A STUDY OF PINOCYTOSIS IN HUMAN MONOCYTES DURING THEIR DIFFERENTIATION INTO MACROPHAGES IN VITRO

by

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Abstract

A report appeared in 1982 suggesting a sharp increase in pinocytic activity in human monocytes as they mature into macrophages *in vitro*. The thesis describes a detailed examination of this issue.

For isolating high yields of monocytes from blood, a density-gradient method using Percoll was found to be the most suitable. Monocytes, cultured in a defined medium supplemented with horse and fetal calf sera, were found to differentiate into macrophages within one week. This maturation was monitored and confirmed by a number of criteria: an increase in, cell size, in cell protein, in protein-to-DNA ratio, in intracellular hexosaminidase activity, and in hexosaminidase secretion in response to opsonized zymosan; a decrease in peroxidase activity, a change in morphology, and the development of fluoride-resistance to cytochemically visualised esterase activity.

A method for quantifying pinocytosis was developed, using various ^{125}I-labelled tracer macromolecules: polyvinylpyrrolidone in the absence and presence of suramin; vinylamine-vinylpyrrolidone copolymer; and polystyrene beads, 100 or 1100nm in diameter. It proved difficult to quantify reliably the cellular uptake of ^{125}I-labelled polyvinylpyrrolidone alone. Satisfactory results were obtained with the other substrates: uptake was progressive (and usually linear) with time and in most cases was inhibited by colchicine. The polystyrene beads were captured at the highest rates.

The pinocytic activity of monocytes was studied at daily intervals over the 7 days of their *in vitro* maturation, using ^{125}I-labelled

polyvinylpyrrolidone (in the presence of suramin (500μg/cm^3 as marker substrate). There was a 2- to 3-fold increase between Day-0 and Day-3, but the significance of this result is uncertain, owing to the major donor-dependent variations observed in the data. Similar experiments using vinylamine-vinylpyrrolidone copolymer as substrate yielded no evidence of an increase in pinocytic activity during maturation.

CONTENTS

CHAPTER 1

Introduction

1.1. ENDOCYTOSIS

1.1.1. Introduction

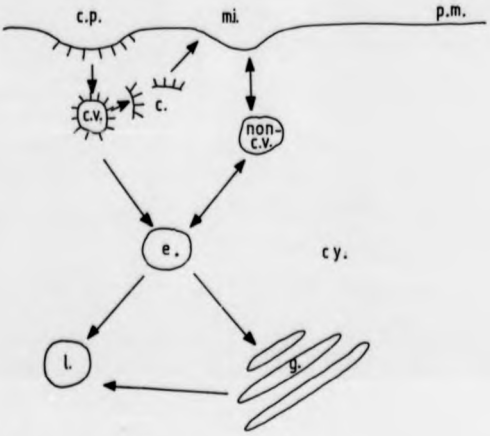
The term endocytosis encompasses two well-known processes. The first, pinocytosis, was first described by Lewis in 1931 and is often termed "cell drinking", since it is how the cell internalises extracellular fluid and any contained low molecular weight soluble material. The second process, phagocytosis or "cell eating", was initially described by Metchnikoff (1883) and refers to the ingestion of particles. Pinocytosis is thought to occur in nearly all cell types. It appears to be a constitutive phenomenon (see below Section 1.1.4.). Pinocytosis plays a central role in cell physiology, being responsible for the entry into the cell of many substances (reviewed by Schneider et al., 1985). For example various proteins, macromolecules, peptide hormones, glycoproteins and enzymes are taken up pinocytically for cell growth, nutrition and development.

In contrast, phagocytosis is not an ongoing process but needs to be triggered by contact of the cell with the substrate. Cells performing phagocytosis are mostly specialized cell types such as amoeba which phagocytose to feed, and polymorphs and mononuclear phagocytes which are responsible for the removal (by phagocytosis) of bacteria, foreign proteins, cell debris and dead red blood cells. Such cells are termed "professional phagocytes".

A number of comprehensive reviews have been published in recent years concerning endocytosis, including Besterman & Low 1983; Duncan & Pratten, 1985; Mellman, 1984; Steinman et al. 1983; and Willingham & Pastan, 1984.

Figure 1.1.

Schematic representation of possible sequences of events during pinocytosis



c.p.	coated pit
m.i.	membrane invagination
p.m.	plasma membrane
c.v.	coated vesicles
c.	coats (recycled)
non-c.v.	non-coated vesicles
e.	endosomes
l.	lysosomes
g.	golgi
cy.	cytosol

In both pinocytosis and phagocytosis extracellular material is internalized via vesicular structures formed at the cell surface. With pinocytosis, if a substrate is captured by the vesicle simply along with the extracellular fluid, then it is said to enter by fluid-phase pinocytosis. However if the substrate is concentrated in the vesicle owing to an affinity for the plasma membrane, then it will enter the cell more rapidly by what is called adsorptive pinocytosis. Ligands entering the cell in this adsorptive manner may do so bound either to non-specific or specific receptors on the plasma membrane. The latter (specific) mechanism is termed receptor-mediated endocytosis. Similarly during phagocytosis, particles that bind to the cell surface (prior to ingestion), may do so in a non-specific way or via a specific receptor.

1.1.2. Pinocytosis

1.1.2.1. Pinocytic events. The possible sequences of events thought to occur during pinocytosis are given in Figure 1.1.. The vesicle or pinosome usually forms in areas of the plasma membrane termed coated pits. Using transmission electron microscopy, coated pits have been visualized as unique, fuzzy, bristle coats situated on the cytoplasmic face of the forming vesicle (Ungewickell & Branton, 1982). The major structural protein of the coat is clathrin, which forms a lattice-work of hexagon and pentagon units (Ockleford, 1976; Pearse, 1982). Coated pits have been seen in most cell-types, exceptions being the macrophage and the erythrocyte. They are thought to be foci in which certain receptors with attached ligand may concentrate prior to internalization (Carpentier et al., 1982). The formed vesicle, which may or may not be coated (often depending on the cell-type), is classically thought to pinch off from the

plasma membrane and migrate into the cytosol. The coat is thought to be lost rapidly (15 seconds to 1 minute) and possibly recycled back to the cell surface. Even though Anderson et al. (1976) have demonstrated coated vesicles in the cytosol by scanning electron microscopy, it has been argued that such structures are still in continuity with the cell surface. Recent evidence indicates that the coat does not leave the cell surface and that only uncoated vesicles enter the cytosol. For example, Wehland et al. (1981) using antibodies to clathrin as intracellular probes, have proved that soluble clathrin is not found around the recently internalized vesicle.

Within the cytosol, pinosomes either fuse with each other or with organelles called endosomes, sometimes called receptosomes or ligandosomes. Some endosomes have a low pH, which is probably due to the operation of an ATP-driven proton pump. This pump was described in endosomal fractions using 9-amino acridine as a probe for pH gradients (Saermark et al., 1985). Endosomes are generally thought to be areas in which dissociation between receptors and ligands occurs (reviewed by Yamashiro & Maxfield, 1984). The endosome has been postulated as a "sorting area" for intracellular traffic of ligands, various receptors and ligands (together or alone) being redirected to other loci within the cell or back to the plasma membrane. Generally, the vesicles leaving the endosome are thought to fuse with the lysosomes and thus the endosome has been termed a prelysosomal organelle. However it has recently been proposed that, although this pinocytic sequence is probably the major route in phagocytes, in most cells many ligands are transferred directly from the endosome to tubules of the transreticular Golgi, and then to lysosomes if appropriate. Evidence for this is that receptosomes do

not generally contain lysosomal enzymes and are not seen to fuse with lysosomes (reviewed by Willingham & Pastan, 1984). It is thought that the Golgi may also represent a major sorting area from which ligands, receptors and plasma membrane components are redirected to other parts of the cell (Willingham & Pastan 1984).

The necessity for membrane recycling during pinocytosis was made evident by Steinman et al. (1976), following his estimate that in macrophages 68 percent of the plasma membrane is internalized per hour. This recycling of membrane was later proved, by Besterman et al. (1981), by following the kinetics of exocytosis of [^{14}C]sucrose from preloaded cells. They showed that most of the fluid internalized was rapidly returned by exocytosis to the extracellular space, via a fast-emptying compartment which was presumed to be the endosome. The remainder is transferred to a much slower-emptying compartment, presumably the lysosomes. Recycling of the plasma membrane has been demonstrated by following the fate of the ectoenzyme 5'-nucleotidase, in various cell-types (reviewed by Widnell, 1983). The enzyme is known to exchange continuously between the cell surface and the interior, and the kinetics of this recycling correlate with the rate of fluid-phase pinocytosis.

1.1.2.2. Mechanisms of substrate-selectivity. Substrates entering the cell in the fluid-phase (i.e. along with the extracellular fluid) do so in a non-selective manner. However substrates are selectively internalized if they have an affinity for either specific or non-specific receptor sites on the cell surface. Specific receptors exist on many cells and these bind avidly with one or more ligands. For example the mannose receptor, which is found on a number of cells including the mononuclear phagocytes, recognises mannose-terminal

glycoproteins (Shepherd et al., 1982). Numerous receptors have been documented, including those for growth factors (Carpenter & Cohen, 1976), lipoproteins (Brown & Goldstein, 1979), transferrin (Karin & Mintz, 1981), and various glycoproteins (Stahl et al., 1980). Ligands binding to these receptors enter the cell at a greater rate than solutes entering in the fluid phase. The substrate's chance of being internalised is often further increased in that many of the receptors are thought to be able to move laterally within the plasma membrane and concentrate in coated pit regions prior to internalization (reviewed in Steinman et al., 1983).

Pinocytosis mediated by non-specific receptors (i.e. adsorptive pinocytosis) was reviewed by Lloyd & Williams (1984). It is generally believed that the adsorptive substrate's increased affinity for non-specific receptors is due to a number of factors, including its hydrophobicity, charge and size. The importance of hydrophobicity was shown by Moore et al. (1977) and also Kooistra et al. (1981) each demonstrating that the hydrophobic, formaldehyde-denatured bovine serum albumin is cleared by the rat visceral yolk-sac (a model system extensively used to study pinocytosis), and by rat peritoneal macrophages, at a much higher rate than native bovine serum albumin. More recently Duncan et al. (1982) showed that incorporation of phenolic residues (greater than approximately 10mol percent) into the plasma expander poly(α,β-N(-2-hydroxy-ethyl)-DL-aspartamide also increased its clearance by the rat visceral yolk-sac. Duncan et al. (1984) also demonstrated in the same system increased pinocytic uptake of polymers of N-(2-hydroxypropyl)methacrylamide, when hydrophobic tyrosinamide residues were incorporated into the polymer.

Evidence for the importance of charge has been given by Kooistra et al. (1981) who showed that rat peritoneal macrophages cleared the basic H4 isomer of porcine lactate dehydrogenase at three times the rate for the negatively charged H4 isomer. Likewise a cationic vinylamine-vinylpyrrolidone copolymer was cleared by the same cells much more efficiently than polyvinylpyrrolidone (PVP) (Pratten et al., 1982). The importance of size was demonstrated by Duncan et al. (1980) using different-sized PVP molecules where, with increasing sized molecules, an increase in affinity and hence clearance by rat peritoneal macrophages was observed.

