

A STUDY OF SURFACE RECEPTORS ON RAT T LYMPHOCYTES

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ABSTRACT

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A double immunolabelling technique was developed to study microscopically the interactions between CD4, CD45 and the T cell receptor on the surface of rat T cells induced by the phenomenon of co-capping.

It was found that both CD4 and CD45 passively co-cap with the actively capped T cell receptor, that the T cell receptor and CD45 passively co-cap with CD4, but that neither CD4 nor the T cell receptor co-cap with CD45. Co-crosslinking and active capping of CD45 with either the T cell receptor or CD4 prevented CD4 or the T cell receptor respectively, from passively co-capping.

These experiments were extended to study the effects of particular antibody crosslinking conditions on T cell proliferation and tyrosine phosphorylation. A correlation was found to exist between receptor distribution and the effects of particular antibody combinations on proliferation and tyrosine phosphorylation. The significance of this with respect to T cell activation is discussed.

Finally, an observation is reported concerning the failure of some cell lines to cap antibody-crosslinked surface molecules. Preliminary investigations into the nature and extent of the phenomenon are described.

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ABBREVIATIONS

APC	antigen presenting cell
ATP	adenosine triphosphate
BSA	bovine serum albumen
cAMP	cyclic adenosine monophosphate
CCD	charge coupled device
Con A	concanavalin A
Ci	curie
DAG	diacylglycerol
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immuno-sorbant assay
FITC	fluorescein isothiocyanate
GDP	guanosine diphosphate
GTP	guanosine triphosphate
gp	glycoprotein
HIV	human immunodeficiency virus
IFN- γ	interferon gamma
Ig	immunoglobulin
IL2	interleukin 2
IL2R	interleukin 2 receptor
IP ₃	inositol 1,4,5-trisphosphate
LFA	lymphocyte function-associated antigen
MHC	major histocompatibility complex

mIg	membrane-bound immunoglobulin
Mr	relative molecular weight
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PI	phosphatidylinositol
PIP	phosphatidylinositol 5-phosphate
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonylfluoride
RNA	ribonucleic acid
SDS	sodium dodecylsulphate
TBS	tris buffered saline
T cell	thymus derived lymphocyte
TCR	T cell antigen receptor
TEMED	N,N,N,N-tetramethylene diamine
TPA	12-O-tetradecanoylphorbol 13-acetate
TRITC	tetramethylrhodamine isothiocyanate

CHAPTER 1

INTRODUCTION

1.1 General features of the immune system.

The function of the immune system is to protect the body from invasion by microorganisms and viruses. To do this two levels of defence have been established. The first of these, the innate immune system, provides a simple non-specific barrier against infection and includes phagocytes and soluble factors present in the secretions from epithelial membranes. The adaptive immune system, on the other hand, consists of a complex array of cells and soluble factors which interact to specifically identify and destroy pathogens. Adaptive immunity, as the name suggests, results in an enhanced immunological response with repeated infection by a particular pathogen.

Specific recognition in the adaptive system is mediated by B and T lymphocytes. These two classes of cells can be distinguished on the basis of their site of differentiation, function and cell surface antigens.

B lymphocytes are generated from the same bone marrow stem cells as T lymphocytes. They express membrane bound immunoglobulin (mIg) on the cell surface which acts as a receptor for antigen in the native form. Internalisation of the antigen-mIg complex ultimately results in the re-expression at the cell surface of a fragment of processed antigen in association with class II major histocompatibility complex (MHC) proteins (see below). A B cell may then be stimulated to secrete antibody specific for the antigen initially recognised. T lymphocytes play a role in this by binding to the processed antigen-MHC and secreting soluble factors which can enhance or suppress the B cell response.

1.2 T lymphocytes.

(a) General characteristics.

T lymphocytes develop from common haemopoietic stem cells which also differentiate into B lymphocytes, platelets, neutrophils, macrophages and other myeloid cell types. Normal resting peripheral T lymphocytes are small agranular cells (6-10 μm diameter) with a large nucleus.

Progenitor T lymphocytes migrate from the bone marrow to the thymus where they undergo maturation. During development in the thymus, the T cells proceed through a series

of maturational stages which can be identified by the expression of surface molecules, in particular CD4 and CD8.

The most immature thymocytes are the CD4⁻ CD8⁻ cells, constituting about 5% of the total population (Trowbridge et al., 1985). These then develop, through an early CD4⁻ CD8⁺ stage (Paterson and Williams, 1987), into CD4⁺ CD8⁺ cells and represent the majority of cells within the thymus (Mathieson and Fowlkes, 1984). The most mature cells express either CD4 or CD8.

CD2 is expressed early in thymic ontogeny (Furley et al., 1986), prior to the TCR-CD3 complex which appears at low levels at the CD4⁺ CD8⁺ developmental stage.

It is during development in the thymus that selection of T cells for antigenic reactivity occurs. The exact mechanism of thymic selection is unknown, but it is thought to depend on deletion of cells which express receptors for self antigens (Kappler et al., 1987) and selection of cells which are able to recognise foreign antigen in the context of self MHC proteins (Zinkernagel, 1978).

On leaving the thymus, the mature T cells migrate to peripheral lymphoid organs such as the lymph nodes and spleen, where they perform the various roles of killing infected cells and modulating the activity of other immune cells.

(b) T lymphocyte subsets.

There are two mutually exclusive T cell subsets, defined by the expression of CD4 and CD8 surface glycoproteins (Reinherz et al., 1979, Reinherz and Schlossman, 1980). It was originally thought that expression of these molecules was strictly related to functional characteristics, in that CD4 expression defined the population of helper T cells, whilst CD8⁺ lymphocytes were cytotoxic/suppressor cells (Reinherz and Schlossman, 1980). Although this is generally the case, it has been demonstrated that some CD4⁺ T cells were capable of specifically killing target cells (Shaw et al., 1981, Biddison et al., 1982) and some CD8⁺ cells are capable of helper function (Swain, 1983). A more accurate definition of the CD4 and CD8 subsets relates to the ability of cells expressing these glycoproteins to recognise foreign antigen in the context of products of the MHC. CD8⁺ cells recognise antigen in the context of class I MHC (Norment et al., 1988, Rosenstein et al., 1989), whilst CD4⁺ cells are restricted by class II MHC (Doyle and Strominger, 1987). The CD4⁺ T cell subset can be further subdivided, based on the expression of CD45 isoforms (see below).

1.3 The $\alpha\beta$ T cell receptor complex.

(a) Structure.

i. The α and β chains.

The antigen recognition structure within the T cell receptor complex is made up of two polypeptides, α and β (Dembic et al., 1986), which are joined by disulphide bonds. They are single membrane-spanning molecules, lying mostly outside the cell, with only a 4-12 amino

acid cytoplasmic domain (Saito et al., 1984). The transmembrane region is positively charged (Davies and Bjorkman, 1988), contrasting with the negatively charged transmembrane region of the the CD3 chains (van den Elson et al., 1985), with which the α and β chains are associated. The ability of the T cell population to recognise almost any antigen is a function of the enormous number of different α and β chains which can be produced by the variable recombination of a number of segments within the α and β genes.

The α gene is encoded on chromosome 1 in both human and mouse and consists of 50-100 variable (V_α) segments, at least 50 joining (J_α) segments and 1 constant (C_α) segment (Winto et al., 1985). The β gene is found on chromosome 6 in the mouse and chromosome 7 in man. It contains a tandem duplication of 1 diversity (D_β) region, approximately 6 J_β segments and 1 C_β segment. Outside this duplication there are about 20 V_β family members in the mouse and up to 100 in the human (Gasgoine et al., 1984).

Recombination events are directed by a short 28-41 base pair region flanking each segment. This consists of a highly conserved palindromic heptamer and a less conserved nonamer separated by a 12 or 23 bp spacer. The flanking region 3' to the V regions contains the 23 bp spacer, while J segments contain a 12 bp spacer in a 5' flanking sequence. The D regions have a 23 bp spacer at the 3' end and a 12 bp spacer at the 5' end. Recombination occurs between a 12 bp and a 23 bp spacer. The two D region genes can also be read in any frame such that over 4000 different β sequences may be generated. Overall more than 10^7 different receptors can potentially be generated (Kronenberg et al., 1986).

ii. The CD3 complex.

Three chains, γ , δ and ϵ constitute the CD3 complex which is directly associated with the α and β chains (Borst et al., 1983). Unlike the chains involved in antigen recognition, the CD3 chains are invariant. The genes for these molecules are located within a 50-300 kb region on human chromosome 11 and mouse chromosome 9, and are thought to have arisen from the duplication of a single gene (Tunnacliffe et al., 1987).

The CD3 chains each consist of a 79-89 amino acid extracellular domain, a 29 amino acid transmembrane region and a 45-55 amino acid cytoplasmic domain; much larger than that of the α and β chains. The γ and δ chains have core protein sizes of approximately 15 kD, but the apparent molecular weight is increased by glycosylation to 25-28 kD and 20 kD respectively in human and 21 kD and 28 kD in mouse. The ϵ chain is not glycosylated and has a molecular weight of 20 kD in man and 25 kD in mouse.

iii. The ζ and η chains.

A majority of antigen receptors on the T cell surface are coupled to a homodimer of a 16 kD molecule called ζ (Weissman et al., 1988. Baniyash et al., 1989). This has a large, 113 amino acid, cytoplasmic domain, a 21 amino acid transmembrane region, and only 9 amino acids exposed on the extracellular surface of the cell. ζ is non-glycosylated and shows no sequence or structural similarity to the α , β or CD3 chains. It is encoded on chromosome 1 as is a related 22 kD molecule, η . Ten percent of all T cell receptors are complexed to a $\zeta\eta$ heterodimer as opposed to a $\zeta\zeta$ homodimer (Baniyash et al., 1988).

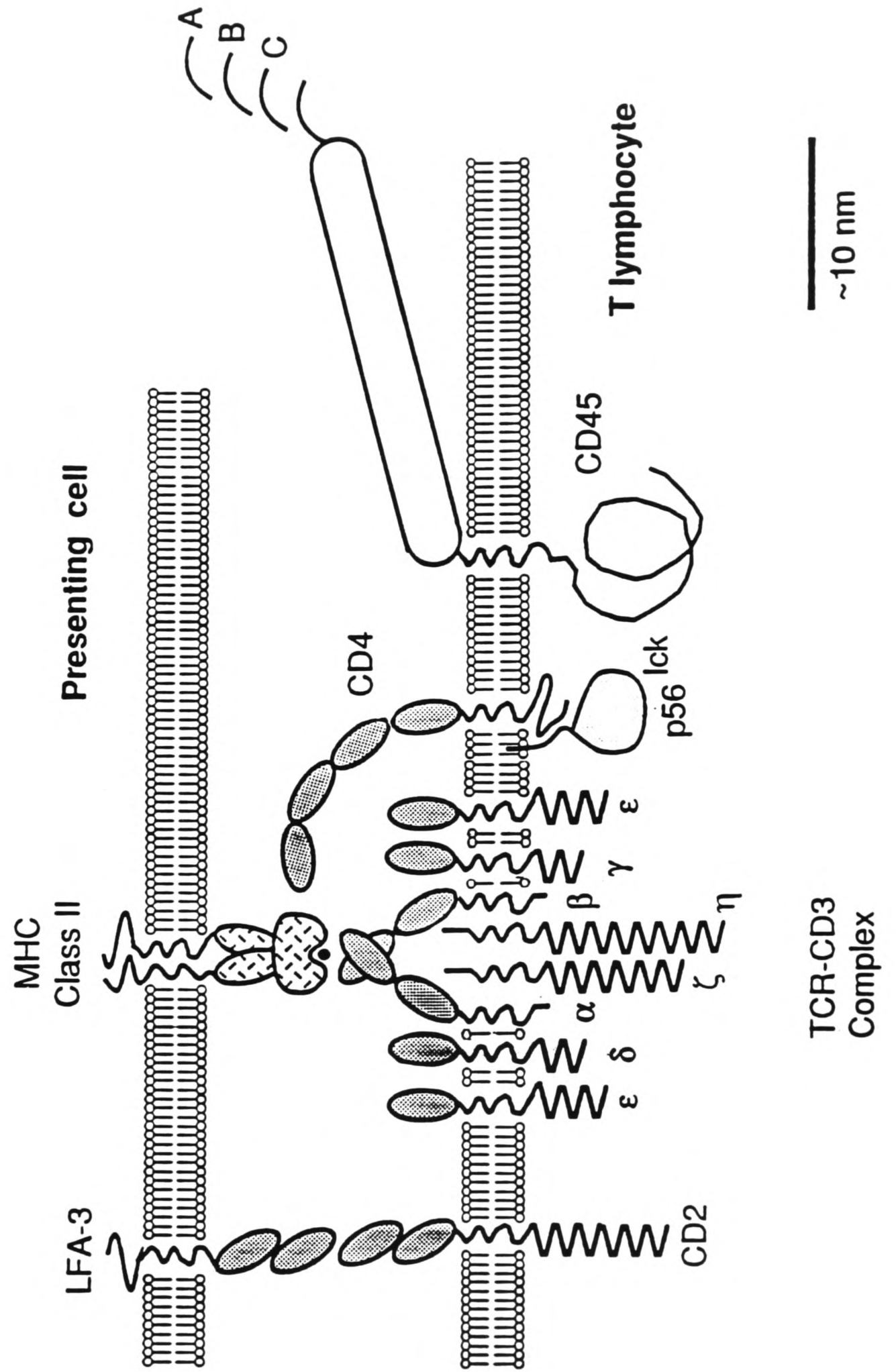


Figure 1.1 Molecules involved in T cell activation

(b) Assembly of the receptor complex.

The way in which the various subunits of the receptor complex associate on the surface of the T cell has not yet been unequivocally determined. Use of specific detergents has resulted in the production of partial complexes of CD3 $\gamma\epsilon$ and $\delta\epsilon$ (Bonifacino et al., 1988) suggesting that these chains may form heterodimers. *IP. with Ab to spec. chains which result in dissociation of some chains* ^{TR100} Meanwhile, covalent crosslinking studies have demonstrated that the β chain could be crosslinked to the CD3 γ chain in human T cells *heterodimerised x later DSP NP40* (Brenner et al., 1985). Because of the structural similarities between α and β and γ and δ it has been proposed that the α chain interacts with the CD3 δ chain (Ashwell and Klausner, 1990). The ζ chain has been shown to form an association with the α and β chains. *also in Bonifacino* A possible structure for the T cell receptor complex is shown in Figure 1.1.

Only complete structures containing all components of the receptor complex are permitted by the cell to arrive at the surface (Koning et al., 1988). Incomplete structures leave the endoplasmic reticulum, where oligomerisation of the components occurs (Alarcon et al., 1988) but are then transported into lysosomes where they are degraded. It appears that the sites of interaction between chains also act as degradation signals. If the chains are joined together in the correct conformation, the degradation signals are masked and so the unit will be transported to the cell surface. If the complex is incomplete, the degradation signals will be exposed and the complex will be broken down (R. Klausner, Biochemical Society of Great Britain, Birmingham meeting, December 1990.). In this respect ζ appears to be the limiting factor as expression is one tenth that of the other chains (Minami et al., 1987). Thus the cell is able to ensure that only fully functional receptor complexes are expressed on the surface.

1.4 T cell recognition of antigen.

The recognition of antigen by the T cell receptor is restricted by the class I and class II gene products of the major histocompatibility complex (MHC). This is a highly polymorphic set of genes which encode for three different classes of protein. Class I is a single Immunoglobulin-like protein associated with a molecule called β_2 microglobulin which is encoded outside the complex. Class II is encoded by the I region of the complex and consists of two chains, also with Ig-like regions. Class III molecules are involved in the complement cascade. The antigen presenting cell (APC) processes the antigen to produce peptide fragments which then bind to the class I or class II MHC molecules (Erb and Feldman, 1975; Babbitt et al., 1985, Watts et al., 1984) and is presented on the cell surface. Class I molecules are restricted to presentation of endogenously derived peptides, whereas the peptides presented by class II molecules are derived exogenously (Braciolo et al., 1987). Determination of the 3D structure of the class I molecule (Bjorkman et al., 1987; Bjorkman et al., 1987) revealed that the antigen binding site is present as a groove running along the top of the molecule.

That the processed antigen is recognised by the T cell receptor α and β chains was demonstrated by experiments in which the DNA encoding the α and β chains from an antigen-specific T cell clone were transfected into T cells with different antigen/MHC reactivities. The transfectants were able to respond to the same antigen/MHC combination as the $\alpha\beta$ DNA donor (Saito et al., 1987; Dembic et al., 1986).

It is thought that the V_{β} region confers some MHC binding specificity (Fink et al., 1986; Saito and Germain 1987; Kappler et al., 1987), while the CD3 chains do not appear to play a role in antigen recognition.

1.5 CD4.

CD4 has been variously called W3/25 in the rat (Williams et al., 1977), T4 or Leu-3 in the human (Reinherz et al., 1979, Terhorst et al., 1980) and L3T4 in the mouse (Dialynas et al., 1983)(Figure 1.1).

It is a 55 kD glycoprotein of 430 amino acids in the rat. There is a large extracellular domain and a 25 amino acid hydrophobic transmembrane region. The cytoplasmic tail is relatively short, consisting of only 38 amino acids (Clark et al., 1987).

CD4 expression has been identified on thymocytes, mature T cells, and macrophages in the rat (Jefferies et al., 1985) and human (Wood et al., 1983), but only on thymocytes and T cells in the mouse (Crocker et al., 1987). It is expressed in a complementary manner to CD8 on T cell subpopulations, and was originally thought to identify the T helper subpopulation, although this is not strictly true (see above and below). It is now more accurate to say that it identifies class II restricted T cells.

Doyle and Strominger (1987) showed that CD4 binds to a non-polymorphic determinant on the class II major histocompatibility antigen, and proposed that the interaction between

these two molecules strengthens binding of the T cell to the antigen presenting cell. However, in these experiments, CD4 was expressed at very high density so the contribution of CD4-class II interactions to cell adhesion in physiological conditions is still uncertain.

The cytoplasmic domain of CD4 is associated with the src tyrosine kinase p56^{lck} (Rudd et al., 1988, Veillette et al., 1988). Treatment of cells with PMA induces dissociation of p56^{lck} from CD4 (Hurley et al., 1989) and to phosphorylation and a reduction in the level of surface expression of CD4 (Acres et al., 1986; Bedinger et al., 1988; Maddon et al., 1988)

CD4 has also been shown to be the receptor for gp120, the exterior envelope glycoprotein of human immunodeficiency virus 1 (HIV-1). HIV bound to CD4 on the cell surface is thought to introduce its RNA into the cytoplasm of the cell by direct fusion of the viral envelope with the plasma membrane (Robey and Axel, 1990)

Many reports have suggested CD4 to be physically and functionally associated with other T cell surface molecules. This area is discussed in detail in Chapters 3,4 and 5 and so will not be pursued here.

1.6 CD45.

(a) General features.

The cluster of differentiation (CD) number 45 (CD45) has been assigned to a family

of high molecular weight glycoproteins (Cobbold et al., 1987) which had previously been referred to in the literature under a variety of other names including T200 (Trowbridge et al., 1976), B220 (Coffman et al., 1981), Ly-5 (Komuro et al., 1975) and leukocyte common antigen (LCA) (Sunderland et al., 1979).
X Leukocyte typing III.
and Weissman ✓

CD45 was identified as one of the major specificities of anti-rat lymphocyte serum (Fabre and Williams, 1977) and was found to be expressed on all cells of haematopoietic origin (Sunderland et al., 1979).

Most thymocytes express a 180 kD form of the molecule while B cells express a 240 kD form. Affinity purification of CD45 from solubilised rat T cells has resulted in the identification of four separate bands by SDS PAGE, with apparent molecular weights of 180, 190, 200 and 220 kD (Brown and Williams, 1982; Woollett et al., 1985).

CD45 is extremely abundant and is estimated to occupy 10% of the lymphocyte cell surface (Williams and Barclay, 1985). The molecule traverses the plasma membrane and has an external domain of approximately 540 amino acids which is poorly conserved, showing only 35% homology between human, rat and mouse (Thomas, 1989). Variation in the size of the external domain is responsible for the production of CD45 isoforms with different molecular weights. This is the result of alternate splicing of exons 4,5 and 6 (Thomas et al., 1987; Saga et al., 1987; Streuli et al., 1987), three of the 33 exons which make up the CD45 gene located on chromosome 1 (Shen, 1986). Potentially, eight mRNAs may be generated and so far six have been identified (Barclay et al., 1987). The alternatively spliced exons 4,5 and 6 are also termed A, B and C respectively, and this nomenclature is used to identify the
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various CD45 isoforms (Figures 1.1 and 1.2).

The remaining portion of the extracellular domain is encoded by exon 3, towards the amino terminal side of the alternately spliced region, and exons 7-15 which encode the region extending to the plasma membrane. Within this 300 amino acid region are two cysteine rich sub-domains.

The CD45 molecule contains a 22 amino acid transmembrane region (exon 16), and the remaining exons (17-33) encode a 705 amino acid cytoplasmic domain with a molecular weight of approximately 80 kD. Unlike the extracellular domain, the cytoplasmic domain is highly conserved, showing 85% homology between species (Thomas, 1989). It was assumed therefore, that the cytoplasmic domain played an important role in CD45 function. This was confirmed by recent observations that the 300 amino acid tandem repeat within the cytoplasmic tail shows homology to human placental protein tyrosine phosphatase (PTPase) (Charbonneau et al., 1988). It has since been demonstrated that CD45 is indeed a PTPase (Tonks et al., 1988) and this is further discussed in chapter 5.

Despite the fact that CD45 was identified over fifteen years ago, attempts to find a natural ligand for the molecule have so far proved unsuccessful. Until the discovery that CD45 was a PTPase, one of the few suggestions as to the function of CD45 came from the work of Bourguignon and colleagues. They found that CD45 can associate with the cytoskeletal protein fodrin (Bourguignon et al., 1985; Suchard and Bourguignon, 1987) and proposed that CD45 acted as a link between other surface molecules and the cytoskeleton.

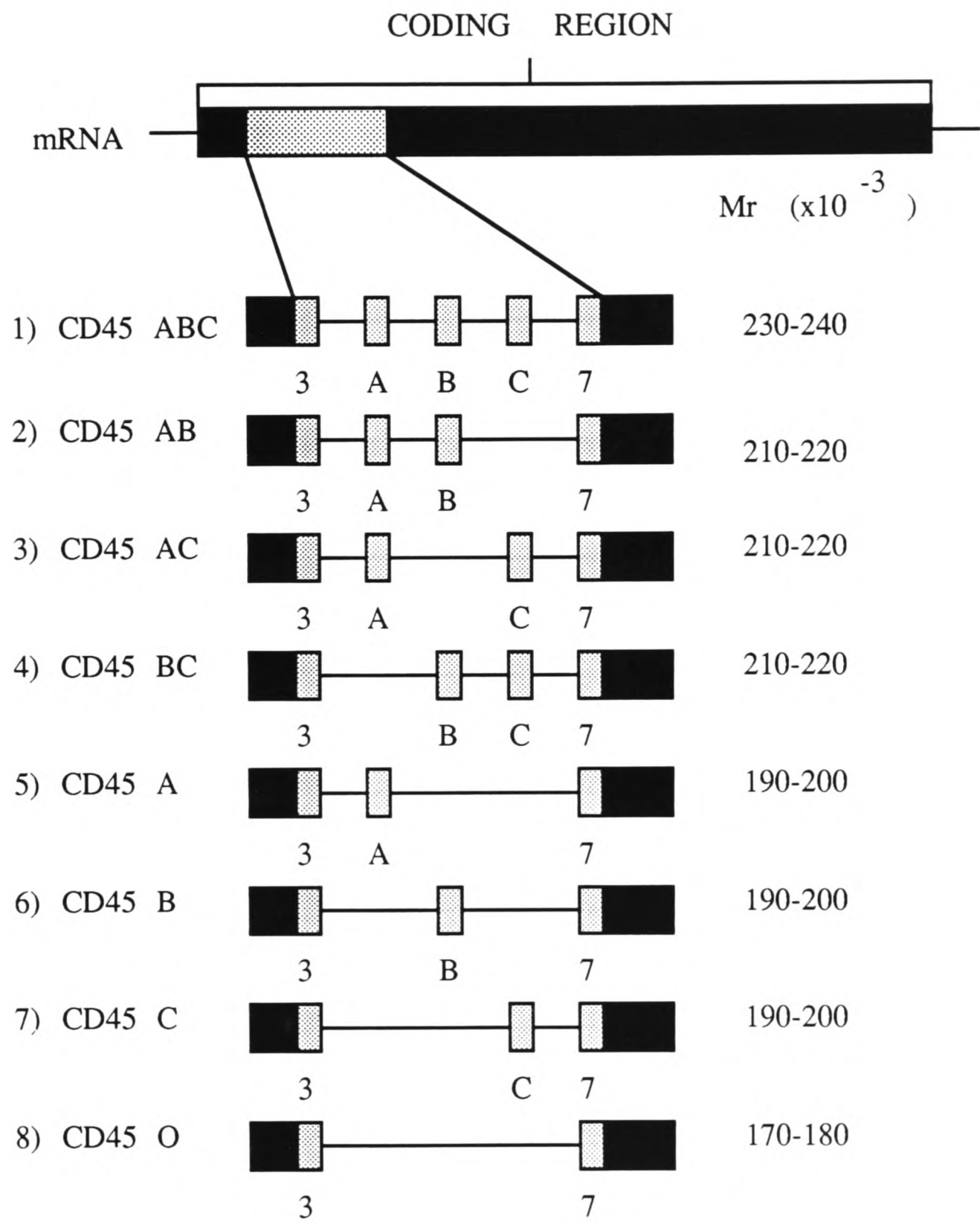


Figure 1.2 Expression of CD45 Isoforms.

The discovery that CD45 isoforms define T cell subpopulations has added a further dimension to the debate on the function of this enigmatic molecule.

(b) CD45 isoforms.

Antibodies directed against CD45 fall into two groups; those that recognise all forms of the molecule, such as OX-1 in the rat, and those that recognise epitopes that are restricted to particular isoforms. The latter group are termed CD45R (Cobbold et al., 1987) and include UCHL-1 and 2H4 in the human (Morimoto et al., 1985; Smith et al., 1986) and OX-22 in the rat (Spickett et al., 1983). *under mammal*

The OX-22 epitope is expressed on all B cells and CD8⁺ T cells and two thirds CD4⁺, but on only 2% of thymocytes (Spickett et al., 1983). The partial sequence of the OX-22 epitope has been determined (Barclay et al., 1987). It was identified as lying in the region of exons B and C and has since been localised to exon C. The OX-22 epitope is therefore not expressed on the low molecular weight isoforms of CD45, which do not contain exon C. *220k*

The separation of CD4⁺ T cells into OX-22⁺ and OX-22⁻ populations relates to the functional characteristics of these two groups. Spickett et al. (1983) showed that parental OX-22⁺ CD4⁺ T cells were able to mediate graft v. host disease (GVHD) in F₁ hybrid rats, but that OX-22⁻ cells had no effect. On the other hand, OX-22⁻ cells were sixteen times more effective than OX-22⁺ cells at providing help for a secondary B cell antibody response *parental sorted cells into irradiated T_H*

(Arthur and Mason, 1986).

In the human system, the 2H4 antibody (Morimoto et al., 1985) defines a CD4⁺ T cell population with similar characteristics to the rat OX-22⁺ cells. For the purposes of this review the population of CD4⁺ cells defined by these and similar antibodies will be described as CD45R⁺. UCHL-1 (Smith et al., 1986) binds to the 180 kD CD45 isoform, termed CD45R0 because none of the alternately spliced exons are expressed. Thus UCHL-1 defines the reciprocal CD4⁺ T cell population to 2H4. An antibody analogous to UCHL-1 is not yet available in the rat system.

The discovery of CD45-isoform-defined sub-populations within the CD4⁺ T cell population led to speculation as to whether these represented separate T cell lineages, or were different maturational states of a single population.

Whilst it was known that activation of rat OX-22⁺ CD4⁺ T cells results in loss of the OX-22 epitope (Paterson et al., 1987) and that phytohaemagglutinin (PHA) stimulation of human CD4⁺ T cells induces loss of 2H4 and concomitant gain of UCHL-1 binding (Akbar et al., 1988), it was not known whether this was a permanent phenotypic change, or if the cells reverted on entering the resting stage.

Evidence that CD45R⁺ CD4⁺ T cells undergo an irreversible phenotypic change upon activation with antigen was suggested by the observation that cord blood from neonates contains less than 5% CD45R0 cells (Sanders et al., 1988). This proportion increases throughout an individual's life (Terao et al., 1988), presumably as an individual is exposed

to an increasing number of pathogens.

Further to this, Powrie and Mason (1989) reported that injection of OX-22⁺ or OX-22⁻ cells into nude rats resulted in expansion of the T cell population to near normal levels. However, regardless of the OX-22 phenotype of the injected cells, most of the expanded T cell population was OX-22⁻ 8-10 weeks after the initial reconstitution, suggesting that conversion to OX-22⁻ phenotype is a unidirectional process.

The nude rats reconstituted with OX-22⁺ CD4⁺ T cells in these experiments were able to mount a significant anti-OVA antibody response, whereas rats reconstituted with OX-22⁻ cells could not. From this it was deduced that the OX-22⁺ population contained unprimed CD4⁺ T cells, some of which expressed the receptor for OVA antigenic determinants, whilst the OX-22⁻ population contained cells which had been primed by previous exposure to antigen. As it is unlikely that the donor T cells would have experienced the OVA antigen prior to being used for reconstitution, there would be no OX-22⁻ CD4⁺ T cells which expressed the anti-OVA receptor. Thus it was proposed that OX-22⁺ CD4⁺ T cells represented the naive population with the collective ability to recognise almost any antigenic determinant. Following activation by antigen, these cells lose OX-22 expression and become memory cells.

Strong doubt has recently been cast on the unidirectional nature of the activation induced phenotypic change from CD45R⁺ to CD45R0 by the work of Bell and Sparshott (1990). In experiments in which nude rats were reconstituted with CD45R⁺ or CD45R0 CD4⁺ thoracic duct lymphocytes, the populations expanded and, in the early stages of observation,

were shown to be CD45R0 phenotype, regardless of the phenotype of the injected cells. These results are essentially identical to those of Powrie and Mason (1989). However, longer term observation of the reconstituted T cell populations demonstrated the emergence of a CD45R⁺ population constituting over one third of the total. Bell and Sparshott provided evidence to suggest that this population was unlikely to be the result of the preferential expansion of a small number of CD45R⁺ contaminants. Future investigations may therefore result in revision of the theory that transfer from naive to memory phenotype is unidirectional.

*pops from two diff mouse strains
CD45R⁻ CD45R⁺ CD45R⁻ CD45R⁺
→ 1 2*

In the mouse system, antibodies to restricted CD45 epitopes have only recently become available (Luqman et al., 1991). Prior to this, two major murine CD4⁺ T cell subsets, T_h1 and T_h2, were identified by analysis of clonal T cell lines (Bottomly, 1988). The T_h2 lines showed many of the characteristics of naive (OX-22⁺) normal T cells, including expression of high molecular weight CD45 isoforms (Kim et al., 1985; Luqman et al., 1991). The T_h1 lines appear to express the CD45R0 isoform.

Exactly how the murine system relates to the rat and human systems is unclear, although it is unlikely that the T_h2 cell lines are directly equivalent to normal naive cells as they will have been repeatedly stimulated by antigen (Powrie and Mason, 1988).

1.7 Other T cell surface molecules.

(a) CD2.

CD2 (also known as T11) was identified as a T cell specific sheep red blood cell

receptor on human T cells (Lay et al., 1971). It is a 50 kD transmembrane nonpolymorphic protein with a 185 amino acid extracellular domain and a 116-117 amino acid cytoplasmic tail (Williams et al., 1987; Sewell et al., 1986; Yagita et al., 1988). The physiological ligand for CD2 is lymphocyte function associated antigen-3 (LFA-3) (Dustin et al., 1987), a widely distributed cell surface glycoprotein believed to be involved in cell adhesion (Springer et al., 1987).

Combinations of anti-CD2 antibodies which are directed against different epitopes on the CD2 molecule, such as OX-54 and OX-55 in the rat, can stimulate T cell proliferation (Clark et al., 1988). When employed singly, these antibodies appear to have little effect. Stimulation of T cells via the CD2 pathway induces many of the activation events associated with stimulation via the TCR (Meuer et al., 1984; Alcover et al., 1986; Pantaleo et al., 1987), but it is not known how these two receptors are associated. CD2 and the TCR are reported not to be physically linked (Reinherz et al., 1982), whilst Schraven et al. (1990) suggest that an association may exist between CD2 and CD45.

CD2 expression does not appear to be necessary for activation via the TCR (Moingeon et al., 1988), although in many cases expression of the TCR/CD3 complex is necessary for activation via CD2 (Breitmeyer et al., 1987, Brockenstedt et al., 1988). Stimulation via CD2 results in serine phosphorylation of CD3 γ (Breitmeyer et al., 1987), thought to be a protein kinase C mediated event (see below). Weissman et al. (1988) did not find any evidence of CD2-induced tyrosine phosphorylation of the TCR associated ζ chain, although, using different anti-CD2 antibodies, Monostori et al. (1990) did. CD3 γ serine phosphorylation and ζ tyrosine phosphorylation are both events associated with TCR-mediated activation (Patel

et al., 1987, Baniyash et al., 1988).

(b) CD5.

The anti-rat CD5 antibody, OX-19 recognises a 69 kD glycoprotein expressed on thymocytes and all peripheral T cells (Dallman et al., 1982; Dallman et al., 1984). Whilst antibody crosslinking of CD5 alone does not appear to induce any stimulatory response (Dallman et al., 1984), anti-CD5 antibodies are able to augment the mitogenic response to PHA or concanavalin A (Dallman et al., 1984) and to anti-CD3 antibodies (Ceuppens and Baroja, 1986).

1.8 T cell activation.

When processed antigen is presented to a T cell in the context of MHC proteins by an antigen presenting cell, a complex series of biochemical events is initiated which ultimately result in transcription of activation associated genes, induction of specialised effector functions and proliferation.

(a) External signals resulting in T cell activation.

Antigen stimulated T cell activation can be mimicked by a number of artificial means. Antibodies directed against the T cell receptor or CD3 are commonly used. In general these

must be crosslinked by secondary antibody, or bound to the Fc receptors on monocyte accessory cells (Van Wauwe et al., 1980), or immobilised on plastic tissue culture plates or Sepharose beads (Williams et al., 1985). There is, however, a wide variation in the requirement for crosslinking and accessory signals between different anti-TCR/CD3 antibodies (Janeway et al., 1988). Some antibodies require no crosslinking, others require crosslinking only (Geppert and Lipsky 1987), while others require crosslinking and additional soluble factors such as interleukin 1 (Williams et al., 1985) or interleukin 2 (Hunig et al., 1989). Determination of the cause of this variation could provide a valuable insight into the mechanism of T cell activation.

Antibodies to many other surface receptors including CD2, CD4, CD5, CD8 and CD28, but not CD45, have also been shown to be capable of inducing at least some of the activation events induced by anti-TCR/CD3 antibody crosslinking (reviewed in Geppert et al., 1990). Whether or not these molecules transmit signals under physiological conditions is unknown, but it may be that activation of T cells by antigen-presenting cells requires the occupation of multiple surface molecules which each contribute to the generation of the overall signal.

Activation of T cells by lectins such as Con A or PHA appears to require the expression of a functional T cell receptor complex (Weiss and Stobo, 1984, Ohashi et al., 1985). Lectins crosslink a range of surface molecules by binding to specific sugar residues. It is possible that they exert their mitogenic effect by heterologous receptor crosslinking.

Phorbol esters in conjunction with calcium ionophores can also induce T cell activation

by mimicking the effect of diacylglycerol, inositol 1,4,5-trisphosphate (IP_3) and inositol 1,3,4,5-tetrakisphosphate (IP_4) on protein kinase C activation and calcium influx respectively (see below).

(b) Intracellular events leading to T cell activation.

Many activation events have been identified, including phosphatidylinositol (PI) metabolism, calcium mobilisation, serine and tyrosine phosphorylation and the initiation of DNA synthesis, but it is not yet clear exactly how they are interconnected. Much of the current theory is based on knowledge of the order in which the activation events occur and the use of agents which specifically affect components of the biochemical processes. This section is therefore intended to give a general overview of our current knowledge of events which occur following stimulation of T cells, so that the studies presented in this thesis can be placed in the correct context.

i. Phosphatidylinositol metabolism.

