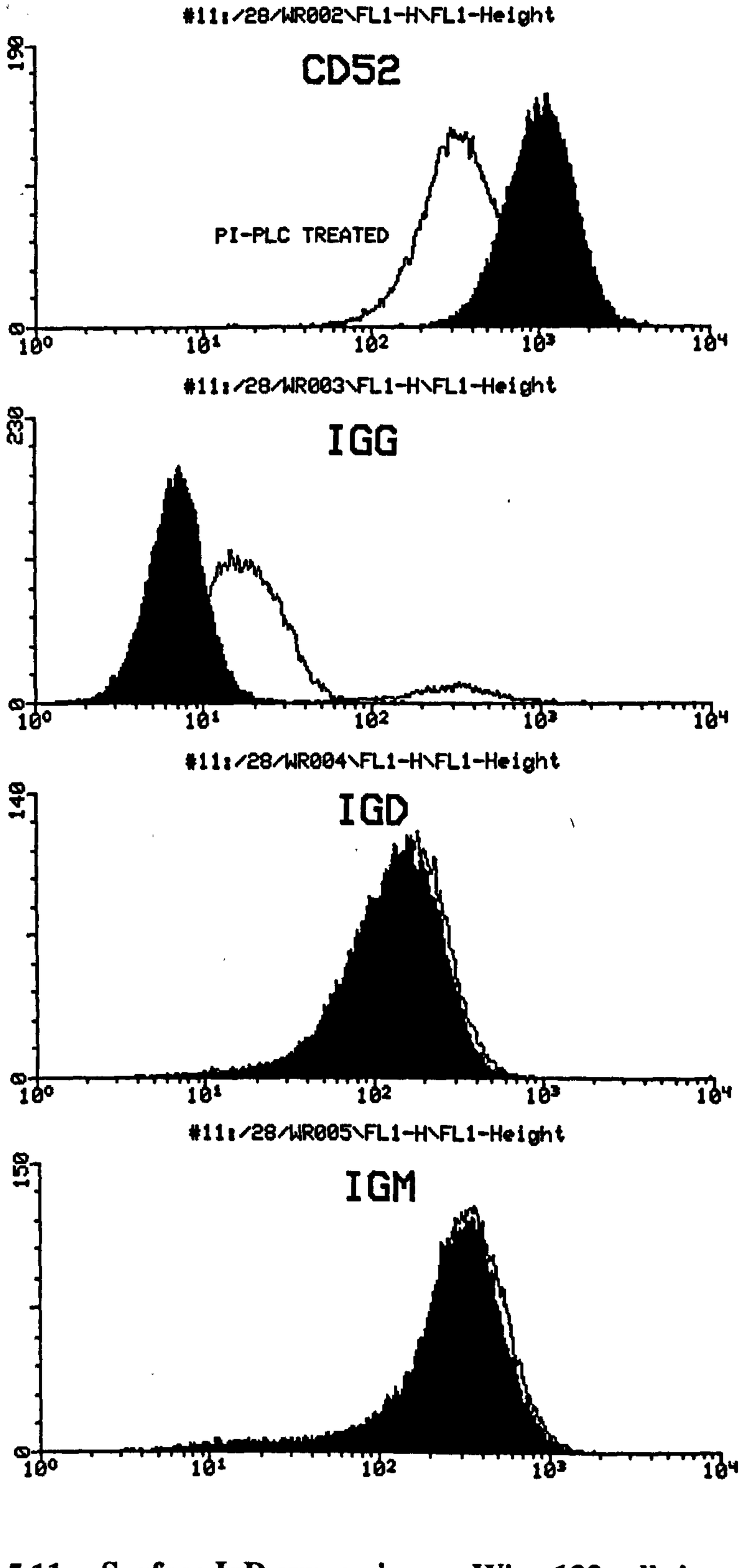


Figure 5.11 Surface IgD expression on Wien 133 cells is not sensitive to PI-PLC treatment.

Wien 133 C1 cells were incubated in the presence or absence of PI-PLC and then stained for expression of CD52, IgG, IgD and IgM using FITC labelled antibodies. Cells were analysed by FACScan using LYSIS II software.

5.3 Discussion

Attempts to generate CD52 negative cell lines by crosslinking CD52 on the surface of the Wien 133 cell line and selecting cells resistant to cell death, generated a panel of cell lines all bearing a bimodal pattern of CD52 expression. The majority of cells in the population being negative, with a small minority expressing levels of CD52 comparable to the parental cell line. Despite the fact that the cells were cloned by two rounds of limiting dilution, the obvious explanation for the phenotype of the CV clones is that they are not truly clonal. However, in a second experiment carried out in the laboratory where the parental Wien 133 cell line was subjected to monocyte killing in the presence of CAMPATH-1 antibody, cells surviving and subsequently cloned, displayed the same pattern of CD52 antigen expression as the CV clones. Errors in cloning technique were ruled out by three different experiments. In the first experiment, a CV cloned cell line was treated with FITC labelled spiked CAMPATH-1H, in the presence of complement, thus generating a CD52 negative population. After a few days back in culture, the cell population resumed its former bimodal pattern of CD52 expression. In a second experiment, CV cells were separated using a FACSort into CD52 high and CD52 low expressors. After a few days in culture the bimodal pattern of CD52 expression returned. In the third experiment, CV cells were separated into CD52 positive and CD52 negative cells using CAMPATH-1 antibody and magnetic beads, with the same outcome. The bimodal pattern of CD52 expression remained stable after months of maintaining the CV clones growing in *in vitro* culture.

The loss of expression of GPI-anchored CD55 and CD59 proteins, as well as CD52, in the CV clones suggests that there is a common defect present at some level between protein translation, assembly and transport of GPI-anchored proteins to the cell surface. ELISA and Western blotting indicated that there were lower levels of CD52 antigen in variant compared to parental cells. In contrast, the RNA message levels of CD52 appeared equivalent in all cell types. This suggests that there is a defect at some level between transcription and translation. However, in contrast to CD52, detection of CD59 protein by Western blotting in CV clone cell extracts, indicates that CD59 is present at equivalent levels to the parental Wien 133 cell line. This suggests that the defect is not due to a

failure in protein translation. The discrepancy between the detection of CD52 and CD59 proteins can be attributed to the antibodies used for detection. All antibodies available against CD52 require the presence of the GPI-anchor for detection (Hale, 1995).

In cases where a signal does not exist for transfer of the protein to a GPI-anchored protein, some proteins eg. LFA-3 and DAF, can exist as conventional transmembrane proteins (Navenot et al., 1996; Spicer et al., 1995). CD59 would appear to be totally dependent on the presence of a GPI-anchor for cell surface expression but as all antibodies available to the antigen require the presence of the intact GPI anchor for detection, it is not possible to conclude likewise for CD52.

The evidence discussed above suggests that the defect in surface expression of GPI-anchored proteins in the CV cell lines occurs at, or, during the synthesis and transfer of the GPI-tail onto the newly formed peptide. This conclusion is supported by the fact that the CV cell lines were selected by using an antibody specific for the CD52 antigen but in the selection process, surface expression of other GPI-anchored proteins, including CD55 and CD59 was lost as well.

CV clones were not sensitive to CAMPATH-1 induced growth inhibition/cell death, compared to the parental Wien 133 cell line. However, the CV clones still displayed the same sensitivity resulting in rapid cell death when treated with anti-IgM. This indicates that the defect was not due to defective signalling in the apoptotic pathway. This also indicates that CD52 is not an essential part of the B cell receptor signalling apparatus (likewise, CD59 can not have a significant role to play in B cell signalling) and sensitivity to growth inhibition/cell death in Wien 133 cells by CAMPATH-1 antibodies, is dependent on high levels of surface expression of CD52.

The high levels of CD52 expression in *ex vivo* CV cells comparable to the Wien 133 parental cell line, suggests that expression of CD52 and/or other GPI-anchored proteins may confer some advantage to survival *in vivo*. The expression of CD55 (DAF) and CD59 may be important as both have roles in protecting cells from the effects of autologous complement attack (Kirschfink, 1997). However, the emergence of CD52 negative T cells

from CAMPATH-1 treated patients suggest that the expression of CD52 and other GPI-anchored proteins is not essential for cell survival *in vivo,* at least in the same species. Studies by Brett et al., 1996a, showed that CD52 negative cells could still be detected in patients, two years after treatment with CAMPATH-1H. Studies looking at CD52 expression in monkeys found that lymphocytes from old world species (baboons and macaques) were recognised by all CAMPATH-1 antibodies tested, but those from five new world species tested were not. Erythrocytes from baboons and macaques were also positive. In cynomolgous and rhesus monkeys, it was found that erythrocytes could be positive or negative for CD52 expression (Hale et al., 1990), suggesting there could be some redundancy in the role/function of CD52.

There are two possible explanations for the observations that *ex vivo* CV cells expressed high levels of CD52 comparable to Wien 133 parental cells. The first explanation is that the *ex vivo* CV cells, could represent the outgrowth of a minority of high expressing CD52 cells. In support of this, there was an obvious difference in the rate of tumour take and the size of tumours recovered from mice implanted with CV cells, compared to those which received Wien 133 parental cells. The rate of tumour take being lower and the size of tumours recovered being smaller in the CV implanted compared to the Wien 133 implanted mice (Peter Topley, personal communication). Arguements against, include the ability of CV cells, treated in the presence of CAMPATH-1 antibodies and complement to deplete all high expressing CD52 cells, prior to implantation in mice, being able to establish tumours with high levels of CD52 expression. The second explanation, is that CD52 expression is regulated and that the defect in CD52 expression is reversible. This would explain why it was not possible to obtain totally CD52 negative cells. Taylor et al., 1997, found that the CV cells were deficient in levels of the precursors required for GPI-anchor biosynthesis. They followed up these observations, looking for mutations in the PIG-A gene, but failed to find any. This is in contrast to findings in PNH (Paroxymal nocturnal haemoglobinaemia) patients. PNH is characterised by abnormal activation of complement on erythrocytes due to a deficiency in GPI-anchored complement regulatory proteins including DAF and CD59; the abnormality is found only in haemopoietic cells and other GPI-anchored proteins are similarly affected (Rosse 1990). In all cases of PNH examined, the defect has been traced

to a mutation in the PIG-A gene, one of the key enzymes involved in GPI-anchor biosynthesis (Takeda et al., 1993; Miyata et al., 1994). The delay in tumour take and reduced size of tumours recovered from mice injected with CV cells, compared to parental Wien 133 cells, could reflect a time lag, during which expression of GPI-anchored proteins. A possible explanation is that as a result of selection, a key protein/proteins involved in regulation of transcription or translation of either CD52 itself, or of proteins involved in GPI anchor synthesis and assembly with nascent polypeptides destined for GPI anchorage, have become limiting. In any one cell, the expression of GPI-anchored CD52 would be restricted by whether, or not, low abundance regulatory proteins contacted their targets. A similar outcome might be expected if a mutation arose in a regulatory protein resulting in decreased efficiency in function. In support of this second explanation, Bessler et al., 1994, found that red blood cells from PNH patients were completely deficient (PNH III) or partially deficient (PNH II) in surface expression of GPI anchored proteins. Both PNH III and PNH II cells were shown to have somatic mutations in the PIG-A gene. However, the mutation in the PIG-A gene in PNH II cells produced a partial rather than a total loss of PIG-A gene function. Taylor et al., 1997, failed to find mutations in the PIG-A gene in both Wien 133 variant cells and negative T cell lines obtained from CAMPATH-1H treated patients. This suggests that the defect in GPI-anchored protein expression in Wien 133 variant cells was not due to mutations in PIG-A. Unlike the Wien 133 variant cells, the T cells were totally CD52 negative and remained so (Brett et al., 1996b). Such cells were observed *in vivo* for up to two years following CAMPATH-1H treatment. It is therefore, likely that the defect in T cells is different from the defect in the Wien 133 variant cells, which appears incomplete and reversible.

The results described have implications for the treatment of lymphoid malignancies as CD52 positive lymphoid cells may lose expression of CD52 and become insensitive to further treatment with CAMPATH-1 antibodies. A recent study by Ginaldi et al., 1998, showed that the levels of CD52 expression in normal and leukemic B and T cells correlated with *in vivo* therapeutic responses to CAMPATH-1H and it has previously been reported that not all B and T cell malignancies express surface CD52 (Dyer et al., 1990). Therefore, although I have been unable to find documented evidence that B cell or T cell negative malignant cells have arisen following CAMPATH-1 therapy, the possibility exists that such cells could arise as a result of antibody mediated selection pressure.

6. Biochemistry of signalling via the CD52 antigen

6.1 Introduction

In common with a number of GPI-anchored proteins expressed in lymphoid cells e.g. Thy-1, Ly-6, CD55 and CD59 (Reviewed by Robinson, 1991; Deckert et al., 1992), studies have shown that CAMPATH-1 antibodies directed against the CD52 antigen can induce T cell proliferation (Chapter 3). In contrast, ligation of the CD52 antigen on tumour cell lines resulted in a profound inhibition of cell growth, followed by apoptosis (Chapter 4). These results suggest that CD52 may play a role in cell signalling.

Studies showed that CAMPATH-1 antibody mediated growth inhibition/apoptosis in the CD52 transfected Jurkat T cell line, was dependent on the presence of a functional T cell receptor (Chapter 4). Likewise, IL-2 production induced by activating T cells via Thy-1 or Ly-6, has been shown to require the presence of a functional TCR complex (Gunter et al., 1987; Sussman et al., 1988).

The TCR complex consists of the variable αβ chains which combine to form the antigen recognition site. The TCR itself has no intrinsic signalling capacity. Following receptor ligation, signals are initiated by changes in phosphorylation of tyrosine residues present on the TCR associated invariable chains, namely, the γ, δ and ε chains, which together form the CD3 complex, and the TCR-zeta homodimer (Reviewed by Weiss and Littman, 1994). The key component of the TCR complex required for signalling through the Thy-1 or Ly-6 molecules, leading to IL-2 production, is the TCR-zeta chain (Wegener et al., 1992; Codias et al., 1992).