1.1.2.3. Requirements for pinocytosis. Although as noted above, pinocytosis is a constitutive activity, many authors have shown that it can be inhibited. By using low temperature, metabolic and cytoskeletal inhibitors, the pinocytic clearance of a number of substrates by various cell-types can be inhibited (Duncan & Lloyd, 1978; Pratten & Lloyd, 1979; Starling et al., 1983). This suggests a requirement for energy and the involvement of the cells contractile machinery during pinocytosis. However it is not yet clear whether energy for pinocytosis is derived from oxidative phosphorylation or only from glycolysis. Although many inhibitors affect both pinocytosis and phagocytosis (see Chapter 8), some may only affect one process or the other. Interferon is interesting, in that in mouse peritoneal macrophages it inhibits the pinocytosis of both horseradish peroxidase and dextran-FITC (demonstrated cytochemically) but enhances the phagocytosis of IgG-coated sheep red blood cells (Wang et al., 1984). Also the inhibition of pinocytosis was only observed in approximately 90 percent of the cells, possibly indicating that a sub-population of the cells was unaffected. It is possible that there is a requirement for calcium ions during

pinocytosis. EGTA, a chelating agent, inhibits pinocytosis in rat peritoneal macrophages (Pratten & Lloyd, 1979). Also trifluoperazine, an antagonist of calmodulin (a protein involved in Ca^{+} transport), inhibits pinocytosis in both fibroblasts and mouse peritoneal macrophages (Shirazi & Dean, 1983). A cellular enzyme transglutaminase may play a role in pinocytosis. The enzyme was shown to be essential for the clustering of receptors in the coated pits of fibroblasts (Davies et al., 1980). In addition, Dean et al. (1984) postulated, in a review article, that the pH of the endocytic vesicles is important for pinocytosis to proceed, since, in a number of cell systems, by raising the intravesicular pH by the incorporation of amines, pinocytosis was inhibited. Dean et al. (1984) concluded that the elevation in pH affected the fusion of endocytic vesicles, therefore also affecting membrane flow, which is necessary for pinocytosis to occur.

1.1.2.4. Stimulators of pinocytosis. Many chemicals are known to stimulate fluid-phase pinocytosis, but in the majority of cases the increase is not due to an increase in pinosome formation but to some other mechanism. For instance trypan blue is known to stimulate the pinocytic clearance of ^{125}I-labelled polyvinylpyrrolidone (PVP) (a fluid-phase marker) by the rat visceral yolk-sac (Roberts et al., 1980). This mechanism involves the dye interacting with the PVP, enabling the latter to enter the cells adsorptively. This is known as "piggy-back" endocytosis, a further example being the increase in the clearance of PVP by rat peritoneal macrophages in the presence of suramin (Pratten & Lloyd, 1983); details of this latter experiment are given in Chapter 4.

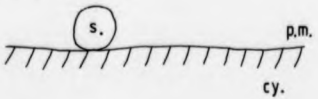
In other instances stimulation may be due to redirecting the flow of a pinocytic substrate so that more accumulates in the lysosomes than is rapidly regurgitated. This has been shown for the pinocytic substrate lucifer yellow, the majority of which is usually rapidly exocytosed. However with mouse peritoneal macrophages, in the presence of phorbol myristate acetate or horseradish peroxidase, the fluorescent probe was seen to accumulate in the lysosomes, its usual rapid exocytosis being greatly reduced (Swanson et al., 1985). One example of a stimulation of pinocytosis was shown by Bennett & Cohn (1966), who simply observed an increase in the number of vesicles formed (microscopically) when horse monocytes were treated with endotoxin, (this technique has however since been criticised, see Section 6.4.6.).

In addition to chemicals modulating pinocytosis, the cell culture system, particularly the serum used, may also affect the process, but this appears to be a substrate- and cell-dependent effect. For example decreasing the serum concentration appears to increase pinocytosis by the rat visceral yolk-sac: a greater increase is seen with an adsorptive substrate (BSA) than with two fluid-phase markers (PVP and sucrose) (Ibbotson & Williams, 1979). However with rat peritoneal macrophages, although a similar effect is seen with an adsorptive marker (colloidal gold), with the fluid-phase marker PVP a decrease in the serum concentration reduces the rate of pinocytosis (Koolstra et al., 1981).

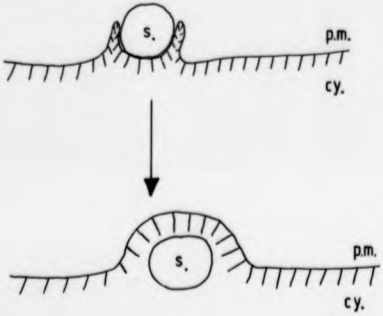
Figure 1.2.

Schematic representation of the sequence of events in phagocytosis

(i) Binding of the substrate



(ii) Ingestion of the substrate



s. = substrate

p.m. = plasmamembrane

cy. = cytosol

1.1.3. Phagocytosis

1.1.3.1. Phagocytic events. The sequence of events in phagocytosis has been described by Besterman & Low (1983); Castle (1984) and Stossel, (1976). There are only two major events (see Figure 1.2.), the first being the binding of the substrate to the phagocyte. For this to occur the substrate needs to be recognized by the cell, this recognition (the mechanism of which is unclear) allowing the substrate to bind strongly to the cell. Ingestion, the second major step follows: pseudopods (that is, finger-like projections of the plasma membrane) begin to surround the particle until the tips fuse at the distal side of the substrate, completely engulfing it.

Griffin et al. (1975) proposed that binding and ingestion represent a continuous process, whereby adjacent sites on the cell surface sequentially bind to adjacent sites on the substrate in a "zipper-like" fashion, until the whole of the substrate is surrounded by plasma membrane. The vesicle or phagosome once formed enters the cell and fuses with lysosomes. Lysosomal hydrolases are released into the phagosome bringing about the enzymic degradation of the engulfed material.

During phagocytosis there is an increase in oxygen consumption by the phagocyte. This respiratory burst was first thought to supply extra energy for the phagocytic process itself, but it is now known that the oxygen is used to produce very reactive oxygen metabolites including hydrogen peroxide, superoxide anion, hydroxyl radicals and singlet oxygen. These products are secreted during phagocytosis and are a useful armament for destroying other cells (e.g. invading bacteria).

1.1.3.2. Mechanisms of substrate-selectivity. As with pinocytosis, specific receptors play an important role in the phagocytic process. Prior to ingestion many substrates are modified (or opsonized) by serum proteins such as complement, immunoglobulins or fibronectin, all of which attach to the substrate itself. The opsonized material then binds to the phagocyte at specific receptor sites, for example for complement or the Fc region of the immunoglobulin. This opsonization often increases a substrate's capacity to be captured. It is not clear if this is simply due to the opsonin binding to both the substrate and the receptor, thus forming a link between the two, or whether the opsonins alter the properties of the particle (for example changing its charge or hydrophobicity). It is evident that factors other than the opsonin binding to the receptor are important, as opsonization does not make all substrates equally susceptible (Michl & Silverstein, 1978). Again like pinocytosis, non-specific receptors exist that mediate phagocytosis, also recognising surface features such as hydrophobicity and charge. Mudd & Mudd (1924) first noted that capsulated bacteria were not ingested as readily by phagocytes as the more hydrophobic unencapsulated strains. More recently hydrophobic forces have also been shown to be important in the phagocytosis of both polystyrene beads and red blood cells, both commonly used in studies on phagocytosis (Capo et al., 1979). With respect to charge, Ono & Awai (1984) showed that phagocytic cells (rat peritoneal and liver macrophages) had surface receptors with specific affinity for anionic particles; these receptors were not found on other somatic cells. Also, Peterson et al. (1984) showed that cationic but not anionic and neutral poly amino acids stimulated the phagocytosis of Staphylococcus epidermidis and suggested that they acted as opsonins.

In addition to the nature of the substrate being important for its capacity to be phagocytosed, the nature of the phagocyte plasma membrane is also important. It is generally thought that a phagocyte will only ingest material that is more hydrophobic than itself. Thrasher et al. (1973) showed that a preparation of lymphokines, which decreased the hydrophobicity of cultured macrophages, caused an increase in the cells' ability to ingest opsonized red blood cells. This effect, which has been reported by a number of authors, is only specific for complement-receptor-mediated phagocytosis and not for Fc-receptor-mediated phagocytosis.

1.1.3.3. Requirements for Phagocytosis. As with pinocytosis, a variety of inhibitors have been used to evaluate the requirements for phagocytosis. It is generally thought that the binding step does not require temperature and energy, but it is clear that both are required for ingestion to occur (Silverstein et al., 1977). The role of calcium during phagocytosis is not very clear. The presence of divalent cation has been shown to be necessary for macrophages to bind with opsonized red blood cells and also the yeast Candida crusei (Uher et al., 1981; Warr, 1980). Parod & Brain (1983) also showed that extracellular calcium promoted the binding of polystyrene beads to hamster alveolar macrophages. With respect to intracellular calcium levels, Lew et al. (1985) showed in human neutrophils using opsonized red blood cells, that complement-receptor-mediated phagocytosis was not affected by changing calcium concentrations, but that Fc-receptor-mediated phagocytosis was, requiring at minimum the physiological level of intracellular calcium for ingestion to occur.

1.1.3.4. Stimulators of Phagocytosis. A number of factors increase phagocytosis, but the mechanisms underlying the stimulation remain undefined. Endotoxin, a substance that was reported to increase pinocytosis (see Section 1.1.2.4.), also increases phagocytosis; this has been shown in bone marrow-derived mouse macrophages (Cooper et al., 1984) and horse monocytes (Bennett & Cohn, 1966). In contrast interferon, although having an inhibitory effect on pinocytosis (see Section 1.1.2.3.), stimulates the phagocytosis of opsonized red blood cells (IgG-coated) by mouse peritoneal macrophages (Wang et al., 1984).

The effect of several stimulatorsof phagocytosis appears to be dependent on the age of the cells. For example Rohnsack et al. (1985) showed that the enhancing effect of lamin on the phagocytosis of complement and IgG-coated red blood cells was most pronounced after human monocytes had been in culture in vitro for five days. Also the enhancing effect of a glucocorticosteroid, dexamethasone, on the phagocytic uptake of IgG-coated red blood cells by bone marrow-derived macrophages was suppressed following 156 hours of in vitro culture (Shezen et al., 1985).

1.1.4. Some unanswered questions

A major difference between the two endocytic processes is the need for a "trigger". Phagocytosis only occurs following the attachment and recognition of a particle, whereas pinocytosis is thought to be an ongoing, constitutive process. The exact nature of the "trigger" for phagocytosis is not clear. However it is thought that intracellular free calcium ions act as secondary messengers mediating phagocytosis. Surface stimuli are known to trigger transient

changes in intracellular free calcium. For example, Young et al. (1984), using the fluorescent indicator Quin 2, showed an increase in free cytosolic calcium ions in the mouse macrophage cell-line J774, following interaction of the cells with ligands for the Fc-receptor. The increase was only partly inhibited by the extracellular presence of the calcium-chelator, ethylene glycol-bis(ß-aminoethylether)N,N,N'N,N'-tetracetic acid (EGTA), indicating that it was due to both calcium influx and mobilization from intracellular stores (not exclusively mitochondria). Furthermore they suggested that localized cytosolic calcium ion gradients were important in generating signals for phagocytosis. The gradients probably occur in areas of the cell in contact with the phagocytosed particle. Sawyer et al. (1985), also using Quin 2, have demonstrated regional differences in the concentration of calcium ions in resting human neutrophils and in those migrating towards and ingesting a particle (opsonized zymosan). Higher levels of free cytosolic calcium were apparent in loci where some relevant cellular function occurred; for example, in the lamellipodium during chemotaxis and the pseudopods and periphagosomal region during ingestion. In contrast with the data of Young et al. (1984), the increase in cytosolic calcium was thought to be mainly due to an influx of extracellular calcium, rather than to both an influx and calcium mobilization. These differences may derive from real differences between the species or cell-types used: Young et al. (1984) used a mouse macrophage cell-line, whereas Sawyer et al. (1985) used human neutrophils.