One of the earliest activation events so far identified is the tyrosine phosphorylation of two molecules with apparent molecular weights of 100 and 135 kD (June et al., 1990). The identity of these molecules is unknown, but it is possible that the higher molecular weight substrate is phospholipase C γ (PLC γ), one of a family of at least five phosphoinositide specific PLC's. This enzyme catalyses the hydrolysis of the phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), into IP_3 and diacylglycerol (DAG) (Michell, 1975; Berridge, 1984). It appears that tyrosine phosphorylation of PLC γ is required for activation of the enzyme (Mustelin et al., 1990), possibly by enabling it to overcome the

PLC γ Ca^{2+} spec. mostly cytosolic

Heads PLCs by \oplus in resp to ligands

inhibition caused by the association of the actin-binding protein profilin, with PIP_2 (Goldschmidt-Clermont et al., 1991). This would also suggest a role for this phospholipid in the control of cytoskeletal organisation (see below).

PIP_2 itself is generated by the action of PIP kinase on PIP, which in turn is generated by the action of a 60 kD membrane protein, type II PI kinase, on phosphatidylinositol (PI). In resting T cells, most of the inositol phosphates are in the form of PI (Berridge, 1984, Majerus et al., 1984), and a state of equilibrium exists between the levels of PI, PIP and PIP_2 .

The products of PIP_2 hydrolysis, IP_3 and DAG, each play important roles in subsequent signalling events. IP_3 induces release of calcium from intracellular stores (Streb et al., 1983) by binding to a receptor on internal cell membranes (Ferris et al., 1989) and is converted to IP_4 by IP_3 -3-calcium/calmodulin-dependant kinase. IP_4 appears to alter the plasma membrane permeability to extracellular calcium (Irvine and Moor, 1986). Diacylglycerol activates the calcium and phosphatidylserine dependant kinase, protein kinase C (PKC) (Sano et al., 1983).

ii. Protein kinase C.

Protein kinase C (PKC) exists as at least eight different but closely related isozymes (Ono et al., 1988). These isozymes differ in their calcium and phospholipid dependency (Sekiguchi et al., 1988; Ono et al., 1988) and intracellular distribution (Kiley and Jaken, 1990; Ito et al., 1988). Thus the multitude of cellular phosphorylation events which have

been attributed to PKC (for review see Nishizuka, 1986), may be under the individual control of the different isozymes.

The enzyme consists of two main domains, a regulatory domain and a catalytic domain. The regulatory domain contains an amino acid sequence which acts as a pseudosubstrate for the catalytic domain (House and Kemp, 1987), suggesting a method by which the kinase could be controlled. Proteolysis by a calcium dependant protease leads to calcium, phosphoserine and DAG independant activation (Kishimoto et al., 1983), presumably by separating the catalytic and regulatory domains.

The exact mechanism of PKC activation by DAG is unknown. However, DAG has the effect of greatly increasing the affinity of PKC for calcium. Thus the enzyme can become fully active without a net increase in intracellular free calcium (Kishimoto et al., 1980).

Tumour promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) and 12-O-tetradecanoylphorbol 13-acetate (TPA) have a molecular structure similar to DAG and can activate PKC directly (Castaga et al., 1982), as can synthetic DAG analogues (Lapetina et al., 1985). The artificial PKC activators are, however, fundamentally different from DAG in that DAG is very rapidly metabolised into inositol phospholipids or arachadonic acid, whereas phorbol esters and DAG analogues persist for much longer periods, resulting in greatly extended PKC activation. Prolonged exposure of cells to phorbol esters results in down regulation of PKC (reviewed in Blumberg et al., 1984).

Protein kinase C phosphorylates serine and threonine, but not tyrosine, residues on a broad range of cellular substrates. These include CD45 (Auturo and Gahmberg, 1987), CD3 γ and ϵ chains (Cantrell et al., 1985), CD4 (Acres et al., 1986), p56^{lck} (Veillette et al., 1988), the protooncogene product *c-raf*, (Siegel et al., 1990) and numerous cytoskeletal components (Patarroyo and Gahmberg, 1984; Burn et al., 1988) (see also review by Kikkawa and Nishizuka, 1986). It is notable that CD2 does not appear to be phosphorylated by PKC (Chatila ^{and Gehen} et al., 1988). PKC has been implicated in the control of many intracellular events including calcium levels (Tsien et al., 1982), pH (Burns and Rozengurt, 1983) and activation of enzymes such as the serine/threonine kinase, *c-raf* (Siegel et al., 1990).

iii. Other protein kinases.

There are two further major groups of protein kinases which, evidence suggests, play important roles in T cell activation. These are the tyrosine kinases and the cyclic nucleotide dependant kinases exemplified by protein kinase A. The role of tyrosine phosphorylation in T cell activation is discussed in detail in chapter 5 and so will not be dealt with here.

Protein kinase A (PKA) is activated by cyclic AMP, which appears to generate an inhibitory signal for T cell activation (Krammer, 1988). Like PKC, PKA phosphorylates serine and threonine residues, and many of the PKC substrates can also be phosphorylated *in vitro* by PKA, but a distinct set of PKA substrates also exists (Krammer, 1988). Effects of PKA include inhibition of PI hydrolysis and of serine phosphorylation of the CD3 γ chain and tyrosine phosphorylation of the ζ chain (Klausner et al., 1987). These effects can be reversed by phorbol esters (Patel et al., 1987), suggesting dominance of PKC activation over PKA.

The role of cyclic GMP dependant kinases (PKG) is poorly defined and the substrates are unknown. They appear to positively affect T cell responses to mitogen (reviewed by Haddon, 1988).

iv. Calcium mobilisation.

The calcium ion concentration in resting T cells is approximately 100 nM (Tsien, 1981). Stimulation with mitogens such as Con A or PHA or with antibodies to the TCR or certain other T cell surface molecules, results in an increase in intracellular free calcium to 200-250 nM (Weiss et al., 1984; O'Flynn et al., 1985; Alcover et al., 1986; Altman et al., 1990). More sensitive studies have been undertaken in the human leukemic T cell line Jurkat. Stimulation of Jurkat cells with anti-CD3 antibodies results in a rapid (within 60 s) fivefold increase in intracellular free calcium which plateaus at approximately 250 nM for at least a further 30 min before returning to basal levels (Imboden and Stobo, 1985). The early transient increase in cytoplasmic calcium is a result of IP_3 - induced release from the endoplasmic reticulum (see above). The sustained calcium increase is the result of influx from outside the cell, and is thought to involve IP_4 (Irvine and Moore, 1986).

Antibodies directed against a wide range of surface receptors including CD2, CD3, CD4, CD5, CD6, CD7, CD8 and MHC class I can also stimulate an increase in intracellular calcium (Ledbetter et al., 1987; Beyers et al., 1989). Stimulation with combinations of CD2 antibodies can ultimately induce proliferation, but when anti-CD4 antibodies are used the increase in calcium is generally not accompanied by a proliferative response (Williams and Beyers, 1989).

prog. inn. ✓

v. GTP binding proteins.

Classical GTP binding proteins (G proteins) consist of an α , β , γ heterotrimer, which dissociates into α and $\beta\gamma$ subunits upon the exchange of α -bound GDP for GTP. The α subunit then binds to and stimulates or inhibits an effector enzyme. The endogenous GTPase activity of the α subunit eventually hydrolyses the GTP to GDP, causing dissociation of α from the enzyme, permitting the heterotrimeric complex to reform (for a review of G proteins see Gilman, 1987).

There is some evidence to suggest that G proteins are involved in T cell activation. This is mostly based on the fact that G protein activators, AlF_4^- and a non-hydrolysable GTP analogue, guanosine 5'-O-(3-thiotriphosphate) ($\text{GTP}\gamma\text{S}$), can reproduce the effects of TCR/CD3-mediated stimulation such as PIP_2 hydrolysis (O'Shea et al., 1987), PKC-mediated CD3 γ and ϵ chain phosphorylation (O'Shea et al., 1987), and IL-2 production (Aussel et al., 1988). Exactly how these, as yet unidentified G proteins might couple the TCR complex to downstream signalling events is unknown.

ALC β - Not S. G. Rice

Another type of G protein is represented by $\text{p}21^{\text{ras}}$ the product of the proto-oncogene *c-ras*. This is a small 21 kD GTP binding protein which has been implicated in coupling receptors to PI metabolism (Michell and Kirk, 1986) and also to events downstream of the PI pathway (Yu et al., 1988). The active form of the molecule is in the GTP-bound state (Field et al., 1987; Satoh et al., 1987; Satoh et al., 1987). Recently Downward et al. (1990) showed that stimulation of T cells with PHA or an anti-CD3 antibody caused $\text{p}21^{\text{ras}}$ to change from the GDP-bound to the GTP-bound state. This effect can also be mimicked by PKC activators but not by calcium ionophores suggesting that PKC is primarily responsible for the

intracellular activation of p21^{ras}. In B cells p21^{ras} has also been demonstrated to co-cap with surface immunoglobulin (Graziadei et al., 1990) suggesting that it is involved in this signalling pathway.

vi. Nuclear events.

The ultimate effect of the cascade of biochemical events resulting from T cell stimulation is the coordinated sequential activation of genes for proliferation and differentiated cellular functions. However, the precise role of these events in conveying a signal to the nucleus is unclear, as there are some reports of IL-2 gene expression and proliferation in the absence of PI hydrolysis and calcium mobilisation (Sussman et al., 1988, Holter et al., 1986).

A recent review by Crabtree (1989) listed over 70 gene products regulated during T cell activation. Even this list is likely to be significantly short of the total number of genes regulated.

Among the genes which show increased transcription during T cell activation are the protooncogenes *c-fos*, *c-myc*, and *c-myb*, the gene for NFAT-1 (nuclear factor of activated T cells) and the genes encoding interferon γ (IFN γ), IL-2, IL-2 receptor (IL-2R), transferrin receptor and histone H3 (Reed et al., 1986, Granelli-Piperno et al., 1986, Kronke et al., 1985)

NF- κ -B
histone promoter
region
IL-2, IL-2R

The exact functions of the proto-oncogenes are unknown. *c-fos* is thought to be involved in the transition of the cell from the G₀ to the G₁ phase of the cell cycle, while *c-myc* may play a role in entry into the S-phase (Heikkila et al., 1987). Both these genes are

activated within 30 min of cell stimulation (Reed et al., 1982) and are termed immediate nuclear activation events. *c-myb*, which encodes a DNA binding protein, is expressed later (16 h) and is regulated in parallel with the histone H-3 gene (Torelli et al., 1985).

NFAT-1 is expressed approximately 20 minutes after stimulation. It is a DNA binding protein which binds to one of the enhancer regions in the IL-2 gene (Shaw et al., 1988, Crabtree, 1989) and is thought to be essential for activation of the IL-2 gene.

The activation of the IL-2 and IL-2R genes is essential for T cell proliferation and requires extended exposure of the T cell to activating ligand (Goldsmith et al., 1988).

vii. Interleukin 2.

The IL-2 gene is activated approximately 45 minutes after T cell stimulation (Wiskocil et al., 1985) and the IL-2R gene at about 2 h (Leonard et al., 1985).

T cell activation initiated by the TCR complex or via CD2 induces the expression of high affinity IL-2 receptors and the secretion of IL-2. Interaction of IL-2 with its receptor results in internalisation of the IL-2/IL-2R complex and induces the activated T cell to enter the S phase.

One of the effects of IL-2 binding to its receptor is phosphorylation of the receptor and of a number of other membrane proteins (Gaulton and Eardly, 1986). There is some evidence to suggest that the IL-2R is associated with a protein kinase (Benedict et al., 1987), but the immediate consequences of receptor induced phosphorylation events are unknown.

IL-2 seems to utilise a different signal transduction pathway to that employed by the T cell receptor, as it does not appear to affect intracellular free Ca^{2+} concentration or PI hydrolysis (Mills et al., 1985; Mills et al., 1986). Evidence for the role of other signalling pathways such as cAMP and G proteins is contradictory, and the exact mechanism of IL-2 mediated T cell activation waits to be determined.

viii. Differential activation of CD4⁺ T cell subtypes.

The identification of CD4⁺ T cell subtypes according to expression of CD45 isoforms has already been mentioned (section 1.6b). It is apparent that there is some distinction between the activation requirements of the CD45R⁺ and CD45R0 T cell subsets, and in their responses to activating ligands.

Byrne et al. (1988) found that CD45R0 cells produced IL-2, expressed IL-2R and proliferated in response to anti-CD3 antibody, whereas CD45R⁺ cells did not. However addition of rIL-2 to the CD45R⁺ cells induced them to express IL-2R, and to proliferate almost as well as the CD45R0 cells.

The proposal that CD45R0 cells produce a greater proliferative response to anti-CD3 antibody than CD45R⁺ cells was contested by Geppert et al. (1990), who suggested that the response observed depends on the time after activation that proliferation is assessed. They reported that a six day incubation period (as opposed to four days used by Byrne et al., 1988) resulted in greater proliferation among the CD45R⁺ cells.

These results would tend to suggest that while CD45R0 T cells proliferate more rapidly

in response to stimulation, CD45R⁺ cells have a greater capacity to proliferate in the long term. The exact nature of this difference remains to be determined.

Also in contrast to the results of Byrne et al., Salmon et al. (1989) found that stimulation of T cell populations with PMA and calcium ionophore or PHA, resulted in high levels of IL-2 mRNA production by CD45R⁺ cells and low levels of IL-4 and IFN- γ mRNA, whilst CD45R⁰ cells produced high IL-2, IL-4 and IFN γ mRNA. Arthur and Mason (1986) found that CD45R⁰ cells did not produce IL-2. However this appeared to depend on the serum content of the incubation medium, as replacing rat serum with human serum resulted in production of IL-2 by CD45R⁰ cells.

The consensus would appear to suggest that CD45R⁰ CD4⁺ cells produce IL-2, IL-4 and IFN γ , and proliferate rapidly in response to an activation stimulus, whereas CD45R⁺ CD4⁺ cells produce IL-2 but not IL-4 and IFN γ , and proliferate more slowly but ultimately more extensively than CD45R⁻ cells.

1.9 Receptor capping.

The first report of surface receptor capping was produced by Taylor et al. (1971). They showed that, following binding of fluorescein conjugated anti-Ig serum to isolated spleen cells and warming to room temperature or 37°C, the labelled receptors collect firstly into small patches and then into a single surface accumulation called a cap.

Lectins and antibodies are equally capable of inducing cap formation, but this is very

much dependant on the valency of the ligand. The study by Taylor et al. showed that univalent Fab antibody fragments were unable to induce cap formation unless a secondary crosslinking antibody was used. Crosslinking is similarly required when most monoclonal antibodies are used. The same appears to apply to lectins, such that the divalent derivative of the lectin Con A is unable to induce capping, whereas the tetravalent form is (Unanue et al., 1972; Yahara and Edelman, 1973; Gunther et al., 1973).

(a) Mechanism of capping.

Although capping was first described twenty years ago, the exact mechanism by which it occurs is not clearly understood.

There are two main theories which attempt to explain how capping occurs. One, the "lipid flow model", implies a passive redistribution of receptors, while the other involves an active mechanism directed by the attachment of surface receptors to the membrane cytoskeleton.

The lipid flow model proposes that there is a continual oriented flow of membrane lipid, with insertion into the plasma membrane at a particular site and removal at another by endocytosis, resulting in a constant cycling (Bretscher, 1976). It is argued that aggregations of protein molecules within the membrane, such as antibody- or lectin-induced patches, would be swept along with the lipid flow, resulting in an accumulation at the site of membrane removal. According to this theory, smaller particles would be able to diffuse freely and so be able to overcome the lipid flow, thereby displaying an essentially random

distribution when not crosslinked.

Although this theory is currently out of favour, several observations have been made for which it is difficult to impose a role for the cytoskeleton. For example, glycolipid, experimentally introduced into the outer leaflet of the plasma membrane can be induced to cap following crosslinking (Stern and Bretscher, 1979). Also, crosslinking of MHC antigens lacking a cytoplasmic domain still results in their capping, even though it is very unlikely that the molecule could be interacting directly with the cytoskeleton (Murre et al., 1984).

It is quite possible that two mechanisms of receptor capping could exist. One passive, directed by lipid flow, or random diffusion of antibody bound membrane proteins, the other active, directed by the cytoskeleton and requiring energy. Indeed, Braun and Unanue (1983) have identified two types of receptor on the basis of capping characteristics. The first group, exemplified by surface immunoglobulin, cap rapidly, require a low degree of crosslinking and are sensitive to metabolic inhibitors such as sodium azide and to microfilament disrupting drugs such as cytochalasin (Taylor et al., 1971; de Petris, 1974). The second group, of which CD45 is an example, cap slowly, require extensive crosslinking and display only limited pharmacological sensitivity (Braun et al., 1982; and C.Turner, 1986 D.Phil. thesis, University of Oxford).

A majority of studies, however, have concerned the role of the cytoskeleton in receptor capping, and it is this that will be discussed further.

(b) Involvement of the cytoskeleton.

Initial indicators that an active, cytoskeletal mediated, mechanism could be responsible for the redistribution of crosslinked receptors, came from the observation that in many cases, capping could be inhibited by drugs such as sodium azide and cytochalasin B (Taylor et al., 1971, de Petris, 1974).

Many groups have reported a partial accumulation of cytoskeletal components under the cytoplasmic face of capped receptors. These include actin (Bourguignon and Singer, 1977, Toh and Hard, 1977, Gabbiani et al., 1977, Oliver et al., 1977, Singer et al., 1978), myosin (Bourguignon and Singer, 1977, Bourguignon and Rozek, 1980), α -actinin (Geiger and Singer, 1979, Hoessli et al., 1980), spectrin (Nelson et al., 1983) and talin (Burn et al., 1988). The fact that these molecules are associated with the caps suggests that they might be involved in linking transmembrane proteins to the actin cytoskeleton, which could then produce the contractile force necessary to draw the receptors together. These observations provide important information on the role of the cytoskeleton in receptor capping.

The lymphocyte cytoskeleton exists as a complex array of interacting proteins which is concentrated immediately beneath the plasma membrane (Schreiner and Unanue, 1976, also see Geiger, 1983 for an extensive review of membrane-cytoskeletal interactions). Due to its highly polymeric state, the cytoskeleton can be isolated as an intact insoluble residue following treatment of the cells with non-ionic detergents (Yu et al., 1983, Weber and Osborn, 1979). This technique has provided further evidence for the involvement of the cytoskeleton in receptor capping. Detergent extraction of the cytoskeleton complex has

revealed ligand induced co-isolation of surface Ig (Braun et al., 1982), the T cell receptor complex (Marano et al., 1989), and Con A receptors (Painter and Ginsberg, 1982, Patton et al., 1989).
det. ind.

*+ Capping & Cytoskeleton
CD45 = CD8 = CD2 molecule
CD45 = TCR
CD45 = TCR induced*

Electron microscopical analysis of capped surface immunoglobulin (De Petris, 1975) and Con A receptors (Albertini and Anderson, 1977) have shown a realignment of actin filaments parallel to the plasma membrane, thus providing a further indication that the cytoskeleton is involved in receptor capping.

Numerous theories have been proposed to explain how the cytoskeleton might direct receptor redistribution. Bourguignon and Singer (1977), proposed the existence of a "protein X" which could provide a common link between crosslinked receptors and the cytoskeleton. Contraction of the cytoskeleton is then proposed to occur in a calcium-dependant manner analagous to muscle contraction. Bourguignon et al. (1985) later claimed to have identified this "protein X" as being a 180 kD transmembrane glycoprotein linked to the cytoskeletal protein fodrin (spectrin) in mouse T lymphoma cells. It is thought that this molecule is CD45. The exact method by which membrane proteins could become attached to the cytoskeleton awaits elucidation, but there are numerous clues as to how the capping mechanism may be controlled, suggesting that it is intimately linked with the process of cell activation.

(c) Control of capping.

It is notable that, in many cases, the procedures used to induce surface receptor capping

also induce PI metabolism, PKC activation and an increase in intracellular free calcium. Therefore, it is not unreasonable to suppose that receptor capping may be under the control of some or all of these components.

PIP₂, produced by the action of PIP kinase on PIP (see section 1.8bi), is capable of dissociating complexes of actin and profilin (profilactin) which leads to the polymerisation of the free actin monomers (Lassing and Lindberg, 1985). It appears that once profilin is bound to PIP₂, PIP₂ can be metabolised to IP₃ and DAG only by the tyrosine phosphorylated form of PLC γ (Goldschmidt-Clermont et al., 1991). PI, PIP and PIP₂ are in equilibrium such that metabolism of PIP₂ will lead to increased PI and PIP metabolism and hence to the production of fresh PIP₂ which could in turn dissociate further profilin-actin complexes. The dissociation constants of the profilin-actin monomer (Stossel et al., 1989) suggest that the profilin released by PIP₂ metabolism would not rapidly reassociate with actin. A similar model to this has recently been proposed by Stossel (1989).

Thus, it is possible that PIP₂ could be involved in the establishment of the contractile network responsible for drawing the surface molecules into the cap.

In order for receptors to be capped by the action of the cytoskeleton, they must first somehow be linked to it. Protein kinase C, activated by DAG, itself one of the products of PIP₂ metabolism, is an attractive candidate for performing this function. Upon activation via phorbol esters (Kraft and Anderson, 1983) or anti-CD3 antibodies (Nel et al., 1987), PKC redistributes from the cytosol to the plasma membrane. Burn et al. (1988) reported that activators of PKC, such as phorbol esters, induced the association of integrins (a family of

integral membrane proteins that act as receptors for extracellular matrix proteins such as fibronectin) with talin, a cytoskeletal component. A transmembrane glycoprotein gp85, identified by Kalormis and Bourguignon (1988), was found to associate with PKC, and PKC activation by the phorbol ester TPA significantly enhanced gp85 affinity for the cytoskeletal protein ankyrin (Kalormis and Bourguignon, 1989), which appears to link spectrin to membrane glycoproteins (Bennett, 1982). Related to this, the link between spectrin and actin via band 4.1 appears to be regulated by PIP₂ (Anderson and Marchesi, 1985).

PKC activators have also been demonstrated to enhance the capping of Con A bound receptors (Patarroyo and Gahmberg, 1984), possibly by enhancing interactions between receptors and the cytoskeleton.

The other product of PIP₂ metabolism is IP₃, which is involved in the release of calcium from intracellular stores. It is well established that calcium is involved in the contraction of both skeletal and smooth muscle, and there is some evidence to suggest that it may be involved in the capping process. Bourguignon and Pressman (1983) found that low concentrations of the ionophore monensin, which promotes the influx of Na⁺ in exchange for K⁺, stimulated receptor capping. It was concluded that the influx of Na⁺ stimulated an increase in intracellular Ca⁺. The calcium binding protein, calmodulin, has also been demonstrated to be associated with cap formation (Nelson et al., 1982, Salisbury et al., 1981).

Capping inhibited by Ca²⁺ antagonists

Further analogies between capping and the calcium mediated contraction of smooth muscle can be drawn from the observation that myosin light chain kinase (MLCK), a 130 kD protein involved in smooth muscle contraction, is phosphorylated during capping (Bourguignon

MLCK (P) in receptor capping
204.

et al., 1981) and preferentially accumulates under Con A-induced caps (Bourguignon et al., 1982). Kerrick and Bourguignon (1984) further demonstrated that use of agents to control MLCK activity similarly affected smooth muscle contraction and con A receptor capping.

MLCK
Ca²⁺ + G₁₂
activated

Although there are no definitive reports concerning the biochemical pathways controlling surface receptor capping, it is apparent that there is a body of circumstantial evidence indicating a link between the intracellular processes that occur following T cell stimulation, and capping. Thus PIP₂ may be involved in the establishment of a cytoskeletal network, PKC may induce receptor-cytoskeleton associations, and contraction may be mediated by calcium in a manner similar to that of smooth muscle. The close association between the two may partly explain the difficulty in identifying the capping mechanism.

(d) Physiological significance of capping.

Whilst capping often occurs under conditions which lead to cell activation, there is little evidence to indicate that capping is necessary for activation. It would be logical to suppose that a reaction which requires energy and uses cellular resources to redistribute the cytoskeleton, must be important in the physiology of the cell. It should be remembered however, that using soluble, crosslinked antibodies to stimulate T cells and to induce receptor capping results in an entirely artificial situation in which, theoretically, the whole population of a particular receptor species is crosslinked. It could therefore be argued that active receptor redistribution is an artifact induced by this excessive receptor crosslinking,

and designed as a "garbage clearance" mechanism, to rid the cell of unwanted aggregates.

Kupfer, Singer and their colleagues have addressed this argument in an elegant series of experiments using a specific antigen expressed on the surface of antigen presenting cells (APC) to bind to the receptors on T cell clones (Kupfer et al., 1987a; Kupfer et al., 1987b; Kupfer and Singer, 1989a; Kupfer and Singer, 1989b). They have shown that specific direct interaction between a T cell and an APC resulted in: (i) redistribution of the T cell receptor and CD4 to the site of interaction (Kupfer et al., 1987b); (ii) the accumulation of talin under the membrane of the T cell where it is in contact with the APC (Kupfer et al., 1987a and b); (iii) the reorientation of the microtubule organising centre and the golgi apparatus inside the T cell to face the contact region with the APC (Kupfer et al., 1986). In this case, only a restricted population of receptors is initially bound, suggesting that redistribution of receptors does occur under physiological conditions. The redistribution of the cytoskeletal protein talin and the fact that redistribution is antigen-specific, suggests that an active mechanism is involved, as opposed to receptors accumulating at the point of contact as a result of binding to the appropriate ligand on the APC during random diffusion.

Further evidence for a physiological role for capping comes from the work of Weder and Parker (1976), who demonstrated that lymphocyte activation responses could be inhibited by cytoskeletal disrupting agents. One interpretation of this is that the disruption of the cytoskeleton prevented the coordinated redistribution of surface receptors, thereby preventing the interactions necessary for full activation. More recently, Geppert and Lipsky (1990) have undertaken similar work and shown that induction of proliferation and IL2 production in CD4⁺ T cells by antigen-presenting cells or anti-CD3 antibodies is inhibited by cytochalasins

B and D, drugs known to inhibit capping. As with many studies in which drugs are shown to inhibit a particular response, one cannot be absolutely certain that the drug is not affecting substrates other than the known target, in this case the microfilament network.

1.10 Aims of this thesis.

The rapidly increasing information on the role of tyrosine phosphorylation in T cell activation and the discovery that many surface receptors are associated with kinases and phosphatases (see chapter 5), suggests that these molecules may interact with each other during the process of T cell stimulation. Particularly interesting in this respect are the T cell antigen receptor (TCR), CD4 and CD45 which have been suggested to be physically (see chapter 3) and functionally (see chapter 4) associated.

The aim of this thesis therefore, is to determine whether these molecules become physically ~~h~~associated during specific antibody crosslinking, and to relate this to the effect on cell activation and tyrosine phosphorylation events. In doing this, an attempt is made to present a role for the active redistribution of surface membrane proteins (capping) in T cell activation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Buffers.

(a) Phosphate-buffered saline

Phosphate-buffered saline (PBS) pH7.4 comprised 1.7g Na_2HPO_4 (BDH), 0.43g NaH_2PO_4 (BDH), 18.12g NaCl (BDH) made up to 2 litres with distilled H_2O .

(b) Tris-buffered saline.

Tris-buffered saline (TBS) contained 10mM Tris (BDH) and 155mM NaCl and was titrated to the required pH with HCl.

2.2 Experimental animals.

DA rats bred in the animal house of the Department of Zoology, Oxford were used.

2.3 Tissue culture medium.

RPMI 1640 medium

10.43g RPMI 1640 powdered tissue culture medium (Gibco) was dissolved in 1 litre Analar water (BDH). To this was added 2g NaHCO₃ and 10ml 10000 u/ml penicillin-10000 u/ml streptomycin solution (Gibco). The solution was then filtered under sterile conditions through a Falcon 7105 0.22 micron bottle top filter into 100ml bottles. When required heat inactivated foetal calf serum (FCS) was added to 10%.

2.4 Antibodies used.

The monoclonal antibodies used in these experiments are described in table 2.1 below. Polyclonal antibodies were generally obtained from Sigma or Serotec, and are described within the relevant chapters.

2.5 Preparation of a single cell suspension of rat lymph node cells.

The rat was killed by exanguination following etherisation and the mesenteric and cervical lymph nodes immediately removed. Following washing in RPMI medium plus 10% FCS the lymph nodes were pressed through a stainless steel sieve using the plunger of a 5ml plastic disposable syringe. The resulting cell suspension was passed through a whisp of cotton wool to remove aggregates. The cells were then washed twice in RPMI medium plus 10% FCS by centrifugation at 400xg (1660 rpm, Jouan CT422) for 5 minutes. If sterile cells were required, the rat was dissected in a class II tissue culture hood using sterile instruments. The steel sieve was autoclaved and the syringe plunger washed with methanol before use.

Antibody	Antigen	Source	Details
OX-1	rat CD45	Dunn School*/ Serotec	Mouse IgG1, recognises all isoforms. ref: Sunderland et al., 1979.
R73	rat α/β TCR	T. Hunig*/ Serotec	Mouse IgG1, recognises a non -polymorphic determinant on α/β receptor. ref: Hunig et al., 1989.
W3/25	rat CD4	Dunn School*/ Serotec	Mouse IgG1, ref: Williams et al., 1977.
OX-19	rat CD5	Serotec	Mouse IgG1, ref: Dallman et al., 1982.
OX-8	rat CD8	Serotec	Mouse IgG1, ref: Brideau et al., 1980.
56A	human β -spectrin	produced in Shotton lab.	Isotype unknown, does not cross-react with rat lymphocytes.
F10-89-4	human CD45	Serotec	Mouse IgG2a, recognises all isoforms. ref: Dalchau et al., 1980
UCHT1	human CD3	Serotec	Mouse IgG1, ref: Beverly and Callard, 1981.
DFT1	human CD43	Serotec	Mouse IgG1
B-H1	human CD2	Serotec	Mouse IgG1

Table 2.1 Monoclonal antibodies used.

* **Antibody Sources:**

Dunn School; MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, South Parks Road, Oxford.

T. Hunig; Professor T Hunig, Gen-Zentrum der Universitat Munchen, Max-Planck Institut fur Biochemie, Munchen, Germany.

Serotec; 22 Bankside, Station Approach, Kidlington, Oxford.

2.6 Isolation of rat peripheral blood lymphocytes.

(a) Production of Ficoll/Triosil density 1.09

Solution A: 14% ficoll (Sigma) in distilled water.

Solution B: 33% triosil (Sigma) in distilled water.

96ml of solution A was mixed with 40 ml of solution B and the density checked at room temperature using a hydrometer. The solution was sterilised by passage through a 0.45 micron filter, aliquoted and stored at 4°C until use.

(b) Isolation of cells

5ml DA rat blood containing 100ul 1mg/ml heparin (sigma) was diluted 1:1 with RPMI 1640 medium . 3ml sterile Ficoll-triosil lymphocyte separation medium, density 1.09, was aliquotted into two 25ml conical bottomed screw top tubes (Sterilin) and 6ml blood gently pippetted on top of the separation medium so as to maintain a clear distinction between the two layers. The tubes were then centrifuged at 700g (1900rpm, Jouan CT422) for 30 minutes

at 24°C. The lymphocytes were then removed from between the two layers using a pasteur pipette and washed in RPMI medium.

2.7 Isolation of human peripheral blood lymphocytes.

Human peripheral blood lymphocytes were isolated using the same procedure as for rat except that Histopaque 1083 separation medium (Sigma) was used instead of Ficol-triosil.

2.8 Isolation of T cells

(a) Depletion of B cells using nylon wool

0.12g nylon fibre (Travenol) was soaked in 0.2M HCl for 10min and then washed in five changes of 50ml distilled water to remove the acid. The dried nylon wool was loosely packed into a 2ml disposable syringe barrel and, if required sterile, autoclaved. The column was then washed with 20ml RPMI plus 10% FCS then, with the top and bottom sealed, incubated at 37°C for 10 minutes and washed again with medium. 3×10^7 lymphocytes prepared either as in 2.6 or 2.7 were suspended in 0.5ml RPMI plus 10% FCS and added to the column which was then sealed at the bottom and a subsequent 0.5ml medium added. After incubating the sealed column at 37°C for 30 minutes the nylon fibre was eluted with 4ml medium at about 1ml/min to obtain the T cells.

(b) Depletion of B cells by panning

The viability of the cells was assessed by trypan blue (BDH) exclusion and was always found to be greater than 80% by this method. The purity of the T cell population was determined by incubating cells with the murine anti-alpha/beta T cell receptor antibody R73

(a gift of Dr T. Hunig, Munich, FRG. and Serotec) followed by fluorescently labelled goat anti-mouse IgG (experimental procedure as in Section 3.2). The cells were then viewed under a Zeiss epifluorescence microscope and found to be greater than 95% T cell receptor positive.

The technique of panning was also used to increase the proportion of CD4⁺ T cells using the anti-rat CD8 antibody OX-8.

2.9 Preparation of fixatives

(a) Paraformaldehyde

The required percentage of paraformaldehyde was prepared by stirring the paraformaldehyde powder with 50ml PBS at 70°C in a large conical flask. After the paraformaldehyde had dissolved 1 or 2 drops of 1N NaOH was added to clear the solution. This was cooled and further cleared by filtration through a Whatman No.1 filter paper. The fixative was then adjusted to the required pH.

(b) Glutaraldehyde

Glutaraldehyde was made fresh as required from a 50% stock solution (Agar Scientific).

2.10 Antibody purification by protein A-sepharose affinity chromatography.

(Ey et al., 1978)

(a) Reagents.

(i) 0.25 g Protein A coupled to sepharose CL-4B (Sigma)

(ii) 0.1 M sodium phosphate pH 8.0 (BDH)

- (iii) 0.1 M citric acid (BDH)
- (iv) 0.1 M sodium citrate pH 6.0 (BDH)
- (v) 2M tris base (BDH)
- (vi) 1 ml crude ascites.

The protein A separese was rehydrated in Sodium phosphate buffer and washed twice by centrifugation at 200g for 5 min (Jouen CT 422 cetrifuge).

Columns were made using 2ml plastic syringes with a piece of porous inert plastic as a gel support. 1ml of the protein A coupled beads was poured into the column . The rubber end of the syringe plunger was removed and a 1.1x50 syringe needle with the point removed was passed through it. The rubber end was then inserted into the top of the syringe barrel so that the tip of the needle was pointing upwards. A second needle with the tip removed was put on the end of the syringe and tubing fitted to each of the needles.

The column was then washed with 5 ml 0.1 M phosphate buffer, 2 ml citric acid solution, then another 5 ml phosphate buffer. The pH of the ascites was then adjusted to 8.0 by the addition of 2 M tris base and diluted with 1 ml of phosphate buffer. This was then applied to the column and the eluate collected. The column was then washed with 0.1 M phosphate buffer and 0.5 ml fractions collected. The absorbance at 280 nm was monitored and, when it had returned to basal levels, the IgG1 bound to the protein A was specifically eluted with 0.1 M sodium citrate, pH 6.0. Again 0.5 ml fractions were collected, and the fractions containing the purified antibody were identified by determining the absorbance at 280 nm. The antibody containing fractions were then pooled and dialysed overnight against

PBS.

2.11 Production of IgG Fab fragments.

(a) Reagents

- (i) 0.1 M sodium phosphate pH 7.0, containing 0.01M cysteine and 2 mM EDTA (BDH)
- (ii) 10 mg purified IgG in 0.1 M sodium phosphate buffer
- (iii) Papain (Sigma)
- (iv) Protein A sepharose column (see above)

(b) Procedure

1 mg of papain was dissolved in 100 μ l 0.1 M phosphate buffer, the 50 μ l of this was added to the IgG. The solution was then gently mixed and incubated at 37°C overnight. The Fab fragments were separated from the Fc fragments and undigested antibody on a protein A sepharose column, as described above. In this instance however, the unretarded fraction was retained, as this contained the Fab fragments, whilst the Fc fragments and undigested antibody bind to the protein A.

2.12 Enzyme-linked immunosorbant assay (ELISA)

The ELISA was generally used to test hapten modified antibodies. 50 μ l of dilutions of modified and unmodified protein were added per well of a 96 well flexible flat bottomed vinyl microtitre plate (Falcon) in triplicate. This was then left overnight at 4°C or for two

hours at room temperature and followed by washing x3 with PBS containing 0.05% Tween 20 (SIGMA) and 0.02% NaN_3 . The plates were then tapped on tissues to remove the remaining tween and blocked for at least two hours at room temperature by the addition of 200ul/well of 1% BSA in PBS. Following aspiration of the BSA the plates were washed x 3 with 0.05% Tween. Antibody directed against the hapten molecule was added at a concentration of 1-10ug/ml, 50ul/well diluted in PBS plus 0.1% BSA and incubated for 1 hour at room temperature. Following aspiration of antibody and washing as before, peroxidase conjugated antibody directed against the secondary antibody was added at a dilution of 1/1000 in PBS plus 0.1% BSA, 50ul/well and left at room temperature for 1 hour.

