Aspects of the cell biology of antigen processing for MHC II

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by

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ABSTRACT

Antigen processing for presentation by antigen presenting cells (APCs) expressing class II major histocompatibility complex molecules (MHC II) involves partial digestion of antigen, binding of antigen-derived peptides to MHC II, and intracellular trafficking of both antigen and MHC II. In this thesis, two major aspects of these intracellular events have been studied.

A flow cytometric assay was developed to measure fluid-phase endocytosis by dendritic cells, potent APCs which have been shown to have poor lysosomal function, and which have been suggested to have a low level of endocytic activity. The assay used a two compartment model to measure the separate activities of early endosomes and late endosomes. Comparison of dendritic cells with different B lymphocyte APCs showed that endocytic traffic through late endosomes, some of which are thought to be related to the site of peptide loading, was similar in dendritic cells and B cells. Thus, the low endocytic activity reported elsewhere was not detected in this project.

Organelles containing MHC II were studied in a cell-free system using membranes from disrupted APCs. These organelles were studied by density gradient centrifugation and separated from total membrane by immuno-isolation on magnetic beads. Density gradient centrifugation demonstrated that organelles containing newly synthesised MHC II had a marginally higher density than early endosomes. Immuno-isolated MHC II was found to co-isolate with endocytic markers, in particular markers of early endosomes. This indicates that MHC II is widely distributed throughout the endocytic pathway, including early endosomes, even though it is not widely thought that antigen binds MHC II in early endosomes. In addition, immuno-isolated MHC II was enriched for newly synthesised molecules, which indicates that the organelles where peptide loading is thought to occur were immuno-isolated. In initial experiments, immuno-isolated newly synthesised MHC II was used to perform some of the biochemical events of antigen processing.
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CHAPTER 1. INTRODUCTION TO THE CELL BIOLOGY OF ANTIGEN PROCESSING FOR PRESENTATION BY MHC II

The cellular immune system has been the focus of much research since the discoveries of lymphocyte recirculation, clonal selection, and acquired tolerance over 30 years ago. Subsequent discoveries have revealed that antigen presenting cells convert antigen into a specific form for recognition by the cellular immune system. This conversion is termed antigen processing. Antigen processing is a complex series of intracellular events which make use of previously unknown pathways. Therefore, antigen processing is as much in the realm of cell biology as immunology. This review of antigen processing for presentation by MHC II deals with the subject as follows:

A. antigen processing is placed in context: (i) within the overall immune response, (ii) by contrasting different cells which process for presentation by MHC II;

B. the background for antigen processing for MHC II is described: (i) antigen processing for MHC I; (ii) the endocytic pathway, (iii) the enzymes which degrade exogenous antigens;

C. the molecular and cellular functions for antigen processing for MHC II are summarised: (i) the interactions of MHC II and peptide, (ii) the cell biological interactions of MHC II;

D. in the light of current knowledge of antigen processing, unresolved aspects and future directions are discussed: (i) the phenomena which indicate a role for processing in immune regulation, (ii) the intracellular sites where antigen is processed, (iii) the experimental approaches for studying the cell biology of MHC II, including cell-free methods, (iv) the aims of this project.
A. THE CONTEXT OF ANTIGEN PROCESSING

(i) ANTIGEN PROCESSING AS A PART OF THE IMMUNE RESPONSE

The cellular immune system: T cells recognise antigen in the context of MHC. The immune system is capable of recognising and responding to non-self using primitive, non-adaptive mechanisms. However, on their own these responses are inadequate for defence against microbial colonisation. In phylogenetically more advanced animals, the immune system has evolved an adaptive arm, the importance of which has recently been publicised by the appearance of Acquired Immune Deficiency Syndrome. The adaptive arm of the immune system also recognises and responds to non-self, but differs from the non-adaptive arm by its capacity for amplification and memory. The adaptive immune system has two divisions: humoral and cellular, which communicate with each other closely. The humoral immune system recognises antigen with antibodies synthesised by B cells. The antigens are intact, i.e. in the native state. Thus, foreign structures can be detected in solution or on the surface of either invading micro-organisms or host cells.

The cellular immune system functions in the limited context of intracellular interactions. The effector cells are termed T cells, and the cell recognised by a T cell is an antigen presenting cell (APC). The ligand on APCs for T cells was determined to be the product of major histocompatibility complex class I and class II genes (MHC I and MHC II) (Zinkernagel and Doherty, 1974; Shevach and Rosenthal, 1973; Rosenthal, 1978). There is more than one locus within each MHC class, the products of the different loci being termed isotypes. Within each locus, there is a large amount of polymorphism, each allele being termed an allotype. The differences between allotypes are smaller than the differences between isotypes.

MHC II was also shown to control the level of response of in-bred laboratory animals to foreign antigens, being called the "Immune response", or Ir genes (McDevitt et al., 1972; Benacerraf and McDevitt, 1972). In addition, the response of an individual animal's T cells to antigen was shown to occur only in the context of antigen presenting cells expressing the same MHC as that animal, this phenomenon being termed MHC restriction (Zinkernagel and Doherty, 1974). Therefore, antigen recognition by T cells was shown to require the co-recognition of both MHC and antigen, and theoretically could be achieved by either one or two receptors. Within the last 10 years it has been
shown that there is a single T cell receptor, structurally similar to an immunoglobulin Fab, for both MHC and antigen (Haskins et al., 1983; Chien et al., 1984).

The different functions of T cytotoxic and T helper cells are related to the tissue distribution of MHC I and MHC II. Two major T cells subsets were identified by the mutually exclusive expression of CD4 and CD8 surface markers. The CD4\(^+\) subset, termed T helper cells, is responsible for delayed-type hypersensitivity, and amplification of antibody and cytolytic responses. The CD8\(^+\) subset, termed T cytotoxic cells, is responsible for killing virally infected and tumour cells. The functional division of these subsets is correlated with the requirement of T helper cells for APC which are MHC II\(^+\), whereas T cytotoxic cells require APC to express MHC I. The molecular basis for this has been demonstrated recently from the binding of CD4 to MHC II and CD8 to MHC I. Without this extra binding, the activation of T cells is diminished (Bierer et al., 1989). In addition, CD4 and CD8 have signal transduction functions which are important for T cell signalling (Janeway, 1992a). Therefore, CD4 and CD8 act as co-receptors for the interaction of T helper and cytotoxic cells with APCs bearing MHC II and MHC I respectively.

The tissue distribution of the two classes of MHC differ widely. MHC I is expressed by almost all nucleated cells. In contrast, MHC II is expressed primarily by cells in the immune system (B cells, dendritic cells, activated macrophages, activated T cells and thymic epithelium) as well as some cells outside the immune system. Therefore, most MHC I\(^+\) cells are MHC II\(^+\). The term APC would lose its currency if applied to antigen presentation by MHC I, and has traditionally been reserved for cells expressing MHC II. The distribution of MHC I and MHC II indicates the differing roles of the T cell subsets. T cytotoxic cells scan all nucleated cells, killing those with abnormal expression of virus- or tumour-associated genes. T helper cells scan APCs within the immune system, controlling the level and nature of responses to antigens the APCs have acquired from microorganisms or other cells. A co-ordinated immune response is largely the result of T helper function, for example at the level of cytokine secretion - different T helper cell subsets secrete specific patterns of cytokines (Mosmann and Coffman, 1989).
T cells recognise short peptides derived from protein antigens. The form of antigen recognised by T cell receptor in the context of co-recognition of MHC is not the same as the form recognised by antibody. Denatured antigens can be recognised by T cells but fail to be detected by antibody. The antigen recognised by antibodies (termed B cell epitopes) are generally the 3-dimensional configuration of tertiary structure (Atassi, 1975), not necessarily protein. In contrast, the antigens recognised by T cell receptors (T cell epitopes) are the primary structures of proteins.

The earliest evidence was that antigen recognised by T cells was in the form of fragments intimately associated with MHC (Benacerraf, 1978). Subsequently it was shown that short peptides derived from internal sequences within an antigen could substitute for epitopes (Shimonkevitz et al., 1983 and 1984). The importance of antigen-derived peptides was confirmed by the demonstration of binding with moderate affinity between short peptides containing T cell epitopes and the MHC II molecules for which these epitopes were restricted (Babbitt et al., 1985; Buus et al., 1986). Antigens which elicit antibody responses but cannot elicit T cell responses are either very small (termed haptens) or large, non-protein polymers (termed T-independent antigens); in both cases the antigen cannot form short peptides.

Peptide binds to a groove on the apical surface of MHC. MHC molecules consist of 4 extracellular domains with 2-fold rotational symmetry. Two membrane-proximal domains are part of the immuno-globulin superfamily and form a V-region-like heterodimer. Two membrane-distal domains are similar to each other and form a closely linked heterodimer, but are dissimilar to any other known polypeptide. MHC I is formed by a heavy chain (≈44 kDa), which contains both the membrane-distal domains (α1, α2) and one membrane-proximal domain (α3). The fourth domain of MHC I is provided by β2-microglobulin (β2m, 12 kDa), encoded outside the MHC. MHC II is formed from two chains, α (≈33 kDa) and β (≈28 kDa), each with one membrane-distal domain (α1 and β1) and one membrane-proximal domain (α2 and β2).

The precise mechanism of antigen recognition emerged from the structure of an MHC I molecule which was solved by X-ray crystallography (Bjorkman et al., 1987a). This enabled models to be made for the structure of MHC II (Brown et al., 1988), and the
interaction of MHC I with T cell receptor (Bjorkman et al., 1987b). The membrane-distal domains together form a longitudinal groove on the apical surface of the molecule. The floor of this groove is constructed from a β-pleated sheet, and the two walls of the groove consist of α-helices. In the groove, material was detected which was consistent with a mixture of peptides. This is the only site with such clear peptide binding capacity. In addition, the residues which are most responsible for polymorphism between allotypes form the walls and floor of the groove. This indicates that the groove is indeed the antigen binding site. More recent studies have shown that a characteristic set of peptides is bound to each allotype in its apical groove.

The manner in which T cell receptors recognise MHC and peptide has been hypothesised to be via direct contacts between the germ-line-encoded CDR1 and CDR2 regions of the T cell receptor and the α-helices of MHC, while antigen is recognised by CDR3, which is extremely variable due to V(D)J joining and random nucleotide addition (Davis and Bjorkman, 1988). In confirmation of this, specific interactions, such as acid-base pairs, have been demonstrated between residues of CDR3 and MHC-bound peptide (Jorgensen et al., 1992). The conformation of an epitope is largely determined by polymorphic residues in the MHC groove to which it binds. Thus, the basis of alloreactivity across small allelic differences is the appearance as non-self of self peptides in altered conformations (Nathenson et al., 1986; Lechler et al., 1990; Cotner et al., 1991). The solution of the structure of crystals of the MHC/peptide/T cell receptor complex is awaited.

Theoretical minimum number of residues in T cell epitopes. The repertoire of T cell receptors created by rearrangement has been suggested to have in the order of $10^7$ specificities (Kronenberg et al., 86). On this basis the T cell receptor must contact at least 5 residues ($20^5 \approx 3 \times 10^6$). MHC binding must be less specific than T cell binding to allow a wide range of pathogen-derived molecules to be presented. Assuming that the smallest pathogens such as viruses, which may encode 1000 residues or less, might be expected to yield at least one epitope, MHC binding might impose specificity on 2 residues ($20^2 = 400$). Therefore, the shortest peptide which can fit the theoretical requirements of APC and T cell recognition need have at least $5 + 2 = 7$ residues.
The definition of antigen processing. Protein antigens are recognised in the form of a short peptide bound to an MHC molecule. The series of events for both antigen and MHC which bring this about is termed antigen processing. The subsequent interaction with a T cell is termed antigen presentation.

Antigen processing of exogenous antigen is intracellular. The minimal molecular requirements for antigen processing are the mechanism to expose T cell epitopes, and the expression of MHC II which binds the epitopes. Antigen processing also involves intracellular traffic of antigen and MHC II. The need for internalisation of antigen was suggested by the finding that processing was sensitive to lysosomotropic agents such as NH\textsubscript{4}\textsuperscript{+} and chloroquine which neutralise lysosomal acidic pH (Ziegler and Unanue, 1982). This indicated that antigen processing might occur in lysosomes. Mild fixation of APCs abolishes antigen processing, while not affecting presentation either of antigen processed prior to fixation, or of peptides (Shimonkevitz et al., 1983). In addition, processing was shown to require cellular energy expenditure (Ziegler and Unanue, 1981; Werdelin and Buus, 1983). Therefore, a complex intracellular event is required to process antigen.

Processed antigen is not usually released from inside one APC back to the extracellular medium for other APCs to present (Harding et al., 1990; Steinman, 1991). This shows that either epitope loading is internal or that epitopes are always kept associated with membrane and thus their loss is prevented. Prevention of loss of soluble epitopes to the extracellular medium has the advantage of preventing binding to MHC II on the surface of nearby B cells, with the induction of incorrect B-T pairing (Mitchison, 1971). Internalisation of antigen prior to degradation circumvents this difficulty by exporting only those T cell epitopes which are bound to MHC II.

It has been proposed that dendritic cells are unable to process antigen but acquire processed fragments from macrophages (Inaba et al., 1981). Such synergistic processing has not been found subsequently (Inaba et al., 1990; Pure et al., 1990). Therefore, all available evidence suggests that antigen processing occurs inside the presenting APC. There are cell surface proteinases which might be capable of processing antigen (Kenny, 1977; Buus and Werdelin, 1986a), but their role remains unclear. The plasma membrane of APCs remains important as a site for targeting antigens (see section B. (ii)), and as
the likely site where synthetic peptides bind, with the therapeutic possibility to modulate specific immune responses, either for stimulation (Milich et al., 1987; Berzofsky et al., 1991) or for inhibition (Wraith et al., 1989).

Different antigens are presented to T helper and T cytotoxic cells: there are two intracellular pathways of antigen processing. The separation of function between T cytotoxic and T helper cells requires that a different set of antigens must be presented to each subset of T cells. For example, virally infected cells must be recognised by T cytotoxic cells, while an APC must present a virus to T helper cells without itself being infected with the virus. This is achieved by the presentation of endogenous antigens synthesised in the cytosol of the cell by MHC I, and of exogenous antigens acquired from outside the cell by MHC II.

MHC I presents cytosolic molecules (Townsend et al., 1986). Processing is insensitive to disrupting lysosomal function (Morrison et al., 1986), but is dependent on protein export (Yewdell and Bennink, 1989; Nuchtern et al., 1989). MHC I does not enter the endocytic pathway (Neefjes et al., 1990). In contrast, MHC II presents exogenous molecules (Germain, 1986; Yewdell and Bennink, 1990; Long and Jacobson, 1989). Processing is less dependent on protein export than MHC I (Harding and Unanue, 1989; Adorini et al., 1990; St.Pierre and Watts, 1990), and MHC II intersects the endocytic pathway (Neefjes et al., 1990; Neefjes and Ploegh, 1992a). The general rule separating these two pathways has notable exceptions (Carbone and Bevan, 1990; Jaraquemada et al., 1990; Rock et al., 1990).

With two classes of MHC, and a corresponding division in the source of antigens, there are two pathways of antigen processing. For MHC I the pathway is: (1) synthesis in cytosol, (2) degradation, (3) translocation into ER, (4) binding to MHC I, (5) export to plasma membrane. For MHC II the pathway is: (1) uptake by APC, (2) traffic into MHC II^{+ve} endosome, (3) degradation, (4) binding to MHC II, (5) export to plasma membrane. The pathways are kept separate by the absence of peptide binding to MHC II in the ER, and the targeting of MHC II to endocytic compartments. Otherwise, the basic schemes of the pathways differ only in the order of degradation and delivery to the MHC binding compartment. The biochemical similarity of peptide binding to the two classes
of MHC is demonstrated by those peptides which bind to MHC of both classes (Choppin et al., 1990; Hickling et al., 1990; Perkins et al., 1989). The dissimilarity is therefore largely a product of the differing cell biological roles of MHC I and II.

Only a small fraction of the MHC molecules of an APC express a single epitope. It has been shown by analysis of peptide/MHC complexes extracted from APCs that the copy number of a particular complex required to stimulate a T cell is surprisingly low. Estimates using T hybridomas as responding cells indicate between 50 and 300 complexes per APC are sufficient for T cell responses, i.e. 0.01% to 0.1% of the total MHC (Demotz et al., 1990; Harding and Unanue, 1990a; Christinck et al., 1991). There are a number of peptides represented at much higher levels than this, for example in one MHC II species, 6 peptides occupy 50%-75% of the binding sites (Rudensky et al., 1991a). It has been suggested that T cell clones are more sensitive than T hybridomas, and that in vivo responses may be to as few as 10-20 complexes (Germain, 1991). This would mean that MHCs could present well over 1000 specificities simultaneously.
A(ii) THE CELLS WHICH PROCESS AND PRESENT VIA MHC II

Macrophages. Macrophages consist of a heterogenous population of bone-marrow derived, phagocytic cells found in many tissues, for example Kupffer cells lining hepatic sinusoids. Unstimulated macrophages are MHC II⁺. Expression of MHC II and the second signal are both induced by bacterial cell wall components and inflammatory cytokines such as gamma-IFN and IL-1 (Weaver and Unanue, 1990; Janeway, 1992b). Antigen uptake can occur via several routes. Fluid-phase endocytic traffic is the least efficient, but is at a higher rate than in other cell types (Besterman et al., 1981). Receptor-mediated endocytosis of antigens opsonised with IgG or complement can occur via receptors for Fc, C3b and C4b receptors (Gammon et al., 1987; Arvieux et al., 1988; Gosselin et al., 1992). In addition, scavenger receptors with a broad, ill-defined specificity recognise and enhance uptake of desialylated molecules and polyanions (Rohrer et al., 1990; Naito et al., 1991). Finally, phagocytosis delivers particulate antigens, such as whole micro-organisms, into an MHC II⁺ compartment with many properties of late endosomes (Harding and Geuze, 1992).

The highly active degradative pathway of macrophages does not imply a greater capacity for processing of all antigens than less active cells (Chain et al., 1986). In contrast to dendritic cells, macrophages may act as APCs in the periphery, since they respond to activation by inhibition of migration, and secretion of centrally and locally acting cytokines, including IL-6, IL-1 and TNF, which can amplify the immune response.

B cells. Resting B cells process and present specific antigen, and activated B cells can respond to several antigens (Chesnut and Grey, 1981; Chesnut et al., 1982). MHC II expression is constitutive on B cells. Expression of the co-stimulatory molecule B7/BB1 is induced by bacterial and other inflammatory molecules (Liu and Janeway, 1991). Cooperation between B and T lymphocytes specific for the same antigen is an important part of the adaptive immune response (Mitchison, 1971; Lanzavecchia, 1988). Initial models were of simultaneous recognition by B and T cells, but since T cell epitopes are the result of antigen processing within B cells, recognition must be sequential (Lanzavecchia, 1988). B cells take up and processes antigen; epitopes are presented to T cells; antigen-specific T cells in turn stimulate the presenting B cell. Therefore, to achieve antigen-specific B-T cooperation, the epitopes presented by a B cell must only
be from an antigen which the B cell's membrane immunoglobulin (mIg) binds. This is
teological evidence for the importance of mIg in antigen presentation by B cells.

The importance of mIg has been directly demonstrated by the presentation of antigen
by specific B cells at antigen concentrations as low as \( \approx 10^{-12} \text{M} \), upto 10,000x less than by
irrelevant B cells (Pierce et al., 1988; Lanzavecchia, 1985 and 1990). Once a response to
infection is underway, B cells are especially important when the antigen concentration
is low, when other APCs cannot take up sufficient antigen. As the infection is overcome,
antigen levels fall still further to a point where secreted antibody is in excess of antigen,
so there is no free antigen to be taken up. This mechanism for terminating successful
immune responses explains the prophylactic effect of passive immunisation with anti-D
in Rhesus disease of the newborn.

In the absence of ligand, mIg (approximately \( 10^5 \) per cell) recycles between pools of
roughly equal size on the plasma membrane and in early endosomes, with an average
cycling time of 15 minutes (Davidson et al., 1990). After antigen binding, mIg is removed
from the cell surface. Polyvalent antigen/mIg complexes traffic into late endosomes and
lysosomes (Tony et al., 1985), the same route as other cross-linked receptors (for example
transferrin receptor, TfR) (Ekblom et al., 1983). For monovalent antigen/mIg, endocytic
routing is also altered away from recycling (Davidson et al., 1990). The signals for mIg
routing are in its transmembrane and short cytoplasmic domains, and in the molecules
associated with mIg (Reth et al., 1991) which are regulated by phosphorylation.

Antigen processing by specific B cells is extremely efficient (Lanzavecchia, 1985). EBV-
transformed B cells specific for Tetanus-toxoid (TT) presented antigen after a 48 hour
incubation at antigen concentrations at which 0.05% of mIg was occupied with antigen
(Lanzavecchia, 1990), equivalent to 50 tetanus toxoid molecules per cell. Assuming
clearance of surface mIg-TT complexes every 15 minutes (Davidson et al., 1990), the
uptake of approximately 10,000 surface bound TT molecules was sufficient for
presentation. Assuming delivery of all these complexes for processing, and that T cells
recognise 50-300 specific MHC/peptide complexes, processing by these APC yielded a
single epitope from 0.5%-3% of antigen. The mechanisms which lead to this efficiency
include: (1) co-localisation of internalised mIg with MHC II (Pletscher and Pernis, 1983);
(2) mIg-bound antigen at a high concentration in the plane of the membrane; (3) protection from further processing by mIg of T cell epitopes within partially degraded antigen.

Antigen is delivered, by mechanisms (1) and (2), into a compartment where processing is efficient. There is ample evidence that mechanism (3) leads to the control of processing by the fine specificity of the B cell’s mIg (Ruud et al., 1986; Davidson and Watts, 1989; Davidson et al., 1990). The B cell epitope is protected from degradation, although other portions of antigen are lost and mIg is cleaved into Fab/antigen remnant + Fc. Similar antibody-directed processing is seen for uptake via FcR. In a study using macrophages as APCs, a panel of monoclonal antibodies specific for a single antigen were used to enhance responses by a panel of T cell clones specific for the same antigen. However, some combinations of antibody and T cell clones were not enhancing. Antibody-directed processing, with the protection of T cell epitopes near to B cell epitopes may explain the proximity of B and T cell epitopes on some antigens (Milich et al., 1986).

Migratory dendritic cells. Dendritic cells are the most potent in vitro APC, in particular for primary responses. The first dendritic cells to be described were those in the epidermis, which have taken the name of their discoverer in 1868, Paul Langerhans. Dendritic cells are now known to form a small population in almost all organs. The precursors of tissue dendritic cells are a small population of MHC^+ peripheral blood mononuclear cells which differentiate into tissue dendritic cells in response to GM-CSF (Inaba et al., 1992) or in response to the combination of TNFα and GM-CSF (Caux et al., 1992). The induction of tissue dendritic cells may be a way in which these cytokines amplify local immune responses. After differentiation these cells become MHC II^+ve, with multiple cytoplasmic extensions which contact a large number of nearby parenchymal cells. Langerhans’ cells contain unique, membrane bound, acidified organelles, named Birbeck Granules, which are in contact with the endocytic pathway (Stossel et al., 1990). Dendritic cells are non-phagocytic (Girolomoni et al., 1990), do not have many electron-dense lysosomes (Stossel et al., 1990), and are correspondingly poor at terminally degrading foreign material (Chain et al., 1986). The poor lysosomal function is associated with the a failure to process some but not all antigens.
The tissue stage of dendritic cells is only an intermediate stage prior to migration and maturation. This is enhanced by non-specific tissue injury either by direct signalling from injured parenchymal cells, or via cytokines, possibly TNFα. Upon activation, dendritic cells migrate into lymphatics, and draining secondary lymphoid organs (Steinman, 1991), where they reside as interdigitating dendritic cells in T cell areas for several days before dying (Macatonia et al., 1987; Larsen et al., 1990). The maturation of tissue dendritic cells has been studied by in vitro culture of Langerhans’ cells (Schuler and Steinman, 1985; Romani et al., 1989; Aiba and Katz, 1991). Cell biological aspects of the maturation include the cessation of synthesis of II and MHC II, with undiminished expression of cell surface MHC II (Pure et al., 1990; Kampgen et al., 1991). In addition, a reduction in the ability to process antigens upon maturation has been deduced from the loss of acidification of endocytic organelles and Birbeck granules (Stossel et al., 1990). Therefore, antigen acquired and processed at the early stages of activation in the periphery is retained for presentation after migration. Thus, mature dendritic cells are less able to process antigen encountered after leaving the periphery.

Dendritic cells’ potency as APCs derives from high level expression of MHC I, MHC II, B7/BB1 and the adhesion molecules CD11a, CD18 and CD54 (LFA-1, ICAM-1 and LFA-3), and from low levels of cell surface glycoprotein sialylation (King and Katz, 1990; Young et al., 1992). Intracellular MHC II has been found throughout the endocytic pathway, as well as in non-endocytic vesicles (Arkema et al., 1991). The reduced surface sialylation may enhance (1) uptake of cationic antigens (Apple et al., 1988), (2) specific immune recognition of MHC/peptide (Boog et al., 1989), (3) clustering with T cells which occurs prior to engagement of MHC II by T cell receptor and CD4 (King and Katz, 1990). Dendritic cells present antigen to and stimulate T cytotoxic cells without ensuing lysis, and may therefore provide an important site for T helper cells to control T cytotoxic cell responses in three cell clusters (Mitchison and O’Malley, 1987). In support of this role for dendritic cells, dendritic cells have been suggested to be the APCs capable of presenting exogenous antigens by MHC I (Carbone and Bevan, 1990; Rock et al., 1990), although this activity has not been detected in all systems (Lopes and Chain, 1992).

Dendritic cells resident in primary lymphoid tissues, APCs without facilitated antigen
uptake. Although some dendritic cells in primary lymphoid tissues are post-migratory following inflammatory responses, interdigitating dendritic cells are detected at all times in lymphoid tissue, not only after immune stimulation (Kamperdijk et al., 1985). This non-stimulated population is likely to include the cells which acquire antigens from the circulation (Crowley et al., 1990). Further evidence in favour of additional subsets of splenic dendritic cells comes from the heterogeneity of dendritic cell markers in the spleen (Aiba and Katz, 1990; Agger et al., 1992). Unlike macrophages, splenic dendritic cells have very low levels of cell surface receptors for antigen: i.e. opsonin or scavenger receptors (Steinman, 1991; Naito et al., 1991). This implies that non-specific means of uptake including fluid-phase endocytosis may be an important mechanism of antigen entry for splenic dendritic cells (Janeway, 1992b).

However, splenic dendritic cells have been shown to have poor fluid-phase endocytic activity, especially in comparison with macrophages (Kapsenberg et al., 1986; Inaba et al., 1990). Although evidence for co-operation between macrophages and dendritic cells was obtained some years ago, and taken to imply the transfer of pre-processed antigen (Inaba et al., 1981), this finding has not been repeated (Inaba et al., 1990; Pure et al., 1990). Therefore, how might non-migratory splenic dendritic cells acquire antigen? It is possible that the claim of endocytic inactivity is inaccurate, since these studies measured uptake after long pulses of a fluid-phase marker. From kinetic studies of endocytosis in macrophages and fibroblasts, a pulse of several hours or more leads to saturation of endosomes and uptake into lysosomes (Besterman et al., 1981; Swanson et al., 1985). Relatively poor lysosomal function clearly does not inhibit processing of all antigens by dendritic cells. Therefore, it is important to measure the traffic of fluid-phase markers through pre-lysosomal compartments.

In Chapter 3, a technique to assess fluid-phase endosomal traffic in dendritic cells is described. Fluid-phase traffic through early and late endosomes was shown to be similar to several B cell APCs. This confirms that splenic dendritic cells can take up antigen by fluid-phase endocytosis.

Other cells. The expression of MHC II and antigen processing in the thymus (thymic epithelium and dendritic cells) is necessary for the T cell repertoire to distinguish self
from non-self (Robertson et al., 1991). In addition, the expression of MHC II can be induced on many cell types. Within the immune system, activated human, but not rodent T cells express MHC II and are able to process and present antigen (Hewitt and Feldmann, 1989), although the physiological importance of this is not known. On cells outside the immune system, for example pancreatic islet cells, expression of MHC II is induced as part of a non-specific inflammatory response, and may be responsible for auto-immune responses to (normally) sequestered antigens (Todd et al., 1985; Bottazzo et al., 1985). MHC II is also expressed on activated endothelial cells exposed to gamma-IFN, where it may be involved in delayed-type hypersensitivity responses (Pober and Cotran, 1990). MHC II is found at all times on gut epithelium, although its function is not known.

A wide range of cell types express MHC II under some circumstances. Transfection of other cell types also demonstrates that often the only additional requirement for a cell to process and present antigen is the expression of MHC II and Ii. Therefore, the other molecules involved are constitutively expressed in the large majority of cells, implying that antigen processing uses many facets of the pre-existing exocytic, endocytic and degradation pathways.

**Mechanism of antigen presentation.** The binding between MHC/peptide and T cell receptor together with CD4/8 is usually insufficient to activate T cells (Springer et al., 1987). There are a number of adhesion molecules expressed on T cells with a paired, complementary protein on the target cells. Examples of such pairs are CD2/LFA-3, LFA-1/ICAM-1. These multiple interactions occur prior to engagement of T cell receptor by MHC, and are thought to be a means of focusing T cells to their targets. Other interactions may also determine the outcome of the encounter between T cell and target. There is a second signal which is required for presentation to lead to T cell activation rather than T cell anergy. This signal is delivered by B7/BB1 on APCs, the ligand for which is CD28 on T cells (June et al., 1990).

The strength of the antigen-non-specific interaction determines the amount of antigen-specific interaction which must occur. For T cell hybridomas and other secondary T cell responses, the requirements for the antigen-specific component are less stringent than
for naive cells because of greater antigen-non-specific interaction (Cerottini and MacDonald, 1989). Therefore, secondary responses require less antigen processing before successful presentation.

**MHC II expression and antigen processing capacity in an APC are related to its role in antigen presentation.** The immune response is partly regulated by control of APC functions: MHC II expression; second signal expression; and, in some cases, the antigen processing machinery. The main APC types have distinctive roles for which they have adapted different patterns of regulation of APC function.

Macrophages are particularly suited to process particulate antigens. Thus, they are likely to be the predominant APC for bacteria. To avoid presentation of self cellular components, for example apoptotic bodies, the expression of both MHC II and second signal is only induced in the presence of pathogen-related molecules.

The important aspect of B cells as APCs is to recruit T cell help for the production of Ig by focusing T helper cells onto the very same antigens that are recognised by the B cells' mIg. The constitutive expression of MHC II, compared to macrophages, may allow initiation of secondary immune responses after antigen uptake by resting memory B cells. It has remained uncertain whether resting B cells can elicit primary T helper responses. However, a recent study shows that this interaction can occur, mediated via the induction of B7/BB1 (Koulova et al., 1991).

The role of migratory dendritic cells is to scan parenchymal cells of non-lymphoid organs for abnormal protein synthesis, and present antigens in primary lymphoid tissues. Although the second signal is constitutively expressed, these cells do not contact large numbers of T cells until inflammatory signals lead to migration. The maturation process is the best-documented example of down-regulation of the antigen-processing machinery, in this case to preserve a cohort of previously processed antigen.

The only APCs in primary lymphoid organs which constitutively express the second signal are the resident dendritic cells, which also are the APCs with the most limited means of antigen uptake. Therefore, their role may be to present soluble antigens without prior
non-specific recognition, possibly detecting pathogens which do not express molecules such as lipopolysaccharide, mannan and dsRNA. In addition, these cells may be particularly involved in responses to viruses, the pathogens which are the least able to induce a second signal.
B. THE BACKGROUND TO ANTIGEN PROCESSING FOR MHC II

(i) ANTIGEN PROCESSING FOR MHC I

MHC I binds 8-9mers. The crystal structure of MHC I was shown to contain peptide in the apical binding groove (Bjorkman et al., 1987 a and b). Since then, it has been shown that MHC I retains low molecular weight material during immuno-precipitation. Separation on reverse-phase HPLC reveals a large number of peptides (for example: Van Bleek and Nathenson, 1990; Rotzschke et al., 1990), although even more peptides which bind with low affinity may be lost during the precipitation. Some MHC I molecules yield mostly 9mers; others yield 8mers. These peptides are much shorter than was previously expected (Townsend et al., 1986). This length of the processed epitope is very similar to the theoretical minimum of 7 residues which could contain sufficient information for specific immune recognition by both T cell receptor and MHC (see above), demonstrating the efficiency of the tertiary complex of MHC-peptide-T cell receptor. One or two residues have been found to be fairly invariant for all peptides binding to a particular MHC I species, and have been termed anchor residues (see below).

The peptide is in an extended confirmation (Madden et al., 1991), not an α-helix as was once postulated (DeLisi and Berzofsky 85, Rothbard and Taylor, 88). The peptide backbone has key interactions at its N- and C- termini, which fix the peptide into position. Peptide side-chains of certain residues, particularly the anchor residues, fit into well-defined side-pockets in the peptide-binding groove (Garrett et al., 1989). An individual side chain is selected by the lining of the side-pocket, which is partly made up of the polymorphic residues within MHC I. In this way different MHC molecules are able to select different groups of peptides.

The structures of several MHC I-peptide complexes have now been solved (Fremont et al., 1992; Matsumura et al., 1992; Zhang et al., 1992; Silver et al., 1992; Guo et al., 1992; Madden et al., 1992). These structures demonstrate that peptide can form a bulge away from the floor of the binding groove 4-5 residues from the N-terminus, this being the site in the peptide which accommodates the single residue variation in length between different complexes for the same MHC I molecule. An important, but subtle feature of peptide binding is that the conformation of the α-helices which form the sides of the
The bound peptides have an MHC-dependent motif. In a single species of MHC I, the eluted peptides have been sequenced as a pool. An overall pattern, or motif, is apparent, which is specific for an individual MHC I species (Falk et al., 1991). Anchor residues are represented by a single amino acid, or a limited set of related amino acids. Less strict requirements are imposed on other positions, at which the amino residues are thought to interact less specifically with MHC. A third group of positions contains a large range of amino acids, these positions being proposed to interact not with MHC but with T cell receptor.

More recently, HPLC has been combined with mass spectroscopy to achieve better resolution of peptides eluted from MHC I, and to sequence the major species. The results confirm that peptides are mainly derived from cytosolic proteins, such as the human nuclear protein p68 (Jardetzky et al., 1991; Hunt et al., 1992a). In addition, peptides derived from signal sequences have been detected (Hunt et al., 1992a; Henderson et al., 1992).

A different method of determining the range of peptides bound by MHC I has been to synthesise a wide range of 8-9mers which are all related to a suggested motif (Schumacher et al., 1992). The peptides which bind can be eluted from MHC and visualised in 2 dimensions (HPLC and TLC) to produce a "fingerprint" for that MHC molecule. Point mutations in the MHC molecule alter peptide binding by loss and gain of certain specificities.

Production, translocation and binding to MHC I of 8-9mers. There is very little data on the cytosolic enzymes which process antigen. It has been suggested that the ubiquitin pathway, a common cytosolic degradation system, can lead to MHC I processing (Townsend, 1987; Monaco, 1992a). However, multiple routes are possible since non-ubiquitinated antigens introduced into the cytosol are also presented (Moore et al., 1988). Recent studies of the MHC II locus have uncovered two components of the low
molecular mass polypeptide (LMP), a cytosolic multi-chain proteolytic complex (Monaco, 1992a) which might deliver peptides from the ubiquitin degradative pathway to the translocation step. Despite the candidacy of these molecules for a role in MHC I processing, their importance has recently been thrown into doubt (Arnold et al., 1992), and their role is unclear.