In contrast to phagocytosis, pinocytosis is thought of as an ongoing process, with no apparent trigger. Substrates are thought to be captured by the cell simply because they are present in the forming

pinosome. However, an important question remains unanswered concerning the constitutive nature of pinocytosis: what keeps pinocytosis going? As noted above, pinocytosis involves major membrane events and is dependent on metabolic energy and extracellular calcium. To describe it as constitutive does not imply that it is untriggered, merely that we do not know what continuous intracellular signals are required to allow pinocytosis to proceed.

It is well established that an increase in intracellar free calcium ions mediates a number of cell responses (Campbell, 1985). Recent work by Koenig (1983a) provides evidence that free cytosolic calcium is involved in controlling several transport processes, including pinocytosis. Rat kidney cortex slices were used to study various intracellular changes during the β-adrenergic stimulation of the pinocytosis of horseradish peroxidase. Stimulation of pinocytosis by testosterone involved an increased influx of extracellular calcium and a mobilization of cytosolic calcium from intracellular (mainly mitochondrial) stores. Also for the maintenance of the basal rate of endocytosis, extracellular calcium was required (Goldstone et al., 1983a). Koenig's group also showed that, upon pinocytic stimulation by testosterone, a transient increase in ornithine decarboxylase (the rate-regulating enzyme for the synthesis of polyamines) was observed. Also a sustained increase in intracellular polyamine concentrations was seen, which was needed for the stimulation of pinocytosis, along with the transient increase in intracellular-free-calcium mentioned above (Koenig et al., 1983b). They hypothesized that the polyamines served as messengers to generate calcium signals by increasing free cytosolic calcium (via influx and mobilization as described earlier). The resultant rise in calcium ions triggering the rapid increase in pinocytosis. In support of this theory, the same effect was observed

following stimulation by the ß-adrenergic agonist isoproterenol (Koenig et al., 1983a), and was shown to be inhibited by the ß-adrenergic antagonist propanalol (Goldstone et al., 1983b). Koenig's work could be criticised in that only single time-points were used to quantitate rates of pinocytosis (thus giving no indication of the kinetics of the stimulation). Also slices of kidney cortex were used; presumably the preparation of these would yield heterogenous samples in terms of cell damage and thickness. In addition the substrate used was not ideal: horseradish peroxidase would be rapidly degraded once pinocytosed by the lysosomal system. Nevertheless the results are very interesting and are discussed further in Chapter 9.

An alternative approach to identifying the intracellular trigger for pinocytosis is to study a cell-type in which pinocytosis is not present initially but "starts up" during the cell's differentiation. One can then study events surrounding this onset period.

To my knowledge, only one such cell-type is known, the monocyte, in which pinocytosis has been reported to develop as the cell differentiates in vitro into the mature cell, the macrophage. Bennett & Cohn (1966) in a study on the isolation and properties of horse monocytes, reported a single experiment, in which monocytes were observed to have little or no microscopically discernible pinocytic activity for the first 20 hours of culture in vitro, after which vesicle formation, fusion and movement became progressively more active. As previously mentioned (see Section 1.1.2.4.), this pinocytic activity was augmented in the presence of endotoxin. Likewise, in a major study of changes in lipoprotein metabolism during the development of human monocytes (from both normal and

familial hypercholesterolaemic subjects), Knight & Soutar (1982), report a single experiment in which pinocytosis was monitored as the cells differentiated. They showed the cellular uptake of PVP to be low on Day 2 of culture, but to increase by approximately five-fold by Day 5, then remaining constant up to Day 15, (N.B. cells referred to as Day 0 are freshly isolated cells, Day 1 being 24 hours old, and so forth). The process was not studied further.

The object of the work reported in this thesis (described more fully in Section 1.2.8.), was to study to what extent, if any, pinocytosis occurred in the young, developing monocyte, and to examine events surrounding this onset. (Even at this early stage of the thesis, it should be mentioned that another report other than those of Bennett & Cohn (1966) and Knight & Soutar (1982) exists: Van Furth et al. (1979) showed no great difference in the percentage of cells pinocytosing when comparing monocytes, either freshly isolated or cultured in vitro for 48 hours, with freshly isolated macrophages.)

The monocyte and macrophage belong to the mononuclear phagocyte system, and the next section of this Introduction describes the characteristics of this cell-lineage.

1.2. THE MONONUCLEAR PHAGOCYTE SYSTEM

1.2.1. Introduction

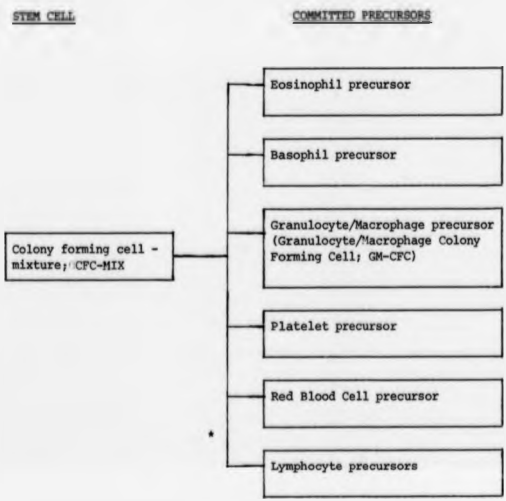
The mononuclear phagocyte system, previously known as part of the reticuloendothelial system, includes all highly phagocytic mononuclear cells and their precursors. Briefly, the precursor cells are found in the bone marrow, and these give rise to monocytes in the

blood, which mature into macrophages. The macrophages are found either free or fixed in various tissues, and have been given various names according to their location (for example Kupffer cells are liver macrophages). The system has been reviewed recently by Van Furth & Sluiter (1983).

The macrophage is generally thought to be the major functional cell in this system with respect to host defence. The functions of macrophages have been reviewed by Cline et al. (1978). The macrophage is believed to act as a scavenger cell at sites of inflammation, removing cellular debris; to be involved in the induction and the regulation of the immune response, where it interacts with various other cells (e.g. lymphocytes); to be the body's main defence against a number of microorganisms, particularly those with significant intracellular stages in their life-cycles (e.g. Plasmodium spp.); and to play an important role in controlling the development and spread of neoplastic cells. Macrophages are sometimes described as "activated" or "stimulated". Activated macrophages are cells present in animals that have been sensitized to specific antigens (e.g. endotoxin). As their name implies, they are generally more active than normal (or resident) macrophage populations. For example only the "activated" cells possess antitumour activity. The term "stimulated" macrophage is used to describe cells obtained from an animal following the administration of a non-specific irritant (e.g. mineral oil). "Activated" and (to a lesser degree) "stimulated" cells have a greater phagocytic capacity than the resident macrophages (Edelson et al., 1975; Finbloom, 1985, and North, 1978).

Figure 1.3.

Haemopoietic Cell Compartments in Man

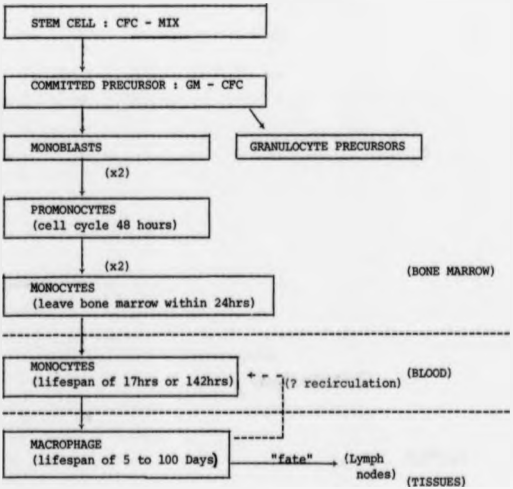


*In the mouse lymphocyte precursors arise from an equivalent stem cell to the CFC-MIX, the assumption is that lymphoid cells arise from the CFC-MIX in man, but unlike the other precursors this has not yet been proved. (Reference: Dexter, 1983).

Figure 1.4.

The Origin, Kinetics and Fate of Human Mononuclear Phagocytes:

The Classical View



NB At each stage upto the monocyte, division and differentiation occur. Monocytes do not proliferate but differentiate into the macrophage which is also non-proliferative.

In order to aid in their functional tasks, macrophages secrete a number of products. These have been reviewed by Davies & Bonney (1979), and Page et al. (1978) and include various enzymes; enzyme inhibitors, for example α_2-macroglobulin which inhibits proteases; and a variety of biologically active factors, including prostaglandins, colony-stimulating factor, complement components, pyrogen and interferon.

1.2.2. The origin, kinetics and fate of the mononuclear phagocytes

In man, all blood cells originate from a common stem-cell which is found in the bone marrow. The current view of this haemopoietic system, as reviewed by Dexter (1983), is given in Figure 1.3.. The stem-cell is capable of self-renewal by mitosis and is pluripotent, i.e. able to give rise to various progenitor cells. Each progenitor cell is highly proliferative and usually committed to producing precursor cells of only one cell lineage. However the progenitor cell that gives rise to the mononuclear phagocytes also gives rise to the granulocyte lineage; it is therefore called the granulocyte-macrophage colony-forming cell (GM-CFC).

Various procedures have been used to study the kinetics and migration of mononuclear phagocytes from the bone marrow through to the tissues, including in vivo labelling of cells with [^{3}H]thymidine and the injection of fluorescently-labelled bone-marrow cells into recipients (Van Furth & Cohn, 1968; Volkman, 1976). Data from these approaches resulted in the "classical" scheme shown in Figure 1.4.. This subject has been reviewed recently by Dougherty & McBride (1984), who make it clear that certain points are still debated; these will be outlined below.

It is accepted that the stem-cell divides and differentiates, giving rise to committed progenitor cells (GM-CFC). These likewise give rise either to cells committed to the granulocyte lineage or to monoblasts which are then committed to the macrophage lineage. The monoblast divides, giving rise to two promonocytes. Promonocytes divide 48 hours later each giving rise to two monocytes. In general one promonocyte generation only is thought to exist, but Boll (1974) described a further cell-division to yield two generations of promonocytes in man. The monocytes leave the bone marrow, entering the blood, usually within 24 hours of their formation. The period monocytes remain in the blood is about 142 hours (Van Furth & Sluiter, 1983). The monocytes leave the blood at random, differentiating into macrophages in the tissues. The time macrophages are thought to remain in the tissues is variable, ranging from 5 to 100 days. Macrophages are generally thought not to proliferate, and the populations are thought to be maintained by an influx of monocytes. A small degree of local proliferation is often apparent thought to be due to the presence of immature dividing cells (i.e. promonocytes). It is not clear whether macrophages can recirculate back into the blood during their lifespan, but eventually they are thought to migrate to the lymph nodes. Van Furth & Sluiter (1983) showed macrophages not to be present in the efferent lymph. They are therefore presumed to die in the lymph nodes.