TCR ligation induces conformational changes leading to the activation of src family tyrosine kinases, lck and fyn. Within seconds, lck and fyn phosphorylate ITAMs (immunoreceptor tyrosine activation motifs) on the TCR invariable chains. Phosphorylated ITAMs provide docking sites for proteins, including the tyrosine kinase ZAP-70. ZAP-70 phosphorylates another adaptor protein, LAT. LAT enables

recruitment to the membrane and activation of the phospholipase PLC-γ (Zhang et al., 1998). PLC-γ converts the membrane component, phosphatidylinositol 4,5-biphosphate (PI 4,5 P2), into two second messengers, diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG remains attached to the inner side of the plasma membrane, from where it recruits and activates protein kinase C (PKC). IP_3 binds to fyn phosphorylated receptors on specialised parts of the endoplasmic reticulum, leading to the release of intracellular stores of calcium. Both PKC and calcium serve to activate a number of proteins (Reviewed by Cantrell, 1996; Reth and Wienands, 1997; Brdicka et al., 1998).

In B cells, the sequence of signalling events following receptor ligation follows a similar pattern to that seen in T cells, although differing in some of the key proximal signalling molecules. The B cell receptor (BCR) complex comprises surface immunoglobulin which is associated with the signal transducing disulphide linked Ig-α (CD79α) and Ig-β (CD79β) heterodimer. Src family kinases, including blk, fyn, lck and lyn, associate with the BCR in unstimulated cells (Cambier et al., 1994). Upon receptor ligation, one of these, or a combination, phosphorylates ITAM motifs on CD79α and CD79β. This leads to an increase in src kinase association and activity as well as enabling the tyrosine kinase, syk, to bind to the phosphorylated ITAMS. Syk phosphorylates both PLC-γ1 and PLC-γ2, the latter of the two predominating in B cells. In common with T cells, activation of small G binding proteins initiate MAP kinase cascades which in turn activate transcription factors controlling novel protein synthesis. Like the TCR, stimulation of the BCR yields alternative outcomes depending on the context of the primary signal. Thus coligation of CD19 positively regulates B cell activation, possibly via its association and activation with PI3-kinase (analogous to CD28 in T cells). B cell activation is negatively regulated by coligation of the surface receptor with CD22 and CD32 (FcRγII) (Reviewed by DeFranco, 1997).

In primary resting T cells, a signal through the TCR alone is insufficient to drive the cells to proliferate. Full cell activation, as measured by the ability of the cell to produce IL-2, requires further costimulatory signals. These can be provided by ligation of CD28 with

its cognate ligands on the APC, B7.1 or B7.2, or alternatively by antibodies directed to CD28 (Hutchcroft and Bierer, 1996). Ligation of CD28 leads to the recruitment and the activation of other lipid kinases, including phosphatidylinositol 3-kinase (PI-3K), which uses PI as a substrate to generate PI(3,4)P3 and PI(3,4,5)P3, and sphingomyelinase, a phospholipase, specific for sphingomyelin, which generates ceramides (Chan and Ochi, 1995).

Tyrosine kinase activity proximal to both CD3 and CD28 surface receptors leads to the recruitment of small GTP binding proteins (GTPases), including ras, rho and rac. These proteins are able to activate multiple effector pathways including MAP kinase cascades and culminate in the activation of transcription factors such as NFAT, Fos, Jun and AP-1. These are translocated to the nucleus where they bind to response elements, initiate transcription of proteins and drive T cell proliferation (Alberola Ila et al., 1997).

Numerous reports have shown that antibodies directed against GPI-anchored proteins, including Ly-6, Thy-1, CD24, CD48, CD55 and CD59, are also capable of activating signalling pathways, including changes in tyrosine phosphorylation and calcium fluxes (Stefanova and Horejsi, 1991). In lymphocytes, GPI anchored proteins cluster in detergent insoluble glycosphingolipid enriched domains, termed DIGS (Parton and Simons, 1995). Extraction and biochemical analysis of these clusters has shown that in addition to GPI-anchored proteins, these macromolecular complexes contain members of the src family tyrosine kinases, including lck and fyn. Following ligation of the GPI anchored proteins, there is an increase in associated tyrosine kinase activity (Stefanova et al., 1991; Stefanova and Horejsi, 1991; Cinek and Horejsi, 1992).

For some GPI-anchored proteins, eg. Qa-2, Ly-6 and DAF (CD55) antigens, studies have shown that by expressing these antigens with a conventional transmembrane region, they lose their ability to activate cells. This suggests that the GPI-anchor is essential for their function (Robinson et al., 1989; Su et al., 1991; Shenoy-Scaria et al., 1992). In addition, studies by Shenoy-Scaria, 1992, showed that the association of tyrosine kinase activity with DAF was dependent on the presence of the GPI anchor and, that if it was replaced

with a transmembrane domain, associated tyrosine kinase activity was no longer detected. However, this does not apply to all GPI-anchored proteins, as studies looking at signalling via CD73 demonstrated. Both a GPI-anchored, and a transmembrane, form of the protein were capable of inducing cell activation in Jurkat T cells (Resta et al, 1994).

The issue which remains unresolved, is how GPI-anchored proteins which are located on the outer leaflet of the cell membrane can activate kinases which are located in the cytoplasm. Therefore, for GPI-anchored proteins to be involved in signal transduction, there must be a mechanism (eg. adaptor protein/proteins, internalisation of GPI-anchored proteins) whereby signals received by ligand binding on the outside of the cell are transduced to the inside.

Investigations were therefore, initiated to see, firstly, whether ligation of CD52 could initiate changes in tyrosine phosphorylation as an indication that CD52 can mediate signalling; and, secondly, if so, was there any evidence of the presence of an adaptor protein via which intracellular signalling could be achieved.

6.2 Results

6.2.1 Effect of anti-CD52 antibodies on tyrosine phosphorylation in resting CD4+ T cells

In order to look at the effect of ligating CD52 on tyrosine phosphorylation, resting CD4+ T cells were purified from peripheral blood. Cells were incubated on ice with no antibody, CAMPATH-1G (CD52 specific) alone, anti-CD3 alone or anti-CD3 together with CAMPATH-1G, for 30 minutes. Excess primary antibody was removed by washing cells in PBS and then a secondary crosslinking anti-globulin antibody was added. Cells were transferred to a waterbath at 37°C and incubated for 5 minutes before washing once more in PBS. Cells were resuspended in PBS and then lysed by addition of reducing SDS PAGE sample loading buffer. Samples were analysed by SDS PAGE followed by western blotting. Specific tyrosine phosphorylation was observed using the 4G10 mouse monoclonal anti-phosphotyrosine antibody and ECL. Results are shown in figure 6.1.

Several proteins are rapidly phosphorylated following the addition of anti-CD3 antibody alone. This includes proteins at approximate molecular weights of 20, 36, 70, 120 and 150 kDa. Crosslinking the rat anti-CD3 with a secondary antibody further enhances the phosphorylation of the 36 kDa band. A similar sized protein to the 36 kDa protein is phosphorylated if CD52 alone is crosslinked with secondary antibody but is not present when CD52 is added in the absence of a secondary antibody. Coligation of CD3 and CD52 and crosslinking with a secondary antibody increases the phosphorylation of the 36 kDa band to a level higher than that seen with any of the previous treatments, suggesting that the addition of CAMPATH-1G is augmenting the signal produced by anti-CD3.

6.2.2 Coligation of CD52 and CD3 in resting CD4+ T cells leads to a phosphorylation pattern comparable to that seen when CD4 and CD3 are coligated.

The CD3 coreceptor, CD4, is associated non covalently via its cytoplasmic domain with the src family kinase lck (Glaichenhaus et al., 1991). Lck is also found in large protein complexes, which include GPI-anchored proteins (Stefanova et al., 1991). Therefore, it was of interest to determine whether coligating CD52 with CD3 resulted in a similar pattern of phosphorylated proteins when compared to that seen when CD4 and CD3 were coligated. The results indicated that crosslinking CD4 leads to a different pattern of phosphorylation compared to when CD52 or CD3 are crosslinked. In particular, crosslinking CD4 induces phosphorylation of a band at approximately 34 kDa compared to the 36 kDa band seen when CD52 or CD3 are crosslinked (figure 6.2). The main difference between CD52 and CD3 crosslinking is that the overall phosphorylation is much weaker in the case of CD52 compared to CD3. Co-crosslinking CD52 with CD3, or CD3 with CD4, produces a synergistic enhancement of the overall tyrosine phosphorylation. In particular, the band at 36 kDa appears to be phosphorylated to a greater extent with both CD3 and CD4 co-crosslinking and CD3 and CD52 co-crosslinking. In addition, the band at 20 kDa is weakly phosphorylated with anti-CD3 alone, but there is a strong synergy increasing the phosphorylation of this band with anti-CD3 and anti-CD4, and anti-CD3 and anti-CD52 combinations. Overall, the pattern of tyrosine phosphorylation seen with the two different paired combinations appears very similar.

6.2.3 CD52 crosslinking induces tyrosine phosphorylation in a T cell clone

The same experiment was performed using the T cell clone MF14. Results are shown in figure 6.3. As with CD4+ T cells purified from peripheral blood, several proteins showed enhanced phosphorylation after crosslinking with anti-CD3; these included proteins with molecular weights of approximately 36, 70, 80, 120 and 150 kDa. Ligation of CD52 alone had no effect on phosphorylation. However, crosslinking CD52 with a secondary antibody resulted in increasing phosphorylation of proteins at molecular weights of 70 and

120 kDa. Coligation of CD52 with CD3 together with a secondary crosslinking antibody produced a band at approximately 36 kDa. Ligation of CD4 alone without secondary crosslinking induces phosphorylation of several proteins, the intensity of which can be increased by further crosslinking. Co-crosslinking of both CD4 and CD52 with CD3 resulted in a synergistic increase in tyrosine phosphorylation of several different proteins.

6.2.4 Immunoprecipitation of phosphorylated proteins in T Cells following CAMPATH-1H activation

In order to try to identify individual proteins which become phosphorylated following CAMPATH-1 activation of CD52+ve CD4+ T cells, 4G10 anti-phosphotyrosine antibody was used to immunoprecipitate proteins. 5×10^6 CD4+ T cells cells were treated with CAMPATH-1G alone, CAMPATH-1G together with secondary anti-rat IgG, rat anti-CD3 together with anti-rat IgG, or anti-rat IgG alone; the primary antibody was incubated with the cells on ice for 30 minutes and excess antibody removed by washing the cells in PBS. Secondary crosslinking IgG was added and the cells transferred to 37°C for 5 minutes. Cells were then washed prior to lysis. Each lysate was mixed with 4G10 antibody-agarose suspension. Bound tyrosine phosphorylated proteins were visualised by Western blot analysis and detection with the 4G10 anti-phosphotyrosine antibody. Results are shown in figure 6.4. CAMPATH-1G alone does not induce any protein phosphorylation above that seen in cells treated with anti-rat IgG alone; CAMPATH-1G crosslinked with anti-rat IgG and anti-CD3 together with anti-rat IgG give a similar pattern of protein phosphorylation, including bands at 96-200 kDa, 70-96 kDa and 46-69 kDa; a band at approximately 36 kDa appears in both tracks but is stronger when anti-CD3 is crosslinked compared to CAMPATH-1G.

The 4G10 immunoprecipitates were probed unsuccessfully with antisera directed against ZAP-70, lck and PI-3 kinase. It was therefore not possible to positively identify any of the phosphorylated species which appeared following co-crosslinking of CD52 and CD3.

6.2.5 Effect of herbimycin A on signalling in CD4+ T cells

Crosslinking the CD52 antigen has been shown to induce changes in tyrosine phosphorylation. Experiments were, therefore, carried out to determine whether signalling via the CD52 antigen can be blocked by the tyrosine kinase inhibitor, Herbimycin A. CD4+ T cells purified from peripheral blood were incubated in the presence or absence of Herbimycin A for 24 hours before addition of antibodies. The effects of treatment with the drug were investigated in both T cell proliferation assays and on protein phosphorylation.

The effect of Herbimycin A on cell proliferation, after 5 days culture, following activation in the presence of CAMPATH-1H antibody together with secondary crosslinking antibody and PMA is shown in figure 6.5. The growth of cells is totally inhibited by pretreatment of cells with Herbimycin A at 0.3 μM. However, 0.3 μM Herbimycin A significantly reduces but does not completely inhibit CD3 and CD52 induced tyrosine phosphorylation (figure 6.6). In the presence of Herbimycin A, the basal phosphorylation pattern of CD4+ T cells is reduced. In particular, the phosphorylation of the upper two bands of a triplet around 55 kDa, as well as proteins between 69-96 kDa.

6.2.6 Ligation of CD52 does not induce a calcium flux.

Ligation of GPI-anchored CD59 on human neutrophils (Morgan et al., 1993) and Thy-1 on murine T cells (Gunter et al., 1986) have been shown to induce the release of calcium from intracellular stores. Calcium is required for activation of a number of proteins including the calcium/calmodulin dependent calcineurin phosphatase, the target of cyclosporin A, and downstream transcription factors. In chapter 3, Cyclosporin A was shown to inhibit CD4 + T cell proliferation induced in the presence of CAMPATH-1 antibodies. In chapter 4, Cyclosporin A was shown to partially inhibit CAMPATH-1 antibody induced growth inhibition in the Wien 133 B cell line, although the data in this case, suggests that the drug is blocking a growth inhibition effect mediated by the secondary crosslinking antibody and the contribution of CD52 in the growth inhibition

signal is independent of calcineurin. The ability of CD52 to mediate a calcium flux was, therefore, investigated.