Some mutant cell lines selected for low MHC I expression present peptides but not endogenous antigens (Townsend et al., 1989; Hosken and Bevan, 1990), due to lack of peptide supply for MHC I binding (Schumacher et al., 1990). The genetic defect in the mutant cell lines has been detected as a deletion within the MHC II locus (Deverson et al., 1990), which contains 2 largely homologous ATP-binding cassette proteins (Trowsdale et al., 1990; Spies and DeMars, 1991). It has been postulated, but not proven, that these proteins are, like other members of this family, membrane transporters. The dimer formed by the two polypeptides, termed peptide supply factor (PSF), may translocate 8-9mers from the cytosol into the ER (Spies et al., 1992). In the absence of PSF, MHC I epitopes are not recovered from a total cell lysate (Falk et al., 1990). Therefore, these short peptides are either not created or rapidly degraded in the absence of MHC I. Signal sequence-derived peptides tend to be longer than 8-9 residues, indicating that the usual length of epitopes is imposed by pre-ER processing. In the rat, which has less polymorphic MHC I genes than mouse and human, allelic variants have been detected in the PSF genes (Monaco, 1992b). The PSF alleles have been shown to lead to loading of MHC I with different sets of peptides, thus enhancing overall MHC polymorphism (Powis et al., 1992).

Increasing the length of peptides from 8-9mers leads to rapid loss of affinity. For example, an impurely synthesised peptide of 12 or 16 residues bound only weakly to MHC I, while a minor contaminant of 9 residues, which was not formed by degradation after MHC binding, was recovered from MHC I (Schumacher et al., 1991). Therefore, longer peptides have a much reduced affinity for the binding site. This is likely to be caused by the failure of longer peptides to be bound by MHC I at both termini of the peptide back-bone. A longer peptide may bind, but one or both termini are incompletely bound, the off-rate is high, and any conformational changes to trap peptide cannot occur.
It has been shown that MHC I expression can be stabilised on PSFnull cells by peptide, \( \beta_2 \text{m} \), or low temperature. The mechanism underlying this stabilisation is the acquisition of a temperature-stable conformation after binding both \( \beta_2 \text{m} \) and peptide (Ljunggren et al., 1990; Schumacher et al., 1990; Townsend et al., 1990; Elliott et al., 1991), which shows that peptide has a structural role. As discussed below, peptide plays a similar, though lesser role, in determining MHC II structure.

Presentation of endogenous antigens by MHC I is inhibited by treatment of cells with Brefeldin A, a fungal product which has pleiotropic actions on several compartments in the cell, including preventing exit of newly synthesised proteins from the Golgi (Nuchtern et al., 1989; Yewdell and Bennink, 1989; Cox et al., 1990). This shows that only newly synthesised MHC I binds peptide. The most favoured site for this binding is the ER.

**Exogenous antigens are sometimes presented by MHC I.** It has generally been found that cytosolic loading is required for presentation of antigen to CD8\(^{+}\) T cells via MHC I (for example: Lopes and Chain, 1992; Collins et al., 1992). However, exceptions have been reported. For instance, an MHC II\(^{+}\) sub-population of splenocytes does not need cytosolic loading of antigen (Rock et al., 1990). A further exception to the rule has been described for cell-associated antigens, which are processed *in vivo* for presentation by MHC I (Carbone and Bevan, 1990). This indicates a pathway involving phagocytosis of cells, and subsequent presentation of their contents by MHC I. Although the APCs involved in either of these exceptions have not been identified, dendritic cells have been suggested as probable candidates.
**E. (ii) THE ENDOCYTIC PATHWAY**

The uptake and traffic of antigen by APCs are important steps for antigen processing. Macrophages have been used as model cell types in the study of endocytosis, therefore the endocytic pathway of macrophages is well characterised. The endocytic pathways of B cells and dendritic cells have not been studied to such a large extent. B cells are small in size with a high nuclear to cytoplasmic ratio. Dendritic cells have until recently been available only in small numbers. However, the difficulties in studying natural APCs has been by-passed by transfecting cell types such as fibroblasts and HeLa cells with MHC II + Ii (Lechler et al., 1985; Bakke and Dobberstein, 1990; Lotteau et al., 1990). The finding that these neo-APCs can process and present antigen indicates that the basic cellular mechanisms of APCs are not specialised. The major specialisations of an APC are the expression of MHC II and of receptors for antigen or antigen-containing complexes, which make use of pre-existing endocytic molecular mechanisms. Therefore, the properties of the endocytic pathways in APCs might be deduced from studies of the endocytic pathways of other cell types.

Although certain functions of the endocytic pathway are now well understood, the underlying nature of the endocytic pathway is still quite uncertain. The main theoretical schism is whether an individual endosome matures through various stages (maturation model), or whether there are pre-existing compartments with vesicular traffic as for the Golgi (vesicular shuttle model) (Murphy, 1991; Griffiths and Gruenberg, 1991). It is possible that the seeming contradictions are explained by the use of different cell types. In particular, rapidly growing tissue culture cells may be expected to differ from growth arrested, explanted cells. Lack of agreement extends to the nomenclature for endocytic subcompartments. The terms used here are similar to those used by other immunologists (Brodsky, 1992), as well as cell biologists who favour maturation (Dunn and Maxfield, 1992) and vesicular shuttle (Gruenberg et al., 1989).

**Uptake of antigen.** Antigens with cell surface receptors are taken up by invagination of plasma membrane bearing the receptor into pits which then form inside-out vesicles. There are two types of pit/vesicle: clathrin-coated and non-clathrin coated. Clathrin is a multimeric cytosolic protein with light and heavy chains that form triskelia joined together in a pentagonal/hexagonal lattice (Pearse, 1988). The lattice apposes the plasma
membrane to form a clathrin-coated pit, in which a range of plasma membrane receptors are concentrated. Some receptors such as Tfr and LDL receptor, which are important for the nutrition of all cells, are among those which interact with clathrin constitutively, as is mIg. For other receptors the interaction is controlled. For example, EGF receptor only interacts with clathrin after EGF binding, and is down-regulated by the subsequent internalisation. Receptor phosphorylation is a common way of controlling localisation of receptors, by controlling interactions between the receptor and the cytoskeleton. Apart from receptors concentrated in clathrin-rich pits, there is sufficient space in the pits for the random inclusion of all other molecules, except those held away by interactions with the cytoskeleton, such as CD4/p56 \(^{ck} \) (Pelchen-Matthews et al., 1991).

The interaction with clathrin is indirect, being mediated via the hetero-tetrameric adaptin complex (Pearse, 1988; Ahle et al., 1988), and possibly via other proteins as well (Ahle and Ungewickell, 1990). Adaptin is able to recognise elements of clathrin as well as a motif in the cytoplasmic tail of some receptors. This motif often contains a tyrosine (Jing et al., 1990), but the tyrosine is not obligatory. The recognition element is thought to be a tight \( \beta \)-turn with an exposed loop containing an aromatic residue (Vaux, 1992). Invagination of clathrin-rich pits is achieved by increasing numbers of pentagonal interstices to produce spheres approximately 100nm in diameter. The clathrin is then removed by an uncoating ATPase, to yield vesicles which can fuse with early endosomes.

Non-clathrin coated plasma membrane pits have been identified as the route of entry for molecules such as ricin and GPI-linked proteins (Sandvig et al., 1987; Bamezai et al., 1992). This route of entry has been difficult to study given both the similarity of these pits to un-invaginated plasma membrane, and the lack of distinguishing features for the vesicles after internalisation. However, the recent identification of specific proteins coating the pits during their formation is likely to herald new insights into this pathway (Rothberg et al., 1992).

Pinocytosis, literally drinking by the cell, is mediated by both sorts of vesicles. This is the route for non-specific uptake of exogenous molecules. Random inclusion in the lumen is described as fluid phase endocytosis. Molecules which rely on fluid phase uptake are, by definition, those which do not interact with other mechanisms of uptake in a
productive fashion, and therefore are taken up with the lowest efficiency. Low affinity binding with the plasma membrane, for example by interaction between a cationic molecule and surface sialic acids, leads to non-specific adsorptive endocytosis by inclusion of plasma membrane into internalised vesicles (Apple et al., 1988).

Whereas clathrin-coated vesicles are a universal port of entry in all cells including yeast and protozoa (Singer and Riezman, 1990; Coppens et al., 1988), the uptake of particulate antigens by phagocytosis is restricted to specialised cells such as macrophages. After initial surface binding via a limited set of receptors for Fc, complement and bacterial saccharides, an actin-dependent mechanism engulfs the particles and forms a phagosome. Phagosomes enter the endocytic pathway at the stage of late endosomes (Harding and Geuze, 1992).

**Early endosomes.** The most important function of early endosomes is to rapidly sort and then to recycle the vast majority (97% of membrane molecules, ≈80% of fluid-phase molecules) back to the cell surface with an average transit time of <3-10 minutes (Besterman et al., 1981; Schmid et al., 1988). The structure of early endosomes is a complex mixture of vesicles and tubules with a volume around 1% of total cytoplasmic volume but 20% of the area of the plasma membrane (Griffiths et al., 1989). Vesicular early endosomes 300-500nm in diameter contain internal membrane vesicles, and are termed multivesicular bodies (MVBs). MVBs in the periphery of the cell are attached to a large, cupped cisterna for which no function has been identified (Hopkins, 1983; Gruenberg et al., 1989; Griffiths, 1992).

Newly endocytosed fluid-phase marker is localised at the rim of MVBs in the periphery of cells; later on the marker is seen throughout the lumen of more central MVBs (Storrie et al., 1984). Video microscopy has shown that MVBs move slowly (0.05 μm/sec) towards the pericentriolar area (Hopkins et al., 1990). In some cell types it has been shown that molecules which are destined for trafficking to late endosomes are associated with the internal membranes rather than the limiting membrane (Felder et al., 1990). Although this is not seen in all systems (Griffiths, 1992), it is good evidence that early endosomal sorting can occur in MVBs.
Molecules destined for recycling are located in the tubules 30-50nm in diameter extending from MVBs (Rome, 1985; Geuze et al., 1987). Post-sorting molecules are found in tubular recycling early endosomes near the Golgi (Yamashiro et al., 1984; Dunn et al., 1989; Stoorvogel et al., 1989). In some cells the tubular extensions of different MVBs do not join (Marsh et al., 1986), but in some areas of other cell types they join to form a tubular reticulum (Hopkins et al., 1990; Tooze and Hollinshead, 1991). No early endosomal function is correlated with traffic along tubules as opposed to vesicular traffic along a pre-determined pathway between tubular extensions. Treatment with Brefeldin A creates a tubular reticulum which includes early endosomes and trans-Golgi network (TGN) (Wood et al., 1991). This finding indicates that the TGN and early endosomes are closely linked, but the function of this pathway has not been examined.

During migration to the centre of the cell the internal membranes of an MVB increase. At the same time the accessibility to some endocytosed markers decreases, although the accessibility to rapidly trafficking membrane molecules such as TfR does not decrease (Stoorvogel et al., 1991; Dunn and Maxfield, 1992). In the view of the maturation model, it is proposed that MVBs progress to a stage where they lose all recycling molecules. This is followed by loss of all tubular extensions, and then the MVB traffics to the late endosome. The trafficking MVB is equivalent to a carrier vesicle in the vesicular shuttle model.

Early endosomes have a partly overlapping set of proteins with both plasma membrane and late endosomes, but also contain some unique species (Schmid et al., 1988; Beaumelle et al., 1990). A large family of rab proteins, ras-like small GTP-binding proteins, controls membrane traffic within the entire endo- and exo-cytic pathways. rab5 and rab4 are found on non-identical subsets of early endosomes but not on late endosomes (Chavrier et al., 1990; Van Der Sluijs et al., 1991). These two proteins have differing functions, controlling aspects of entry in early endosomes and recycling from them respectively (Marsh and Cutler, 1993).

The sorting mechanism in MVBs is highly efficient. For example, TfR in some cell types is recycled many 100s of times (Omary and Trowbridge, 1981). The efficient sorting of TfR has been used by some workers to define early endosomes as the TfR+ve
compartment (Beaumelle et al., 1990). Traffic to late endosomes is promoted by aggregation (Ekblom et al., 1983; Mellman et al., 1984; Ukkonen et al., 1986; Gruenberg and Howell, 1987), and by dissociation from the membrane into the lumen of the MVB (Brown et al., 1983; Tycko et al., 1983). Dissociation is often the result of the acidic environment. The pH is higher than in late endosomes because of partial inhibition of the proton pump by the positive charge imported by Na\(^+\),K\(^+\)-ATPase (Cain et al., 1989; Fuchs et al., 1989). Na\(^+\),K\(^+\)-ATPase is not present in late endosomes. The acidity is important for TfR to unload its Fe\(^{3+}\) (Dautry-Varsat et al., 1983).

Although the sorting mechanism has not been unravelled, it is likely to involve repeated low efficiency sorting events which during maturation of an MVB lead to overall high accuracy (Dunn et al., 1989). Possible sorting mechanisms include physico-chemical responses to changing membrane curvature, from the concavity of the tubular portions to the convexity of the internal membranes.

In the context of antigen processing, it is important to note that some surface receptors, particularly mIg and other antigen receptors, are sorted more rapidly by the early endosome, and therefore enter the late endosome more rapidly (Brodsky, 1992). In addition, it is important to note that there is heterogeneity between cell types. For example, TfR recycling is inhibited by ≤20 °C block in erythroid cells and macrophages (Sipe et al., 1991; Harding and Unanue, 1990b). It is therefore necessary to be cautious in applying findings in non-APCs to the study of antigen processing.

Late endosomes. Late endosomes are the intermediates between early endosomes and lysosomes. This can be judged from their pH (≈5.5) and bouyant density, both of which are intermediate (Storrie et al., 1984; Mellman et al., 1986; Griffiths et al., 1990). Fusion of carrier vesicles with late endosomes, as for other microtubule-mediated transport events (Kelly, 1990), is blocked by temperature below 20 °C (Gruenberg et al., 1989). The late endosome is a large tubulo-cisternal structure with internal membranes (Rabinowitz et al., 1992; Geuze et al., 1988; Griffiths et al., 1990). The late endosome has been proposed to be a single copy organelle (Griffiths, 1992).

The late endosome contains the majority of the cell's mannose-6-phosphate receptors
(MPRs) (Bleekemolen et al., 1988; Geuze et al., 1988; Griffiths et al., 1988). Some areas of late endosomes lie in close apposition to the TGN, which is where MPR is mainly located (Tassin et al., 1990). MPRs bound to lysosomal hydrolases (which are specifically tagged with mannose-6-phosphate) leave the TGN in clathrin-coated vesicles. These vesicles differ from plasma membrane derived vesicles through use of a different set of adaptin proteins (Ahle et al., 1988). After fusing with the late endosome, the acidic pH causes dissociation of enzyme. MPRs then recycle to the Golgi by vesicular traffic (Goda and Pfeffer, 1988). The phosphate is cleaved from the lysosomal hydrolases, preventing rebinding to MPR and recycling to the TGN.

Fluid-phase marker enters the late endosome after approximately 20-30 minutes (Gruenberg et al., 1989). Apart from the presence of MPR and the kinetics of entry, the late endosome can be specifically identified by the presence of rab7 (Chavrier et al., 1990). The late endosome is otherwise biochemically similar to lysosomes with high levels of lysosomal glycoproteins and hydrolases (Griffiths, 1992). Late endosomal function is a combination of hydrolysis and recycling, with a high proportion of fluid-phase marker being recycled from late endosomes to the cell surface after entry via early endosomes (Besterman et al., 1981; Strous et al., 1985; Swanson et al., 1987).

Lysosomes. In fibroblasts lysosomes are larger than the early and late endosomes together (Griffiths et al., 1989). In different APCs the size of the lysosomal compartment varies widely: well-developed in macrophages, poorly developed in B cells and dendritic cells. The membrane largely consists of highly glycosylated proteins resistant to hydrolysis, termed lysosome-associated membrane proteins (LAMPs) (Fambrough et al., 1988). The lumen contains hydrolases which have condensed by a pH-dependent mechanism into an electron dense matrix (Buckmaster et al., 1988).

Lysosomes were originally conceived as the end-point of uni-directional traffic towards hydrolysis. In support of this view, they are more acidic and have a greater bouyant density than late endosomes, and the kinetics of entry are slower (≥60 minutes). However, the linear view has been challenged by the striking biochemical similarities with the late endosome (Griffiths, 1992) and the discovery of dynamic interactions between lysosomes, possibly indirectly via the late endosome (Deng and Storrie, 1988;
Lippincott-Schwartz and Fambrough, 1987). Therefore, lysosomes may be specialisations of the late endosome, and separation of lysosomes and late endosome may be incomplete (Griffiths, 1992). The unique environment of lysosomes would nevertheless lead to condensation of the hydrolases.

**Connection between endocytic and exocytic pathways.** As discussed above, there are recycling early endosomes in close physical proximity with the TGN. Functional contact has been demonstrated for TfR (Cresswell, 1985; Stoorvogel et al., 1988; Neefjes et al., 1990) and ricin (van Deurs et al., 1988). This intersection between endocytic and exocytic compartments has been proposed as a site where endocytosed antigens encounter MHC II. However, the entry of transferrin is slow (Griffiths and Simons, 1986), and the entry of fluid-phase endocytic markers into the TGN is so inefficient as to be largely undetectable (M. Marsh, pers. comm.). For efficient antigen processing to occur via entry to the TGN, antigen would have to utilise an unknown carrier function.

**Maturation versus vesicular shuttle.** Current evidence indicates that there is some maturation and some vesicular shuttling. Incoming vesicles fuse with a semi-permanent early endosomal tubulo-cisternal network containing MVBs. An MVB which matures as it transits through the early endosomal compartment for approximately 15 minutes before exiting as a carrier vesicle to the late endosome. The late endosome has many properties of lysosomes and might communicate with them, possibly via maturation (Roederer et al., 1990).
B. (iii) THE ENZYMES WHICH PROCESS EXOGENOUS ANTIGEN

The constitutive expression in the large majority of cell types of the molecular machinery for antigen processing suggests that antigen processing uses the basic cellular degradative pathway. The vastly greater amount of antigen taken up compared to that presented indicates that antigen processing may be a by-product of normal degradation, and that several, redundant mechanisms might have the necessary activity. However, there are differences between APCs, even when closely related (Michalek et al., 1989). One source for this heterogeneity may be degradative enzymes.

The effector molecules of the processing apparatus may have important roles within degradative compartments. Therefore their susceptibility to proteolysis is an important facet of their function. Immunoglobulin is relatively resistant to proteolysis, which makes it an ideal carrier for antigen to degradative compartments. In addition, MHC α and β chains are relatively resistant to degradation compared to Ii (Blum and Cresswell, 1988).

Processing determines which T cell epitopes are exposed. The processed fragments of exogenous antigens are approximately 2 kDa in size (Demotz et al., 1989; Chicz et al., 1992). Therefore considerable degradation occurs during antigen processing. However, the extent of degradation must be limited to the production of oligopeptides longer than or equal to 15 residues, i.e. degradation must be partial. In any event successful processing must achieve the exposure of an MHC-binding structure (i.e. a T cell epitope) by degradation and/or other changes. The difficulty with which a T cell epitope is exposed is not related to the overall size of the antigen. Small, tightly folded oligopeptides need to be processed (Regnier-Vigouroux et al., 1988). Similarly, some short peptides might contain an epitope for one MHC II molecule, but are folded in such a way as to require further processing to bind a different molecule (Fox et al., 1988; Brett et al., 1988). On the other hand, large proteins may contain a naturally exposed T cell epitope and thus not require processing, for example fibrinogen, molecular weight 330 kDa (Lee et al., 1988).

The exact sequence of cleavages and other changes has not been established for any antigen. The importance of this sequence is that it facilitates the exposure of some epitopes but not others, and might destroy some of the epitopes but leave others
undamaged. For example, in a study of hen egg lysozyme, a single cleavage greatly enhanced both rotational mobility and presentation of an epitope near the cleavage site, even though the protein remained held in a single unit by disulphide bonds (E. Sercarz, pers. comm.). Therefore, the accessibility of an epitope is crucial.

In order to address specific questions regarding individual cleavages in antigen degradation a suitable system has to be developed. The use of highly simplified antigens offers some advantages in narrowing the range of relevant enzymes (Buus and Werdelin, 1986b), but this situation is possibly over-simplified. Even processing of small polypeptides such as EGF and insulin generates considerable complexity (Schaudies et al., 1987; Delovitch et al., 1988). Therefore, the globular proteins which are commonly used, such as ovalbumin, hen egg lysozyme and myoglobin, may be too large to demonstrate the exact order of individual cleavage steps.

**Peptides are produced by a combination of endoproteinases, exoproteinases, and other enzymes.** Peptide bonds are degraded in two ways: endoproteinases cleave in the middle of a peptide chain; exoproteinases cleave from the termini of a peptide chain. Both types of enzymes have preferred residues at the site of cleavage. For example, cathepsin D is thought to cleave between residues 2 and 3 of a 7 residue motif (Van Noort and Van der Drift, 1989). The importance of endoproteinases in antigen processing has been demonstrated by mimicking processing in vitro with endo-proteinases such as trypsin, or with agents which cleave internally such as cyanogen bromide (Shimonkevitz et al., 1983).

The main families (cysteine, aspartyl, serine and metallo proteinases) are defined by the biochemical moiety at the active site. The major cellular endoproteinases involved in protein catabolism are the cysteine proteinases cathepsins B, H and L, and the aspartyl proteinase cathepsin D (Barrett, 1977). There is an additional aspartyl proteinase, cathepsin E, which is much less well characterised than cathepsin D (Azuma et al., 1989; Jupp et al., 1988), but which is the dominant aspartyl proteinase in some murine APCs (Bennett et al., 1992).

Exoproteinases remove terminal residues singly or in pairs. The best evidence for their importance is the finding that a single epitope expressed by an APC consists of many different peptides bound to MHC II, all of which share the epitope, but which differ
because of heterogenous N- and C- termini (Chicz et al., 1992). The heterogeneity of the peptide termini indicates the involvement of exoproteinases. However, the role of these enzymes is unclear, since antigens of all sizes can bind to MHC II. The removal of extra residues at the extremes of a peptide may prevent the re-alignment of the peptide within the binding groove, which would lead to the presentation of a second epitope overlapping with, or adjacent to, the first (Kurata and Berzofsky, 1990).

The only non-proteinase activity to be definitely associated with antigen processing is the reduction of disulphide bonds (Jensen, 1991; Collins et al., 1991), which has been identified as a lysosomal function, although the enzyme responsible is not known. Reduction of disulphide bonds in vitro substitutes for in vivo processing of many other antigens (Streicher et al., 1984; Regnier-Vigouroux et al., 1988; Sette et al., 1989a). However, the significance of this is doubtful, because these antigens are likely to undergo partial proteolysis prior to entering lysosomes.

**Endoproteinase inhibitors are blunt tools for the identification of processing enzymes.**

The role of endoproteinases in antigen processing has largely been ascertained using endoproteinase inhibitors. In many systems these inhibitors block processing but not presentation. The identity of the processing enzyme involved has been established with the greatest accuracy for simple, repetitive polymers where it is feasible that only one cellular enzyme is capable of performing the required cleavage (Buus and Werdelin, 1986b). However, most studies have used inhibitors with a broad spectrum, inhibiting all proteinases from one or more families (Yoshikawa et al., 1987; Shaw and Chain, 1989; Diment, 1990), in particular leupeptin (Streicher et al., 1984; Puri et al., 1986; Yoshikawa et al., 1987; Bhardwaj and Colston, 1988; Eisenlohr et al., 1988).

Leupeptin has pleiotropic effects on cells which include the reduction of transport to dense lysosomes from early endosomes, which was detected in rat hepatocytes (Tolleshaug and Berg, 1981) and ascribed to "constipation" of the lysosomes. Leupeptin leads to an accumulation of MVBs, with the appearance of inhibition of MVB fusion with the late endosome (Griffiths, 1992). The lack of knowledge of how this effect is brought about indicates that any deductions from experiments using leupeptin must be made with extreme caution. For example, the effect on endosome-to-lysosome transport
might well explain the effects of leupeptin on the proteolysis of LI (Blum and Cresswell, 1988; Pieters et al., 1991; Neefjes and Ploegh, 1992a), and this might underly the failure of leupeptin-treated cells to process many different antigens.

Positive identification of endosomal processing enzymes has been difficult. Apart from the use of inhibitors, enzymes have been positively identified by their ability to carry out the necessary processing steps in vitro, and by localisation to the intracellular compartments responsible for processing. The degradation of a protein to reveal an epitope in vitro has been demonstrated for ovalbumin and myoglobin. The presentation of ovalbumin 323-339 is dependent on an aspartyl proteinase: both cathepsins D and B fragment ovalbumin in vitro, but only cathepsin D exposes the epitope (Diment, 1990). The epitope is on a fragment which is glycosylated, molecular weight ≈12 kDa (Rodriguez and Diment, 1992; K. Bennett and B. Chain, unpublished results). For myoglobin, cathepsins D and B in vitro account for nearly all the cleavages observed in vivo (Van Noort et al., 1991). Cathepsin D acts first, followed by C-terminal trimming of epitopes by cathepsin B.

The localisation of proteinases is classically lysosomal, which is consistent with their optimal function at acid pH. However, antigen processing may not involve entry into lysosomes. A role for endosomes in processing is indicated by the failure of mutant cells with a defect in endosomal but not lysosomal acidification to process antigen (McCoy et al., 1989). In support of this, proteolytically active cathepsins D and B have been detected in endosomes (Diment and Stahl, 1985; Diment et al., 1988), by a combination of the kinetics of degradation (within 10 minutes) and the use of inhibitors. These enzymes have also been detected by immuno-electron microscopy (immuno-E.M.), although the antisera used might bind pro-enzymes, and do not necessarily indicate active enzymes.

The difficulty in obtaining positive identification of a processing enzyme is illustrated by further studies of ovalbumin in A20 cells. Because of the sensitivity to pepstatin, it was initially proposed that cathepsin D was responsible (Chain et al., 1988; Rodriguez and Diment, 1992). However, the predominant aspartyl proteinase in A20 cells was subsequently found to be cathepsin E, and it is this enzyme which processes ovalbumin,
as shown by a specific inhibitor for cathepsin E which does not affect cathepsin D (Bennett et al., 1992). In A20 cells, cathepsin E is found in an un-identified low density compartment but is absent from the high density lysosomes which contain cathepsin D. This is the first report of a non-ubiquitous enzyme involved in antigen processing, and is evidence that enzymes contribute to APC heterogeneity.

The evidence for processing in early endosomes is weak. It has been claimed that early endosomes rather than late endosomes contain proteinases because of the rapid kinetics of entry. In support of this, there are some examples where cathepsin B activity has been encountered within 2-3 minutes of endocytosis, traffic not being blocked by ≤20 °C (Roederer et al., 1987; Bowser and Murphy, 1990). In addition, cathepsin D and other hydrolases traffic through early endosomes en route for late endosomes (Ludwig et al., 1991). However, cathepsin D activity encountered within 10 minutes of endocytosis (Diment and Stahl, 1985; Diment et al., 1988) lies beyond a 17-20 °C block (Mayorga et al., 1989), and is therefore likely to be in late endosomes. Furthermore, the enzymes recognised by immuno-E.M. in vesicles after 2 minutes internalisation of mIg (Guagliardi et al., 1990; Brodsky and Guagliardi, 1991) are accessed more rapidly and to a much greater extent by internalised mIg than by internalised Tf (Brodsky, 1992). Therefore, mIg may be an exception to the general rule of early endosome kinetics, with rapid access to the late endosome.

Processing enzymes are anti-processing enzymes. In many cases differential sensitivity of epitopes to proteinase inhibitors has been demonstrated: an inhibitor which prevents presentation of one epitope does not inhibit presentation of another epitope. In some cases proteinase inhibitors enhance presentation of epitopes (Buus and Werdelin, 1986b; Streicher et al., 1984; Yoshikawa et al., 1987). Therefore, in the absence of inhibitor epitopes are destroyed by proteinases. These proteinases are probably the same as those responsible for processing of other epitopes, but in this context they are identified as anti-processing enzymes.

The role of extracellular degradative enzymes is unclear. Extracellular processing, as discussed elsewhere, is not seen as a general rule, and theoretically might interfere with specific B-T pairing. However, enzymes which function at neutral pH have been detected
in serum (McCoy et al., 1988; Guo et al., 1992) and on the plasma membrane (Kenny, 1977), including APCs (Buus and Werdelin, 1986a; Chain et al., 1989; De Bruijn et al., 1992). The general role of these enzymes has not been determined. However, their existence indicates that processing might occur under unexpected conditions.

Future directions: How is partial degradation achieved? The phenomenon of anti-processing shows that the goal of partial degradation requires a delicate balance between sufficient degradation to expose an epitope and subsequent destruction. This balance would be affected by protection of an epitope by binding, either to MHC II or to other peptide binding molecules. One question which remains unanswered is whether each enzyme encountered in the cell by an antigen degrades it maximally, i.e. does an enzyme which exposes an epitope and which is capable of then destroying that epitope by anti-processing always do so in vivo? For example, ovalbumin 323-339 might be exposed by cathepsin B, but also might be destroyed by it; other ovalbumin epitopes might be exposed and destroyed by cathepsin D. The answer to this question has a direct bearing on the importance of the role of trafficking during processing as opposed to MHC-directed processing in generating epitope hierarchy. To this end, experiments to determine the influence of MHC II on the degradation of antigen by proteinase should be undertaken.
C. THE MOLECULAR AND CELLULAR FUNCTIONS OF ANTIGEN PROCESSING

(i) THE INTERACTIONS OF MHC II WITH Peptide

Structural comparison with MHC I. Although the binding of peptide to MHC was demonstrated in MHC II before MHC I (Babbitt et al., 1985), MHC II does not form crystals as easily and the exact nature of its binding to peptides is therefore less clear. On the basis of its similarity with MHC I, epitopes for MHC II could be as short as 8-9 residues, but differences at both ends of the groove imply that MHC II epitopes may be longer. As for MHC I, side-pockets defining anchor residues on bound peptides, together with interactions with the peptide backbone, are expected to contribute to a peptide motif.

MHC II binds 13-17mers derived mainly from molecules in the endosomal membrane. The analysis of peptides eluted from MHC II has recently been achieved (I-A^b: Rudensky et al., 1991a I-A^c: C. Janeway, pers. comm., I-A^d: Hunt et al., 1992b, DR3: Chicz et al., 1992, DR3 in T2 cells: Riberdy and Cresswell, 1992). Peptides from MHC II are 13-25mers, mostly around 15 residues, which is significantly longer than the 8-9mers in MHC I. The major peaks of peptides eluted from MHC II can represent a considerable proportion of the total peptide recovered (50 and 75% for the 6 most prominent peptides from I-A^b and I-E^b respectively). However, the estimate of the total number of epitopes presented by a single MHC II species in a single APC was still as high as 500-1000 (Germain, 1991). The number of epitopes might vary between different MHC species, for example I-A^d has a greater number of peptides than I-A^b (Hunt et al., 1992b). More accurate data on the number of epitopes displayed might be available in the future using more sensitive techniques for detection of individual peptides, such as ion trap mass spectroscopy (D. Hunt, pers. comm.).

The major peptides recovered are mostly derived from endosomal membrane molecules: MHC II itself, MHC I, Ii, mlg, transferrin receptor, Na^+-K^+-ATPase, and (in mouse) MMTV env proteins. The only exogenous proteins which have been found to produce a major peptide are BSA, apolipoproteins and fetuin. Therefore, MHC II predominantly presents proteins synthesised endogenously, though unlike MHC I these proteins are endosomal not cytosolic. The entry of cytosolic proteins into the endocytic pathway via autophagocytosis (Dice, 1990) may explain how cytosolic antigens are occasionally
presented by MHC II (Jaraquemada et al., 1990; Nuchtern et al., 1990).

Several forms of each of the major peptides are recovered from MHC II. These forms are nested sets with variable extensions around a minimal sequence. In some MHC II molecules only the C-terminus shows variation, indicating that the N-terminus is fixed (Rudensky et al., 1991a), whereas for other MHC II molecules both termini are variable (Hunt et al., 1992b; Chicz et al., 1992). This confirms that the binding groove of MHC II is open at both ends.

The sequences of eluted peptides fit a motif (Chicz et al., 1992) similar to the motifs detected for MHC I (Falk et al., 1991), however motifs are more difficult to establish in the presence of variable N-termini. An alternate method of establishing the motif has been the innovative technique of a plasmid expression library (Hammer et al., 1992). 2 x 10^7 random nonamers expressed at the exposed N-terminal of a minor phage protein were selected by repeated rounds of direct binding to MHC II. The motif of sequences selected in this manner largely fitted the motif established by eluting peptides from the same MHC II species (Chicz et al., 1992).

Conformational changes occur in MHC II. MHC II has been found in an SDS resistant form, which resists denaturation by 2% SDS at temperatures below 50-60°C (Billing et al., 1976; Cresswell, 1977). On SDS-PAGE gels these α/β dimers run with an apparent molecular weight ≤90% of true molecular weight, and have therefore been termed compact dimers. Compact dimers have been found to contain peptide (Wettstein et al., 1991; Sadegh-Nasseri and Germain, 1991; Stern and Wiley, 1992). Compact dimers do not contain li (Germain and Hendrix, 1991), and are formed in endocytic compartments (Neefjes and Ploegh, 1992a). Compact dimers can be denatured by acid pH, which also leads to loss of bound peptide. Under conditions which denature compact dimers, an intermediate with 100% of the expected molecular weight is first formed, termed a floppy dimer (Dommair et al., 1989). Further denaturation yields individual α and β chains. Interestingly, peptide is still associated with these individual chains (Dornmair et al., 1989; Tampe et al., 1991). Reduction and re-oxidation have the same effect as pH changes on MHC II peptide binding (Dornmair and McConnell, 1990), which confirms that pH change is able to bring about alterations in conformation.
Empty MHC II molecules do not form compact dimers. Empty MHC II has been obtained from two sources. Affinity-purified MHC II, which presumably contains peptide, has been incubated at low pH, separated from eluted peptides, and reconstituted without peptide in the presence of detergent micelles (Wettstein et al., 1991; Sadegh-Nasseri and Germain, 1991). Alternatively, MHC II has been expressed in and purified from insect cells (Stern and Wiley, 1992).

The function of compact dimer formation is unclear. The ease with which MHC II molecules form compact dimers varies between individual molecules (Germain and Hendrix, 1991). I-A\(^d\), which forms a small amount of compact dimers, is rescued from rapid degradation (t\(^d\) < 4hr) by peptides which form compact dimers (t\(^d\) > 8hr), but not by other peptides. Therefore, the role of compact dimer formation may be to rescue both MHC and peptide from degradation. It might be that for I-A\(^d\), and similar MHC molecules, there is an adaptation to the increased degradation by increased synthesis. The functional impact of compact dimer formation may be to prevent peptide exchange after a pulsed exposure (for example in migrating APCs) allowing presentation of the same epitopes for longer and hence to more T cells. Some MHC II molecules, for example I-A\(^d\), do easily not form compact dimers \textit{in vitro} (R. Germain and J. Miller, pers. comm.). I-A\(^d\) has a greater number of peptides bound to it than I-A\(^b\) (Hunt et al., 1992b). Therefore, lack of compact dimer formation may allow recycling empty or occupied \(\alpha/\beta\) to bind a wider range of peptides, possibly including peptides encountered outside the major antigen processing compartment.

The conformational change for MHC I which is both peptide dependent and temperature sensitive, and hence is analogous to compact dimer formation in MHC II, is necessary for antigen presentation. In contrast, the conformational change for MHC II has not been shown to be a requisite for successful antigen processing or presentation, and its biological significance is unclear.
C(ii) THE CELL BIOLOGICAL INTERACTIONS OF MHC II

MHC II is co-synthesised with invariant chain (Ii). MHC II presents peptides derived from exogenous antigens which are processed in the endocytic pathway. MHC II reaches the endocytic pathway via the ER and Golgi, but does not tend to present the peptides which bind MHC I in the exocytic pathway. Expression of MHC II α and β in most cell types is sufficient for the presentation of endogenous antigens (Sekaly et al., 1988), and exogenous peptides (Peterson and Miller, 1990). However, α and β alone do not lead to presentation of all exogenous antigens (Stockinger et al., 1989; Hammerling and Moreno, 1990). A third molecule, the invariant chain (Ii), is required. Ii is thought to have several functions: MHC II folding in the ER; protecting the MHC binding site from peptides in the ER; and routing MHC II out of the default exocytic pathway into endosomes. Ii is a non-polymorphic type II glycoprotein. The predominant form in mouse is 31 kDa (p31), and 33 kDa in humans (p33). Expression of Ii is largely co-regulated with MHC II, although it is encoded outside the MHC (Kappes and Strominger, 1988).