The substrate solution was made as follows; 4.86ml 0.1M citric acid (BDH) was added to 5.14ml 0.2M sodium phosphate (BDH) and to this was added 4mg O-phenylene diamine (Sigma) followed by 4ul 30% hydrogen peroxide (Boots the Chemists). 100ul of this solution was then immediately added to each well and as soon as the colour had developed, 200ul 0.5M sulphuric acid was added per well to stop the reaction. The plate was then read on a Titertek Multiscan ELISA plate reader, absorbance 2 and filters 4 and 8.

2.13 Biotinylation of proteins

(a) Reagents

- (i) Dimethylformamide (DMF)(Sigma)
- (ii) N-hydroxysuccinimidobiotin (NHS-biotin)(Sigma)
- (iii) 0.1M NaHCO_3
- (iv) 2 x PD-10 columns
- (v) glass tubes

(b) Transferring protein to conjugation buffer.

A PD-10 column was pre-equilibrated with 0.1M NaHCO₃ and 2ml of protein solution was added and eluted with 0.1M NaHCO₃. 0.5ml fractions were collected and the protein appeared in fractions 7 and 8. These were pooled and the protein concentration determined by absorbance at 280 nm.

(c) Conjugation

0.25 ml of 0.1M NHS-biotin dissolved in DMF was added per 1ml of protein solution and incubated in a glass tube at room temperature for 1 hour.

(d) Separation of biotinylated antibody from unbound NHS-biotin.

The biotinylated protein was then separated from the free NHS-biotin by passing the mixture through a PD-10 column pre-equilibrated with PBS, and eluting with PBS. 0.5ml fractions were collected and the conjugated protein was generally found in fractions 7 and 8 which were pooled.

The labelled antibody was tested by ELISA assay and stored at -20°C.

2.14 TNP modification of proteins

(a) Reagents

- (i) trinitrobenzenesulphonic acid (TNBS) (BDH)
- (ii) PD-10 G25 column (Pharmacia)
- (iii) PBS

(b) Conjugation

2ml protein at 0.5mg/ml in PBS was added to 2ml 20mM TNBS in PBS

neutralised with 1M NaOH, and incubated at 37°C for 1 hour.

The TNP modified protein was separated from the free TNP by passing the mixture through a PD-10 G25 column as described above for biotinylated proteins. The TNP modified protein was then tested by ELISA assay and stored at -20°C.

2.15 Fluorescein conjugation of antibodies

(a) Reagents

- (i) Conjugation buffer: 5.8ml of a 5.3% Na₂HCO₃ solution was added to 10ml of 4.2% NaHCO₃ and one part of this mixture was mixed with nine parts of 0.15M NaCl pH 9.5 at room temperature.
- (ii) Fluorescein isothiocyanate (FITC) (Sigma)
- (iii) 2 x PD-10 G25 columns (Pharmacia)
- (iv) PBS

(b) Desalting of antibody for transference into conjugation buffer.

The antibody was desalted by passage through a PD-10 (pre-equilibrated with conjugation buffer) and eluted with conjugation buffer. A maximum volume of 2.0ml of antibody solution was desalted in this way. 1.0ml fractions were collected and the protein appeared in fractions 3 and 4. These fractions were pooled and the pH checked to be 9.5.

(c) Conjugation

FITC was dissolved in the conjugation buffer to give a concentration of 1mg/ml, 0.3ml of FITC solution was added to each ml of protein solution (at 1-2mg/ml). This mixture was incubated for 3 hours at room temperature in the dark.

(d) Separation of FITC-antibody from unbound FITC

A PD-10 column was equilibrated with PBS. The FITC mixture was loaded onto the column and eluted with PBS. 1ml fractions were collected. The absorbance at 280nm was measured, and the conjugated protein was found in fractions 3 and 4. These fractions were pooled and the pH checked to be 7.0 to 7.5. The efficiency of conjugation was calculated by the method of Weir (1973) and should be between 0.5 and 1. The labelled antibody was stored at -20°C.

2.16 Gold conjugation of antibodies

(a) Adjusting the pH of the gold sol

15 nM colloidal gold sol was purchased from Biocell. Colloidal gold is unstable in the presence of electrolytes, but it can be stabilised by the addition of protein. The ideal pH for conjugation of gold to IgG is 9.0, however, measuring the pH of the sol without first stabilising the gold will damage the electrode. 5 ml of the sol was therefore stabilised by the addition of excess IgG (> 5µg/ml sol). The pH was then measured and the amount of NaOH required to adjust the pH to 9.0 was recorded. This information was then used to adjust the pH of the unstabilised gold sol.

(b) Determining the minimum amount of IgG required to stabilise the sol

200 µl samples of the sol were aliquoted into six LP4 tubes. A 50 µg/ml solution of the antibody was made and 0, 5, 10, 15, 20 or 25 µl samples were added to the tubes. The mixture was then left at room temperature for 1-2 minutes, then 25 µl of 10% NaCl was added to each tube. The minimum protein concentration required to stabilise the gold could be determined from the lowest concentration of antibody at which the colour of the sol did not turn blue.

(c) Conjugation

The antibody was transferred to water by passing it through a PD10 column, pre-equilibrated with distilled water, and eluting with water. The stabilisation concentration of the antibody was then added to the gold sol at pH 9.0 and incubated at room temperature for 5 minutes. 1 ml of 0.1% BSA was then added to ensure stabilisation of all the gold particles.

(d) Purification of IgG-gold

The preparation was concentrated by centrifugation at 15,000 g for 45 min in a Beckman 70Ti rotor. This produced a pellet consisting of a large loose part and a small tightly-packed part. The supernatant was removed and the loose pellet resuspended in PBS in 1/25 of the original volume of sol used. A 10%-30% glycerol gradient, pH 7.2, containing 0.1% BSA was then made in a Beckman 70Ti rotor tube using a Bio-rad gradient former. The resuspended pellet was then gently layered on top of this. The gradient was then centrifuged at 5000 g for 10 minutes. This resulted in the separation of a dark-red band, about half way down the tube, which contained the purified IgG-gold. This was then dialysed against five changes of PBS and stored at 4°C.

2.17 SDS Polyacrylamide Gel electrophoresis (Laemmli, 1970)

Slab gels were run using a Bio-Rad vertical slab electrophoresis cell. 1.5mm thick gels were used.

(a) Reagents

- (i) 30% w/v acrylamide, 0.8% w/v Bis-acrylamide (Bio-Rad Ltd) in distilled water.
- (ii) 10% w/v sodium dodecyl sulphate (Bio-Rad Ltd) in distilled water.
- (iii) 10% w/v ammonium persulphate (Bio-Rad Ltd) in distilled water.
- (iv) TEMED (N,N,N',N' - tetramethylethylene diamine; Bio-Rad Ltd)

(v) 0.75 M Tris-HCl pH 8.8.

(vi) 1.00 M Tris-HCl pH 6.8.

(b) Preparation of gels

(i) Separating Gel

The gel was prepared by mixing the volumes of the solutions in order as given in the table below. The gel was de-gassed for 30 minutes then poured between glass plates, a 2mm layer of propan-2-ol overlaid on top and left to polymerise.

	<u>10%</u>	<u>15%</u>
30% Acrylamide plus		
0.8% Bis acrylamide	20ml	30ml
0.75 M Tris-HCl pH 8.8	30ml	26.4ml
10% SDS	0.6ml	0.6ml
10% Ammonium persulphate	0.3ml	0.3ml
Distilled water	9.1ml	2.7ml
TEMED	40ul	40ul

(ii) Stacking gel - 3% acrylamide

The propan-2-ol was poured off the gel and the top washed several times with distilled water. The surface was then dried by inserting a sheet of blotting paper between the glass plates and running the edge just above the surface of the gel. The stacking gel was prepared by mixing the solutions in the order shown in the table below.

30% Acrylamide plus	
0.8% Bis-acrylamide	1.6ml
1 M Tris HCl pH 6.8	2.0ml
10% SDS	0.16ml
10% Ammonium persulphate	80ul
Distilled water	12.14ml
TEMED	24ul

The stacking gel was de-gassed for 30 minutes and applied to the surface of the separating gel. The well forming combe was then added and the gel allowed to polymerise for 1.5 to 2 hours.

(c) Running buffer.

0.025 M Tris, 0.192 M glycine, 0.1% SDS in H₂O pH 8.3.

(d) Sample buffer

1.0 ml 0.5 M Tris-HCl pH 6.8 (BDH)

0.8 ml glycerol (Sigma)

1.6 ml 10% SDS (Sigma)

0.4 ml 2-mercaptoethanol (Sigma)

4.0 ml distilled water

(e) Procedure

Protein containing samples were mixed with an equal volume of sample buffer, and

heated at 100°C for 2 minutes in a water bath. Both the upper and lower compartments of the electrophoresis apparatus were filled with running buffer and the samples were applied to the individual wells of the stacking gel using a 100ul Hamilton syringe. Gels were electrophoresed from the negative to the positive electrode at 50V overnight until the line of the marker dye approached the bottom of the gel. The gel was then removed from the electrophoresis apparatus and stained for protein as described.

(f) Protein stain (Weber and Osborn, 1969)

- (i) Coomassie blue stain: 1 g Coomassie brilliant blue
 182 ml methanol
 182 ml distilled water
 36.8 ml glacial acetic acid

Gels were stain in the above solution at 37°C for 30 minutes

- (ii) Destaining solution: 75ml glacial acetic acid
 50 ml methanol
 875 ml distilled water

Gels were left in this solution until excess stain had diffused out.

(g) Molecular weight estimation

The relative molecular mobility of the bands was determined by comparison with the mobility of the bromophenol blue marker and the molecular weight estimated by using standard curves constructed with marker proteins (Weber and Osborn, 1969).

The standards used (with Mr in parenthesis) were:

Phosphorylase B	(97,000)
Albumin	(66,000)
Ovalbumin	(45,000)
Carbonic anhydrase	(31,000)
Trypsin inhibitor	(21,000)
Alpha lactalbumin	(14,400)

All standards were obtained from Bio-Rad.

2.18 Western blotting

(a) Reagents

- (i) transfer buffer: 25mM Tris, 192mM glycine and 20% glycine pH 8.3.
- (ii) Whatman 3mm filter paper cut into 2 x 18cm squares.
- (iii) 0.1um nitrocellulose filter paper cut into a 18cm square.
- (iv) Bio-Rad "Trans-blot" blotting apparatus.

(b) Transfer of proteins

The transfer of proteins to nitrocellulose paper was achieved using the method of Towbin et al. (1979) as modified by Burnette, (1981).

The gel cassette was opened-up in a sandwich box containing about a 3 cm depth of transfer buffer. One "Scotch-bright" pad was placed on the lower half of the cassette and immersed in transfer buffer. A piece of filter paper was then placed on top of the Scotch-bright pad and soaked in the buffer. The nitrocellulose was then immersed in the buffer and placed on top of the filter paper. The sandwich was completed by placing the gel on top of the nitrocellulose, followed by a second piece of filter paper and a second Scotch-bright pad.

Care was taken to ensure that there were no air bubbles in between any of the layers. Finally the cassette was closed and inserted into the "Trans-blot" cell with the nitrocellulose lying towards the positive terminal side of the gel.

Transfer was achieved overnight at 4°C at 40 V.

(c) Immunolabelling of transferred proteins

After transfer of the proteins was complete the nitrocellulose was cut around the gel, including the cut-off corner, and rinsed in PBS. It was then blocked for at least one hour at 37°C with 3% BSA/PBS followed by two washes in PBS. If antibody was scarce the nitrocellulose was then laid on a piece of clingfilm on the bench and 2ml of antibody (either as neat culture supernatant or purified ascites at 1-10 ug/ml diluted in 0.1% BSA/PBS) was layered over the top of the filter using a bent pasteur pipette. Damp tissue was placed around the edge of the filter and a sandwich box placed over the top. After incubation for at least two hours at room temperature the antibody could be recovered using the cling film, and reused. If antibody was readily available the nitrocellulose was placed in a plastic bag, containing 10 ml of culture supernatant or diluted ascites, which was then heat-sealed and placed on a rocker.

Following incubation with antibody the nitrocellulose was washed five times in PBS and left for one hour in 30ml of a 1/1000 dilution of sheep anti-mouse peroxidase antibody (Dakopatts) in 0.1% BSA/PBS. The peroxidase labelled antibody was detected using 60mg chloro-1-naphthol in 20ml methanol made up to 120ml with 0.1 M tris buffered saline pH 7.5 with the addition of 60ul 30% hydrogen peroxide just before use. The substrate mixture was added to the nitrocellulose after it had been washed extensively in PBS. When purple band(s) appeared on the filter the reaction was stopped by washing the filter several times

in distilled water.

(d) Total protein stain of blotted proteins

To enable molecular weight estimations of specifically labelled proteins on immunoblots a series of molecular weight standards were run in parallel on the gel and transferred to the nitrocellulose paper. The piece of nitrocellulose carrying the standards was cut off and stained for two minutes with amido black stain (Schaffner and Weissman, 1973) comprising;

0.1% Amido black (Bio-rad)

43% methanol

10% glacial acetic acid

in distilled water.

and destained in;

5% methanol

7% acetic acid

Staining of transferred standards provides a more accurate determination of the molecular weight of the blotted proteins than staining a strip of gel containing the standards with coomassie blue, as the nitrocellulose filter is less susceptible to changes in dimensions than the polyacrylamide gel during the respective staining and destaining procedures.

CHAPTER 3

CO-CAPPING STUDIES

3.1 Introduction

There are three fundamental lines of evidence which suggest that CD4 and CD45 may be involved in the generation of intracellular signals via the TCR/CD3 complex. Firstly, the recent evidence that CD4 is associated with the protein tyrosine kinase p56^{lck} (Barber et al., 1989; Rudd et al., 1988; Veillette et al., 1988) and that CD45 is a protein tyrosine phosphatase (Tonks et al., 1988; Charbonneau et al., 1988) suggest that they may be involved in the tyrosine phosphorylation events which occur following stimulation via the T cell receptor.

Secondly, studies of the effect of receptor crosslinking on cell activation have shown that both CD4 and CD45 can alter the level of response following co-crosslinking with the

T cell receptor (Ledbetter et al., 1988; Owens et al., 1987; Ledbetter et al., 1987; Emrich et al., 1988). Both of these areas are described more fully in Chapters 4 and 5.

The third major area of investigation into the associated roles of CD4, CD45 and the T cell receptor has involved studies into the physical associations between these molecules on the surface of both normal T cells and T cell lines.

Many researchers have made use of the fact that, following crosslinking of a specific receptor by antibodies, there is a specific reduction in the level of surface expression of this receptor (termed down-modulation). Simultaneous comodulation of other receptors has been taken to imply a physical association between the two. For example, Andersson et al. (1988) used an acetic acid wash to remove radiolabelled antibodies bound to receptors on the surface of T cells following CD3 or CD4 crosslinking. They report that, following CD3 crosslinking, 10% of CD4 is internalised and conversely, that 5% of CD3 is internalised after CD4 crosslinking. This led them to propose an association of CD4 and CD3 molecules with a stoichiometry of 2:1, which is similar to the ratio of these molecules on the cell surface. CD45 crosslinking did not lead to the internalisation of CD3 or CD4 and the reverse experiment was not reported.

In apparent contradiction to this, Rivas et al. (1988), using flow cytometry to measure the level of cell surface receptor labelling, found normal T cells did not show a reduction in CD4 surface labelling following CD3 crosslinking, except when the cells had previously been stimulated by PHA.

A possible explanation for this difference may lie in the antibodies used. Saizawa et al. (1987) found that the ability of an anti-CD3 antibody to induce comodulation of CD4 related directly to its ability to activate D10.G4.1 (D10) T helper cells. High potency antibodies induced CD4 comodulation, whereas low potency antibodies did not. In agreement with Andersson et al. (1988), a stoichiometry of 2:1 CD4:CD3 was inferred. Saizawa et al. further suggested that an association between CD4 and the T cell receptor is induced by conformational change of the T cell receptor following receptor ligation. As evidence for this, it was shown that incubation of D10 cells with the Fab fragment of anti-CD3 antibody followed by crosslinking with whole anti-CD4 antibody induced cell activation, whereas the anti-CD3 Fab fragment alone did not. The Fab fragment was suggested to have induced CD3/CD4 association enabling the CD3 crosslinking required for activation to be subsequently brought about by the anti-CD4 antibody.

If CD4 and the T cell receptor complex do associate, the site of interaction may lie in the region of the $V\alpha$, $C\beta$ and $CD3\epsilon$ chains, as antibodies directed against epitopes on these molecules inhibit CD4/T cell receptor association (Janeway et al., 1987). Alternatively, this may be the site of interaction between the T cell receptor and an intermediary molecule which itself binds to CD4.

Stimulation of T helper cell lines by specific antigen has been shown to have a similar comodulatory effect on CD4 as stimulation with antibodies (Weyand et al., 1987). It appears that the results for CD4 can be extended to CD8, as this molecule has been demonstrated to behave in a similar manner (Takada and Engleman, 1987).

The results of these studies have been used to suggest that T cell receptor ligation or crosslinking is required for a physical association with CD4 to occur. However, affinity chromatography of detergent cell lysates using anti-CD4 antibody resulted in the co-isolation of a small amount of the T cell receptor which could be prevented by pre-immunoprecipitation with an anti-T cell receptor antibody (Gallagher et al., 1989). This would suggest that some stable CD4:TCR complexes exist prior to receptor ligation. Further evidence for this was obtained by fluorescence resonance energy transfer (FRET) analysis (Chuck et al., 1990). A stable association was found to exist on 54% of HPB-ALL cells which had been maintained at 0°C in the presence of the metabolic inhibitor sodium azide.

Fluorescence energy transfer was also used by Mittler et al. (1989). Contrary to the report by Chuck et al., they found that significant energy transfer between CD3 and transfected human CD4 occurred on a mouse L3T4 negative T cell hybridoma only after CD3 ligation by antibody and warming to 37°C.

Thus it is apparent that there is some disagreement as to whether a permanent association between CD4 and the T cell receptor occurs, or whether one is induced by receptor crosslinking.

An alternative method for identifying associations between receptors is to study their relative distribution on the cell surface using fluorescently labelled markers.

Kupfer et al. (1987) used labelling with fluorescently tagged antibodies to show that both CD4 and the T cell receptor accumulated at the contact site between specific cell

couples, but neither collected at the contact site when irrelevant antigen was presented. This argues in favour of an active, TCR ligation-induced association, since the ligand for CD4, class II MHC, is present on the antigen presenting cell in non-specific as well as specific cell couples.

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As well as visualisation of receptor redistribution in cell couples, the phenomenon of surface receptor capping on isolated cells (Taylor et al., 1971) (described in detail in Chapter 1) can also be employed. Use of antibodies conjugated to a fluorochrome with a particular absorption and emission spectrum, such as fluorescein isothiocyanate (FITC), enables the cap to be visualised under the microscope using epifluorescent illumination. If further movement of the surface molecules is then prevented by mild fixation with paraformaldehyde, the distribution of a second surface molecule may be determined using specific antibodies conjugated to a fluorochrome with a different absorption and emission spectrum, such as tetramethylrhodamine isothiocyanate (TRITC). Passive co-redistribution (hereafter termed "co-capping") of the second receptor with the first would suggest an association between the two.

Kupfer and Singer (1988) used this technique to show that CD4 co-caps with the T cell receptor on D10 T cells, when the clonotypic antibody 3D3 is bound. This antibody has the unusual property, still not understood, of being able to activate and induce capping on D10 cells without the requirement for crosslinking. When non-activating anti-T cell receptor antibodies were used, no co-capping of CD4 was observed, even after crosslinking. Similar results were presented by Rojo et al. (1989).

Co-capping was used in one of the few published investigations into associations between CD45 and CD4 and the T cell receptor (Dianzani et al., 1990). They report that only the low molecular weight CD45 isoform co-caps, suggesting that a physical association between CD45, CD4 and the TCR occurs only in cells with the memory phenotype.

Two other investigations into the association of CD45 with other T cell surface molecules have recently appeared in the literature. Schraven et al. (1990), used the bifunctional crosslinking reagent, dithiobissuccinimidylpropionate (DSP), to demonstrate that CD45 and CD2 were associated on the surface of human peripheral blood T cells. Using a similar technique, Volarevic et al. (1990) demonstrated the association of CD45 with the TCR, and with the glycosyl-phosphatidylinositol linked molecule, Thy-1, on murine cells. The extensive studies into associations between CD4 and other T cell surface molecules is not matched by work on CD45.

It is apparent that there is a significant amount of evidence to suggest that CD4, CD45 and the TCR somehow physically associate on the surface of a variety of cell types. Whilst surface receptor associations can be studied in a variety of ways, there are a number of advantages in using the co-capping technique with isolated cells, as opposed to other techniques mentioned earlier. Comodulation studies do not discount the possibility that the modulation of CD4 with the T cell receptor is coincidental and doesn't necessarily require direct association between the receptors. Conversely, FRET analysis and affinity chromatography provide greater evidence that associations occur, but no information on the distribution of the molecules on the cell surface. Direct visualisation of specific cell-cell couples would appear to be an ideal method for studying surface receptor associations, except

that results from T cell lines cannot necessarily be extrapolated to normal cells (see Chapter 6).

Consequently, it was decided to employ the co-capping technique on isolated normal rat T cells to study the associations between CD4, CD45 and the T cell receptor. This enables the distribution of these receptors to be determined, under conditions which can result in the production of intracellular signalling events (Williams and Beyers, 1989).

Rat cells were used because very little is known about the behaviour of receptors on the rat T cell surface and because antibodies directed against a number of rat T cell surface molecules were readily available from the Sir William Dunn School of Pathology, South Parks Road, Oxford (courtesy of Prof. Alan Williams, Head of the MRC Cellular Immunology Unit).

The use of these mouse monoclonal antibodies, however, posed problems in the development of the double immunolabelling technique, as there were a number of points at which non-specific binding could potentially occur. Firstly, following crosslinking of the first mouse monoclonal antibody with anti-mouse IgG, the second mouse monoclonal antibody would be able to bind to free sites on the anti-mouse antibody. Secondly, a further anti-mouse layer to label the second mouse monoclonal would also bind to the first mouse monoclonal giving a false impression of the distribution of the molecules.

Various approaches were used to circumvent this. The problem of the second mouse monoclonal antibody binding to the first layer anti-mouse IgG was solved by including a

blocking step in which free binding sites on the anti-mouse antibody were blocked by mouse immunoglobulin. The optimal concentration for this was 0.1 mg/ml. It was thought that blocking with whole mouse Ig might sterically inhibit access of antibodies used in subsequent labelling, to their epitopes. However, Fab fragments of mouse immunoglobulin, created by papain digest as described in Materials and Methods, did not noticeably improve labelling of the second receptor.

Attempts to eliminate the binding of the second anti-mouse antibody to the first mouse monoclonal initially focused on the use of a second mouse monoclonal directly conjugated to the fluorochrome, according to the method described in Chapter 2, thereby eliminating the need for a second anti-mouse layer. However at low FITC to protein ratios the level of fluorescence was too low to see clearly, and at higher ratios some non-specific binding occurred, possibly due to the effect of the fluorochrome molecules on the active site.

Thus, as an alternative approach, hapten modified antibodies were tested. Modification of the second mouse monoclonal antibodies with trinitrophenol (TNP) (as in General Materials and Methods) did not eliminate non-specific binding, as the anti-TNP antibodies used to detect them also bound to the primary layers. Further affinity purification could not improve this.

The most successful system involved attachment of biotin to the second monoclonal antibody, and subsequent visualisation with FITC or TRITC conjugated streptavidin. The alternative use of anti-biotin antibodies (Sigma) resulted in some non-specific binding.

Work by Turner et al. (1988), demonstrated that sequential labelling and capping of different receptor species, by specific monoclonal antibody and polyclonal secondary antibody, resulted in both molecular species capping to the same place. If one intends to infer associations between receptors from the passive co-capping of one species following the active capping of another, then secondary capping events must obviously be prevented. This can be achieved by fixing the cells with paraformaldehyde which effectively crosslinks and immobilises the molecules on the cell surface. One problem with this is that excessive paraformaldehyde can functionally alter some epitopes and thereby inhibit antibody binding. To avoid this, the minimum concentration of paraformaldehyde required to prevent capping was determined, and this was found to 0.05%. In order to be absolutely sure that no secondary movement of receptors was occurring, 0.1% paraformaldehyde was used in these experiments.

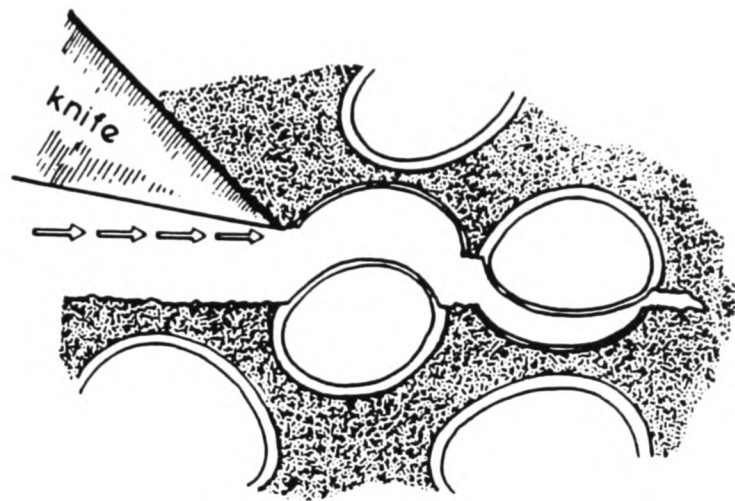
The co-capping experiments were extended to study the distribution of one of the three molecules following the co-crosslinking and capping of the other two. This was intended to provide information on physical associations to complement the co-crosslinking studies of several groups including Ledbetter et al. (1988), Emmrich et al. (1988) and Owens et al. (1987) in which the effect on cell activation was studied.

Attempts were also made to gain further information on the organisation of co-capped receptors within the cap using the technique of label-fracture and electron microscopy, developed by Pinta da Silva and Kan (1984). This involves labelling and capping receptors as if for viewing under the light microscope, except that the antibodies used are conjugated

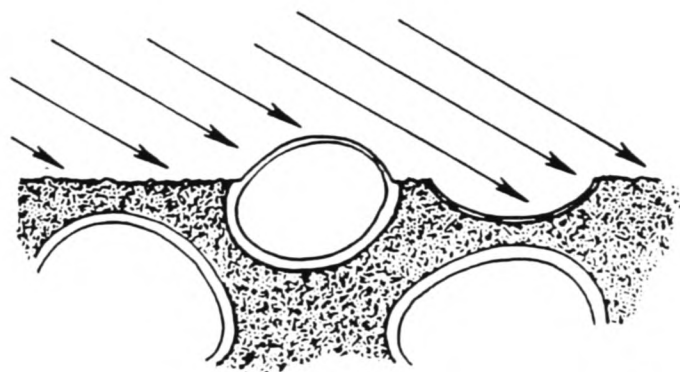
to different sized gold particles instead of fluorochromes. The cells are then cryoprotected, frozen and fractured in a freeze-fracture device. A platinum/carbon replica of the fractured surface is made which is then viewed under the electron microscope.

The freeze-fracture replica technique is based on the fact that when a cooled knife passes through a block of ice, a plane of fracture will run just ahead of the knife edge. When this fracture plane reaches a frozen cell it will travel along the path of least resistance which often lies in between the leaflets of the membrane lipid bilayer. (Fig 3.1). A fine layer of platinum discharged over the fractured surface at an oblique angle produces a shadowed replica. This is then strengthened by a coat of carbon. The replica can then be floated off the sample, gently washed to remove debris and viewed under an electron microscope. Normally, biological tissue is removed from the replica by bleach, but since this would also strip the gold particles from the outer surface of the E-face fractures, in the label-fracture technique washing is limited to distilled water. This results in cell debris being left attached to P-face fractures, which are thus ignored.

Initially attempts were made to use specific monoclonal antibodies which had been directly conjugated to gold particles (see Materials and Methods). This would have been preferable as the gold particles are much closer to the receptors and thus would more accurately have labelled their position. However, as with the fluorescence studies, the level of labeling was too low and consequently the biotin-streptavidin system was used.



a) Fracture



b) Replication



c) Washing

Figure 3.1 Principles of freeze-fracture. (a) Gold-labelled frozen cell suspension is fractured with a cooled knife. (b) Fractured specimen is shadowed with a thin platinum/carbon layer, then strengthened with a layer of carbon. (c) Replica is floated onto water and gently washed. Cell debris remains attached to the P-face.

3.2 Methods.

(a) Fluorescence co-capping studies following capping of a single receptor species.

T cells were isolated from 6-12 week old DA rats by either nylon wool filtration or negative panning as detailed in General Materials and Methods. The antibodies used to label the primary cell surface antigen were OX 1 spent culture supernatant (anti-pan CD45), W3/25 ascites (anti-CD4), R73 ascites (anti- $\alpha\beta$ TCR), 56A culture supernatant (anti-human spectrin, used as a negative control antibody) and OX 19 ascites (anti-CD5). These are described more fully in chapter 2. The spent culture supernatants were used at a final dilution of 1/2, and the crude ascites were diluted to approximately 50-75 μg IgG1/ml in RPMI 1640 tissue culture medium.

10^6 T cells in RPMI medium were added to plastic LP3 tubes (Luckham Ltd.) and centrifuged for 5 min at 400 g, 4°C, in a Jouen CT422 centrifuge. The supernatant was then removed by aspiration and the cells resuspended by vortexing. 100 μl of diluted antibody was then added. After incubation on ice for 45 min, the tubes were topped up with 1 ml medium and washed x3 by centrifugation. Prior to this, FITC conjugated goat anti-mouse IgG (Sigma) had been diluted 1/30 in RPMI+ 10% DA rat serum and incubated at 4°C for 30 min to remove any non-specific binding. The cells were then resuspended in 100 μl of the diluted anti-mouse antibody and incubated for 45 min on ice. After washing as before, the cells were resuspended in 200 μl medium and placed in a 37°C incubator for 15 min. This time had previously been shown to be sufficient for a majority of cells to cap the crosslinked receptors (C. Turner, 1986. D. Phil. thesis, University of Oxford). At the end of this period, the tubes

were returned to ice and 200 μ l ice-cold 0.2% paraformaldehyde (Agar Scientific) in PBS was added.

After allowing 30 min on ice to fix the cell surface molecules, the cells were washed x3 in PBS and resuspended in 100 μ l 0.1 mg/ml mouse immunoglobulin (Sigma) in PBS for 30 min on ice. The cells were then washed x3 in PBS. Antibodies to the second receptor being studied had been previously conjugated to biotin according to the method in Chapter 2. A concentration of 20 μ g/ml biotinylated antibody was used and 100 μ l of this dilution was added to each tube. Following incubation on ice for 45 min, the cells were washed x3 in PBS, resuspended in 100 μ l of a 1/30 dilution of TRITC-conjugated streptavidin (Serotec) and incubated for 45 min on ice. They were then washed x3 in PBS. The protocol used for surface labelling is summarised in Figure 3.2.

(b) Fluorescence co-capping studies following co-crosslinking of two receptor species.

Use was made of the fact that all the antibodies used were developed in mice. 10^6 cells were therefore sequentially incubated with antibodies as before, except that antibodies to two different molecules were used together as a cocktail in the first incubation. These antibodies were diluted so as to give a final concentration of each which was the same as if they had been added individually. They could then be simultaneously cross-linked simply by adding an anti-mouse IgG secondary antibody. The remainder of the labelling procedure for a third antigen was as before.

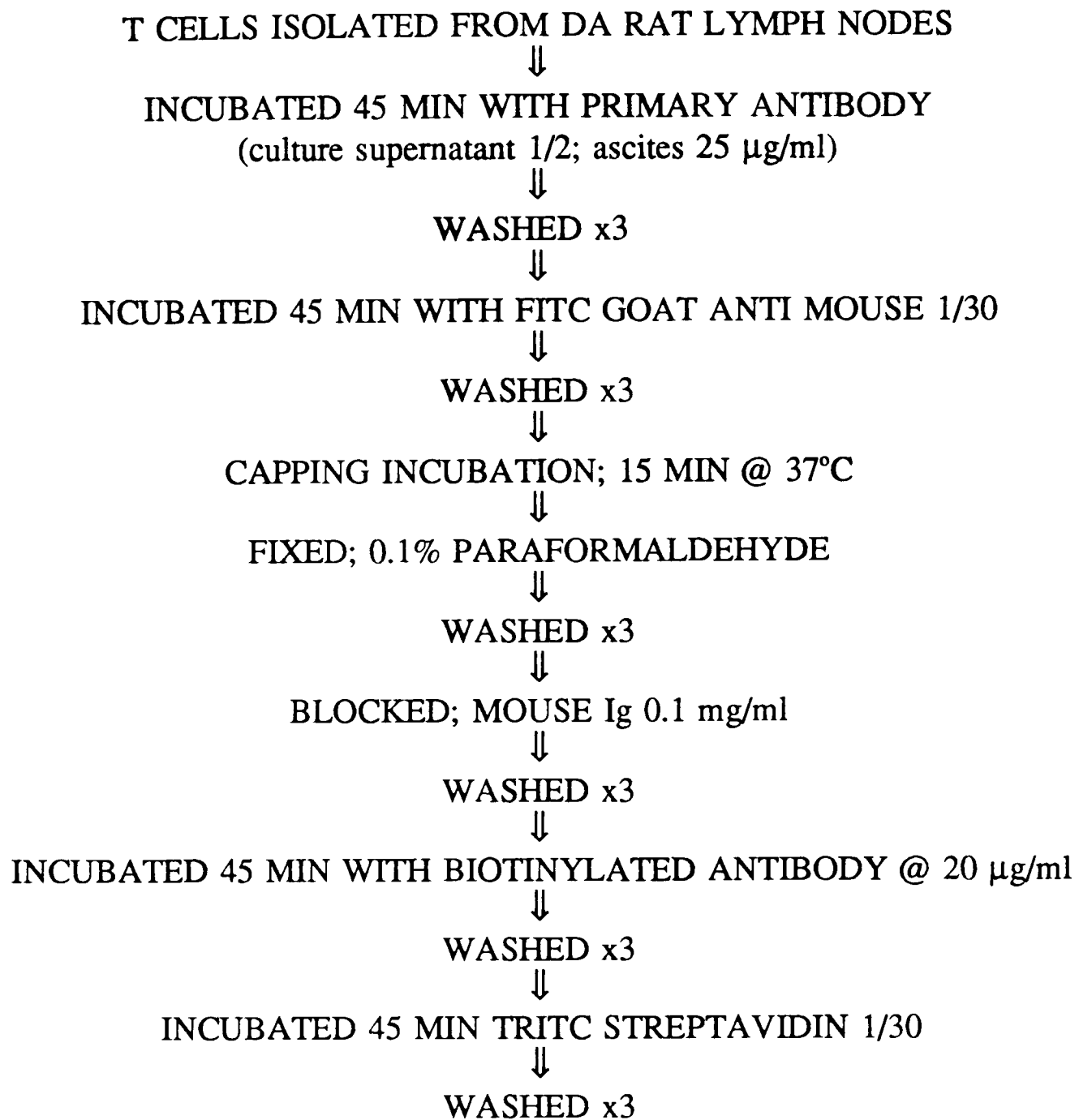


Figure 3.2. Summary of protocol used for double immunofluorescence labelling.

(c) Preparation of slides for observation.

After the cells had received the final wash, the supernatant was removed by aspiration, the pellet resuspended by vortexing and 0.5 ml of "Citifluor" anti-fade reagent (Agar Scientific) in PBS was added to each tube. After further vortexing, 12 µl of the cell

suspension was placed on a glass microscope slide and a glass coverslip gently placed over the top. The edges of the coverslip were sealed with nail varnish and the cells were viewed under a Zeiss Standard 18 microscope fitted with epifluorescence illumination, an HB50 mercury arc lamp and appropriate filter sets for FITC and TRITC (Table 3.1) using a x63/1.4 planapo oil immersion lens.

Colour photography was performed using AGFAchrome RS1000 slide film, exposed at 1000 ASA. Exposure times were typically 15-60 seconds.