Purified CD4+ T cells were loaded with calcium sensitive dyes and then incubated for 1 minute in the presence of secondary crosslinking antibody. Results shown in figure 6.7, indicate that the crosslinking antibody alone had no effect on calcium release. After one minute, addition of anti-CD3 induced a rapid rise in the level of intracellular calcium. In contrast, addition of CAMPATH-1H alone in the presence of secondary crosslinking antibody had no effect on calcium release. Like CAMPATH-1H, neither anti-CD4 antibody alone, nor anti-CD28 alone, in the presence of a secondary crosslinking antibody, mobilised calcium from intracellular stores (Data not shown). However, in combination with anti-CD3, anti-CD4 synergised to give an overall higher level of calcium release. CAMPATH-1H added together with anti-CD3 reduced the rise in calcium compared to that seen with anti-CD3 alone. The reduced rise in calcium seen with anti-CD3 in the presence of CAMPATH-1H antibody, required co-ligation. Crosslinking CD3 with a secondary antibody which did not crossreact with the humanised anti-CD52 antibody, resulted in the same profile of calcium release seen when CD3 alone was crosslinked. The overall profiles of calcium release induced by ligation of CD3 alone, CD4/CD3 and CD52/CD3 were similar in that all showed a very rapid initial calcium flux which equally rapidly disappeared. In contrast, CD3/CD28 resulted in a much slower rise in the initial calcium signal but this was sustained over the ten minute time period looked at.

6.2.7 CD52 ligation induces phosphorylation in the Wien 133 B cell lymphoma cell line

In previous chapters, experiments have been described where CAMPATH-1 ligation of CD52 on the B cell lymphoma cell line, Wien 133 induces apoptosis. The same effect can be triggered by ligation of IgM, the B cell surface receptor (BCR), which induces rapid cell death. Anti-IgM induced apoptosis in B cell lines has been shown to be accompanied by an increase in protein tyrosine phosphorylation (Igarishi et al., 1994;

Beckwith et al., 1991; Mizuguchi et al., 1992). Therefore, having demonstrated in chapter 4, that ligation of CD52 can induce apoptosis, it was of interest to determine whether CD52 ligation on the Wien 133 cell line, lead to an increase in protein phosphorylation. The effect of IgM or CD52 ligation in the Wien 133 B cell line, expressing high levels of CD52 antigen was compared to the CV5 cell line in which 90 per cent of the population is CD52 negative and only 10 per cent of the population expresses the CD52 antigen at a level comparable to the Wien 133 cell line. Results are shown in figure 6.8.

There is an obvious difference between the basal levels of tyrosine phosphorylation in the two cell lines. In the Wien 133 cell line, there is a predominant doublet at around 55 kDa as well as several bands of higher molecular weight. In the CV5 cell line, the same pattern of basal phosphorylation is apparent but at a much lower level. In addition, in both the Wien 133 and the CV5 cell lines, treatment with anti-IgM induces a high level of phosphorylation on multiple protein bands. In contrast, ligating CD52, in the presence of secondary crosslinking antibody, induces weak phosphorylation of multiple bands in the Wien 133 and CV5 cell lines. There is a much smaller increase in the protein phosphorylation in the CV5 cell line following CD52 crosslinking and this probably reflects the fact that only 10% of the cells in this population are CD52 positive. IgM induced phosphorylation in the CV5 cell line is also reduced compared to the Wien 133 cell line.

6.2.8 Immunoprecipitation of CD52 antigen and associated proteins from Wien 133 cells

In order to look for a possible adaptor protein associated with CD52, the Wien 133 B cell line was chosen. This is because in addition to the fact that the Wien 133 cell line expresses the CD52 antigen at a high density, high cell numbers could be obtained.

Wien 133 cells (1×10^8) were metabolically labelled by culturing them overnight in the presence of medium containing ^{35}S-Methionine and ^{35}S-cysteine (ProMix). Cells were lysed either in the presence of NP40 or N-octyl glucoside detergent, insoluble material

was removed by microcentrifugation and then proteins in the lysates were immunoprecipitated with CAMPATH-1H (Fab)2 immobilised on sepharose beads or anti-CD4 (Fab)2 immobilised on sepharose beads as a negative control (Wien 133 cells are negative for CD4 cell surface expression) or sepharose beads alone. The beads were washed extensively to remove nonspecifically bound proteins and then SDS PAGE sample loading buffer was added. Proteins were separated by SDS PAGE electrophoresis and then visualised by autoradiography. The results are shown in figure 6.9. The CAMPATH-1H immunoprecipitation enriched for bands at approximate molecular weights of 70, 96 and 110 kDa. In repeated experiments, the 110 kDa band was consistently coprecipitated with the CD52 antigen whereas the 70 kDa band was not always present. The same bands were visible in both NP40 and N-octyl glucoside lysed samples indicating that there was no difference between the two detergents in terms of efficiency of isolating CD52 associated proteins. However, in contrast, the presence of the CD52 antigen following immunoprecipitation with CAMPATH-1H antibody, as demonstrated by Western blot analysis, was clearly detected in CAMPATH-1H immunoprecipitations of N-octyl glucoside lysed cells but only a faint signal was detected in immunoprecipitations of NP40 lysed cells. No band corresponding to CD52 was detected in anti-CD4 immunoprecipitates of cell lysates indicating that under the conditions used specificity had been achieved (Figure 6.10). It should be noted that the high molecular weight proteins detected in CAMPATH-1 immunoprecipitations could represent proteins bearing similar epitopes to CD52, rather than associated proteins.

6.2.9 Western blot detection of lyn in Wien 133 cells

Studies in T cells by Stefanova et al., 1991, have shown that GPI-anchored proteins are found together with the src tyrosine kinases, lck and fyn, in large detergent resistant complexes and have suggested that the association has implications in GPI-anchored protein cell signalling. In B cells, the predominant src family tyrosine kinase is lyn. Therefore, western blots of immunoprecipitates of CD52 and associated proteins were probed with anti-lyn to look for the specific presence of the kinase. Results are shown in figure 6.11. A doublet at between 55-58 kDa is visible in both N-octyl glucoside and NP40 detergent total cell lysates, but this band is not enriched for following CAMPATH-1H antibody immunoprecipitation suggesting that at least prior to activation, CD52 and

lyn are not closely associated. The fainter 70 kDa band appearing on the blots in CAMPATH-1H immunoprecipitates, following detection with anti-lyn antisera which is not visible in the total cell lysate material may be due to a cross reactive protein enriched for by the CD52 immunoprecipitation.

6.2.10 Protein sequence analysis of CD52 associated proteins

Attempts were made to identify the CD52 associated proteins by protein sequence analysis. Cell numbers were increased and lysed in the presence of N-octyl glucoside. Following CAMPATH-1H $(Fab)_2$ immunoprecipitation, proteins were separated by SDS PAGE, transferred to PVDF Problot membrane, stained with Coomassie and excised for protein sequencing. No protein sequence data for the CD52 associated protein was obtained.

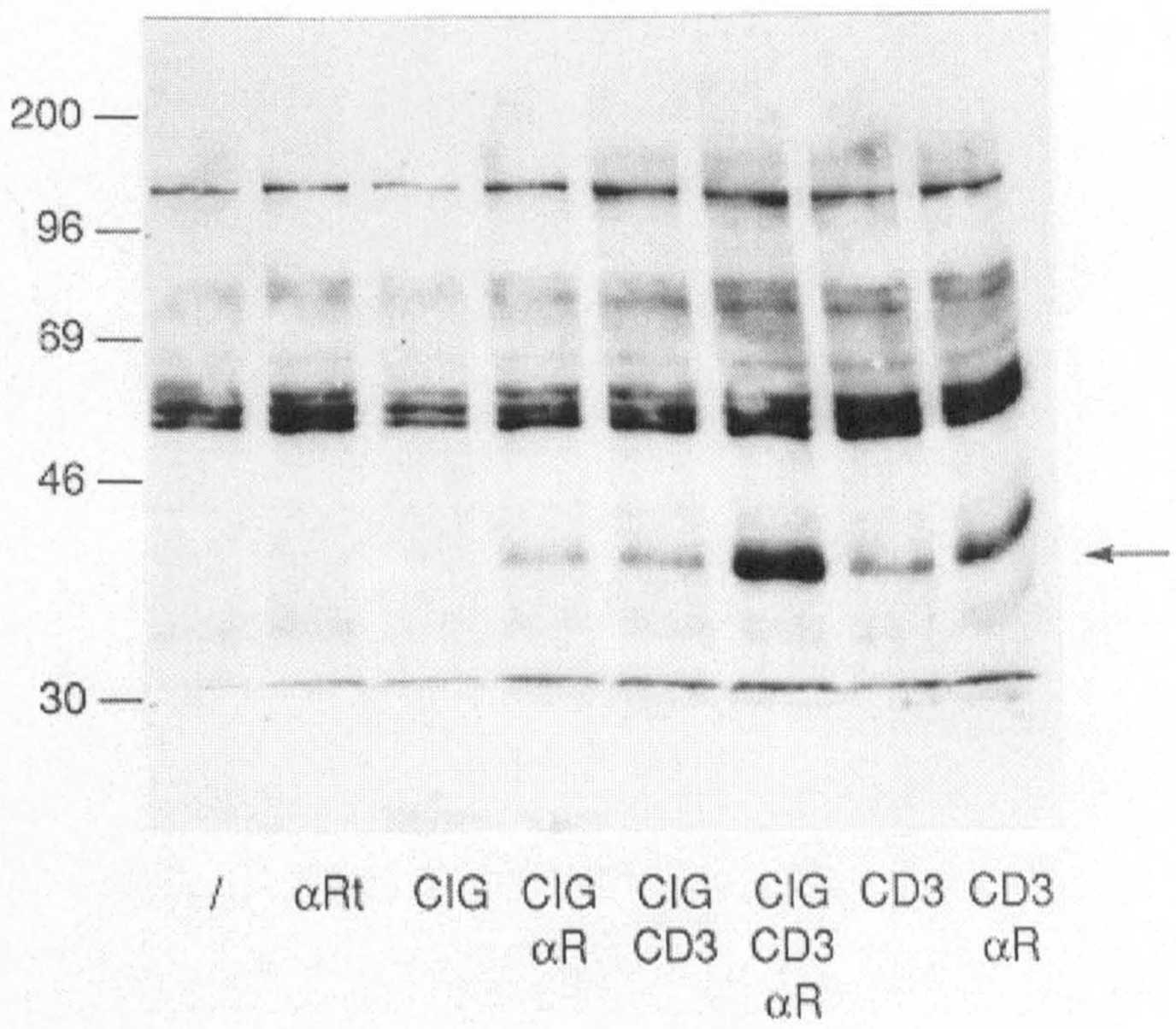


Figure 6 1 CD52 ligation induces tyrosine phosphorylation in CD4+ T cells.

Resting CD4+ T cells (1 x 10^6/20 µl) purified from peripheral blood were incubated on ice for 30 minutes, in the presence of no antibody (/), CAMPATH-1G (C-1G), rat anti-CD3 (CD3), or C-1G together with anti-CD3. Excess antibody was removed by washing and cells were then resuspended +/- anti-rat IgG (αR) in 20 µl volume and incubated at 37°C for 5 minutes. All antibodies were added at a final concentration of 20 µg/ml. The reaction was stopped by the addition of 20 µl SDS PAGE sample loading buffer. The equivalent of 5 x 10^5 cells (=20 µl volume) was loaded per track onto a 7.5% SDS PAGE gel. Following transfer to nitrocellulose membrane, tyrosine phosphorylated proteins were visualised using 4G10 anti-phosphotyrosine antibody and ECL detection.

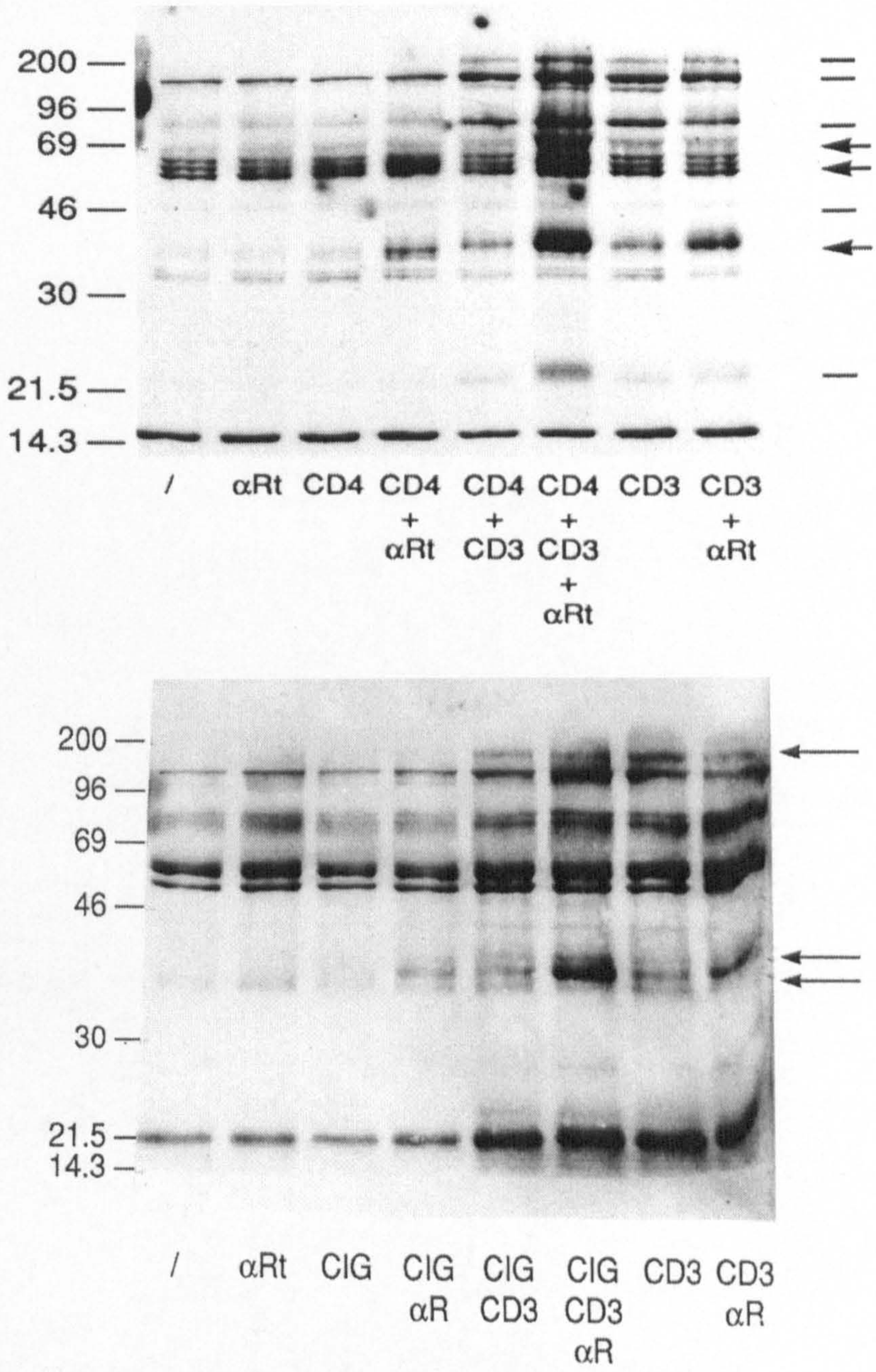


Figure 6.2 The pattern of CD52 and CD4 induced tyrosine phosphorylation is comparable.