MHC II α and β chains, both type I membrane glycoproteins, are exported from the ER and through the Golgi within 30 minutes of synthesis. However, MHC II dimers (termed α/β) are not expressed on the cell surface for a further 2 to 3 hours (Blum and Cresswell, 1988; Neefjes et al., 1990). This delay is caused by interaction with Ii, since α/β is exported directly to the cell surface in the absence of Ii (Lotteau et al., 1990). The interaction between α/β and Ii to produce α/β/Ii is mediated by their extracellular domains (Kjer-Nielsen et al., 1990), and occurs shortly after translocation into the ER (Kvist et al., 1982).

Ii assists folding of MHC II. Ii self-associates into trimers due to an interaction in the lumenal region (Marks et al., 1990). These trimers non-covalently bind α and β chains to yield a nonamer, (α/β/Ii)_3, which can then release α/β dimers (Roche and Cresswell, 1990a; Roche and Cresswell, 1991). Dimers of different isotypes can be found in a single nonamer (Cresswell, 1992). The Stokes' radius of Ii_3 is only marginally less than the Stokes' radius of (α/β/Ii)_3 (Roche et al., 1991). Therefore, Ii_3 may provide a framework within which it assists the correct folding of α and β, i.e. a chaperonin-like function. In support of this function, Ii has been shown to facilitate assembly of mis-matched α/β pairs (Layet and Germain, 1991). In addition, some conformational differences have been
noted on MHC II molecules in the absence of Ii (Peterson and Miller, 1990; Rath et al., 1992).

**Ii controls peptide binding.** In vitro studies indicate that the binding to \( \alpha/\beta \) of either Ii or peptide is mutually exclusive (Roche and Cresswell, 1990a; Teyton et al., 1990). The mutual exclusivity of peptide and Ii binding has also been demonstrated in vivo (Murphy et al., 1992). One model of the altered affinity is direct blocking of the antigen binding site by a loop of Ii, after the loss of which peptide can bind (Elliott et al., 1987). An alternative model is that Ii imposes a low affinity conformation on the peptide binding site so that there is continuous exchange of peptides, and hence loss of weakly bound peptides during experimental purification procedures. In this model, loss of Ii leads to retention of previously bound peptide (Hammerling and Moreno, 1990). In support of this model, it has been shown that \( \alpha/\beta/Ii \) can bind peptide, although the affinity is x30-50 lower than the affinity of \( \alpha/\beta \) for peptide (Roche and Cresswell, 1990a; Roche et al., 1992). The development of techniques that detect weakly bound peptides is required to decide between these two models.

**Invariant chain directs MHC II to endosomes.** Ii is synthesised in excess over \( \alpha \) and \( \beta \) (Kvist et al., 1982; Machamer and Cresswell, 1984). The route of export for excess p33 is not via the Golgi, but into degradative vesicles with lysosomal properties, suggestive of autophagosomes to some workers (Marks et al., 1990; Lotteau et al., 1990), but assigned as endosomes by others (Bakke and Dobberstein, 1990). The signal for this traffic includes residues 10-16 of the 32 amino acid cytoplasmic domain of p31. This signal is obscured by the correct association of Ii with \( \alpha \) and \( \beta \), but not obscured by association with \( \alpha \) chain alone. Therefore, this pathway allows degradation of excess Ii or complexes, including mis-folded \( \alpha \) or \( \beta \).

Successfully folded \( \alpha/\beta/Ii \) is routed into endocytic compartments rather than the plasma membrane. This trafficking is brought about by a second signal in the cytoplasmic domain of p31, within residues 17-29, since Ii without any cytoplasmic domain is exported directly to the plasma membrane. The mechanisms for Ii directed traffic of MHC II are found in many cell types transfected with MHC II. However, these mechanisms are not universal, since MHC II traffic is unaffected by Ii in mouse L cells, which process antigen
without transfection of Ii (Salamero et al., 1990a; Lechler et al., 1985). The nature of the endocytic compartments in which \(\alpha/\beta/Ii\) resides is discussed below.

**Ii has an ER retention signal.** Approximately 10% of human Ii has a cytoplasmic domain 15 amino acids longer (p35) due to an alternative initiation site. This N-terminal extension contains an ER retention signal, which is dominant over other signals in Ii (Lamb et al., 1991): one p35 molecule in an MHC nonamer retains the whole complex. A delay in export may allow more time for correct assembly of the complex and correct folding of \(\alpha\) and \(\beta\), which may be more important for human MHC II than mouse because of the increased number of isotypes and hence of mismatched \(\alpha/\beta\) pairing. Replacement of the p35 by p33 would then allow export from the ER.

Murine Ii traffic has been studied less than human Ii. Endosomal targeting is essentially similar although murine Ii does not have the optional ER signal (Cresswell, 1992). Instead p31 expressed on its own is retained for some time in the ER before export (Simonis et al., 1989), and so there is an ER retention signal in murine p31. This finding is also seen in human cells, where excess p33 is exported from the ER inefficiently (Lamb and Cresswell, 1992).

**Ii dissociation follows Ii degradation in endosomes.** The trafficking signals in Ii's cytoplasmic domain have a co-ordinated sequence of action that leads to endocytic localisation of correctly assembled \(\alpha/\beta/Ii\). Ii's signals dominate over the targeting of \(\alpha/\beta\) to the plasma membrane, which is finally revealed after the dissociation of Ii, and which may not require active signalling since cell surface expression may be a default pathway. Complexes of \(\alpha/\beta\) with mutated Ii which does not express the endosomal target sequence are located on the plasma membrane (Roche et al., 1992). The Ii in these complexes is not degraded and does not dissociate from \(\alpha/\beta\).

Ii dissociation is brought about by a series of proteolytic cleavages of the luminal domain. The first cleavage, by an unidentified enzyme, leaves an intermediate of 25 kDa (p25) which is still attached to, and causes endosomal localisation of, \(\alpha/\beta\). p25 is degraded by a leupeptin sensitive proteinase(s), and then dissociates (Pieters et al., 1991; Neefjes and Ploegh, 1992a). It has been suggested that the cysteine proteinase cathepsin
B is the enzyme responsible for this (Reyes et al., 1991; Roche and Cresswell, 1991). No proof for this is available, because of the pleiotropic effects of leupeptin. α/β is also exposed to proteolytic enzymes, but is less sensitive to proteolysis that Ii (Blum and Cresswell, 1988).

The activity of Ii degradation varies between different MHC II⁺ve cells. In "professional" APC the proteolysis is so rapid that all intermediates are difficult to detect by metabolic labelling; in the presence of leupeptin, p25 builds up and the other intermediates are clearly seen (Blum and Cresswell, 1988), implying that leupeptin only partially blocks proteolysis. In cells where the importance of MHC II expression is less clear, such as melanoma cells, intermediates are readily detectable even in the absence of leupeptin (Pieters et al., 1991).

**High level Ii expression creates large Ii⁺ve vesicles.** Cells transfected with Ii in high copy number contain large vacuoles which are Ii⁺ve and contain some endosomal markers. These vesicles are created solely by high level Ii expression. Transfectants with lower copy number contain a similar number of smaller vesicles (T. Nilsson, pers. comm.). The vesicles have variously been called autophagosomes (Lotteau et al., 1990), endosomes (Bakke and Dobberstein, 1990) and macro-endosomes (R. Germain, pers. comm.). The exact nature of these vesicles is not apparent. However, their large size indicates a possible function of Ii: the inhibition of onward flow in the endocytic pathway, for example by aggregate formation. The inhibition of flow at limited sites might create micro-environments which represent semi-permanent specialised antigen processing compartments.

**Free Ii is found at the cell surface.** One reason that MHC II was discovered before Ii was the failure of Ii to co-precipitate with cell surface MHC II (Shackelford and Strominger, 1980). Although it was thought that Ii dissociated from MHC II inside the cell and remained intracellular (Kvist et al., 1982), it has been found at the surface on many APCs (Koch et al., 1982; Wraight et al., 1990; Poirier, 1992), particularly in B cells. In some cell lines the surface Ii has been found to be associated with α/β (Koch et al., 1991). This may derive from the minority of p31 which is not correctly sorted away from the plasma membrane (Lotteau et al., 1990). The role of this surface α/β/Ii in unclear.
II has many modifications. Approximately 10% of II has an additional extra-lumenal domain due to differential exon splicing, yielding p41 (Koch, 1988). Some APCs, such as A20 cells, express less p41 than this. The extra domain contains a thyroglobulin repeat element, the significance of which is unclear. It is equally unclear what role p41 plays in antigen processing, although it has been reported that the presentation of some antigens but not others is highly dependent on the expression of p41 (J. Miller pers. comm.). In addition, the traffic of p41 has been shown to be different from that of p31 (Teyton and Peterson, 1992).

Modifications to p31 which have been reported are: addition of chondroitin sulphate, palmitylation of the cytoplasmic domain, and phosphorylation. p31 linked to chondroitin sulphate is transported rapidly to the cell surface, and acts as an accessory molecule in primary T cell activation (J. Miller, pers. comm.). Palmitylation has been hypothesised, by analogy with the transferrin receptor, to have a function in the endocytic pathway (Koch, 1988). However, no clear function for these modifications is known (Cresswell, 1992; Teyton and Peterson, 1992).

Overall role of II - a promoter of antigen processing. II has many adaptive functions for processing of exogenous peptides by MHC II, but it is still not an absolute requirement (Hammerling and Moreno, 1990; Peterson and Miller, 1990). Therefore, II's role in antigen processing is partial, as a promoter of antigen processing: II may allow binding by peptides in the exocytic pathway, but with low affinity; α/β/II may not traffic to endocytic compartments not also entered by α/β, but II may lead to a longer residence in these sites. Natural II⁻ APC are now available for the first time, using II⁺ mice ("II knockout") rather than non-APCs transfected with α/β (D. Mathis, pers. comm.). Cell surface expression of α/β is low, but is induced by an undefined signal during in vitro culture. APCs present peptides well, and some whole antigens are presented better than by wild type APCs. Other antigens are presented less well. The in vivo effectiveness of an II⁺ immune system, as judged by the antibody response to antigen, is partially compromised, with reduced primary but unaffected secondary responses. Therefore, this important study agrees with the conclusions from previous work that II is a partial, not an absolute, requirement.
The associations of MHC II are not limited to invariant chain. MHC II interacts with a number of other molecules which do not show co-ordinated expression with MHC II, and may be constituents of most nucleated cells. Because of their omnipresence, these molecules have been difficult to identify, and none has been ascribed a definite function.

**Peptide binding protein (PBP72/74).** A membrane protein molecular weight 72-74 kDa has been isolated from APCs by its ability to specifically bind to a pigeon cytochrome c peptide, hence termed PBP72/74 (Lakey et al., 1987). In this system PBP72/74 was required for processing of whole antigen. On the basis of its membership of the family of HSP70 proteins (Vanbuskirk et al., 1989), it has been proposed that PBP72/74 is a chaperonin which holds peptide after antigen degradation in an extended conformation suited to later MHC binding (DeNagel and Pierce, 1992). In support of this, PBP72/74 is located in an endosomal compartment which is α/β/II^+ve (VanBuskirk et al., 1991).

**Phospholipids.** APCs treated with phospholipase have been demonstrated to lose the ability to present (Falo et al., 1986). Although the interpretation of these experiments is still unclear, the most likely explanation is that the lipid environment affects MHC conformation, which has been demonstrated in vitro (Dornmair et al., 1989; Luescher and Unanue, 1990; Roof et al., 1990).

**An important additional molecule remains undefined: chaperonin or peptide supply factor (PSF)?** Two separate mutant cell lines have been reported which present peptide, but are defective in antigen processing. These cells express α/β with an altered conformation, which is SDS-sensitive. Both cells have a deletion closely linked to the MHC II locus.

One mutant cell line produces empty α/β which binds and presents exogenous peptides with increased efficiency (Mellins et al., 1990; Mellins et al., 1991). It has been proposed that the lost function is a chaperonin which affects the conformation of MHC II, and might be involved with the conformational changes that follow II dissociation (Peterson and Miller, 1990).

The other mutant cell line has a similar phenotype (Riberdy and Cresswell, 1992). Many
hours after synthesis of α/β, Ii-derived peptides co-elute from more than 10% of the α/β. The peptides are all in a nested set up to 24 amino acids long, from residues 81-104 of the lumenal domain (Riberdy et al., 1992). Peptides from the same region of Ii have been eluted from three other MHC II molecules (Rudensky et al., 1991a; Chicz et al., 1992; Hunt et al., 1992b). All of the α/β/Ii81-104 was SDS-sensitive, even after low pH treatment in the absence of other peptides. In contrast, SDS-resistant, compact dimers were formed, and Ii81-104 was lost, after low pH treatment in the presence of other restricted peptides. Two explanations of these data are possible.

(1) Ii81-104 may be a common proteolytic product of Ii which binds to the peptide binding groove of many MHC II species. Because Ii degradation by necessity occurs with a high ambient concentration of MHC II, the peptide will tend to be well represented amongst those eluted from MHC II. In the mutant cell line a failure of the antigen processing machinery, for example reduced delivery of peptides to the processing compartment, might cause increased predominance of Ii81-104. This explanation also predicts that certain peptides do not allow the floppy dimers to convert to compact dimers, even in vitro. The location of this gene at the same genetic locus as PSF for MHC I and the similar phenotype to PSFnull mutants provide circumstantial evidence that the missing gene is a PSF for MHC II.

(2) Ii81-104 is the region which associates with MHC II in the ER, binding outside the antigen binding groove, and altering the binding groove's conformation to reduce affinity for epitopes. The mutation affects either the mechanism which dissociates Ii from α/β (a chaperonin function) or delivery of α/β/Ii to the site where dissociation can occur (a targeting function). Whichever of these functions is lost, the correct conformation for high affinity epitope binding cannot be adopted.

These models are testable by determining the binding site of MHC II for Ii81-104. The clarification of the mutation in these cell lines is anticipated to lead to the discovery of further molecule(s) important for antigen processing.

Recycling MHC II has a limited role in antigen processing. To fulfil its function, MHC II must encounter exogenous antigen which has been endocytosed. One way in which this
might be achieved is by uptake of antigen and MHC II together from the cell surface into the endocytic pathway. Since II is mainly intracellular, this endocytosed MHC would be α/β not α/β/Ii. Endocytosis of MHC II is seen in murine B cells, in human dendritic cells (Arkema et al., 1991), but not in macrophages (Harding and Unanue, 1989) or, according to most studies, in human transformed B cells (Guagliardi et al., 1990; Neefjes et al., 1990; Davis and Cresswell, 1990). Therefore, there are only some APCs which recycle MHC II. Sensitive techniques have revealed small, rapidly recycling internal pools in human B cell lines for both MHC I and MHC II (Reid and Watts, 1990).

It has been suggested that a normal sub-population of α/β in many APCs (identified as floppy dimers) is empty of peptide (Germain and Hendrix, 1991), and hence is likely to be responsible for peptide binding during recycling. However, it has been shown that MHC II which is sensitive to SDS is not necessarily empty (for example: Lanzavecchia et al., 1992). Therefore, the majority of, if not all, recycling MHC II can only bind peptide by peptide exchange. Evidence for such peptide exchange is based on the finding that the half-life of MHC II/peptide complexes in APCs is shorter than the half-life of the MHC II molecules themselves (Harding et al., 1989; Pedrazzini et al., 1991; Poirier, 1992). In addition, it has been shown that the loss of MHC II/peptide complexes can be enhanced by other peptides which bind to the same restriction element (Pedrazzini et al., 1991). These findings were obtained with APCs expressing I-A^d, which does not easily form compact dimers. Thus, it may be that some peptide exchange can occur in floppy dimers, although the experimental conditions which use high peptide concentrations are unlikely to be encountered in vivo.

Inhibitors of protein synthesis and protein export both inhibit MHC II processing (Harding and Unanue, 1989; Adorini et al., 1990; St.Pierre and Watts, 1990). Although this inhibition has not been proven to be mediated via altered MHC II traffic, it has been taken as evidence of the importance of newly synthesised MHC II in the endocytic pathway. Subsequently it has been shown that in the very same human B cell lines that have rapid MHC recycling (see above) α/β/peptide complexes contain newly synthesised, not recycling, MHC II (Davidson et al., 1991). Therefore, peptide binding to recycling MHC is limited to floppy dimers and certain APCs only.
D. UNRESOLVED ASPECTS AND FUTURE DIRECTIONS

(i) PHENOMENOLOGY OF ANTIGEN PROCESSING

The cellular experiments which led to the discovery of the active role of APCs identified several phenomena by which antigen processing appears to regulate the immune response. This phenomenology has laid the theoretical framework for many of the subsequent experiments addressing the underlying molecular and cell biological mechanisms of processing. In some areas theory still awaits experimental (dis)proof.

Determinant selection. The Ir gene effect has been hypothesised to be mediated by determinant selection, whereby MHC II selects peptides created during antigen processing for later presentation to T cells (Benacerraf, 1978; Rosenthal, 1978). This hypothesis is contrary to ideas that Ir gene effects are caused by holes in the T cell repertoire (Ishii et al., 1981; Clark and Shevach, 1982). The determinant selection hypothesis is supported by the findings that the peptides presented vary between different MHC molecules (Livingstone and Fathman, 1987), and that the polymorphism of MHC is mainly associated with the peptide binding groove (Bjorkman et al., 1987a and b; Brown et al., 1988). Thus, in some experiments most epitopes bind strongly to their restriction element, and only a small minority bind strongly to other MHC molecules (Buus et al., 1987).

A comparison of the peptides eluted from different MHC II molecules, shows that the major epitopes are different with the notable exception of I^{81-104}, for which a separate binding site on MHC II may exist (Riberdy et al., 1992). This is further evidence for determinant selection.

The importance of determinant selection has been challenged by other experiments which show degenerate binding of peptides to a large number of allotypes, and even to different isotypes for both MHC I and II (Sette et al., 1989b; Chen and Parham, 1989; Roche and Cresswell, 1990b; Chen et al., 1990; Choppin et al., 1990; Hickling et al., 1990). Explanations of this cross-reactivity include: (1) some of the peptides used contain more than one binding site (Panina-Bordignon et al., 1989); (2) immobilised peptides used in solid-phase assays show less specificity than gel filtration experiments; (3) subtle kinetic differences in MHC binding can be associated with considerable differences in
immunogenicity (Roche and Cresswell, 1990b).

An additional explanation for cross-reactive binding is that many epitopes do indeed bind to several MHC molecules, especially at the high concentrations used in experiments, but that the epitopes are not generated by cellular processing mechanisms. This indicates that determinant selection may interact with degradation in a complex manner leading to MHC-directed processing, which is discussed below.

Intermolecular competition. The series of steps by which an antigen is processed and presented by an APC to a T cell provides multiple opportunities for competition by other antigens. This was first demonstrated by the inhibition of an antigen-specific T cell response by a closely related antigen, the competition occurring at the level of the APC (Werdelin, 1982). Many mixtures of antigens restricted to the same MHC molecule show this competitive inhibition (Werdelin, 1986). It has been shown that almost any mix of proteins, such as that found in serum, can compete with exogenous antigen (Lorenz et al., 1990). Competition has also been shown between peptides binding to MHC II \textit{in vitro} (Buus et al., 1987; Lamont et al., 1990a) and \textit{in vivo} (Acorini et al., 1988; Lamont et al., 1990 a and b).

Self proteins provide a large number of competitors, both from outside the cell, for example from serum (Lorenz and Allen, 1988; Lorenz et al., 1990; Rosloniec et al., 1990), and from proteins endogenous to the APC (Hammerling and Moreno, 1990; Rudensky et al., 1991 a and b). It has been shown that in the absence of serum the level of competition is reduced to less than 15% (Harding and Unanue, 1990a), which indicates the dominance of exogenous proteins. The overall competition is at such a level as to leave 5-10% of MHC II empty for peptide binding in many APCs (Babbitt et al., 1985; Watts and McConnell, 1986; Sadegh-Nasseri and McConnell, 1989; Demotz et al., 1989; Stern and Wiley, 1992). The existence of this empty pool of MHC II has important therapeutic implications for peptide vaccines. However, experimental observation of 5-6% empty MHC II (Babbitt et al., 1985) did not agree with model calculations based on studies of the same cells that 2% were empty (Harding and Unanue, 1990a). The inaccuracy of the model implies that there are still some unknown variables. For example, low affinity peptides might dissociate during MHC purification thus generating
apparently empty molecules.

Receptor-mediated uptake of antigen reduces the competitive effect of non-specific protein unless the uptake of non-specific protein is also enhanced by receptor mediated uptake (Lorenz et al., 1990). Therefore competition depends on the efficiency of trafficking in the antigen processing pathway.

There is no evidence for direct competition for intracellular transport mechanisms or for competition for degradative enzymes, although such evidence might be hard to obtain given that antigen processing is only a minor by-product of normal intracellular processes. Inhibition of antigen processing in ways other than competition, termed interference, has been detected in some cases (Leyva-Cobian and Unanue, 1988).

For competition between peptides to occur as described above, the competitor peptide or protein has to be present at high concentrations (≈1000 times) compared to the specific peptide. This is explained by the mechanism of the competition, which has been termed blockade (Gautam et al., 1992). Because an epitope can activate specific T cells after binding only 0.01-0.1% of MHC II molecules, the available pool of empty MHC II (≈5-10%) must be occupied almost entirely by the competitive inhibitor.

Recent experiments indicate a different form of competition which is ≈100 times more efficient than blockade of MHC II binding. This is only found with peptides which are very similar to the epitope itself, for example containing a single substitution (De Magistris et al., 1992). The substituted peptide binds to MHC, is non-immunogenic for specific T cells, and inhibits presentation at only 3-10 fold excess. The inhibition is specific for that epitope, and is not mediated by a negative or anergic signal. This more efficient form of competition has abundant therapeutic possibilities because of its high efficiency and specificity (Sakai et al., 1989; Wraith et al., 1989).

Epitope hierarchy. Antigens in vivo tend to induce a T cell response to one or two major, or dominant, epitopes (MHC II: many examples including Gammon et al., 1987; Berzofsky et al., 1988; MHC I: Berzofsky, 1991, L. Gao, H. Stauss, B. Chain, manuscript in preparation). A larger number of minor, or non-dominant, epitopes are responsible
for a small proportion of the response. In addition, there are cryptic epitopes which elicit no response in the context of the whole antigen, but which are immunogenic in their own right, i.e. a peptide containing that epitope can elicit a response to itself. The existence of epitopes with different strength of immune response within the same antigen is termed an epitope hierarchy. In general, cryptic epitopes do not have a lower affinity for MHC, and the T cell responses to these peptides are strong. The responses to peptides similarly do not distinguish between major and minor epitopes. Therefore, epitope hierarchy is largely unexplained by either determinant selection or holes in the repertoire.

Although the T cell repertoire does not bring about hierarchy, manipulations of the T cell repertoire have been used to indicate the mechanisms underlying hierarchy. The difference between primary and secondary T cell responses, the latter being more sensitive, has been utilised to show that after inducing a primary response to a minor epitope, the epitope can become major (Shivakumar et al., 1989). These erstwhile minor epitopes were therefore minor by virtue of a relative failure to be processed, and hence a lower level of the epitope on APCs, which is sufficient for secondary but not primary responses. In contrast, absolute failure to be processed cannot be circumvented by peptide vaccination (Milich et al., 1987).

As a corollary of this effect, it has been shown that tolerance induction is dependent on immunological hierarchy: only the major epitopes are processed at sufficient levels to induce tolerance, cryptic epitopes of self antigens not being tolerised (Gammon and Sercarz, 1989). T cells which recognise cryptic self determinants might be the source of auto-immune responses (Gammon et al., 1991), especially as chronic immune responses enhance antigen presentation (Lehmann et al., 1992).

It is thought that much of immunological hierarchy is brought about by differential processing of epitopes (Gammon et al., 1987; Berzofsky et al., 1988). During the course of antigen degradation, some epitopes may be exposed early and remain until late, others may be exposed only briefly before they are destroyed by further (anti)-processing. The differences in ease of exposure of an antigen may be caused by the secondary structure of degradation intermediates which affect accessibility for MHC binding. The immunogenicity of some epitopes is dependent on a critical residue only when whole
antigen is used as immunogen, but not when the epitope is in the form of a short peptide (Brett et al., 1988; Vacchio et al., 1989). In such cases, an interaction between amino acid residues in the epitope and residues outside the epitope, termed hindering structures, is implicated. In other examples, the residues critical for immunogenicity are all distant from the epitope in terms of primary structure, although they are presumably spatially close in the secondary structure (Kovac and Schwartz, 1985). Secondary structures have been shown to be relative, not absolute, inhibitors of processing (Shastri et al., 1986).

It has also been noted that epitope hierarchy varies between genetically identical individuals. This has been attributed both to differing T cell memory to cross-reactive epitopes encountered during infections, and to the in vitro outgrowth at random of a small number of clones which have successfully competed for limiting amounts of IL-2 (Gammon et al., 1990).

**Epitope hierarchy and MHC-directed processing for MHC II.** The generation of epitope hierarchy is simply explained in terms of the epitope which is most easily exposed by processing has the highest effective concentration and is therefore dominant. However, as discussed below, the simple concept that ease of processing determines hierarchy does not account for all the observations.

A major way in which hierarchy might also be created is MHC-directed processing: MHC II may be present in the degradative compartment and may alter the way in which epitopes are exposed. Thus, hierarchy is a result of the complex interactions between all three of antigen, proteinase and MHC II. The sequence in which epitopes are exposed is likely to be important, rather than selection of epitopes by direct competition. This concept has the virtue of parsimony: an already well-documented reaction (MHC + epitope) might explain hitherto unclear results.

MHC II molecules have been found to present peptides derived from themselves and other MHC II molecules (Murphy et al., 1989; Rosloniec et al., 1990). These peptides are among the most predominant epitopes eluted from many different MHC II species, and the phenomenon is seen in all major APC types (Murphy et al., 1992). This suggests that MHC II is present in a compartment where degrading enzymes act. This is not surprising
in the light of the requirement for Ii degradation to achieve dissociation from MHC II. However, it also indicates that antigen degradation occurs in the presence of MHC II. This is circumstantial evidence for MHC-directed processing, as it is unlikely that any other mechanism would provide such high levels of epitopes for newly synthesised α/β/Ii to bind.

MHC-directed processing explains the phenomenon of "intramolecular competition", in which two epitopes restricted by different MHC molecules are both processed and presented well by APCs expressing only their own restriction element, but only one epitope is presented by APCs expressing both MHC molecules (Berkower et al., 1985; Gammon et al., 1987). Although described as "intramolecular competition", there is only scant evidence for competition between two MHC molecules for a single antigen intermediate on which both epitopes are exposed (Wang et al., 1992). Rather, the sequence in which the epitopes are exposed might lead to MHC-directed processing. A degradative intermediate is likely to contain multiple epitopes of which only one is exposed. This first epitope can bind to MHC II, and become dominant. After the dominant epitope binds to MHC it is protected from further degradation (Werdelin, 1986; Unanue and Allen, 1987; Donermeyer and Allen, 1989; Mouritsen et al., 1992).

The possible mechanisms for the loss of other epitopes after MHC binds to the dominant epitope are: (1) alteration of traffic of the degradative intermediate by MHC binding so that the other epitopes are not exposed, or are exposed only when MHC binding is not possible; (2) degradation of other epitopes in the intermediate is accelerated by MHC binding, perhaps caused by conformational changes in the antigen. Experiments to investigate these mechanisms have not been done.
THE SITE(S) OF ANTIGEN PROCESSING FOR MHC II

Much of the data regarding the biochemistry and cell biology of antigen processing for MHC II has been discussed above. In this section, the different parts of the pathway taken by exogenous antigen are assessed for their major roles in processing, for which the basic requirements are taken to be: (1) the exposure of an epitope within the context of a degradative intermediate, (2) the presence of empty α/β dimers after Ii dissociation and (3) an environment in which epitope can bind to α/β.

The role of the plasma membrane is for antigen targeting. Targeting of antigens to plasma membrane molecules enhances presentation variably, depending on the target molecule (Lanzavecchia, 1985 and 1990; Pierce et al., 1988). All isotypes of mIg (IgM, D, G and E) are uniformly good targets. Target molecules which are not endocytosed do not enhance presentation. Some receptors enhance presentation for some antigens by some APCs, but do not act uniformly; these include transferrin receptor (see below) and MHC I. The variability for MHC I might relate to it being endocytosed in some but not all cell types (Vega and Strominger, 1989).

Some antigens associate with MHC II in early endosomes. The presence of α/β/Ii in early endosomes is controversial. α/β/Ii has been localised in early endosomes labelled by mIg internalised for 2 minutes by immuno-E.M. (Guagliardi et al., 1990). However, the same protocol repeated on the same cell type did not lead to these results (Neefjes and Ploegh, 1992b). In addition, the definition of the MHC II** compartment as early endosomes may have been inadequate (see section B.(iii)). Therefore, the presence of both α/β/Ii and proteinases in early endosomes is not proven.

Even in the absence of α/β/Ii, the existence of a small but substantial pool of empty α/β dimers in most APCs (Germain, 1991) which recycle to some extent (Harding et al., 1989; Reid and Watts, 1990) might provide the opportunity for some peptide loading in early endosomes. The finding that, whereas mIg-targeted antigen requires newly synthesised MHC, pinocytosed antigen can be presented by old MHC (Kakiuchi et al., 1990), has been taken to suggest, without supporting evidence, that processing occurs in early endosomes (Brodsky, 1992). However, this appears to be unlikely, given the kinetics of antigen processing.
Despite the lack of direct evidence for the involvement of early endosomes, there appears to be a role nevertheless. Firstly, TfR, a marker of early endosomes is an efficient target for enhancing processing of some antigens (Lanzavecchia, 1990), but not others (Pierce et al., 1988). The variability might indicate that for some, but not all antigens delivery to early endosomes is sufficient to bring about processing. In these cases the site of peptide binding to MHC might include early endosomes, but due to kinetic considerations (see below) is likely to involve subsequent traffic to other compartments (Brodsky, 1992).

The strongest evidence of a role for early endosomes are the major epitopes eluted from MHC II which are derived from molecules not normally found outside plasma membrane and early endosomes. TfR-derived epitopes have been found in all three published reports, and an Na⁺,K⁺-ATPase-derived epitope has been found in one of the three (Rudensky et al., 1991a; Hunt et al., 1992b; Chicz et al., 1992). These epitopes each represent 5-10% of total MHC II-associated peptides, therefore they involve a reasonably large proportion of the receptor molecules. There are two possible explanations: (1) these receptors are sufficiently numerous and contain strong epitopes by chance; during degradation of receptors at the end of their life these epitopes bind MHC in late parts of the endocytic pathway. (2) these receptors interact with MHC II in early endosomes in such a way as to enhance their processing. This explanation has a more rational basis: an epitope on TfR or Na⁺,K⁺-ATPase might be easily exposed by processing in early endosomes, and bind to α/β/β2m with low affinity in early endosomes. The mechanism for subsequent processing might include traffic to late endosomal compartments (Brodsky, 1992). This explanation leads to predictions regarding (1) the conditions under which epitopes in TfR and Na⁺,K⁺-ATPase are exposed and (2) altered traffic of TfR in the presence of MHC II, both of which are testable.

The kinetics of processing indicate traffic beyond early endosomes. It is well established that there is a time lag between antigen binding to an APC and the appearance of processed T cell epitopes (Harding and Unanue, 1989; Lanzavecchia, 1987 and 1990). The most convincing studies have used B cells specific for tetanus toxoid as APCs. The earliest appearance of processed epitopes is detected after 45-60 minutes by calcium fluxes in tetanus toxoid-specific T cells (Roosnek et al., 1988). Intermediate antigen
concentrations lead to a small T cell response after 60 minutes, which rises to a maximal response by 3-4 hours. Concentrations of antigen 100-fold higher have the same lag period, but a more rapid rise in strength of response thereafter, maximal levels being reached at two hours. The results are similar for antigen delivery via targeting to TIR, with a lag of 45 minutes. These findings contrast with peptide presentation, which is immediate (Roosnek et al., 1988), and with whole antigen presentation by B cells after fluid phase uptake, which has a lag of 8 hours (Eisenbörhr et al., 1988; Gosselin et al., 1988; Lakey et al., 1988). The 45-60 minute time lag is not related to the concentration of one of the reactants, and is thus unlikely to be caused by a biochemical reaction. The rapidity of rise after the lag is related to antigen concentration, and therefore verifies the assumption that more antigen leads to more complexes. The lag of 45-60 minutes implies that processing requires a cell biological event of this duration. This is a strong argument against early endosomes being the site of antigen processing.

Is there a specialised processing compartment? It has been proposed that the majority of antigens are processed in a single compartment or specialised sub-compartment. In B cell APCs, organelles have been identified which contain high levels of MHC II, with low levels of MHC II outside these areas (Neefjes et al., 1990; Peters et al., 1991). The properties of these MHC II-rich vesicles include proximity to the TGN (Neefjes et al., 1990), and expression of late endosomal and lysosomal markers in a combination not exactly like either late endosome or lysosome (Peters et al., 1991). In macrophages, two different MHC II-rich compartments have been detected (Harding et al., 1990). One of these compartments has characteristics of late endosomes and lysosomes, the other consists of MHC II-rich peripheral sacs, not seen in other APC types. The peripheral compartment appears to be internalised ruffles of plasma membrane. The faster access to such a compartment might reflect the ability of macrophages to process antigen more rapidly than B cells, with a lag of only 15-30 minutes (Harding and Unanue, 1989). In dendritic cells, MHC II was found throughout the endocytic pathway, and in non-endocytic vesicles proposed to have a storage capacity for newly synthesised MHC II (Arkema et al., 1991).

Since most cell types which express MHC II (experimentally, after co-transfection of α, β and Ii) are capable of processing, the genesis of a unique organelle for antigen
processing in B cells must derive from the expression of α, β, Ii. An example of such a mechanism would be if Ii affected the local environment at a single point in the endocytic pathway, generating unique properties. Entry into processing compartments is enhanced by certain receptors, especially mIg, and is therefore heterogeneous between APC types. Entry into the compartment might be particularly difficult for some antigens, especially after uptake in the fluid-phase. For dendritic cells, which express low levels of opsonin receptors, non-specific membrane adsorption and even fluid-phase uptake might be important (Janeway, 1992b).

**Processing of some epitopes requires entry into lysosomes.** Some antigens have been shown to traffic via lysosomes prior to presentation. Antigen encapsulated in liposomes which are stable at acidic pH (≥4.5) and release antigen only in lysosomes are efficient at targeting antigen for processing in comparison to liposomes which release antigen in early endosomes (Harding et al., 1991a). In addition, the enzymes which reduce intrachain disulphide bonds, a necessary step in the production of some T cell epitopes, are found only in lysosomes (Collins et al., 1991). Since little or no MHC II is detected in lysosomes (Harding et al., 1990; Neefjes et al., 1990; Peters et al., 1991), partially processed antigen might have to be transported in a retrograde fashion from lysosomes to late endosomes. Alternatively, in the light of the relative resistance of α/β to proteolysis, it may be that MHC traffics rapidly through lysosomes, where it can rescue epitopes. Since all processing events, including peptide binding, are more rapid in lysosomes, this might be the only site where some epitopes are generated. However, lysosomes are a high risk environment where a high proportion of both antigen and MHC would be destroyed.

**Summary: processing occurs throughout the endocytic pathway.** The overall view proposed here is that all parts of the endocytic pathway contain both MHC II and antigen, and can therefore be the sites of antigen processing. Different epitopes might be processed in different sites. One major determining factor is the means of endocytosis, which is particularly important for mIg targeting in B cells and phagocytosis in macrophages. It is likely that targeted antigen delivery increases efficiency of processing by leading to higher concentrations of antigen in processing compartments. Apart from the expression of different antigen receptors, there are other unknown differences
between APCs which cause heterogeneity in processing, for example Y-Ae expression in the thymus (Murphy et al., 1992).