Although the most common view is that all macrophages are derived from monocytes, recent evidence by a number of authors supports a non-monocytic origin for tissue macrophages (i.e. monocytes only giving rise to inflammatory (exudate) macrophages). Daems & DeBakker (1982) studied the origin of mouse peritoneal macrophages, using both immunocytochemical and [^{3}H]thymidine labelling/autoradiography

Table 1.1.

Factors thought to regulate the proliferation of mononuclear phagocytes

Factor	Source	Effect	Reference
Factor increasing monocytopoiesis- FIM	Macrophages	Stimulates promonocyte proliferation	Van Furth and Sluiter, 1983.
Monocyte production inhibitor; MPI	Serum, during 2° antibody response	Inhibits promonocytes	(as above)
Migration inhibition factor; MIF	Lymphocytes	Inhibits monocyte to macrophage transition, also retrodifferentiation of macrophages	Sorg et al., 1984.
Colony stimulating activator; CSA	Macrophage leukaemic cell-lines	Stimulates mononuclear phagocyte production	Ascensao and Mickman, 1984.

techniques. Their conclusions were that:- peritoneal macrophages derive from locally dividing progenitor cells which arise from specific bone-marrow stem cells not of the granulocyte/macrophage lineage. In support of a non-monocytic origin for peritoneal macrophages, several authors have described and characterized a bone-marrow cell that is different from the progenitor cell GM-CFC and is capable of giving rise *in vitro* to macrophage populations but not to granulocytes (Chen *et al.*, 1979; Metcalf, 1982).

1.2.3. Regulation of the mononuclear phagocyte system

A number of regulatory steps have been demonstrated *in vitro* using human bone-marrow cells which, when cultured in soft gel, give rise to both granulocyte and macrophage colonies. In order for the stem-cells to give rise to the progenitor cell GM-CFC, growth factors are required from conditioned medium (Johnson & Metcalf, 1977). The growth factor colony-stimulating-factor (CSF) was then shown to be necessary for the GM-CFC to form granulocyte or macrophage colonies. CSF has recently been reviewed by Stanley *et al.* (1983). The biological effects of CSF are mediated by a specific receptor. In addition to the proliferative effects of CSF, it is thought to stimulate the survival and differentiation of various mononuclear phagocytes, i.e. bone-marrow-derived macrophages, and peritoneal macrophages. A secondary role for CSF has been postulated, related to the activation of mature macrophages, for it is able to increase their cytocidal capacity.

A number of other factors are thought to be involved in regulating the proliferation of mononuclear phagocytes and some of these are listed in Table 1.1.. It is also evident that many factors either

Table 1.2.

Factors affecting the maturation of mononuclear phagocytes

Factor	Effect	Target cell	Reference
Phorbol esters	Stimulatory	Monoblast cell-line (human)	Hattori et al., 1983.
Retinoic acid	Stimulatory	Monoblast cell-line (human)	Hattori et al., 1983.
Interferon	Inhibition	human monocytes	Becker, 1984.

inhibit or stimulate the subsequent maturation of the monocyte into the macrophage: a few of these are listed in Table 1.2., and include substances such as interferon, retinoic acid and phorbol esters. In addition, a number of disease states have been shown to inhibit the maturation of monocytes to macrophages; these include leukaemia, carcinoma and various inflammatory diseases (DeMulder et al., 1983; Van Furth & Van Zwet 1983, and Yoda et al., 1984). Many inflammatory conditions also cause an increase in the number of immature mononuclear phagocytes in the blood and tissues. Past disease states can also affect the cells. For example, Salahuddin et al. (1982) developed an in vitro system for long-term cultures of human monocytes and showed that the presence of Epstein-Barr virus antibody in the blood drastically reduced the success rate of his cultures (only 1 in 10 were successful).

1.2.4. Heterogeneity in mononuclear phagocytes

There is a wealth of data on macrophage heterogeneity, a subject that has recently been reviewed by Dougherty & McBride (1984). Two types of heterogeneity have been characterised. The first exists between macrophage populations from different anatomical sites: this inter-population heterogeneity has been reviewed by Walker (1982), who described how cells from different sites may have different properties. Most studies have involved comparison between alveolar and peritoneal macrophages. For example, the former cell was shown to gain energy from oxidative phosphorylation, the latter from glycolysis. The second type of heterogeneity is seen among cells from the same site, i.e. intra-population heterogeneity. Differences may be morphological, functional or metabolic.

The question must arise as to the causes of such heterogeneities. Two possibilities exist. The first is that distinct subsets exist, i.e. cells with predetermined tissue destinations and/or functional capabilities. There is no conclusive evidence to back this suggestion. The second possibility is that only one cell-type exists which can adapt to the demands of different environments. This latter view was amplified by Dougherty & McBride (1984), who suggested that heterogeneity could arise from such an adaptive cell due either to modulation by various environmental/inflammatory stimuli or by changes which occurred during the differentiation of the cell. Thus in the latter case heterogeneity would be due to the presence of different-aged cells in a population, with unique age-dependent characteristics. Many changes do occur as monocytes mature and these are outlined in the next section.

There is evidence that the inter-population heterogeneity described is due to cells being modulated by local stimuli. For example Bar-Eli et al. (1981) showed that enzyme expression of bone-marrow precursor cells could be readily altered by changes in the micro-environment.

1.2.5. Differences between monocytes and macrophages

Although there have been a number of studies with laboratory animals, there is relatively little information on the differences, in form and function, between circulating monocytes and tissue macrophages in man. This is chiefly due to the difficulty of obtaining human macrophages. However, Van Furth et al. (1979), using a number of parameters, compared blood monocytes with tissue-macrophages isolated either from the peritoneal cavity of

women undergoing laparoscopy, or from the skin following "skin-window" preparations (where macrophages are allowed to adhere to glass coverslips placed on skin abrasions). Cells were maintained for short periods attached to coverslips within a Leighton tube culture system. Tissue-macrophages (Giemsa-stained) were much larger than monocytes and exhibited a greater degree of spreading, and a greater proportion possessed rounded nuclei rather than the indented nuclei apparent in monocyte populations. Cytochemical differences were seen, almost all of the monocytes possessing peroxidase activity, compared with 63 percent of the peritoneal macrophages (this decrease in peroxidase activity was thought to represent the loss of the enzyme from the older cells (see later, Section 5.1.3.). However 97 percent of skin-macrophages demonstrated peroxidase activity, although this number rapidly decreased to 80 percent following culture in vitro for a 6 hour period. It was concluded that the skin-macrophages represented young macrophages, i.e. recently derived from circulating monocytes. When esterase activity was measured (cytochemically), although equal numbers of both populations possessed activity, the macrophages stained more weakly. Also, esterase staining was shown to be inhibited by sodium fluoride only in the monocytes.

The most striking difference was found when comparing the cells' ability to phagocytose via the complement-receptor (using complement- and IgM-coated erythrocytes): only 16 percent of monocytes, but 74 percent of macrophages, phagocytosed via this receptor. This could not be attributed to the number of receptors present in the two populations, as there was little difference in the numbers. The ability to phagocytose via the Fc- receptor did not differ between the two populations. Also, the cells' ability to pinocytose dextran

sulphate (determined microscopically) differed only slightly; this is discussed later in Section 1.2.7.8..

In a more limited study, Biondi et al., (1984) described the presence of previously unreported surface-antigens (recognized by two new monoclonal antibodies BMM1 and PAM1) on both breast-milk and pulmonary macrophages, but not on blood monocytes, or indeed other mononuclear phagocytes (obtained from different sites). The expression of these antigens on pulmonary macrophages, along with the disappearance of antigens more common to the macrophage-lineage, indicated a significant heterogeneity at the membrane level among cells from different anatomical sites. This concept of heterogeneity is described in Section 1.2.4., and its importance in studying the developing monocyte is discussed later in Chapter 9. It is clear from such studies that it is very difficult to obtain normal, tissue macrophages routinely from healthy volunteers. The cells have usually been isolated from patients undergoing surgery, and so any underlying disease state could affect the cells' characteristics. Likewise, the isolation of both skin and peritoneal macrophages appears to select for a young exudate-population. Such cells may in fact have been "stimulated" by the isolation procedure. Also cells isolated from different anatomical sites may be very different to each other. Because of such problems, most studies on the developing monocyte involve the isolation of the readily available blood monocyte, which is allowed to mature in vitro into the monocyte-derived macrophage.

The transition from monocyte to macrophage in vitro is thought to parallel the in vivo situation. This was demonstrated by Hammerström (1979), who compared human monocytes with human peritoneal

macrophages, as each was maintained *in vitro*. The two freshly-isolated populations showed differences in the initial stages of culture, the macrophages having greater effector cell function (e.g. the ability to degrade phagocytosed *Candida albicans*, and to suppress DNA synthesis of target cells) than the monocytes. After a few days in culture the monocytes developed similar effector functions and morphological characteristics to the freshly-isolated macrophages. Ideally it would have been interesting to compare the two populations from the same donor, to avoid any donor variation, but this was not ethically possible owing to the risk of causing anaemia.

1.2.6. Changes during the monocyte-to-macrophage transition *in vitro*

1.2.6.1. Introduction. Although few authors have characterised differences between freshly isolated populations of human monocytes and macrophages, many have reported changes occuring during the *in vitro* transformation of monocytes into macrophages. Surprisingly, only one short review (Musson, 1984) has been published.

1.2.6.2. Morphological changes. Lewis, in 1925 first cultured and described the development of macrophages from a mixed population of white blood cells in incubated hanging drops of human blood. She noted that after about four days macrophages developed; these were much larger than the other cells and were phagocytic towards red blood cells. Cell-separation and culture techniques have developed considerably since Lewis's day, enabling the development of macrophages *in vitro* from isolated blood monocytes, and allowing the study of numerous other parameters.

Morphological changes that occur during the monocytes' differentiation into macrophages *in vitro* include increased numbers of lysosomes (Goldstein, 1954) and lipid vacuoles (Goldstein & McCormick 1951), and an increase in membrane ruffling and in the numbers of microvillous projections and filopodia (Zuckerman *et al.*, 1979). Most strikingly the cells become larger as they mature (Johnson *et al.*, 1977). Zuckerman *et al.* (1979) in agreement with others described the mature cells as a heterogenous population in relation to shape and size. The morphological characteristics are discussed in detail in Section 3.1.5..

1.2.6.3. Changes in structural markers

The expression of various surface antigens, defined by using monoclonal antibodies, is thought to change as monocytes differentiate into macrophages *in vitro*. For example the membrane marker 63D3 decreased, and yet the proportion of cells expressing the HLA-DR antigen increased markedly after Day 0, and then decreased (Haimovitz *et al.*, 1983). These authors were careful to point out that the expression of both of these markers was modulated by the presence of different sera, and the latter also by exposure of the cells to lymphokines and interferon.

1.2.6.4. Biochemical changes. Johnson *et al.* (1977) have demonstrated a close correlation between the cell numbers and the protein content of a monocyte population cultured *in vitro*. Both parameters decreased by approximately 50 percent of the initial values by Day 4, after which the cell number continued to decrease (very slowly), whereas the protein content steadily increased. These data indicate that the protein content per cell remains stable up to

Day 4, after which it increases. Similar data have been reported by many authors (Musson et al., 1980; Nakagawara et al., 1981; O'Dorisio et al., 1984).