Resting CD4+ T cells (1 x 10^6/20 µl) purified from peripheral blood were incubated on ice for 30 minutes, in the presence of no antibody (/), CAMPATH-1G (C-1G), rat anti-CD3 (CD3), rat anti-CD4 (CD4), C-1G together with anti-CD3, or anti-CD3 together with anti-CD4. Excess antibody was removed by washing and cells were then resuspended +/- anti-rat IgG (αR) in 20 µl volume and incubated at 37°C for 5 minutes. All antibodies were added at a final concentration of 20 µg/ml. The reaction was stopped by the addition of 20 µl SDS PAGE sample loading buffer. The equivalent of 5 x 10^5 cells (= 20 µl volume) was loaded per track onto a 10% SDS PAGE gel. Following transfer to nitrocellulose membrane, tyrosine phosphorylated proteins were visualised using 4G10 anti-phosphotyrosine antibody and ECL detection.

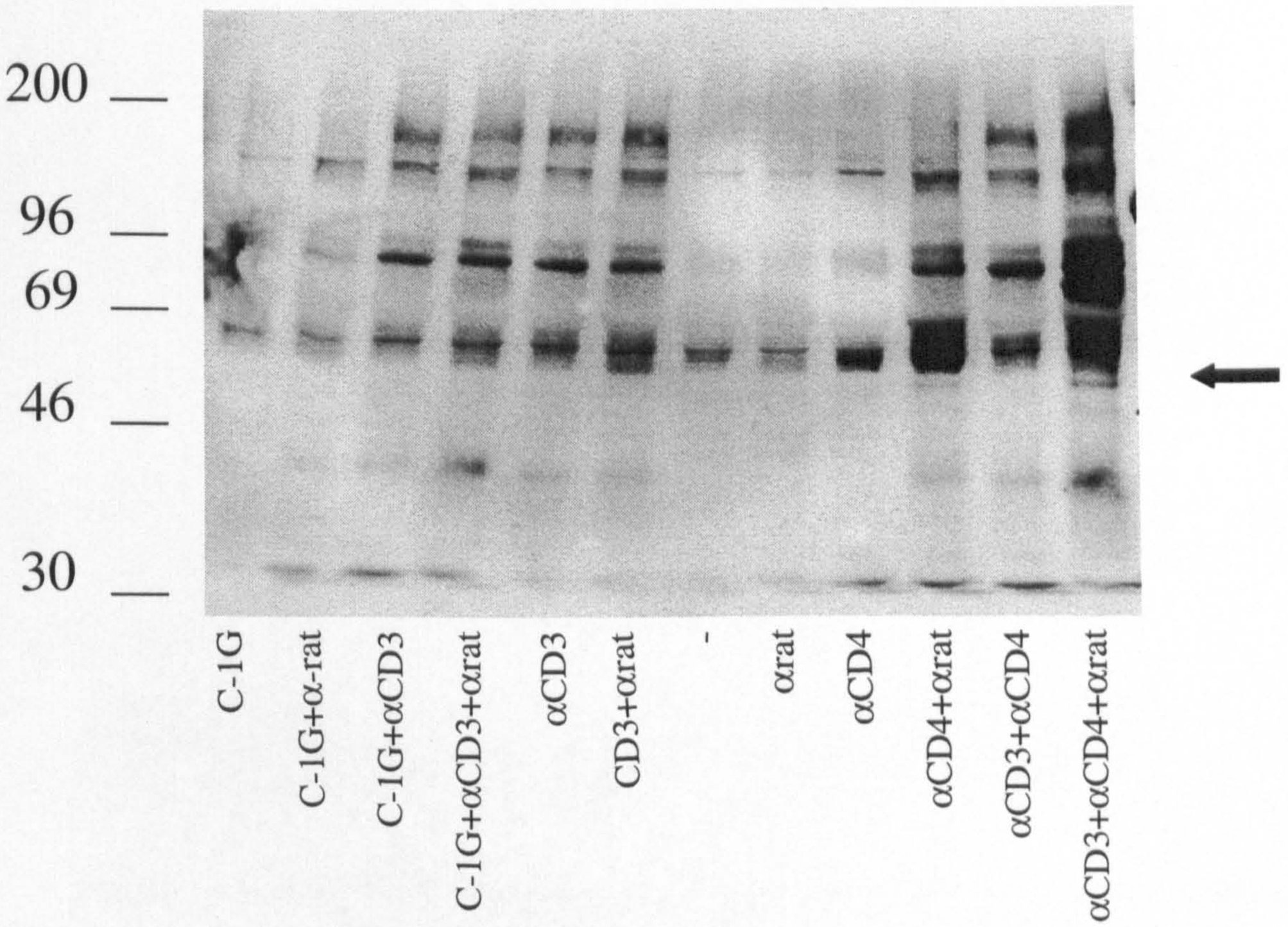


Figure 6.3 CD52 ligation induces tyrosine phosphorylation in the MF14 T cell clone.

T cell clone MF14 cells, were incubated on ice for 30 minutes, in the presence of no antibody, CAMPATH-1G (C-1G), rat anti-CD3 (αCD3), rat anti-CD4 (αCD4), C-1G together with anti-CD3, or anti-CD3 together with anti-CD4. Excess antibody was removed by washing and cells were then resuspended +/- anti-rat IgG (αrat) in 20 µl volume and incubated at 37°C for 5 minutes. All antibodies were added at a final concentration of 20 µg/ml. The reaction was stopped by the addition of 20 µl SDS PAGE sample loading buffer. The equivalent of 5 x 10^5 cells (=20 µl volume) was loaded per track onto a 7.5 % SDS PAGE gel. Following transfer to nitrocellulose membrane, tyrosine phosphorylated proteins were visualised using 4G10 anti-phosphotyrosine antibody and ECL detection.

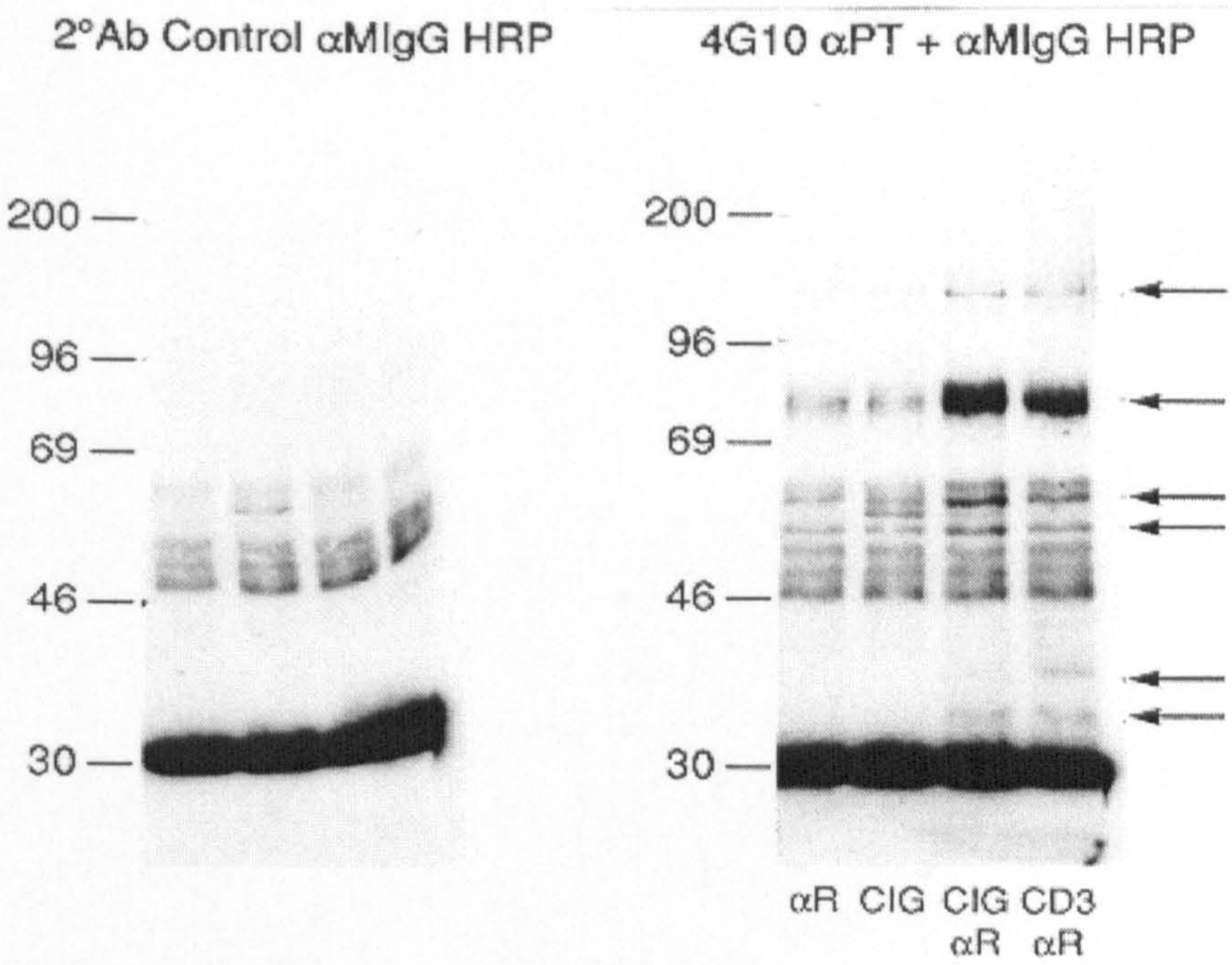


Figure 6.4 Immunoprecipitation of tyrosine phosphorylated proteins from CD52 activated cells differs from CD3 activated cells.

CD4+ T cells (5 x 10^6/immunoprecipitation) purified from peripheral blood were incubated for 30 minutes on ice with no primary antibody, CAMPATH-1G (C-1G), or rat anti-CD3 (CD3). Cells were washed in PBS and resuspended in 20 μl volume in the presence or absence of anti-rat IgG (αR), as indicated on the figure. All antibodies were added at a final concentration of 20 μg/ml. Cells were incubated at 37°C for 5 minutes and then lysed in 1% N-octyl glucoside lysis buffer. The lysates were incubated with 4G10 anti-phosphotyrosine antibody coupled to agarose. Bound proteins were eluted by addition of SDS PAGE sample loading buffer and separated by 7.5 % SDS PAGE gel. Following transfer to nitrocellulose, tyrosine phosphorylated proteins were visualised by using 4G10 anti-phosphotyrosine antibody and ECL detection.

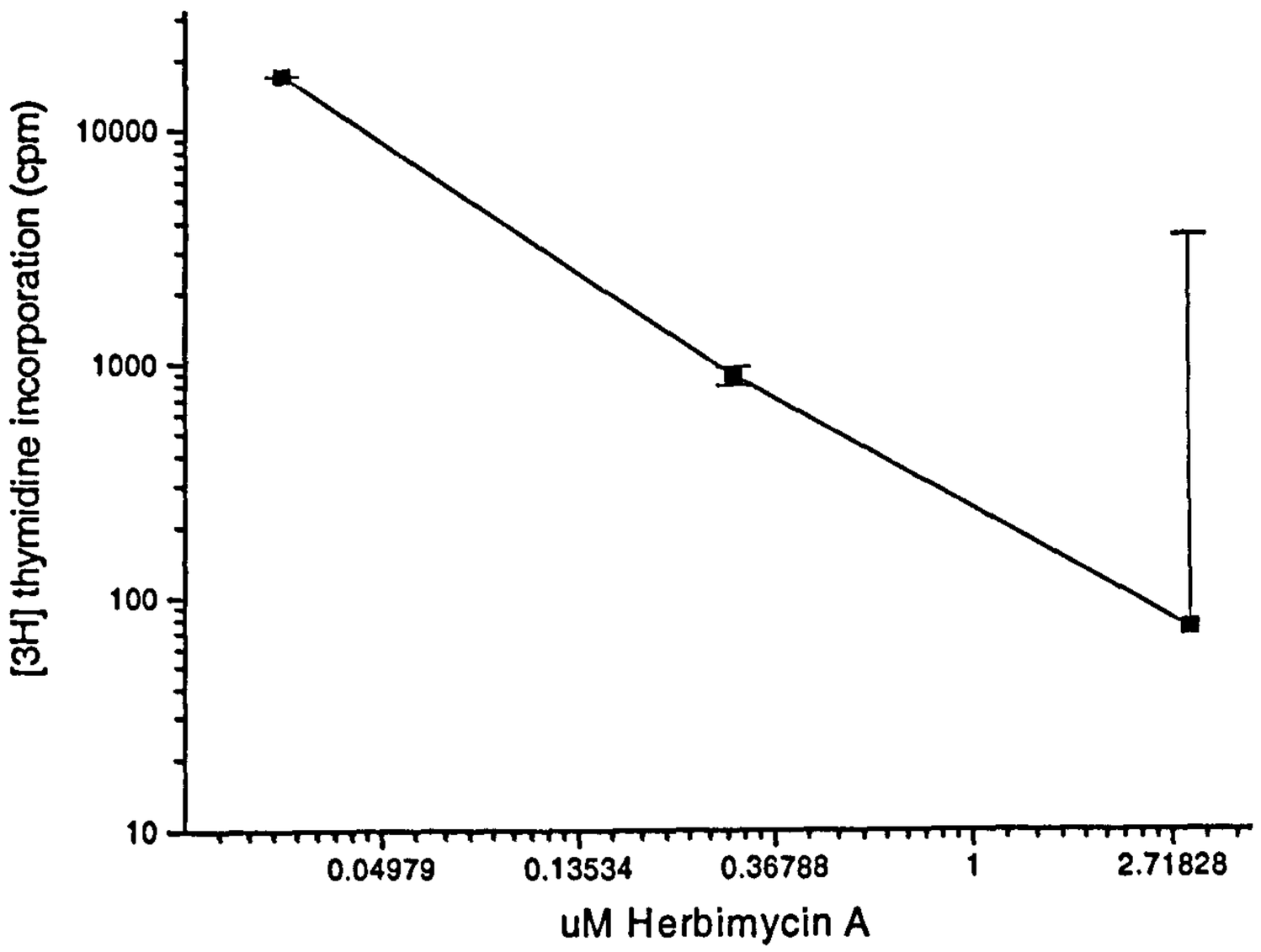


Figure 6.5 Herbimycin A partially inhibits CD52 induced CD4+T cell proliferation.