The other major factor determining the site of processing is the varying requirement for degradation. Antigens and MHC II are both delivered into early endosomes, where proteolytic activity is low. Epitopes which require minimal processing might be exposed rapidly, but at this stage the only empty α/β are recycling, floppy α/β dimers, the amount of which varies between MHC species. In the absence of high levels of floppy dimers, antigen intermediates with an epitope exposed might bind α/β/Ii with low affinity, and thereafter traffic with α/β/Ii rather than recycle immediately to the cell surface. At an intermediate point in the endocytic pathway, the degradative activity will be balanced so as to degrade Ii rapidly, but not to affect α/β itself. At this point there will be a high concentration of empty α/β to bind epitopes which have already bound with low affinity in early endosomes, and sufficient degradative activity to generate a large variety of additional epitopes. Such a site, which may vary between APCs, is likely to be where most MHC binding occurs. Epitopes which require the most processing will be exposed late in the pathway, possibly in lysosomes. In these compartments, the concentration of empty α/β is likely to be low, and the likelihood of degradation increases for both the epitope and α/β.

At both the beginning and at the end of the pathway the requirement for partial degradation cannot be balanced with the conflicting needs for degradation of Ii and preservation of both α/β and the epitope. In the middle of the pathway, a reasonable balance between these needs might be achieved. It is intriguing to hypothesise that one of the functions of MHC II is to create the environment in which its other functions can best be performed. This function may be like that of resident Golgi enzymes, which appear to be an important factor in preserving the unique environment of each Golgi cisterna (G. Warren, pers. comm.).
D. (iii) EXPERIMENTAL APPROACHES TO ANTIGEN PROCESSING

Functional experiments are highly sensitive. Experimental approaches for locating the sites of antigen processing include those which use subsequent presentation to T cells as the read-out. Since as few as 50-300 out of $>10^5$ MHC II molecules are recognised by T cells, such functional experiments are much more sensitive than any other current technique.

Some functional experiments use biochemical inhibitors or genetic variations to investigate a particular aspect of processing. However, it is difficult to selectively alter just one cell function. For example, chloroquine inhibits antigen degradation (Ziegler and Unanue, 1982) by removing intracellular pH gradients. Low pH may be required for antigen degradation, invariant chain dissociation and compact dimer formation. However, chloroquine also prevents re-expression of recycling molecules (Strous et al., 1985), an effect which could override the other pH-dependent functions. An even more striking example is leupeptin, which has been presumed to suppress Ii degradation by its inhibition of cathepsin B (Blum and Cresswell, 1988; Pieters et al., 1991; Neefjes and Ploegh, 1992a). However, not only does leupeptin have a broad spectrum of proteinase inhibition, but it also slows the delivery of all early endosomal contents to late endosomes (Tolleshaug and Berg, 1981; Griffiths, 1992), a highly relevant step for antigen processing.

Other problems with functional experiments include the possibility of redundancy in several aspects of antigen processing. Therefore, the importance of a biochemical event might be masked by the use of pathways which are otherwise minor. In addition, T cell responses plateau above a certain number of complexes per APC ($\approx1000$). Responses must therefore be titrated carefully (Lanzavecchia, 1985; Pedrazzini et al., 1991).

Antibodies which might reveal functional aspects of processing are powerful tools for future experiments. Antibodies identify 3-dimensional structure, but not function. However, there are well-defined molecular changes that are associated with functional aspects of antigen processing. Antibodies which discriminate between conformations of MHC II have not been used for intracellular studies. Such antibodies might be of little value in E.M. due to loss of conformational epitopes during processing. The intracellular
Degradation of invariant chain has been studied by detecting the ratio of its ectoplasmic to cytoplasmic domain using confocal microscopy (Romagnoli et al., 1992). Antibodies to proteinases that distinguish active enzyme from the proenzyme have not yet been used. Processing intermediates of the antigen itself would also be useful subjects of study. In many cases the intermediates are unknown. There are at present two reported cases of antibodies to defined peptide epitopes bound to specific MHC alleles (Rudensky et al., 1991b; Reay et al., 1992). These antibodies are powerful tools in cell biological studies to determine the site of complex formation (Murphy et al., 1992).

Histological experiments. E.M. has been used to visualise intracellular MHC II by immuno-labelling. MHC II-positive parts of the endocytic pathway have been identified either by immuno-gold labelling of molecules in the endocytic pathway (TfR, MPR, LAMP-1) or by endocytic tracers (ferritin, BSA-gold, HRP). Other molecules which might take part in antigen processing, such as proteinases, have been co-localised with MHC II. In addition, multi-colour, confocal fluorescent microscopy has been used to study endocytic traffic, including traffic of MHC II (Salamero et al., 1990a; Lotteau et al., 1990). This technique is technically less demanding than E.M., but it yields lower resolution.

Histological methods provide a static view of dynamic cell functions but do not detect the rate of flux. Therefore, fluxes such as rapid recycling (for example: internalisation of MHC II in human B cells) may not be detected unless cell function is perturbed to block one part of the cycle. In addition, antigen processing may not occur in a simple sequence along a linear pathway. Events such as the exposure and binding of epitopes may occur in many parts of the overall pathway.

Biochemical experiments. Isolated molecules, any pair of antigen, MHC II and proteinase, have been studied in vitro, crudely recreating antigen processing. It still remains for MHC-directed processing to be tested by mixing all three molecules together. However, these experiments cannot recreate the cellular environment, in particular: the lipid bilayer and interactions with other, unknown proteins.

Biochemical observation of antigen processing by whole cells is usually made difficult.
by the vast excess of antigen and MHC II compared to an individual α/β/peptide complex. Labelling of along the entire length of an antigen, for example by iodination or methylation (Davidson et al., 1991; Marsh et al., 1992), allows detection of epitopes in association with MHC II. Detection of unlabelled antigen eluted from MHC by tandem mass spectroscopy is less sensitive than functional experiments, although future mass spectroscopic techniques might improve sensitivity up to 100-fold (D. Hunt, pers. comm.). Processing can also be followed by the conformational changes in the MHC II molecule itself.

**Cell-free systems.** Cell-free systems reconstitute cellular functions using fractionated cells which allow manipulation of the system to an extent not possible with intact cells. Cells can be altered minimally, to yield perforated or semi-intact cells, or disrupted to produce post-nuclear supernatant (PNS) or cytosol-free membranes which can then be fractionated. Fractionation of membranes can be according to different physical properties or by immuno-identification with antibodies. The cell-free approach has not been widely applied to antigen processing. However, a cell-free model system for antigen processing would be useful in two types of experiments: (1) to identify the processing compartments, and (2) to determine the biochemistry of processing in the cellular environment (Shaw and Chain, 1989).

(1) **Identification of processing compartments.** Processing vesicles should be endocytic, MHC II⁺ and proteinase⁺. The classical separation technique is density gradient centrifugation (de Duve, 1975). However, endosomes tend to share the same density as other compartments such as Golgi, ER and plasma membrane. Therefore, although useful for initial separation, centrifugation does not produce pure endosomes. Separation of processing vesicles from other vesicles therefore requires a technique that recognises individual molecules or functional properties using specific antibodies. Examples of such techniques include flow cytometry (Murphy, 1985), and immuno-isolation (Richardson and Luzio, 1988; Howell et al., 1989a). An advantage of immunological techniques is that they are successful with cells grown long-term in culture, which are more difficult to homogenise and fractionate by density than *ex vivo* cells, such as rat liver - the classical tissue for density gradient centrifugation studies. Vesicles identified as endocytic, MHC II⁺ and proteinase⁺ could be used to determine which molecules co-localise
(2) **Biochemistry of processing in a cellular environment.** Processing vesicles could be used to perform antigen processing in a cell-free environment that is very similar to that of the intact cell. For example, the correct lipid environment would be preserved, rather than using molecules in solution or in micelles. In a cell-free system, the events of antigen processing could be detected by biochemical changes to MHC II and antigen. It would be difficult to measure processing by subsequent presentation to T cells, because the ectoplasmic domains of MHC II point into the lumen of endocytic vesicles.

The benefit of cell-free processing is that the conditions can be controlled. For example, in the study of epitope hierarchy, there is currently no direct evidence for the presence of MHC in the same compartment as the enzymes which expose epitopes. To investigate to what extent all the functions for processing are present in a single vesicle, it would be important to prevent communication between vesicles, which would otherwise reconstitute normal intracellular traffic (Gruenberg and Howell, 1989). In a cell-free system fusion between vesicles can easily be prevented, for example by removal of cytosol or by immuno-isolation onto a solid support.
D. (iv) THE AIMS OF THIS PROJECT

In this project, two different areas of study were pursued. Firstly, endocytosis of exogenous antigens was studied (Chapter 3). It is a paradox that dendritic cells, which are the most potent APCs, have been reported to have a relatively inactive endocytic pathway. The assays used previously on dendritic cells did not produce quantitative analyses, and did not study the kinetics of endocytosis. Therefore, an assay system was developed to compare endocytic traffic through early and late endosomes in different APC types. The separation of early and late endocytic traffic was achieved by kinetic analysis of exocytosis.

The development of a cell-free system to study the cell biology of APCs was the ultimate goal of the second part of the project. Cell-free systems are powerful tools for dissection of the biochemical events of complicated intracellular processes, and have not been applied to APCs. The study of the interaction of antigen degradation and epitope binding to MHC II would be facilitated by the purification, if only partial, of vesicles in which antigen processing occurs. After confirming the suitability of a B cell APC for such studies, by examining its intracellular endocytic and MHC II pathways (Chapter 4), a technique was developed to isolate MHC II^{+ve} vesicles which included those in which antigen processing is thought to occur (Chapter 5), and which might be capable of reproducing in a cell-free system some of the steps of antigen processing which occur within APCs (Chapter 6).
CHAPTER 2. MATERIALS AND METHODS

Chemicals, animals and cells. All chemicals were obtained from Sigma, UK, unless otherwise stated. Peptides were synthesised at the Imperial Cancer Research Fund, London. Mice and rabbits were obtained from the Imperial Cancer Research Fund colony, Clare Hall, Potters Bar.

A20-2J cells were a kind gift of P. Marrack, Denver, USA. AK34 cells were a kind gift of A. Venkitaraman, Cambridge, UK. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) (made up with half of the designated bicarbonate) supplemented with 5% heat-inactivated fetal calf serum (FCS, Gibco), and 50μM 2-mercapto-ethanol (complete medium) in a humidified incubator containing 5% CO₂ at 37°C. A20 cells were split after reaching confluence (approximately 10⁶ cells/ml). When subconfluent A20 were moderately adherent to tissue culture plastic, and were dislodged by physical stress.

CHAPTER 3. Flow cytometric assay for endocytosis of lucifer yellow (LY) by A20 cells. 5 x 10⁶ A20 cells in suspension were incubated in DMEM, buffered with HEPES and supplemented with 5% FCS and 50μM 2-mercapto-ethanol (DMEM/FCS) for 30-90 minutes. The cells were divided into five groups, and pre-warmed lucifer yellow 10mg/ml (dipotassium salt, LY) was added to four of the groups to a final concentration of 3.3mg/ml for 2, 6, 20 and 60 minutes at 37°C. Excess cold DMEM/HEPES at 4°C was added to all five groups to stop endocytosis. At this point, pre-cooled LY was added to the fifth group (0 minutes). All subsequent steps were performed at 4°C. Cells were centrifuged at 300g, and the pellet resuspended in DMEM/HEPES. This wash step was repeated three times. Cells were finally resuspended in 1ml DMEM/HEPES/FCS. 30μl samples were submitted to flow cytometry in pre-cooled tubes surrounded by ice-cold water on a FACScan flow cytometer (Becton Dickinson, UK). Acquisition of ≥5000 events at up to 400 events per second was complete in less than 15 seconds.

After acquisition, a dot-plot gate was placed on the size (forward scatter) and granularity (side scatter) axes to exclude clumped and dead cells. LY was detected in the green channel (fluorescein optics, excitation 488nm, emission 520-560nm). The median green fluorescence of the live cells was given on a log₁₀ scale, and converted to a linear scale.
The median value was chosen in preference to the mean because it was less affected by small populations of highly bright dead or dying cells.

**Flow cytometric assay for exocytosis of LY by A20 cells.** The chase period was commenced by submerging the tubes containing cells in water kept at 37°C, with frequent inversion to redistribute heat. The chase was commenced for each group at 30 second intervals. 30μl samples from the tubes were taken as described above starting one minute after the temperature was raised to 37°C, and samples from all five groups were assayed within 2½ minutes. In this fashion, all groups were initially assayed every 3 minutes from 1 to 25 minutes of the chase. Subsequent assays from 30 to 120 minutes chase were performed at increasingly prolonged intervals, upto approximately 10 minutes. Cells remaining after 120 minutes were checked for viability by trypan blue exclusion.

**Flow cytometric assay with rhodamine dextran (RD).** The flow cytometric assay as described above was adapted for RD by using non-fixable RD (70kDa). Impurities were removed from RD by two passes through Sephadex G50 (Pharmacia), followed by extensive dialysis and lyophilisation. RD was used in place of LY at a final concentration of 5-10 mg/ml.

5 x 10^6 A20 cells, incubated in 1ml DMEM/HEPES/FCS for 75 minutes, were stained for 15 minutes with 1,1’-dihexadecyloxacarbocyanine perchlorate (Di.O, stock 2.5mg/ml in ethanol; Molecular Probes, Oregon, USA) final concentration 13μg/ml. These cells were washed at 4°C as above, divided and added to each of the five RD-pulsed groups. RD and Di.O were detected in the orange channel (phycoerythrin optics, excitation 488nm, emission 560-600nm) and green channel respectively.

Because the RD and Di.O axes were not completely orthogonal, the absolute values of RD were marginally affected by the presence of Di.O. Thus, for cells pulsed for 0 minutes, the value [RD⁺₀ - RD⁻₀] was a non-zero constant, where RD⁺ and RD⁻ are the linearised median RD fluorescence in flow cytometer units after t minutes pulse for RD⁺ve and RD⁻ve cells respectively. Therefore, for RD⁺ve cells pulsed for 2 to 60 minutes, cell-associated RD was calculated from:

\[
[RD⁺_{2, 6, 20 or 60} - RD⁺₀] - [RD⁻_{2, 6, 20 or 60} - RD⁻₀].
\]
Use of Di.I^{*re} controls to calculate background for LY. Assay for LY endocytosis and exocytosis was as above, with the addition of a further $5 \times 10^6$ A20 cells, stained for 15 minutes of 1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate (Di.I, stock 10mg/ml in ethanol; Molecular Probes, Oregon, USA) final concentration 50µg/ml. These cells were added to each of the five LY-pulsed groups. Di.I was detected in the orange channel. Cell-associated LY was calculated, as for RD, from:

$$[LY^+_{2, 6, 20 \text{ or } 60} - LY^+_0] - [LY^-_{2, 6, 20 \text{ or } 60} - LY^-_0]$$

Conversion of flow cytometer units to absolute volume. Flow cytometer fluorescence units were converted to the volume of LY per cell in µm$^3$. A20 cells were pulsed with 1.7 or 3.3mg/ml LY for 60 minutes and washed as above. Aliquots of cells were taken for cell counting and flow cytometry. Flow cytometry produced a value on an arbitrary, linear scale. This value was linearly proportional to the concentration of LY in the labelling solution.

The remaining cells were lysed in 1% NP40 at 4°C for 60 minutes. Nuclei were removed by centrifugation for 5 minutes at 850g. Post-nuclear supernatant (PNS) was assayed fluorimetrically for LY (excitation 383-393nm, emission 522-532nm) on a luminescence spectrometer (LS50, Perkin Elmer, UK). The content of the labelling solution per A20 cell was calculated by comparison of PNS with known concentrations of LY. Assuming that LY was a true marker for fluid, and was neither concentrated nor diluted by any cellular mechanisms, this value was the volume of endosomes accessed by LY during the 60 minute pulse. This value was independent of the concentration of LY in the labelling solution.

Calibration of flow cytometry units was achieved by calculating the flow cytometry units per mg/ml of LY in the labelling solution. This value was then divided into the absolute volume of endosomes accessed by LY to produce a conversion factor from flow cytometer units into absolute volume. This experiment was repeated on 4 separate days with the result:

$$\text{conversion factor} = 0.29 \ (n=4, \ s.d. = 0.03)$$

where volume = conversion factor x flow cytometer units / [LY]

(units: µm$^3$) (arbitrary scale) (mg/ml)
Comparison of the uptake of four fluid-phase markers. 3-5 x 10^7 A20 cells in suspension were incubated in DMEM/HEPES/FCS for 30-90 minutes. The cells were divided into 5 groups, and a pre-warmed mixture of four endocytic markers added for 2, 6, 20 and 60 minutes (pre-cooled for 0 minutes group, as above) to give a final concentration of LY 3.3mg/ml, RD 3.3mg/ml, [^14]C-sucrose 5μCi/ml (Amersham) and [^3]H-dextran (70 kDa) 1μCi/ml. Endocytosis was stopped and cells were washed as above. Aliquots were removed to check viability and count cells, and for flow cytometry. Remaining cells were lysed and PNS was assayed for each of the four markers: LY and RD by fluorimetry (RD: excitation 555-562.5nm, emission 570-580nm), [^14]C-sucrose and [^3]H-dextran by dual channel scintillation counting (LKB-1214, Wallac, UK).

Endocytosis by A20 cells across a range of LY concentrations. 1 x 10^6 A20 cells were divided into eight aliquots and incubated with LY at doubling concentrations from 52μg/ml to 6.7mg/ml at 37°C for 60 minutes. Cells were washed and assayed for LY content by flow cytometry.

[^14]C]-sucrose exocytosis after 6 and 60 minutes endocytosis. 2.5 x 10^8 A20 cells were incubated in DMEM/HEPES/FCS for 30-90 minutes. The cells were divided into 3 groups, and pre-warmed [^14]C-sucrose added for 6 or 60 minutes to a final concentration of 20μCi/ml. Endocytosis was stopped and cells were washed as above. Each group of cells was divided into ten aliquots, which were chased at 37°C for 0-120 minutes before washing, lysing and assaying for cell-associated ^14C. In addition, ^14C in the chase supernatant was assayed, confirming that total ^14C remained constant.

Sequential pulses of LY and RD. 6 x 10^6 A20 cells were incubated in DMEM/HEPES/FCS for 60 minutes, washed and divided into 6 groups. Two groups of cells were then incubated for 20 minutes at 37°C with LY (final concentration 5mg/ml), two groups with RD (10mg/ml), one group with both LY and RD and a negative control group with neither. Endocytosis was stopped, cells were washed and an aliquot was assayed by flow cytometry. LY and RD axes were made orthogonal. A further 20 minute incubation was performed. For one of the groups incubated with LY, RD (10mg/ml) was included in this incubation. Similarly LY (5mg/ml) was added to one of the groups incubated initially with RD. Otherwise the first label(s) was allowed to chase out. Cells
were washed and a further aliquot was assayed by flow cytometry. This was followed by a further 20 minute chase and then a 150 minute chase, with assays at the end of both periods.

**Adaptation of the flow cytometric assay for dendritic cells.** Dendritic cells were purified from CBA spleen cells, according to an adaptation of the method of (Ellis et al., 1991). A suspension of washed spleen cells was centrifuged upwards through a multi-step iso-osmolar percoll gradient. Percoll (Pharmacia) was made iso-osmolar by mixing 10 parts to 1 part of 10 x Hanks' balanced salt solution, and buffered with 20mM HEPES (pH 7.4). This mixture was termed 100% percoll. Dilutions to produce percoll at less than 100% were made in 1 x Hanks balanced salt solution, 20mM HEPES (pH 7.4). A pellet of spleen cells was resuspended in 70% percoll, 5 spleens in 1ml for one gradient, eight gradients in total. Over this, layers were poured of 60%, 50% and 30% percoll, 1ml, 1.5ml and 1.5ml respectively. The gradient was spun at 850g for 15 minutes, with slow deceleration. Low density leukocytes (50%-30% interface) were washed free of percoll and cultured in tissue culture flasks in complete medium for 2 hours. Non-adherent cells were removed by repeated, gentle washing in DMEM/HEPES pre-warmed to 37°C. Remaining cells were cultured over-night. Detached cells at 1-2 x 10⁶/ml were rosetted for 30 minutes with 0.5% sheep red blood cells (SRBC, Imperial Cancer Research Fund, Clare Hall, UK) sensitised with the maximum non-agglutinating dose of anti-SRBC serum (Capell, UK). Cells were layered onto 3ml ficoll-hypaque, and spun at 850g for 20 minutes, with slow deceleration. 40 spleens yielded 2-5 x 10⁶ cells at the interface above the ficoll-hypaque. These cells were enriched for dendritic cells, while macrophages, B cells and dead cells moved through the ficoll-hypaque.

The endocytosis assay was carried out as for A20 cells with a single adaptation, in that after the first wash at 4°C cells were resuspended and stained with anti-mouse Ig linked to the hetero-conjugate phycoerythrin-Texas Red, detected in the red channel (excitation 488nm, emission 620-660nm) (Becton Dickinson). Upto 50% of cells were positive by this staining, and were excluded by setting a gate during acquisition.

**Confocal microscopy of dendritic cells.** Dendritic cells were pulsed with fixable RD (10mg/ml, Molecular Probes, Oregon USA) for 6 or 60 minutes and then chased for 0
or 30 minutes. All further steps were at 4°C. Cells were stained with anti-Ig to detect B cells. Cells were washed with phosphate-buffered saline (PBS), adhered to poly-D-lysine coated multi-well slides for 5 minutes and fixed with paraformaldehyde for 10 minutes. Confocal microscopy was performed on an Axioscop (Zeiss) with sub-micrometer confocal optics (MRC Lasersharp 500, BioRad).

The ratios of cell diameters were obtained from forward scatter by flow cytometry. These values were compared to A20 cells, for which absolute cell diameter was also obtained by examination of 100 cells using an eye-piece graticule.

**Adaptation of the flow cytometric assay for B cells.** Resting B cells were obtained from spleens of either CBA or DBA/2 mice. A multi-step iso-osmolar percoll gradient was used, as for dendritic cells. A suspension of washed spleen cells in 70% percoll, 4 spleens in 1ml for one gradient, was over-layered with 70%, 60%, and 0% percoll, 1ml per layer. The gradient was spun at 850g for 15 minutes, with slow deceleration. High density leukocytes (70%-60% interface) were washed free of percoll and treated with a mixture of antibodies 3.168 (anti-CD4), RL172.4 (anti-CD8) and rabbit complement (Buxted) to lyse T cells. The resulting population was >97% membrane immunoglobulin (mIg) positive, and less than 3% expressed CD3. Activated B cells were obtained by incubating resting B cells with lipopolysaccharide (30μg/ml) for 72 hours. The resulting cells were 100% positive for mIg. The endocytosis assay carried out as for A20 cells.

**CHAPTER 4. Labelling of transferrin (Tf) with 125I.** Human iron-saturated transferrin (Tf) was labelled with 125I to yield 125I-Tf. The chloramine T method was used with 1mCi of 125I per 100μg of Tf. After removal of free 125I by gel filtration, 99.9% of radioactivity was TCA precipitable. Gamma-counts were measured in a 16-well counter (NE1600, Nuclear Enterprises).

**Labelling of Tf with horse radish peroxidase (HRP).** Tf was labelled by conjugation with horse radish peroxidase (HRP) using the bifunctional cross-linker 3-(2-pyridyldithio)-propionic acid N-hydroxysuccinimide ester (SPDP) to yield Tf-HRP. HRP (10mg) was mixed with a 30-fold molar excess of SPDP (2.4mg) in 1ml of PBS for 30 minutes at room temperature, before gel filtration on a Sephadex G25 column in 0.1M sodium
acetate, 0.1M sodium chloride (pH 4.5). HRP was then treated with dithiothreitol (DTT, final concentration 50mM) for 30 minutes at room temperature before being passed through Sephadex G25 in PBS (pH 8.0). Tf (10mg) was also reacted with SPDP (1.2mg), and then filtered through Sephadex G25 in PBS (pH 8.0). HRP and Tf were mixed for 30 minutes at room temperature, and then reaction by-products were removed by further gel filtration in PBS (pH 7.4).

Since unconjugated Tf and HRP were not removed by this method, the molar ratio of the proteins (1 x Tf : 2 x HRP) was designed to ensure that a majority of Tf bound one HRP, and that there was a minimum of free HRP, which could be taken up by non-specific membrane adsorption, and hence enter late endosomes.

Disruption of A20 cells. A20 cells were pelleted and resuspended in homogenisation buffer (HB: 250mM sucrose, 2mM CaCl₂, 0.5mM MgCl₂, 4mM EGTA, 10mM tris/HCl pH 7.4). The ratio of Ca²⁺ to EGTA was calculated to produce a similar concentration of free Ca²⁺ as found inside cells (≈100nM) (algorithm of B. Gomperts, U.C.L.). Cells were pelleted, and resuspended in HB at 3-10 x 10⁷ per ml. Protease inhibitors were added: leupeptin (100μg/ml) and pefabloc (Pentapharm, 240μg/ml), a water-soluble, non-toxic analogue of phenylmethylsulphonyl fluoride. The cell suspension was passed forty times through a stainless steel chamber, diameter 8.020mm, containing a ball-bearing diameter 8.010mm (European Molecular Biology Laboratory, Heidelberg, Germany). The chamber was washed out with HB to collect remaining material. Disruption of cells was confirmed after addition of trypan blue to a 1μl sample.

Labelling of plasma membrane and early endosomes with transferrin (Tf). A20 cells were pre-incubated for 60 minutes in DMEM/HEPES/1% bovine serum albumin (BSA) to remove cell surface and internal bovine Tf previously taken up by the cells from FCS. Cells were then labelled for various times with ^{125}I-Tf (25-50ng per 10⁶ cells, i.e. 2-4 x 10⁵ Tf molecules per cell) or with Tf-HRP (1μg Tf per 10⁶ cells, i.e. 8 x 10⁵ Tf molecules per cell) at either 4 °C or 37 °C to label plasma membrane or early endosomes respectively. For plasma membrane labelling, cells were washed repeatedly in DMEM/HEPES by centrifugation at 300g for 2 minutes at 4 °C. For early endosomal Tf labelling, all further steps were performed at 4 °C after addition of excess cold DMEM/HEPES. Cells were
pelleted once. Iron was stripped from surface Tf by resuspension of the cell pellet for 5 minutes in a mild acid wash (25mM acetic acid, 125mM NaCl, 5mM CaCl$_2$, 14mM NaOH – pH 4.75). This did not affect cell viability. This was followed by centrifugation and resuspension of the cells in PBS (pH 7.4) for 10 minutes, during which time cell surface Tf dissociated because of the reduced affinity of iron-depleted-Tf for TfR (Dautry Varsat et al., 1983). Cells were washed three times thereafter in PBS. The most efficient pH to strip iron was determined from the maximum removal of $^{125}$I-Tf from cell surface labelled cells (93%) without loss of cell viability.

**Labelling with fluid-phase HRP.** A20 cells were pelleted and resuspended in DMEM/HEPES/1% BSA containing HRP (5mg/ml) for various times (pulse) at 37°C. Endocytosis was stopped by addition of excess cold DMEM/HEPES, and the cells pelleted. The cells were resuspended in DMEM/HEPES/1% BSA for a further period (chase) before exocytosis was stopped by addition of excess cold DMEM/HEPES. In experiments to label late endosomes only, the pulse and chase periods were both of 15 minutes duration. Tf labelling was performed during the chase period in some cases.

**Measurement of HRP.** HRP was assayed in flat-bottomed 96-well microtitre plates. 50µl of sample was mixed with 50µl of O-phenylene-diamine (OPD) solution. OPD was made up at 1mg/ml in 25mM citric acid, 50mM Na$_2$HPO$_4$, 11mM HCl (pH 5.0), 0.015% H$_2$O$_2$. 0.5% NP40 was added to the assay solution to lyse membrane vesicles when measuring latent enzyme. After incubation at room temperature for 20-60 minutes, absorbances were read at 450nm. Background values were obtained from multiple wells containing medium and OPD only. A positive control, consisting of HRP ≈10ng/ml, was present in each row of the plate used for samples.

**Measurement of β-hexosaminidase.** β-hexosaminidase was assayed in flat-bottomed 96-well microtitre plates. 25µl of sample was mixed with 25µl of solution containing 1.7mg/ml p-nitrophenyl N-acetyl-β-D-glucosaminide in 50mM citrate, pH 4.8. After incubation at 37°C for 150 minutes, the reaction was stopped and colour developed by addition of 200µl of a solution containing 166mM glycine, 104mM Na$_2$CO$_3$, 84mM NaCl, pH10.7. 0.5% NP40 was added to the assay solution to lyse membrane vesicles when measuring latent enzyme. Absorbances were read at 405nm. Background values were
Measurement of galactosyltransferase. (Method adapted from Bretz and Staubli, 1977). 80µl of assay mix (40mM sodium cacodylate pH 6.6, 17.5mg/ml ovomucoid, 40mM 2-mercaptopo-ethanol, 0.2mM UDP-galactose, 0.2% triton X-100, 2mM ATP, 40mM MnCl₂ and 0.5µCi/ml [³H]-UDP-galactose) was incubated with 20µl sample for 30 minutes at 37°C. The reaction was stopped with 1ml of ice cold 1% phosphotungstic acid/0.5M HCl (PTA/HCl). After 10 minutes on ice, the sample was centrifuged at gMAX=13000 for 7 seconds, and the supernatant aspirated. The pellet was resuspended and washed 3-times with 1ml PTA/HCl, then once in 1ml ice-cold 96% ethanol. The pellet was solubilised in 250µl 0.4M tris, 0.8% SDS. 200µl of the sample was removed into a scintillation vial, and 3ml aqueous scintillation fluid added before β-counting.

Centrifugation of PNS into a 7.4ml percoll gradient. Upto 1ml of PNS was layered onto 28% percoll (7.4ml of 28% percoll in HB). The gradient was spun at 30,000 r.p.m.(gAV=62,000) for 25 minutes at 4°C in a pre-cooled TFT65.13 rotor in a Centrikon T-2070 ultracentrifuge, with maximum deceleration (method adapted from Stoorvogel et al., 1991). The gradient was fractionated from the bottom through a narrow capillary tube, to yield 10 fractions of approximately 840µl each. Due to lack of uniform function by the fraction collector, the number of fractions varied from 9.3 to 11. These results were standardised by rescaling the fractions so that top of the gradient would be fraction 10, maintaining the bottom of the gradient as fraction 1. Therefore, where the number of fractions = F, the nᵗʰ fraction would be rescaled to give n’ where:

\[ n' = \left(\frac{(n - 1) \times 9}{(F - 1)}\right) + 1 \]

This rescaling was also performed for comparing results from large and small gradients (see figure 1, below). To accompany this rescaling of the x-axis, the y-axis was rescaled so that all graphs were comparable with each other, based on the expected 10 fractions. Therefore, "% marker per fraction" was rescaled so that the area under the graph equalled 10 x F %. In experiments where cytosolic marker in fractions 9 and 10 was excluded, the mean value of fractions 1-8 was 12.5% and the sum of the values for these fractions equalled 12.5 x F %.

The density of each fraction was calculated from the apparent sucrose concentration, as
ascertained using a refractometer designed to measure sucrose concentrations. The apparent sucrose concentration of certain known solutions containing varying amounts of sucrose and percoll was used to derive the relationship between density and apparent sucrose concentration (see table 1). The densities across three different percoll gradients used in this study (7.4ml/28%/10 fractions, 8.8ml/25%/20 fractions and 30ml/27%/28 fractions) are shown in figure 1.

For assay of HRP and β-hexosaminidase, the high concentrations of percoll in the lowest fractions formed an opaque suspension after prolonged exposure to pH 5. Therefore, a gradient containing no cells was used to obtain negative control percoll fractions.

**Gel filtration of PNS used to determine the release of endocytic [¹⁴C]-sucrose.** A20 cells were pelleted and resuspended in DMEM/HEPES/1% BSA containing [¹⁴C]-sucrose (6μCi/ml) for 60 minutes at 37°C. Cells were disrupted as above. Membrane vesicles were removed from cytosol by gel filtration through sepharose S2B gel (Pharmacia) which was pre-blocked with 1% BSA and unlabelled A20 PNS. A 10ml column of pre-swollen S2B gel was used for 1ml samples of PNS, and 0.5ml fractions were taken. Membranes eluted in the void fractions, which were cloudy.

The separation of membranes from cytosolic proteins by S2B gels was judged to be adequate from a separate experiment using cells in which the membranes had been labelled with Di.I. Fluorescein isothiocyanate (FITC) conjugated to ovalbumin (FITC-ova) was added to PNS from these cells, and the mixture passed through a 30ml S2B column, with 1ml fractions. Membranes were recovered in fractions 6-9, which contained less than 2% of FITC, the peak of which was recovered in fractions 20-35.

**Colorimetric assay for protein on percoll gradient samples.** 1.6 x 10⁸ A20 cells were disrupted and the PNS spun into 28% percoll as described above. Protein was measured using the microassay version of a protein-dye binding colorimetric assay (Bradford, 1976). Percoll interfered with the colorimetric assay, and therefore was removed by centrifugation at 90,000 r.p.m.(gAv=330,000) for 15 minutes at 4°C in a pre-cooled TLA-100-3 rotor in a Beckman TL100 ultracentrifuge. Prior to the centrifugation, membranes were lysed by addition of NP40 (final 0.1% v/v). The measured amount of protein in
fractions 1-8 was at the limit of the sensitivity of the assay, being 2-30 μg total protein per fraction.

**35S incorporation assay for cellular protein.** 2 x 10^7 A20 cells were cultured for 18 hours in 20 ml of medium containing 40 μCi of mixed 35S-amino acids. This mixture consisted of 85% 35S-methionine, 15% 35S-cysteine (Translabel, ICN Flow). For such long-term labelling the medium used was 90% DMEM without methionine and cysteine, 5% FCS, and 5% complete DMEM, plus 50 μM 2-mercapto-ethanol. Approximately 30% of radioactivity was incorporated, and cell viability was maintained. Before disruption of cells, unconjugated label and newly synthesised proteins were chased for one hour in complete DMEM with added 1 mM methionine and 1 mM cysteine.

**Centrifugation of PNS into a 30 ml percoll gradient.** PNS (1 ml) was mixed with percoll in HB (29 ml), to give a final concentration of 27% percoll and centrifuged at 17,000 r.p.m. (gav = 22,000) for 105 minutes at 4 °C in a pre-cooled TFT70.38 rotor in a Centrikon T-2070 ultracentrifuge (method adapted from Galloway et al., 1983). The gradient was fractionated from the bottom through a narrow capillary tube, to yield 28 fractions of approximately 1070 μl each.

**Re-centrifugation of early endosomes.** The early endosomes of A20 cells were labelled with 125I-Tf, and PNS prepared as above. PNS was diluted 50:50 in 28% percoll to yield 14% percoll before centrifugation into a 7.4 ml 28% percoll gradient. Fractions 4, 6 and 8 of this gradient were made up to 14% percoll and re-centrifuged into three further 7.4 ml 28% percoll gradients.

**Assessment of recycling of 125I-Tf.** A20 cells were incubated with 125I-Tf for 5 or 80 minutes and surface label stripped as above. Aliquots of the cells pulsed for 5 minutes were re-incubated (chased) for 10, 30 and 90 minutes in the presence of deferoxamine mesylate (final concentration 33 μg/ml). Cells were washed (without repeated stripping), and PNS prepared from all groups of cells and centrifuged into 7.4 ml 28% percoll gradients as above.

**Raising antisera to peptide and FITC.** Peptides were synthesised consisting of the C-
terminal 12 residues of I-Aα (α_cyt) and of H-2L^ (MHC I_cyt) (see figure 22). For each peptide, keyhole limpet haemocyanin (KLH, 2mg) was mixed with peptide (2 mg) in 1ml of PBS (pH 7.4) and added to 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (19.2mg), incubated at room temperature for 4 hours, then extensively dialysed against fresh PBS. FITC (1mg, from a 10mg/ml stock in dimethylsulphoxide) was similarly conjugated to BSA (2mg). Rabbit antisera were raised using complete Freund's adjuvant for the first vaccination, and incomplete Freund's on multiple occasions 4-6 weeks apart over several months.

**Enzyme-linked immunosorbent assay (ELISA) to test antisera.** To test the anti-α_cyt response, microtitre wells (Falcon) were coated overnight at 4°C with α_cyt (100μg/ml in 0.1M Na_2CO_3), washed (with PBS/0.05% Tween 20, three times), blocked at room temperature for 1 hour with casein (casein Hammarsten (BDH), 2%w/v in PBS with 0.1% azide), washed, incubated at 37°C overnight with dilutions of serum (± excess free α_cyt) in casein, washed, incubated for 90 minutes at 37°C in anti-rabbit globulin conjugated to alkaline phosphatase (diluted 1 in 500 in casein), washed, developed at 37°C for 2-4 hours in p-nitrophenyl phosphate (5mg per 9ml PBS plus 1ml diethanolamine), and read at 405nm. To test anti-FITC responses, FITC-ova was used in the coating step.