In general, as monocytes mature into macrophages they are more metabolically active. Bennett & Cohn (1966) noted an increase in glucose utilisation during the differentiation process. Much later, DeMulder et al. (1983) noted an increase in three enzymes of intermediary metabolism, namely glucose-6-phosphate dehydrogenase, phosphohexose isomerase and isocitrate dehydrogenase. The intracellular levels of a number of other enzymes rise as monocytes mature in vitro, including lactate dehydrogenase and creatinine kinase (O'Dorisio et al., 1984; Loike et al., 1984). Creatinine kinase activity was not detected until the fifth day in culture, after which it increased dramatically.

Using cytochemical staining techniques, Salahuddin et al. (1982) demonstrated an increase in non-specific esterase (NSE) activity as macrophages aged in vitro. Musson (1983) further showed that monocyte NSE staining became resistant to the inhibitor sodium fluoride as the cells matured. Cyclic nucleotide levels have also been studied in maturing monocytes by O'Dorisio et al., (1984), who demonstrated a 17-fold increase in cAMP, cGMP remaining relatively constant. The cAMP:cGMP ratio increased dramatically from 2:1 in monocytes to 9:1 in macrophages, suggesting that cAMP may be an important mediator of cell differentiation.

Macrophages are active secretory cells and various enzymes such as plasminogen activator and angiotensin-converting enzyme are secreted to a greater extent as monocytes mature in vitro (Ragsdale & Arend,

1981; Andreason et al., 1983). The secretion of the latter enzyme is only detected in cells cultured beyond Day 7. The secretion of several mediators of host defence alters as monocytes mature in vitro, the most well studied being complement. Cole et al. (1982) reported an increase in both the synthesis and secretion of complement (C2) during the monocyte/macrophage transition. However the increase was not found to approach the level seen in mature macrophages directly isolated from breast milk, indicating that factors other than those provided in vitro could be required for the maximal development of complement synthesis in monocytes.

A number of other products are only secreted by monocyte/macrophages older than Day 3 to 4. These include fibronectin (which has been proposed as an opsonin), and alpha-2-macroglobulin (Alitalo et al., 1980; Movi et al., 1977). Older cells also secrete a unique over-sulphated galactosaminoglycan (Kolset et al., 1983); this secretion was later shown to be prevented if the cells were grown on fibronectin-coated dishes rather than plastic dishes (Kolset et al., 1984). The importance of the culture environment with respect to the various changes is discussed in Chapter 9.

1.2.6.5. Functional changes. The maturation of monocytes into macrophages is associated with many changes in the cells' functional abilities. These include a decrease in chemotactic activity (Van Der Meer et al., 1982); a deficit in accessory cell function (Mayerik et al., 1983); and enhanced phagocytic and cytotoxic ability (Hammerstrøm, 1979, and Wuest et al., 1981).

The cytotoxic ability of maturing monocytes has been well studied using normal or neoplastic target cells. Results however are very

contradictory and appear to be affected by factors such as the type of serum present. For example Musson (1983) and Andreesen et al. (1983) both noted an increase in the cytolytic ability of monocytes over a 7-day culture period, although the timing of the increase differed slightly. However Becker (1983) using the same target cell as Musson found that the cytotoxic ability of monocytes was lost following overnight culture. The difference may have been due to the type of serum employed, for Musson used autologous serum and Becker used heterologous AB positive (human) serum. In addition Becker noted that the cytotoxic ability could be maintained by using fetal calf serum, which Andreesen also used, and could be enhanced by both interferon and endotoxin. Wuest et al. (1981) also noted an increase in the cytotoxic activity of monocytes in culture, but noticed considerable donor-to-donor variation, a point which is discussed further in Chapter 9.

1.2.7. Endocytosis-related changes during the monocyte-to-macrophage transition in vitro

1.2.7.1. Introduction. We now turn to the changes in particular features of monocyte/macrophage populations in vitro, that will become the main focus of this investigation. In an earlier Section (1.1.4.) we have already reported that pinocytosis appears to develop during monocyte maturation, and this observation is now discussed more fully in the context of other endocytic events.

1.2.7.2. Changes in plasma membrane enzymes. Changes in the activities of ectoenzymes are of interest, as the plasma membrane is thought to recycle constantly during pinocytosis and is also intimately involved in the phagocytic process. For example the

activity of the ectoenzyme 5'-nucleotidase has been correlated with phagocytic function: its activity decreases upon phagocytic challenge from that measured in unchallenged cells, and this decrease relates to the size of the phagocytic challenge (Johnson et al., 1977). 5'-nucleotidase turnover at the cell surface also correlates well with the rate of fluid-phase pinocytosis (see Section 1.1.7.1.). Data on the activity of 5'-nucleotidase in monocyte/macrophage cultures are contradictory: Johnson observed an 11-fold increase by Day 2, with no further increase in cells grown up to Day 4. However Knight & Soutar (1982), Musson et al. (1980) and O'Dorisio et al. (1984), although confirming the initial increase, reported a subsequent decline in the activity, the enzyme levels being less by Day 14 than those observed in the freshly-isolated cells.

The enzyme transglutaminase has also been implicated in endocytosis. Its role is not clear, but it has been postulated to regulate the clustering of receptors into coated pits (see Section 1.1.2.3.), and also possibly to play a role in Fc-mediated phagocytosis in macrophages (Schroff et al., 1981). The level of this enzyme increases 50-fold during the maturation of the monocyte in vitro (Murtaugh et al., 1984).

1.2.7.3. Changes in lysosomal enzymes. As monocytes mature into macrophages there is an increase in the intracellular activity of a number of lysosomal enzymes including, ß-glucuronidase, ß-glucosaminidase and leucine-aminopeptidase (Becker, 1984; Musson, 1983; Newman et al., 1980). An increase in acid phosphatase is also apparent, up to 60-fold between Day-1 and Day-7 monocyte/macrophages (Newman et al., 1980), but the reported timing of the increase varies greatly between authors: Zuckerman et al. (1979) noted that the

increase occurred on Day 5, whereas Becker (1984) observed the increase on Day 1.

Increases in the secretion of lysosomal enzymes have been reported during the differentiation of monocytes to macrophages, most notably in the secretion of lysozyme. Lysozyme secretion increases by 10-fold up to Day 7 and by 30-fold up to Day 14 (Zuckerman et al., 1979).

In contrast to the reports showing an overall increase in lysosomal enzyme activity during monocyte maturation, Johnson et al. (1977) showed an initial and sustained decrease in myeloperoxidase activity over the first three days in culture. This decrease paralleled the disappearance of peroxidase granules monocyte cultures (Gordon et al., 1974). The role of this enzyme in mononuclear phagocytes is discussed in Section 5.1.3..

The release of lysosomal hydrolases from various mononuclear phagocytes has been shown to be greatly stimulated by certain phagocytic stimuli, i.e. those with chronic inflammatory potency such as zymosan particles . This response is not seen in freshly isolated monocytes but occurs after several days in culture (e.g. Knight & Boutar, 1982, Musson et al., 1980). Further details of these experiments are given in Section 5.1.2..

1.2.7.4. Fc-receptor-mediated phagocytosis. All mononuclear phagocytes possess surface receptors for the Fc region of immunoglobulin, i.e. the "Fc-receptors". Fc-receptors recognise material opsonized with the IgG class of antibody such as IgG coated-erythrocytes (EIgG). It is generally agreed that the ability

to endocytose via the Fc-receptor increases as monocytes differentiate *in vitro* (Andreesen *et al.*, 1983; Musson, 1983; Newman *et al.*, 1980): the actual percentage of cells ingesting EIgG usually remains stable, but the number of EIgGs ingested per cell increases.

The increase in Fc-receptor-mediated phagocytosis often occurs around Day 3 of the monocyte/macrophage cultures. Weust *et al.* (1981) showed that this increase in phagocytosis was due to an increase in the number of receptors, rather than to increased receptor avidity. In addition a new type of Fc-receptor recognized by the monoclonal antibody 3G8 has been shown to appear on Day 3 on a proportion of the monocyte/macrophage cell population (Fleit *et al.*, 1982). This new receptor was also expressed on 55 percent of freshly isolated pulmonary macrophages.

In contrast to these increases, Haimovitz *et al.* (1983) reported the stable expression of the Fc-receptor during the monocyte-to-macrophage transition. They also noted an effect of serum on its expression, in that cells cultured in human serum, as opposed to fetal calf or bovine serum albumin, did not express the receptor to as great an extent.

1.2.7.5. Complement-receptor-mediated phagocytosis. Unlike the Fc-receptor, which is expressed by all mononuclear phagocytes, the complement receptor is often only expressed at certain stages of differentiation of the cell. Three distinct receptors exist, CR_1, CR_2 and CR_3, each recognizing different forms of the third component of complement. The expression of the CR_1-receptor is stable during the maturation of monocytes *in vitro* (Inada *et al.*, 1983, Haimovitz *et al.*, 1983). With these studies only Day-0, Day-1 and Day-7 cells

were tested, and therefore any change between Day 1 and Day 7 may have been undetected. However the expression of the CR_2-receptor was shown to appear in monocytes following the first three hours in culture (Inada et al., 1983). With the CR_3-receptor an initial decline in its expression was noted in monocyte/macrophage cultures, followed by a recovery which commenced on Day 3, maximal expression being reached by Day 10.

Phagocytosis via the complement receptor is usually measured using erythrocytes coated with IgM and complement. It is generally thought that the ability to ingest complement-coated erythrocytes via the complement-receptor appears during the maturation of monocytes into macrophages, usually around Day 3 (Newman et al., 1980; Musson, 1983). As indicated earlier, Van Furth et al. (1979) showed that a higher proportion of human peritoneal macrophages ingested complement-coated erythrocytes than did blood monocytes. Newman et al. (1985) recently obtained human peritoneal macrophages and examined complement-receptor-mediated phagocytosis in fractions of increasing maturity (separated by 1g velocity sedimentation). They showed a maturation-dependent ability to phagocytose via the complement-receptor, in agreement with the previous studies.

1.2.7.6. Non specific phagocytosis. Studies on non specific phagocytosis by maturing monocyte cultures are not very common and often contradictory. The discrepancies may be due to the different substrates used or to differences in culture systems (i.e. adherence v. suspension cultures). For instance Van Der Meer (1982) using suspension cultures of human monocytes showed no significant increase in the cells' ability to ingest two Staphylococcus spp. over a 21-day period. However a number of authors using adherent cultures noted an

increase in phagocytic ability as monocytes matured: for example Odegaard et al. (1984) using Candida albicans as the substrate, Bennett & Cohn (1966) using Staphylococcus albus and Speert & Silverstein (1985) using zymosan. Interestingly the ingestion of zymosan commences and the mannose-receptor appears on Day 3 in monocyte/macrophage cultures. This, and the fact that the uptake of zymosan is inhibited by yeast mannan, lead Speert & Silverstein (1985) to conclude that the particle is phagocytosed via mannose-receptors.

1.2.7.7. Receptor-mediated endocytosis (pinocytosis). As monocytes mature into macrophages the expression and activity of a number of receptors involved in receptor-mediated endocytosis changes. For example by measuring radiolabelled degradation products of various ^{125}I-labelled low density lipoproteins (LDL), it has been shown that the activity of the LDL-receptor, which binds native LDL, decreased as the monocytes matured, whereas the activity of the modified LDL-receptor which recognizes modified forms of LDL, increased (Fogelman et al., 1981, Knight & Soutar 1982). Knight & Soutar (1982) pointed out that the increase in the activity of the latter receptor, coincided with the increase in phagocytic ability of the cells.