Fluorochrome	Exciter filter	Chromatic beam splitter	Barrier filter	Catalogue number (Zeiss)
FITC	BP 485/20	FT 510	BP 515-565	487717
TRITC	BP 546/12	FT 580	LP 590	487715

Table 3.1. Fluorescence microscope filter sets.

(d) Counting of capped and co-capped cells.

At least 140 cells were counted for each antibody combination in each experiment. If the fluorescent label appeared as a diffuse continuous ring around the cell, the antigen was deemed to be uncapped. If the fluorescence appeared localised to a single region on the cell surface the antigen was counted as being capped. Co-capping was said to have occurred if the fluorescent label of the second antigen was superimposed on the cap of the first antigen.

(e) Measurement of antibody labelling.

To determine whether the level of antibody labelling varied according to whether or not a molecule was co-capped, cells were labelled as previously described except that 1 μCi [^{125}I]-labelled streptavidin (Amersham) in 100 μl PBS was used as the final step. The cells were washed x6 in PBS by centrifugation and resuspended in 200 μl PBS. The radioactivity was then counted in a LKB 1270 "Rackgamma II" gamma counter.

(f) Freeze-fracture of immuno-gold labelled cells.

(i) Preparation of labelled cells for freeze-fracture.

Cells were labelled as for viewing under the fluorescence microscope except that anti-mouse IgG conjugated to 5 nm gold particles and streptavidin conjugated to 15 nm gold particles (Biocell) was used. After the final washing regime, the cells were fixed in 200 μl 5% glutaraldehyde/PBS at 4°C overnight, washed once in PBS by centrifugation and resuspended in 200 μl 30% glycerol in PBS for one hour at room temperature. After this time the cells were centrifuged once again and most of the supernatant removed so as to leave a concentrated cell suspension. A small amount of the sample was sucked into the pasteur pipette and, under a microscope, a drop of the cell suspension was placed on a cleaned gold Balzers specimen support and immersed for 15 seconds in liquid Freon-22, cooled to near its freezing point (-160°C) by liquid nitrogen. The frozen sample was then taken out of the Freon, shaken to remove any remaining liquid and rapidly transferred to a specimen holder in a bath of liquid nitrogen.

(ii) Freeze-fracture and replication.

The freeze-fracture chamber of a Balzers BAF 301 freeze-fracture apparatus was evacuated to 2×10^{-7} mbars and cooled to -170°C . The chamber was vented and the frozen samples were inserted via a port on the front of the equipment. The vacuum was then restored and the platinum and carbon electron beam evaporation guns each pre-fired at 60 mA and 2000 V for 5 seconds. The specimen stage was then warmed to -120°C and the knife cooled with circulating liquid nitrogen until the film of vacuum grease covering the knife could be seen to craze. The knife comprised a single-edged carbon steel razor blade held in the jaws of the microtome assembly. The cooled knife was then lowered so that it just touched the ice lying over the specimen surface. It was then further lowered such that next time it was passed over the specimen it would cause fracturing across the top of the specimen. This was then repeated a number of times until the knife had removed the top half of the hemispherical frozen specimen droplet, exposing a large smooth fractured surface. The fractured surface was then immediately replicated by depositing 95% platinum/5% carbon at an angle of 45° with the gun being operated at 100 mA and 2200 V. The deposited thickness of Pt/C was measured by a quartz crystal thin film monitor, on a sensitivity range of 300 Hz full scale, and the shadowing was terminated after a frequency change of 180 Hz, which corresponded to 2 nm of Pt/C. The replica was subsequently strengthened by shadowing with carbon at an angle of 90° . The carbon gun was operated at 110 mA and 2400 V. 120Hz was deposited which corresponded to 15nm of carbon. The fractured replicated specimens were then removed from the machine, the replica floated onto distilled water and gently washed five times by transfer to fresh water every hour. It was then picked up on an acetone-cleaned hexagonal 400 mesh copper grid and viewed in a Philips EM400 transmission electron microscope, operated conventionally at 80 kV.

3.3 Results.

(a) Specificity of antibody labelling.

The mouse monoclonal anti-human spectrin antibody 56A, which does not bind to the surface of rat T cells, was used in place of the OX-1, W3/25, R73 and OX-19 antibodies in each co-capping combination, both in its biotinylated and native forms. In no case was there any labelling with either the FITC- and TRITC-conjugated goat anti-mouse IgG or the FITC- and TRITC-conjugated streptavidin suggesting that non-specific labelling was not occurring (Fig 3.3a and b). This conclusion is also supported by the observation that where co-capping did occur, it tended to be complete. If non-specific binding was occurring and the second molecule was not in fact co-capped, one would expect still to see peripheral labelling with perhaps only an increase in intensity in the cap region.

(b) Co-capping studies following the capping of a single receptor species.

It was found that on $46 \pm 7\%$ of CD4⁺ T cells, CD4 co-capped with the T cell receptor (Fig 3.4a and b), and that CD45 co-capped on $73 \pm 5\%$ of cells (Fig 3.5a and b). $49 \pm 3\%$ of CD4⁺ cells co-capped the TCR with CD4 and CD45 co-capped with CD4 on $67 \pm 10\%$. Neither CD4 nor the TCR co-capped with CD45 (Fig 3.6a and b and 3.7a and b). CD5 did not co-cap with CD4, CD45 or the TCR and none of these molecules co-capped with CD5. The results of three representative experiments are summarised in Table 3.2 below.

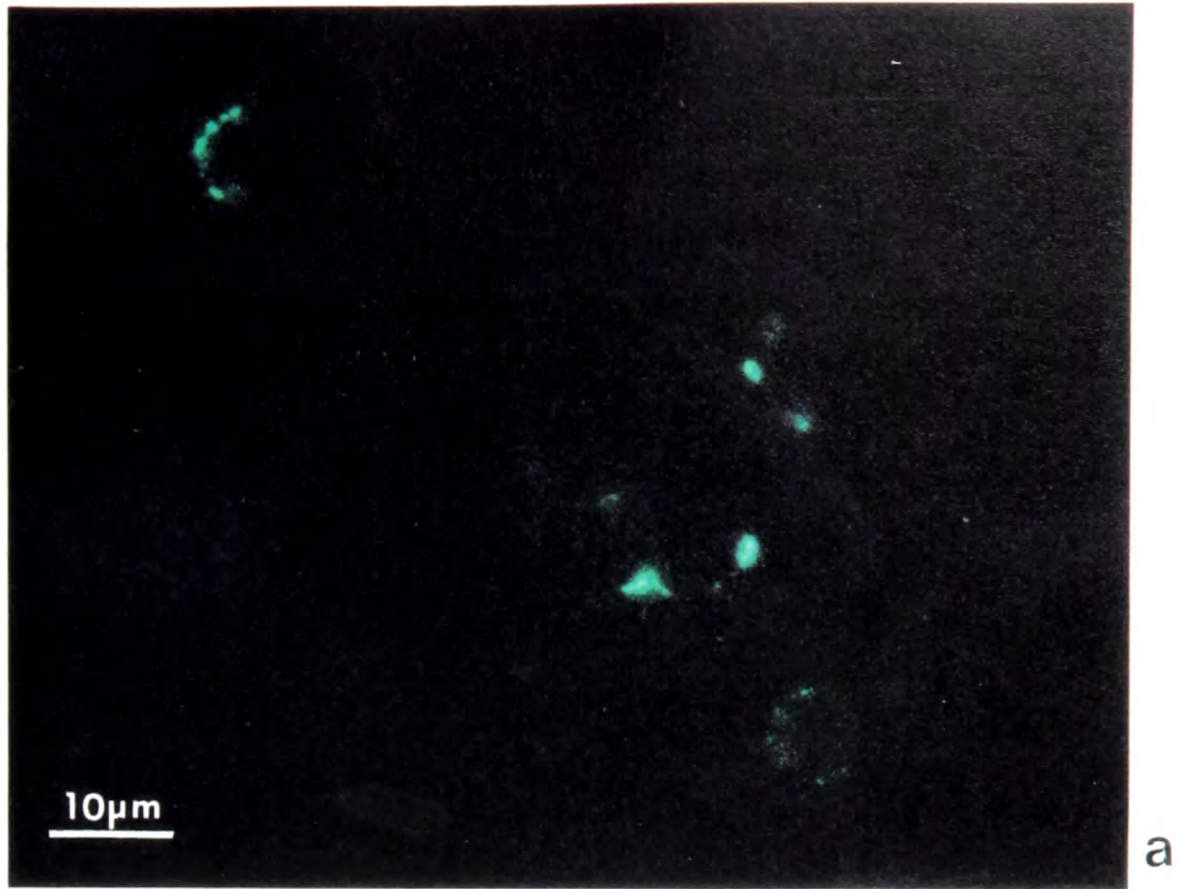


Figure 3.3 Specificity of antibody labelling. (a) Cells labelled with OX-1 followed by [FITC]-conjugated anti-mouse and incubated at 37°C for 15 minutes. (b) Same cells subsequently labelled with 56A[Biotin] followed by [TRITC]-conjugated streptavidin.

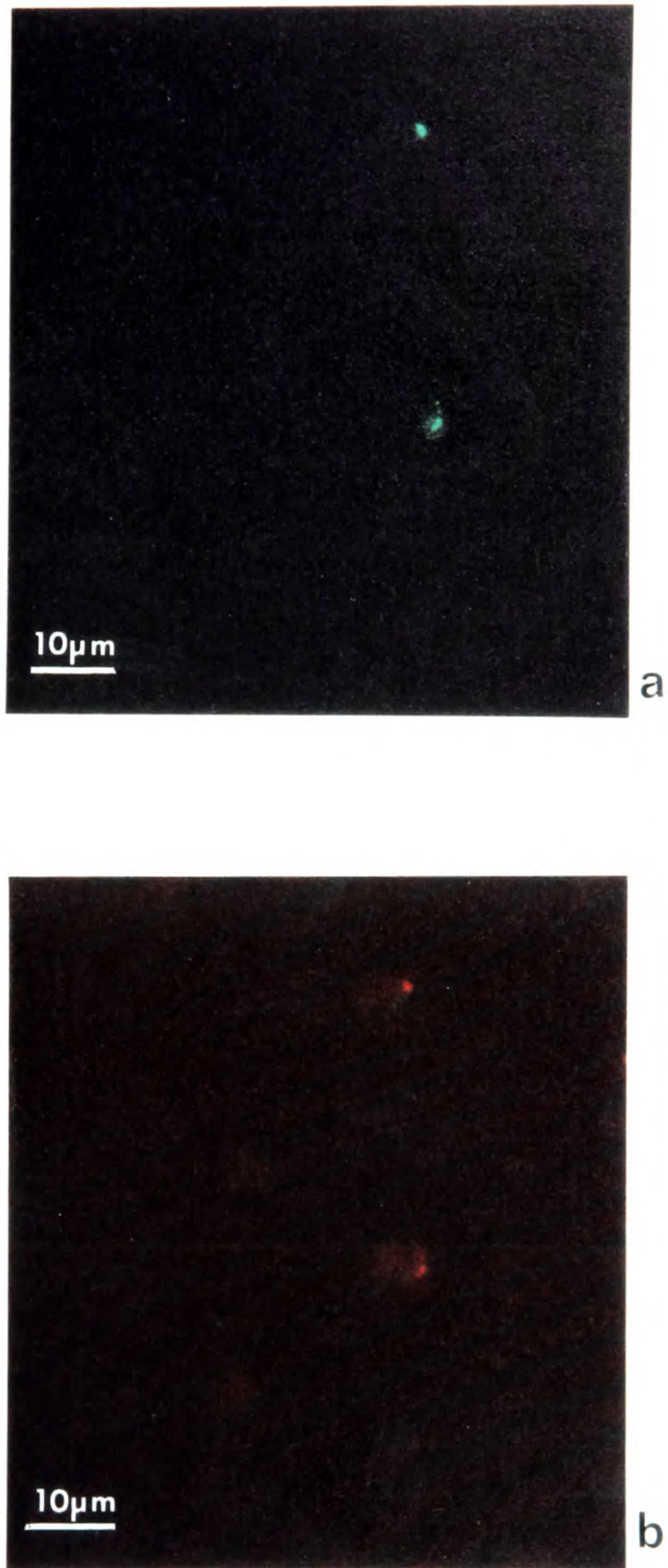


Figure 3.4 CD4 co-capped with the T cell receptor. (a) Cells labelled with R73 followed by [FITC]-conjugated anti-mouse and incubated at 37°C for 15 minutes. (b) Same cells subsequently labelled with W3/25[Biotin] followed by [TRITC]-conjugated streptavidin.

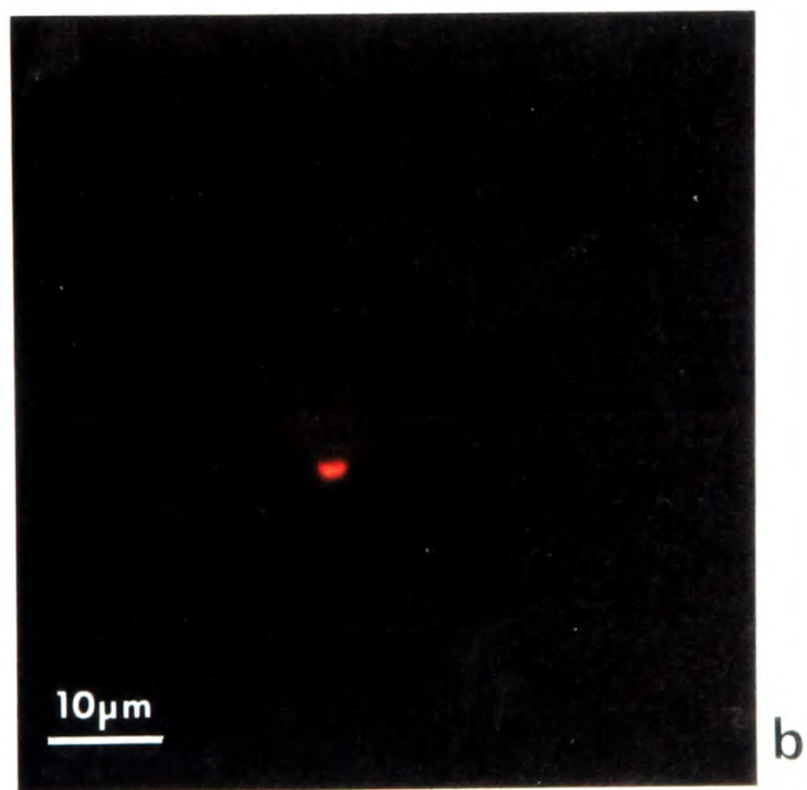
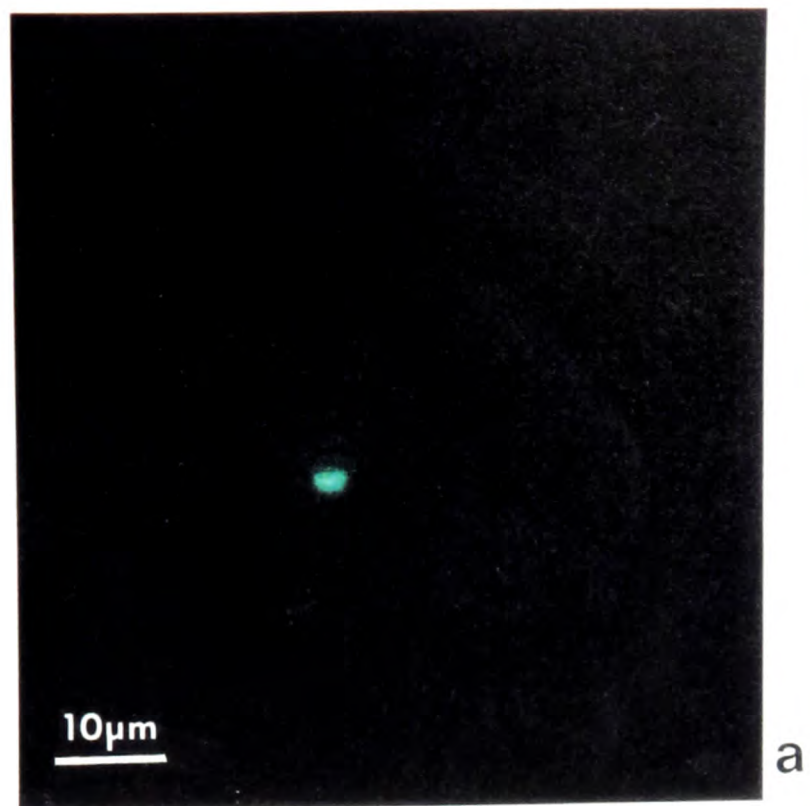


Figure 3.5 CD45 co-capped with the T cell receptor. (a) Cells labelled with R73 followed by [FITC]-conjugated anti-mouse and incubated at 37°C for 15 minutes. (b) Same cells subsequently labelled with OX-1[Biotin] followed by [TRITC]-conjugated streptavidin.

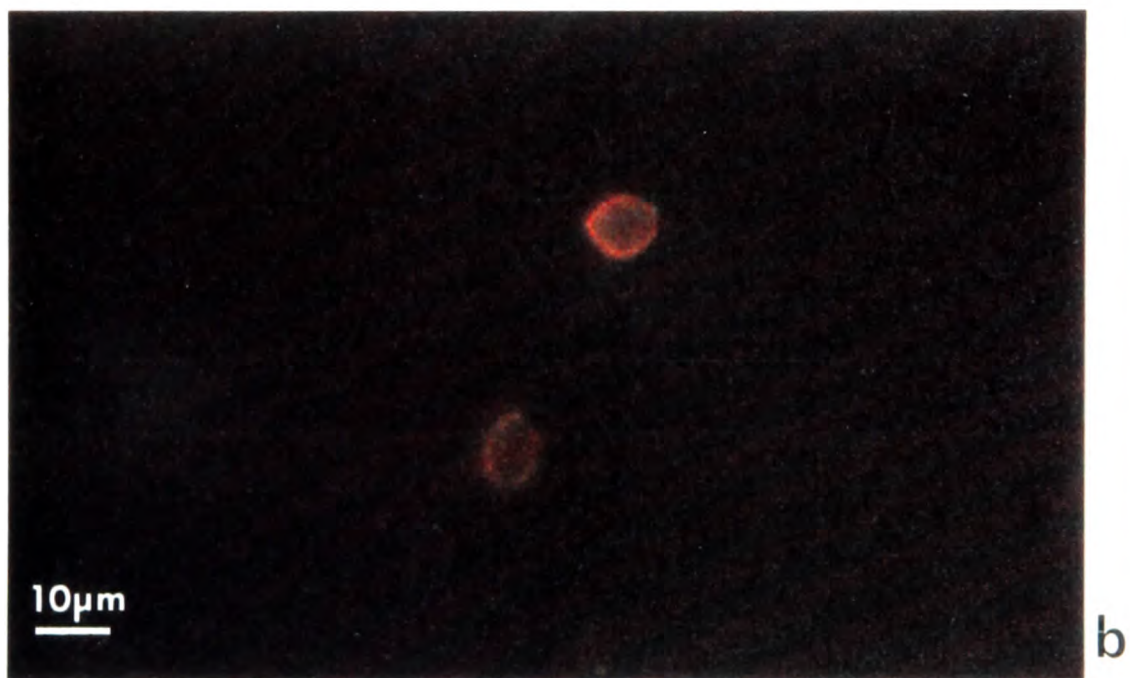
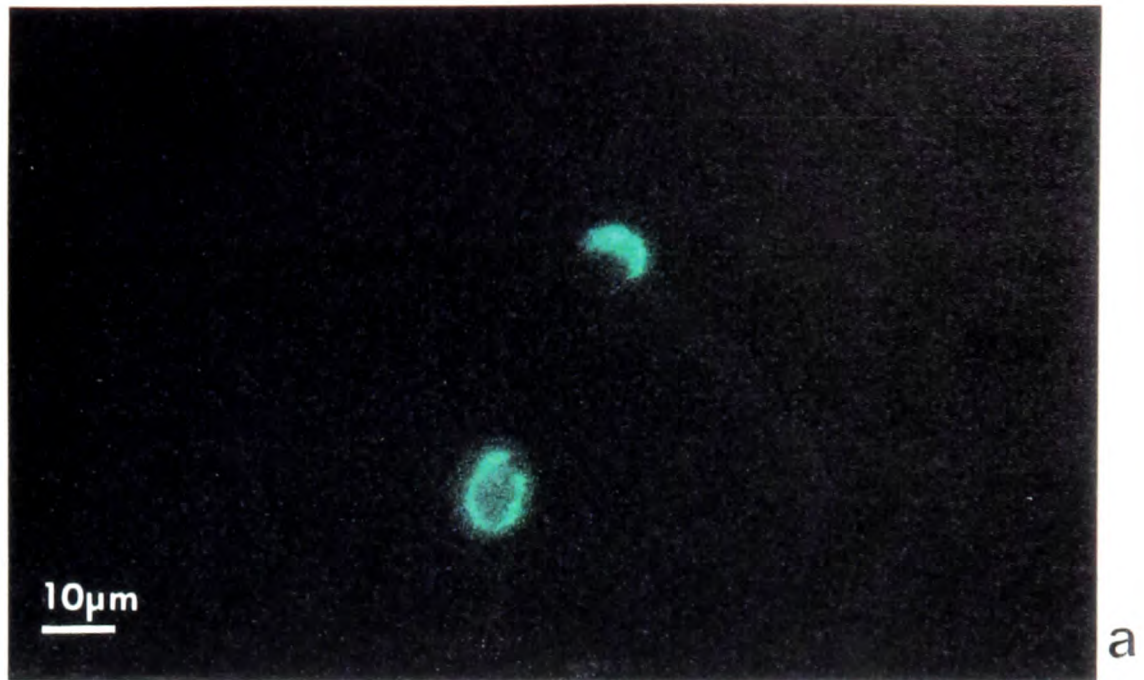


Figure 3.6 CD4 not co-capped with CD45. (a) Cells labelled with OX-1 followed by [FITC]-conjugated anti-mouse and incubated at 37°C for 15 minutes. (b) Same cells subsequently labelled with W3/25[Biotin] followed by [TRITC]-conjugated streptavidin.

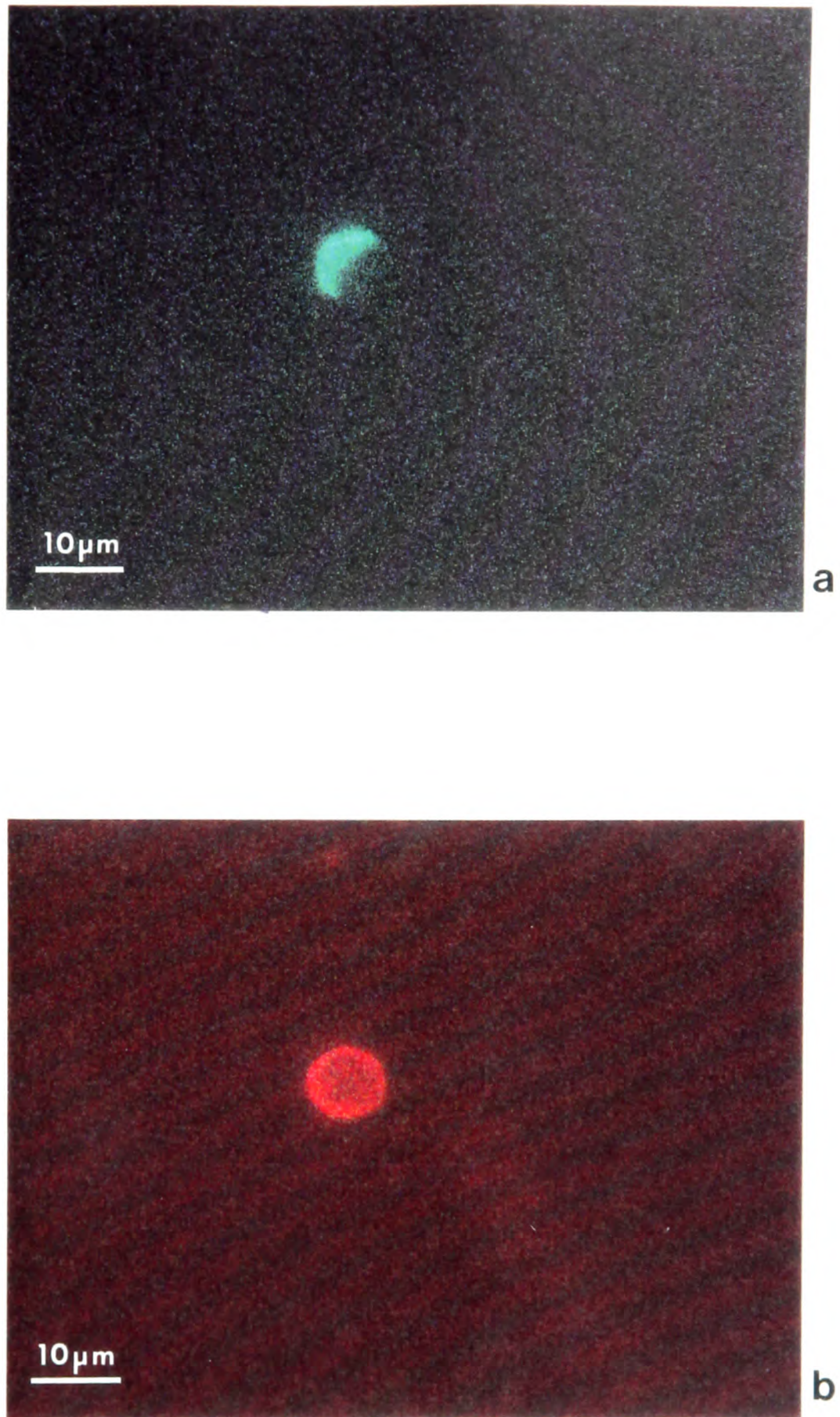


Figure 3.7 T cell receptor not co-capped with CD45 (a) Cells labelled with OX-1 followed by [FITC]-conjugated anti-mouse, then incubated at 37°C for 15 minutes. (b) Same cells subsequently labelled with R73[Biotin] followed by [TRITC]-conjugated streptavidin

Actively capped molecule	Secondary molecule	Percentage co-capped calls			Mean \pm SEM
		Exp 1	Exp 2	Exp 3	
TCR	CD4	54%	37%	48%	46 \pm 7%
TCR	CD45	71%	80%	68%	73 \pm 5%
TCR	CD5	0%	4%	ND	2 \pm 2%
CD4	TCR	47%	54%	46%	49 \pm 4%
CD4	CD45	75%	81%	57%	71 \pm 10%
CD4	CD5	2%	5%	4%	4 \pm 1%
CD45	TCR	4%	5%	3%	4 \pm 1%
CD45	CD4	6%	7%	4%	6 \pm 1%
CD45	CD5	2%	1%	5%	3 \pm 2%
CD5	TCR	0%	2%	ND	1 \pm 1%
CD5	CD4	4%	6%	ND	5 \pm 1%
CD5	CD45	12%	7%	ND	10 \pm 3%

Table 3.2. Summary of co-capping data for three separate experiments.

(c) Co-capping studies following the co-crosslinking of two receptors species.

When CD4 and the T cell receptor were co-crosslinked and capped together, CD45 was co-capped on approximately 70% of cells (Fig 3.8a and b); a similar percentage to when CD45 was found co-capped with individually capped CD4 or TCR. If the TCR was co-crosslinked with CD45 however, CD4 did not co-cap (Fig 3.9a and b), whereas co-crosslinking the TCR with CD5 did result in CD4 co-capping on approximately 50% of cells. Co-crosslinking CD4 with CD45 left the TCR diffusely distributed, whilst CD4 co-

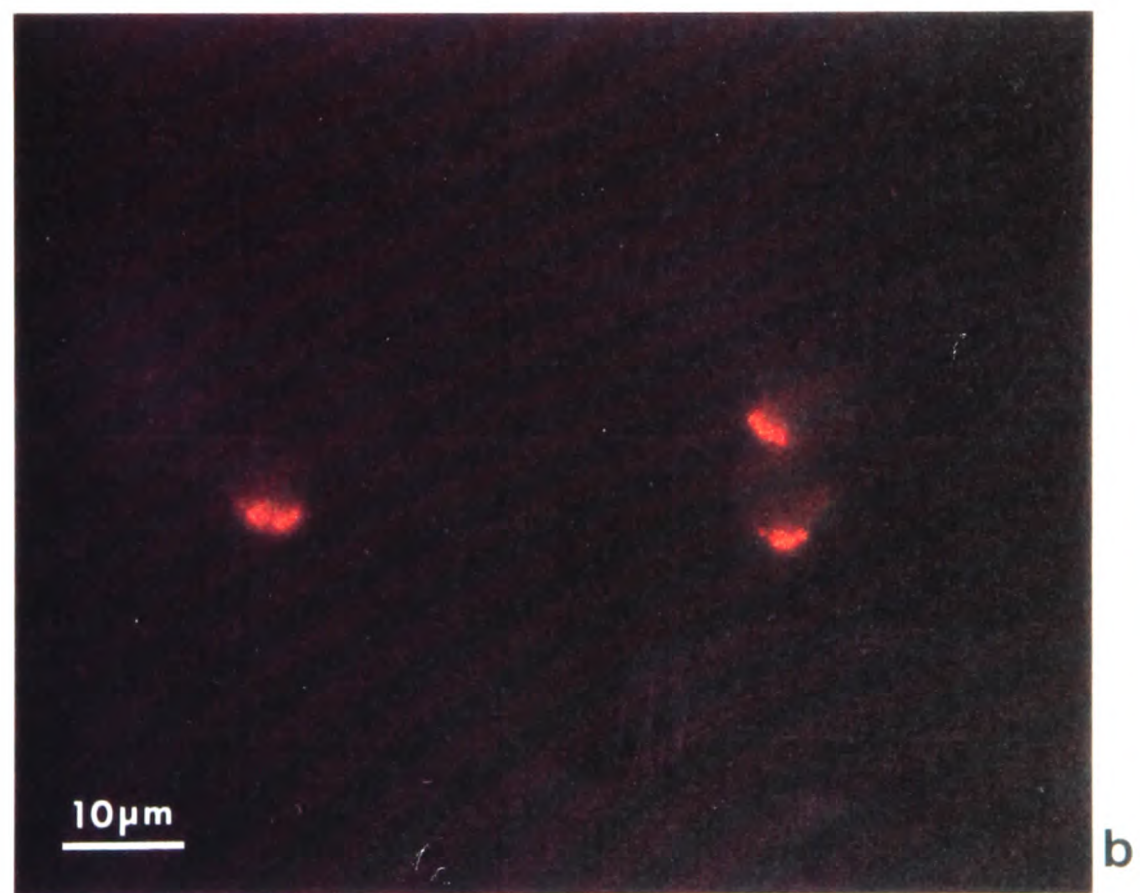
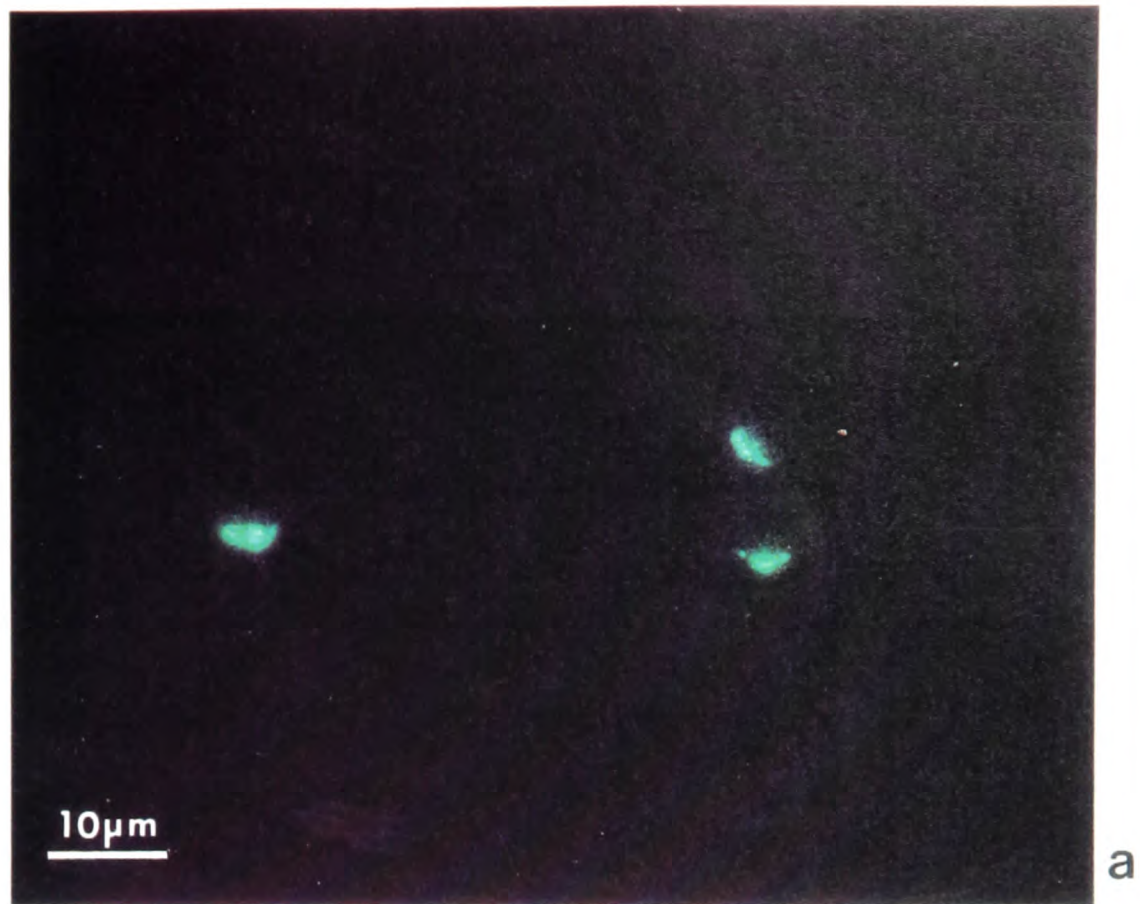


Figure 3.8 CD45 co-capped with co-crosslinked CD4 and TCR. (a) Cells labelled with W3/25 and R73 followed by [FITC]-conjugated anti-mouse and incubated at 37°C for 15 minutes (b) Same cells labelled with OX-1[Biotin] followed by [TRITC]-conjugated streptavidin.

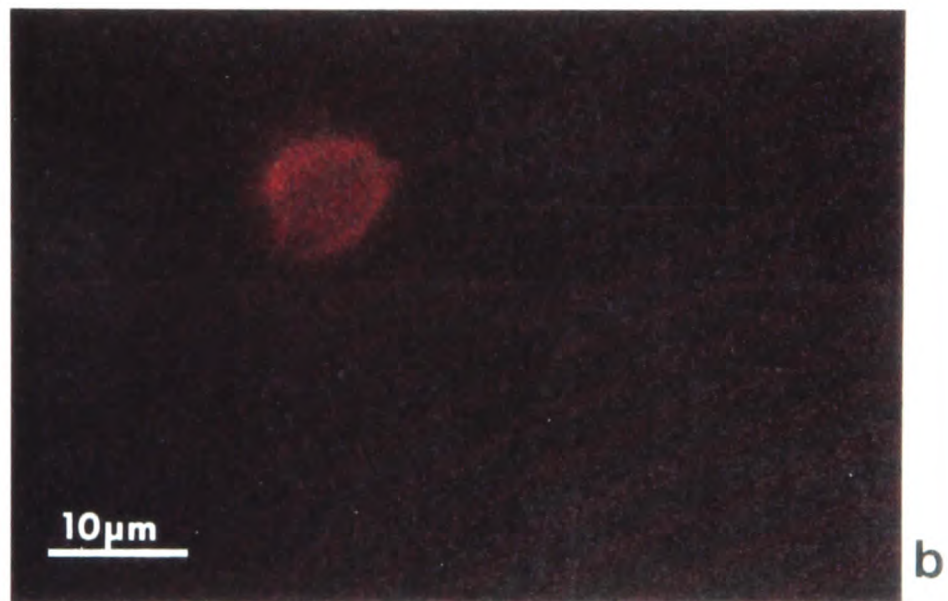
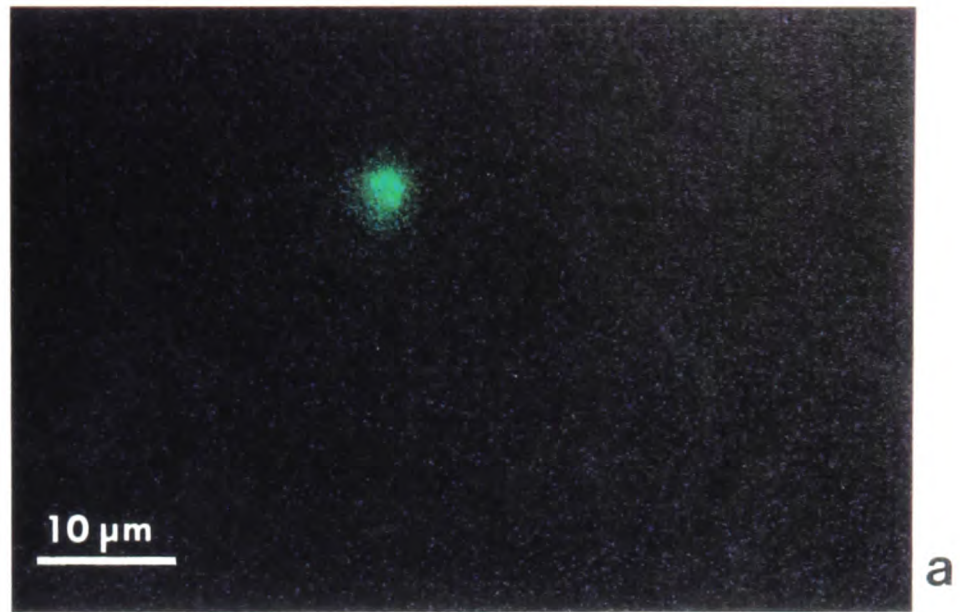


Figure 3.9 CD4 not co-capped with co-crosslinked TCR and CD45. (a) Cells labelled with R73 and OX-1 followed by [FITC]-conjugated anti-mouse and incubated at 37°C for 15 minutes. (b) Same cells labelled with W3/25[Biotin] followed by [TRITC]-conjugated streptavidin.

crosslinked with CD5 caused the TCR to redistribute to the capped region. These results are summarised in Table 3.3 and were shown to be true in five separate experiments.