**Preparation of solid matrix support for protein.** Cyanogen bromide-activated sepharose (Pharmacia, 1g dry weight) was pre-swollen in 1mM HCl for 15 minutes (making 3.5ml of gel), mixed for 2 hours at room temperature with protein (20mg, dialysed into coupling buffer: 0.1M NaHCO_3, 0.5M NaCl), washed with coupling buffer (50ml, three times), blocked for 2 hours at room temperature (with 0.1M tris/HCl pH 8.0, 0.5M NaCl), rinsed alternately with acid (0.1M Na acetate pH 4.0, 0.5M NaCl) and blocking buffer, and resuspended in PBS.

**Affinity purification of anti-FITC and anti-α_cyt antisera.** To enable specific binding of antibody to a solid matrix without excessive amounts of antigen which lead to high avidity and loss of antibody during elution, FITC and α_cyt were coupled at a one to one molar ratio to ova and BSA respectively, using the same methods as above (i.e. 0.2mg FITC for 20mg ova; 0.4mg α_cyt for 20mg BSA).
Sepharose gel (2.5ml) coupled indirectly to hapten was pre-treated with 2.5ml 1M propionic acid (pH 3.0), neutralised in 2M tris, washed into PBS, then mixed with serum (25ml) for two hours at room temperature. Gel was washed for 30 minutes (in 25mM tris pH 7.4, 0.5M NaCl), then treated with 5ml propionic acid. 5μl of 0.5ml fractions was tested for pH and acidic fractions rapidly neutralised with 2M tris. Fractions with peak protein concentration (estimated from absorbance at 280nm) were pooled and dialysed against PBS. For anti-FITC (3mg protein/ml), 45% of the added antibody activity was recovered, as shown by the quenching of FITC fluorescence by anti-FITC. For anti-α_cyt (1mg protein/ml) approximately 20% of activity was recovered, as measured by ELISA.

\[ ^{125}\text{I} \] labelling of A20 cells. 10^7 A20 cells were washed twice in ice cold PBS (complete, pH 7.4), resuspended in 100μl PBS, mixed with \[ ^{125}\text{I} \] (0.5mCi carrier free NaI, Amersham), 2μg lactoperoxidase, 180μg glucose and 2.5 units of glucose oxidase for 45 minutes at 37°C. The reaction was stopped with cold iodide (2ml of KI at 1.25mg/ml in DMEM). Cells were washed in cold DMEM, lysed for 15 minutes at 4°C (in 150mM NaCl, 5mM EDTA, 50mM tris/HCl pH 8.0, plus proteinase inhibitors: pefabloc and leupeptin (final concentrations 0.24mg/ml and 0.1mg/ml respectively).

Immunoprecipitation. The following antibodies were directly coupled to sepharose (as above) for some experiments: anti-α_cyt, TIB120, In-1. In addition, protein A-sepharose (Pharmacia) was used to precipitate endogenously synthesised Ig. All precipitations and washes were performed at 4°C. The method for direct precipitations was as follows: PNS was pre-cleared twice for 1 hour with sepharose coupled to normal rat Ig (20μl of 50% slurry per 10^7 cells), and antigen was precipitated by addition for 1-2 hours of sepharose directly coupled to antibody, or normal rat Ig as negative control (10-20μl of 50% slurry per 10^7 cell). For indirect precipitations: PNS was pre-cleared with sepharose-protein A, antibody or normal rat/rabbit Ig (negative control) was added for 1 hour (approximately 1μg per 10^7 cells), and antigen was precipitated by further sepharose-protein A or species specific antiglobulin-agarose. Beads were washed as follows: 30 minutes in high salt (0.5M NaCl, all wash buffers contained 10mM tris/HCl pH 7.2 and 0.5% NP40) plus 1% BSA, transferred to a fresh tube, washed in high salt once more, low salt (no NaCl) twice, and normal salt (0.15M NaCl) twice. The final pellet was resuspended in an equal volume of 2x sample buffer to give a final concentration of 2% sodium dodecyl sulphate.
(SDS), 62.5mM tris/HCl pH 6.8, 10% glycerol, 0.2mg/ml bromophenol blue, ± 0.77M 2-mercapto-ethanol. Beads in sample buffer were boiled for 5 minutes and then centrifuged at $g_{\text{MAX}} = 13000$ for 10 minutes.

**Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE).** Minigel apparatus (Protean II, Biorad) was used to pour and run 10-15% polyacrylamide gels using tris/glycine buffers according to the method of Laemmli. Gels were fixed, treated with scintillant (Amplify, Amersham), dried, and exposed for autoradiography at $-70^\circ\text{C}$. In some cases densitometry was performed using NIH Image 1.47 software.

**$^{35}$S metabolic labelling for immunoprecipitation.** A20 cells were cultured for 1 hour at $37^\circ\text{C}$ in medium without methionine and cysteine, containing FCS which had been dialysed against PBS to remove free amino acids, followed by a pulse for 15-60 minutes in the same medium containing $^{35}$S-methionine (Amersham) or mixed $^{35}$S-amino acids (Translabel, ICN Flow). $^{35}$S-methionine was usually used at 0.2mCi per $10^7$ cells. Labelling was stopped by excess cold DMEM, and the cells were washed once. For chase of metabolic label, cells were resuspended in complete DMEM with added 1mM methionine and 1mM cysteine. For labelling of proteins in the ER (no chase) the short pulse (15 minutes) was followed by a chase of 5 minutes to reduce the amount of free $^{35}$S-methionine associated with the cells. After chase periods from 0-18 hours, cells were washed a further two times before being lysed as for $^{125}$I labelling above.

**Pronase treatment of MHC II.** 2.5 x $10^6$ A20 cells pulse-chase labelled with $^{35}$S-amino acids were washed into 1ml of cold DMEM/HEPES, and pronase (1mg) added. The cells were rolled at 45 minutes, during which time a fraction of the pronase adhered to the cells and walls of the tube. The cells were washed three times in DMEM/HEPES, and lysed as above. The samples were divided for precipitation of MHC II by anti-$\alpha_{\text{c}}$ and Ig by protein A.

**Precipitation of surface MHC II.** 2.5 x $10^6$ A20 cells, which had been pulse-chase labelled with $^{35}$S-amino acids, were resuspended in 0.5ml DMEM/HEPES/1% BSA and incubated for 30 minutes at $4^\circ\text{C}$ with polyclonal rabbit anti-MHC II (10$\mu$l) or normal rabbit serum (negative control). Cells were washed three times in DMEM/HEPES, lysed
and PNS stored at -70 °C. After thawing, protein A-sepharose was used to precipitate mlg ± surface MHC II after pre-clearing with sepharose coupled to normal rat Ig. TIB120-sepharose was added to the supernatants left from protein A precipitation.

**Precipitation of MHC II from percoll gradients.** 10⁷ A20 cells were labelled with ³⁵S-amino acids at higher levels than usual (1mCi per 10⁷ cells) for either 16 hours plus 1 hour chase or 20 minutes plus 70 minute chase. Cells were disrupted in a stainless steel chamber and PNS centrifuged into a 7.4ml 28% percoll gradient yielding 10/11 fractions, ≈800μl each, as above. Fraction 1 was discarded since its high density prevented recovery of sepharose beads by centrifugation, and since very few membrane markers entered this fraction. Membranes in fractions 2-10/11 were lysed by addition of NP40 (200μl 1%NP40, 2%BSA in PBS). Normal mouse serum (1 in 200) and normal Ig (final 0.5mg/ml) were added to each fraction after pre-clearing with a mixture of anti-rat and anti-rabbit agarose, prior to addition of anti-α cytokine and indirect precipitation with anti-rabbit agarose. The addition of normal serum and Ig was designed to prevent any binding of A20-derived Ig to the agarose.

**CHAPTER 5. Identification of inverted vesicles containing MHC II on a percoll gradient.** 2.5 x 10⁷ A20 cells were disrupted and the PNS incubated with 1μg of ¹²⁵I-anti-α cytokine for 60 minutes at 4 °C. Membranes were separated from free label by gel filtration on sepharose 2B. 0.4ml of membranes in HB were layered onto 8.8ml 25% percoll, which was centrifuged at 23,200 r.p.m. (gAv=33,000) for 60 minutes at 4 °C in a pre-cooled MSE10x10 rotor in a Prepspin ultracentrifuge (method adapted from Stoorvogel et al., 1987). The gradient was fractionated by removal from the bottom through a narrow capillary tube, to yield 20 fractions of approximately 460μl each. To identify the fractions of this gradient entered by plasma membrane vesicles, whole A20 cells were incubated with ¹²⁵I-anti-mlg for 30 minutes at 4 °C, washed, disrupted, and the PNS centrifuged into percoll.

**Immuno-isolation on magnetic immuno-adsorbent by anti-α cytokine.** The immuno-adsorbent (ImAd) had a binding capacity of 5-10μg IgG per mg, which contained 6.5 x 10⁷ beads, with a surface area of 16cm² (Dynal). Prior to use, ImAd was pelleted for ≥2 minutes using a magnetic rack designed for use with Eppendorf tubes (Dynal). ImAd was then
resuspended gently in wash buffer (WB: 1% BSA in 150mM NaCl, 2mM CaCl$_2$, 0.5mM MgCl$_2$, 4mM EGTA, 10mM tris/HCl pH 7.4). This wash process was repeated. Finally, ImAd was resuspended in WB plus 1% normal mouse serum at 6.8 x 10$^8$ beads per ml.

PNS (1ml from 7 x 10$^7$ A20 cells) was incubated for 2 hours at 4 °C with anti-α$_{cyt}$ (1μg per 2 x 10$^7$ cells). An additional 0.1μg of $^{125}$I-anti-α$_{cyt}$ per 2 x 10$^7$ cells was included in the incubation. Membranes were separated from free antibody by gel filtration on sepharose 2B. Membranes labelled with antibody (1ml) were divided into aliquots (100μl, PNS derived from 7 x 10$^6$ cells) in Eppendorf tubes (capacity 1.5ml). Varying amounts of ImAd were added, from 3.7 x 10$^4$ to 9.5 x 10$^6$ beads per 10$^6$ A20 cells (i.e. PNS derived from 10$^6$ cells), i.e. 2.6 x 10$^5$ to 6.7 x 10$^7$ beads per aliquot. ImAd and PNS were mixed slow end-over-end rotation in a plastic rotor for 2 hours at 4 °C. ImAd was pelleted, PNS supernatant was removed, and ImAd washed twice. $^{125}$I was measured in PNS supernatant, the first wash solution, and washed, coated ImAd.

**Di.I labelling of isolated vesicles for flow cytometry.** 18 hours before use A20 cells were labelled with Di.I. 2 x 10$^7$ cells in 10ml DMEM/HEPES/FCS at 37 °C plus Di.I (final concentration 20μg/ml) for 10 minutes. Cells were removed from free Di.I by centrifugation, and incubated overnight. Isolation was carried out as above, with anti-FITC as a negative control for anti-α$_{cyt}$, and 1 x 10$^6$ beads per 10$^6$ A20 cells. To inhibit immuno-isolation, either KLH-α$_{cyt}$ (10μg), or KLH-MHC I$_{cyt}$ (50μg) were added to the mixture of PNS and anti-α$_{cyt}$. Coated ImAd was examined by fluorescent microscopy using Texas Red optics (Zeiss) and by flow cytometry using FACScan. Di.I was detected in the orange channel.

**Immuno-isolation of early endosomes.** A20 or AK34 cells were labelled with Tf* for 5-80 minutes at 37 °C followed by acid stripping of surface label. Membranes from disrupted cells were separated from cytosol by gel filtration on a sepharose 2B column in WB. Membranes were incubated for 1 hour at 4 °C with anti-α$_{cyt}$ (1μg per 2-6 x 10$^6$ cells) or negative control serum (anti-FITC or normal rabbit Ig). Isolation was carried out for 1 hour as above, with 5-11 x 10$^6$ beads per 10$^6$ A20 cells. Repeated isolations were performed by mixing the PNS supernatant from the previous isolation with a further aliquot of ImAd.
Immuno-isolation under different ionic conditions. Immuno-isolation of PNS from A20 cells labelled with $^{125}$I-Tf in early endosomes was performed as above, except that the membranes were maintained in HB during gel filtration. Aliquots of membranes were diluted 1:1 with HB or iso-osmolar solutions containing choline chloride or NaCl (both containing 1% BSA, 2mM CaCl$_2$, 0.5mM MgCl$_2$, 4mM EGTA, and 10mM tris/HCl pH 7.4) or these solutions already diluted 1:1 with HB. In addition, a duplicate of the sample containing NaCl included ATP and a regenerating system (final concentrations from a x100 stock: 1mM ATP, 10mM MgCl$_2$, 8mM creatine phosphate, 31U/ml creatine phosphokinase, pH 7).

Direct immuno-isolation. ImAd (15mg) was incubated with antibody (anti-α$_c$yt or anti-FITC, 100μg) overnight at 4°C in PBS (1ml). ImAd was washed three times in PBS/1% BSA, with rotation for 30 minutes between each wash. PNS from Di.I labelled A20 cells was mixed with ImAd coated with specific antibody at 1.4 x 10$^5$ beads per 10$^6$ cells.

Effect of free anti-α$_c$yt. PNS was incubated for 1 hour at 4°C with anti-α$_c$yt (1μg per 8 x 10$^6$ cells) or negative control serum (normal rabbit Ig). Isolation was carried out for 1 hour as above, with 1.6 x 10$^6$ beads per 10$^6$ A20 cells. Repeated isolations were performed by mixing the PNS supernatant from the previous isolation with a further aliquot of ImAd.

Acid stripping of vesicles in PNS. PNS from A20 cells labelled with $^{125}$I-Tf at 4°C for 60 minutes was divided into two aliquots. One aliquot was acid stripped, while the other remained as a control. Acid stripping was performed by addition of acetic acid to reduce pH to 4.7, and deferoxamine mesylate to chelate free iron (final concentration 33μg/ml). After incubation for 5 minutes pH was neutralised with tris, and excess cold apo-transferrin added (final concentration 10μg/ml). This solution was left for 10 minutes before membrane-bound $^{125}$I-Tf was separated from free label.

Immuno-isolation of multiple intracellular compartments from a percoll gradient. 5-15 x 10$^7$ A20 cells were labelled as before for the incorporation of $^{35}$S-amino-acids into cellular protein, pulse-chase endocytosis of HRP into late endosomes, and internalisation of $^{125}$I-Tf into early endosomes followed by surface stripping. PNS from these cells was
centrifuged into a 7.4ml 28% percoll gradient, yielding 10 fractions. Fractions were divided into two, and incubated with 0.5µg of either anti-α_cyt or normal rabbit Ig for 90 minutes. Fractions were diluted 1:1 with WB containing 1% NMS and isolation was carried out for 1 hour as above with ImAd 1-1.6 x 10^7 beads per fraction. Repeated isolation was performed by mixing the PNS supernatant from the first isolation with a further aliquot of ImAd.

**Electron microscopy.** 5 x 10^7 A20 cells were incubated in DMEM/HEPES/1% BSA for 60 minutes at 37°C, washed and incubated for 15 minutes at 4°C in DMEM/HEPES/1% BSA containing ^125^I-Tf and 10nm colloidal gold conjugated to R17.217, a monoclonal IgG2a rat anti-mouse transferrin receptor (gold-AMTR, a kind gift of C. Futter). This antibody does not interfere with the binding of Tf to TfR. The gold label had been stored at 4°C, and was pelleted, resuspended and filtered just prior to use at final A_{250}/cm ≈ 10. Cells were washed free of unbound gold-AMTR, and incubated for 5 minutes at 37°C, before being cooled back to 4°C. Plasma membrane ^125^I-Tf was acid stripped, cells were disrupted, PNS centrifuged into 7.4ml 28% percoll, fractions taken, and immuno-isolation by anti-α_cyt or normal rabbit Ig performed as above. ImAd coated with vesicles was washed twice in WB, transferred into a fresh tube, pelleted, resuspended in 0.5ml PBS, and fixed with an equal volume of 1% glutaraldehyde (Taab Laboratories), 0.2M sodium cacodylate pH 7.2 at room temperature. Samples were then postfixed in 1% w/v osmium tetroxide in 0.1M cacodylate buffer, dehydrated in a series of graded solutions of ethanol and embedded in Epon 812 resin (Taab Laboratories). Sections were cut on a Reichert-Jung ultramicrotome, mounted on carbon/formvar-coated slot grids, stained for 15 minutes with 3% w/v uranyl acetate then 5 minutes with lead citrate and viewed at 60kV in a Philips CM12 electron microscope.

**Immuno-isolation with In-1.** PNS from 2 x 10^7 A20 cells labelled with ^125^I-Tf in early endosomes was divided into four aliquots, and incubated with anti-α_cyt, normal rabbit Ig, In-1 or normal rat Ig (1µg per 5 x 10^6 cells). Immuno-isolation of the first two samples was performed with 2.8 diameter beads coated with anti-rabbit Ig (5 x 10^6 per 10^6 A20 cells), and for the other two samples with 4.5µm diameter magnetic beads coated with sheep anti-rat Ig (3 x 10^6 per 10^6 A20 cells). The larger beads coated with anti-rat Ig
were more rapidly pelleted because of their larger size, but were otherwise similar.
Second and third isolations were performed by mixing the PNS supernatant from the
previous isolation with a further aliquot of ImAd.

After the third isolation, the PNS supernatants from the In-1 and normal rat Ig samples
were further divided in two, each half being incubated with anti-α_{cyt} or normal rabbit Ig
(1μg per 2.5 x 10^6 cells) and then vesicles isolated with ImAd coated with anti-rabbit Ig
(2.4 x 10^6 per 10^6 A20 cells).

Precipitation of MHC II from isolated vesicles. A20 cells were labelled with ^35S-amino-
acids for 20 minutes (0.2mCi per10^7 cells). Cells were washed into chase medium and
chased for 0, 1, 3 and 14 hours. For each group: cells were disrupted, membranes
separated from cytosol by gel filtration, vesicles isolated by anti-α_{cyt} or negative control
antiseraum (affinity-purified rabbit anti-irrelevant peptide = MHC I_{cyt}). PNS supernatant
was lysed and MHC II precipitated. ImAd coated with vesicles was incubated with
rotation for 30 minutes in WB containing NP40, to lyse vesicles. During this time
anti-α_{cyt}/MHC II complexes remained attached to ImAd. These ImAd beads were then
washed in varying salt concentrations and boiled in sample buffer for SDS-PAGE of
eluted proteins as for sepharose beads.

CHAPTER 6. Degradation of II. 4 x 10^7 A20 cells were incubated overnight in leupeptin
(20μg/ml). These cells, and a group of control cells, were pulsed with ^35S-amino-acids
(0.1mCi per 10^7 cells) for 1 hour with 2 hours chase. For the cells incubated in leupeptin,
all subsequent incubations contained leupeptin. Membranes were then isolated with
anti-α_{cyt} (1μg per 2 x 10^7 cells, plus 8.4 x 10^5 beads per 10^6 cells). For the control cells,
leupeptin was omitted from the homogenisation step. ImAd coated with vesicles was then
treated with a buffer to dissociate MHC II from its surface. The dissociation buffer
contained 10% normal rabbit serum, 0.1mg/ml α_{cyt} peptide and 20μg/ml KLH-α_{cyt}, which
blocked unoccupied sites on ImAd and competed with binding of MHC II to anti-α_{cyt} and
with binding of anti-α_{cyt} to ImAd. After blocking unoccupied sites on ImAd, vesicles were
lysed by the addition of NP40 0.1%.

The material from ImAd for the two groups was further sub-divided into two. The
material from cells which had not been exposed to leupeptin was incubated at either 4°C or 37°C for 90 minutes. The material from cells which had been exposed to leupeptin was incubated at 37°C for 90 minutes with either further leupeptin (100μg/ml) or added cathepsin B (0.5mg/ml, activated by dithiothreitol 0.5mM). At the end of this incubation, all samples were returned to 4°C, and precipitation performed with In-1. The supernatants remaining after this were then re-precipitated with TIB120.

The PNS supernatant from the cells treated with leupeptin (i.e. material not isolated by anti-α crystallin onto ImAd) was lysed and precipitated by In-1. After completion of the precipitation and wash steps, but prior to boiling in lysis buffer, sepharose beads were divided into two, and either maintained on ice, or incubated at 37°C for 2 hours with cathepsin B (0.5mg/ml, activated by dithiothreitol 0.5mM). Sepharose was then washed into sample buffer and processed for SDS-PAGE.

**Peptide loading of MHC II.** MHC II directly immuno-precipitated onto sepharose beads by TIB120 was washed into solution containing tris-acetate pH 4.5, and ova 323-339 (1.6mg/ml, i.e. 1mM), and incubated at 37°C for 10 minutes or 2 hours. Tris was added to raise the pH to ≈7.5, then sample buffer without 2-mercapto-ethanol was added to the samples for 30 minutes at room temperature. Sample buffer was then removed from the sepharose beads by centrifugation, and divided into two, one half being boiled for 5 minutes.
Table 1. Calibration of sucrose refractometer

The sucrose concentration of various solutions was compared to the apparent sucrose concentration as observed by refractometer. The density of HB was 1.030g/cm³ and the density of percoll (100% in water, i.e. as supplied) was 1.130. Therefore, the density (D) of a sample of HB containing an undefined amount of percoll which had an apparent sucrose concentration of s (%w/v) was given by:

\[ D = 1.030 + (0.13 \times (s - 7.0) / 11) \]

<table>
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<th>actual [sucrose] (%w/v)</th>
<th>apparent [sucrose] (refractometer %)</th>
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<tr>
<td>HB</td>
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<tr>
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<td>100% percoll in HB</td>
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Figure 1: The range of density of percoll gradients used in this study

The density of fractions from three different gradients were compared, density being calculated from refractometry (see table 1). The different gradients had from 10 to 28 fractions, but were rescaled to fit 10 fractions (see text).
CHAPTER 3. FLUID-PHASE ENDOCYTOSIS BY DENDRITIC CELLS

Introduction. Aspects of the endocytic pathway which are worthy of study include structural specialisations which are relevant to APC function. For example, Birbeck granules in Langerhans' cells are a structural specialisation of the endocytic pathway (Stossel et al., 1990), with no known role. Other specialisations may be at the functional level, for example, mIg in B cells has been demonstrated to deliver antigen for efficient processing, while dendritic cells appear to have no specific receptors for antigen. In addition, there are many differences between APCs for which the mechanism is not known; for example, the varying expression of Y-Ae in thymic APCs by processing of I-E^b for presentation by I-A^b (Rudensky et al., 1991b).

Dendritic cells are the most potent in vitro APC, and yet their capacity to take up antigen by endocytosis has been called into question (see chapter 1). This paradox has not been addressed by detailed studies of endocytic traffic in dendritic cells. In addition, studies comparing the nature of the endocytic pathway in different types of APCs have not been performed. The reasons for this lack of information is that techniques to study endocytosis have required large numbers of cells, and dendritic cells are available only in small numbers.

Previous attempts to measure fluid-phase endocytosis by dendritic cells have compared them with macrophages over long pulses (Inaba et al., 1990; Pure et al., 1990). Long pulses tend to saturate endosomes and stress endocytic uptake into lysosomes. Therefore, poor long-term uptake by dendritic cells reflects poor lysosomal function (Chain et al., 1986; Stossel et al., 1990). However, lysosomal function may be unimportant for antigen processing, which may occur in a compartment related to late endosomes (Peters et al., 1991). Therefore, it is important to measure the traffic of fluid-phase markers through pre-lysosomal compartments. This can be achieved by kinetic analysis of endocytosis over minutes rather than hours.

In this chapter, a technique to investigate endocytic traffic in APCs is described. This technique measures fluid-phase endocytic (pinocytic) traffic, the relevance of which may vary according to the role of the APC in vivo. Thus, it has been proposed that dendritic cells may rely more on fluid-phase endocytosis than B cells and macrophages (Janeway,
1992b). Although using fluid-phase uptake as a measure of antigen uptake has the
 disadvantage that this is not the most important route for antigen uptake in all APCs,
it has the advantage that all APCs can process and present soluble antigens via this
route, although with varying efficiency (Chesnut and Grey, 1981; Chain et al., 1986).
Therefore, antigens can gain access to the processing compartment after pinocytosis by
all APCs. Measurement of other endocytic routes which are not shared between APCs
would not allow any comparisons to be drawn. In addition, a fluid-phase marker used to
measure traffic in late endosomes has the advantage that fluid-phase contents of early
endosomes are sorted into late endosomes more than membrane bound contents of early
endosomes (Rome, 1985; Geuze et al., 1987).

Endocytic parameters can be obtained by a kinetic analysis of endocytosis and exocytosis
in a two compartment model. Fluid-phase uptake is curvilinear: initial rapid uptake tails
off, usually over 15-30 minutes, and is followed by slower uptake thereafter over a period
of several hours. The non-linearity is caused by efflux (exocytosis) of marker which
increases in parallel with endocytosis. The most detailed studies have investigated
exocytosis following variable periods of endocytosis (i.e. chase following pulse), which has
the following characteristics: (1) loss of marker is initially rapid followed by a long lasting
slower phase; (2) the absolute amount of marker lost in the rapid phase of the chase
rises with the duration of the pulse, but reaches a maximum level; (3) the proportion of
marker lost in the rapid phase of the chase has a maximum with the shortest pulses and
is less for longer pulses (Besterman et al., 1981; Swanson et al., 1985).

These findings are compatible with a two compartment model for the endocytic pathway.
In this model the following assumptions are made:

(i) The endocytic pathway is considered as two sets of homogeneous organelles, early
endosomes (EE) and late endosomes (LE). This division fits with the best current model
of the endocytic pathway (Griffiths and Gruenberg, 1991; Murphy, 1991; Griffiths, 1992).
Early endosomes and Late endosomes each form two reticular organelles; traffic is from
early to late endosomes via carrier vesicles.

(ii) Lysosomes can be considered as extensions from late endosomes; two-way traffic
between them has kinetics much slower than those of early endosomes and late endosomes. Therefore for this assay, the separate existence of lysosomes can be ignored.

(iii) Exocytosis of marker from both compartments is proportional to the amount of marker and a rate constant ( = k\textsubscript{out}\textsuperscript{EE} or k\textsubscript{out}\textsuperscript{LE}). Therefore, in the absence of incoming marker, the concentration of marker and the rate of exocytosis both exponentially approach zero, with half-life ($t\textsubscript{1/2}$) = ln 2/k\textsubscript{out}; while in the presence of a constant rate of incoming marker the rate of exocytosis exponentially approaches the rate of entry, again $t\textsubscript{1/2}$ = ln 2/k\textsubscript{out}. Therefore, the situation at steady state, when filling is maximal, can be derived from the kinetics and extent of uptake over the first 60 minutes:

$$EE_{\text{max}} \approx EE_{60} \ (\text{as} \ 60 \gg t\textsubscript{1/2}\textsuperscript{EE})$$
$$LE_{\text{max}} = \frac{LE_{60}}{(1 - e^{-ln \ 2x60/t\textsubscript{1/2}LE})}$$

(see appendix)

(iv) Marker in early endosomes is either immediately recycled or enters late endosomes, from which it is exocytosed without re-entering early endosomes.

The entire model is derived from Besterman \textit{et al.}, 1981, and is summarised in figure 2.

**Previous assays for endocytosis are not suitable for APCs; possible advantages of a flow cytometric assay.** Previously described assays for exocytosis have used easily detectable markers (fluorescent or radioactive) and large numbers of purified cells. For example, endocytosis by macrophages has been studied using [$^{14}$C]-sucrose (Besterman \textit{et al.}, 1981) and lucifer yellow (Swanson \textit{et al.}, 1985). However, not all APC types are available in large numbers. Dendritic cells in particular form a minor population of many different tissues, and less than 5 x 10\textsuperscript{6} can be obtained from the spleens of 50 mice.

The requirement for less cells is met by a flow cytometric assay, since flow cytometry can measure low levels of fluorescent label in hundreds of individual cells per second. Thus, a snap-shot of a dynamic cellular process can be obtained frequently enough to follow rapid events such as recycling in early endosomes. In addition, by use of antibodies labelled by other fluorophores, a flow cytometric assay can be adapted to measure fluid-phase endocytosis in impure populations of cells.
Flow cytometric assay for endocytosis applied to A20 cells. Initial studies were performed on the A20 murine cell line, derived from a Balb/c (H-2^d) B cell lymphoma. 5 groups of A20 cells were pulsed with lucifer yellow (LY) 3.3 mg/ml at 37°C for 0, 2, 6, 20 and 60 minutes. Over 0 to 20 minutes pulse endocytosis of LY was rapid, followed by a slower phase from 20 to 60 minutes (figure 3).

After cooling the cells and removing the labelling solution, LY did not decrease in cells maintained at 4°C for several hours. On warming cells back to 37°C, LY decreased rapidly within 1 minute, the rapid decrease being followed by a much slower phase of LY loss beyond 30 minutes (figure 3). The rapid phase was prominent after short pulses, for example 2 and 6 minutes, while the slower phase increased in prominence with duration of pulse. Exocytosis by A20 cells therefore followed the expected characteristics of the two compartment model seen in macrophages and fibroblasts. By fitting an exponential curve to the values from 30 to 120 minutes chase, the half-life of the slow phase of exocytosis was found to be 102 minutes.

The assay was also applied to uptake of rhodamine dextran 70kDa (RD). This was treated to remove low molecular weight impurities which have strong membrane interactions, and which may enter cells by crossing membranes (Preston et al., 1987). Endocytosis and exocytosis of RD and LY were similar, except that from 30-120 minutes chase exocytosis of RD was much slower (half-life 285 minutes, figure 4a) than had been seen for LY (figure 3). Long-term retention of RD was seen even if the initial pulse had only been for 2 minutes. The likely cause of this slow decrease of RD was detection of RD after it had been exocytosed from cells, either in the medium surrounding cells as they passed through the flow cytometer, or possibly due to membrane adhesion and maybe even re-uptake of the exocytosed RD. The detection of RD in this manner masked its loss from inside cells.

Accurate controls are required to assess the slow phase of exocytosis. Since it was apparent that RD was detected after being exocytosed, the technique was adjusted to control for this effect. A20 cells pulsed with RD for 0, 2, 6, 20, or 60 minutes were mixed with an equal number of A20 cells which were RD^-w^, but which had been labelled with the lipophilic dye Di.O. This dye was detected on an orthogonal axis to RD and did not
partition back into the medium to cross-over to the RD\textsuperscript{**} cells. The two groups of cells were completely separated according to Di.O content (figure 5a). During the chase, a significant rise in the RD values of the Di.O\textsuperscript{**} cells was seen over the first 30 minutes (figure 4b). These changes were used to calculate the amount of RD that was being detected after exocytosis, and hence the RD that remained inside the cell after endocytosis (figure 4b). The true exocytosis of RD from 30 to 120 minutes chase was found to be similar to that of LY, half-life 123 minutes.

For LY uptake, a similar adjustment was made, except that the control cells were labelled with Di.I, from which LY was easily separable (figure 5b). During the chase, there were much less background changes in the Di.I\textsuperscript{**} cells due to detection of LY than had been seen for RD, and the half-life of exocytosis of LY from 30 to 120 minutes was 85 minutes (also see figure 8). Therefore, by monitoring background changes it was shown that RD, but not LY, was detected in a cell-associated manner after exocytosis.

In further assays of LY endocytosis, flow cytometer fluorescence units were converted to the volume of LY per cell in \( \mu m^3 \) (see chapter 2).

**Some fluid-phase markers are adsorbed onto plasma membrane.** Many fluorophores have been used as fluid-phase markers, in addition to radioactive isotopes and enzymes. The differences between these markers are largely caused by variation in non-specific adsorption to the plasma membrane. The suitability of a marker can vary between cell types. For example, horse radish peroxidase (HRP) has been used as a fluid phase marker in BHK cells, in which it was demonstrated by E.M. to be in the lumen of endocytic vesicles (Griffiths et al., 1989). In contrast, the uptake of HRP by peritoneal macrophages was inhibited by mannan (Swanson et al., 1985), indicating membrane interaction in these cells.

To determine the suitability of LY and compare it with other markers, A20 cells were incubated for 0-60 minutes with a mixture of four endocytic markers: LY, RD, \([^{14}C]\)-sucrose and \([^{3}H]\)-dextran (70 kDa). After the pulse, the absolute volume of each marker per cell was calculated (figure 6). The uptake of RD was greater than that of LY and \([^{14}C] \)-sucrose. This difference was particularly evident over the first 2 minutes, and
continued for 20 minutes. In addition, exocytosis of RD was exceptionally rapid over the first minute. Therefore, there appeared to be a pre-early endosomal compartment with very rapid kinetics. This indicates that RD is adsorbed onto the plasma membrane. In contrast, LY and \[^{14}C\]-sucrose did not appear to be adsorbed onto membrane. Further evidence for this was obtained from LY uptake during 60 minutes, which was linearly proportional to LY concentration over the range 0.5-6.7 mg/ml, from which it can be concluded that there was no saturable interaction between LY and the plasma membrane.

Cell-associated \[^{3}H\]-dextran was high after a brief incubation of label with A20 cells at 4°C (without any incubation at 37°C, = 0 minutes group) and rose rapidly over 20 minutes pulse at 37°C, at which time the content of \[^{3}H\]-dextran was 50 times that of LY or \[^{14}C\]-sucrose. The enhanced uptake of \[^{3}H\]-dextran compared to \[^{14}C\]-sucrose was also seen in the membrane fraction of homogenised cells, and was therefore caused by a membrane interaction by \[^{3}H\]-dextran or an impurity, not by the uptake into the cytosol of an \[^{3}H\]-labelled saccharide.

\[^{14}C\]-sucrose is concentrated within late endosomes and lysosomes. The uptake of LY and \[^{14}C\]-sucrose was similar, but not the same: after 60 minutes the uptake of \[^{14}C\]-sucrose was greater (figure 6). This difference has been observed previously in BHK cells (Griffiths et al., 1989).

To investigate the handling of \[^{14}C\]-sucrose further, A20 cells were pulsed with \[^{14}C\]-sucrose for 6 and 60 minutes and chased for varying periods (figure 7). The exocytosis of sucrose showed a greater dominance of the slow phase than was seen for LY. The kinetics of late endosomal traffic were slower for \[^{14}C\]-sucrose (\(t^{1/2}_{LE} = 320\) minutes) than for LY (\(t^{1/2}_{LE} = 85\) minutes, see below). In addition, the maximum content of late endosomes was much higher using \[^{14}C\]-sucrose (113\(\mu\)m\(^3\)) than with LY (14.2\(\mu\)m\(^3\), see below). Indeed, the content of \[^{14}C\]-sucrose in late endosomes was equivalent to 15% of the entire cell volume. No compartment of this size exists ultrastructurally. In respect of late endosomal traffic, RD behaved similarly to LY. A similar pattern of endocytosis of sucrose was seen in macrophages (Besterman et al., 1981). The large amount of sucrose in late endosomes may be related to its ability to vacuolate lysosomes (Montgomery et
al., 1991), by an unknown mechanism, possibly by generating hyperosmolar regions with high [¹⁴C]-sucrose concentrations. LY might not undergo such concentration because of its two negative charges.

The marginally different handling of [¹⁴C]-sucrose and LY during 60 minutes pulse (figure 6, and compare figures 4 with 7) indicates that entry into lysosomes occurs within 60 minutes of uptake. Therefore, the two compartment model is not completely accurate, and might be improved by a three compartment model (Besterman et al., 1981). However, it was not possible to maintain cell viability or accuracy of the negative control beyond 2 hours chase, so lysosomal kinetics could not be analysed separately.

The two compartment model using LY as a fluid-phase endocytic marker. From the above, it appears that LY is an ideal fluid-phase endocytic marker. After taking background variation into account as above, the data were next analysed to separate LY in early endosomes from LY in late endosomes by simplified curve stripping (figure 8). The data from cells pulsed for 60 minutes were used to calculate the kinetics. Cells pulsed for shorter periods had similar kinetics, but the fluorescence values were lower, hence less accurate for curve drawing.