CD4+ T cells (1 x 10^5/well) purifed from peripheral blood, pretreated for 24 hours +/- Herbimycin A at a range of concentrations from 0 - 3 mM, were cultured for 5 days in the presence of CAMPATH-1H together with anti-human IgG and PMA. Antibodies were added at a final concentration of 20 μg/ml. PMA was added at a final concentration of 10 ng/ml. During the last 18 hours of culture, [^{3}H]-thymidine was added to the wells. Results are expressed as percentage of control growth, in the absence of drug.

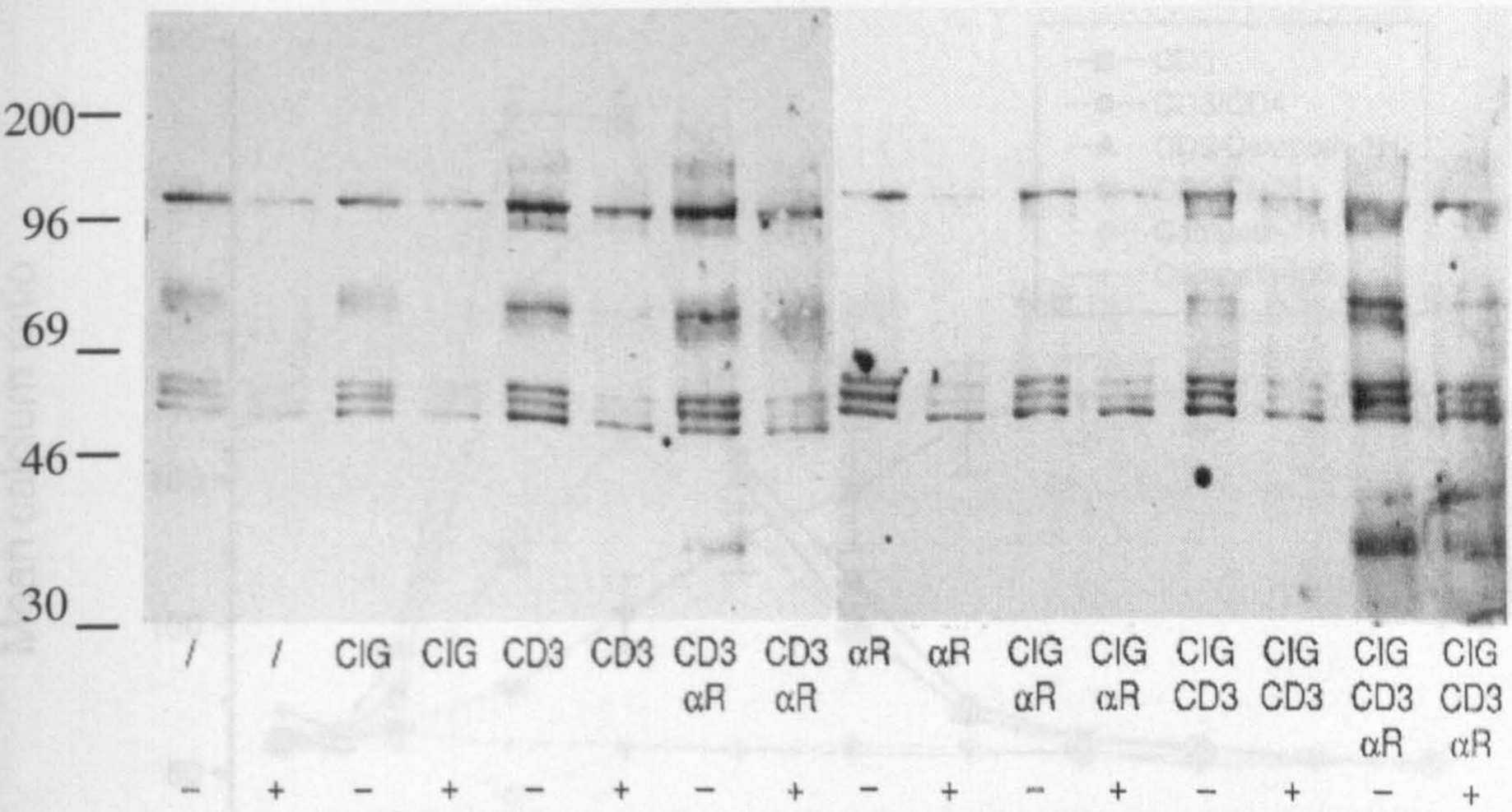


Figure 6.6 Herbimycin A partially inhibits CD52 induced tyrosine phosphorylation in CD4 + T cells.

CD4 + T cells were incubated in the presence or absence (+/-) of Herbimycin A at 0.3 μM final concentration for 24 hours. Cells were then incubated with no antibody (/), CAMPATH-1G (C-1G), rat anti-CD3 (CD3) or C-1G together with anti-CD3 on ice for 30 minutes. Excess antibody was removed by washing cells in PBS. Cells were resuspended in the presence or absence of anti-rat IgG (αR). All antibodies were added at a final concentration of 20 μg/ml. Cells were incubated at 37°C for 5 minutes. The reaction was stopped by the addition of 20 μl SDS PAGE sample loading buffer. The equivalent of 5 x 10^5 cells was loaded per track onto a 7.5% SDS PAGE gel. Following transfer to nitrocellulose membrane, tyrosine phosphorylated proteins were visualised using 4G10 anti-phosphotyrosine antibody and ECL detection.

Calcium ratios of antibody stimulated human CD4+ cells.

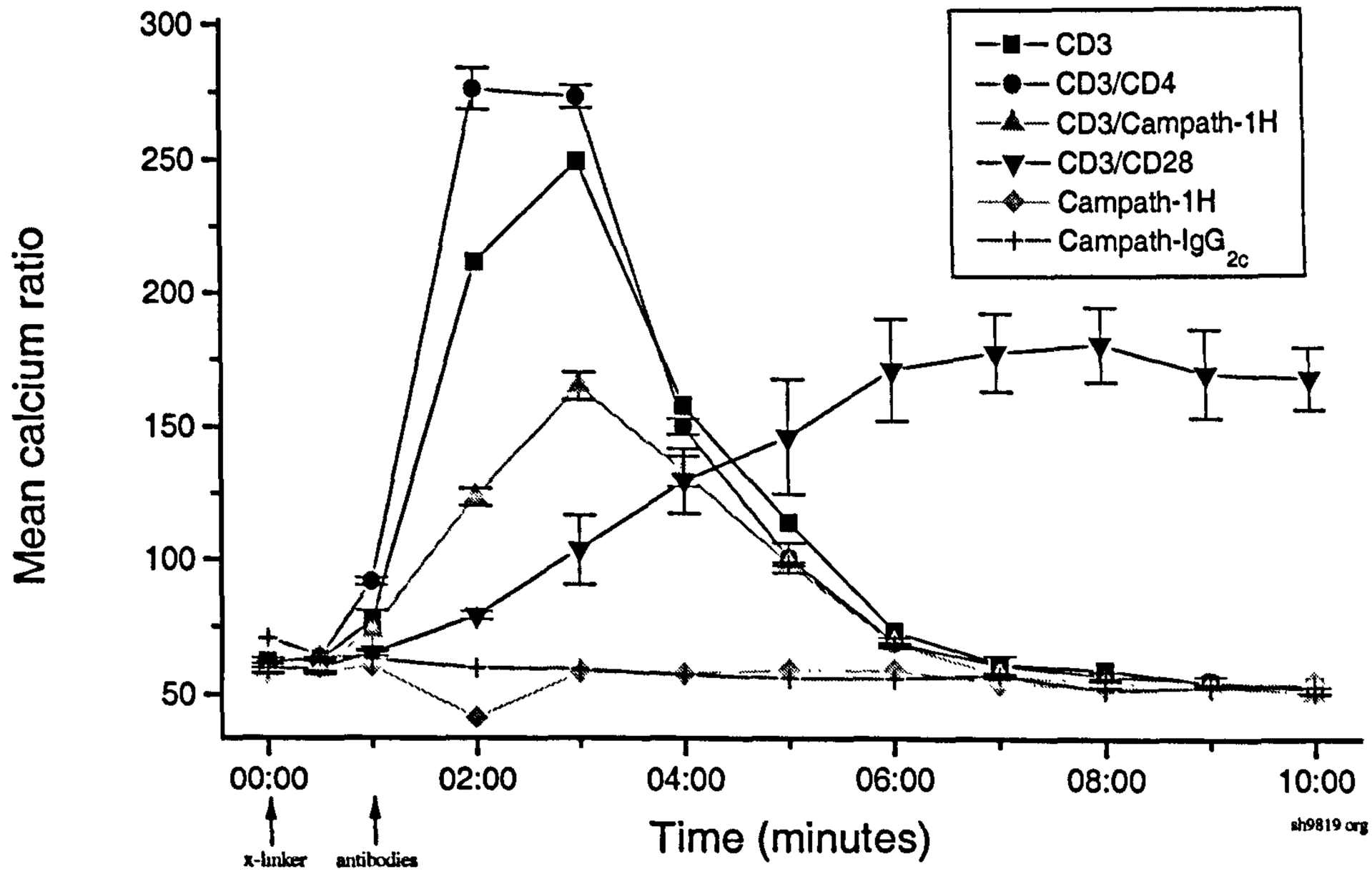


Figure 6.7 CAMPATH-1H does not synergise with anti-CD3 to induce a calcium flux in CD4+ T cells.

Fluo-3 AM and Fura-red AM loaded CD4+ T cells were suspended at 1 x 10^6/ml in RPMI/ 30 mM HEPES and prewarmed at 37°C before transferring to a Coulter XL-MCL flow cytometer for data acquisition. Anti-mouse IgG (crossreactive with both mouse and human IgG) was added at time 0 and incubated with the cells for 1 minute before adding anti-CD3 alone, CAMPATH-1H alone, IgG_{2c} alone, anti-CD3 together with CAMPATH-1H, anti-CD3 together with anti-CD4 or anti-CD3 together with anti-CD28. All antibodies were added at a final concentration of 20 µg/ml.

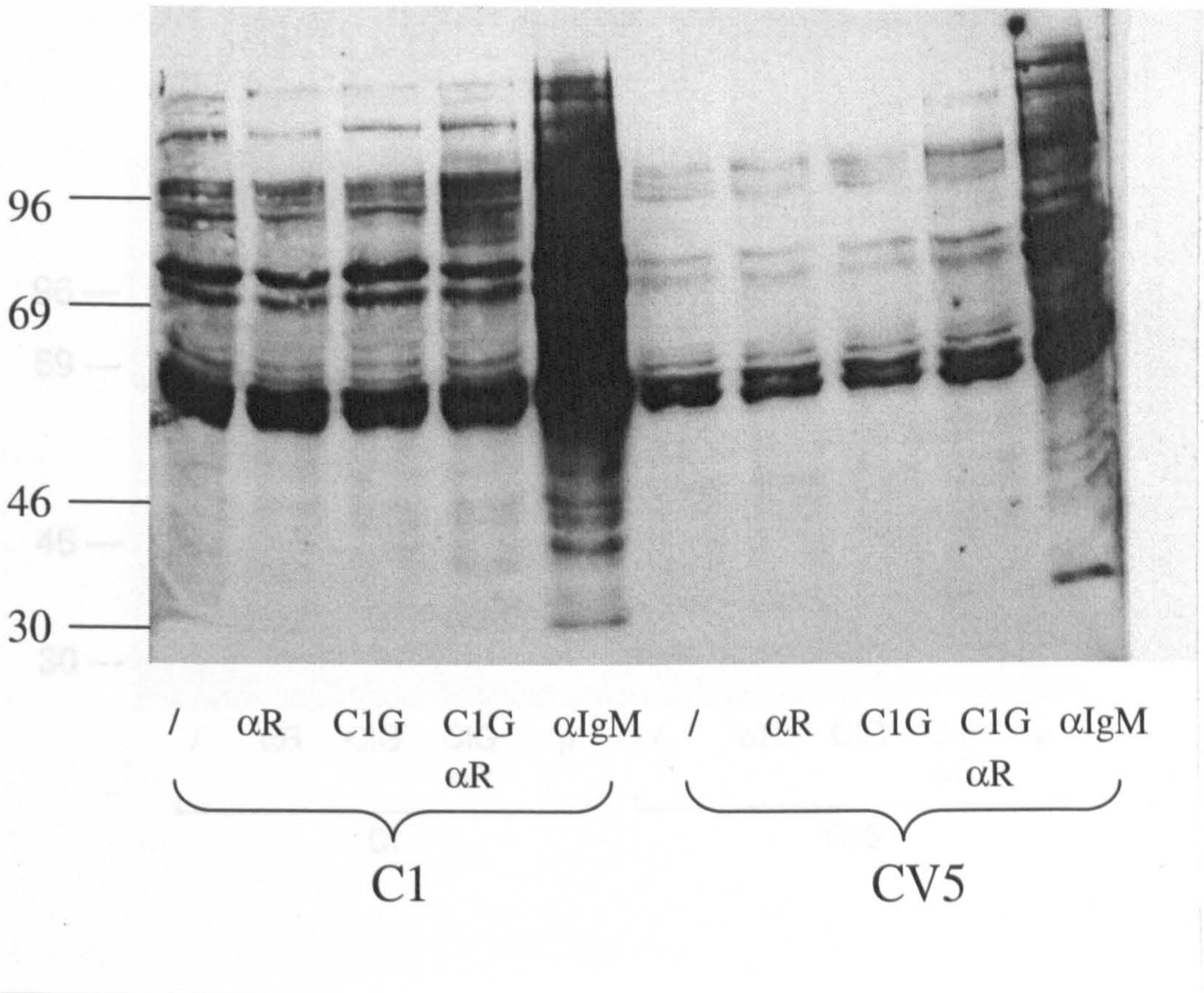


Figure 6.8 CD52 ligation induces tyrosine phosphorylation in Wien 133 B cells: Comparison of the levels of tyrosine phosphorylation induced by crosslinking the CD52 antigen in the Wien 133 B cell line compared to the CV5 cell line.