Actively capped molecules	Secondary molecule	Co-capping
TCR+CD4	CD45	YES
TCR+CD5	CD45	YES
TCR+CD5	CD4	YES
TCR+CD45	CD4	NO
CD4+CD5	TCR	YES
CD4+CD45	TCR	NO
CD45+CD5	TCR	NO
CD45+CD4	CD4	NO

Table 3.3. Summary of effects of co-crosslinking receptors on co-capping.

(d) Measurement of antibody labelling.

The reason CD45 prevents co-capping when it is co-crosslinked to the TCR or CD4 could be a result of the large amount of CD45 on the cell surface. When capped by crosslinked antibodies this could produce a protein meshwork over the cap region which would sterically inhibit access of the W3/25 or R73 antibodies and streptavidin to the co-capped molecules. To test this possibility, the level of [¹²⁵I]-labelled streptavidin bound to biotinylated W3/25 was measured following capping of either the TCR alone or the TCR co-crosslinked with CD45. In the former case CD4 is co-capped, in the latter it is not. The level

of ^{125}I -streptavidin bound was found to be not significantly different (Table 3.4).

Actively capped molecule(s)	Secondary antibody	Degree of [^{125}I]Strp labelling*
TCR	W3/25 [biotin] (CD4 co-capped)	2737±304
TCR+CD45	W3/25 [biotin] (CD4 not co-capped)	3136±543
TCR	56A [biotin] (negative control antibody)	505±15

Table 3.4. Quantitation of [^{125}I] streptavidin binding.

* Results are expressed as mean counts per min \pm SEM (N=3).

(e) Freeze-fracture studies of receptor co-capping.

It is somewhat puzzling that, when CD45 is co-capped with either the TCR or CD4, the distribution of CD45 appears very similar to that of the actively capped molecule, even though there is considerably more CD45 on the cell surface. One possible explanation for this may lie in a report by Andre et al. (1990) who show that, because of differences in fluorescence intensities, a partially capped molecule may appear completely capped, since the eye or the camera is unable to pick out a low level of peripheral fluorescence compared to the intense fluorescence of the cap. It was partly to test this that the label-fracture experiments were undertaken.

Fig 3.10a and b shows CD45 co-capped with the TCR. The large gold particles, labelling the actively capped TCR, appear to be concentrated in four areas within the cap,

with the small particles, identifying co-capped CD45, randomly dispersed throughout the cap region. The partitioning of label within the cap was not always observed however, and sometimes the TCR appeared randomly distributed. Gold labelling outside the cap was never observed suggesting that CD45 completely co-caps with the TCR. Unfortunately, when CD45 was capped no gold labelling of CD4 or the TCR was obtained, either peripherally or within the cap region (Fig 3.11). When cells were incubated with antibodies in the presence of sodium azide, to prevent capping and maintain the receptors in a diffuse distribution, only very low labelling with anti-CD45 was observed and no labelling with anti-CD4 or anti-TCR.

This suggested that label was being lost from the replica surface, particularly when molecules were diffusely distributed and so less cross-linked by antibodies. Increases in the glutaraldehyde fixative concentration and fewer washes of the replica were tested, but no improvement in the level of gold labelling was achieved.

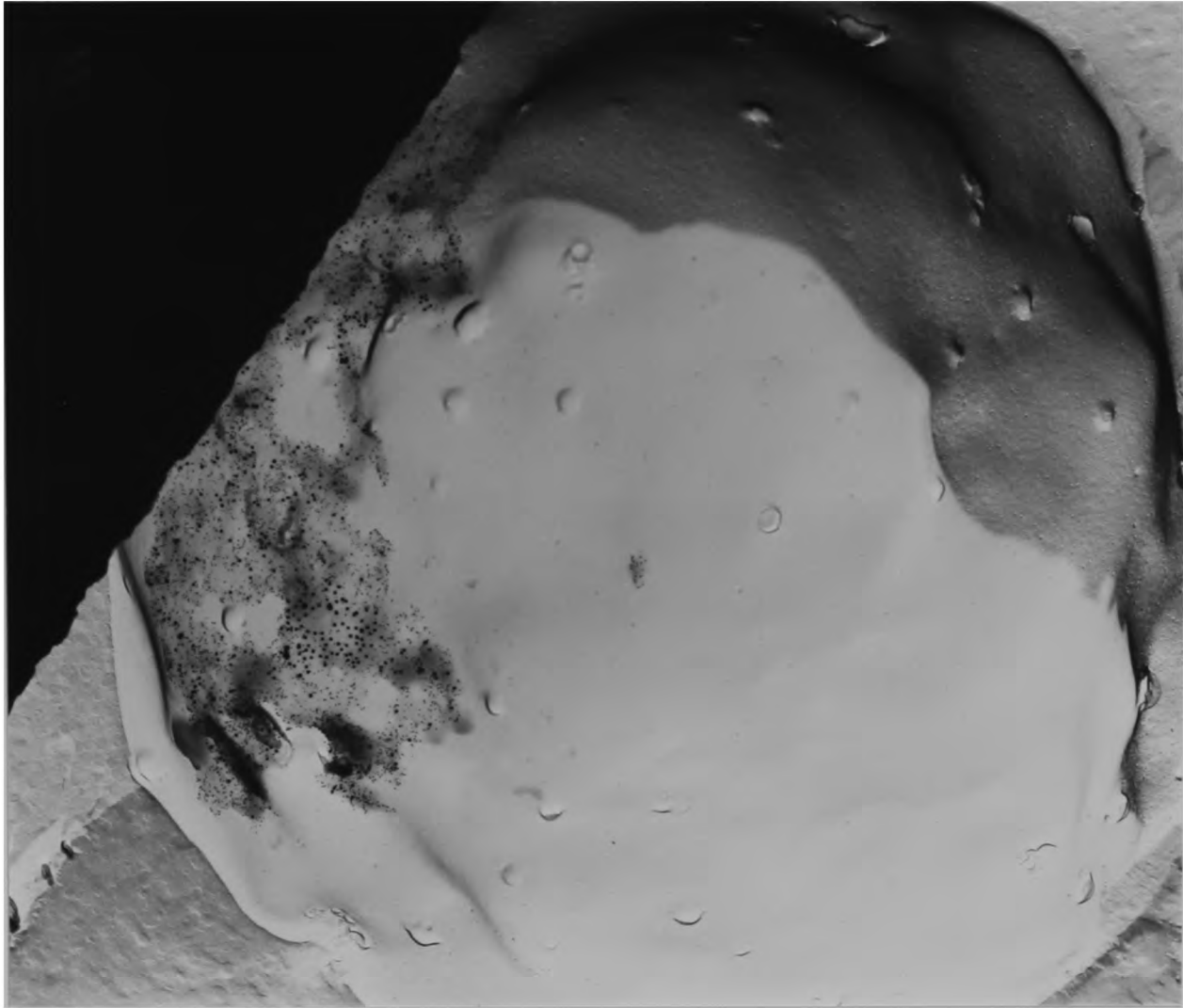


Figure 3.10 Immunogold labelled freeze-fracture replica showing CD45 co-capped with the T cell receptor. Cells were labelled with R73 followed by 15 nm gold-conjugated anti-mouse and incubated at 37°C for 15 minutes. They were then labelled with OX-1[Biotin] followed by 5 nm gold conjugated streptavidin. (a) magnification x . (b) magnification x

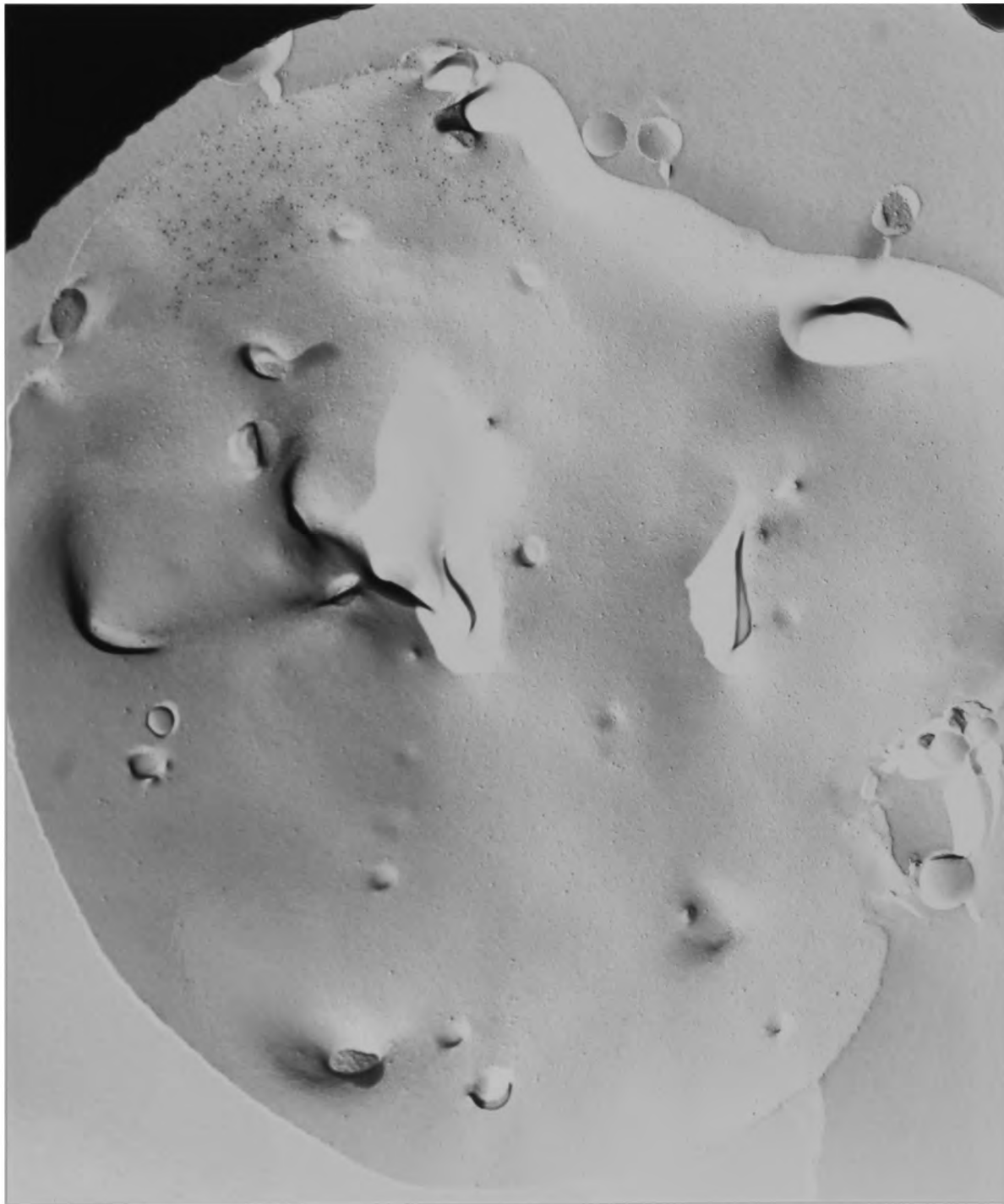


Figure 3.11 Loss of gold labelling of secondary antigen following CD45 capping. Cells were labelled with OX-1 followed by 5 nm gold conjugated anti-mouse and incubated at 37°C for 15 minutes. They were then labelled with R73[Biotin] followed by 15 nm gold conjugated streptavidin.

3.4 Discussion.

As mentioned previously, capping receptors with antibodies is an unphysiological procedure, in that theoretically all receptors are bound and crosslinked whereas in cell-cell couples only a proportion of the receptors would be bound. However, clustering of receptors at the point of cell contact does occur (Kupfer and Singer, 1988) and co-capping studies can provide useful information on receptor associations.

When the TCR is crosslinked and capped, CD45 and CD4 also redistribute into the cap region. Similarly, both CD45 and the TCR co-cap with CD4 but neither the TCR nor CD4 co-cap with CD45. This suggests that either the three molecules exist on the T cell surface in a complex which is broken down by OX-1 binding and/or crosslinking but not by CD4 or TCR crosslinking, or the molecules exist independently and an association is formed following the ligation and/or crosslinking of CD4 or the TCR, but not by crosslinking CD45.

This co-capping work is similar, in some respects, to a recent report by Dianzani et al. (1990) on murine T cells. Here co-capping studies are taken to the next logical step by studying the role of CD45 isoforms. These have been discussed fully in section 1.6b, and so will only be briefly summarised here. CD4⁺ T cells express a number of different CD45 isoforms with molecular weights ranging from 180-220 kD (Brown and Williams, 1982). Antibodies, such as OX-22, which recognise an epitope encoded by the C exon (Spickett et al., 1983), one of the alternately spliced exons within the rat CD45 gene, have been shown to identify a sub-population of CD4⁺ T cells with the characteristics of naive cells, with respect to antigen recognition.

Dianzani et al. use an antibody called 16A to identify sub-populations of CD4⁺ T cells according to expression of CD45 isoforms. 16A binds to an epitope on high molecular weight isoforms within the region encoded by exon B, and is claimed to identify the population of naive CD4⁺ T cells. They propose that co-capping of CD45 and CD3 with CD4 is restricted to the 16A⁻ subpopulation of CD4⁺ T cells, representing the memory population.

Results already presented in this section (Table 3.2) indicate that co-capping does not occur on all cells within the rat CD4⁺ T cell population and therefore may be restricted to a sub-population determined by expression of a particular CD45 isoform. A direct comparison between these results and those of Dianzani et al. is not possible as OX-1 binds to a common CD45 epitope and therefore does not identify a sub-population of CD4⁺ T cells. However, like 16A, OX-22 is claimed to separate CD4⁺ T cells into naive (CD45R0) and memory (CD45R⁺) phenotypes, with approximately 1/3 cells in the OX-22⁻ (CD45R0) population (Powrie and Mason, 1989). If the same situation occurs in the rat as in the mouse, and CD45 and CD3 only co-cap on CD45R0 cells, then one would expect approximately 1/3 of an unfractionated CD4⁺ T cell population to co-cap these molecules in the rat.

Reference to Table 3.2 indicates that this is not the case, as 49% of unfractionated CD4⁺ T cells co-cap CD3 with CD4, and 71% co-cap CD45 with CD4. From this data therefore, it is unlikely that co-capping of the molecules studied occurs only on memory cells.

It is notable that after fractionation of the CD4⁺ cells with 16A antibody, Dianzani et al. do not observe an absolute correlation between CD45 isoform expression and the co-

capping of CD3 with CD4. In fact, in the 16A⁺ population, 27% of cells co-cap. If the co-capping data from the fractionated populations is pooled and weighted according to representation in an unfractionated population, 44% of all CD4⁺ cells co-cap CD3 with CD4. This figure is very similar to that reported for rat cells here.

The lack of correlation between expression of the memory phenotype and co-capping is not entirely surprising. If the associations between different surface molecules are somehow directed by particular CD45 isoforms, then the fact that T cells have the potential to express up to eight different molecular species of CD45, suggests that the interactions which occur are far more complicated than that proposed by Dianzani et al. The production of antibodies to specific CD45 isoforms could help resolve this problem.

Returning to the remainder of the observations presented in this chapter, when CD45 is co-crosslinked with the T cell receptor CD4 does not co-cap. Similarly when CD45 and CD4 are co-crosslinked the TCR does not co-cap. However, if the TCR or CD4 are co-crosslinked with CD5, the pattern of co-capping is the same as if the molecules are crosslinked on their own. This would suggest that the failure to co-cap is either (1) a property of OX-1 binding to CD45, or (2) an effect of CD45 being brought into close association with the TCR or CD4.

One possible explanation for these observations, based on the first of these options, is as follows; CD45, CD4 and the T cell receptor may exist independently on the cell surface and an association between the molecules may occur following ligand binding and/or

crosslinking of the T cell receptor or CD4, possibly via the external region of the low molecular weight CD45 isoform. CD45 has been shown to associate with the cytoskeletal protein fodrin (Bourguignon et al., 1985; Suchard and Bourguignon, 1987), so initiation of capping through contraction of cytoskeletal components could result in CD45 carrying along CD4 and TCR, so that all three molecular species become concentrated into a single area.

The OX-1 antibody may therefore bind to an epitope on CD45 involved in the interaction with CD4 and the TCR thereby preventing association between the receptors. Secondary crosslinking would still result in coaggregation of CD45 with whichever molecule it was being crosslinked. The multiple isoforms of CD45 provide a basis for selective redistribution of surface molecules dependant on where they bind to on the CD45 extracellular domain. Thus different signals could be transmitted through the formation of different receptor combinations.

If this hypothesis is correct, then OX-1 should be able to inhibit the co-capping of CD4 with the TCR, or vice versa, regardless of whether it is co-crosslinked. This can be tested by incubating the cells with R73 or W3/25 whole antibody, and Fab fragments of OX-1. Crosslinking could then be carried out with an Fc specific anti-mouse antibody, which should bind only to the R73 or W3/25 whole antibodies. After capping, the distribution of the second molecule could be determined using the standard biotin/avidin system.

The alternative hypothesis suggests that CD45 prevents co-capping by being brought into close proximity with CD4 or the TCR, and thereby exerts a negative influence. The obvious way in which such an effect could be exerted is through the tyrosine phosphatase

activity of CD45. This is much more difficult to resolve, as so little is known about the biochemical pathways controlling capping. There are a number of questions which must be answered;

1. Is the mechanism of capping the crosslinked TCR alone the same as when TCR and CD45 are co-crosslinked?

It is possible that two capping mechanisms exist, one active and the other not (see section 1.9a). Crosslinking the TCR alone could initiate tyrosine phosphorylation events which ultimately lead to cytoskeletal mediated capping of the TCR, and co-capping of CD45 and CD4. The close association of a tyrosine phosphatase with the receptor complex, such as occurs when the TCR and CD45 are co-crosslinked, could prevent these phosphorylation events and thereby prevent active capping. The TCR and CD45 would still be able to cap by a different, passive mechanism.

PLC δ - ty^(P)

2. Does CD4 co-cap with the T cell receptor (or vice versa) because of an induced association between CD4 and CD45, and because CD45 actively "carries" CD4 into the cap?

There is no evidence to suggest that CD4 associates with the cytoskeleton. It is possible that another molecule, such as CD45 acts as a linker. A CD4/CD45 association could be induced by an initial TCR mediated tyrosine phosphorylation event which could be inhibited by CD45 tyrosine phosphatase.

The limited knowledge on this area means that speculation is almost boundless. It is apparent that fundamental questions concerning the control of receptor capping must first be answered. This is likely to be a difficult task, as the answers have remained elusive for over two decades.

Finally, it is unfortunate that the freeze-fracture work did not produce any further insight into the nature of the co-capping which was observed under the light microscope, as the preparation of samples for freeze-fracture is extremely time-consuming. Although the problem of the loss of label from the cell surface may have eventually been solved, it was decided that the results potentially available from the development of double immunogold label-fracture, did not justify the time that would be required. The double immunogold label-fracture were therefore not pursued further.

In conclusion, the results presented in this chapter agree with observations made on human and murine cells (see section 3.1), and indicate that physical associations between CD4, CD45 and the TCR occur on the surface of rat T lymphocytes. The significance of these findings in relation to T cell proliferation and intracellular tyrosine phosphorylation events will be further discussed in Chapter 7.

CHAPTER 4

EFFECT OF ANTIBODY-MEDIATED RECEPTOR CROSSLINKING ON PROLIFERATION

4.1 Introduction.

Physiological activation of resting T cells occurs via the interaction of the T cell receptor complex with antigen and MHC molecules on the surface of an antigen presenting cell. This interaction results in a sequence of events which include tyrosine phosphorylation (Klausner et al., 1987; Hsi et al., 1989), inositol phospholipid metabolism (Imboden and Stobo, 1985; Imboden et al., 1987) a rise in intracellular free calcium (O'Flynn et al., 1985; Shapiro et al., 1985), production of interleukins and expression of their receptors (Efrat and Kaempfer, 1984; Weiss et al., 1984; Leonard et al., 1985), and proliferation (Marack and Kappler, 1986; Weiss et al., 1986). All these criteria can be used as a measure of cell activation.

Physiological activation can be mimicked in a number of ways. For example, mitogenic lectins, which are plant-derived molecules, bind to particular sugar residues and thereby crosslink a number of groups of surface molecules. Many have a powerful effect on cell activation.

Monoclonal antibodies directed against the CD3 complex (Van Wauwe et al., 1980) or the $\alpha\beta$ T cell receptor (Kaye et al., 1984) are also capable of activating T cells and provide a much more specific method of studying cell activation as most monoclonal antibodies are specific for a particular surface molecule. T cells can also be activated by a combination of antibodies to the CD2 molecule (Meuer et al., 1984). The role of this so-called "alternative pathway" of T cell activation is unclear and is currently the subject of intensive research.

It has already been mentioned in Chapters 1 and 3 that both CD4 (or CD8) and CD45 may play a role in activation via the TCR complex. Evidence suggesting a physical association between these molecules has been extensively detailed in Chapter 3 and their role in tyrosine phosphorylation events leading to activation will be discussed in Chapter 5. This section aims to describe the way in which the roles of CD4 and CD45 in activation via the TCR complex may be studied by examining the effect of antibodies to these molecules on proliferation and other activation events.

There is much variation in the results of previous studies into the effects of anti-CD4 and anti-CD45 antibodies on activation via the receptor complex. This appears to depend on such things as the antibodies and cells used, and the experimental conditions employed.

These studies may be broadly divided into two groups; those in which CD4 or CD45 are ligated separately from the T cell receptor, and those in which they are co-crosslinked.

There is a fundamental difference between these two lines of approach. In the first group one is simply investigating the effect of antibody binding to the receptor in question, whereas in the second group, effects may also be due to the fact that the antibody target is being forced into close proximity with the TCR complex.

An example of how variable results on antibody induced T cell activation can be, is shown by two reports on this subject from the same group. Ledbetter et al. (1985) reported that the proliferative response to immobilised anti-CD3 could be greatly enhanced by the addition of anti-CD45 antibodies to the culture medium. In a later paper however, (Ledbetter et al., 1988) using the same cells and antibodies, soluble anti-CD45 was said to have no effect on T cell proliferation. The only apparent difference between these experiments was that the anti-CD3 antibody was bound to Sepharose beads when stimulation with anti-CD45 was observed, and was coated on plastic microtitre wells when anti-CD45 had no effect. The difference may therefore relate to the amount of CD3 crosslinking, as this would be expected to be greater when the antibodies were immobilised on the plates. Also using anti-CD3 coated Sepharose beads, Martorell et al. (1987) found augmentation of proliferation with eleven different anti-CD45 antibodies in solution. The way in which these antibodies are exerting their effect is however, somewhat suspect as at least one of the anti-CD45 antibodies was stimulatory for a CD45⁻ cell line.

A comparison of the apparently contradictory results of Ledbetter et al. (1985) and

Ledbetter et al. (1988) with a study by Owens and Fasekas de St Groth (1987), on the effects of soluble anti-CD4 antibodies on activation, lends credence to the possibility that the amount of CD3 crosslinking affects the influence of antibodies directed against other receptors. They found that anti-CD4 antibodies in solution would inhibit the T cell response to anti-CD3 bound to sepharose beads or coated on plastic microtitre plates. In the latter case however, the anti-CD4 was only inhibitory when the concentration of anti-CD3 bound to the plate was reduced such that, in the absence of anti-CD4, the level of activation was the same as for anti-CD3 bound to sepharose beads. It was proposed that anti-CD4 antibodies disrupt the formation of TCR/CD4 complexes required for activation and that this requirement could be overcome by a high avidity TCR/ligand interaction, i.e. when anti-CD3 is immobilised at high concentration.

Interestingly, Bank and Chess (1985) showed that only certain soluble anti-CD4 antibodies would inhibit activation of T cells by anti-CD3 bound to sepharose beads. This was interpreted as being due to the transmission of a negative signal following the binding of particular antibodies to CD4.

An alternative explanation could be that inhibitory antibodies bind to the site of interaction between CD4 and the TCR complex (or an intermediary molecule), whereas non-inhibitory antibodies do not. This idea has been proposed with respect to OX-1 (anti-CD45), as an explanation of the inhibitory effect of TCR/CD45 co-crosslinking on CD4 co-capping (see Chapter 3).

Thus it appears that the stimulatory effect of crosslinking the T cell receptor complex with immobilised antibodies can be enhanced by the addition of soluble anti-CD45 antibodies

and inhibited by soluble anti-CD4 antibodies. This effect however, varies according to the amount of TCR crosslinking, and with the antibodies used.

Physically crosslinking CD4 or CD8 with the TCR complex may be physiologically relevant as the receptor complex and CD4 (or CD8) may bind to the same antigen/MHC complex on the antigen presenting cell and thereby brought into close physical association. It is interesting therefore, that when anti-CD4 antibodies are co-crosslinked with anti-CD3 antibodies, they appear to have the opposite effect to when they are in solution.

Emmrich et al. (1988) produced heteroconjugates of anti-CD4 and anti-CD3 antibodies which stimulated T cells under conditions in which homoconjugates of the anti-CD3 antibody had no effect. There have been a number of other reports in which, under the same conditions, co-ligation of CD4 and the TCR complex enhances activation, whereas separate ligation is inhibitory (Owens et al., 1987; Emmrich et al., 1986; Jonsson et al., 1989).

Whilst there are physiological reasons to suspect that an association between CD4 and the TCR occurs, the natural ligand for CD45 is unknown and until recently (Dianzani et al., 1990; Volarevic et al., 1990) there have been no reports of investigations into an association between it and the TCR. Probably as a consequence of this, studies on the effect of anti-CD45 antibodies on activation have concentrated on the use of soluble antibodies. One co-crosslinking study by Ledbetter et al. (1988) reported that co-immobilisation of anti-CD45 with anti-CD3 antibodies was inhibitory both to the increase in intracellular free calcium that follows the crosslinking of the TCR complex, and to proliferation. As with CD4, in a majority of cases anti-CD45 antibodies appear able to exert opposite effects on activation

depending on whether they are co-crosslinked with the TCR complex, or bound separately.

The antibody crosslinking employed in many of the activation studies described above, would generally have induced the receptors to redistribute into a cap (Taylor et al., 1971). In the previous chapter, it was demonstrated that, with the particular antibodies used, CD4, CD45 and the T cell receptor redistribute on the cell surface in a manner determined by which receptor(s) are crosslinked. There appears to be no universal rule as to the effect of antibodies to a particular receptor, or combination of receptors, on activation, so it would be interesting to relate the antibody induced redistribution of the receptors to the effect these antibodies have on T cell activation.

As mentioned earlier, there are a number of ways by which activation can be measured. One of these involves measurement of the amount of [³H]-thymidine incorporated into the DNA of replicating cells. This gives an indication of the relative abilities of antibodies to induce cells to proliferate. Incubating the cells with [³H]-thymidine during the period when stimulus-induced DNA replication is expected to occur, ensures that the maximum difference between background DNA synthesis and stimulus-induced synthesis is observed.

The experiments were performed in two ways: firstly as a direct repetition of the co-capping experiments described in Chapter 3, using soluble monoclonal antibodies crosslinked by secondary antibody, and secondly by using antibodies immobilised on plastic microtitre plates. The different conditions were employed to allow wider comparison of results and to compare the effects of crosslinking receptors over the whole surface of the cell, with crosslinking only a proportion as is the case when immobilised antibodies are used. The

concentrations of antibodies used were in the same range as used in the co-capping experiments so as to enable direct comparison.

4.2 Methods.

(a) Purification of antibodies.

Antibodies were purified on a protein A sepharose (Sigma) column as described in Chapter 2.

(b) Sterilisation of antibodies and equipment.

Dissecting instruments, the tea strainer used to isolate lymph node cells and the syringe containing nylon wool used in T cell isolation were all sterilised in an autoclave. PBS and RPMI 1640 tissue culture medium were sterilised by passage through 0.22 μm Falcon 150 ml bottle top filters under vacuum. Antibodies were diluted to the required concentration and sterilised by filtration through Acrodisc 0.2 μm syringe filters. All other equipment used was sterile when purchased.

(c) Isolation of sterile CD4⁺ lymph node T cells.

6-8 week old DA rats were etherised and pinned out in a class II tissue culture hood. Following washing in methanol, the rat was killed by exanguination and the cervical and mesenteric lymph nodes were removed using sterile instruments. T cells were isolated as described in Chapter 2, and CD4⁺ cells were subsequently isolated by negative panning using sterile 90 mm petri dishes (Nunc) coated with OX 8 (anti-CD8) antibodies as described in chapter 2.

(d) Control reagents.

As a negative control, the anti-human spectrin antibody 56A was used in place of the anti-TCR, anti-CD4 or anti-CD45 antibodies (see Chapter 2 for details on the antibodies). The mitogenic lectin concanavalin A (Con A)(Sigma) was used as a positive control. This was diluted to 5-50 µg/ml and used in an identical manner to the test antibodies.

(e) Effect of soluble antibodies on [³H]-thymidine incorporation.

Sterile screw-top cryotubes (Nunc), normally used for storing cells in liquid nitrogen, were used as a container in which to incubate the cells. The inside of the tubes was firstly blocked with sterile 1% BSA in PBS for 1 h to prevent the antibodies ^{adsorbing} sticking to the walls. After rinsing the tube with sterile PBS, 50 µl antibody, or a cocktail of antibodies, at concentrations ranging from 10-100 µg/ml, was added, followed by 10⁵ CD4⁺ T cells in 50 µl RPMI medium. After incubation for 45 min on ice the cells were washed x3 in RPMI medium by centrifugation at 400 g for 5 min followed by aspiration of the supernatant and resuspension in 1 ml medium. After the final wash the supernatant was aspirated and the cells resuspended in 100 µl sterile goat anti-mouse IgG diluted 1/30 in RPMI + 2% DA rat serum and 50 units/ml recombinant human interleukin 2 (rhIL2) (Boehringer Mannheim) or 100 µl RPMI + 2% rat serum and 50 units/ml rhIL2. The tubes were then put into a 37°C CO₂ incubator for 48 h. After 40 h, 1 µCi of [³H]-thymidine (Amersham) was added to each tube. At the end of the incubation period the cells were washed x6 with 1 ml PBS by centrifugation as before. After the final wash the cells were resuspended in 1 ml PBS and the cell suspension was sucked out of the tube using a glass pasteur pipette and added to 4

ml "Optisafe" liquid scintillation fluid (Pharmacia) in plastic scintillation vials. The amount of [³H]-thymidine incorporated into the cells was then measured using a 1215 Rackbeta liquid scintillation counter (Pharmacia-LKB).

(f) Effect of immobilised antibodies on [³H]-thymidine incorporation.

Sterile plastic 96 well-flat bottomed tissue culture plates (Costar) were coated with antibodies by incubation with 50 µl of diluted sterile purified ascites for 1 h at room temperature. The wells were then washed x3 by spraying them with sterile PBS followed by aspiration of the remaining buffer. The wells were then blocked to prevent other protein binding by incubation for 1 h at room temperature with 100 µl sterile 1% BSA in PBS. 10⁵ CD4+ T cells suspended in 100 µl RPMI + 2% rat serum and 50 units/ml rhIL2 were then added to each well. The cells were then left for 48 h in a 37°C CO₂ incubator and 1 µCi ³H thymidine was added to each well for the final 8 h. The cells were harvested by sucking the contents of the wells through glass fibre filters and washing for 10 seconds with PBS using a Nunc Cell Harvester 8. The filters were then air dried and the discs containing the fibre discs onto which the cells had been transferred were put into 4 ml "Optisafe" scintillation fluid and the beta radioactivity was counted as before.

4.3 Results.

(a) Standardisation of [³H]-thymidine incorporation between experiments.

There was some slight variation in the absolute amounts of [³H]- thymidine incorporated in different experiments. In order to simplify comparisons between experiments, the level of proliferation for each antibody was standardised against the relevant negative control in which cells were incubated with the anti-human spectrin antibody 56A, or with 56A plus anti-mouse IgG. This was done by dividing the number of counts for the test antibody(s) by the number of counts recorded for the negative control. Thus a value of 1.0 indicates a level of [³H]-thymidine incorporation identical to that of the control, and a value of 2.0 would indicate twice the control level. The level of [³H]-thymidine incorporated into cells incubated with the control antibody ranged from 600-1300 counts per minute.

(b) Effects of soluble antibodies.

The negative control antibody, 56A, did not induce an increase in [³H]-thymidine incorporation above background levels. Incubation with Con A, on the other hand, instigated a 5-10 fold increase. When cells are incubated with the mouse anti-rat $\alpha\beta$ T cell receptor antibody R73 alone, the level of ³H thymidine incorporation is the same as when they are incubated with the control antibody 56A (Table 4.1). However, when R73 is crosslinked by a secondary anti-mouse antibody, the level of proliferation increases by 3-5 times that of the control. This result is in agreement with that of Hunig et al. (1989). Neither OX 1 (anti-CD45) nor W3/25 (anti-CD4) had any effect on proliferation, when used alone or in

combination with anti-mouse crosslinking antibody.

Antibody used	Dilution ($\mu\text{g/ml}$)	Secondary antibody	Level of proliferation*		
			Exp. 1	Exp. 2	Exp. 3
R73	10	no	1.08 \pm 0.1	0.99 \pm 0.2	ND
R73	50	no	0.93 \pm 0.1	1.14 \pm 0.4	1.08 \pm 0.3
R73	100	no	ND	0.98 \pm 0.1	0.84 \pm 0.1
R73	10	yes	3.01 \pm 0.4	5.00 \pm 0.5	2.99 \pm 0.3
R73	50	yes	4.30 \pm 0.3	4.82 \pm 0.4	3.19 \pm 0.4
R73	100	yes	4.94 \pm 0.4	5.17 \pm 0.6	3.96 \pm 0.4
W3/25	10	yes	1.23 \pm 0.1	1.09 \pm 0.1	ND
W3/25	50	yes	1.42 \pm 0.1	1.17 \pm 0.1	ND
W3/25	100	yes	1.32 \pm 0.1	0.98 \pm 0.1	ND
OX-1	10	yes	1.00 \pm 0.1	1.23 \pm 0.2	1.09 \pm 0.2
OX-1	50	yes	1.55 \pm 0.2	1.07 \pm 0.1	1.18 \pm 0.1
OX-1	100	yes	0.89 \pm 0.1	1.24 \pm 0.2	1.07 \pm 0.1
OX-19	10	yes	1.25 \pm 0.1	1.23 \pm 0.2	ND
OX-19	50	yes	1.07 \pm 0.1	1.32 \pm 0.1	ND
OX-19	100	yes	1.31 \pm 0.1	1.15 \pm 0.1	ND

Table 4.1. Effect of soluble antibodies on [^3H]-thymidine incorporation relative to negative control. *: mean \pm SEM, N=4.