Given that early endosomes had emptied by 30 minutes chase, LY from 30 minutes onward were in late endosomes only. An exponential curve drawn through these points, \( t^{\text{LE}}_1 = 85 \) minutes, produced the values for content of late endosomes during 0-30 minutes chase by extrapolation. Hence the values for early endosomes content during 0-30 minutes chase could be fitted to a separate exponential curve, \( t^{\text{EE}}_1 = 4.2 \) minutes. For all groups of cells, the amount of LY in early endosomes and late endosomes was calculated (figure 9). This two compartment analysis shows that early endosomes filled with a half-life of \( \approx 5 \) minutes, while late endosomes filling was almost linear over 60 minutes. The similarity of the \( t^{\text{EE}}_1 \) for emptying and filling fulfils the prediction of the exponential model for endocytic traffic.

Another prediction of the two compartment model is that both rapid and slow filling/emptying occur at the same time. This was investigated by sequential pulses with LY and RD, in either order, followed by chase (figure 10). The presence of a second
marker did not affect endocytosis or the pattern of exocytosis: rapid early then slow late. For example, while LY was emptying slowly, RD which had been pulsed after the LY was emptying rapidly.

The estimates of $EE_{\text{max}}$ and $LE_{\text{max}}$ were $5.6\mu m^3$ and $8.6\mu m^3$ respectively. The total endocytic volume was 2% of the estimated volume of A20 cells (diameter = $11.4\mu m$, volume = $\pi d^3/6$). This is similar to the proportion of BHK cell volume occupied by endosomes estimated from serial thin E.M. sections (Griffiths et al., 1989).

The flow cytometric assay can be adapted for dendritic cells. Splenic dendritic cells were purified according to a long-established method using centrifugation on layered gradients, differential adhesion, and rosetting (Ellis et al., 1991). This required 24 hours in vitro culture and only yielded small numbers of cells. The purity of these cells was as low as 50%, as judged by staining with anti-Ig, which showed contaminating B cells. These cells were excluded from the flow cytometric assay by positive staining with anti-Ig coupled to the paired fluorophore phycoerythrin-Texas Red, which was detected as a third colour on the flow cytometer, along with LY and Di.I (figure 11). Colour compensation for this combination of fluorophores was different from the compensation for LY and Di.I alone, and was not completely successful. Hence, even though the LY$^{\text{+ve}}$ and LY$^{\text{-ve}}$ populations were kept separate (figure 11), changes in LY were not fully compensated on the Di.I axis. Endocytosis by dendritic cells was detected in this impure population by staining the contaminants, therefore avoiding disturbing dendritic cell function by cross-linking any surface markers.

To directly visualise the endocytic compartments of dendritic cells labelled cells were examined by confocal microscopy. Standard fluorescence microscopy was not suitable for these cells due to the small amount of cytoplasm and spherical shape. To perform microscopy the cells were fixed. During this procedure LY leaked across membranes and was seen throughout the cells, including in the nuclei. This compared with a restricted cytoplasmic distribution in unfixed cells examined by standard fluorescence microscopy using LY optics. The leakage might have partly been the result of the low molecular weight of LY (522 Da). Therefore, fixable RD (Molecular Probes) was used to label cells. This marker behaved similarly to non-fixable RD, although with greater membrane
Dendritic cells were labelled with fixable RD for 6 or 60 minutes and then chased for 0 or 30 minutes (figure 12). After the brief pulse, diffuse staining was seen throughout the cytoplasm. Note the linear plasma membrane staining on the right-hand side of the cell. Chase of the marker led to minimal punctate staining. After the longer pulse, staining was more concentrated in one pole of the cell. Chase led to considerable punctate staining, also with one pole dominating. These patterns of staining were consistent with the two compartment model, with diffuse reticular EE, and pericentriolar, vesicular LE.

**Dendritic cells are endocytically heterogeneous.** After endocytosis of LY for 60 minutes, the range of LY content was wide (figure 11) compared to A20 cells (figure 5) and resting and activated B cells, although the range of cell sizes, as judged by forward scatter, was not any wider. This demonstrates heterogeneity in endocytic activity among dendritic cells. The heterogeneity was most clearly detected after 60 minutes endocytosis, and was maintained after a further 120 minutes chase, indicating that the contents of late endosomes were the source of variability.

The variability in function of late endosomes may reflect the different functions of dendritic cells in the spleen. Migratory dendritic cells enter the spleen after non-specific stimulation in non-lymphoid organs, and are thought to carry with them antigen that has been acquired in these organs for presentation in primary lymphoid tissue. These dendritic cells therefore have little need for traffic through late endosomes. A second population of dendritic cells resides in the spleen and acquires antigens from the circulation. These cells require active traffic beyond early endosomes into late endosomes for delivery of antigen to the site of antigen processing. The existence of resident (not recently activated) splenic cells may explain the finding that freshly obtained splenic dendritic cells can mature in a similar fashion to freshly obtained Langerhans' cells (Girolomoni et al., 1990).

**Two compartment analysis for dendritic cells shows relatively high levels of late endocytic traffic compared to other APCs.** Two compartment analysis of endocytosis...
using the flow cytometric assay with LY was performed on dendritic cells, resting B cells and activated B cells. For this analysis dendritic cells were treated as a single population. The maximum LY content in early endosomes and late endosomes was calculated for each of the these APC types (figure 13). The kinetics of LY traffic, together with the fraction of the cells occupied by endosomes, are given in table 2. Similar results to those obtained with LY were obtained with non-fixable RD, although the relative size of early endosomes was increased.

The kinetics of the two compartments were similar for all four cell types (table 2). The size of the early endosomes, and the absolute traffic through them, varied considerably (figure 13). However, the volume of early endosomes as a proportion of total cell volume was fairly constant. The absolute size of late endosomes was less variable between cell types, and therefore occupied a much higher proportion of cell volume in the smaller cells: resting B cells and dendritic cells. These results therefore show that fluid-phase traffic through late endosomes, was at a relatively high level in splenic dendritic cells.

Macrophages are the only APC type not suitable for this assay, because of their tendency to adhere. However, a comparison of results using $[^{14}C]$-sucrose shows that macrophages are roughly twice as active than A20 cells (macrophages LE$^{\text{max}}$ = 37% cell volume, $t^{\text{LE}} \approx 3$ hours; A20 cells LE$^{\text{max}}$ = 15% cell volume, $t^{\text{LE}} \approx 5$ hours). Therefore, fluid-phase endocytic traffic through late endosomes, which are closely related to the antigen processing compartment, is similar in all APC types including dendritic cells. The poor long-term accumulation of endocytic markers by dendritic cells can be explained by their poor lysosomal function compared to macrophages. This indicates that a smaller proportion of the contents of late endosomes in dendritic cells are routed into lysosomes than in macrophages, but does not reflect a similar difference in traffic through the pre-lysosomal compartment.

In summary, the flow cytometric assay has shown that splenic dendritic cells after 24 hours in culture are endocytically active, with heterogeneous traffic through late endosomes. Therefore, the antigens which have been shown not to be processed by dendritic cells in vitro are likely to have been taken up to the normal extent by endocytic traffic. For these antigens, the phenotypic changes that splenic dendritic cells undergo
in culture (Girolomoni et al., 1990) may have prevented processing, or the proteinases expressed by dendritic cells may be insufficient to expose the epitopes of these antigens (Wettstein et al., 1991; Harding et al., 1991a and b).
Figure 2: the two compartment model for endocytosis into early and late endosomes

Early and late endosomes are considered as the only endocytic compartments, with one-way traffic from early to late. Rapid mixing of contents within each compartment is assumed to lead to exponential kinetics of filling (during a continuous pulse) and emptying (during chase). The model and the rate constants K1 (units: \( \mu m^3/sec \)), k2, k3 and k4 (units: sec\(^{-1}\)) are adapted from Besterman et al., 1981.
Figure 3: Endocytosis and exocytosis of LY by A20 cells

A20 cells were pulsed with LY for 0, 2, 6, 20 and 60 minutes, washed and LY content determined by flow cytometry. Exocytosis of LY was then measured by repeated sampling over a chase period of up to 120 minutes. Data are from a single representative of 3 experiments.
Figure 4: Background adjustment for exocytosis of RD
Exocytosis was followed for 120 minutes following endocytosis of RD for 60 or 6 minutes. (a) uncorrected RD values are given, equivalent to \[RD_{6,60} - RD_0\]. Half-life beyond 30 minutes chase = 285 minutes.
(b) the dotted lines are equivalent to \[RD_{6,60} - RD_0\], which showed an increase over the first 30 minutes. These values were used to correct the RD values, which are equivalent to \[RD_{6,60} - RD_+ - (RD_{6,60} - RD_0)\]. Half-life beyond 30 minutes chase = 123 minutes. See methods for notation.
Figure 5: Flow cytometric separation of mixed populations of A20 cells
A20 cells had been pulsed with fluid-phase endocytic marker and mixed with an equal number of control A20 cells stained with a lipophilic dye with complementary fluorescence to the endocytic marker. The mixture of cells was analysed by flow cytometry.

(a) fluid-phase marker - rhodamine dextran (RD), control cells - Di.O;
(b) fluid-phase marker - lucifer yellow (LY), control cells - Di.I;
Plots show 4000 events, after gating for size and granularity. The axes are logarithmic, spanning 4 decades.
Figure 6: Comparison of endocytosis of four different fluid-phase markers by A20 cells over a 0, 2, 6, 20 and 60 minute pulse

The volume per cell occupied by each marker is shown, except for $^3$H-dextran values (off scale), which are: 2 minutes - 227$\mu$m$^3$, 6 minutes - 424$\mu$m$^3$, 20 minutes - 870$\mu$m$^3$ and 60 minutes - 850$\mu$m$^3$. Data are the mean of three experiments; error bars show one standard deviation.
Figure 7: $^{14}$C-sucrose exocytosis from A20 cells

A20 cells were pulsed with $^{14}$C-sucrose for 6 or 60 minutes, and then chased for varying periods up to 120 minutes. Loss of label was similar whether determined by measuring label remaining in cells or label lost into the supernatant.
Figure 8: Separation of early and late endosomal contents by analysis of exocytosis

After 60 minutes endocytosis, exocytosis was biphasic. Points from 30 to 120 minutes chase were used to draw an exponential curve, half-life 85 minutes, which was taken to represent the contents of late endosomes. The difference between total and late endosomes was taken to represent the contents of early endosomes, and a second exponential curve was drawn through these data, half-life 4.5 minutes. LY in cells labelled for 2 minutes was exocytosed with the same, rapid kinetics as seen for early endosomes, indicating that the late endosomes were not significantly filled during this pulse.

Data are from a single representative of three experiments.
Figure 9: Two compartment analysis of endocytosis by A20 cells over 60 minutes
LY was endocytosed for 0, 2, 6, 20 and 60 minutes by A20 cells. Kinetic analysis of exocytosis, as in figure 8, allowed separation of early and late endosomal contents. Data are from a single representative of three experiments.
Figure 10: Detection of fast and slow emptying/filling in the same cell at the same time
A20 cells were pulsed for 20 minutes, either prior to start (i.e. -20 to 0 minutes) or
during the first 20 minutes, with LY and/or RD, as described in the key above. RD and
LY content of cells were measured by flow cytometry at 0, 20, 40 and 60 minutes.
Figure 11: Flow cytometric analysis of dendritic cells

Dendritic cells had been pulsed with the fluid-phase endocytic marker LY and mixed with an equal number of control cells stained with Di.I. The mixture of cells was analysed by flow cytometry. A live gate was imposed to exclude mIg^+ve cells, which were positive on a third fluorescent axis. There is a subpopulation of cells with high LY (which is not fully compensated on the Di.I axis, because the use of three colours required imperfect colour compensation settings).

Plots show 4000 events, after gating for size and granularity. The axes are logarithmic, spanning 4 decades.
Figure 12: Dendritic cells labelled with endocytosed RD

Dendritic cells were pulsed with fixable RD and chased as follows:

**top left:** pulse 6 minutes, no chase - diffuse cytoplasmic staining; plasma membrane staining on right side of cell.

**top middle:** pulse 60 minutes, no chase - heavy staining of one pole of cells plus diffuse cytoplasmic staining. This cell was much brighter than after 6 minutes pulse (top left). To be recorded at a similar intensity, the original image was recorded at an eighth of the exposure (**top right**), and the final image intensified 8-fold, hence its granularity.

**bottom left:** pulse 6 minutes, chase 30 minutes - minimal punctate staining remains.

**bottom middle:** pulse 60 minutes, chase 30 minutes - punctate staining, mainly in one pole of the cell, much heavier than after 6 minutes pulse (bottom left).

Selected cells were mIg*'. Images were confocal to ≤1μm, taken through the widest part of the cell. Scale bar (**bottom right**) = 10μm.
Figure 13: Volume of LY in early and late endosomal compartments of four different APCs

Volumes per cell are the projected maximal filling, assuming the kinetics of filling are the same as those of emptying. (For calculation of $E_{\text{max}}$ and $L_{\text{max}}$ see text).

Values are the means of three experiments (A20 cells and resting B cells) or two experiments (activated B cells and dendritic cells).

Error bars show one standard deviation.
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Table 2: Kinetics of endocytic traffic of LY in four APC types

Cell diameter was obtained by comparison of forward scatter from flow cytometry to A20 cells, which were measured directly.

Half-lives were obtained as in figure 8.

% early routed to late was calculated from: \( \frac{100 \times LE_{\text{max}} \times t_{\frac{\text{EE}}{2}}}{EE_{\text{max}} \times t_{\frac{\text{LE}}{2}}} \) (see appendix)

Traffic of LY through each endosomal compartment was calculated as: \( 60 \times \text{size} \times \ln 2 / t_{\frac{1}{2}} \).

% cell volume occupied by endosomes was calculated assuming cell volume = \( \pi/6 \times d^3 \).
CHAPTER 4. CELL-FREE STUDIES OF THE ENDOCYTIC AND INTRACELLULAR MHC II PATHWAYS

Introduction. The endocytic and MHC II pathways were studied in disrupted A20 cells. Other, similar studies have used non-APC types, including HeLa cells (Lotteau et al., 1990) and fibroblasts (Bakke and Dobberstein, 1990; Romagnoli et al., 1992). These are easier to study because of their greater size and lower nuclear-to-cytoplasmic ratio. These cells have been transfected with α/β and/or Ii. However, transfected cells do not always function identically to natural APCs (Salamero et al., 1990). Therefore, A20 cells have the advantage of being a more natural APC, although their malignant phenotype may have an effect on antigen processing. A20 cells constitutively express high levels of Ia\(^d\), and process exogenous antigens for presentation (for example: Shimonkevitz et al., 1983; Eisenlohr et al., 1988; Shaw and Chain, 1989; Bikoff, 1992; Hunt et al., 1992b; Poirier, 1992). The specificity of mIg expressed by A20 cells is unknown, therefore all nominal antigens are handled equally for processing. Another advantage of using A20 cells in cell-free studies is that results can be correlated with other studies on their enzymology (Bennett et al., 1992; Rodriguez and Diment, 1992).

In chapter 3 it was shown that the endocytic pathway of A20 cells was kinetically similar to the endocytic pathways of macrophages and other APCs including dendritic cells. Here, A20 cells were homogenised and membrane vesicles centrifuged into density gradients. In the first instance it is important to ascertain that the system yields intact vesicles from the organelles under study, in this case the MHC II\(^{+ve}\) elements of early and late endosomes and lysosomes. The origin of endocytic vesicles must be apparent after disruption of the APC. In addition, MHC II within the vesicles must be identifiable. This chapter is divided into two sections, in which A. the endocytic pathway, and B. the intracellular pathway of MHC II are characterised.

A. CHARACTERISATION OF ENDOCYTIC VESICLES FROM A20 CELLS
The endocytic pathway of A20 cells was studied to determine whether the expected division into early and late endosomes and lysosomes could be made after cell homogenisation. Early endosomes are commonly defined biochemically by the presence of transferrin receptors (TfR), which cycle rapidly between early endosomes and the cell surface. Labelling with transferrin (Tf) does not alter the rapid (≤10 minutes), efficient
(≥99%) recycling of TfR via clathrin coated vesicles, the limiting membrane of MVBs, tubular extensions from MVBs, early endosomal tubular reticulum, and recycling early endosomes. Labelling with Tf has the advantage that label remaining on the cell surface can be easily stripped.

The lag for antigen processing of 45-60 minutes seen in human and murine B cells (Roosnek et al., 1988; Harding and Unanue, 1989) indicates that the antigen processing compartment lies beyond early endosomes and may therefore be a specialised late endosome, as suggested by Peters et al. 1991. Therefore, the approach used in this study to identify late endosomes was to pulse-chase with the fluid-phase marker horse radish peroxidase (HRP). The timing of the pulse and chase were designed to commence filling of late endosomes, to prevent filling of lysosomes and to empty out early endosomes. Conformation of the results obtained here might be obtained using endogenous markers located in late endosomes, such as mannose-6-phosphate receptors or rab7.

A20 cells have been shown to have relatively few dense lysosomes (I. Olsen, pers. comm.). Those which are present can be identified by the large number of acid hydrolases, such as β-hexosaminidase. This enzyme has been found outside the classical lysosomal distribution, for example in BHK cells (Waheed et al., 1988), indicating residence in a pre-lysosomal compartment. Such β-hexosaminidase is relevant for antigen processing, since the enzyme was found in both late endosomes and MHC II-rich organelles in human B cells (Peters et al., 1991). Therefore, the β-hexosaminidase⁽ vesicles in A20 cells which are not dense lysosomes are of interest.

**Disruption of A20 cells.** Cells in long-term culture are more difficult to disrupt than ex vivo cells (Howell et al., 1989b). For lymphoid cells such as A20 cells, the difficulty is increased because of their small size. Thus, the conditions required to disrupt cells by nitrogen cavitation were so severe as to leave few intact endosomes. Therefore, A20 cells were disrupted more gently, by repeated passage through a 5μm clearance in a steel homogenising chamber. The efficiency of disruption as monitored by trypan blue was 80-95%. Disruption produced some trypan blue⁻ vesicles 1-2μm in diameter, and large numbers of particles less than 1μm in diameter which were barely detectable by phase microscopy.
Rapid separation of vesicles from 3 endocytic compartments by density gradient centrifugation. Post-nuclear supernatant (PNS) of A20 cells was layered on top of, and centrifuged into, a 7.4ml self-forming gradient of 28% percoll, which was then divided into 10 fractions, from the bottom upwards. Molecules not associated with membrane vesicles remained in the top two fractions, whereas membrane markers entered the gradient. Figure 14 shows the profile of A20 plasma membrane, after labelling cells with $^{125}$I-Tf at $4^\circ$C. $^{125}$I peaked in fraction 7. In contrast, FITC dextran added to PNS did not associate with any membranes, and remained in fractions 9 and 10 (figure 14). Since plasma membrane produces low density vesicles, the gradient clearly separates low density vesicles from cytosol.

A20 cells were double labelled with $^{125}$I-Tf in early endosomes only, and HRP in late endosomes. Cells were disrupted and the PNS separated from nuclei and intact cells. The PNS was spun into a self-forming percoll gradient, thus separating membrane-associated marker (fractions 1-8) from free marker (fractions 9 and 10). The profiles of early endosomal, late endosomal and lysosomal markers, using endogenous $\beta$-hexosaminidase as the lysosomal label, were obtained. The profiles were reproducible and characteristic (figure 15a). Results with one label were not affected by the presence of the other labels. The proportion of the markers in fractions 9 and 10 was: $^{125}$I-Tf - 30%, HRP - 40%, $\beta$-hexosaminidase - 15%; this amount of marker had leaked into the cytosol during disruption. The results are similar to other systems where 30-60% of fluid-phase HRP and lysosomal enzymes were released during cell homogenisation (Galloway et al., 1983; Diment and Stahl, 1985; Ukkonen et al., 1986). In addition, the profile of galactosyltransferase, a marker for the trans-Golgi and TGN (Nilsson et al., 1993), was determined. The distribution of this enzyme was similar to that of TfR (see figure 37e).

Since different proportions of each marker were in the top fractions, the profiles of membrane-associated marker are not immediately comparable. Therefore, the profiles were rescaled so that fractions 1 to 8 totalled 100% (figure 15b). Lysosomes (peak fractions 1 to 4) were denser than early endosomes (peak fractions 6 and 7) with some overlap. Late endosomes had intermediate density (peak fractions 2-7), overlapping to a large extent both other compartments. Early endosomes were marginally more dense than plasma membrane vesicles (see figure 14), as in other cell types (Mellman et al.,
For all of $^{125}$I-Tf, HRP and $\beta$-hexosaminidase, the nuclear pellet formed after disruption contained 20-30% of the label associated with membranes. This is similar to other data on cells in long-term culture where up to half of a marker is lost in the nuclear pellet (Howell et al., 1989a). This is caused by binding of organelles to the cytoskeleton, which collapses around the nucleus, and which is more developed in immortalised cells than in cells ex vivo.

The integrity of endocytic vesicles is maintained. In a cell-free system, it is important that the vesicles formed are intact, so their normal functions can be maintained. The degree of damage was determined from the latency of enzyme within each endocytic compartment. Latency of enzymes, an historic milestone in the discovery of lysosomes (de Duve, 1975), occurs when enzymatic activity in cell lysates appears on the addition of detergent, or after many hours at 4°C. Latency therefore indicates that enzymes are retained within intact membrane vesicles.

All percoll fractions were assayed for enzyme activity with and without addition of detergent, thus measuring the accessibility of enzyme to a substrate molecule, weight less than 500 Da. Early endosomes were studied using Tf-HRP in place of $^{125}$I-Tf. In the top two fractions none of the enzymes were latent, which confirms the presence of cytosol without endocytic vesicles. The latency of the three endocytic markers in other fractions was: Tf-HRP 60%, HRP 60%, and $\beta$-hexosaminidase 85%. Latency was unaffected by overnight incubation at 4°C.

For early endosomes, the label used was membrane-bound, so leakage would be unlikely to occur even if the organelle were broken open. However, after a 5 minute pulse with Tf-HRP the majority of the marker was found in the top 2 fractions, with the membrane-associated marker in fractions 1-8 showing the expected density and latency (figure 16a). The HRP present in fractions 9 and 10 was shown to be free HRP mixed in with the Tf-HRP hetero-conjugate. Free HRP adhered to the cell surface non-specifically, but its low affinity binding was highly reversible. Hence, if the 5 minute pulse of Tf-HRP was followed by a 5 minute chase, free HRP was lost, but Tf-HRP was not. The proportion
of Tf-HRP in the top fractions was much reduced by this 5 minute chase (figure 16b).

Tf in fractions 9 and 10 may derive from cell surface molecules that dissociate from TfR after cell disruption, rather than in the preceding wash steps. Leakage of early endocytic marker during disruption was therefore assessed using fluid-phase HRP endocytosed for 2 to 3 minutes. 40% of this HRP was in the cytosol layer. Therefore, early endosomes and late endosomes tended to break open to a similar extent, which was greater than for lysosomes. This may be explained by the complex, reticular nature of these compartments, and implies that the vesicles formed by cell disruption might not carry out all functions of the intact organelle.

When a low molecular weight fluid-phase marker was used in place of HRP to ascertain leakage from late endosomes during cell disruption a different result was obtained. After 60 minutes endocytosis of [14C]-sucrose, which is then mainly in late endosomes (see figure 7), 90% of label was released from membranes during cell disruption. This was determined by gel filtration through sepharose S2B, rather than by centrifugation. The disparity between sucrose and HRP may result from the difference in molecular weight, indicating that small holes, which may be short-lived, are created in late endosomes when A20 cells are disrupted.

HRP traffics through endocytic vesicles with increasing density. Endocytosis involves the movement of molecules in the fluid-phase through a series of organelles of increasing density (Storrie et al., 1984; Murphy, 1991). To investigate this traffic in A20 cells, HRP was endocytosed for increasing periods, in all cases the pulse being followed by a chase period to allow dissociation of a vast excess of HRP non-specifically adhering to plasma membrane. Figure 16 shows the effect of a 5 minute chase after a 5 minute pulse with less than 0.04mg/ml free HRP. HRP at 5 mg/ml completely obscured internalised label unless a chase period was included. For late endosomal filling, the chase period was also required to allow early endosomes to empty.

After endocytosis for 2-3 minutes, HRP was in low density vesicles, peak fraction 6 (figure 17). This confirms that endosomes defined as early on kinetic grounds have the same density as early endosomes defined by the presence of TfR. After 6-8 minutes
endocytosis HRP was in vesicles of intermediate density, peak in fraction 5 with a significant amount in fractions 2-4. On kinetic grounds, it would be expected that marker at this stage would still be in early endosomes (see chapter 3). Therefore, this finding indicates either that HRP entered late endosomes more rapidly than RD and LY (chapter 3), or that there is a sub-compartment of early endosomes with increased density. After the longest period of endocytosis, 15-75 minutes the peak of HRP was in high density vesicles, peak in fraction 3 (compare with β-hexosaminidase, peak in fraction 2). On kinetic grounds, this HRP would be expected to be mainly in late endosomes, with some entry into lysosomes.

In the light of the above findings, the flat profile of HRP after 15-30 minutes of endocytosis (figure 17) can be explained as showing a mixture of label in both intermediate density vesicles, as seen after 6-8 minutes, and high density late endosomes and lysosomes, as seen after 15-75 minutes.

The profile of cell-derived protein on percoll gradients. The profile of protein on the percoll gradient was similar whether measured colorimetrically (Bradford, 1976) or by long-term incubation with low levels of ^S-amino acids, followed by a 1 hour chase (figure 18). The peak of protein was in fractions 5 to 7, which indicated that the majority of membranes were light. Light membranes include Golgi, ER and plasma membrane.

Difficulties were incurred for both techniques. The colorimetric assay required the lysis of vesicles to allow removal of percoll from each sample. In addition, values obtained were at the limit of sensitivity for the assay. For ^S labelling, the proportion of label in fractions 9 and 10 was 65%. This was lower than the result obtained by gel filtration of PNS from ^S labelled cells, where 94% of label was in the cytosol. In addition, the fraction of protein in the cytosol as measured by colorimetry was 90%. Therefore, it is likely that ^S entered the gradient in a form not associated with membrane vesicles, although the mechanism for this is not known. Whatever the mechanism, this renders suspect the use of ^S as a marker for protein.

The overlap seen on percoll gradients between early endosomes and lysosomes is partly a function of gradient's size. The small overlap between early endosomal and lysosomal
markers on the percoll gradient (figure 15b) might indicate that the protocol used to form a gradient is inadequate to achieve complete separation. To investigate this, membranes were also separated on a 30ml percoll gradient, i.e. four times larger. The density profile of the small and large gradients was quite similar (see figure 1). The overlap between $^{125}\text{I-}\text{Tf}$ and $\beta$-hexosaminidase was slightly reduced on the larger gradient (figure 19). Therefore, the conditions under which the gradient formed made a slight difference to the separation of markers. The persisting overlap of lysosomal and early endosomal markers in the intermediate fractions of this gradient was also seen in some previous studies using the same gradient (Galloway et al., 1983). However, in other experiments a different form of overlap was seen, with a minor amount of lysosomal marker in low density vesicles (Diment and Stahl, 1985). The different types of overlap might be explained by differing properties of endosomes from different cell types. The overlap seen in A20 cells may reflect their relative lack of true lysosomes.

**Early endosomes have sub-populations with distinct densities.** The peak of $^{125}\text{I-}\text{Tf}$ on the percoll gradient demonstrates that early endosomes are mostly light vesicles (figure 15b). However, 5-10% of $^{125}\text{I-}\text{Tf}$ was found in fractions 1-4. This sub-population may represent an artefact due to poor resolution by the density gradient. However, the use of a larger gradient did not alter this finding (figure 19). This finding was further tested by centrifuging PNS into one percoll gradient, and then re-centrifuging membranes from individual percoll fractions into a second gradient. As shown in figure 20, $^{125}\text{I-}\text{Tf}$ from the low density peak (fraction 6) of the first gradient re-entered fraction 6 in the second gradient. In comparison, $^{125}\text{I-}\text{Tf}$ from the intermediate density tail (fraction 4) mainly re-entered intermediate density fractions, and $^{125}\text{I-}\text{Tf}$ in the lightest vesicles (fraction 8) predominantly re-entered that position. Therefore, the location of individual vesicles on the percoll gradient is a permanent feature of those vesicles, and not an artefact. This implies that there is a sub-population of early endosomes (TfR$^{+ve}$ vesicles) which can be identified by its higher density.

**Transferrin recycles through vesicles in fractions 1-4.** Endocytic vesicles with intermediate and high density are potentially important in antigen processing. The 5-10% of TfR would not imply a role for early endosomes in antigen processing unless this proportion of TfR were shown to be in the recycling pool. The alternative would be that
these TfR represent traffic to lysosomes for degradation. *A priori* this is unlikely, since $^{125}$I-Tf labelling was for 15 minutes, during which time a single episode of recycling would have been completed, delivering only $\leq 1\%$ of TfR for degradation (Octave et al., 1981).

To determine whether TfR in fractions 1-4 were recycling, the density profile of $^{125}$I-Tf after a 5 minute pulse was compared to the profiles after chase of this label for upto 90 minutes and after a continuous pulse for 80 minutes (figure 21). During the chase, the absolute amount of $^{125}$I-Tf initially decreased rapidly. The proportion of $^{125}$I-Tf in fractions 1-4 rose from 7 to 20%. The longer pulse increased total cell labelling by 25%, with 6% of label in fractions 1 to 4. These results indicate that Tf rapidly saturates binding to TfR, and is rapidly lost. Some selective retention of TfR in fractions 1-4 was seen only after prolonged chase, not after prolonged pulse. The significance of this result is unclear, but it may indicate a subtle difference in recycling of TfR through vesicles which enter fractions 1 to 4 compared to majority of TfR. Therefore, the overall finding was that the majority of TfR in fractions 1 to 4 is recycling.

**Conclusions.** The main conclusions of the studies of the endocytic pathway presented here are that the APC cell line A20 yielded intact endocytic vesicles with similar properties to other cell types. Vesicles could be adequately separated according to density on a short percoll gradient. There were sub-populations of early endosomes with a different density from the main bulk of early endosomes. It was not possible to say whether this sub-population of vesicles, and possibly similar sub-populations in the other two compartments, differed from the main population because of a discrete separation of function, or because of an extreme of normal variation within a unified population.
Figure 14: Separation of cytosol and membranes on a percoll gradient

PNS from A20 cells, which had been surface labelled with $^{125}\text{I}$-Tf, was centrifuged into a self-forming percoll gradient. FITC-dextran was added to PNS as a marker of free, i.e. non-membrane bound, molecules in the cytosol. Fraction 10 contained 90% of FITC-dextran.
Figure 15: Profiles of endocytic markers on a percoll gradient

The percentage of internalised transferrin, HRP and β-hexosaminidase in each fraction was calculated in two different ways:

(a) total fractions 1 to 10 = 100%
(b) fractions 1 to 8 (membrane-associated marker) = 100%
Figure 16: Distributions of HRP and Tf-HRP after pulse with or without chase
A20 cells were pulsed for 5 minutes, followed by:
(a) no chase. Fractions 9 and 10 contain >50% of total HRP, indicating the retention of free HRP.
(b) chase for a further 5 minutes. Fractions 9 and 10 contain 20% of total HRP. Free HRP has been lost.
Figure 17: Position of a fluid-phase marker according to duration of endocytosis

HRP was endocytosed for 1 to 60 minutes by A20 cells, which were then washed and chased in HRP-free medium for a further 2 to 15 minutes. Thus, the times for which cells had been exposed to HRP prior to disrupting the cells was 2-3 minutes, 6-8 minutes, 15-30 minutes and 15-75 minutes.

β-hexosaminidase values are the mean of three separate experiments (error bars show one standard deviation).

Values have been rescaled so that fractions 1 to 8 total 100%.
Figure 18: Profiles of membrane-associated protein, measured by two different techniques
Values have been rescaled so that fractions 1 to 8 total 100%. Fractions 9 and 10 are not shown. These fractions contained 70% and 90% of total protein measured by $^{35}$S incorporation and colorimetry respectively.
Figure 19: Overlap between early endosomal transferrin and β-hexosaminidase as a function of gradient size.

Overlap of early endosomal marker (internalised $^{125}$I-Tf) and lysosomal marker (β-hexosaminidase) occurred between fractions 4 and 7 of the small (7.4ml, 10 fractions) gradient. The overlap was reduced, but nonetheless present, in the large (30ml gradient, 28 fractions - rescaled to fit the same axes as the small gradient).
Figure 20: The range of densities displayed by early endosomes
PNS from A20 cells, the early endosomes of which had been labelled with \(^{125}\)I-Tf, was centrifuged into a percoll gradient. Membranes in fractions 4, 6 and 8 from this original gradient were re-centrifuged into three further gradients.
Figure 21: Assessment of recycling of $^{125}$I-Tf through dense vesicles

Cells were pulsed with $^{125}$I-Tf for 5 or 80 minutes, and stripped of surface label. Cells pulsed for 5 minutes were also chased for 10, 30 and 90 minutes in the absence of label, in the presence of deferoxamine. PNS from each group of cells was centrifuged into a percoll gradient. The values given are:

(a) total membrane-associated $^{125}$I-Tf (fractions 1 to 8), plus solid shading = $^{125}$I-Tf associated with high density membranes (fractions 1 to 4).

(b) $^{125}$I-Tf on high density membranes as a percentage of total.
B. INTRACELLULAR PATHWAY OF MHC II IN A20 CELLS

The intracellular traffic of proteins can be studied using disrupted cells which are fractionated by the techniques developed above. The need to identify MHC II from the outside of intact intracellular vesicles (see chapter 5) required the development of an antibody to MHC II's cytoplasmic domain, which was developed, purified and shown to be MHC II specific. Other findings regarding the intracellular pathway of MHC II were: MHC II traffic was found to be similar to other APCs, although few complex dimers formed during the delay in export to the cell surface, a finding seen in other APCs expressing I-A\* (Germain and Hendrix, 1991); the density distribution of MHC II on percoll gradients was compared to that of endocytic markers: total cellular MHC II was found in vesicles of all densities, with a similar density to plasma membrane; a cohort of MHC II approximately 80 minutes post-synthesis was found in vesicles of slightly higher density than this; these vesicles may be the site of antigen processing.

Recognition of I-A\(\alpha\) by antiserum to a peptide from its cytoplasmic domain. The cytoplasmic domain of I-A\(\alpha\) contains 15 residues. The sequence is conserved for most haplotypes (Kaufman et al., 1984), and is dissimilar to I-E\(\alpha\) (figure 22). A peptide consisting of the C-terminal 12 amino acids of I-A\(\alpha\) was coupled to KLH as carrier, and rabbit antiserum was raised. The serum was then affinity-purified on peptide coupled to BSA to yield anti-\(\alpha_{\text{cyt}}\). The activity of the serum as detected by ELISA was completely blocked by excess peptide. In addition, the ELISA assay was used to estimate the affinity of anti-\(\alpha_{\text{cyt}}\) for the peptide.

To confirm the specificity of anti-\(\alpha_{\text{cyt}}\), the plasma membrane of A20 cells was lactoperoxidase-labelled with \(^{125}\)I. Two bands were immuno-precipitated, corresponding to I-A\(\alpha\) and B (apparent molecular weights 31 and 37 kDa, figure 23, lane 1). TIB120, a monoclonal antibody specific for both I-A\(d\) and I-E\(d\) (ATCC), precipitated the same two bands (lane 2). The effect of boiling on MHC II precipitated by TIB120 was shown (compare lanes 2 and 3): a band at 60 kDa seen in the unboiled sample (lane 3) was not seen in the boiled sample.