Shepherd et al. (1982) followed the clearance of radiolabelled mannose-terminating ligands by the mannose-receptor in various mononuclear phagocyte populations. She showed that human alveolar macrophages ingested via the mannose-receptor, whereas the human monocyte-like cell-line U937 did not. Also freshly isolated monocytes did not express the receptor until Day 2, the activity being greatest on Day 5, then decreasing slightly over the next 2

days. Shepherd suggested that this reduction in mannose-receptor activity after Day 5, may correlate with the cells progressing beyond the state of normal macrophages, since activated macrophages also exhibit decreased receptor activity (Rzekowitz et al., 1981).

1.2.7.8. Fluid-phase pinocytosis. Unlike the endocytic processes just described, very little has been reported on fluid-phase pinocytosis as monocytes mature into macrophages in vitro. It has already been described (see Section 1.1.4.), how pinocytosis is thought by two authors to "start up" during monocyte differentiation. Bennett & Cohn (1966), isolated horse monocytes from blood using albumin gradients, and maintained the cells in "Leighton-tubes" in medium supplemented with 40 percent newborn calf-serum. They described how vesicle formation and fusion were not morphologically discernible until after the first 20 hours of culture in vitro. Between 20 and 72 hours, the pinocytic activity became progressively more active. Knight & Soutar (1982), isolated human monocytes from the blood and "buffy-coats" of normal and familial hypercholesterolaemic subjects, using the gradient material Ficoll-hypaque. The cells were maintained in plastic wells in medium supplemented with 20 percent autologous serum, the medium being changed on Day 3. Pinocytic activity was assessed by measuring the uptake of ^{125}I-labelled polyvinylpyrollidone (PVP), a fluid-phase marker. They described how Day-2 cells demonstrated a low level of pinocytic activity, yet by Day 5 cells were capable of taking up five times more PVP, than the younger cells. There was no further increase in pinocytic activity over the next 10 days, and no difference was observed between the normal and diseased subjects.

In contrast to these two studies, one study is reported in which such a drastic increase in pinocytic activity is not evident as the cells mature. Van Furth *et al.* (1979) cultured human blood monocytes (isolated using Ficoll-hypaque gradients), for up to 48 hours in "Leighton tubes" in medium supplemented with 20 percent heat-inactivated newborn calf-serum, renewing the medium daily. Pinocytosis was determined morphologically using dextran sulphate over this period. Only a very slight difference in the pinocytic ability of the cell population was seen, approximately 70 percent of cells pinocytosing on Day 0 after 6 hours incubation with the substrate: 92 percent after 24 hours and 95 percent after 48 hours incubation respectively. It was also reported that the percentage of monocyte precursor cells (the promonocytes), which pinocytosed, was similar to the monocytes. With the fully differentiated cell the macrophage, depending on the site of isolation, 70 to 85 percent were found to be pinocytically active.

1.3. AIMS OF THESIS

As discussed earlier (see Section 1.1.4.) it is not clear from the data, whether or not pinocytosis develops as monocytes mature. The data of Knight & Soutar (1982) and Bennett & Cohn (1966), both suggest that a dramatic increase in pinocytic activity occurs as monocytes mature: Van Furth *et al.* (1979), indicated only a slight increase in the number of pinocytically active cells. The primary aim of this study is thus to use the *in vitro* monocyte-to-macrophage model, to resolve the doubt surrounding the onset of pinocytosis in the developing monocyte, and to study events surrounding this possible onset.

The problem in using such a model, is that one needs to be certain that the cells do in fact mature in the *in vitro* culture system employed. It is evident from the previous Sections (1.2.6. and 1.2.7.), that a wide variety of changes occur as monocytes mature into macrophages *in vitro*. Unfortunately many of the changes reported are in contradiction to each other, and may only be expressed by a proportion of the cell population; these therefore do not represent good "maturation markers". However, from the changes documented, it is apparent that several are common to a number of the studies. There has therefore developed a concensus of opinion as to which changes may be used to ascertain monocyte maturation. Such markers include the cells generally becoming larger and more well spread; the loss of peroxidase activity; the acquired ability to phagocytose via the complement-receptor; an increased secretion of lysosomal enzymes, and a further increase in enzyme release upon phagocytic challenge; an increase in the protein content per cell (following the first few days in culture); and the cytochemical demonstration of non-specific-esterase activity becomes resistant to inhibition by sodium fluoride (Andreesen *et al.*, 1983; Johnson *et al.*, 1977; Musson *et al.*, 1980; Van Der Meer *et al.*, 1982; and Zuckerman *et al.*, 1979).

A major part of my thesis is concerned with studying many of the parameters given above, in my cell-system, to be certain that the monocytes are truly maturing into macrophages.

CHAPTER 2

Experimental Methods

2.1. INTRODUCTION

Many of the methods used are outlined below but, for some of these methods, the exact details of the procedures are given in the appropriate Chapters. A few of the methods employed are described solely in the relevant Chapter. Unless otherwise stated, Analar-grade chemicals were used and operations performed at room temperature. Aseptic techniques were employed where necessary. Glassware was sterilized at 1.05kg/cm^3 for 15 minutes, and medium 199 and various additives sterilized with filter systems (supplied by Millipore Corporation, Massachusetts U.S.A. and Sartorious Instruments Ltd., Surrey, U.K.).

2.2. EXPERIMENTAL METHODS

2.2.1. Separation of human blood on density gradients of Percoll

The method used to obtain blood monocytes using the density-gradient material Percoll (supplied by Pharmacia Fine Chemicals A.B., Uppsala, Sweden), was as described by Pertoft et al. (1980). (Later, a modified version of this method was adopted, which is detailed in Section 4.5.). Human blood (20cm^3) was collected into evacuated glass tubes (10cm^3) by venipuncture of donors, who were already giving blood for routine blood testing. The blood was transferred into a glass container (20cm^3) containing three bent paperclips and gently agitated (15 minutes) to defibrinate it (fibrin adheres to the clips thus preventing clot formation).

Into duplicate conical plastic tubes (12cm^3), Percoll (4cm^3; adjusted to a density of 1.076gm/cm^3 in 0.15M NaCl) was placed. Defibrinated

blood (7cm^3 aliquots) was carefully layered over the Percoll and the tubes centrifuged (15°C; 800g; 30 minutes). This caused the red cells and granulocytes to sediment to the base of each tube. Above them were the separation fluid and the serum (uppermost), and at the interface were found mononuclear cells in a clearly discernible band. These cells were collected using a Pasteur pipette and washed (100g; 10 minutes) in medium 199 (prewarmed to 37°C; supplied by Gibco Europe Ltd., Uxbridge, U.K.). The cell pellets were resuspended and combined in a known volume (usually 1 to 2cm^3) of prewarmed culture medium (medium 199 containing 100,000 units penicillin-streptomycin; fungizone 2.5$\mu g/cm^3$; gentamycin 200$\mu g/cm^3$; fetalcalf and horse serum (heatinactivated, 10 percent w/v of each); supplied by Flow Laboratories Ltd., Hertfordshire, U.K.) and the suspension termed the "mononuclear cell preparation". Small portions of this "mononuclear cell preparation" were used to determine cell viability and the monocyte yield and purity, as described in Section 2.2.5. and 2.6.5., the remainder was introduced into a culture system as described in Section 2.2.7..

2.2.2. Separation of human blood on density gradients of Nycodenz

The method used to obtain blood monocytes using two different density Nycodenz (supplied by Nyegaard & Co., Oslo, Norway) gradients, was as described by Bøyum et al. (1983). Human blood (10cm^3) was collected into evacuated plastic tubes (5cm^3) containing disodium EDTA (14mg), by venipuncture of donors who were giving blood for routine blood testing. In duplicate, 3cm^3 portions of Nycodenz (either with a density of 1.078g/cm^3 or 1.069g/cm^3) were placed into conical plastic tubes (12cm^3). Anticoagulated blood (3cm^3 aliquots) was layered over the Nycodenz, and the tubes centrifuged (250g; 15 minutes). Red

cells and granulocytes sedimented to the bottom during centrifugation, directly above which was the separation fluid with a plasma layer uppermost. At the interface of the latter two was found a band of mononuclear cells. These bands were collected and the cells washed in medium 199 (prewarmed to 37°C; 10cm^3; 1000g; 10 minutes). The supernatants were discarded and duplicate cell pellets were combined and resuspended in a known volume of culture medium (usually 1 to 2cm^3; prewarmed to 37°C). These "mononuclear cell preparations" were used to determine cell viability and the monocyte yield and purity, as described in Section 2.2.5. and 2.2.6..

2.2.3. Separation of human blood using dextran

The method used to obtain blood monocytes by "dextran-sedimentation" was as described by Goldblatt et al. (1978). Human blood (20cm^3) was collected into evacuated plastic tubes (10cm^3) containing the anticoagulant heparin (100 units, supplied by Sigma Chemical Co., Dorset, U.K.) , by venipuncture of donors who were giving blood for routine blood testing. In duplicate, dextran (4cm^3; 4.5 percent w/v in PBS; m.w. 500,000; supplied by Sigma Chemical Co., Dorset, U.K.) was placed into conical-bottomed plastic tubes(20cm^3). Heparinised blood (10cm^3) was added to the dextran, and the mixture incubated (37°C; 20 minutes). The red cells sedimented to the bottom of the tube, leaving a white-cell-rich plasma above. This plasma was transferred to conical-bottomed plastic tubes (20cm^3). Equivolume medium 199 was added containing heparin (5 units/cm^3) and the tubes centrifuged (600g; 20 minutes). The supernatants were discarded and the cell pellets washed in plain medium (10cm^3) by centrifuging as above. The supernatants were discarded, and the pellet mixed with NH_4Cl solution (9cm^3 of 0.83 percent w/v NH_4Cl and 1cm^3 Tris buffer

(pH 7.65)), and allowed to stand for 8 minutes at 37°C to lyse any remaining red blood cells. The lysed red blood cells were removed by a further centrifugation (as above). The duplicate cell pellets were combined by resuspension in a known volume of culture medium (usually 1-2cm^3). This "mononuclear cell preparation" was used to determine cell viability and the monocyte yield and purity, as described in Sections 2.2.5. and 2.2.6..

2.2.4. Determination of the total white-cell count and the percentage of monocytes in human blood

The total number of white blood cells in freshly drawn human blood (defibrinated, heparinized or containing EDTA) was determined using a Coulter counter (Model ZB1, Coulter Electronics Ltd., Hertfordshire, U.K.). The percentage of monocytes was determined by examining Giemsa-stained blood smears. Blood smears were prepared on grease-free microscope slides. Smears were air-dried and fixed in methanol (100 percent; 15 minutes). The methanol was then replaced with the Giemsa stain (1 drop of Giemsa stain in 1cm^3 of water; 20 minutes). Smears were washed with distilled water and examined microscopically (x400 and x1000). The percentage of white cells recognized as monocytes (i.e. large cells with a single purple kidney-shaped nucleus and pale blue cytoplasm) was calculated, following the examination of 200 white blood cells. Having the total number of white cells in the blood and the percentage of these which were monocytes, it was possible to calculate the initial number of monocytes in the blood samples.