When R73 was co-crosslinked with W3/25 or OX 19 (anti-CD5), the level of [^3H]-thymidine incorporation was the same as when R73 alone was crosslinked (Table 4.2). However, if R73 was co-crosslinked with OX 1 there was a marked inhibition of proliferation. Generally this reduced the level of [^3H]-thymidine incorporation to about 50%

of the increase seen with crosslinked R73 alone. The relative concentrations of the co-crosslinked antibodies did not appear to significantly affect the level of inhibition. Co-crosslinking OX 1 with W3/25 did not increase proliferation above basal levels.

(c) Effect of immobilised antibodies.

The effect of antibodies coated on plastic plates appeared to be identical to that observed for antibodies in solution. Lymphocytes in wells coated with R73 showed a 5-7 fold increase in proliferation over lymphocytes in wells coated with 56A, OX-1 or W3/25. This level of stimulation was reduced by approximately 50% by co-immobilising R73 with OX-1, but not by co-immobilising R73 with either 56A or W3/25, neither of which had any effect. The results of three representative experiments are shown in Table 4.3.

(d) Effect of simultaneous incubation with immobilised and soluble antibodies.

In the co-crosslinking experiments described in (c) above, the antibodies were pre-mixed and bound to the plates, prior to the addition of the cells. On a number of occasions however, one of the antibodies was omitted from this mixture, such that the plates were coated with only one antibody. In these cases, the second antibody was added either at the same time, or some 15 min after, the addition of the cells. All antibody concentrations were 50 µg/ml.

Antibodies used				Level of proliferation*		
A	Conc. (µg/ml)	B	Conc. (µg/ml)	Exp.1	Exp.2	Exp.3
R73	10	-	-	4.37±0.5	4.21±0.4	3.90±0.4
R73	50	-	-	4.61±0.5	4.77±0.4	4.12±0.5
R73	100	-	-	4.79±0.6	4.56±0.4	ND
R73	10	OX-19	10	ND	5.64±0.6	4.10±0.4
R73	10	OX-19	50	ND	5.15±0.3	4.67±0.5
R73	10	OX-19	100	ND	4.67±0.5	4.78±0.4
R73	50	OX-19	10	ND	4.89±0.6	4.17±0.2
R73	50	OX-19	50	ND	5.00±0.4	5.13±0.6
R73	50	OX-19	100	ND	5.25±0.4	4.30±0.4
R73	100	OX-19	10	ND	4.31±0.5	4.17±0.4
R73	100	OX-19	50	ND	4.09±0.6	4.97±0.4
R73	100	OX-19	100	ND	5.01±0.5	4.56±0.5
R73	10	W3/25	10	5.03±0.6	4.87±0.5	4.99±0.5
R73	10	W3/25	50	4.35±0.5	4.81±0.2	4.71±0.6
R73	10	W3/25	100	4.67±0.5	ND	4.98±0.5
R73	50	W3/25	10	5.43±0.5	5.01±0.3	4.47±0.4
R73	50	W3/25	50	5.15±0.4	4.10±0.5	5.18±0.5
R73	50	W3/25	100	4.97±0.5	4.61±0.5	5.31±0.7
R73	100	W3/25	10	4.18±0.5	4.75±0.3	3.92±0.4
R73	100	W3/25	50	4.88±0.6	4.67±0.6	4.71±0.5
R73	100	W3/25	100	4.76±0.4	5.71±0.3	ND
R73	10	OX-1	10	2.15±0.3	1.98±0.3	2.37±0.2
R73	10	OX-1	50	2.87±0.2	1.78±0.2	2.00±0.2
R73	10	OX-1	100	2.01±0.2	1.73±0.2	1.98±0.2
R73	50	OX-1	10	2.61±0.3	2.22±0.3	2.45±0.2
R73	50	OX-1	50	2.35±0.2	2.17±0.3	ND
R73	50	OX-1	100	ND	1.86±0.2	2.01±0.2
R73	100	OX-1	10	2.65±0.1	2.08±0.2	3.15±0.5
R73	100	OX-1	50	2.14±0.2	1.79±0.2	2.43±0.2
R73	100	OX-1	100	ND	2.17±0.4	2.11±0.1

Table 4.2. Effect of co-crosslinking soluble antibodies on the level of proliferation relative to the negative control.

*: mean ± SEM, N=3.

Antibodies used				Level of proliferation*		
A	Conc. (µg/ml)	B	Conc. (µg/ml)	Exp.1	Exp.2	Exp.3
R73	10	-	-	6.17±0.5	5.98±0.7	6.34±0.6
R73	50	-	-	6.67±0.4	6.24±0.6	6.89±0.8
R73	100	-	-	6.14±0.5	6.40±0.6	5.13±0.6
W3/25	10	-	-	1.07±0.1	1.06±0.1	ND
W3/25	50	-	-	0.97±0.1	1.23±0.2	ND
W3/25	100	-	-	1.43±0.2	1.11±0.1	ND
OX-1	10	-	-	1.09±0.1	0.81±0.2	ND
OX-1	50	-	-	0.89±0.1	1.24±0.2	ND
OX-1	100	-	-	0.88±0.1	1.20±0.2	ND
R73	50	56A	10	6.34±0.5	6.57±0.7	7.04±1.0
R73	50	56A	50	6.45±0.6	6.11±0.6	5.79±0.6
R73	50	56A	100	ND	6.00±0.6	5.87±0.7
R73	50	W3/25	10	5.91±0.7	6.24±0.5	6.87±0.8
R73	50	W3/25	50	5.71±0.5	6.23±0.6	6.35±0.6
R73	50	W3/25	100	5.15±0.6	5.78±0.6	6.50±0.5
R73	50	OX-1	10	3.17±0.4	4.12±0.3	2.99±0.3
R73	50	OX-1	50	2.87±0.4	2.95±0.3	3.02±0.2
R73	50	OX-1	100	2.01±0.2	2.69±0.3	2.75±0.3

Table 4.3. Effect of immobilised antibodies on the level of proliferation relative to the negative control.

*: mean ± SEM, N=3.

Table 4.4 shows that when plates were coated with R73 alone and OX 1 was added at the same time as the cells, there was a similar level of inhibition of [³H]-thymidine incorporation as when the plates are pre-coated with both antibodies. On the other hand, when OX 1 was added after the addition of the cells to the R73 coated wells, there was no inhibition. W3/25 added at the same time as the cells, or 15 min later, did not affect the level of proliferation to R73.

Immobilised antibody	Soluble antibody	Time of soluble antibody addition	Level of proliferation*
R73	-	-	5.56±0.5
R73	OX-1	with cells	2.48±0.4
R73	OX-1	after cells	4.78±0.4
R73	W3/25	with cells	5.78±0.6
R73	W3/25	after cells	5.13±0.2

Table 4.4. Effect of immobilised and soluble antibodies on the level of proliferation relative to the negative control.

*: mean ± SEM, N=3.

4.4 Discussion.

In these experiments the presence of IL2 was required to induce a measurable response to the crosslinked antibodies. This is consistent with the report of Hunig et al. (1989) who detected only a small response to R73 in the absence of IL2. The IL2 gene is normally activated approximately 45 minutes after T cell stimulation (Wiskocil et al., 1985). It is possible that a secondary signal is required to stimulate IL2 production which is not provided by R73. Such a signal may be provided by co-crosslinking CD4 with the TCR but this was not tested.

When the T cell receptor is bound by R73 antibody and crosslinked with an anti-mouse antibody, there is an increase in the level of [³H]- thymidine incorporation. From the results in Chapter 3, we know that, under these conditions, the T cell receptor redistributes within the membrane to form a cap and CD4 and CD45 are passively co-redistributed. The same clustering of receptors occurs when CD4 is bound by W3/25 and crosslinked by secondary antibody. However, in this case there is no stimulation of proliferation. Also, co-crosslinking CD45 with the TCR complex leaves CD4 diffusely distributed and, under these conditions, there is an inhibition of ³H thymidine incorporation.

These results suggest that all three receptors may need to be associated for antibody mediated activation to occur. When CD4 is excluded from that group by co-crosslinking CD45 with the T cell receptor, full activation does not occur. The fact that inhibition is not complete may mean that only a subpopulation of CD4⁺ cells are inhibited. It is possible that this group is represented by those cells that are capable of co-capping CD4 with the T cell

receptor, although there is no direct evidence for this.

Co-localisation of CD4, CD45 and the TCR, however, is not enough to stimulate proliferation, as is evidenced by the lack of response to CD4 crosslinking. It would appear that the TCR must be bound for activation to occur.

These results can be interpreted in an alternative way. Whilst the co-capping data presented in the previous chapter suggests that co-crosslinking CD45 with the TCR prevents CD4 from co-capping, the possibility that a small proportion of CD4 molecules are functionally associated with the TCR under these conditions, cannot be excluded. In this case, the observed inhibition of proliferation may not be due to the exclusion of CD4 from the cap, but may be the result of a negative effect exerted by CD45 co-crosslinked with the TCR. A similar interpretation may be made if it transpires that CD4 does not need to physically associate with the TCR in order to play a role in activation.

Some groups have observed an augmentation of activation when CD4 and the TCR complex are co-crosslinked (Emmrich et al., 1988; Owens et al., 1987; Jonsson et al., 1989). The antibodies used under the conditions employed in these studies reveal no such effect. This apparent contradiction may be due to the concentrations of R73 used to stimulate the cells. Owens et al. (1987) found an augmentation of response only at low, sub-optimal, concentrations of anti-TCR antibody. Such concentrations were not tested in these experiments, as it was intended make them directly comparable with the co-capping data. Consequently, the concentrations of antibody used apparently induced maximal stimulation.

Another possible explanation for the failure to observe enhanced stimulation when CD4 and the TCR are co-crosslinked comes from the work of Jonsson and Eichman (1990). They report that the enhanced response of co-crosslinking CD3 with CD4 observed by some workers, is in fact a function of the time point at which measurements of activation are taken and due to an ^{acceleration} increase in the kinetics, not the magnitude, of the response. In the experiments described in this chapter, only very large changes in the kinetics would have been observed as cells are incubated with [³H]-thymidine for eight hours.

The results presented in this chapter are consistent with the theory presented in Chapter 3, that the binding site of the antibody may influence receptor associations. To summarise, it was proposed that OX-1 (anti-CD45) bound to the site of interaction between CD45 and the TCR and CD4. This model also implies that W3/25 and R73 do not interfere with these associations as both R73 and W3/25 are able to induce co-clustering of all three molecules.

Consistent with this model are the observations on the effects of incubating the cells with immobilised R73 and soluble OX-1 or W3/25. When OX 1 is allowed to bind CD45 before the cells are incubated with immobilised R73, the effect is inhibitory, whereas incubation with OX 1 after exposure to R73 has no effect. If however, soluble W3/25 is added to the cells prior to exposure to R73, there is no observable effect on activation. Under the proposed model, pre-incubation of the cells with OX-1 would block the association of CD45 with CD4 and the TCR complex, there would be no co-capping and no activation. W3/25 does not affect these molecular interactions so pre-incubation with this antibody has no effect on co-capping or activation. Once R73 has bound to the T cell receptor, it is assumed that the associations between CD4, CD45 and the TCR complex have already been

induced and therefore cannot be affected by subsequent addition of OX-1.

Support for this model comes from the work of Bank and Chess (1985). They found that the anti-CD4 antibody OKT4C, in soluble form, inhibited proliferation in response to Sepharose bound anti-CD3 antibody, but that antibodies such as OKT4 and OKT1 directed against other epitopes on CD4 did not. If OKT4C bound to the site of interaction with CD45 and/or CD3 then it would inhibit the formation of the receptor associations which the model implies are necessary for activation. Thus OKT4C could be said to be acting in a similar way to OX 1.

The fact that the results using soluble and immobilised antibodies are similar implies that crosslinking or co-crosslinking only a proportion of receptors, which may be physiologically relevant, produces the same effect on activation as ligation of receptors over the whole surface, as occurred in the co-capping studies. At the time of the initial co-capping experiments, it was not possible to view the distribution of receptors when immobilised antibodies were used. This is because conventional microscopes cannot focus through a cell to the area of contact between the cell and antibodies immobilised on plastic plates. The out-of-focus-blur to which conventional microscopes are subject means that it is impossible to distinguish between diffuse and capped receptor distribution on the top or bottom surfaces of the cell. This is why all conventional photographs of capped cells show the cap located on the side of the cell. However, the development of confocal microscopy (in our laboratory) means that in future it will be possible to view caps situated on the top or bottom of the cell and so to determine whether the co-capping results presented in chapter 3 are also

true when immobilised antibodies are used. The results of Kupfer and Singer (1987) and Andre et al. (1990), using cell-cell couples indicate that capping and co-capping do occur when only a proportion of receptors are crosslinked. It is therefore not unreasonable to apply the same model to activation studies using either soluble antibodies or immobilised antibodies.

CHAPTER 5

EFFECT OF ANTIBODY-MEDIATED RECEPTOR CROSSLINKING ON TYROSINE PHOSPHORYLATION

5.1 Introduction.

Discussion of results in the previous two chapters has focussed largely on how antibody binding might affect interactions between CD4, CD45 and the T cell receptor, and how this relates to cell proliferation. Another factor which should be taken into account is the importance of tyrosine phosphorylation in cell signalling and the recently discovered tyrosine phosphatase activity of CD45, and the tyrosine kinases associated with CD4 and the T cell receptor (Tonks et al., 1988; Rudd et al., 1988; Veillette et al., 1988; Samelson et al., 1990).

The relatively low levels of phosphotyrosine in cells compared to phosphoserine and phosphothreonine (Hunter and Sefton, 1980) meant that intracellular phosphotyrosine was detected much later than the other two phosphoamino acids (Eckhart et al., 1979). Consequently, the importance of tyrosine phosphorylation in cell signalling has only recently become apparent. Tyrosine kinases, responsible for the addition of phosphate to tyrosine

residues, have been more extensively studied than the tyrosine phosphatases which reverse the process. The former can be loosely divided into two groups, those kinases whose genes have become part of retroviruses, and transmembrane kinases, exemplified by growth factor receptors. (for reviews see Hunter and Cooper, 1985; Hunter, 1987; Yarden and Ullrich, 1988).

The effect of tyrosine phosphorylation on cell metabolism can be seen in that overexpression of retroviral associated tyrosine kinases can lead to neoplastic transformation of normal cells (Marth et al., 1985) whilst production of cell lines with mutated epidermal growth factor (EGF) and insulin receptors which lack kinase activity, resulted in a loss of ligand induced response (Honegger et al., 1988; Rosen et al., 1987). Tyrosine dephosphorylation has similarly been shown to affect cell proliferation (Livanainan et al., 1990)

Particular interest in tyrosine phosphorylation with respect to T cell activation arose from the discovery that the ζ chain associated with the TCR:CD3 complex is phosphorylated on tyrosine residues following activation of cells with antigen (Samelson et al., 1986), anti-receptor antibodies (Patel et al., 1987), anti-Thy 1 antibodies (Klausner et al., 1987), and more recently anti-CD2 antibodies (Monostori et al., 1990). Other, as yet unidentified molecules have also been shown to be tyrosine phosphorylated following T cell stimulation with antigen and anti-receptor antibodies. Phosphorylation of 100 kD and 135 kD substrates occurs within 10 seconds of stimulation (June et al., 1990a) and later phosphorylation of substrates with approximate molecular weights of 36 kD (Patel et al., 1987), 53 and 62 kD (Hsi et al., 1989) and 68 kD (Jin et al., 1990) have been reported. Phosphorylation of the ζ

chain is thought to occur 15-30 minutes after the initial activation event (Hsi et al., 1989).

Such observations implicate tyrosine phosphorylation in the process of cell activation. Further evidence was obtained by June et al (1990b), who showed that the drug herbimycin A, which inhibits protein tyrosine kinase activity, prevented T cell receptor mediated events including phosphatidyl inositol (PI) hydrolysis. The authors suggested that tyrosine phosphorylation precedes PI turnover based on the observation that herbimycin A inhibition could be partially overcome by phorbol esters, which activate protein kinase C. The fact that tyrosine phosphorylation of the 100 and 135 kD substrates precedes PI hydrolysis adds further weight to this argument (June et al., 1990a).

In contrast to the work with herbimycin, Trevillyan et al. (1990) found that the tyrosine kinase inhibitor genistein prevented IL 2 production in response to anti-CD3 antibodies which could not be overcome by phorbol ester treatment. The drug appeared to have no effect on receptor mediated PI hydrolysis and it was therefore suggested that genistein was not affecting activation via this pathway.

Prior to these experiments, Samelson et al.(1986) showed that the ζ chain is constitutively tyrosine phosphorylated in T cells from animals with a lymphoproliferative disorder resulting from the *lpr* or *gld* mutations, again providing circumstantial evidence that tyrosine phosphorylation is important in the control of T cell proliferation.

Investigations into the kinases responsible for the tyrosine phosphorylation events associated with TCR mediated activation, has centred upon the protein products of two members of the *src*

proto-oncogene family, p59^{fyn} and p56^{lck}.

Fyn kinase was described in 1986 by Kawakami et al. and Semba et al.. Recently Samelson et al. (1990) demonstrated that, under certain conditions, kinase activity could be immunoprecipitated with the T cell receptor, and suggested an association between the TCR and p59^{fyn}. Fyn was shown to be capable of phosphorylating the ζ chain and a number of other substrates, but little else is currently known about its role in T cell activation.

The 56 kD lck kinase has been more extensively studied than fyn, and consequently there is a greater body of evidence indicating that it plays a role in T cell activation. Expression of p56^{lck} is predominantly restricted to T lymphocytes, where it is the most abundant *src* family member expressed (Marth et al., 1987). Low levels of p56^{lck} have also been found in thymocytes and B cells (Perlmutter et al., 1988). In common with other *src* tyrosine kinases, the amino terminus of p56^{lck} is myristoylated and, through this, is associated with the cytoplasmic face of the plasma membrane (Buss and Sefton, 1985).

p56^{lck} was first linked to the control of T cell proliferation from observations on the lymphoma cell line LSTRA (Casnellie et al., 1982) which shows unrestricted growth, increased tyrosine phosphorylation (Casnellie et al., 1983) and a 50 fold increase in p56^{lck} expression (Marth et al., 1988a). Marth et al. (1988b) further showed that a point mutation at tyrosine 505, which is phosphorylated in resting T cells, led to an increase in phosphorylation at another tyrosine residue, 394, as well as increased kinase activity and neoplastic transformation of NIH 3T3 fibroblasts. This suggested that phosphorylated tyrosine 505 negatively regulates the kinase activity of p56^{lck} and that tyr 394 is an autophosphorylation site, a theory which was supported by the work of Amrein and Sefton (1988).

There are also changes in the serine phosphorylation state of p56^{lck} following stimulation of T cells, which result in a change in its apparent molecular weight to 59 kD (Veillette et al., 1988). Such observations providing further fuel to speculation that p56^{lck} is involved in cell activation.

Interest in p56^{lck} entered a new phase with the discovery by two groups that the enzyme is complexed to CD4 and CD8 molecules (Rudd et al., 1988; Veillette et al., 1988). CD4 and CD8 were already strongly suspected of being involved in T cell receptor mediated signal transduction (for review see Rudd, 1989), so the discovery that they were associated with a tyrosine kinase led to a flurry of activity in which attempts were made to fit this knowledge into developing concepts on the role of tyrosine phosphorylation in T cell activation.

Barber et al. (1989) showed that p56^{lck}, isolated using anti-CD8 antibodies, was able to phosphorylate the ζ chain, as well as the CD3 γ , δ and ϵ chains, *in vitro*. This was also shown to be true for CD4 associated p56^{lck} (Rudd, 1989). Tyrosine phosphorylation of the ζ chain was also demonstrated following crosslinking of CD4 by antibodies (Veillette et al., 1989). This was related to a concurrent increase in kinase activity associated with p56^{lck}. Interestingly, monovalent Fab fragments of anti-CD4 antibodies did not exert this effect, suggesting that oligomerisation of CD4 molecules is necessary in the same manner as is required for activation of the EGF receptor kinase (Gill et al., 1984; Yarden and Schlessinger, 1987). Crosslinking the TCR or Thy 1 did not increase p56^{lck} kinase activity, although the short incubation period used (1 min) does not preclude the possibility that p56^{lck} may show increased activity at a later time point.

Given that the kinase activity of p56^{lck} is probably controlled by the phosphorylation state of Tyr-505 (Marth et al., 1988), it is likely that a tyrosine phosphatase is involved in the regulation of this enzyme. A candidate for this has been found in the transmembrane glycoprotein CD45, the cytoplasmic domain of which was recently found to be a tyrosine phosphatase. This discovery arose

from the initial isolation and sequencing of human placental protein tyrosine phosphatase (PTPase) 1B (Tonks et al., 1988a; Tonks et al., 1988b). A search for related sequences revealed two domains, I and II, within the 705 amino acid tail of CD45 which show 54% and 48% homology respectively with the 35 kD placental tyrosine phosphatase, when conservative substitutions are taken into account (Charbonneau et al., 1988). Further work by this group demonstrated that CD45 was indeed capable of dephosphorylating tyrosine residues (Tonks et al., 1988c). The tyrosine phosphatase activity has since been localised to domain I (Streuli et al., 1989), although it is possible that domain II may have tyrosine phosphatase activity for different, as yet untested, substrates.

A number of other PTPases have been identified since the discovery of tyrosine phosphatase activity associated with CD45. One of these is LAR, which has a cytoplasmic domain homologous to CD45 and an external domain similar to neural cell adhesion molecule (NCAM) (Streuli et al., 1988; Cool et al., 1989; Charbonneau et al., 1989). The family of membrane bound PTPases is currently expanding rapidly (Alexander, 1990).

A number of reports have recently appeared concerning the affect of CD45 on the phosphorylation and activation state of p56^{lck}. Characterisation of the enzyme activity revealed that, unlike placental PTPase, CD45 exhibited substrate selectivity *in vitro*, and one of the substrates was p56^{lck} (Tonks et al., 1990). Ostergaard et al. (1989) demonstrated an increase in the phosphorylation of p56^{lck} at tyrosine 505 in mutant cell lines that fail to express CD45. Mustelin et al. (1989) found that loss of CD45 expression was associated with a decrease in p56^{lck} kinase activity. These observations were related to later stages of T cell activation by Pingel and Thomas (1989), who reported that CD45 negative cell lines failed to proliferate in response to antigen or anti-CD3 antibodies. The cells were still able to proliferate in response to interleukin 2, indicating that CD45 is involved specifically with T cell receptor mediated activation.

The restriction of CD45 tyrosine phosphatase activity to the plane of the membrane, along with a considerable proportion of p56^{lck} and p59^{fyn} kinase activity, implies the existence of a mechanism of control over enzyme:substrate interactions. These enzymes, associated with the cytoplasmic domains of CD45, CD4 and the T cell receptor, could be brought into contact with their respective substrates by co-ligation of the receptors, such as occurs when CD4 and the T cell receptor bind to the same antigen/MHC class II complex. Alternatively, the co-capping of CD45 and CD4 with the TCR following TCR crosslinking (as demonstrated in chapter 3) would also serve this purpose.

Having established the distribution of these three molecules following their separate crosslinking, or co-crosslinking in various combinations, and related this to the effect this has on proliferation, it seems pertinent in the light of the information detailed above, to attempt to further relate the distribution of the receptors under particular antibody crosslinking conditions to the effect on tyrosine phosphorylation.

A series of experiments were therefore undertaken, based on the crosslinking and capping studies described in chapter 3, in which cells were incubated with antibodies and allowed to cap, solubilised, and the proteins separated by SDS polyacrylamide gel electrophoresis. The tyrosine phosphorylated proteins were then identified, following transfer to nitrocellulose filters, by probing with anti-phosphotyrosine antibodies.

In chapter 4, the presence of IL2 was necessary in order to induce a measurable proliferative response to crosslinked antibodies. To determine whether this affect tyrosine phosphorylation during the time when the receptors are redistributing, the experiments were carried out in the presence and absence of IL2.

Because surface receptor capping occurs over a period of time, the pattern of tyrosine

phosphorylation was determined at intervals from 0-32 minutes and, for comparison, the distribution of the crosslinked receptors was determined at each of these time points. In order to view the receptor distribution more precisely, the cells were viewed using a cooled slow-scan charge coupled device (CCD) camera, mounted on a Zeiss microscope with facilities for epifluorescent illumination.

The CCD image is digitised, so it can be manipulated so as to eliminate the weaker signals, thereby leaving a "skeleton" image of the more concentrated areas of label on the cell surface. Thus clustering of receptors becomes apparent under conditions where, when the CCD camera is not used, the surface labelling appears dispersed due to the effect of out-of-focus blurr.

The CCD camera was also used to photograph the immunoblots, thereby enabling an image of weaker bands to be recorded.

5.1 Methods.

(a) Incubation of cells with antibodies.

DA rat lymph node cells were enriched for CD4⁺ T cells as described in chapter 2. 10⁷ cells in RPMI medium were added to plastic 1.5 ml eppendorf tubes (Scotlab). The cells were pelleted by centrifugation for 2 min in a Beckman benchtop microfuge (approximately 8,500 g) and the supernatant removed by aspiration. Antibodies were then added to the cells diluted in 50 µl medium so as to achieve the final concentrations detailed in table 5.1.

Antibody	Form	Dilution
R73 (anti-TCR)	Purified ascites	50 µg/ml
W3/25(anti-CD4)	Spent culture supernatant (S/N)	1:2
OX 1 (anti-CD45)	S/N	1:2
R73 + OX 1	Purified ascites S/N	50 µg/ml 1:2
R73 + W3/25	Purified ascites S/N	50 µg/ml 1:2
OX 1 + W3/25	S/N S/N	1:2 1:2

Table 5.1 Summary of antibodies used.

After addition of the primary antibodies, the cells were incubated on ice for 45 minutes, then washed three times by addition of 1 ml medium, centrifugation for 2 min in the Beckman microfuge

and removal of supernatant. Goat anti-mouse IgG (Sigma) was diluted 1/30 in RPMI medium and 30 μ l of this was added to each tube. The tubes were then placed in a 37°C incubator for between 0 and 32 min.

(b) Solubilisation of cells.

At each time point a tube was removed from the incubator and 30 μ l of solubilisation buffer was added. This consisted of 1% SDS, 0.8 mM sodium vanadate, 0.8 mM EDTA, 20 mM sodium fluoride, 20 mM sodium pyrophosphate, 0.6 M sodium chloride, 0.2 mM PMSF and 100 μ g/ml leupeptin in 20 mM Tris/HCl pH 8.0. The sample was then boiled for 5 min.

(c) Preparation for SDS PAGE

60 μ l of 2x reducing sample buffer (see chapter 2) was then added, and the sample was boiled for a further 2 min. Once the sample had been allowed to cool, a small pellet containing nuclear material would settle to the bottom of the tube. This was removed with the tip of a Hamilton syringe and discarded. Failure to do this resulted in smearing of the bands on the gel. The remaining sample was then loaded onto a 12.5% SDS polyacrylamide gel, and the proteins were separated by electrophoresis as previously described (Chapter 2).

Proteins were then transferred to 0.2 μ m nitrocellulose filter paper using a Bio-Rad "Trans-Blot" transfer system as described in Chapter 2.

(d) Identification of tyrosine phosphorylated proteins.

Following protein transfer, the nitrocellulose filter was washed in PBS and blocked with 2% BSA in PBS for 2 h at room temperature. The filter was then washed several times in PBS and placed in a plastic bag. To this was added 10 ml of a combination of anti-phosphotyrosine antibodies, PY20 and PY69 (purified ascites from ICN Ltd.) which had each been diluted to 1 µg/ml in PBS plus 0.1% BSA and preincubated for 30 min at 4°C to remove any non-specific activity. The bag was then sealed and left on a rocker overnight at 4°C.

Following the incubation with anti-phosphotyrosine antibodies, the filter was removed from the bag and washed six times with PBS. 30 µl peroxidase conjugated goat anti-mouse Ig (Dako) was added to 30 ml PBS + 0.1% BSA and pre-incubated for 30 min at 4°C. This was then added to the washed filter and left for 2 h at room temperature on a rocker.

The nitocellulose was then washed six times with PBS and the tyrosine phosphorylated bands were finally visuallised using chloro-1-naphthol as the peroxidase substrate, made up as described in Chapter 2.

(e) Specificity of PY20 and PY69 anti-phosphotyrosine antibodies.

To check that the antibodies PY20 and PY69 were specifically labelling phosphorylated tyrosine residues in these experiments, three samples of 10^7 CD4⁺ T cells were incubated for 30 min on ice with R73 (anti-TCR) antibody, washed in PBS, then incubated for 4 min at 37°C with 30 µl anti-mouse IgG as described in section 5.2 (a). Following lysis of the cells, separation of the proteins by SDS PAGE, and transfer to 0.2 µm ntrocellulose filters as previously described (Section 5.2 (b and

c)), the filter was cut into three strips each containing one of the separated T cell lysates. These were then blocked with 2% BSA in PBS for 2 h at room temperature. PY20 and PY69 antibodies were diluted to 1 µg/ml in PBS plus 0.1% BSA in three separate tubes. Each antibody dilution was then supplemented with one of the following; 1 µM phosphotyrosine, 1 µM phosphoserine or 1 µM phosphothreonine (all from Sigma) and incubated at 4°C for 30 min. The antibodies were then added to the nitrocellulose filters and subsequent treatment was as previously described.

(f) Effect of IL2 on tyrosine phosphorylation

The experiments were repeated according to the method described above (Section 5.1a) except that the cells were incubated at 37°C in 30 µl goat anti-mouse IgG diluted to 1/30 in RPMI medium containing 50 units/ml recombinant human IL2 (Boehringer).

(g) Determination of receptor distribution at each stage of the time course.

Following incubation of the cells at 37°C with the TRITC-conjugated goat anti-mouse Ig and immediately prior to the addition of solubilisation buffer, the cells were vortexed and 3 µl of cell suspension was removed and added to 1 ml ice cold 1% paraformaldehyde solution in PBS. 1 h later, the cells were isolated by centrifugation at 400 g for 5 min and resuspended in 100 µl "Citifluor" anti-fadant (Agar Scientific) in PBS. 12 µl of this cell suspension was then placed on a glass slide and a coverslip was placed on top. The coverslip was then sealed with nail varnish.

The cells were viewed using a photometrics cooled CCD array camera mounted on an inverted Zeiss IM35 epifluorescence microscope using relevant filter blocks (as described in section 3.2) and a Zeiss Plan-Apo x63 oil immersion lens.

At least 150 cells were counted for each time point and each cell was categorised as follows;

UNCAPPED Diffuse distribution of label.

SMALL PATCHES More than four concentrations of label over the cell surface.

LARGE PATCHES Less than four larger concentrations of label.

CAPPED One main area of label.

The percentage of cells in each of these groups at each of these time points was then calculated.

The same procedure was carried out when CD4, CD45 or a combination of receptors was crosslinked.

5.3 Results.

(a) Effect of R73 alone, anti-mouse Ig alone and crosslinked OX-1 and W3/25 on tyrosine phosphorylation.

If the cells were incubated with R73 (anti-T cell receptor) antibody for 30 min on ice and then incubated at 37°C without the addition of anti-mouse crosslinking antibody, before solubilisation and analysis by SDS PAGE and immunoblotting, there was no detectable tyrosine phosphorylation of any bands. Identical results were achieved when the cells were incubated at 37°C with anti-mouse Ig alone or with anti-mouse Ig subsequent to incubation on ice with OX-1 (anti-CD45) antibody.

If the cells were incubated with W3/25 (anti-CD4) antibody, followed by incubation with anti-mouse Ig at 37°C, tyrosine phosphorylation of a single band running at approximately 86 kD was apparent. The labelling of this band gradually increased in intensity throughout the 32 min time period (Fig. 5.1). There was no detectable increase in the tyrosine phosphorylation level of molecules running at 56 kD or 21 kD which could represent p56^{lck} and ζ respectively. A band running at approximately 50 kD is visible in Figure 5.1 and on other immunoblots presented herein. This is the heavy chain of the anti-mouse Ig which shows up on the photographs of the blots because it is TRITC conjugated.

(b) Effect of crosslinking R73 on tyrosine phosphorylation.

Figure 5.2 shows the profile of tyrosine phosphorylated proteins in CD4⁺ T cells at various time points following the addition of anti-mouse antibodies to crosslink pre-bound anti-TCR antibody R73. The detection method used in these experiments reveals a number of bands which become tyrosine phosphorylated and dephosphorylated throughout the 32 min time course.

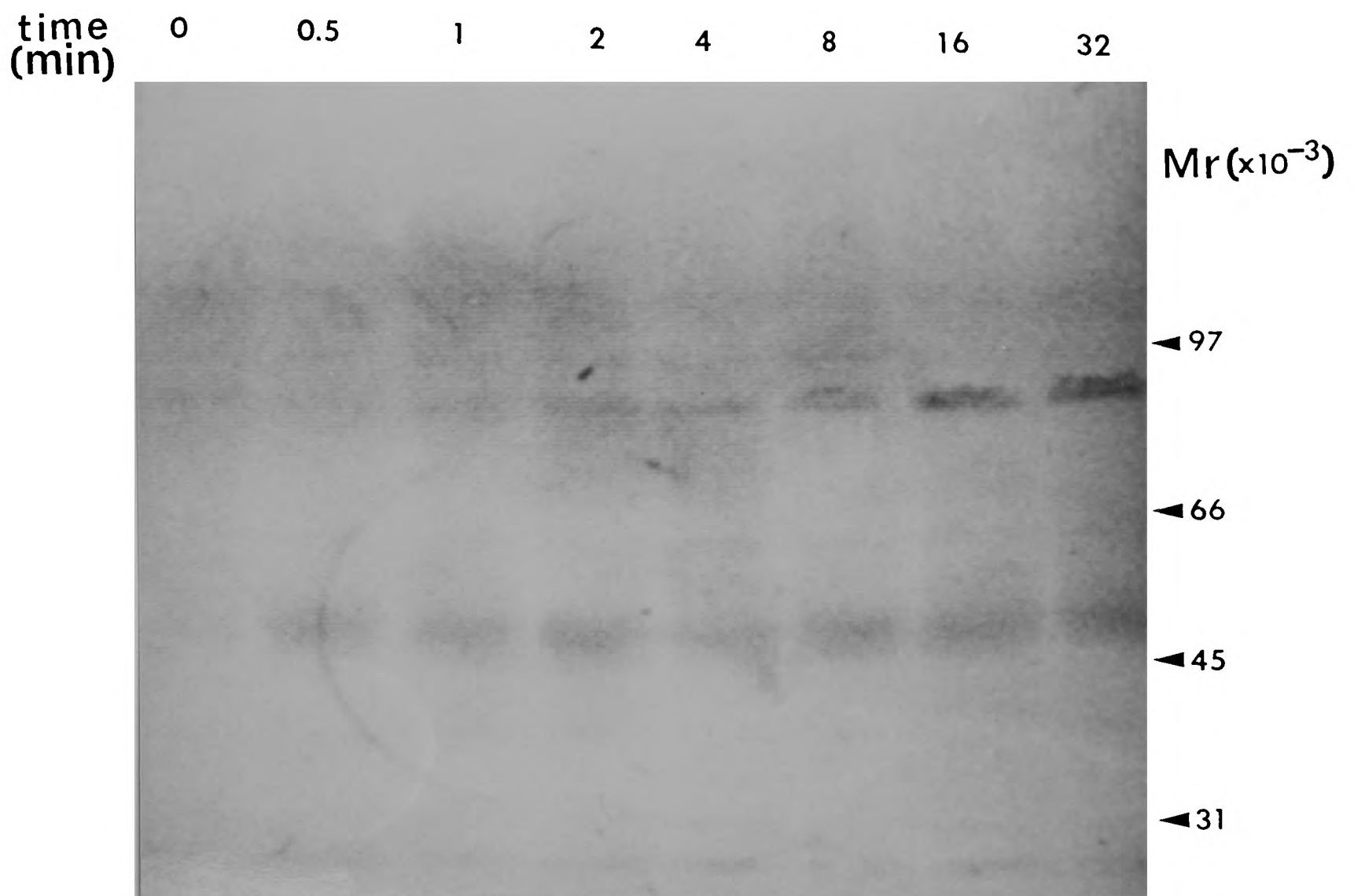


Figure 5.1 Effect on tyrosine phosphorylation, of incubating cells with W3/25 antibody followed by incubation with anti-mouse antibody at 37°C for 0-32 minutes. Cells were lysed at the specified time and the proteins separated by SDS-PAGE on a 12% gel. After transfer to nitrocellulose filters, tyrosine phosphorylated proteins were identified using anti-phosphotyrosine antibodies.