To look at intracellular MHC II, metabolic labelling was undertaken. In the first instance it was shown that the specificity for \(\alpha/\beta\) was maintained. A20 cells were pulsed briefly
with $^{35}$S-amino acids and then chased overnight, so that all MHC II had reached the cell surface as $\alpha/\beta$ (see below). Precipitation with anti-$\alpha_{cyt}$ produced the expected two bands (figure 23, lane 4). The predominance of I-AB reflected the higher number of sulphur containing residues (3 methionines and 5 cysteines) compared to I-A$\alpha$ (2 methionines and 3 cysteines). Using A20 cells metabolically labelled for 30 minutes and chased for 60 minutes, anti-$\alpha_{cyt}$ precipitated three bands (lanes 5 and 6), as did TIB120, though $\alpha$ and $\beta$ were wider (lanes 7 and 8). The third, intermediate band (apparent molecular weight 33) corresponded to $\iota$ (see below). The $\iota$ band was equally densely stained as $\alpha$ and $\beta$. Because of large number of sulphur-containing residues in $\iota$ (13 methionines and 1 cysteine) in comparison to $\alpha$ and $\beta$ (Kaufman et al., 1984), this indicates the precipitation of less $\iota$ than of $\alpha$ and $\beta$, i.e. the precipitation of $\alpha/\beta$ as well as $\alpha/\beta/\iota$. A band of 25 kDa was seen in lanes 7 and 8 (arrow), this probably corresponding to partially degraded $\iota$ still bound to $\alpha/\beta$ (Blum and Cresswell, 1988). This band is not seen in lanes 5 and 6, probably because less counts were loaded onto these lanes.

MHC II which had been precipitated by TIB120, denatured in SDS and re-precipitated by anti-$\alpha_{cyt}$ showed only the heavier of the bands, the $\alpha$ chain (lanes 9 and 10). Therefore, anti-$\alpha_{cyt}$ bound to I-A$\alpha$ in the context of both $\alpha/\beta/\iota$ and $\alpha/\beta$.

The above data show that anti-$\alpha_{cyt}$ binds to I-A after detergent lysis. However, the short cytoplasmic tail of I-A might be inaccessible when inserted in a lipid bilayer. Therefore, binding of anti-$\alpha_{cyt}$ to I-A in A20 membranes was also examined. $^{125}$I-anti-$\alpha_{cyt}$ was incubated with A20 cells which had been disrupted in the steel homogenising chamber as above. Gel filtration to separate membranes from cytosol showed that a proportion of anti-$\alpha_{cyt}$ bound to A20 membranes (data not shown). AK34 cells, which are MHC II-ve derivatives of A20 cells (Venkitaraman et al., 1987), were used to produce membranes by the same means. These MHC II-ve membranes were not bound by $^{125}$I-anti-$\alpha_{cyt}$. Therefore, anti-$\alpha_{cyt}$ bound to MHC II in A20 cell membranes.

In summary, anti-$\alpha_{cyt}$ recognises the polypeptide chain I-A$\alpha$ in all of the different complexes formed by MHC II.

**Precipitation of $\iota$ by the monoclonal antibody, In-1.** In-1 is a rat monoclonal IgG2b.
antibody (Koch *et al.*, 1982) specific for the cytoplasmic domain of murine Ii (Lipp and Dobberstein, 1986). The antibody's specificity was confirmed by immuno-precipitation from metabolically labelled A20 cells (figure 24, lane 2). Of the bands precipitated by In-1, Ii (apparent molecular weight 34 kDa) predominated, with minor bands for I-Aα and β. This shows that Ii was synthesised in excess over α and β in A20 cells as in other cell types (Cresswell, 1992). Other bands with molecular weight 21, 22 and 25 kDa were precipitated by In-1 (figure 24, lane 2, arrows). These are normal intermediates in Ii degradation (Blum and Cresswell, 1988; Pieters *et al.*, 1991). The 25 kDa band corresponds to the band seen in figure 23, lanes 7 and 8. To show that In-1 recognised Ii in the context of MHC trimers, In-1 was used to pre-clear a sample for subsequent precipitation by TIB120, which detected mainly α/β, with only a small amount of Ii, indicating the removal of most α/β/Ii (figure 24, lanes 3 and 4; compare with figure 23, lanes 5 to 8). The effect of boiling on this α/β is shown by comparison of lanes 3 and 4: a faint 67 kDa band seen in the unboiled sample (lane 4) was partially sensitive to boiling (lane 3).

**Post-synthetic modifications to MHC II in A20 cells.**

(1) **Changes in molecular weight.** A20 cells were metabolically pulse-chase labelled, the chase period varying from 0 to 18 hours, before precipitation of I-A*αβ* by anti-α*αβ* (figure 25). During the first hour of chase there was an apparent increase in molecular weight for β chain. Over this time the Ii band became stronger with an apparent decrease in molecular weight for Ii. The changes in molecular weight for β and Ii are likely to reflect carbohydrate modifications during passage through the Golgi. The increased amount of Ii detected might indicate that Ii adopted a configuration with higher affinity for In-1 after synthesis. Over the next 2 hours there was an apparent increase in molecular weight for α chain. The mechanism for this change is unclear, but may reflect increased sialylation of MHC II as it recycles through the TGN.

(2) **Dissociation and degradation of Ii.** The dissociation of Ii was barely detectable at 2 hours chase (figure 25, lane 3), was approximately half complete at 3 hours chase (lane 4), continuing at 4 hours chase (lane 5), and had reached completion by 18 hours chase (lane 6).
It has been noted previously that \( \text{II} \) is more sensitive to proteolytic degradation than \( \alpha \) and \( \beta \) (Blum and Cresswell, 1988). A similar observation for \( \text{I-A}^d \) from A20 cells was made by mild proteolysis of pulse-chase \( \alpha/\beta/\text{II} \) samples. \( \text{II} \) was completely degraded by low levels of pronase into products of 24 kDa and \( \leq 12 \) kDa (figure 26). The selective action of pronase was shown by the partial resistance of \( \alpha, \beta \), and Ig to this treatment.

(3) Turnover of MHC II. The amount of cellular \( \alpha \) and \( \beta \) did not decrease significantly during 1-4 hours chase, while \( \text{II} \) decreased to 50% of its original level (by densitometry). Thereafter \( \alpha/\beta \) levels declined only slowly (see below). This indicates that most \( \alpha/\beta \) in A20 is destined to be expressed on the cell surface. This differs from the finding for \( \text{I-A}^k \) in CBA spleen cells, a sizeable proportion of which was rapidly degraded while the remainder formed compact dimers (Germain and Hendrix, 1991).

The half-life of MHC II in A20 cells was estimated by densitometry of the \( \beta \) bands in figure 25 as \( \approx 20 \) hours. Therefore, the average life-span of \( \alpha/\beta \) in A20 cells is \( \approx 30 \) hours (from: \( k = \ln 2 / t_\frac{1}{2} \)). The life-span of \( \alpha/\beta/\text{II} \) in the processing compartment of A20 cells was estimated as \( \approx 2\frac{1}{2} \) hours, assuming a Golgi transit time of 30 minutes, and average export of \( \alpha/\beta \) to the surface 3 hours post-synthesis calculated indirectly from the dissociation of \( \text{II} \) (figure 25), and also directly - see below). On the basis of the duration of stay in each compartment, the proportions of MHC II in the exocytic (pre-processing) pathway, intracellular (processing) compartment(s), and cell surface (post-processing) plus recycling (possible epitope exchange) compartment approximates to 2%, 8%, and 90% respectively. The minor population (\(< 10\%) in the intracellular compartment is the focus of antigen processing research.

(4) Formation of compact dimers. A band at 67 kDa was seen after 18 hours chase after boiling (lane 6, arrow). The nature of this band is unclear. Its appearance after 18 hours chase but not 1-4 hours chase indicates the band may be compact \( \alpha/\beta \) dimers. Although boiling might be expected to entirely denature \( \alpha/\beta \) dimers, similar partial resistance to boiling has been demonstrated elsewhere (Davidson et al., 1991). However, the size is larger that SDS-resistant compact dimers seen elsewhere, molecular weight \( \approx 60 \) kDa (Sadegh-Nasseri and Germain, 1991).
Other experiments detected bands which might be compact dimers in A20 cells. MHC II on the cell surface and in the endocytic pathway yielded a band of approximately 60 kDa which was seen only in the unboiled sample (figure 23, lanes 2 and 3). This band was of similar density to the remaining MHC II bands, indicating the presence of roughly half of surface MHC II in this form. In addition, MHC II dimers 2-3 hours post-synthesis showed a band of 67 kDa which was partially sensitive to boiling (figure 24, lanes 3 and 4). In these experiments sample buffer contained a high concentration of 2-mercaptoethanol. Although it is possible that this would denature compact dimers by reducing intra-chain disulphide bonds (Dornmair and McConnell, 1990), such denaturation has not been detected in other experiments (Germain and Hendrix, 1991).

Further supporting evidence for the relevance of the high molecular weight band seen after 18 hours chase was obtained by comparison with dimers created by in vitro loading of peptide (see Chapter 6). Therefore, it is possible that I-A^d α/β/peptide complexes were detected as dimers with a stable conformation.

(5) Other high molecular weight complexes. The 75 kDa band (figure 23, lanes 7 and 8, and figure 24, lanes 3 and 4) was specifically isolated by TIB120, since it was much less prominent in parallel control precipitations with anti-α_m (figure 23, lanes 5 and 6) or negative serum and In-1 (figure 24, lanes 1 and 2). The nature of this band was not investigated further, but it could possibly be the chaperonin, PBP72-74 (DeNagel and Pierce, 1992), or β trimers (Gorga et al., 1987).

MHC II is retained inside A20 cells for at least 2 hours. Beyond the TGN, MHC II is diverted from the exocytic pathway taken by the bulk of cell surface proteins such as MHC I and mIg. MHC II remains inside the cells for several hours (Neefjes et al., 1990; Peters et al., 1991). Indirect evidence for this delay, during which II dissociates and peptides derived from the endocytic pathway bind, was seen above (figure 25).

Direct measurement of MHC II export cells was obtained from pulse-chase metabolically labelled A20 cells. In control cells, mIg was precipitated with protein A-sepharose, showing IgL and IgH at constant amounts from 0 to 3 hours chase (figure 27). Note that IgL appeared as a doublet of molecular weight 30 and 31 kDa close to where I-AB would
be seen. In the test cells, cell surface MHC II was identified by incubating whole cells with polyclonal rabbit antiserum prior to lysis, followed by precipitation by protein A-sepharose of both mIg and surface MHC II bound by rabbit antibody. Beyond 2 hours chase surface MHC II was detected as a faint band at 34 kDa and a broad smear upto 38 kDa (lanes 6 and 8 compared to 5 and 7). This is the expected pattern of MHC II (bands at 31, 32 and 34 kDa, and a smear from 34 to 38 kDa (figure 27, lane 9) given that IgL obscured the 31 and 32 kDa bands. One reason for the faintness of MHC II bands may have been that freeze-thawing dissociated a large proportion of MHC II-antibody complexes. This was confirmed by further precipitation with TIB120 of the supernatants remaining after protein A-sepharose treatment, which demonstrated almost equal amounts of MHC II in control and test samples.

These data confirm that MHC II resides in the cell for at least 2 hours prior to export to the cell surface. The low sensitivity of this assay does not exclude the possibility that α/β/II trafics from the TGN to the cell surface before being rapidly internalised into the endocytic pathway. The exact pathway taken by MHC II could be more clearly determined by metabolic labelling and chase at ≤20°C, at which temperature export from the TGN is blocked in many cell types (Griffiths and Simons, 1986). On release of the block the wave of exported molecules might be followed with relative ease.

At steady state MHC II resides mostly in vesicles of low density. To determine the nature of vesicles containing MHC II, A20 cells were metabolically labelled for 16 hours with ^35S-amino acids followed by a one hour chase, thereby labelling MHC II throughout the biosynthetic pathway. PNS was spun into a percoll gradient and fractions taken (note: 11 fractions rather than 10). MHC II was precipitated from individual fractions by anti-α cyt (figure 28). The peak of MHC II was in fractions 6-8. This low density peak coincides with the position of plasma membrane, and therefore confirms that ≈90% of MHC II is on the plasma membrane or possibly recycling into endosomes. After longer exposure of the gels, it was shown that MHC II was also in the dense fractions 1 to 4. Therefore, the intracellular pathway of MHC II in A20 cells produces vesicles with a range of densities.

MHC II in fractions 1-4 does not show a higher II content than seen in fractions 6 to 8
(figure 28), which indicates that it is not all α/β/II in processing compartment(s), but that some of this MHC II is recycling through endosomes after leaving the processing compartments. Note that plasma membrane vesicles would not be detected in fractions 1 to 4, while early endosomes would (see figures 14 and 15).

Interestingly, MHC II was also precipitated from the cytosolic fractions (10 and 11), in which II was relatively more prominent. It is unlikely that this MHC II has been cleaved from the membrane, since the loss of the trans-membrane and cytoplasmic domains would render the molecules 3-4 kDa lighter. One possible explanation of this phenomenon is that a compartment of the exocytic pathway forms very low density vesicles which do not enter the percoll gradient. Similar very low density vesicles have been shown to be derived from a transport vesicles between ER and Golgi (Lodish et al., 1987), and TGN-derived vesicles (Cutler and Cramer, 1990; Stoorvogel et al., 1991). The position of these vesicles in the MHC II pathway remains unclear, but they are likely to be in the exocytic pathway since some MHC II was found in similar very low density vesicles 70-90 minutes after synthesis (see figure 29, fraction 9).

**Newly synthesised MHC II resides in vesicles of intermediate density.** MHC II**<sup>+</sup>** vesicles in fractions denser than plasma membrane may not be of direct relevance to the antigen processing pathway if they contain recycling α/β only. To study α/β/II in the processing compartment(s), a similar experiment to that described above was performed on a cohort of newly synthesised MHC II molecules. Metabolic labelling was for 20 minutes with a chase of 70 minutes. The overall density profile was similar to that seen after labelling the entire biosynthetic pathway (peak fractions 5 to 7, figure 29). However, α/β/II was distributed differently from α/β. Comparison of the II bands and β bands showed that II predominated in fraction 5, while β predominated in fraction 7, and II and β were equivalent in fraction 6 (figure 29). Therefore, a large proportion of labelled molecules were α/β/II residing in vesicles with intermediate density (fractions 5 and 6, not 7).

The remaining MHC II was in the form of α/β after II dissociation, and was in vesicles with the characteristic density of plasma membrane. At steady state these molecules form the vast majority of MHC II, thus explaining why the α/β/II-rich fractions were not detected in the previous experiment. The dissociation of II at 70-90 minutes post-
synthesis is earlier than was evident from precipitation of whole cells (figure 25, and see above). This indicates that the use of an extra dimension (density of vesicle) can lead to a gain in sensitivity to small changes in MHC composition.

The vesicles containing α/β/Ii might be enriched by their characteristic density. The enrichment of α/β/Ii at a density between that of early endosomes and late endosomes (figure 15) is significant vis-à-vis antigen processing because it has been proposed that α/β/Ii resides in a single (possibly specialised) compartment from which recycling α/β is excluded (Peters et al., 1991). The co-localisation of endocytic markers and α/β±Ii in the same vesicle is discussed in the next chapter.
Figure 22: Structure and sequence of murine MHC cytoplasmic domains

The domain structure of MHC class II and class I antigens is given together with the amino acid sequence of the cytoplasmic domains. The sequence given for I-Aα applies to haplotypes k, d, b, f and u.

Residues have been identified using the single letter code: A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr.
Figure 23: Precipitation of MHC II by anti-α<sub>cyt</sub>

**Lanes 1-3:** Surface iodinated MHC II precipitated by anti-α<sub>cyt</sub> (lane 1) and TIB120 (lanes 2 and 3). Samples in lanes 1 and 2 had been boiled, the sample in lane 3 had been incubated at room temperature only. More ¹²⁵I was loaded into lane 2 than the other lanes. Arrow indicates band at 60 kDa which is resistant to SDS but lost on boiling.

**Lane 4:** Metabolically labelled MHC II (pulse 20 minutes, chase 18 hours) precipitated by anti-α<sub>cyt</sub>. Sample had been boiled.

**Lanes 5 to 8:** Metabolically labelled MHC II (pulse 20 minutes, chase 1 hour) precipitated by anti-α<sub>cyt</sub> (lanes 5 and 6) and TIB120 (lanes 7 and 8). Samples in lanes 6 and 8 had been boiled, samples in lanes 5 and 7 had been incubated at room temperature only. Arrow indicates 25 kDa band seen lanes 7 and 8, corresponding to a breakdown product of II (see text). Also note the high molecular weight band in lanes 7 and 8 (75 kDa).

**Lanes 9 and 10:** Metabolically labelled MHC II (pulse and chase both 1 hour) precipitated by TIB120 (lane 10), denatured by boiling in SDS, diluted out of high SDS, and re-precipitated by anti-α<sub>cyt</sub> (lane 9).

M.W. = molecular weight markers in kDa.
Figure 24: Precipitation and of Ii by In-1

Lanes 1 and 2: Metabolically labelled MHC II (pulse 1 hour, chase 3 hours) precipitated by normal rat Ig (lane 1), and In-1 lane 2. Arrows indicate degradation products of Ii at 21, 22 and 25 kDa. Samples had been boiled.

Lanes 3 and 4: Metabolically labelled MHC II (pulse 1 hour, chase 2 hours) pre-cleared once with In-1 directly coupled to sepharose. Remaining MHC II was precipitated with TIB120. The sample in lane 3 had been boiled, while the sample in lane 4 had been incubated at room temperature only. Arrow indicates a 67 kDa band seen in the unboiled sample only. Note high molecular weight band seen in both lanes 3 and 4 (75 kDa).

M.W. = molecular weight markers in kDa.
Figure 25: Post-synthetic modifications to MHC II in A20 cells

Metabolically labelled MHC II (pulse 20 minutes, chase - variable, see below) was precipitated with anti-α_cyt.

Lane 1 - chase 0 hours
Lane 2 - chase 1 hour
Lane 3 - chase 2 hours
Lane 4 - chase 3 hours
Lane 5 - chase 4 hours
Lane 6 - chase 18 hours, arrow indicates a 67 kDa band - see text

Lane 6 was run on a separate gel from the other samples. All samples were boiled. M.W. = molecular weight markers in kDa.
Figure 26: Proteolysis of MHC II

Metabolically labelled MHC II precipitated by anti-α, (lanes 1 and 2) and Ig (lanes 3 and 4) precipitated by protein A from A20 cells pulsed for 20 minutes without chase. Lanes 2 and 4 were treated with low levels of pronase (see methods), while lanes 1 and 3 were untreated. MHC II was degraded almost completely (lane 2), with preservation of β and partial loss of α. Ig was also degraded partially (lane 4). M.W. = molecular weight markers (200, 94, 69, 46, 30, 21 and 14 kDa).
Figure 27: Export of newly synthesised MHC II to the cell surface
Metabolically labelled Ig (lanes 1, 3, 5, 7) and Ig together with surface MHC II (lanes 2, 4, 6 and 8) were precipitated from A20 cells pulsed for 20 minutes and chased for 0 hours (lanes 1 and 2), 1 hour (lanes 3 and 4), 2 hours (lanes 5 and 6) and 3 hours (lanes 7 and 8). MHC II was detected after 2 to 3 hours only. The precipitation of Ig was more efficient than that of MHC II (see text). The pattern expected for MHC II is shown by precipitation of MHC II alone (lane 9).
Figure 28: Precipitation of total cellular MHC II from fractions of a percoll gradient

A20 cells which had been metabolically labelled long-term (pulse 18 hours, chase 1 hour) were disrupted and PNS centrifuged into a 7.4ml 28% percoll gradient. MHC II was precipitated by anti-α from all fractions except #1 (lane number = fraction number). Equal amounts of molecular weight markers (M.W. = 69, 46 and 30 kDa) were loaded on two gels - top = fractions 2 to 5, bottom = fractions 6 to 11. The top gel has been exposed for 4x as long as the bottom gel. Arrows indicate MHC bands. β is close to II* because of upward displacement by a large amount of unlabelled IgL present in the sample.

MHC II is seen in the fractions 10 and 11 (see text).
Figure 29: Precipitation of newly synthesised MHC II from fractions of a percoll gradient. A20 cells which had been metabolically labelled (pulse 20 minutes, chase 70 minutes) were disrupted and PNS centrifuged into a 7.28% percoll gradient. MHC II was precipitated by anti-α_{cyc} from all fractions except #1 (lane number = fraction number). MHC bands as in figure 28 - β* is close to I^* because of upward displacement by a large amount of unlabelled IgL present in the sample. Fractions 2 and 3 (not shown) showed fainter bands than fraction 4.

In fraction 5, I^* is more prominent than β* in fraction 7, I^* is less prominent than β* in fraction 6, I^* and β* are equally prominent.

More MHC II is seen in fraction 9 than in fraction 8 (see text).
CHAPTER 5. A CELL-FREE SYSTEM FOR ANTIGEN PROCESSING: IMMUNO-ISOLATION OF MHC II-POSITIVE ENDOSONES INCLUDING THE PROCESSING COMPARTMENT

Introduction. Immuno-isolation of subcellular compartments is a powerful technique to retrieve a sub-population of vesicles from a diverse mixture based on immuno-recognition (Richardson and Luzio, 1988; Howell et al., 1989a). The technique is particularly suitable if these vesicles cannot be separated from other compartments by conventional cell biological techniques, and if the vesicles have a unique label on their external surface, this label being identified by a specific antibody or lectin. Isolated vesicles can be used to perform experiments of 2 major types: (1) to co-localise other molecules in the isolated compartment (for example Sztul et al., 1991); (2) to perform the functions of the isolated compartment under controlled conditions, in the absence of traffic with other compartments (for example Gruenberg et al., 1989; Salamero et al., 1990b). In this chapter, immuno-isolation of the compartment(s) in which antigen processing occurs was undertaken, and the co-localisation of MHC II with endocytic markers in A20 cells was established.

The unique properties of the vesicles in which antigen processing occurs are not known, especially since a variety of such vesicles may exist. The identification of a vesicle as MHC II^{+ve} was used as the initial, broad selection criterion. MHC II^{+ve} vesicles were isolated following antibody recognition of the cytoplasmic domain of I-Aα. In brief, the following results were obtained: MHC II^{+ve} vesicles were shown to include a large number of early endosomes (Section A); conditions for the isolation procedure were optimised (Section B); parts of the entire endocytic pathway were identified among the population of MHC II^{+ve} vesicles (Section C); finally, the vesicles were shown to be enriched for recently synthesised α/β/Ii rather than recycling α/β (Section D).

A. IMMUNO-ISOLATION OF MHC II^{+ve} ENDOCYTIC VESICLES

A substantial minority of MHC II^{+ve} inverted vesicles have intermediate or high density. The MHC II^{+ve} vesicles which can be identified and isolated immunologically are those which contain MHC II on their limiting membrane. The MHC II vesicles of interest for processing are those which derive from intracellular compartments. These vesicles are
inverted compared to the normal orientation of MHC II on the cell surface. Therefore, the extracellular domain of MHC II is lumenal and the cytoplasmic domain of MHC II is external where it can bind to anti-\(\alpha_{cyt}\). In contrast, the majority of plasma membrane vesicles formed from A20 cells are right-side-out (see section C), and cannot bind anti-\(\alpha_{cyt}\). The only intracellular MHC II\(^{+ve}\) vesicles which cannot be detected by the binding of anti-\(\alpha_{cyt}\) are those in which MHC II is not present on the limiting membrane, but only on internal membranes. Although MHC II may be preferentially sorted onto the internal membranes of MVBs as seen for EGF receptor (Felder et al., 1990), this sorting is unlikely to be complete. In addition, It has been located on the limiting membrane of MVBs (Griffiths, 1992). Therefore, vesicles identified by anti-\(\alpha_{cyt}\) are likely to include all the relevant endocytic vesicles.

To determine the distribution of inverted MHC II\(^{+ve}\) vesicles across a density gradient A20 cells were lysed and PNS incubated with \(^{125}\)I-anti-\(\alpha_{cyt}\). Unbound antibody was removed by gel filtration, and labelled membranes were centrifuged into a percoll gradient. The distribution of antibody was not entirely consistent between experiments, but a substantial minority (30-40\%) of MHC II\(^{+ve}\) vesicles was in high or intermediate density vesicles (figure 30). Some of the \(^{125}\)I-anti-\(\alpha_{cyt}\) in low density fractions would be accounted for by inside-out plasma membrane.

**Anti-\(\alpha_{cyt}\) immuno-isolates MHC II\(^{+ve}\) intracellular vesicles on magnetic immuno-adsorbent.** To achieve immuno-isolation of the vesicles which were recognised by anti-\(\alpha_{cyt}\) (figure 30), these vesicles were separated by binding to a solid support immuno-adsorbent (ImAd) coated with anti-globulin. To avoid centrifugation of ImAd coated with attached vesicles, magnetic ImAd was used. This consists of nonporous, magnetic beads, 2.8\(\mu\)m in diameter, which are uniform in both size and content of magnetic material and are coated with affinity-purified sheep anti-rabbit IgG (Dynal).

Disrupted A20 cells were incubated with anti-\(\alpha_{cyt}\) mixed with a amount of \(^{125}\)I-anti-\(\alpha_{cyt}\). Removal of unbound antibody by passage through a size exclusion gel left 3\% of \(^{125}\)I bound to membranes in the void fraction. Aliquots of these membranes were mixed by rotation with serial dilutions of ImAd coated with sheep anti-rabbit IgG. After this incubation, ImAd was retrieved using a magnet, unbound membranes were removed, and
ImAd was washed briefly by resuspending and repeated retrieval to remove entrapped membranes. ImAd was then resuspended and rotated in buffer alone to remove non-specifically bound vesicles (see below). A maximum of 85% of the $^{125}$I-anti-α cyt was isolated using ≥1.5 x $10^6$ beads per $10^6$ A20 cells (figure 31). Therefore, membrane-bound anti-α cyt was efficiently immuno-isolated.

Having demonstrated that increasing the concentration of ImAd led to greater retrieval of MHC II$^{+ve}$ vesicles (figure 31), it was also shown that a fixed amount of ImAd retrieved a greater proportion of MHC II$^{+ve}$ vesicles when fewer were added, i.e. that the ratio of ImAd to membranes must be high to achieve maximal isolation from the starting material. 15% of $^{125}$I-anti-α cyt was not isolated by excess ImAd. This was unlikely to be the result of damage to Fc epitopes during iodination, since recognition is via polyclonal serum. Therefore, there are some MHC II$^{+ve}$ vesicles which are not isolated. One explanation is that these vesicles have few MHC II molecules, and so bind with a lower affinity to ImAd. Another explanation is that the concentrations of ImAd which led to maximal binding, ≥5 x $10^7$ beads per ml, led to collisions between beads which dislodged vesicles. Binding would plateau below 100% when addition of ImAd led to an increase in off-rate matching the increase in on-rate from the extra binding capacity.

The isolation of A20 vesicles by anti-α cyt was confirmed by several other means. A20 lipids were fluorescently labelled with Di.I, a red lipophilic dye that does not re-partition into the aqueous phase. After overnight incubation so that vesicular traffic distributed Di.I throughout internal compartments, A20 cells were disrupted and isolation carried out as described above. Direct visualisation of fluorescent vesicles attached to ImAd was possible (figure 32). The same sample was studied by flow cytometry, which indicated that the entire population of magnetic beads had bound membrane. This binding was abolished by adding I-Aα cytoplasmic tail 12-mer peptide coupled to KLH, inhibition which was not seen with an irrelevant peptide. These data confirm that the isolation procedure links MHC II on A20 membrane vesicles to ImAd with a high degree of specificity.

Anti-α cyt immuno-isolates MHC II$^{+ve}$ early endosomes. Evidence from many sources indicates that MHC II and TfR co-localise to some extent (Cresswell, 1985; Rudensky
et al., 1991a and b; Chicz et al., 1992; Hunt et al., 1992b). Immuno-isolation was used to determine whether MHC II co-localised with TfR in A20 cells.

The early endosomes of A20 cells were labelled with $^{125}$I-Tf and plasma membrane-associated $^{125}$I-Tf was removed. Immuno-isolation of A20 membranes by anti-$\alpha_{cyt}$ and negative control serum was performed as described above. To maximise isolation, a second aliquot of ImAd was added to non-isolated PNS. Almost 50% of $^{125}$I-Tf was isolated by anti-$\alpha_{cyt}$ compared to less than 10% by the negative control (figure 33). Therefore, 40% of $^{125}$I-Tf was isolated by anti-$\alpha_{cyt}$ above that isolated by negative serum. A third aliquot of ImAd did not lead to any further isolation of $^{125}$I-Tf.

The binding of $^{125}$I-Tf to ImAd via its inclusion in MHC II$^{+ve}$ early endosomes was confirmed by the following findings. Firstly, excess free $^{125}$I-Tf added to the immuno-isolation mixture was not bound by ImAd. Secondly, isolation of early endosomes by anti-$\alpha_{cyt}$ was compared for A20 cells and AK34 cells (MHC II$^{+ve}$): the proportion of $^{125}$I-Tf isolated by anti-$\alpha_{cyt}$ was 47% for A20 cells compared to 7% for AK34 cells. Therefore, early endosomes are specifically immuno-isolated by anti-$\alpha_{cyt}$ interacting with MHC II. Finally, the same proportion of $^{125}$I-Tf was immuno-isolated by anti-$\alpha_{cyt}$ after either a short or long incubation with $^{125}$I-Tf (5 or 80 minutes), which indicates that TfR which are kinetically within 5 minutes of the cell surface co-localise with MHC II.

The isolation of upto 40% of $^{125}$I-Tf by anti-$\alpha_{cyt}$ indicates that MHC II is distributed widely throughout the early endosomal compartment. The maximum recovery of 40% of $^{125}$I-Tf might indicate that the distributions of TfR and MHC II in the early endosomal compartment are overlapping but significantly different.

In summary, by immuno-isolation of $^{125}$I-Tf, MHC II was found to co-localise with 40% of TfR in early endosomes. The data demonstrate that MHC II is present in a large proportion of TfR$^{+ve}$ vesicles, but do not indicate what proportion of MHC II$^{+ve}$ vesicles are TfR$^{+ve}$. In the experiments above, the presence of TfR is the only method used to define early endosomes. Fluid-phase HRP endocytosed for 2-3 minutes might have been used as another marker for early endosomes. However, HRP levels were very low at this time. Therefore, the isolation of a second marker for early endosomes, independent of
B. METHODOLOGICAL ASPECTS OF THE IMMUNO-ISOLATION TECHNIQUE

Because $^{125}$I-Tf was efficiently isolated, $^{125}$I can be monitored non-invasively (without consuming any sample in an assay), this label was routinely used to assess the efficiency of isolation.

Immuno-isolation requires an ionic environment. Established protocols for immuno-isolation require cells to be disrupted in sucrose, and subsequent adsorption on solid support in an ionic environment (Howell et al., 1989a). To determine whether the addition of ions was required in this system, and also which functions of ions were responsible for this effect, isolation of $^{125}$I-Tf in early endosomes was performed in solutions of varying ionic content all of which had the same osmolarity. Isolation failed in the presence of sucrose alone, but addition of 25% iso-osmotic sodium chloride or choline chloride allowed isolation to occur (figure 34). This level of isolation was increased by using 50% iso-osmotic sodium chloride or choline chloride, and was not significantly affected by the addition of ATP. Therefore, the effect of ions was dose-dependent and independent of whether the cation was membrane-permeant or whether ion pumps were active. These results indicate that there was electro-static repulsion between vesicles and magnetic beads which was quenched by increasing the concentration of ions.

Indirect isolation is better than direct isolation. The method used above was indirect immuno-isolation, since antibody (anti-$\alpha_{\text{cyt}}$) was added separately from ImAd (figure 35a). Note that this method leaves residual antibody that will compete for ImAd and that should ideally be removed (see below). The direct method uses ImAd coated with affinity-purified antibody prior to the isolation, and isolates membranes in a single step (figure 35b); it is quicker and uses less antibody and less ImAd. However, as determined by fluorescent microscopy of ImAd using PNS from Di.I labelled cells, the direct method failed to reproducibly isolate MHC II$^{+\text{ve}}$ membranes from A20 cells. This indicated that the cytoplasmic tail of I-A$\alpha$ might be partially buried by a protein layer derived from the cytoskeleton and other cytosolic proteins (Howell et al., 1989b). Anti-$\alpha_{\text{cyt}}$ could therefore not bind to I-A$\alpha$ when there was added steric hindrance due to attachment of the
antibody to ImAd.

**Anti-α cyt is required in excess but free anti-α cyt is inhibitory to isolation.** The isolation of MHC II^"^ vesicles should ideally include membranes which contain only a single MHC II molecule. To achieve this, the occupancy of I-Aα cytoplasmic tail binding sites by anti-α cyt should be close to 100%. The concentration of binding sites in our preparations of A20 membranes is in the order of 10^8M. The K_d of binding anti-α cyt for MHC II is likely to be in this order. Therefore, an excess of anti-α cyt over I-Aα is required if a high proportion of the latter is to be occupied by anti-α cyt, which will lead to excess free antibody.

A by-product of using the indirect method for immuno-isolation was the presence of excess anti-α cyt, which competed with membrane-bound antibody for ImAd. This was demonstrated by performing repeated isolations on the same PNS sample with small aliquots of ImAd. In this manner, 8 rounds of immuno-isolation were performed sequentially. ^125^I-Tf-containing membranes were isolated on aliquots 4-7 of ImAd (figure 36). The finding that the first 3 aliquots of ImAd did not isolate this membrane indicates that when incubated with a mixture of free and membrane-associated anti-α cyt, ImAd bound the former, to the exclusion of the latter. In subsequent rounds, the depletion of competing free anti-α cyt allowed the isolation of anti-α cyt bound to vesicles. This phenomenon is further evidence for the layer of cytoskeletal and cytosolic proteins on the outer surface of vesicles, which would reduce the affinity of anti-rabbit Ig antibodies, as well as inhibiting direct immuno-isolation (see above).

**Removal of cytosol.** In samples where PNS was left for some time at 4°C prior to centrifugation into percoll there was a lesser degree of separation of lysosomal and endosomal markers, which all tended to have an intermediate density. This indicated that cytosol mediated adhesion between vesicles. The mechanism for this was likely to be by non-specific filament polymerisation and deposition. Such adhesion between vesicles would prevent specific immuno-isolation. On this basis cytosol had to be separated from membranes as soon as possible after disruption of cells. Therefore, the removal of free antibody some time after its addition to PNS, which would have been ideal (see above), was not practical. Instead, antibody was added to membranes alone after removal of
Two approaches were used to remove cytosol: gel filtration and density gradient centrifugation. Pelleting of membranes by centrifugation was not useful, since the pellet did not break up to yield individual vesicles. Gel filtration on a size excluding gel (Sepharose 2B) had the advantages of: rapid and efficient separation; ease of scaling up for large quantities of PNS; exchange of solution from sucrose to ionic. The disadvantages of this method were: reduced yield of membranes, which were lost in the gel even after blocking by 1% BSA and A20 PNS; and reduced latency. These effects were probably caused by the physical effects of passage through the gel.

Non-specifically adherent vesicles bind weakly to ImAd. The importance of contaminating, MHC II^{+ve} membranes varies with different types of experiment. For colocalisation of MHC II with a second marker, as in Sections A and C, the specificity must be high. However, for experiments to investigate antigen processing function in a cell-free system within intact vesicles, the major criterion would be that the vesicles are isolated in bulk; in this case the presence of other vesicles on the ImAd is relatively less important, since all vesicles are separated from each other on the surface of the beads, and there is no cytosol to mediate vesicular fusion. To estimate the extent of adhesion, a control group using an irrelevant antiserum, either affinity-purified anti-FITC or normal rabbit Ig, was included in all experiments. The specificity of the isolation was the ratio of membrane isolated by anti-\( \alpha_{cvt} \) to negative control.

Non-specific adhesion was reduced by the addition to the isolation mixture of 1% BSA and 1% normal mouse serum. After isolation and repeated short washes to remove trapped vesicles, the specificity varied from x2-7 for different experiments. ImAd was then rotated in wash buffer for upto 1 hour. The absolute amount of membrane lost during this wash was similar for both anti-\( \alpha_{cvt} \) and negative control samples, thus increasing the specificity to x3-12. This shows that adherent membrane was more weakly attached to ImAd than isolated membrane. ImAd coated with membranes could be left overnight without significant detachment. However, rotation for periods longer than \( \approx 1 \) hour led to loss of membranes with a reduction of specificity. This indicates that collision between beads could dislodge isolated vesicles.
The higher values for specificity were achieved when a greater proportion of marker was isolated. Thus, low specificity values indicated that insufficient ImAd had been added.