Wien 133 cells (1 x 10^6/20 μl) were incubated on ice in the presence of no antibody (/), CAMPATH-1G (C-1G), anti-rat IgG (αR), CAMPATH-1G together with anti-rat IgG, or anti-IgM (μ) for 30 minutes. Cells were then incubated for 5 minutes at 37°C. All antibodies were added at a final concentration of 20 μg/ml with the exception of anti-IgM which was added at a final concentration of 4 μg/ml. The reaction was stopped by the addition of 20 μl SDS PAGE sample loading buffer. The equivalent of 5 x 10^5 cells was loaded per track onto a 7.5% SDS PAGE gel. Following transfer to nitrocellulose membrane, tyrosine phosphorylated proteins were visualised using 4G10 anti-phosphotyrosine and ECL detection.

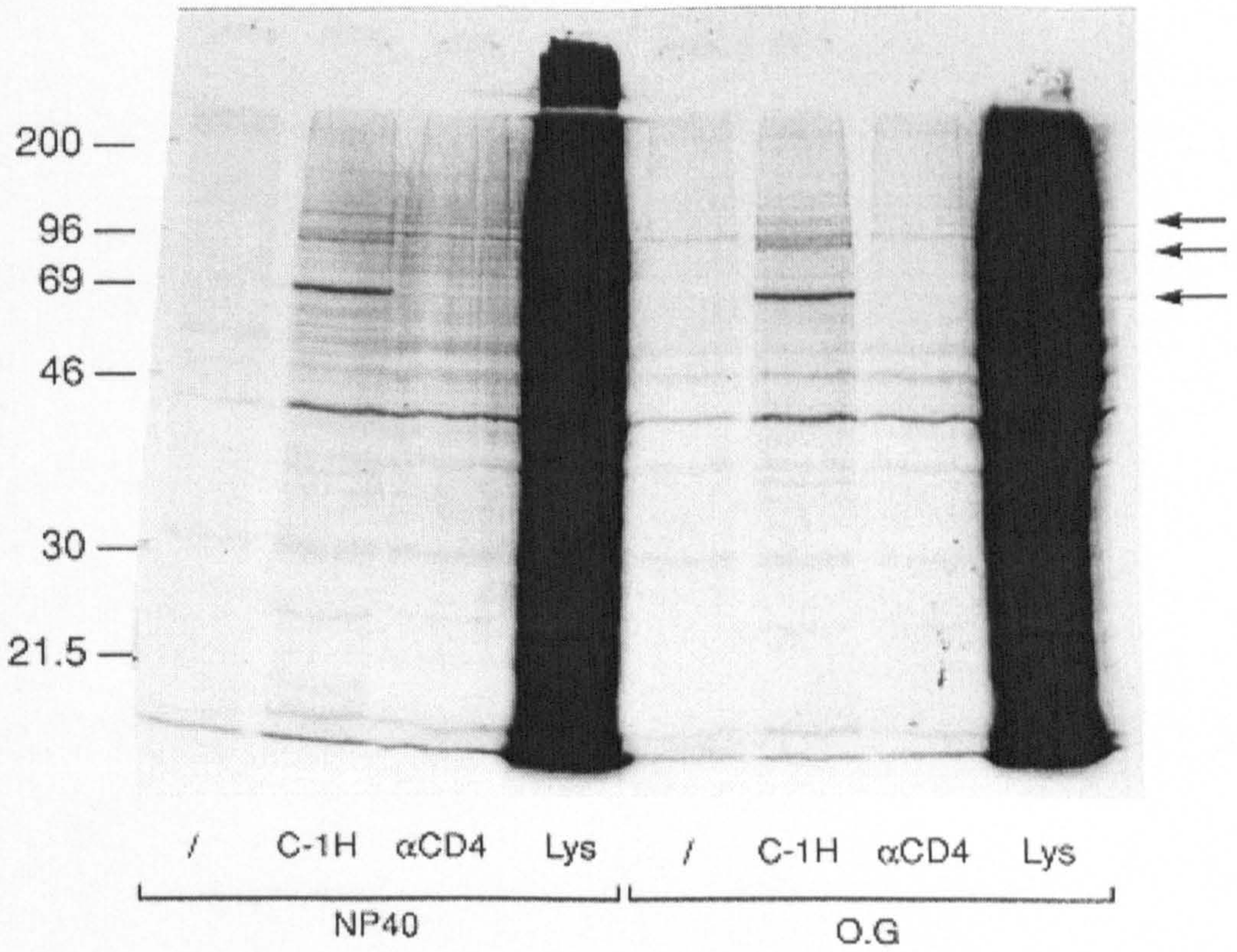


Figure 6.9 CD52 coprecipitates other proteins in Wien 133 B cells.

1 x 10^8 Wien 133 cells, metabolically labelled with [^{35}S] Promix were lysed in either 1% N-octyl glucoside or 1% NP40 lysis buffer and incubated with uncoupled sepharose beads (/), CAMPATH-1H $(Fab)_2$ (C-1H) coupled to sepharose or anti-CD4 $(Fab)_2$ (αCD4) coupled to sepharose. Proteins were eluted with SDS PAGE sample buffer and separated on a 10% SDS PAGE gel. Soluble lysate (Lys) (1 x 10^6 cell equivalent) shows the metabolic labelling of total detergent soluble protein. The gel was dried down and exposed to film for autoradiography.

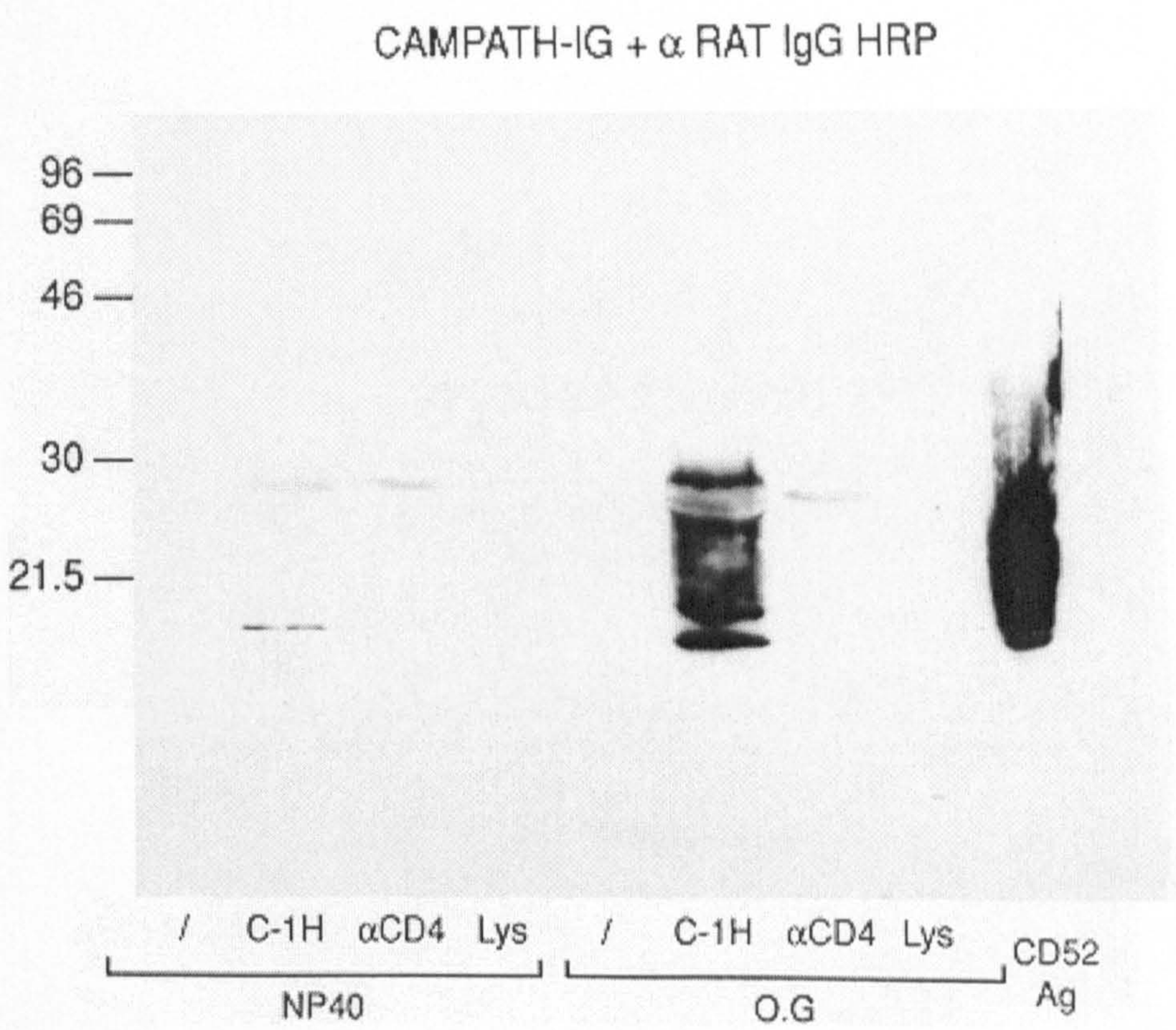


Figure 6.10 Detection of CD52 in CAMPATH-1H immunoprecipitates.

Wien 133 cells were lysed in either 1% N-octyl glucoside or 1% NP40 lysis buffer and incubated with uncoupled sepharose beads (/), CAMPATH-1H $(Fab)_2$ (C-1H) coupled to sepharose or anti-CD4 $(Fab)_2$ (CD4) coupled to sepharose. Eluted proteins were separated by SDS PAGE and transferred to nitrocellulose. Soluble lysate (Lys) = 1×10^6 cell equivalent; purified CD52 antigen (0.0375nmol) was used as a positive control. CD52 was visualised by CAMPATH-1G antibody using ECL detection.

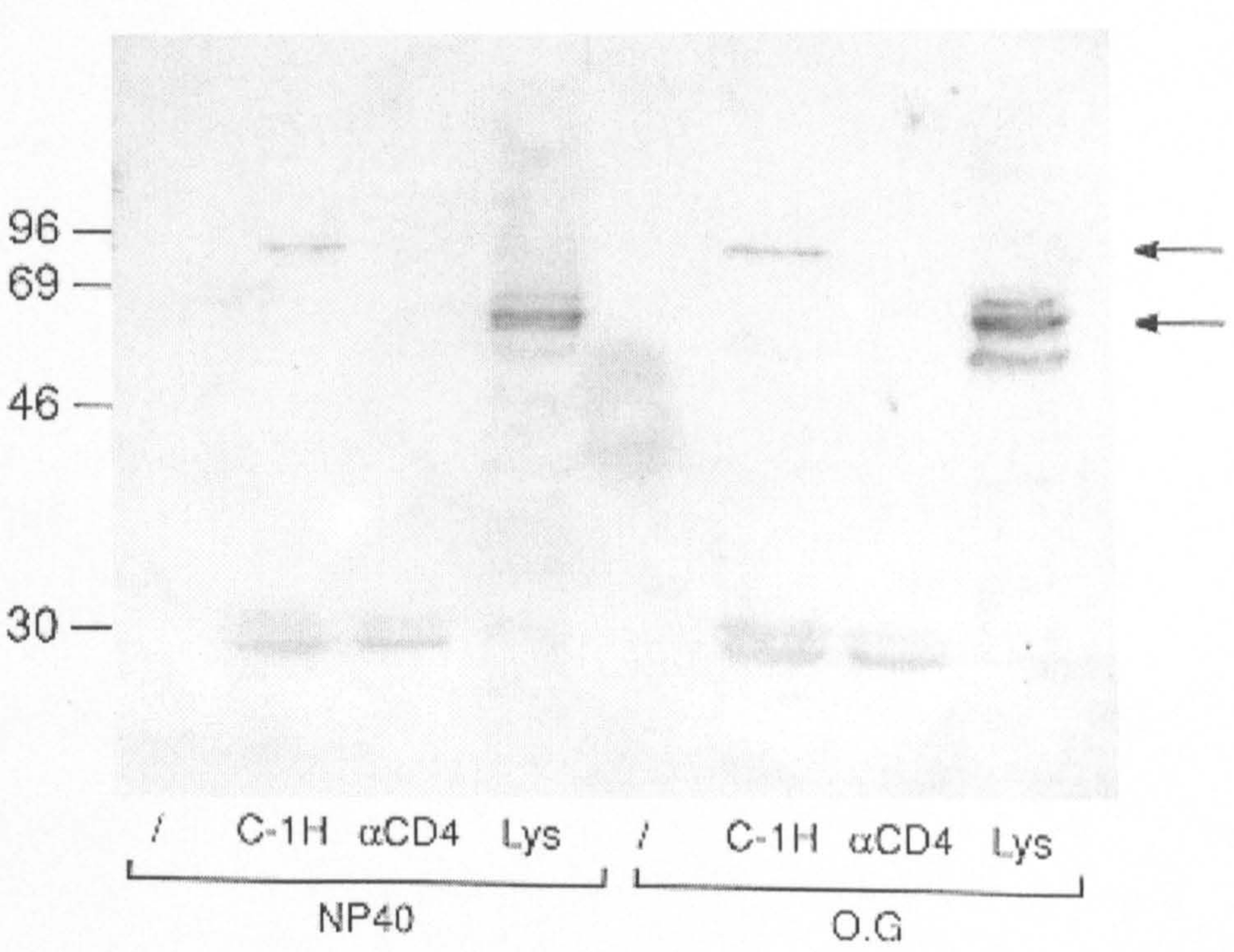


Figure 6.11 CD52 does not coprecipitate lyn.