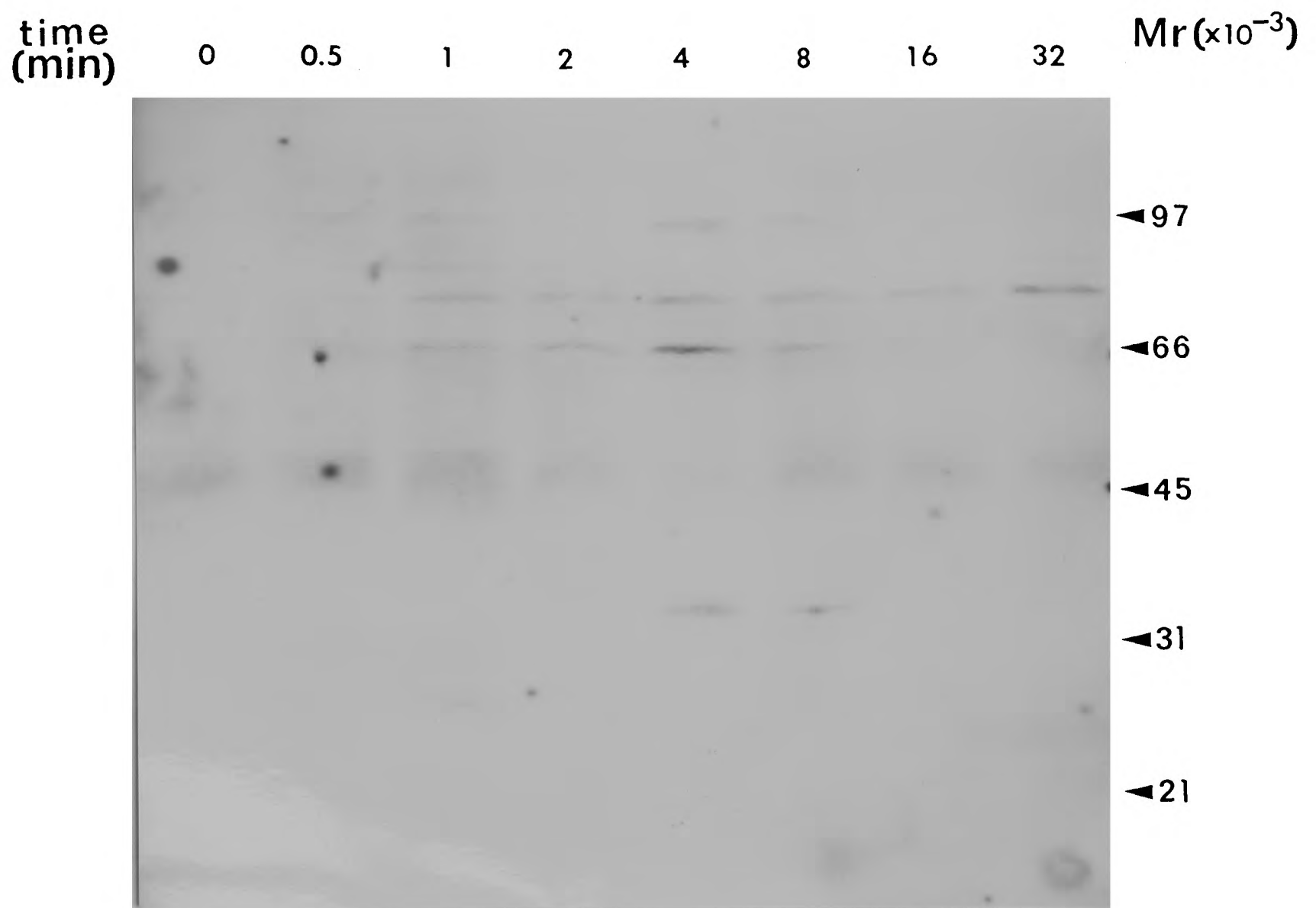


Figure 5.2 Effect on tyrosine phosphorylation, of incubating cells with R73 followed by incubation with anti-mouse antibody at 37°C for 0-32 minutes. Cells were lysed at the specified time and proteins separated by SDS-PAGE on a 12% gel. After transfer to nitrocellulose filters, tyrosine phosphorylated proteins were identified using anti-phosphotyrosine antibodies.

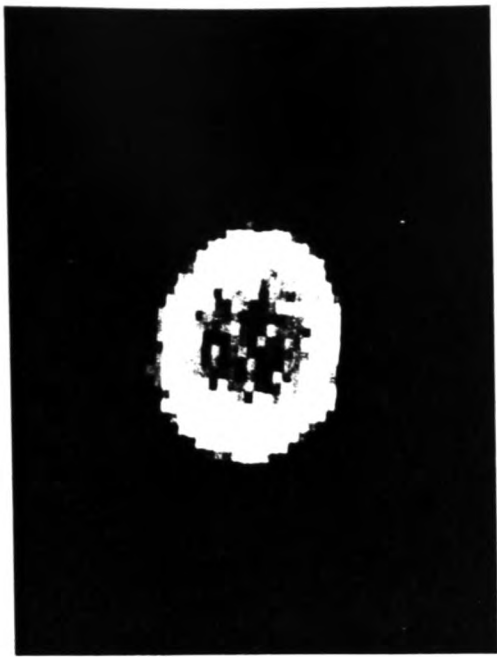
At $t=0$ there is weak phosphorylation of an 86 kD band which gradually increases as the incubation period is extended. At 1 min a faint band appears at 70 kD which reaches maximal intensity at 4 min. At this time two other bands appear at 32 kD and 100 kD which persist until 8 min, after which time tyrosine phosphorylation of the 70 kD band is also no longer detectable.

A faint band running at about 22 kD is apparent in figure 5.2 at 32 min. It is possible that this is the ζ chain but, unlike the other bands, this was not visible on every blot. There was no detectable labelling of any substrate at 56 kD which could be the CD4 associated tyrosine kinase $p56^{lck}$. Two faint bands are also visible from 0-1 minutes. These have molecular weights of approximately 105 and 120 kD.

(c) Distribution of crosslinked receptors.

An example of the distribution of the T cell receptor at selected time points is displayed in Figure 5.3, and Figure 5.4 shows how the percentage of cells in each category changes during the incubation period. From 1-4 min small patches of label are apparent, which increase in size until at 8 min a majority of the cells (71%) show a capped distribution of the TCR. The proportion of capped cells increases slightly at 16 min and at 32 min there is a reduction in the percentage of fully capped cells and a concomitant increase in the proportion of uncapped cells. There appeared to be no reduction in the number of labelled cells during the incubation period, although a quantitative analysis was not undertaken.

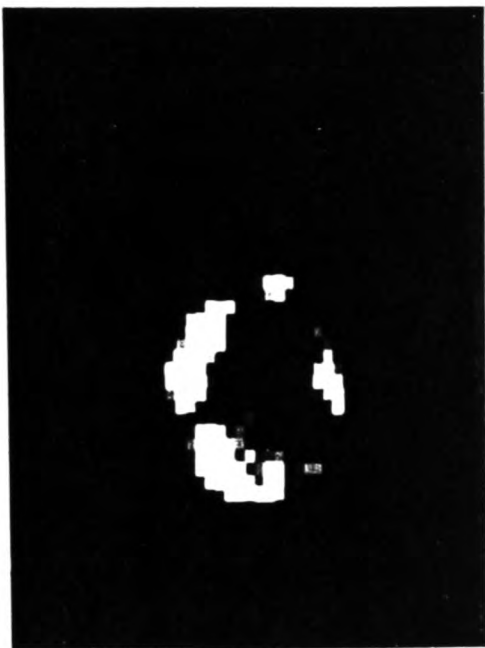
When CD4, CD45 or a combination of receptors was crosslinked the distribution of these molecules was also determined throughout the time course. These appeared to fall into two groups; slow and fast capping. The fast capping group, represented by the TCR, included CD4 and CD4 co-crosslinked with the TCR. The slow capping group was represented by CD45. Figure 5.5 shows that



a



b



c



d

Figure 5.3 Example of the distribution of the antibody crosslinked T cell receptor at various stages during the period of incubation at 37°C. (a) uncapped, (b) small patches, (c) large patches and (d) capped.

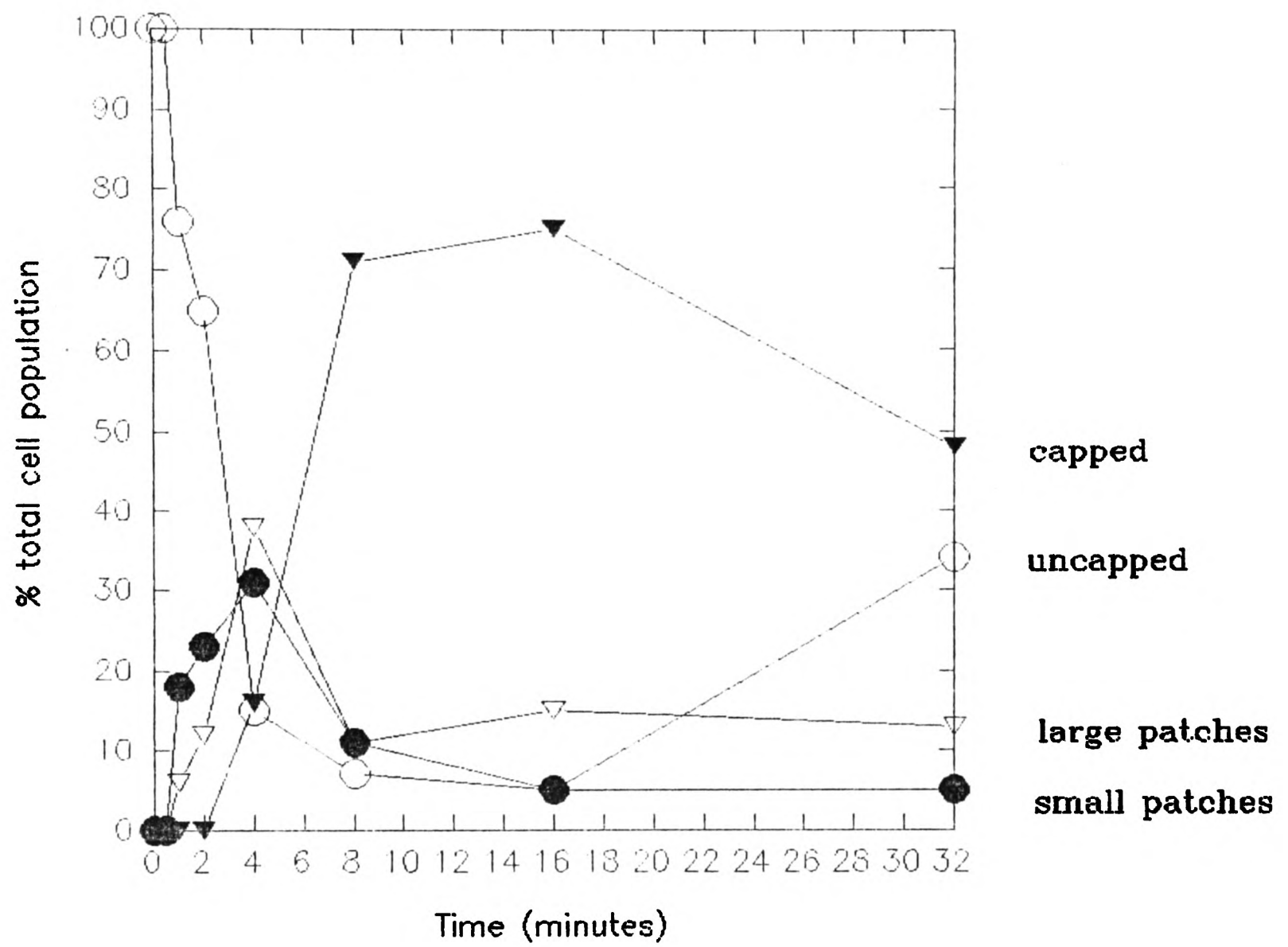


Figure 5.4 Distribution of the T cell receptor during incubation at 37°C, following crosslinking with R73 and anti-mouse IgG.

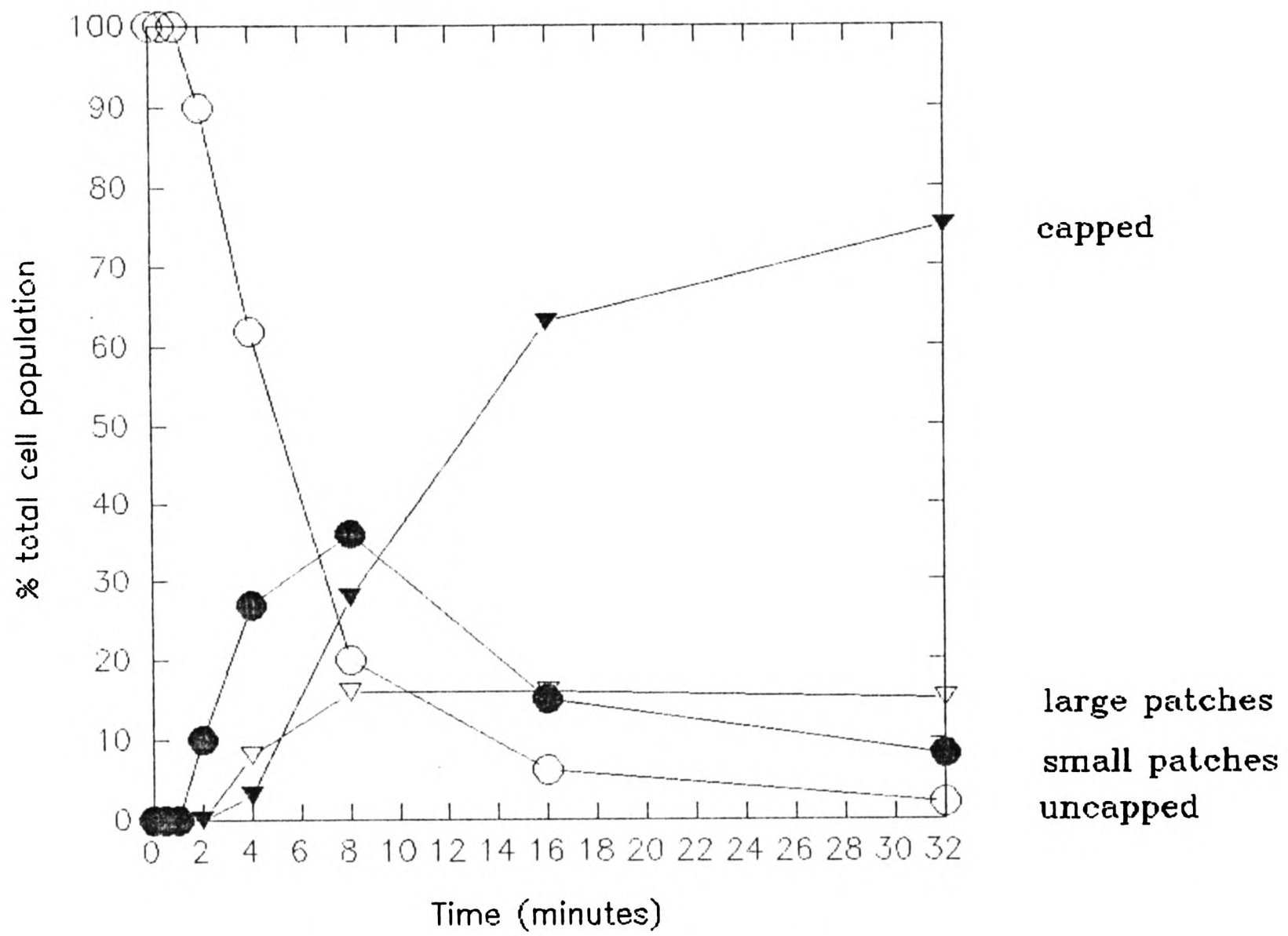


Figure 5.5 Distribution of CD45 during incubation at 37°C following crosslinking with OX-1 and anti-mouse IgG.

capping occurred in a maximum of 75% of cells incubated with OX-1 crosslinked with anti-mouse Ig, but the kinetics was slower than for the TCR. This type of capping also occurred when CD45 was co-crosslinked with CD4 or the TCR.

(d) Specificity of PY20 and PY69 anti-phosphotyrosine antibodies.

When the anti-phosphotyrosine antibodies are preincubated with phosphoserine or phosphothreonine there is no detectable inhibition of labelling of the 32, 70, 86 or 100 kD bands (Fig 5.6 a and b). However, when the antibodies were preincubated with phosphotyrosine labelling of these bands was eliminated (Fig 5.6 c).

(e) Effect of co-crosslinking combinations of receptors on the tyrosine phosphorylation profile.

When CD45 and the T cell receptor were co-crosslinked, there was a marked reduction in the level of tyrosine phosphorylation of the 32, 70 and 100 kD bands (Fig 5.7), but no significant effect on the 86 kD band, the intensity of which increased throughout the time course in the same way as when CD4 or the T cell receptor are crosslinked alone.

Co-crosslinking CD4 with the T cell receptor had the effect of accelerating and prolonging the phosphorylation of the 32, 70, 86 and 100 kD bands (Fig 5.8). It is notable that phosphorylation of the 86 kD band is not detectable at 16 or 32 minutes, whereas crosslinking the TCR alone resulted in detection of this tyrosine phosphorylated band throughout the time course. Tyrosine phosphorylation of a 21 kD substrate was never detected. Interestingly, two further bands were reproducibly present, when CD4 and the TCR were co-crosslinked, which were not detected otherwise. These had molecular weights of 48 and 50 kD and were apparent at 30 seconds. A 50 kD band is also strongly phosphorylated at 2 minutes and more weakly so at 1, 4 and 8 minutes. It is not

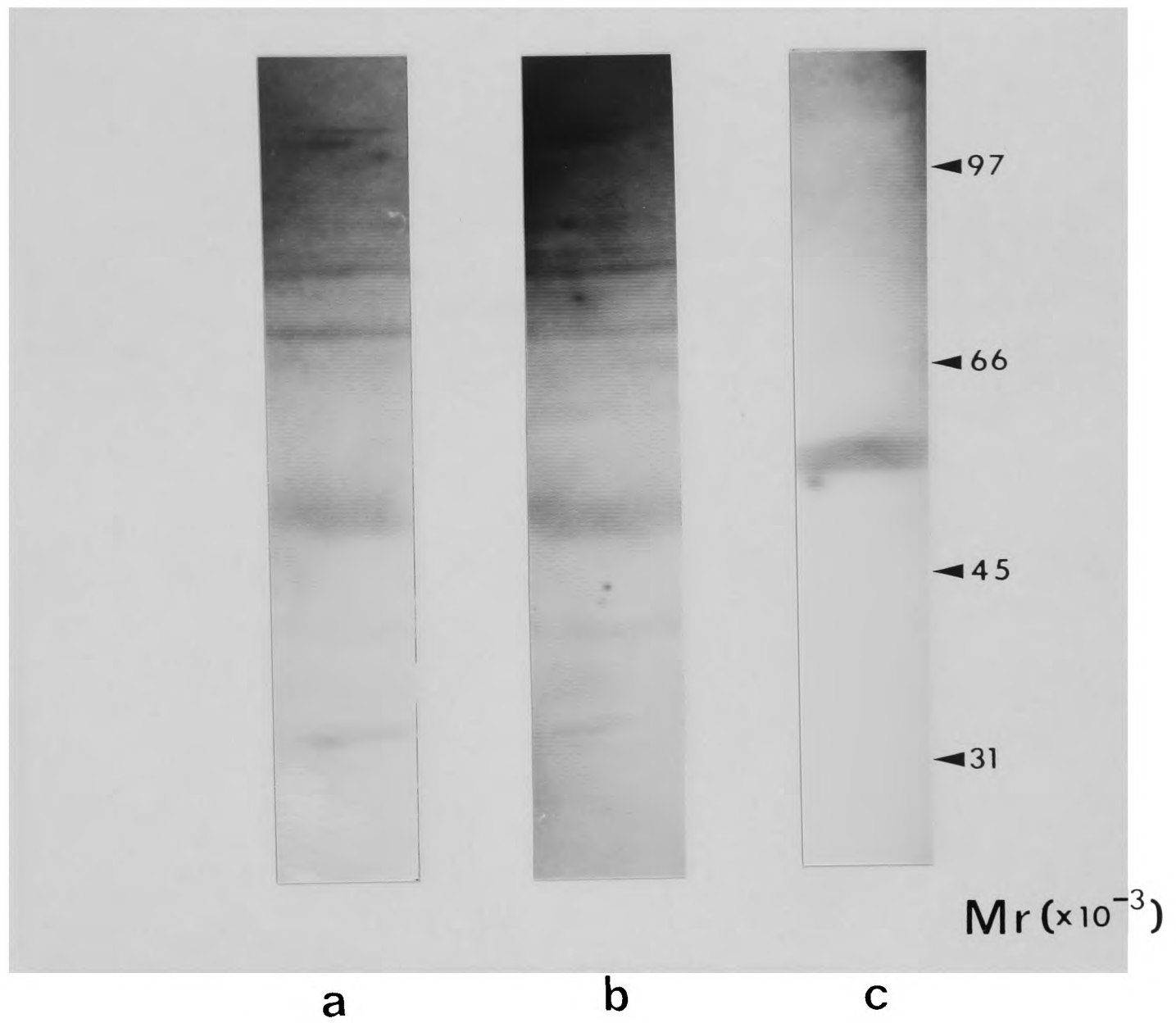


Figure 5.6 Effect of pre-incubating anti-phosphotyrosine antibodies with (a) phosphoserine, (b) phosphothreonine and (c) phosphotyrosine on the labelling of the 32, 70, 86 and 100 kD bands.

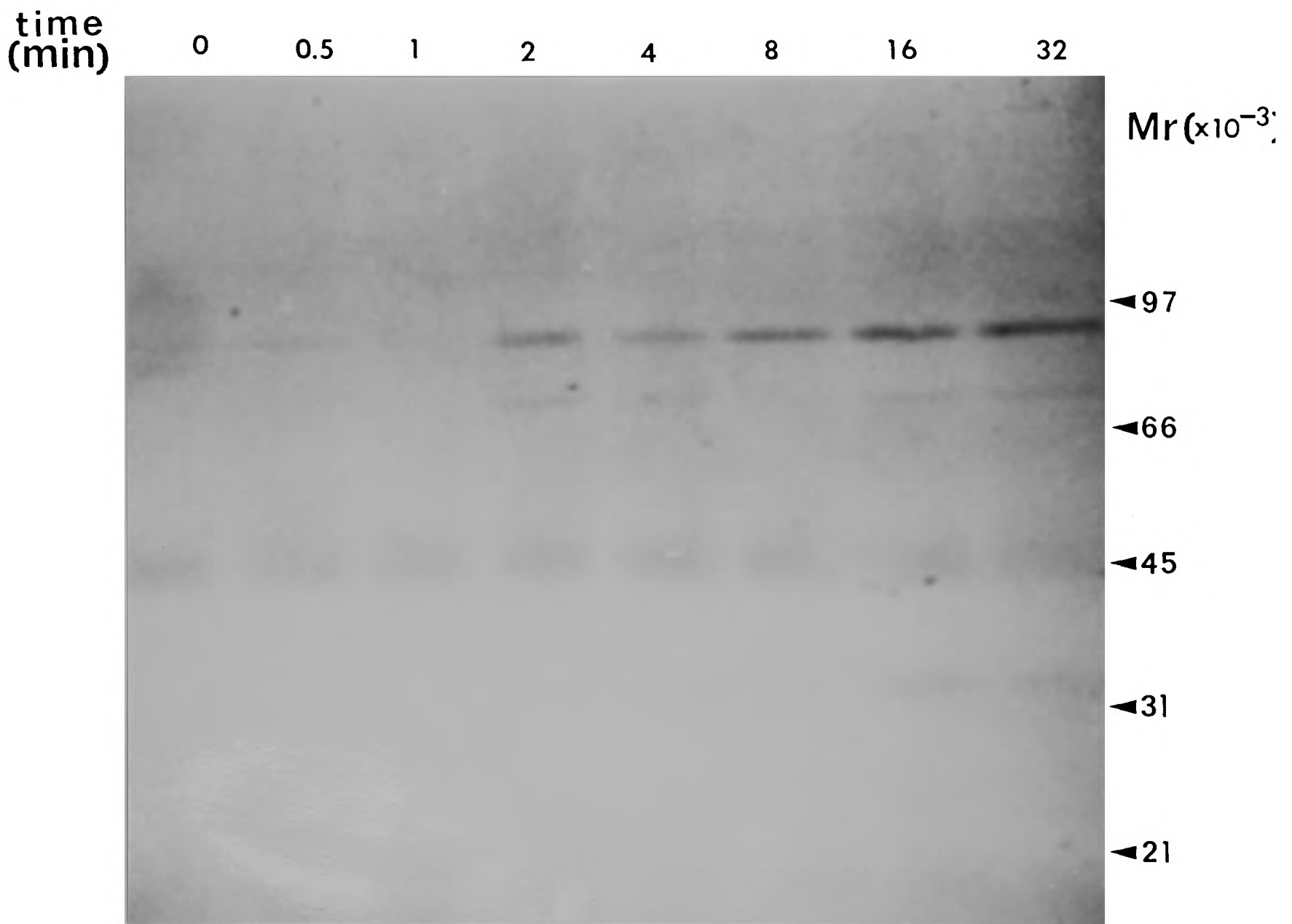


Figure 5.7 Effect on tyrosine phosphorylation, of incubating cells with R73 and OX-1 followed by incubation with anti-mouse antibody at 37°C for 0-32 minutes. Cells were lysed at the specified time and proteins were separated by SDS-PAGE on a 12% gel. After transfer to nitrocellulose filters, tyrosine phosphorylated proteins were identified using anti-phosphotyrosine antibodies.

time
(min)

0 0.5 1 2 4 8 16 32

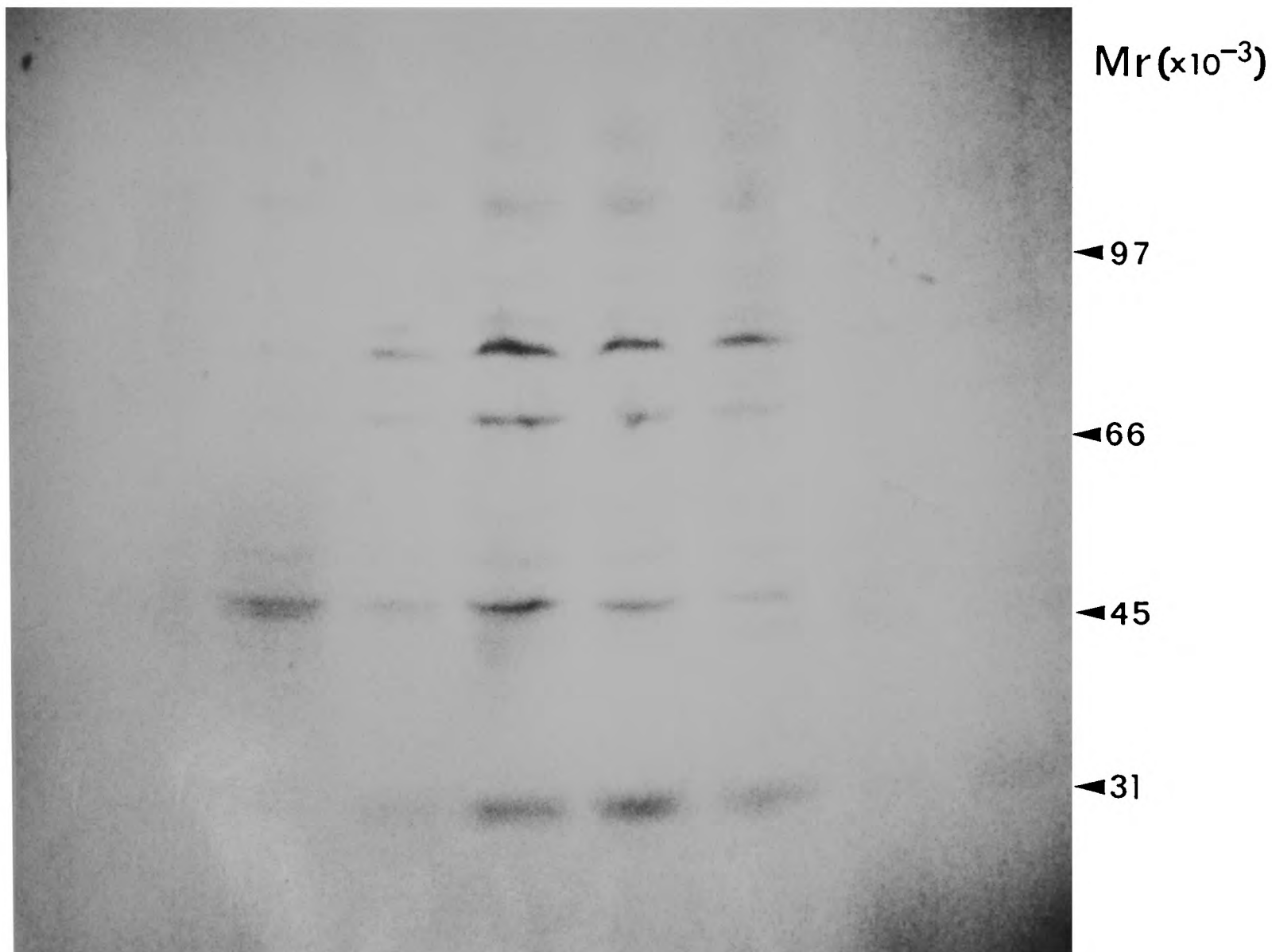


Figure 5.8 Effect on tyrosine phosphorylation, of incubating cells with R73 and W3/25 followed by incubation with anti-mouse antibody at 37°C for 0-32 minutes. Cells were lysed at the specified time and proteins separated by SDS-PAGE on a 12% gel. After transfer to nitrocellulose filters, tyrosine phosphorylated proteins were identified using anti-phosphotyrosine antibodies.

possible to determine from this data whether this is the same substrate which is phosphorylated at 30 seconds.

When CD4 and CD45 are co-crosslinked the tyrosine phosphorylation profile is the same as when CD4 is crosslinked alone (data not shown).

(f) Effect of IL2 on tyrosine phosphorylation.

Incubation of the cells at 37°C in the presence of 50 units/ml IL2 did not alter the tyrosine phosphorylation profile induced by any of the antibody combinations over the 32 minute period studied.

5.4 Discussion.

The results presented in this chapter suggest an association between receptor redistribution and tyrosine phosphorylation. Firstly, the general time-scale of the two events is compatible, although it should be noted that the maximal phosphorylation of band 70 and the appearance of tyrosine phosphorylated bands at 32 and 100 kD occurs at four minutes, before the majority of cells show a capped distribution of the T cell receptor.

A possible cause of this difference could be that when soluble antibodies are used receptors over the whole surface of the cell are crosslinked whereas in physiological conditions only those receptors in close proximity to the antigen presenting cell would initially be crosslinked. Thus it might be expected that local clustering of receptors induced by antibody crosslinking would be sufficient to induce tyrosine phosphorylation events which, in a physiological situation, would require the active redistribution of receptors. The antibody induced capping event could therefore be seen as being the physiological mechanism by which the cell redistributes receptors to the point of cell-cell contact, where they may be liganded, but which is made redundant in these experiments, by the fact that receptors over the whole cell surface are bound.

There are two rates of receptor capping: fast capping, in which the maximum percentage of cells showing a capped distribution of receptors occurs around 8 minutes (Figure 5.4), and slow capping, at which the maximum level is reached at 32 minutes or later (Figure 5.5). There is a correlation between the speed of capping and the state of crosslinking of CD45. When CD45 is crosslinked alone or in combination with CD4 or the TCR, capping is slow. When CD4 or the TCR are crosslinked alone or together, capping is fast.

This difference may simply be a function of the large amount of CD45 on the cell surface

compared to the amounts of CD4 and the TCR. Alternatively two mechanisms of capping may exist: one active requiring the involvement of second messengers and the contractile cytoskeleton, the other passive, a result of clustering of receptors induced by crosslinking with multivalent ligands. The fast capping group of molecules would do so via the active mechanism, while slow capping molecules would do so passively.

The ability of CD45 to change the rate of TCR and CD4 capping from fast to slow may be a function of its tyrosine phosphatase activity. If one of the early tyrosine phosphorylated substrates (June et al., 1990a) is phospholipase C γ , as has been suggested (see Section 1.8b), and tyrosine phosphorylation and activation of PLC γ is important in active receptor capping (see Section 1.9c), then the tyrosine phosphatase activity of CD45 may prevent PLC γ phosphorylation and activation and thereby prevent active capping. The molecules could still cap passively (slowly).

It is possible that the faint bands running at 105 and 120 kD, detectable from 0-1 minute after TCR crosslinking, are the equivalent of the human 100 and 135 kD substrates, reported by June et al. (1990a) to be phosphorylated within 10 seconds of T cell stimulation. One, or both, of these substrates may therefore play a role in the initiation of receptor capping but it is unlikely that their phosphorylation would be a result of actively induced receptor interactions.

Comparison of the effect of crosslinking the TCR alone with that of co-crosslinking CD4 and the T cell receptor provides further evidence implicating receptor aggregation in tyrosine phosphorylation events. Crosslinking the T cell receptor alone results in receptor capping and co-capping of CD4 (as well as CD45) over a period of about 8 minutes. From the previous argument we could infer that important receptor associations occur around four minutes which is when phosphorylation of the 32 kD, 70 kD and 100 kD bands occur. Co-crosslinking CD4 and the T cell receptor accelerates the association between these receptors and we see a corresponding acceleration

in the time course for the tyrosine phosphorylation of these substrates.

When CD45 and the T cell receptor are co-crosslinked, CD4 is excluded from the cap (see Section 3.3). Under these conditions there is an inhibition of tyrosine phosphorylation of bands at 32, 70 and 100 kD. A number of reports have suggested that CD45 is responsible for the dephosphorylation and activation of p56^{lck}. Prevention of a CD45:CD4 association could therefore result in a failure to increase the activity of p56^{lck} which is manifest in the inhibition of certain phosphorylation events.

Fisher et al. (1989) suggested that signal transduction could occur by localised perturbation of tyrosine phosphorylation equilibria due to clustering of receptors. Whilst association of receptors appears to be important in the instigation of tyrosine phosphorylation events, it is evidently not sufficient in itself, as can be seen in the effect of crosslinking CD4 with antibodies. Under these conditions both CD45 and the T cell receptor co-cap. The distribution of receptors on the cell surface is therefore the same as when the T cell receptor is itself crosslinked. There is however, no tyrosine phosphorylation of the 32, 70 and 100 kD bands. This would imply that the T cell receptor must be bound or crosslinked in order for these phosphorylation events to occur and it is not sufficient for CD4, CD45 and the TCR simply to be in the same vicinity.

The identity of the tyrosine phosphorylated molecules is unknown. The 32, 70, 86 and 100, 105 and 120 kD bands have similar molecular weights to tyrosine phosphorylated substrates observed by other groups in response to T cell receptor crosslinking (Hsi et al., 1989; June et al., 1990a; Jin et al., 1990). These experiments were undertaken human cells. This is the first recorded study of tyrosine phosphorylation in rat T cells and so there may be some variation in the apparent molecular weights of the substrates.

In conclusion, crosslinking the T cell receptor with the monoclonal antibody R73 and anti-mouse Ig antibody, results in the tyrosine phosphorylation of a number of molecules which demonstrate similar electrophoretic mobility to those observed in other animals. There appears to be a correlation between the association of CD4 with the TCR, and the tyrosine phosphorylation of several bands.

CHAPTER 6

RECEPTOR CAPPING ON NORMAL CELLS AND CELL LINES

6.1 Introduction.

There is a considerable body of evidence suggesting that the cytoskeleton is involved in the antibody-induced redistribution of membrane molecules, known as capping (Taylor et al., 1971) (see Section 1.9b). If one accepts this, then it is reasonable to assume that a direct or indirect connection must be made between the crosslinked membrane molecules and the cytoskeleton in order for cytoskeletal mediated capping to take place.

The most likely point of interaction is within the cytoplasmic domain of the molecule. However, the wide variation in the cytoplasmic domains of transmembrane molecules creates problems for the idea that linkage to the cytoskeleton is direct. To overcome this, Bourguignon

and Singer (1977) proposed the existence of an intermediary "protein-X" which could act as a common link between crosslinked receptors and the cytoskeleton. Such an association could occur via the extracellular, transmembrane or cytoplasmic domains of the molecules.