C. VESICLES ARE ISOLATED FROM ALL ELEMENTS OF THE INTRACELLULAR PATHWAY OF MHC II

The co-isolation of MHC II with other markers has not been reported previously. In section A, co-isolation of upto 40% of internalised $^{125}$I-Tf by anti-$\alpha_{\text{cyt}}$ showed that MHC II is widely distributed in early endosomes of A20 cells. The same conclusion has been drawn from some E.M. studies of intracellular MHC II (Guagliardi et al., 1990), but not from other, similar studies (Neefjes and Ploegh, 1992b), and is therefore controversial. Given that MHC II is known to traffic via ER, Golgi, plasma membrane and elements of late endosomes and/or lysosomes, a wide range of organelles might be isolated. Therefore, analysis of all membranes isolated by MHC II was undertaken.

Right-side-out plasma membrane vesicles are not isolated by anti-$\alpha_{\text{cyt}}$. Plasma membrane is the compartment which contains the majority of the cell's MHC II (see Chapter 4). Thus, if plasma membrane vesicles were isolated efficiently, they could overwhelm MHC II from intracellular organelles.

Only plasma membrane which forms inside-out vesicles after cell disruption is accessible to anti-$\alpha_{\text{cyt}}$ for immuno-isolation. A20 cells were labelled with $^{125}$I-Tf without internalisation, to label plasma membrane only. Acid stripping of the whole cells led to loss of 93% of marker. After disruption of these cells, acid stripping of vesicles in the PNS led to loss of 85% of marker. The increased retention of $^{125}$I-Tf after acid stripping indicates that a minority (≈10%) of plasma membrane vesicles formed inside-out.

This corresponds to the degree of isolation with anti-$\alpha_{\text{cyt}}$: $^{125}$I-Tf on plasma membrane was isolated to a level 15% of the isolation of $^{125}$I-Tf in early endosomes seen in parallel experiments. Furthermore, after acid stripping of label from right-side-out vesicles, isolation of $^{125}$I-Tf in inside-out plasma membrane vesicles was at the same level as isolation of $^{125}$I-Tf in early endosomes. Therefore, plasma membrane vesicles are unlikely to overwhelm intracellular MHC II $^+$vesicles. This finding was predicted from the earlier finding that out of $^{125}$I-anti-$\alpha_{\text{cyt}}$ which bound to membranes, 30-40% bound to
membranes of intermediate or high density (figure 30).

Lysosomes, late endosomes and Golgi vesicles are immuno-isolated by anti-α cyt. To study the distribution of MHC II throughout the endocytic pathway, A20 membranes from each fraction of a percoll gradient were immuno-isolated with anti-α cyt. Isolation from fractions were ineffective with the first aliquot of ImAd, probably due to the effect of competing free anti-α cyt. A second aliquot of ImAd isolated a reasonable, although sub-maximal, amount of membranes, which were lysed for assay, as were vesicles remaining in the supernatant. Assays were performed for markers of the three endocytic compartments, galactosyltransferase, and for cellular proteins identified by 35S (figure 37).

The isolation of early endocytic and lysosomal markers (figure 37a and c) was similar in that the peak of isolated marker corresponded to the peak of marker in the whole fractions. Therefore, MHC II vesicles were isolated from across the entire range of density, and MHC II was found in the main populations of early endosomes and lysosomes. The same was true to a lesser extent for late endosomes, with more HRP being isolated from fractions of intermediate density (4 to 6) than from the peak of HRP on the gradient (fractions 2 to 7). The extent of isolation across the entire gradient was slightly greater for 125I-Tf than either of β-hexosaminidase or HRP, indicating that MHC II may be more widely spread through early endosomes than the other two endocytic compartments. The main conclusion drawn from this experiment was that MHC II is present throughout the endocytic pathway of A20 cells.

For 35S, there was no apparent correspondence between total and specifically isolated marker (figure 37d). Furthermore, a far greater amount of 35S adhered non-specifically to both ImAd and the walls of the tube in which the isolation was performed than was isolated specifically. Much of this adherent 35S was resistant to 1% NP40, and was released only after boiling in 2%SDS. These findings confirm the suggestion that 35S after long-term labelling entered the percoll gradient in a form not associated with membrane vesicles (see Chapter 4). Because of this problem, it was not possible to determine the enrichment for each endocytic marker relative to total isolated protein.

MHC II was also found in the exocytic pathway by isolation of the Golgi enzyme
galactosyltransferase from the low density fractions in which the majority of the enzyme was found (figure 37e). The level of galactosyltransferase isolated was similar to the levels of HRP and β-hexosaminidase isolated. The isolation of galactosyltransferase is consistent with the finding of large amounts of MHC II and galactosyltransferase in the TGN (Peters et al., 1991, Nilsson et al., 1993).

**Electron microscopy of vesicles isolated by anti-α<sub>cyt</sub>.** It was shown above that anti-α<sub>cyt</sub> isolated a large proportion of 125I-Tf in early endosomes, a lower proportion of markers of late endosomes, lysosomes and trans-Golgi/TGN, and very little of 125I-Tf on right-side-out plasma membrane vesicles. However, the relative numbers of vesicles isolated from each compartment were not determined. For example, it may be that all the early endosomal 125I-Tf was isolated from a minority of early endosomes with concentrated TfR, MVBs being a candidate for such vesicles. To identify individual vesicles, ImAd was examined by electron microscopy. For these experiments, A20 cells were labelled with 10nm colloidal gold coupled to an antibody to mouse transferrin receptor (gold-AMTR) which had been internalised for 5 minutes. Membranes from these cells were separated on a percoll gradient and MHC II<sup>†</sup> membranes isolated from all fractions. The specificity of this isolation, as monitored by 125I-Tf isolation from the same cells, was 5-10-fold. ImAd coated with membranes were submitted for E.M..

The labelling protocol was expected to leave some gold-AMTR on the plasma membrane. Therefore, E.M. was also performed on cells which had not internalised gold-AMTR, to specifically identify the appearance of plasma membrane vesicles. In such an experiment the few gold-containing structures which were isolated were approximately 1μm in diameter and contained no internal membranes.

The ImAd beads had an irregularly indented surface and a poorly defined outer layer, or cortex (figure 38). Internal iron appeared as electron dense deposits upto 30nm in diameter. In addition, the amount of this iron which was deposited at the periphery of beads varied. Together these features indicated that the ImAd was not ideal for E.M. In comparison, ImAd consisting of 4.5μm diameter beads (Dynal) have a smoother outline with a better defined cortical layer (Howell et al., 1989a; Gruenberg et al., 1989).
The vesicles recovered from light membranes (from fraction 7) included two types of vesicle labelled with gold-AMTR. Some vesicles were approximately 1000nm in diameter, contained large amounts of internal membrane, and were labelled with gold-AMTR mainly, but not entirely, at their periphery (figure 38). Other gold-AMTR<sup>+</sup> vesicles were small, tending to contain more than one gold particle (figure 38, inset). Positive identification of all these vesicular profiles early endosomes was made difficult because some vesicles appeared to bind to ImAd indirectly via contact with an intermediate vesicle (figure 38, left hand panel, top). Such indirect binding was not seen in ImAd samples after isolation with a negative control antiserum, and it was presumed that anti-α<sub> cyt</sub> acted as a bridge between MHC II<sup>+</sup> vesicles, not all of which were early endosomes.

Vesicles recovered from dense membranes (from fraction 3) included some small, gold-AMTR<sup>+</sup> vesicles (figure 39, top left panels), as well as other small vesicles which contained no gold (figure 39, bottom left panel). In addition, membranes which appeared to originate from mitochondria and other large structures were detected (figure 39, right hand panels). This contamination is consistent with the specificity of isolation being 5-10-fold.

Overall, these results indicate that the TfR-containing vesicles were isolated by anti-α<sub> cyt</sub>. However, quantitative studies of the distribution of MHC II in the endocytic pathway were not carried out.

D. ISOLATION BY ANTI-α<sub> cyt</sub> ENRICHES FOR RECENTLY SYNTHESISED MHC II

Vesicles throughout the endocytic pathway of A20 cells have been shown to contain MHC II. Among these, the vesicles where antigen processing occurs might be identified by the presence of recently synthesised α/β/Ii rather than recycling α/β (Neefjes et al., 1990; Davidson et al., 1991; Neefjes and Ploegh, 1992a). In this section, experiments are described which demonstrated the presence of α/β/Ii in early endosomes by the isolation of internalised <sup>125</sup>I-Tf via In-1. In addition, recently synthesised α/β/Ii was isolated efficiently by anti-α<sub> cyt</sub>, whereas recycling α/β was isolated to a much lower extent.

Early endosomes contain Ii. The presence of Ii in A20 membrane vesicles was identified
via its cytoplasmic domain using In-1. PNS from A20 cells with $^{125}$I-Tf in early endosomes was incubated with In-1, and li$^+$ vesicles isolated with ImAd coated with anti-rat IgG. In-1 isolated some $^{125}$I-Tf (7%), therefore, li is present in some early endosomes (figure 40a).

The isolation of $^{125}$I-Tf by both In-1 and anti-$\alpha_{cyt}$ does not prove that li and $\alpha/\beta$ are present in the same vesicles. Free, non-degraded li has been shown previously to be present on the surface of A20 cells (Poirier, 1992), and may recycle into early endosomes. The distinction between $\alpha/\beta$ and li present in the same early endosomes, and $\alpha/\beta$ and free li in separate early endosomes was made by sequential isolation of labelled early endosomes In-1 then with anti-$\alpha_{cyt}$. After maximal isolation by In-1, removing 9% of $^{125}$I-Tf, and by normal rat serum, removing 2% of $^{125}$I-Tf, the two samples were further divided and incubated with either anti-$\alpha_{cyt}$ or normal rabbit Ig before repeated immuno-isolation (not maximal). Prior specific isolation by In-1 of only 7% of $^{125}$I-Tf reduced the amount of $^{125}$I-Tf subsequently isolated by anti-$\alpha_{cyt}$ from 9% to 5% (figure 40b). This indicates that the minority of early endosomes which were isolated by In-1 were largely a sub-population of the early endosomes isolated by anti-$\alpha_{cyt}$. Therefore, the early endosomes in which li was detected also contained $\alpha/\beta$.

Anti-$\alpha_{cyt}$ efficiently isolates vesicles containing recently synthesised $\alpha/\beta/li$. To determine the nature of the MHC II in vesicles isolated by anti-$\alpha_{cyt}$, metabolically labelled MHC II was precipitated from isolated membranes. A cohort of MHC II was labelled by a 20 minute pulse with $^{35}$S-amino acids, followed by 0, 1, 3 or 14 hour chase, disruption of cells and isolation by anti-$\alpha_{cyt}$ and a negative control antiserum. The isolated vesicles were then lysed, and MHC II precipitated. In addition, MHC II in the supernatant (i.e. not isolated on ImAd) was precipitated. Figure 41 shows the isolation of MHC II molecules after a chase period of either one or three hours. After 1 hour chase, a significant proportion of labelled $\alpha/\beta/li$ was isolated (figure 41a, lane 1 "ImAd +") which is reflected in the loss of $\alpha/\beta/li$ from the supernatant (figure 41a, lane 3 "S/N +"). Therefore, a cohort of MHC II 1 hour post-synthesis was in a compartment which was isolated efficiently. After 3 hours chase, anti-$\alpha_{cyt}$ yielded only faint MHC II bands, and the supernatant did not show significant reduction in MHC II due to isolation (figure 41b). At 14 hours chase the isolation of $\alpha/\beta$ was similarly inefficient. This indicates that
during 1 to 3 hours post-synthesis α/β was exported to the cell surface, and fits with previous findings that dissociation of Ii from α/β occurred between 1 and 3 hours chase (figure 25), and that plasma membrane vesiculated mainly right-side-out (see above) and was therefore isolated with low efficiency.

Results in Section C showed that the proportion of plasma membrane TfR that was available for isolation by anti-αcry (i.e. in inside-out vesicles) was less than 15%, and that these inside-out vesicles were isolated with the same efficiency as early endosomes. In the absence of recycling, uptake 15% of α/β may therefore be isolated. The results here indicate that the proportion of α/β isolated by anti-αcry was 15% or less of the α/β/Ii isolated (compare figures 41a and b). Therefore, α/β on inside-out plasma membrane vesicles the plasma membrane may have accounted for all of the α/β isolated (figure 41b), in which case recycling α/β was not isolated to any significant extent.

This result indicated that very little α/β was isolated by anti-αcry. On the other hand, a previous result indicated that a large proportion of early endosomes contained α/β but not Ii, because In-1 isolated much less early endosomal 125I-Tf than was isolated by anti-αcry in a parallel experiment (7% compared to 40%, figure 40a). However, the low isolation by Ii might have been caused by methodological differences, such as lower affinity of In-1 for its binding site, because In-1 is monoclonal compared to polyclonal anti-αcry, or lower affinity of ImAd for In-1 compared to anti-αcry. Alternatively, a small amount of α/β may recycle into TfR-rich early endosomes, and bring about their isolation.

Conclusion. Isolation of MHC II^{++} vesicles was achieved by anti-αcry. These vesicles were enriched for recently synthesised α/β/Ii rather than α/β. In addition, the vesicles contained a higher proportion of an early endosomal marker than of markers of late endosomes, lysosomes or trans-Golgi/TGN. Therefore, not only was isolation of MHC II^{++} vesicles achieved from the entire intracellular pathway of MHC II, but early endosomes were found to contain a large amount of MHC II. In addition, there were indications that a proportion of this MHC II was in the form of α/β/Ii. These findings are therefore consistent with the hypothesis that early endosomes play an important part in antigen processing cells (Brodsky 92), with both newly synthesised and recycling
MHC II being delivered to early endosomes and possibly binding epitopes, but only $\alpha/\beta$II being routed from early endosomes to late endosomes. Future experiments might aim at identifying vesicles as containing both $\alpha/\beta$II and endocytic markers by immunoprecipitation of MHC II following endocytosis of lactoperoxidase, or by immuno-isolation using an antibody to the cytoplasmic domain of TfR.
Figure 30: Density gradient of vesicles identified by anti-α cyt, compared to plasma membrane

In two separate experiments, A20 PNS was incubated with $^{125}$I-anti-α cyt prior to removal of free antibody by gel filtration. A significant minority of label was detected in fractions 1 to 5. Plasma membrane labelled with $^{125}$I-anti-mIg was found mainly in fractions 6 to 8, with little in fraction 5 or below. The number of fractions has been rescaled to 10. In addition, values have been rescaled so that fractions 1 to 8 total 100%.
Figure 31: Isolation of MHC$^{\text{ve}}$ vesicles by anti-$\alpha_{\text{Cyt}}$

Immuno-isolation of a fixed amount of A20 membranes was performed using doubling dilutions of ImAd. A small amount of the anti-$\alpha_{\text{Cyt}}$ used for the isolation (10%) had been labelled with $^{125}$I. Antibody not bound to membranes was removed prior to isolation by gel filtration. Therefore, $^{125}$I isolated by ImAd was all bound to membrane vesicles.
Figure 32: Direct visualisation of immuno-isolated MHC II*ve membranes
A20 cells were labelled with Di.I for 10 minutes 18 hours prior to disruption. Membranes from these cells were isolated by anti-α cyt (top and middle rows), or by anti-FITC (bottom row). Left hand panels, phase contrast; right-hand panels, fluorescence, using Texas Red optics.
More fluorescence is seen after isolation by anti-α cyt (compare top and bottom, right-hand panels). At higher magnification, it can be seen that several vesicles were isolated by a single bead, and that some beads were aggregated into pairs as a result of an intervening layer of (presumably MHC*ve) vesicles.
Figure 33: Specific immuno-isolation of early endosomal marker by anti-α

Two rounds of isolation of A20 membranes were performed after labelling of the cells with $^{125}$I-Tf, and removal of cell surface label by acid stripping. A total of 40% more $^{125}$I-Tf was isolated by anti-α than by the negative control serum. The specificity of isolation (ratio +ve/-ve) was 6-fold.

Data are the mean of values obtained from two experiments (bars show standard error).
Isolation performed in iso-osmolar solution containing

- 100% sucrose
- 50% sucrose, 50% Na Cl
- 50% sucrose, 50% Choline Cl
- 75% sucrose, 25% Na Cl
- 75% sucrose, 25% Choline Cl
- 50% sucrose, 50% Na Cl + ATP regenerating system

Figure 34: Immuno-isolation in varying ionic environments

Immuno-isolation was performed in iso-osmotic solutions containing sucrose (100% = 250mM, 75% = 190mM, 50% = 125mM), the remainder of the solution consisting of 150 mM sodium chloride or choline chloride. A duplicate of the 50% sodium chloride group contained an ATP regenerating system. All solutions were buffered for pH and free divalent cation concentration.
Figure 35: Comparison of indirect and direct immuno-isolation

(a) **Indirect**: a two-step procedure requiring (i) binding of antibody to membranes, then (ii) binding of membranes to ImAd, this step being inhibited by free antibody, which can be removed by gel filtration or centrifugation.

(b) **Direct**: (i) antibody is coated onto ImAd, which can then be stored. (ii) Coated ImAd is used in a single-step procedure. Although this is more efficient in terms of antibody and ImAd used, steric hindrance between ImAd and antibody reduces access to partially buried epitopes.
Figure 36: Immuno-isolation of membranes during repeated isolation with sub-optimal amounts of ImAd

PNS was prepared from A20 cells labelled with endosomal $^{125}$I-Tf. Successive rounds of immuno-isolation were performed with anti-$\alpha_{\text{cys}}$ or negative control serum using a small amount of ImAd for each round, and passing remaining PNS to the next round. The data shown are the differences between $^{125}$I-Tf recovered by anti-$\alpha_{\text{cys}}$ and that recovered by negative serum.
Figure 37: Immuno-isolation of markers from a percoll gradient (see legend overleaf)
For each of five cellular markers the amount of marker specifically isolated in a single (non-exhaustive) round of immuno-isolation (hollow circles) is compared to the total marker per fraction (solid triangles). The total amount of marker isolated across the gradients were: β-hexosaminidase: 6.7%; HRP: 7.1%; transferrin: 9.5%; 35S-amino acids: 1.3%; and galactosyltransferase: 8.5%.

Values have been rescaled so that fractions 1 to 8 total 100%.
Figure 38: Electron micrography of isolated vesicles from a light fraction of the percoll gradient

Membranes from A20 cells labelled with gold-AMTR at 37°C for 5 minutes were separated on a 7.4ml 28% percoll gradient. MHC II^{+} membrane isolated from fraction 7 are shown.

Left hand panel: magnification x 14,000; right hand panel and inset: magnification x 52,500.

The gold-AMTR^{+} vesicles included some approximately 1000nm in diameter (left and right panels), which contained large amounts of internal membrane, and were labelled with gold-AMTR mainly, but not entirely, at their periphery. In addition, small gold-AMTR^{+} vesicles were seen, containing more than one gold particle (inset).

The large gold-AMTR^{+} vesicle at the top of the left-hand panel is not in direct contact with a bead in this section, but may have been isolated via contacts with other, smaller vesicles.
Figure 39: Electron micrography of isolated vesicles from a dense fraction of the percoll gradient

Membranes from A20 cells labelled with gold-AMTR at 37°C for 5 minutes were separated on a 7.4ml 28% percoll gradient. MHC II⁺ve membranes isolated from fraction 3 are shown.

All panels: magnification x 52,500.

Vesicles included some small, gold-AMTR⁺ve vesicles (top left panels, x2). Other small vesicles which contained no gold were also seen (bottom left panel). In addition, membranes which appeared to originate from mitochondria and other large structures were detected (right hand panels).
Figure 40: Immuno-isolation of early endosomal marker by In-1

A20 cells were labelled with internal $^{125}$I-Tf. PNS from these cells was isolated by anti-$\alpha_{\text{cys}}$ and/or In-1 and negative control antisera for each antibody.

(a) Initial exhaustive immuno-isolation with anti-$\alpha_{\text{cys}}$ isolated 40% of $^{125}$I-Tf. In-1 isolated 7.5% of label, i.e. 18% as much as anti-$\alpha_{\text{cys}}$. The data shown are the differences between $^{125}$I-Tf recovered by anti-$\alpha_{\text{cys}}$/In-1 and that recovered by negative sera.

(b) Membranes remaining after isolation with In-1 or negative control serum [from (a)] were then non-exhaustively isolated with anti-$\alpha_{\text{cys}}$. In this isolation, 9% of $^{125}$I-Tf was recovered from the sample without prior isolation (negative control), while 5% of $^{125}$I-Tf was retrieved from the sample with prior isolation.
Figure 41: Isolation of newly synthesised MHC II by anti-α\textsubscript{cyt}.

A20 cells were metabolically labelled for 20 minutes and chased for (a) 1 hour and (b) 3 hours. PNS prepared from these cells was immuno-isolated by anti-α\textsubscript{cyt} (lanes marked "+") or by anti-FITC (lanes marked "."). MHC II was precipitated from isolated membranes (lanes marked "ImAd") and from the remaining membranes in the supernatant (lanes marked "S/N").

(a) 1 hour after synthesis, approximately 50% of MHC II\textsuperscript{*} was isolated on ImAd, as shown by its appearance in "ImAd +" and from its loss from "S/N +".

(b) 3 hours after synthesis, very little MHC II\textsuperscript{*} was isolated ("ImAd +"), also shown by the similarity of "S/N +" and "S/N -".

M.W. = molecular weight markers (200, 94, 69, 46, 30, and 20 kDa).
CHAPTER 6. A PRELIMINARY STUDY OF CELL-FREE ANTIGEN PROCESSING FUNCTION USING MATERIAL OBTAINED BY IMMUNO-ISOLATION

Introduction. In Chapter 5 it was shown that a high proportion of recently synthesised $\alpha/\beta/\text{Ii}$ was isolated by anti-$\alpha_{\text{cyt}}$. Therefore, vesicles derived from the antigen processing compartment were isolated, although they were not purified to homogeneity. Since these vesicles are intact (see chapter 4), they may contain molecules which perform important functions for antigen processing. In this chapter preliminary experiments are described to determine whether immuno-isolation could be followed by functional assays for antigen processing.

Firstly, using all the vesicular material isolated by anti-$\alpha_{\text{cyt}}$, vesicles attached to ImAd were lysed by detergent, and the supernatant used to demonstrate Ii degradation. Secondly, formation of compact dimers after peptide loading was shown to be minimal for I-A$^d$ and I-E$^d$. In this regard, the indications were that A20 cells were not of the ideal MHC haplotype for further experiments.

Ii is degraded by immuno-isolated proteinases. Degradation of MHC II isolated by anti-$\alpha_{\text{cyt}}$ was examined. A20 cells were either grown as usual (figure 42, lanes 1,2,7 and 8), or treated with leupeptin overnight to prevent Ii degradation in vivo (figure 42, lanes 3,4,9 and 10). Both groups of cells were then pulsed with $^{35}$S-amino-acids for 1 hour and chased for 2 hours, and membranes were isolated by anti-$\alpha_{\text{cyt}}$. The immuno-isolated material was then dissociated from ImAd, and incubated as follows: cells without leupeptin - incubated at either 4°C (lanes 1 and 7) or 37°C (lanes 2 and 8); cells with leupeptin - incubated at 37°C either without (lanes 3 and 9) or with cathepsin B (lanes 4 and 10). Note that any cathepsin B in this immuno-isolated material would have been inhibited by leupeptin (in lanes 3 and 9), but that free leupeptin had been washed away and so exogenous cathepsin B would not be inhibited (in lanes 4 and 10). Ii-containing complexes were precipitated from all four samples by In-1 (figure 42, lanes 1 to 4). After this clearing of $\alpha/\beta/\text{Ii}$, remaining $\alpha/\beta$ in all four samples was precipitated by TIB120 (figure 42, lanes 7 to 10). As a comparison, total cellular material was precipitated by In-1 (lane 5) and treated with cathepsin B (lane 6).

In this experiment, precipitation of total cellular MHC II by In-1 produced two major bands, corresponding to I-A$\alpha$ and Ii (lane 5). The relative faintness of I-A$\beta$ was unexplained. Cathepsin B treatment of MHC II precipitated by In-1 (lane 6) showed equivalent reduction in $\alpha$ chain to the loss of Ii. This indicates that proteolysis of Ii led
to loss of α when the precipitation was via In-1. By comparison, precipitation via α led to loss of Ii only (see figure 26).

MHC II precipitated by In-1 from immuno-isolated material also showed two bands corresponding to I-Aα and Ii (lanes 1 to 4). The effect of incubation of MHC II together with eluted material from ImAd at 37°C can be seen by comparing lane 1 (4°C) and lane 2 (37°C): degradation of Ii resulted from the incubation at 37°C. The effect of resistance of MHC II to incubation at 37°C per se, and the susceptibility of immuno-isolated MHC II to cathepsin B can both be seen by from lanes 3 and 4. In lane 3 (37°C, cellular cathepsin B inhibited by leupeptin) the same amount of MHC II was recovered as from cells treated at 4°C (compare with lane 1). Therefore, raised temperature alone did not lead to loss of MHC II. In lane 4 (37°C, with exogenous cathepsin B) there was a reduction in MHC II (compare with lanes 3 and 1). Therefore, cathepsin B was capable of degrading isolated MHC II complexes as seen in lane 2.

α/β dimers precipitated sequentially after α/β/Ii from these samples showed characteristic α and β bands more prominently than Ii (lanes 7 to 10), as shown in chapter 4 (figure 24, lanes 3 and 4). No effect on the amount of α/β precipitated was detected as a result of incubation at 37°C with cell-derived (co-isolated) cathepsin B (lane 8) compared to incubation at 4°C (lane 7). In addition, no effect on the amount of α/β precipitated was detected as a result of incubation at 37°C with exogenous cathepsin B (lane 10) compared to incubation at 37°C without active cellular cathepsin B (lane 9). This indicates that Ii was selectively lost due to incubation at 37°C with co-isolated material (lane 2) or due to exogenous cathepsin B (lane 4), whereas α/β was unaffected.

These results indicate that the isolated membranes contained a proteinase which degraded Ii. Therefore, an antigen processing activity was detected in material immuno-isolated by anti-α cyt. Further experiments could use intact vesicles to determine the extent of co-localisation of Ii and the proteinase. Such experiments might be optimised by using a chase period of 1 hour rather than 2 hours.

Compact dimers do not form after peptide-loading of I-A^d. The formation of compact dimers follows peptide loading in some systems (Sadegh Nasseri and Germain, 1991), and is therefore a useful biochemical feature for monitoring antigen processing (Germain and Hendrix, 1991; Davidson et al., 1991; Lanzavecchia et al., 1992; Neefjes and Ploegh, 1992a; Ribet et al., 1992). It was shown in chapter 4 that I-A^d in A20 cells forms
compact dimers to a moderate extent (see figure 23), which is similar to the findings for I-A\textsuperscript{d} in splenocytes (Germain and Hendrix, 1991).

The formation of compact dimers \textit{in vitro} was studied by incubating metabolically labelled MHC II with a peptide restricted to I-A\textsuperscript{d}: ova 323-339 (Shimonkevitz \textit{et al.}, 1983; Hunt \textit{et al.}, 1992b). In two separate experiments MHC II attached to sepharose beads was incubated at 37°C firstly at pH 4.5, then at neutral pH for 15 minutes, before dividing the samples for SDS-PAGE (without 2-mercapto-ethanol) with and without boiling. The two experiments differed in the duration of exposure to peptide at low pH: either 10 minutes or two hours. The low pH should cause a large increase in both dissociation of previously bound peptides and binding of ova 323-339.

A20 cells labelled with a 1 hour pulse and a 1 hour chase provided MHC II which was incubated with peptide for 10 minutes. A faint, well-defined band was seen (figure 43, lane 1), which was partially sensitive to boiling (lane 2). The apparent molecular weight (61 kDa) was likely to be an under-estimate, since the band was displaced down the gel by a large amount of BSA. In this experiment, MHC II molecules not treated with peptide did not show the 61 kDa band (see figure 23, lanes 5 to 8).

MHC II in cells labelled with a 1 hour pulse and a 2 hour chase provided a mixture of $\alpha$/$\beta$/Ii and $\alpha$/$\beta$. $\alpha$/$\beta$/Ii was removed as in figure 24. The remaining $\alpha$/$\beta$ was incubated with peptide for 2 hours. A band was seen (figure 43, lane 3), which was partially sensitive to boiling (lane 4) and had an apparent molecular weight of 67 kDa. In this experiment, MHC II molecules not treated with peptide showed the same SDS-resistant band, but to a lesser extent (figure 24, lane 4). The high molecular weight band in this experiment was more prominent than that seen in the previous experiment (figure 43, compare lanes 3 and 1). This difference might result less from the difference in duration of the incubations, and more from the higher proportion of MHC II complexes in lanes 3 and 4 which were $\alpha$/$\beta$ as opposed to $\alpha$/$\beta$/Ii, which binds peptide with a much lower affinity (Roche and Cresswell 1990a).

It is likely that the band formed by peptide loading is identical to the band seen in boiled samples from metabolically labelled cells after 18 hours chase (figure 25, lane 6), since the bands have the same molecular weight and the same partial sensitivity to boiling. Therefore, I-A\textsuperscript{d} in A20 cells can form SDS-resistant $\alpha$/$\beta$/peptide complexes, but with higher than the expected molecular weight for compact dimers (Sadegh Nasseri and Germain, 1991) and some resistance to boiling.
These results differ from a previous study which demonstrated compact I-A\textsuperscript{d} dimers of molecular weight \(\approx 60\) kDa which were completely sensitive to boiling (Dornmair \textit{et al}., 1989). This difference might be explained by partial destabilisation of compact I-A\textsuperscript{d} by either 2-mercapto-ethanol in the tissue culture medium, or the manipulation of extracted MHC II in the absence of lipid micelles, together with a greater tendency of I-A\textsuperscript{d} to dissociate than other species of MHC II (Germain and Hendrix, 1991). This tendency is confirmed by observations which include: (1) the half-lives of peptide complexes with both I-A\textsuperscript{d} and I-E\textsuperscript{d} on live and fixed A20 cells and \textit{in vitro} are quite short and can be shortened by competing peptides (Pedrazzini \textit{et al}., 1991; Poirier, 1992). (2) Compact I-A\textsuperscript{d} dimers are more sensitive to temperature than I-E\textsuperscript{k} (Dornmair \textit{et al}., 1989).

In further experiments it would be interesting to attempt to form compact dimers using A20 cells which express a more suitable MHC haplotype. I-A\textsuperscript{k-ve}/I-A\textsuperscript{d-ve} A20 cells have been made in this laboratory (Poirier, 1992).

**Future directions for the cell-free assay of antigen processing.** The sub-cellular localisation of peptide loading to MHC II is not known. As discussed in chapter 5, it is likely that the vesicles in which peptide loading occur were isolated, although the preparation also contained vesicles from other intracellular MHC II\textsuperscript{+ve} compartments. Antigen processing could not be detected in this system by the appearance of compact dimers. Nevertheless antigen processing could be monitored by using labelled antigen and examining the recovery of intact or partially degraded antigen after immuno-isolation with anti-\(\alpha\)\textsubscript{ cyt} (Davidson \textit{et al}., 1991; Marsh \textit{et al}., 1992). Antigens used in such experiments could be those for which the pattern of degradation has already been studied, for example ovalbumin or insulin. Alternatively antigens could be designed specifically, for example transferrin, HRP or anti-mouse Ig could be linked by sequences with known proteinase sensitivity to known epitopes. Such a system could also be used to study the enzymes which co-localise with MHC II by varying the susceptibility of the linker sequences.

Finally, the spectrum of molecules associated with MHC II in antigen processing could be studied by raising antisera to isolated vesicles and using standard techniques to clone molecules from an A20 expression library.

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Figure 42: Degradation of Ii after immuno-isolation
A20 cells were either grown as usual (lanes 1,2,7 and 8), or treated with leupeptin overnight to prevent Ii degradation in vivo (lanes 3,4,9 and 10). Both groups of cells were pulsed with $^{35}$S-amino-acids for 1 hour and chased for 2 hours, and membranes isolated by anti-α$_{cyt}$. The immuno-isolated material was then dissociated from ImAd, and incubated as follows:

- **cells without leupeptin** - incubated at either 4°C (lanes 1 and 7), or 37°C (lanes 2 and 8);
- **cells with leupeptin** - incubated at 37°C either without (lanes 3 and 9), or with cathepsin B (lanes 4 and 10).

Ii-containing complexes were precipitated from all four samples by In-1 (lanes 1 to 4). After this clearing of α/β/Ii, remaining α/β was precipitated by TIB120 (lanes 7 to 10). Also shown: total cellular Ii precipitated by In-1: untreated (lane 5), and treated with cathepsin B (lane 6).
**Figure 43: Incubation of I-A<sup>d</sup> with peptide to form compact dimers**

**Lanes 1 and 2:** MHC II from metabolically labelled A20 cells (1 hour pulse, 1 hour chase) was precipitated by TIB120, and incubated with peptide (ova 323-339) at pH 4.5 for 10 minutes. A faint, well-defined band (61 kDa, arrow) was seen which was partially sensitive to boiling. The band may be displaced down the gel by a large amount of BSA (69 kDa).

**Lanes 3 and 4:** MHC II from metabolically labelled A20 cells (1 hour pulse, 2 hours chase) was pre-cleared of α/β/Ii with In-1, and α/β precipitated by TIB120. α/β was incubated with peptide (ova 323-339) at pH 4.5 for 2 hours. A well-defined band (67 kDa, arrow) was seen which was partially sensitive to boiling. This band is more prominent than that seen after only 10 minutes incubation (compare lanes 3 and 1).
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APPENDIX

The following papers have been published, or are in preparation in connection with the work described in this thesis:


Explanation of equations on pages 85 and 106

For a compartment filling with first order kinetics (i.e. a constant rate of influx and a rate of efflux proportional to the content), the filling will be exponential. At time = t:

\[
\text{content} = \text{maximum} \times (1 - e^{-kt}), \quad \text{where} \quad k = \text{rate constant of emptying.}
\]

Rearranging this equation: maximum = \( \{\text{content at time t}\} / (1 - e^{-kt}) \).

In addition, at half-maximal filling (when \( t = t_{1/2} \)), \( 1 - e^{-kt_{1/2}} = 1/2 \), thus \( e^{-kt_{1/2}} = 1/2 \), so that \( k t_{1/2} = \ln 2 \), and so \( k = \ln 2 / t_{1/2} \).

Substituting this value for \( k \), and with \( t = 60 \) minutes, the equation obtained is:

\[
\text{maximum} = \left\{\frac{\text{content at 60 minutes}}{(1 - e^{-\ln 2 \times 60 / t_{1/2}})}\right\} \quad \text{(see p 85)}.
\]

When \( t_{1/2} \) is small in comparison to \( t \), the value of \( \{\ln 2 \times t / t_{1/2}\} \) becomes high, and \( e^{-\ln 2 \times t / t_{1/2}} \) tends to zero. Therefore, \( \text{EE}_{\text{max}} = \{\text{content at 60 minutes}\} \).

The proportion of efflux from EE that reaches LE equals flow through LE divided by the total flow through EE. Using the values defined in the model in figure 2, this proportion is:

\[
\frac{\{k2 \times \text{EE}_{\text{max}}\}}{\{k2 + k3\} \times \text{EE}_{\text{max}}}.
\]

At steady state, the rate of influx into LE equals the rate of efflux; therefore,

\[
\{k2 \times \text{EE}_{\text{max}}\} = \{k4 \times \text{LE}_{\text{max}}\}.
\]

In addition, \( (k2 + k3) = \ln 2 / t_{1/2}^{\text{EE}} \) and \( k4 = \ln 2 / t_{1/2}^{\text{LE}} \).

By substituting these values in the first equation, the proportion of efflux via late endosomes is equivalent to:

\[
\frac{\ln 2 \times \text{LE}_{\text{max}} / t_{1/2}^{\text{LE}}}{\ln 2 \times \text{EE}_{\text{max}} / t_{1/2}^{\text{LE}}}.
\]

Rearranged and expressed as a percentage, this yields the equation used in Table 2:

\[
\% \text{ EE traffic routed to LE} = 100 \times \left\{\frac{\text{LE}_{\text{max}} \times t_{1/2}^{\text{EE}}}{\text{EE}_{\text{max}} \times t_{1/2}^{\text{LE}}}\right\} \quad \text{(p. 106)}
\]