Wien 133 cells were lysed in either 1% N-octyl glucoside or 1% NP40 lysis buffer and incubated with uncoupled sepharose beads (/), CAMPATH-1H $(Fab)_2$ (C-1H)coupled to sepharose or anti-CD4 $(Fab)_2$ (CD4) coupled to sepharose. Eluted proteins were separated by SDS PAGE and transferred to nitrocellulose. Soluble lysate (Lys) = 1×10^6 cell equivalent. The nitrocellulose membrane was probed with rabbit anti-lyn antibody followed by ECL detection.

6.3 Discus sion

It has been clearly demonstrated that ligation of CD52 on resting CD4+ T cells, a T cell clone and the Wien 133 B cell lymphoma line, can induce changes in protein tyrosine phosphorylation. In addition, immunoprecipitation of the CD52 antigen in the Wien 133 cell line, resulted in the coprecipitation of a number of high molecular weight proteins which might include a putative adaptor protein by means of which a GPI-anchored protein could relay signals to the cell interior. The identity of these proteins was not ascertained. Therefore, further experiments to positively identify these proteins would be potentially valuable in order to elucidate how CD52, and other GPI-anchored proteins, are able to activate cells.

Malek et al. (1994) proposed three possible models for intracellular signaling by GPI-anchored proteins. The first is the interaction of the GPI-anchored protein with a transmembrane protein. The high molecular weight proteins (approximate molecular weights, 70, 96 and 110 kDa) which coprecipitated with CD52 in CAMPATH-1H immunoprecipitations from unactivated Wien 133 cells, indicate that CD52 is capable of interacting with other proteins. These proteins provide possible candidates for an adaptor, or adaptors, for linking CD52 to intracellular kinases and signal transduction pathways. Todd and Petty, 1996, reviewed evidence that proteins including GPI-anchored CD16 (FcRγIII receptor) associate with transmembrane proteins containing the integrin common β chain, namely, the two complement receptors CR3 and CR4, and LFA-1. CD59 and CD55 coprecipitate with an 80 kDa protein.

The second model proposed by Malek et al., 1994, is that the cleavage of the GPI-anchor itself, from the protein moiety, could act as a secondary messenger. An example in support of this model was provided recently, by studies showing that free GPI released by cleavage of malarial surface coat proteins could induce nitric oxide synthase expression in macrophages (Tachado et al., 1996). The identification of a cell associated GPI-specific phospholipase D which released CD55 from HeLa cells, as well as heparan sulphate proteoglycan from bone marrow stromal cells, provides evidence that the mechanisms exist for physiological release of GPI-anchored proteins (Metz et al., 1994). Cleavage of

GPI anchors via phospholipase D releases phosphatitidic acid, a potent mitogen. There is no evidence provided by any of the biochemical studies performed that CD52 could mediate its effects via this model. However, in chapter 3, it was shown that during T cell activation, when the IL-2 receptor was upregulated, the CD52 antigen was concurrently downregulated.

The third model suggested by Malek et al., 1994, is that GPI-anchored proteins become internalised and can themselves activate intracellular signalling pathways. Both murine Thy-1 and Ly-6A/E become internalised following activation (Bamezai et al., 1989; Bamezai et al., 1992). As mentioned above, in chapter 3, it was shown how CD52 was downregulated following both polyclonal and antigen specific activation. The mechanism by which CD52 was downregulated was not explored. The two alternatives are firstly, CD52 downregulation was as result of internalisation and possible degradation, or alternatively, the CD52 antigen was shed into the external medium, possibly releasing secondary messengers, as described above, in the second model.

In addition to the three models proposed above by Malek et al., the lateral mobility afforded by the GPI-anchorage could enable the associated kinases to be brought into close proximity to the TCR signalling machinery allowing a synergy or amplification of the TCR signal. The pattern of phosphorylation seen following crosslinking of CD52 in CD4+ T cells, appeared very similar to that observed when CD3 was crosslinked, albeit the intensity of the protein phosphorylation seen was much less in CD52 crosslinked cells, compared to CD3 crosslinked cells. This is in contrast to the phosphorylation pattern which was seen following anti-CD4 crosslinking. This suggests that CD52 ligation could be feeding into the same pathway as CD3 ligation, or alternatively, that both proteins form part of similar signalling protein complexes. In support of this, when CD52 and CD3 are co-crosslinked, the pattern of phosphorylation appears to be synergistic. This indicates that although both anti-CD3, and CAMPATH-1 antibodies, have been used at saturating concentrations, signalling via either one alone, is not maximal in terms of phosphorylation at the time point examined. This is supported by recent data from Tuosto et al. (1998), who showed that protein phosphorylation in Jurkat T cells, induced by ligation of the TCR alone, could be further enhanced when both the TCR and CD28 were ligated.

Further evidence for CD52 signalling utilising TCR pathways is suggested by studies using CD52 transfected Jurkats (chapter 4). These studies showed that CAMPATH-1 mediated growth inhibition required coexpression of a functional T cell receptor. This has also been shown for other GPI-anchored proteins eg. Thy-1 in Jurkat T cells (Gunter et al., 1987). The fact that CAMPATH-1 ligation did not induce a calcium flux in CD4+ T cells, whereas CD3 ligation did, suggests that CD52 ligation is not using the full complement of signalling pathways emanating from the TCR. In contrast, crosslinking other GPI-anchored proteins eg. Thy-1, has been shown to induce a rise in intracellular calcium levels (Barboni et al., 1991; Gunter et al., 1986). This implies that there is some specificity in signalling via GPI-anchored proteins and that they don't all signal via a common mechanism.

There were clear differences in both the basal levels and the signals seen following receptor ligation in terms of tyrosine phosphorylation when comparing the Wien 133 cell line and the CV5 cell line. This has parallels with evidence showing that GPI-anchored Ly-6 negative cells are defective in protein phosphorylation (Lee et al., 1994). One possible explanation is that the large detergent resistant clusters that have been reported containing both GPI-anchored proteins and src family kinase members are absent (see below). One purpose these clusters might serve is to concentrate the kinases to the membrane where they would be in a position to enhance signalling following receptor ligation. This could explain the differences in the anti-IgM induced phosphorylation between the Wien 133 and the CV5 cell line.

GPI-anchored proteins associate in large detergent resistant insoluble complexes with src family kinases (Stefanova et al. 1991, Cinek and Horejsi, 1992). In addition, antibody crosslinking of GPI anchored proteins eg. Thy-1, CD48, CD55 and CD59 can trigger tyrosine kinase activity (Stefanova, 1991). The ability of CD52 to induce tyrosine phosphorylation in both resting CD4+ T cells and the Wien 133 B cell line, indicates that likewise, CD52 can activate tyrosine kinases. Phosphorylation induced via CD52 ligation in CD4+ T cells, could be partially inhibited by the src family tyrosine kinase inhibitor, Herbimycin A (Uehara et al., 1989). The partial inhibition suggests that Herbimycin A is not effective against all tyrosine kinases involved in CD52 mediated protein

phosphorylation and that other tyrosine kinases could be involved eg. syk family kinases, including syk and ZAP-70. Studies by Deckert et al. 1995, looking at the effect of CD59 crosslinking in Jurkats, observed ZAP-70 phosphorylation.

The use of two different detergents for CD52 immunoprecipitation showed that whereas similar proteins coprecipitated with the CAMPATH-1 antibody under conditions used, the CD52 antigen itself was not readily detected on western blots, using NP40 in the lysis buffer. In contrast, N-octyl glucoside enabled detection. This suggested that NP40 might be conveying other properties on the CD52 antigen either preventing CD52 from binding to the nitrocellulose or failing to solubilise CD52. The fact that additional proteins could be seen in CAMPATH-1H immunoprecipitates, but not in the presence of anti-CD4 antibody, suggested that the former was the reason why CD52 could not be detected. Alternatively, individual proteins are solubilised differentially in different detergents (Mayor and Maxfield, 1995; Parolini et al., 1996). Therefore, octyl glucoside could preferentially solubilise CD52, and the high molecular weight proteins seen with both NP40 and octyl glucoside could represent proteins which crossreact with CAMPATH-1H.

In summary, CAMPATH-1 antibodies have been shown to induce changes in protein phosphorylation in peripheral CD4 + T cells and the Wien 133 B cell lymphoma line. CAMPATH-1 antibodies did not induce a calcium flux in peripheral CD4+ T cells. The increased tyrosine phosphorylation in peripheral CD4+ T cells, in response to CD52 ligation, was at least, in part, due to src family kinase activation, demonstrated by the use of a src family kinase inhibitor, Herbimycin A. This correlated with a decrease in cell proliferation. In the Wien 133 B cell lymphoma line, CD52 antigen has been shown to associate with high molecular weight proteins which could be involved in transducing a signal across the membrane into the cytoplasm. These proteins provide a useful starting point for further investigations into CD52 and its role in cell signalling.

7. Discussion.

7.1 Relevance of studies to clinical trial data.

Studies in normal T cells have shown that CAMPATH-1 antibodies, together with a second signal mediated cell proliferation and cytokine release. In both a B cell line (Wien 133) and a CD52 transfected T cell line (Jurkat), crosslinking CD52 with CAMPATH-1 antibodies, together with a secondary anti-globulin antibody, resulted in growth inhibition and apoptosis. Biochemical studies in both normal T cells and the Wien 133 B cell line, showed that CD52 ligation lead to an increase in overall protein tyrosine phosphorylation. These results suggest that ligation of CD52 by CAMPATH-1 antibodies activates signalling pathways and that the functional outcome of CD52 ligation depends on the activation state of the cell. Therefore, it is proposed that in addition to ADCC and complement mediated lysis (Xia et al., 1993; Crowe et al., 1992), another mechanism by which cell depletion could be achieved *in vivo* by CAMPATH-1H, is via apoptosis.

CAMPATH-1 antibody induced apoptosis in both the Wien 133 and CD52 transfected Jurkat T cell lines, required secondary crosslinking. *In vivo,* CAMPATH-1 antibodies bound to CD52 antigen on target cells, could be effectively crosslinked via Fc receptors on cells, including NK, macrophages and dendritic cells (Abbas et al., 1994; Banchereau and Steinman, 1998). CAMPATH-1 antibodies are extremely efficient at depleting blood borne tumours, but relatively ineffective for treating solid tumour masses (Dyer et al., 1989) and this has been attributed to the ratio of Fc receptor bearing cells: target cells being insufficient (Hale et al., 1985). Therefore, whether lymphocyte depletion is mediated by ADCC or apoptosis, the presence of Fc receptors on neighbouring cells is an important component of the mechanism.

The studies carried out in normal T cells, in which crosslinking CD52 in the presence of a second signal, induced cell proliferation, could explain the observations made by Brett et al., 1996, regarding the phenotype of re-emerging T cells, following CAMPATH-1 therapy. FACS analysis revealed that these cells were all positive for the T cell memory surface marker CD45 RO and had upregulated surface expression of HLA-DR. This

suggested that these cells had been recently activated (Young et al., 1997). An alternative explanation for the presence of T cells bearing the hallmark of recent activation is that the antibody itself is being processed and presented, leading to T cell activation. In support of this, patients treated with CAMPATH antibodies have been shown to mount strong anti-idiotype responses (Isaacs, 1992). The generation of anti-idiotypic antibodies could provide another means, in addition to Fc receptor interactions, by which CAMPATH-1 antibodies bound on target cells could be crosslinked. This is not a likely scenario *in vivo,* as CAMPATH-1 antibodies are not likely to persist in circulation for the extended period it would take to generate anti-idiotype specific antibodies required for crosslinking.

Administration of CAMPATH-1 antibodies to patients has lead to a first dose reaction characterised by the production of the pro-inflammatory cytokines, TNF and IFN-γ (Moreau et al., 1994). Studies by Wing, 1996, suggested that the principal source of these cytokines are NK cells. However, as mentioned above, CAMPATH-1 antibodies have been shown to be capable *in vitro,* of inducing T cell proliferation and this is accompanied by the release of cytokines, including IL-2, IL-4, IFN-γ and TNF (Chapter 3).