2.2.5. Determination of cell viability in "mononuclear cell preparations"

The viability of the white blood cells in the "mononuclear cell preparations" was determined using the trypan blue-exclusion test (Tullis, 1953); trypan blue does not penetrate into living cells. In a small tube 10 drops of the "mononuclear cell preparation" was placed, trypan blue (1 drop of 1 percent w/v) was added and the mixture incubated (37°C; 10 minutes). The suspension was examined microscopically (x200); stained cells were deemed dead and unstained cells viable.

2.2.6. Assessing the number of white blood cells and the monocyte purity and yield in "mononuclear cell preparations"

Using Neubauer counting chambers, white blood cell counts were performed (in duplicate) on samples of the "mononuclear cell preparations" (examined microscopically at x200). The percentage of white cells that were monocytes was determined by microscopical examination of 200 white cells in Giemsa-stained smears. These smears were prepared as described in Section 2.2.4. for blood smears, but the stain was applied for a shorter period (7 minutes).

The yield, or percentage of monocytes recovered in the "mononuclear cell preparations" from the original blood sample, was estimated according to Leb et al. (1983).

$$\% \text{ Yield} = \frac{\text{no. of white blood cells in "mononuclear cell preparation"}^{a} \times \text{\% purity of monocytes in "mononuclear cell preparation"}^{b}}{\text{initial number of monocytes in the blood}^{c}} \times 100$$

where

(a) determined by counting the number of white blood cells in the "mononuclear cell preparation" using a Neubauer counting chamber.

(b) determined by microscopic examination of Giemsa-stained smears of the "mononuclear cell preparations".

(c) the total white blood cell count in blood was determined using a Coulter counter. It was assumed that 5 percent* of these cells were monocytes.

*The actual percentage of monocytes in the white cell population was determined in Giemsa-stained preparations. The reason for assuming this value to be 5 percent is discussed later (Section 3.3.1.).

2.2.7. Seeding of "mononuclear cell preparations" and procedure to obtain monolayer cultures of monocyte/macrophages

"Mononuclear cell preparations" were suspended in culture medium (see Section 2.2.1.) to a final density of 1×10^6 cells per cm^3. Into either Leighton tubes (containing glass or plastic coverslips), or individual wells of a 24-well Linbro plastic culture plate (supplied by Flow Laboratories Ltd., Hertfordshire, U.K.), were placed $1cm^3$ portions of cell suspensions. Incubation of the vessels was commenced at 37°C in an atmosphere of air: CO_2 (95 percent: 5 percent). After a 2.5 to 3 hour pre-incubation period, most of the monocytes have settled in their vessel and adhered to either the coverslip or the base of the well. Non-adherent cells were removed at this time by gently aspirating off most of the culture medium, using a long-form Pasteur pipette attached to a mechanical pump. Into each vessel was placed $1.0cm^3$ of culture medium (pre-warmed to 37°C) which was immediatedly removed by gentle aspiration. This washing procedure was repeated twice more, and finally $1.0cm^3$ of culture medium added to each vessel. Incubation was continued at 37°C in air: CO_2 (95 percent:5 percent). Those cells remaining in culture beyond one week had half of their culture medium replaced by $0.5cm^3$ of (warmed) fresh culture medium at this point and, if necessary, at weekly intervals thereafter.

2.2.8. Methods for identifying monocyte/macrophages in culture

2.2.8.1. Morphological identification. On Day 1 and Day 7, monocyte/macrophages growing in Linbro wells were air-dried, fixed and stained with Giemsa stain, as described in Section 2.2.4.. The stained monolayers were examined microscopically (x 1500); 200 cells

were examined and the percentage of cells identified as monocyte/macrophages (as described earlier) recorded.

2.2.8.2. Cytochemical identification. On Day 1 and Day 7, monocyte/macrophages grown in Leighton tubes (containing glass coverslips), were tested for α-naphthyl acetate esterase activity, as described in the Sigma Technical Bulletin No. 90. It was necessary to use a glass surface, since the acetone in the fixative would dissolve a plastic surface. Briefly, the coverslips were removed and placed in a Coplin jar (dark glass), to which fixative was added (citrate-acetone-methanol; 30 seconds; room temperature). The coverslips were removed and washed gently with deionized water and air-dried (approximately 20 minutes). The coverslips were returned to the Coplin jar, to which the "staining reagents" had been added and incubated (37°C; 30 minutes). The coverslips were removed, washed in running tap-water (3 minutes) and counterstained in Mayer's haematoxylin solution (10 minutes). The coverslips were washed in tap-water, air-dried, mounted in DPX and examined microscopically (x1500). The presence of black granulation indicates esterase activity, a characteristic of mononuclear phagocytes. (N.B. lymphocytes very rarely possess esterase activity). Sodium fluoride (1.5mg/cm^3; supplied by Sigma Chemical Co., Dorset, U.K.) was included in the staining reagent in matched controls. This is known to inhibit esterase activity in monocytes but not in the mature macrophages (Musson 1983).

2.2.8.3. Functional identification. On Day 1 and Day 7, the phagocytic ability of monocyte/macrophages in Linbro wells was assessed according to the method of Michl et al. (1976). Polystyrene beads (10µl; 1.0µm diameter; supplied by Sigma Chemical

Co., Dorset, U.K.) were added to cultures for 120 minutes. The cells were then washed twice with PBS in the usual way, then fixed with gluteraldehyde (2 percent in PBS; 60 minutes; room temperature). The monolayers were examined microscopically (x1500): 200 cells were examined and they were deemed monocyte/macrophages if they had internalized four or more polystyrene beads. (N.B. lymphocytes are not phagocytic).

2.2.8.4. Photomicrography of monocyte/macrophages during culture. On Day 0 and Day 7, monocyte/macrophage cultures, grown in Linbro wells were washed twice with PBS (prewarmed to 37°C), air-dried, fixed and stained with Giemsa stain as described in Section 2.2.4.. Stained cells were examined microscopically and photographed (x300) using a 35mm camera attachment (loaded with a panatomic 7x film), and fitted with an automatic exposure unit (20 ASA).

2.2.9. Assessing the number of adherent monocyte/macrophages during culture

Monocyte/macrophage cultures, growing in each type of culture vessel (i.e. Linbro wells and Leighton tubes with glass or plastic coverslips), were examined on various days, using an inverted microscope fitted with a Whipple eyepiece (x3' ,). The number of adherent cells was determined according to Johnson et al. (1977): direct cell counts were made in eight randomly-selected areas of a standard size (i.e. the area within the large square of the Whipple eyepiece). The mean number of adherent cells was expressed as a percentage of the adherent cells counted on Day 0.

2.2.10. Quantitation of cell-size during culture

Cells were examined microscopically (x300), using an inverted microscope fitted with an eyepiece graticule. The arbitrary size (in units) was determined daily for 40 cells (randomly selected). A stage micrometer was used to calibrate the graticule eyepiece, thus allowing the units to be converted into micrometers.

2.2.11. Counting cell-nuclei

The method described by Unkeless and Eisen (1975) was used to obtain cell lysates containing nuclei. Cells were carefully washed with three changes of ice-cold PBS (pH 7.3, 1.0cm^3) using a Pasteur pipette attached to a mechanical pump to aspirate the washings. Triton X-100 (0.5 percent v/v in PBS; containing 5 millimoles/litre of ethylene diaminotetra acetic acid; 0.1cm^3) was added. After 5 minutes the lysate was removed and diluted in isoton (Coulter Electronics Ltd., 10cm^3).

The number of nuclei in cell lysates were counted using a Model ZBI Coulter counter fitted with a 70μm orifice, set with an amplification of 32 times, an aperture current of 1/2 mA, and a lower threshold limit of 9 (arbitrary units). The latter value was calculated for human monocytes (Day-1 cells) using a standard calibration procedure; this value determined the size level above which counts were recorded, and thus avoided small particles such as dust being counted.

2.2.12. Determination of cell-DNA

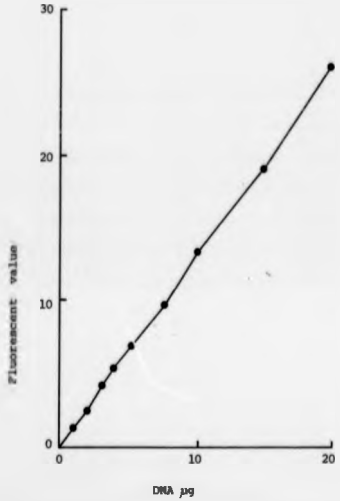
The method described by Erwin et al. (1981) was used to estimate DNA. Cells were washed as described in the previous Section (2.2.11.). To lyse the cells, water (250µl) was added and cells sonicated (amplitude 10µ; 15 seconds) on an ice-bath using an M.S.E. sonicator. Cell samples were transferred into heat-resistant Luckhams tubes using a Pasteur pipette, trichloroacetic acid (TCA) added (10 percent, w/v; 250 µl) and the samples stored overnight at 4°C, to ensure complete precipitation of acid-insoluble material. Samples were then centrifuged (20 minutes; 4°C; 3000 rpm). The supernatants were carefully removed using a Pasteur Pipette and discarded. The precipitates were suspended in TCA (5 percent; 4°C; 500µl) by vortexing. Samples were hydrolysed at 90°C for 30 minutes and cooled on ice. Samples were centrifuged (as described above), and the supernatants containing solubilised DNA aldehydes removed and saved at 4°C for DNA determinations. The precipitates were retained at 4°C for protein determinations by the method of Lowry et al. (1951).

DNA determinations were carried out within 4 hours. To the samples were added 100µl portions of a freshly prepared solution of 3,5-diaminobensoic acid (200 mg/cm^3; decolorized with norite) for 1 hour (60°C). To stop the reaction hydrochloric acid (1 molar, 2cm^3) was added. The fluorescence was determined using a Perkin-Elmer 3000 fluorimeter (excitation filter 380nm; emission filter 520nm).

Standard solutions (1-20µl) of DNA (Type 1; calf thymus; Sigma Chemical Co., Dorset, U.K.; 1mg/cm^3 in 1 molar ammonium hydroxide) were prepared and taken to dryness at 60°C. TCA (5 percent; 500µl)

Figure 2.1.1.

A typical standard curve of fluorescence against DNA concentration

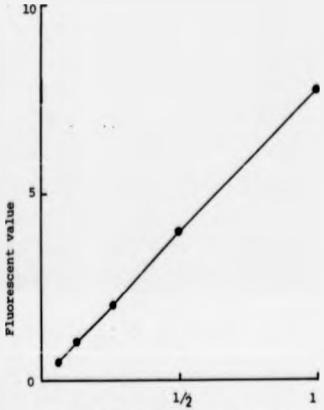


Each point represents the mean of triplicate determinations.

The exact details of this experiment are given in Section 2.2.12.

Figure 2.1.2.

Relationship between the dilution of a sample and the fluorescence, for the DNA assay



Each point represents the mean of triplicate determinations.

The exact details of this experiment are given in Section 2.2.12.

was added to each preparation and these were hydrolysed as described above, and subjected to the assay procedure in order to construct a standard curve. Combined samples of water (250 µl) and TCA (10 percent, w/v 250µl) were also included in the assay to confirm that neither contributed greatly to the fluorescence measured. Investigations were also carried out to confirm that the reaction rate was directly proportional to the amount of DNA included in the assay. For the latter, several Day-0 samples of the solubilised DNA aldehydes were pooled and diluted in TCA (5 percent).