The opportunity to investigate the role of the cytoplasmic domain in receptor capping arose through the availability of a number of transfected Jurkat cell lines created by Albert Beyers (MRC Cellular Immunology Unit, Sir William Dunn School of Pathology). cDNA encoding for rat CD2 with truncated cytoplasmic domains was transfected into a clone of the human leukaemic T cell line Jurkat (He et al., 1988), enabling the effect of a shortened cytoplasmic domain on the capping of rat CD2, to be tested.

As the data presented in this chapter shows, all rat CD2 transfected into the Jurkat cells failed to cap, regardless of whether the transfectant expressed the whole rat CD2 molecule, or mutant CD2 with a truncated cytoplasmic domain. This could have been a consequence of the inability of rat CD2 to function in human cells, with respect to capping. Surprisingly however, when human CD2 capping was tested on wild type CD2⁺ Jurkat cells, this too failed to cap. Given that the redistribution of surface receptors may play an important role in cell signalling (see Section 1.9d), the failure of wild-type Jurkat cells to cap CD2 was of considerable interest. It was therefore decided to investigate this phenomenon further.

The capping characteristics of CD2 on normal T cells were unknown, so one possible explanation was that the failure to cap might be a property of CD2 itself, unrelated to cell type. To test this, CD2 capping on normal human peripheral blood (PB) T cells was studied. Using the same antibodies which failed to cap CD2 on the Jurkat cells, crosslinked CD2 on

human PB T lymphocytes capped effectively. Thus there appeared to be a difference in CD2 capping between normal cells and Jurkats. It was therefore decided to determine whether other Jurkat cell surface molecules behaved in a similar manner.

A possible explanation for the difference between CD2 capping on normal T cells and Jurkats could lie in the degree of antibody crosslinking or the capping incubation period required for capping in the different cell types. There are reports in the literature claiming that the capping kinetics of surface Ig varies with different strains of mice (Fram et al., 1976) and with different species of rabbit (de Groot and Wormmeester, 1981). Also, different molecules on the same cell require different levels of crosslinking for capping to occur. For instance, excessive crosslinking of the very abundant rat thymocyte membrane molecule Thy-1, prevents capping, while extensive crosslinking of CD45 is necessary for capping to occur (Turner, 1986).

To test this, CD2 capping on peripheral blood lymphocytes and Jurkats was compared using a range of antibody concentrations and capping incubation times. Finally, a preliminary investigation was made of receptor capping in a number of other cell lines, to determine whether failure to cap crosslinked receptors was related to the ability to proliferate indefinitely.

6.2 Methods.

(a) Cells and Antibodies.

Jurkat E6.1 cells were transfected with mutated rat cDNA to produce three cell lines; CY 6, expressing CD2 missing all of the cytoplasmic domain except for the cluster of basic residues immediately following the transmembrane region; CY 40, bearing 40 cytoplasmic residues but missing the last two thirds of the cytoplasmic domain; and CY whole, containing the complete rat CD2 sequence (He et al., 1988). These lines and Jurkat E6-1 wild type cells were obtained from Albert Beyers (MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford). Jurkat is a human T cell leukaemia and the E6-1 wild-type line was originally developed by Weiss et al. (1984).

The C58 rat T cell line was developed by the inoculation of C58 murine thymocytes into neonatal rats. It was proposed that the rat leukaemic cells which arose were the result of infection by a murine leukaemia virus (Geering et al., 1966). The cells were obtained from Mary Newton (Nuffield Department of Surgery, John Radcliffe Hospital, Oxford.)

Several Epstein Barr virus transformed B cell lines were obtained from Shirley Ellis (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford.). The cell lines were created by incubation of normal human B cells with the supernatant from an infected B95/8 marmoset cell line (F. Gotch, 1987. D. Phil thesis, University of Oxford). The donors of the original B cells which were transformed to create the lines, kindly donated blood so that capping in the transformed cells could be compared with the normal untransformed parent

B cells. Peripheral blood lymphocytes were isolated and enriched for B cells by negative panning as described in Chapter 2, using the anti-CD3 antibody UCHT1 to remove T cells.

The antibodies used were F10-89-4 (anti-human CD45), UCHT1 (anti-human CD3), DFT1 (anti-human CD43), B-H1 (anti-human CD2), ST-AR33 (anti-human IgG), OX-34 (anti-rat CD2), OX-19 (anti-rat CD5) and OX-1 (anti-rat CD45). These have been described previously in Section 2.4.

(b) Counting patched and capped cells

Cells were prepared for viewing as previously described (Section 3.2c). A cell was deemed to be uncapped if the fluorescent label was diffusely distributed, patched if the label was isolated into a number of clusters over the cell surface, and capped if it was concentrated in a single area.

(c) Testing the capping characteristics of native and transfected CD2 on Jurkat cells, and comparison with CD2 capping on normal human peripheral blood T lymphocytes.

10^6 cells from each of the transfected Jurkat lines and the same number of E6-1 Jurkat cells or normal human peripheral blood lymphocytes (isolated as detailed in Chapter 2), were incubated for 45 min on ice, with 50 μ l of either OX-34, anti-rat CD2 purified ascites at 50 μ g/ml, or B-H1 anti-human CD2 as appropriate. The cells were then washed by centrifugation as previously described (Section 3.2). FITC-conjugated goat anti-mouse IgG (Sigma) was diluted 1/30 in RPMI medium containing 10% human serum and pre-incubated for 30 min at 4°C. 100 μ l of this was then added to each tube and incubated for 45 min on

ice. Following a further washing step, the cells were resuspended in 100 μ l medium and incubated for 15 min at 37°C. The samples were then prepared for viewing under the Zeiss epifluorescence microscope as previously described (Section 3.2c).

- (d) Determination of the capping characteristics of other cell surface molecules on Jurkat E6-1 cells and normal human peripheral blood T cells.

Using the same protocol described above, the study was extended to compare the capping of CD3 (UCHT1), CD45 (F10.89.4) and CD43 (DFT1).

- (e) Effect of antibody concentration and capping incubation time on CD2 capping on Jurkat cells and human peripheral blood T cells.

Cells were treated as above, except that concentrations of B-H1 (anti-CD2) ranging from 1-100 μ g/ml, and dilutions of FITC-conjugated goat anti-mouse from 1/10 to 1/100, were used. The effect of varying the 37°C incubation time was also examined.

- (f) Comparison of surface receptor capping on other cells.

For the (two) rat cell lines (C25) and C58, capping of CD2 (OX-34), CD5 (OX-19) and CD45 (OX-1) was tested, and compared with capping in normal rat lymph node T cells (isolated as described in Chapter 2) using the same protocol as described in section 6.2b above. Details of the antibodies and the dilutions used can be found in Chapter 2.

The capping of surface immunoglobulin (sIg) by ST-AR 33 (Serotec), crosslinked with

anti-rabbit Ig (Sigma) + 10% human serum, was used in the comparison between normal and EBV transformed B cells.

6.3 Results.

(a) Effect of antibody crosslinking on the distribution of native and transfected CD2.

Figure 6.1 shows the distribution of transfected rat CD2 (CY6) on Jurkat cells following crosslinking with OX-34 followed by FITC-conjugated anti-mouse IgG, and incubation at 37°C. The labelled CD2, normally diffusely distributed over the cell surface, collects into small patches, but does not redistribute into a single cap except on a small minority of cells (< 4%). The same distribution was observed for transfected CD2 (CY40) and CD2 (CY whole).

When the distribution of native human CD2 on E6-1 Jurkat cells was determined this was found to cap on only 5% of cells (Table 6.1).

However, incubation of normal human peripheral blood T lymphocytes with the same anti-CD2 and anti-mouse antibodies, followed by the same 37°C incubation period, resulted in redistribution of CD2 into a single cap on up to 50% of cells (Figure 6.2).

(b) Capping characteristics of CD3, CD43 and CD45 on normal cells and Jurkats.

The difference in the capping characteristics of Jurkat cells and PB T cells does not appear to be restricted to CD2 alone. Table 6.1 shows that between 50 and 75 % of peripheral blood T cells demonstrated a capped distribution of CD3, CD43 and CD45 following crosslinking by antibodies and incubation at 37°C. However, these molecules were



Figure 6.1 Distribution of transfected rat CD2(CY6) on Jurkat cells. Cells were labelled with OX-34 followed by [FITC]-conjugated anti-mouse Ig and then incubated at 37°C for 15 min.



Figure 6.2 Distribution of CD2 on human peripheral blood T cells. Cells were labelled with B-H1 followed by anti-mouse Ig, then incubated at 37°C for 15 minutes.

capped on less than 6% of Jurkat E6-1 cells.

Antibody	Antigen	percentage capped cells	
		Jurkat E6.1	human PBLs
F10-89-4	CD45	4%	75%
UCHT1	CD3	6%	52%
B-H1	CD2	6%	57%
DFT1	CD43	4%	68%

Table 6.1 Comparison of receptor capping on Jurkat E6.1 cells and normal human PBLs

(c) Effect of antibody concentration and 37°C incubation period on CD2 capping on Jurkat cells and human peripheral blood T cells.

Table 6.2 summarises the effect of antibody concentration and 37°C incubation period on the capping of CD2 on normal T cells and E6-1 Jurkat cells. The percentage of PB T cells on which crosslinked CD2 is capped increases with increasing concentration of anti-CD2 antibody. A dilution of 1/30 anti-mouse secondary antibody is as efficient at inducing cap formation as 1/10, and both are more effective than the 1/100 dilution. Of the 37°C incubation periods tested, 15 min appears to be the optimum time. On the Jurkat cells however, neither the concentration of antibody, nor the length of time for which the cells are incubated at 37°C, affect the percentage of cells showing a capped distribution of CD2.

Conc. B-H1 (anti-CD2) ($\mu\text{g/ml}$)	Dilution anti-mouse	Incubation time at 37°C (min)	% capped cells		
			E6.1 Jurkat	Normal T cells	
1	1/100	5	1	24	
10			8	28	
100			3	31	
1	1/30		6	27	
10			8	28	
100			3	49	
1	1/10		8	21	
10			1	37	
100			3	46	
1	1/100	15	2	35	
10			3	37	
100			0	33	
1	1/30		2	41	
10			8	40	
100			4	57	
1	1/10		3	39	
10			3	42	
100			5	56	
1	1/100		45	1	32
10				4	39
100				5	34
1	1/30	3		29	
10		4		33	
100		2		47	
1	1/10	8		35	
10		3		32	
100		7		51	

Table 6.2 Effect of antibody concentration and capping incubation period on CD2 capping in normal T cells and Jurkats.

(d) Comparison of surface receptor capping on other cells.

The inability to cap antibody-crosslinked receptors does not appear to be a property restricted to Jurkat cells. The results of this preliminary investigation, detailed in Table 6.3, reveal that EBV transformation of normal human B cells correlates with loss of the ability to cap surface immunoglobulin.

The relationship between unrestricted growth and failure to cap is not however, universal, as a significant percentage of rat C58 cells showed a capped distribution of antibody crosslinked CD2, CD5 and CD45 molecules.

Cells	antibody	antigen	% capped cells
Normal rat PBLs	OX-34	CD2	59
	OX-19	CD5	54
	OX-1	CD45	68
C58	OX-34	CD2	36
	OX-19	CD5	38
	OX-1	CD45	29
EBV line from K.W.	ST-AR 33	human IgG	0
K.W.'s normal B cells			74
EBV line from A.M.			0
A.M.'s normal B cells			67
EBV line from A.J.			3
A.J.'s normal B cells			71
EBV line from J.M.			1
J.M.'s normal B cells			67

Table 6.3 Effect of antibody crosslinking on receptor capping in various cell types.

6.4 Discussion.

The results suggest that the capping of surface receptors is somehow related to the proliferative ability of a cell. EBV transformed cell lines and Jurkat cells, both of which demonstrate unrestricted proliferation, do not cap any of the surface receptors studied. On the other hand, using the same antibodies to crosslink the receptors, a majority of normal cells demonstrate the ability to cap.

This finding may be of value in determining both the mechanism of receptor capping, and its functional significance. The discovery that transformation of normal B lymphocytes by Epstein Barr Virus results in the loss of capping ability provides a particularly useful model on which to work. Here, directly related cells can be studied, potentially simplifying the task of identifying significant biochemical differences between the capping and non-capping cell types.

Although receptor capping is not fully understood, there are a number of aspects of the supposed mechanism which relate to phenotypic changes resulting from cell transformation. Transformation-related changes in cellular systems which are also thought to be involved in capping, would therefore seem a logical area for further study.

An obvious area where the cause of the capping differences might be found is the cytoskeleton. There is a distinct difference in the organisation of microfilaments between many normal and transformed cells (Pollack et al., 1975; Goldman et al., 1976; Ulrich and Quinlan, 1983). Perhaps even more significant with respect to capping is the observation by

Matsumura and colleagues, that there are significant differences in the form of tropomyosin expressed between normal and transformed rat, human and mouse cells (Lin et al., 1986; Matsumura et al., 1983). Detailed knowledge of the function of tropomyosin is restricted to its role in skeletal muscle contraction, where it regulates the interaction between actin and myosin in a calcium dependant manner (Hanson and Lowry, 1966; Ebashi et al., 1969). In non-muscle cells, tropomyosin may play a similar role. An alteration in this mechanism could lead to loss of the ability to actively draw receptors together into a cap.

Flanagan and Koch (1978) noted that P3 myeloma cells were unable to cap mIg. As with the cell lines studied in these experiments, the crosslinked sIg did form patches and it was shown to associate with actin under these conditions. This would tend to suggest that a defect in the contractile mechanism itself, rather than in the establishment of receptor-cytoskeletal associations, is responsible for the failure to cap.

It is, of course, perfectly possible and indeed likely that different defects are responsible for the inability to cap in different cell lines. Cell transformation has also been shown to result in changes in the levels of tyrosine phosphorylation (Hunter et al., 1986). Of particular interest is the observation by Sefton et al. (1981) that the cytoskeletal protein vinculin is phosphorylated on tyrosine residues in certain transformed cells. Also, Martin et al. (1986) report an association between transformation and the tyrosine phosphorylation of a spectrin (fodrin)-associated protein with a molecular weight of 36kD. In the experiments reported in Chapter 5, a molecule of similar molecular weight is distinctly tyrosine phosphorylated 4 min after antibody stimulation of rat T cells, around the same time that capping occurs.

Other molecules which have been implicated in the control of receptor capping are protein kinase C and inositol phosphates (see section 1.9c). These may also be involved in cell transformation and in the control of cell proliferation (Kikkawa et al., 1983; Housey et al., 1988; Macara et al., 1984; Macara et al., 1986).

Apart from information on the mechanism of receptor capping, comparison of capping and non-capping cells may provide insight into the physiological significance of the phenomenon.

All of the normal cells used in this study are capable of redistributing antibody-crosslinked membrane molecules. However, nine of the ten cell lines tested, do not appear to have this ability. The inference therefore is that the active redistribution of membrane proteins is somehow associated with the control of proliferation. It is possible that, by bringing particular receptors into close association, capping may function in generating the "off" signal, loss of which could result in cells existing in a permanent state of activation. Livanainen et al. (1990), have demonstrated that reversion of T lymphoblasts to the resting state involves an increase in protein tyrosine phosphatase activity and a concomitant increase in CD45 expression. If CD45 tyrosine phosphatase does exert a down-regulatory effect, then it may need to be brought into contact with the relevant substrates in order to carry out this role. Inability to cap CD45 may prevent such associations from occurring, thereby preventing the dephosphorylation events required for entry of the cell into the resting state.

The study reported here provides only a preliminary investigation into an interesting capping phenomenon. The lack of detailed information on the mechanism and control of

receptor capping means that it is impossible to produce anything other than vague speculation on the observed differences in capping between normal cells and some cell lines. Comparison of cell types along the lines suggested may provide some insight into the phenomenon.

CHAPTER 7

GENERAL DISCUSSION

CD4 and the TCR are associated with tyrosine kinases (Rudd et al., 1988; Veillette et al., 1988; Samelson et al., 1990) and CD45 is a tyrosine phosphatase (Tonks et al., 1988c). All three molecules are assumed to play a role in T cell activation and it is likely that they do so via the interaction of their kinases, phosphatases and associated substrates. Investigation into the associations between these molecules thus provides valuable information concerning their function. One of the ways of studying receptor associations is to employ the technique of co-capping, using immunolabelling of the molecules on the cell surface to determine their distribution.

The co-capping work presented in Chapter 3 may be viewed in two ways: 1) as an isolated investigation into receptor associations, or 2) as an integrated study into the relationship between antibody-induced receptor redistribution and cellular activation events

such as proliferation and tyrosine phosphorylation, which can also be induced by receptor-specific antibodies.

There is an important distinction between these two lines of approach which influences the value placed upon the co-capping data.

In the first case, capping is used as a model system in which passive co-redistribution (co-capping) of one receptor species with another, actively capped, species may imply an association between the two. Such an association could occur in several non-mutually exclusive ways;

a) The co-capping molecules may be physically complexed on the cell surface, such that redistribution of one species automatically results in co-redistribution of the other. This would be the case if the TCR were actively capped. CD3 would co-cap because it exists in a permanent complex with the TCR (Figure 7.1a).

b) The co-capping molecules may exist independently on the cell surface, a complex being formed following the crosslinking of one of the molecular species (Figure 7.1b).

c) The co-capping molecules may exist independently on the cell surface but be attached to the same cytoskeletal network, or become attached upon antibody crosslinking of one of the molecules. The different molecular species could then be drawn independently into the same area (Figure 7.1c).

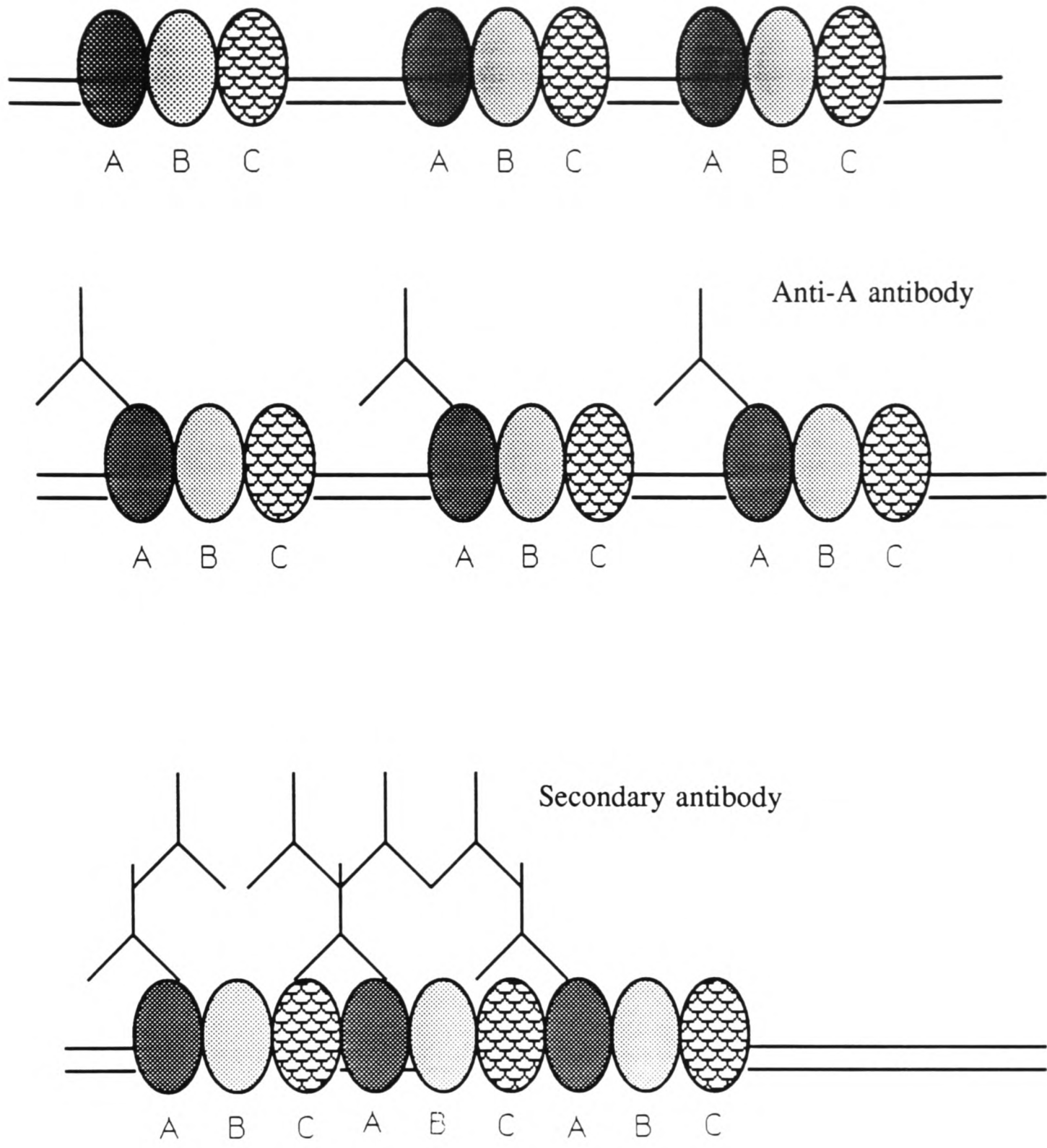


Figure 7.1a (i) Molecules A, B and C are physically associated on the cell surface. (ii) Anti-A antibody binds. (iii) Secondary antibody binds to and crosslinks anti-A antibody. This induces molecule A to cap, also inducing redistribution of B and C.

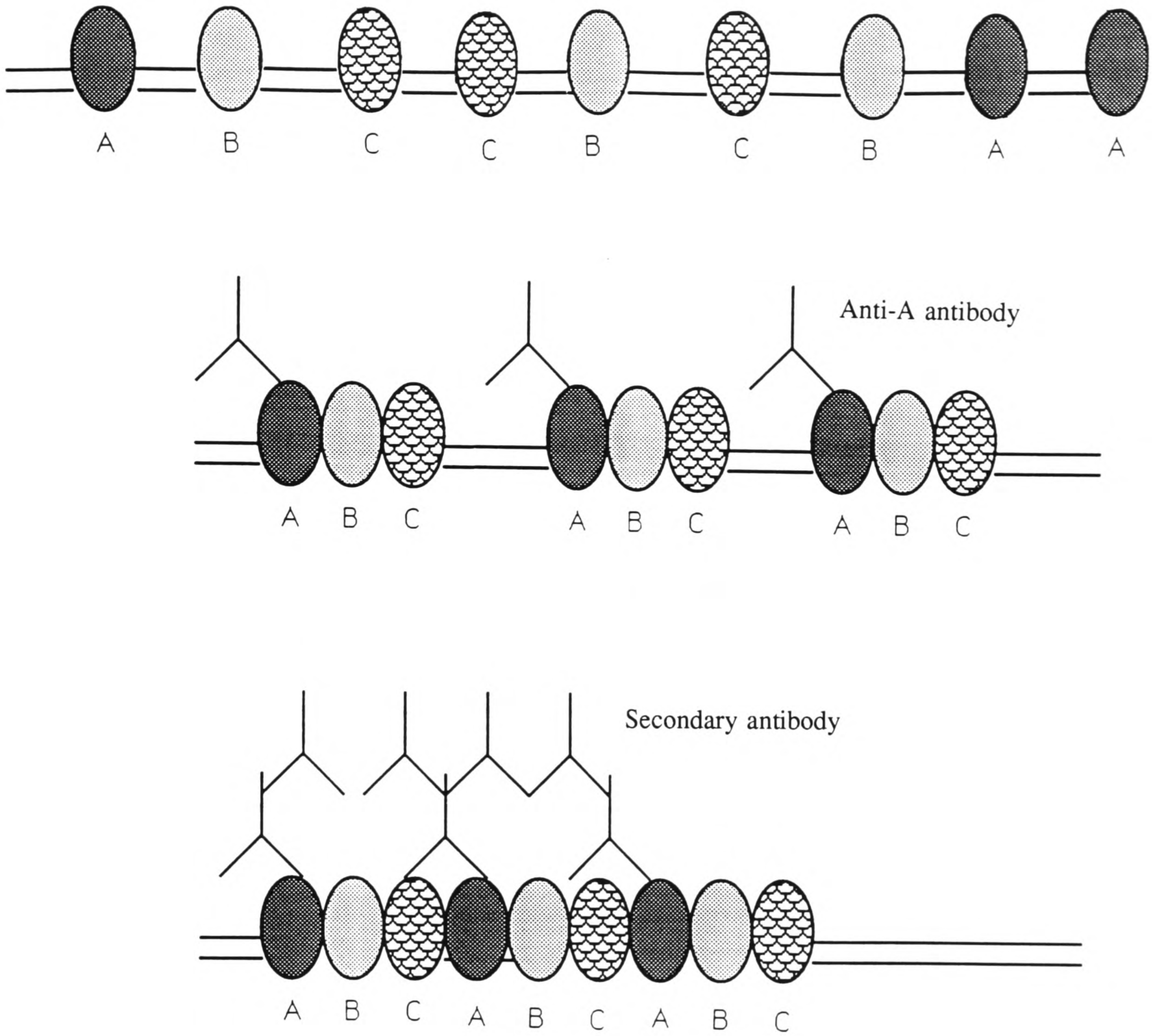


Figure 7.1b (i) Molecules A, B and C are randomly distributed on the cell surface. (ii) Antibody binds to A and induces association between A, B and C. (iii) Secondary antibody binds to and crosslinks anti-A antibody. This induces A to cap, also inducing redistribution of B and C.

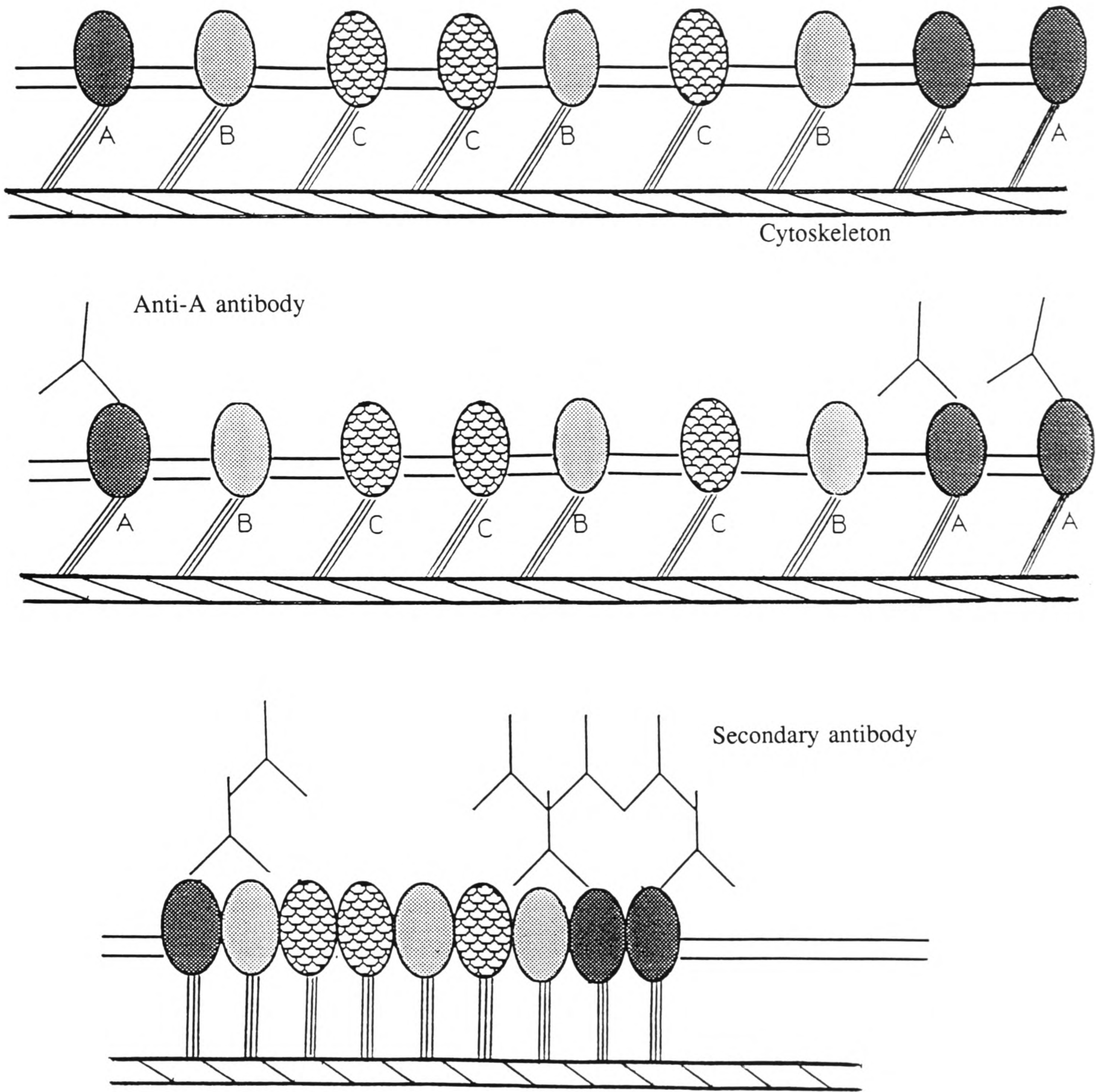


Figure 7.1c (i) Molecules A, B and C are randomly distributed on the cell surface, but linked to the same cytoskeletal network. (ii) Anti-A antibody binds. (iii) Secondary antibody binds to and crosslinks anti-A antibody. This induces contraction of the actin cytoskeleton, causing A to cap along with B and C.

Recently, the work of Dianzani et al., (1990), who showed that co-capping occurs between CD4, CD45 and the TCR on mouse cells, has been cited as evidence that the three molecules associate in a complex on CD4⁺ memory T cells (Klausner and Samelson, 1991; Mackay, 1991). There is, however, no way of determining from co-capping studies alone, the manner in which the observed associations occur. The assumption made by those authors that CD4, CD45 and the TCR are complexed on memory cells is thus not necessarily correct.

If the observed co-capping is a result of inter-molecular associations induced by antibody-mediated receptor crosslinking (Figure 7.1b and c), then much of the significance of the information gained from co-capping studies depends on the physiological significance of capping itself. If capping is an artifact, due to the non-physiological crosslinking of receptors over the entire cell surface, then it is quite possible that the observed co-capping is also an artifact, which does not provide reliable information concerning receptor associations in the native environment.

For this reason the second approach to co-capping results, relating the distribution of receptors to activation events, is more valid. This has been the approach taken in this thesis.

I have used co-capping to determine whether CD4, CD45 and the TCR could associate on the cell surface, and have related this information to the effect of the same experimental conditions on proliferation and tyrosine phosphorylation. For these purposes, the method by which the associations occur is irrelevant. It is the fact that the molecules do or do not interact that is important.

Table 7.1 summarises the data from chapters 3, 4 and 5. It is apparent that a correlation exists between the distribution of CD4, CD45 and the TCR induced by specific antibody crosslinking, and the result of these same experimental conditions on proliferation and tyrosine phosphorylation.

Crosslinked antibody(s)	Distribution of;			Tyrosine phosphorylated substrates	Effect on cell growth
	TCR	CD4	CD45		
R73 (anti-TCR)	capped	capped	capped	32, 70, 86 and 100 kD bands	+++
W3/25 (anti-CD4)	capped	capped	capped	86 kD band only	+/-
OX-1 (anti-CD45)	uncapped	uncapped	capped	none	+/-
R73+W3/25	capped	capped	capped	accelerated phosphorylation of 32, 70, 86 and 100 kD bands	+++
R73+OX-1	capped	uncapped	capped	weak phosphorylation of 32, 70 and 86 kD bands	+
W3/25+OX-1	uncapped	capped	capped	86 kD band only	+/-

Table 7.1 Summary of effect of antibody crosslinking on receptor redistribution, tyrosine phosphorylation and proliferation. Note only the major tyrosine phosphorylated bands with molecular weights of 32, 70, 86 and 100 kD are included as the detection of other bands mentioned in the text was not 100% reproducible.

When the TCR is crosslinked using R73, CD4 and CD45 redistribute to the same area on the cell surface (co-cap), proliferation is enhanced and tyrosine phosphorylation of bands running at 32, 70 and 100 kD occurs at around the same time as patching and capping. If the TCR is co-crosslinked with CD45 using both R73 and OX-1, CD4 is left diffusely

distributed, there is an inhibition of proliferation and of tyrosine phosphorylation. On the other hand, when the TCR is co-crosslinked with CD4, the association which occurs when the TCR is crosslinked alone is accelerated. In response to this we observe an acceleration in tyrosine phosphorylation. There is no increase in proliferation above that measured for crosslinked TCR alone but, as mentioned previously (Section 4.3), there may be an accelerated response which would not be observed under the conditions used. Consistent with this is the report by Jonsson and Eichman (1990) that antibody-mediated co-crosslinking of CD4 and the TCR accelerates IL2 production, but does not result in a net increase in the overall amount produced.

Rudd recently reported that three molecules with molecular weights of 32, 85 and 110 kD could be immunoprecipitated with anti-p56^{lck} antibodies in human cells (Seminar in the MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford, 2-5-91). These bands have been provisionally identified as a GTP binding protein, the regulatory subunit of type III PI kinase and a *raf*-associated molecule, respectively. The molecular weights of these molecules are extremely close to the tyrosine phosphorylated substrates identified in rat cells herein. The fourth major tyrosine phosphorylated band, running at 70 kD, has also been found by Rudd following CD3 crosslinking, but there are as yet no clues to its identity.

The identification of this complex of molecules associated with CD4/p56^{lck} which so closely resembles the tyrosine phosphorylated substrates identified in rat T cells, provides a possible explanation for the inhibition of tyrosine phosphorylation and proliferation seen when CD4 is not co-capped, and an acceleration in tyrosine phosphorylation when CD4/TCR associations are accelerated by co-crosslinking. The molecules could be substrates for the

TCR associated kinase p59^{fyn}. When CD4 is diffusely distributed, p59^{fyn} would not have access to these substrates, whereas when CD4 and the TCR are co-crosslinked, p59^{fyn} and the CD4/p56^{lck}-associated substrates would be brought into contact much more rapidly than when CD4 passively co-caps with the TCR. Alternatively, the CD4/p56^{lck}-associated molecules could be substrates for p56^{lck} itself. They would only be tyrosine phosphorylated when p56^{lck} is activated, possibly by dephosphorylation at tyrosine 505 following association with CD45 tyrosine phosphatase.

One inconsistency in the evidence suggesting that receptor associations are important in T cell activation is the effect of crosslinking CD4 alone. Superficially, the distribution of CD45, CD4 and the TCR is the same as when the TCR is capped. There is a significant difference however, in the effect on T cell activation. When CD4 alone is actively capped, the 32, 70 and 100 kD bands are not tyrosine phosphorylated and there is no enhancement of proliferation. On the other hand, when the TCR is capped, these substrates are tyrosine phosphorylated and the level of proliferation is increased. A simple explanation could lie in the occupation state of the TCR. Under physiological conditions, T cells are activated by the TCR binding to antigen/MHC complex on the presenting cell (see Section 1.8). It is reasonable to assume that the failure to activate cells by anti-CD4 antibodies is therefore a result of the fact that the TCR is not engaged. It is possible that conformational changes resulting from TCR occupancy or crosslinking result in activation of p59^{fyn}, and that this is an essential step in T cell activation.

The co-capping data is therefore consistent with the proliferation and tyrosine phosphorylation data. While it is not possible to overcome the artificial nature of experiments

which use antibodies to stimulate cells, the evidence does suggest that the way in which CD4, CD45 and the TCR are induced to interact is important.

The studies on receptor capping in normal peripheral blood lymphocytes and cell lines provide further circumstantial evidence that receptor redistribution is important in the control of cell proliferation.

In conclusion, this is the first report to relate the interactions between CD4, CD45 and the TCR to the effects that specific antibodies to these molecules have on proliferation and tyrosine phosphorylation. The results are consistent with a recent report by Rudd suggesting that molecules with molecular weights of 32, 85 and 110 kD are associated with the CD4/p56^{lck} complex. I have found in rat cells that when this complex is actively co-crosslinked with the TCR, molecules with similar molecular weights to those reported by Rudd, are rapidly phosphorylated, whereas when CD4 and its associated complex of molecules are not co-redistributed with the TCR (i.e. when the TCR and CD45 are co-crosslinked) there is an inhibition of tyrosine phosphorylation, as well as an inhibition of proliferation.

The proposal of this thesis therefore, is that the T lymphocyte surface molecules CD4, CD45 and the T cell receptor interact by being actively redistributed about the cell surface in response to ligand binding. The nature of this interaction appears to play a significant role in tyrosine phosphorylation events and in the proliferative response.

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