Another side effect of CAMPATH-1 therapy has been the emergence of CD52 negative cell populations (Hertenstein et al., 1995; Osterborg et al., 1997b). Further analysis of these cells revealed that in addition to CD52, the cells were also negative for GPI-anchored CD55 and CD59. Normal individuals have been shown to carry small numbers of cells which are deficient in expression of GPI-anchored proteins (Hertenstein et al., 1995) and the cells which are negative for GPI-anchored protein expression, following CAMPATH-1 treatment, might reflect the expansion of cells from this minority population due to positive selection pressure. Patients affected, therefore, acquire a PNH like phenotype (Paroxysmal nocturnal haemoglobulinaemia) (Rosse, 1990). PNH is an acquired haemolytic disease characterised by abnormal activation of complement on erythrocytes due to a deficiency of GPI-anchored complement regulatory proteins, including DAF and CD59. In addition, varying numbers of lymphocytes can be effected,

although these are not prone to the effects of serum complement, to the same extent as erythrocytes. In all PNH cases looked at to date, the common defect, has been mutations in the PIG-A gene (Takeda et al., 1993). PIG-A is involved in GPI-anchor biosynthesis (Reviewed by Yeh, 1994). Studies in the Wien 133 B cell lymphoma cell line, showed that in the presence of CAMPATH-1 antibody and a secondary crosslinking antibody, the cells surviving treatment, expressed negligible levels of CD52 on their surface. In common with patients who developed T cell populations which became negative for CD52 surface expression (Brett et al., 1996b), other GPI anchored proteins, including CD55 and CD59, were also lost. In some patients, the defect in GPI anchored protein expression was shown to be as a result of a mutation in the PIG-A gene (Hertenstein, 1995). In other patients, although shown to lack mature GPI anchor precursors, no mutation was found in the PIG-A gene (Taylor et al., 1997). Similarly, the CD52 low/negative cells obtained following CAMPATH-1 treatment of the Wien 133 B cell lymphoma cell line, also lacked mature GPI precursors but showed no mutation in the PIG-A gene (Taylor et al, 1997).

Interestingly, RA patients who developed CD52 negative T cell populations, showed an improved disease prognosis compared to those that did not (Brett et al., 1996b). This will be discussed later, when considering the role of CD52 as an adhesion/homing molecule.

7.2 Relevance of studies to the possible function of the CD52 antigen.

The function of the CD52 antigen is still not known. As yet, no ligand has been identified. From the data obtained from studies looking at the effects of CD52 on different cell types three possible roles for the antigen will be discussed:

1. Costimulatory molecule.
2. Homing/cell adhesion molecule.
3. Cell protection molecule.

7.2.1 The role of CD52 in T cell activation.

Studies initiated in resting T cells purified from peripheral blood demonstrate that in common with other GPI-anchored proteins, e.g. Thy-1, Ly-6, Qa-2, CD55, CD59 and CD73 (Robinson, 1991; Resta et al., 1994), ligation of the CD52 antigen can induce peripheral CD4+ T cell proliferation and cytokine production. CD52 induced T cell activation has been demonstrated in both naive (CD45RA+) or memory (CD45RO+) CD4+ T cell subsets and in CD8+ T cells (Chapter 3).

A possible role for CD52 in cell signalling is supported by biochemical studies looking at tyrosine phosphorylation from total CD4+ T cell lysates and Wien 133 B cells, following ligation of CD52 (Chapter 6). The increase in tyrosine phosphorylation observed following CD52 ligation is consistent with reports in the literature that GPI-anchored proteins associate with tyrosine kinases and that following ligation of the GPI-anchored proteins, there is increased kinase activity (Stefanova et al., 1991; Shenoy-Scaria et al., 1992; Cinek and Horejsi, 1992; Garnett et al., 1993; Morgan et al., 1993; Bohuslav et al., 1993).

Studies in CD52 transfected Jurkat T cells suggest that signalling via CD52 is dependent on the expression of a functional TCR complex (Chapter 4). This is in common with GPI-anchored Thy-1, Ly-6 and CD59 proteins (Gunter et al., 1987; Sussman et al., 1988; Bamezai et al., 1988; Korty et al., 1991). This could account for the similarities seen in protein tyrosine phosphorylation patterns when comparing CAMPATH-1G with anti-CD3 and the apparent synergy seen when the two antibodies were co-ligated (Chapter 6)

CD52 may be coupled to multiple signalling pathways. Experiments conducted in the Wien 133 B cell line showed that although Cyclosporin A inhibited the growth inhibition effects induced by the secondary crosslinking antibody alone, the drug had no effect on CAMPATH-1H mediated growth inhibition (Chapter 4). Therefore, signalling via the CD52 antigen in the Wien 133 B cell line resulting in growth inhibition was not dependent on signalling via calcineurin. In contrast, in CD4+ T cells, the IgG2c anti-

CD52 antibody induced proliferation in CD4+ T cells was inhibited in the presence of Cyclosporin A.

CD52, like other GPI anchored proteins does not span the membrane and therefore has no direct association with cytosolic signal transducers. Evidence from CD52 immunoprecipitations in unactivated Wien 133 cells, suggests that the antigen is capable of associating with a number of high molecular weight proteins. These could include a putative transmembrane protein such as CD28 which could explain how GPI anchored proteins can activate cells. GPI-anchored proteins have been shown to associate laterally with members of the β1 integrin family, namely LFA-1, CR3 and CR4. These are all conventional transmembrane proteins and so provide a means by which signals can be transduced to the cell interior (Reviewed by Todd and Petty, 1996; Stockinger, 1997).

An alternative explanation for the ability of CAMPATH antibodies to provide a costimulatory signal in vitro, is that the CD52 antigen is present at very high density on the surface of peripheral T cells (5 x10^5/cell)(Bindon et al., 1988). As a consequence of its ligation, molecules including CD3 and CD28 could be artifactually clustered leading to the activation of associated signalling molecules. This would explain the mitogenicity of the rat IgG_{2c} antibody, as IgG_{2c} isotype antibodies can self aggregate via Fc:Fc interactions (Kummer et al., 1993). In addition, studies in the Jurkat CD52 transfectants showed that inhibition of cell growth was dependent on antigen density.

Ligation of CD52 could activate tyrosine kinases which have been shown in numerous studies to be involved in signalling proximal to both the TCR and CD28 (Rudd et al., 1994). CD4 is regarded as the TCR co-receptor (Janeway, 1992b). It is believed to be involved in the early stages of T cell activation by virtue of covalently associated Lck to its cytoplasmic tail (Glaichenhaus, 1991). Lck appears to play an important role in initiating signalling events, demonstrated by severe defects in TCR proximal signalling in an lck deficient T cell line (Williams et al., 1997). However, Granja et al., 1994, have shown that TCR signalling is not dependent on the presence of CD4 and, therefore, associated lck. These two observations suggest there is an alternative source of lck. One possible source is the associated kinase activity in DIGS (Parton and Simons. 1995). The

lateral mobility afforded by possession of GPI anchorage would allow movement of DIGS to areas of antigen activation. This could enable amplification of the TCR signal and similarly a rapid dispersal of the proteins following signal termination. This could explain the difference observed between CAMPATH and anti-CD4 antibodies in their ability to induce proliferation in CD4+ T cells in the presence of PMA (Chapter 3). In these studies, anti-CD4 crosslinked in the presence of PMA did not induce T cell proliferation, whereas, CAMPATH-1H antibody crosslinked in the presence of PMA did. PMA induces the rapid dissociation of lck from CD4 (Pelchen-Matthews, 1993). Thus, CD4 associated lck may play a role in maintaining basal phosphorylation of ITAMs within the TCR complex. This would allow molecules such as ZAP-70 to bind and become phosphorylated. Further activation could be due to colocalisation of DIGS. Zhang et al., 1998, recently described an adaptor protein, LAT, which is phosphorylated by ZAP-70, but colocalises in DIGS (Brdicka et al., 1998) and this, therefore would provide a means whereby TCR signalling machinery would be brought into close contact with GPI-anchored proteins and associated kinase activity.

The physiological role of DIGS is still unclear and there has been controversy as to whether such domains are merely artifacts caused by detergent extraction procedures (Hooper et al., 1998). Recent evidence has been put forward suggesting that these domains do exist naturally in cells (Varma and Mayor, 1998; Friedrichson and Kurzchalia, 1998). In a T cell line, the effects of CD59 crosslinking were found to be dependent on the antigen being clusterestered in DIGS. Cholesterol chelating agents such as nystatin and filipin which disrupt these domains, resulted in the inability of the proteins to function (Stulnig et al., 1997).

Studies in transgenic mice with a mutation in the PIG-A gene found that the T cells were functional (Takahama et al., 1998). Therefore, GPI anchors are not required for T cell activation. However, Schubert et al., 1995, compared normal and GPI-deficient cells from PNH patients in response to activation via allogeneic EBV transformed B cell lines. They found that there was significantly lower proliferation and cytokine production (IFN-γ) in the GPI deficient cells, whereas CD3 stimulation resulted in comparable levels. They observed no differences in T cell activation when normal T lymphocytes were

stimulated with GPI deficient B cells compared to normals obtained from the same PNH patient. This suggests that B cell expressed CD48, CD58 (LFA-3) and CD59 which have all been shown to be putative ligands for CD2 (Selvarij et al., 1987; Kato et al., 1992; Hahn et al., 1992) are not required in a GPI-anchored form for T cell activation. However, CD58 can be expressed as a transmembrane form and as such, may compensate in addition to other possible undefined costimulatory molecules. Therefore GPI-anchored proteins are not essential for T and B cell interactions but are required for optimal T cell activation.

In support of this, Stefanova and Horejsi, 1991, showed that in immunoprecipitations of CD48, CD55 and CD59 from cells that had been treated with PI-PLC, they could no longer detect tyrosine kinase activity inferring that intact GPI-anchored proteins are required for the association with protein kinases.

Ligation of CD52 by CAMPATH antibodies can clearly induce T cell proliferation *in vitro*. However, the CD52 molecule does not appear to have a role in antigen specific signalling, as the antibody did not block antigen specific activation in a T cell clone, whereas anti-CD4 does. This suggests that the CD52 antigen is not involved in the intimate contacts required between the T cell and the APC in order to get cell activation and is probably not a costimulatory molecule in physiological T cell activation.

7.2.2 A role for CD52 in lymphocyte homing/ cell adhesion

The CD52 antigen is a heavily glycosylated protein which suggests that the sugars may be important for its function and one obvious role would be in adhesion. Lymphocytes utilise adhesion molecules to strengthen cell:cell interactions during antigen presentation eg. CD2/LFA-3, LFA-1/ICAM-1 (Springer, 1990). Adhesion molecules are also used during the process of lymphocyte homing to lymph nodes eg. L-selectin (Bradley et al., 1994).

In support of the CD52 antigen having a role in lymphocyte homing, it is expressed at high levels on cells which recirculate between the peripheral blood stream and lymphoid organs until encountering specific antigen. The antigen is downregulated in both CD52/CD3 activated CD4+ T cells and an antigen activated T cell clone. Downregulation of CD52, together with CD3 and CD4, was observed 24 hours after cell activation. In contrast, surface expression of CD25 as well as the GPI-anchored protein, CD59, was upregulated. This would be the time point at which one would expect T cells and APC in a physiological setting to begin to dissociate (Sara Brett, personal communication).

Therefore, by analogy to L-selectin (Bradley et al., 1994), CD52 could be involved in homing to sites of antigen priming (spleen, lymph nodes) and then be downregulated as a consequence of activation. This would enable cells to migrate back out into the periphery and home to sites of antigen entry. If CD52 were involved in homing to the lymph nodes, this could explain why RA patients who developed CD52 negative T cell populations following CAMPATH-1 therapy, showed a better disease prognosis (Brett et al., 1996b). CD52 negative cells would therefore, no longer be able to efficiently home to the sites of antigen presentation and be activated.

Likewise, CD52 could be involved in spermatozoa 'homing'. It is maybe significant that CD52 is found expressed on seemingly very different cell types, the common feature of lymphocytes and spermatozoa being that they both require motility to function effectively.

7.2.3 A role for CD52 in protection from complement mediated attack.

The generation of CD52 negative cell lines showed that CD52 surface expression was lost concurrently with other GPI-anchored proteins, including CD55 and CD59. Both CD55 and CD59 are involved in protection of cells from the effects of autologous complement lysis (Medof et al., 1984; Davies et al., 1989). When CD52 negative cells were transferred back into an *in vivo* environment and allowed to expand, all the cells recovered were strongly positive for CD52 expression, as well as CD55 and CD59. This suggested that the presence of GPI-anchored proteins was favourable/necessary for growth in an *in* vivo

and potentially hostile environment. CD52 could therefore be involved in protecting cells from host mediated attack, in common with CD55 and CD59.

The possible role of CD52 in protection against autologous complement mediated lysis could also be implied from its localisation in seminal plasma alongside the recognised complement regulatory proteins, CD55 and CD59 (Rooney et al., 1996). Alternatively, it is expressed at extremely high concentrations and so might act purely as a physical barrier/protective mechanism to prevent complement proteins from gaining access and being able to insert into the cell membrane.

In the Wien 133 cell line, all the clones derived from cells placed under selection pressure with CAMPATH antibodies, lost expression of all GPI-anchored proteins looked at, including CD55 and CD59, in addition to CD52. It is possible that there is some interdependency for function in this group of GPI-anchored proteins. CD52 might not be involved directly in binding complement components, but, interfering with CD52 and altering its putative interactions with other surface proteins could explain why CAMPATH antibodies are unusually lytic in the presence of complement (Xia et al., 1993).

7.3 CD52: The Future.

GPI anchors were first described over 20 years ago (Low and Finean, 1977) but their physiological significance is still not understood (Itzhaky et al., 1998). The function of one of the best known GPI-anchored proteins, Thy-1, still remains unclear (Killeen, 1997), despite intensive studies since its discovery in the 1960s (Raff, 1971).

There is still no known ligand for CD52. The studies performed in the Wien 133 B cell lymphoma cell line suggested that a number of high molecular weight proteins co-precipitated with the antigen in unactivated cells. In order to understand the role CD52 is playing in the immune system (if any), the obvious way forward would appear to be the identification of these proteins. Protein microsequencing technology has improved

dramatically over recent years (Lammond and Mann, 1997; Pennington et al., 1997) allowing positive identification of proteins from ever decreasing amounts of material (Lindquist, 1998).

Likewise, the fields of T and B cell signalling are ever evolving (Cantrell, 1998; DeFranco, 1997). Therefore, further insight into which signalling pathways are activated following CD52 ligation will hopefully aid in the understanding of the function of CD52 and its role *in* vivo.

8. References

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