Figure 2.1.1. shows a standard curve of fluorescence against DNA concentration, which may be seen to be linear within a range of 1-20µg DNA. Control samples for the TCA were very low and thus these values were not subtracted from sample values. Figure 2.1.2. shows the relationship between the dilution of a sample and the fluorescence. It may be seen that the extent of the reaction is proportional to the amount of DNA.

2.2.13. Determination of lysosomal enzyme release during phagocytic challenge

Apart from a few minor modifications the experimental procedure described by Knight & Boutar (1982) was used in order to measure lysosomal enzyme release from cells challenged with either opsonized zymosan or (unopsonized) polystyrene beads, and unchallenged cells.

2.2.13.1. Preparation of opsonised zymosan. Zymosan (Sigma Chemical Co., Dorset, U.K.) was suspended in PBS (38mg/cm^3), and 7cm^3 of this suspension placed in a stoppered glass container and boiled for 1 hour, with agitation every 10 minutes. The sterilised solution

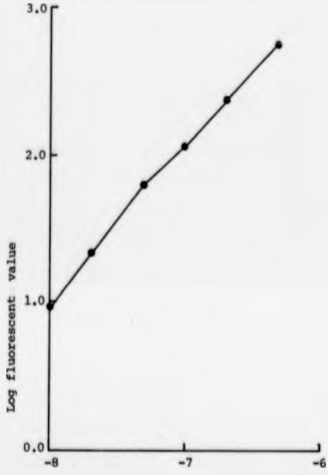
was then washed twice with PBS (1600g; 15 minutes; 7cm^3). Then PBS (7cm^3) and fetal calf serum (14cm^3) were added to the zymosan and opsonization (i.e. coating with complement and IgG) achieved by incubation at 37°C for 30 minutes. Although the procedure described by Knight & Boutar (1982) uses human serum, fetal calf serum was employed instead, since the former has been said to yield opsonized zymosan which is toxic to cells (Leoni, 1985). Once opsonized, the zymosan was washed for a further three times, finally resuspending the pellet in the "experimental medium" (medium 199 containing 2.5mg/cm^3 of bovine serum albumin), to give a final zymosan concentration of 38mg/cm^3. This was stored at -20°C, prior to use.

2.2.13.2. Preparation of polystyrene beads. Polystyrene beads (Sigma Chemical Co. Dorset, U.K.; 1100nm diameter; 100µl) were washed three times, as described above, and finally resuspended in PBS and stored at 4°C. Prior to the experiment cells were washed once more and resuspended in "experimental medium" (described above), to give 40 x 10^8 beads per cm^3.

2.2.13.3. Determination of lysosomal enzyme release. Prior to the experiment, cells were prewashed twice with medium 199 to remove excess serum, finally replacing the medium with the "experimental medium" (0.4cm^3). Incubations at 37°C were commenced by adding either a further portion of "experimental medium" (0.1cm^3), polystyrene beads (20 x 10^8 beads/cm^3 in "experimental medium" ;0.1cm^3) or opsonized zymosan (1.9 mg/cm^3 in "experimental medium"; 0.1cm^3). After 4 hours, the medium was sucked off with a Pasteur pipette and stored at -20°C prior to the determination of hexosaminidase (described below). All cells were then washed twice with medium 199 and lysed with Triton X-100 (0.05 percent v/v;

Figure 2.2.1.

A typical standard curve of fluorescence against 4-methylumbelliferone concentration for the hexosaminidase assay



Each point represents the mean of triplicate determinations.

The exact details of this experiment are given in Section 2.2.13.14.

0.5cm^3); these cell samples were retained at -20°C until assayed for hexosaminidase and protein. Cell protein was determined as described by Lowry et al. 1951, (but the bovine serum albumin standards were diluted in Triton X-100) and hexosaminidase as below.

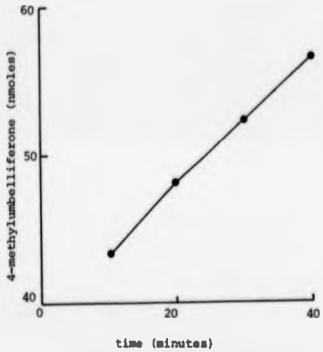
2.2.13.4. Determination of hexosaminidase activity. The method described by Griffiths et al. (1978) was used to determine hexosaminidase activity. Medium and cell samples (stored for less than 3 weeks) were defrosted and 50µl portions placed in plastic cuvettes, to which substrate (2.5mmoles/litre 4 methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in citrate phosphate buffer 0.05 moles/litre; pH 4.3; 0.1cm^3) was added. In some cases the samples assayed were diluted in the buffer (1/2 to 1/10). Incubations were at 37°C for 10 to 40 minutes. The reaction was terminated by adding glycine-sodium carbonate buffer (0.17 moles/litre; pH 10.5; 2.8cm^3) and the fluorescence determined using a spectrofluorimeter with an excitation filter of 365nm and an emission filter of 450nm. Standard samples containing up to 300 nmoles of 4-methylumbelliferone in the glycine carbonate buffer were included in the assay, along with control samples for the "experimental medium" and the Triton X-100 (0.05 percent v/v).

Preliminary investigations were carried out to check that the reaction rate was linear with time and proportional to the amount of enzyme included in the assay. For the latter, cell samples from cells of various ages were pooled and then diluted in buffer.

Figure 2.2.1. shows a standard curve of fluorescence against the concentration of 4-methylumbelliferone, which may be seen to be linear. Control values for the medium and Triton X-100 (0.05 percent

Figure 2.2.2.

Production of 4-methylumbelliferone with time for the hexosaminidase assay, using medium samples from Day-1 monocyte/macrophages

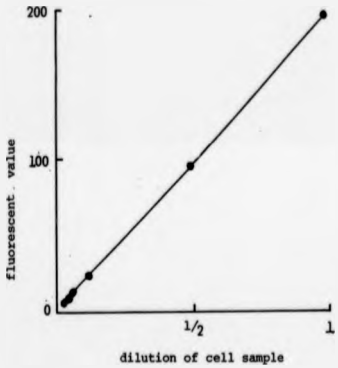


Each point represents the mean of triplicate determinations.

The exact details of this experiment is given in Section 2.2.13.4.

Figure 2.2.3.

Relationship between sample dilution and fluorescence for the hexosaminidase assay using cell samples from Day-7 monocyte/macrophages



Each point represents the mean of triplicate determinations

The exact details of this experiment are given in Section 2.2.13.4.

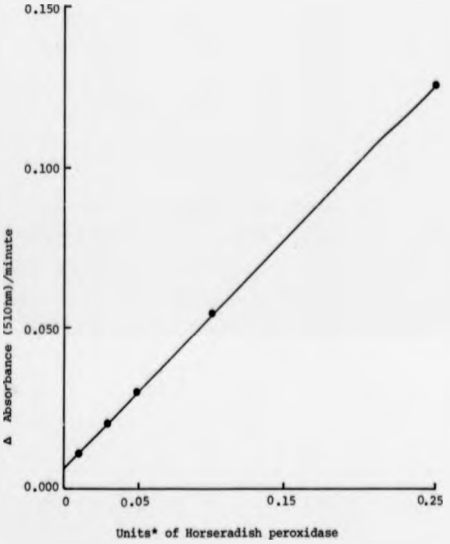
v/v) were low and thus these values were not subtracted from the sample values. Figure 2.2.2. shows the progress curve for incubations up to 40 minutes using medium samples from Day-1 cells. The rate of the reaction increased linearly with time. Figure 2.2.3. shows the relationship between the dilution of a sample and the fluorescence, where cell samples from Day-7 cells were used. It is seen that the extent of the reaction is proportional to the amount of enzyme present.

2.2.14. Determination of peroxidase activity

2.2.14.1. Sample preparation. The medium was collected by aspiration from the cultures to be assayed, carefully removing as much as possible without disturbing the cells. The medium was centrifuged (850g; 7.5 minutes) to remove any cells in suspension. The supernatant and pellet were separated and each kept at 4°C, the supernatant being termed the "medium sample". The remaining cells were carefully washed with serum-free medium (prewarmed at 37°C), six times as described in Section 2.2.11.. Triton X-100 (0.05 percent v/v; 250µl) was added to each well in order to dislodge and lyse adherent cells. After about 7.5 minutes the lysate was sucked out of the well and placed in the centrifuge tube that contained the corresponding pellet derived from the centrifuged culture medium. This solution is termed the "cell sample". In some experiments "cell samples" were obtained by adding water (250 to 500µl) to the washed cells and lysing the cells by sonication (amplitude 10 µ; 15 seconds; 4°C). All samples were kept at 4°C and assayed within 2 hours. To the remaining portions of the sonicated cell samples an equal volume of NaOH (1 mole/litre) was added and these and the Triton lysates were retained for protein determinations.

Figure 2.3.1.

A typical standard curve showing change in absorbance (510nm) against concentration of peroxidase



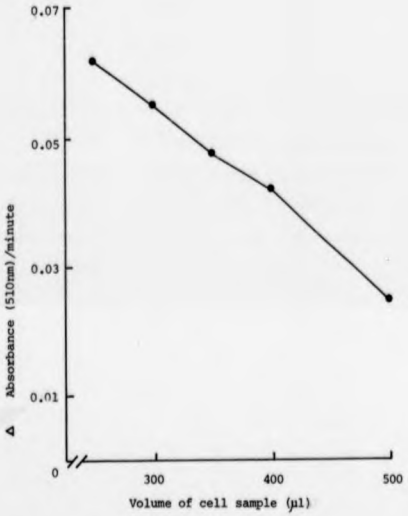
Each point represents the mean of duplicate determinations.

The exact details of these experiments are given in Section 2.2.14.2.

* 1 Unit = The decomposition of 1μmol hydrogen peroxide per minute at 25°C; pH 7.0.

Figure 2.3.2.

Relationship between the volume of the cell sample and the change in absorbance (510nm)



Each point represents the mean of triplicate determinations.

The exact details of this experiment are given in Section 2.2.14.2.

2.2.14.2. Measurement of peroxidase activity. The method described by Worthington Diagnostic Systems (1982) was used to determine peroxidase activity. All samples were assayed in duplicate. Into a plastic cuvette was pipetted aminoantipyrine solution (0.0025 moles/litre in phenol solution 0.17moles/litre; 1.4cm^3), hydrogen peroxide (30 percent aqueous, Sigma Chemical Co., Dorset, U.K.; 0.17moles/litre in PBS 1.5cm^3) was added and the mixture allowed to equilibrate (25°C; 3-4minutes).

Immediately prior to use, a stock solution of horseradish peroxidase (Type I; 1mg/cm^3 ; Sigma Chemical Co., Dorset, U.K.) was further diluted within the range of 0.01 to 0.25 units/cm^3 (1 unit = the decomposition of 1umol hydrogen peroxide per minute at 25°C; pH 7.0). Diluted enzyme or sample (0.1cm^3) was added to the reaction mixture and the increase in absorbance was recorded for 4 to 5 minutes (510nm; 25°C). Portions (0.1cm^3) of Triton X-100, culture medium and water were also included in the assay to ensure that they each did not contribute to the change in absorbance.

The usual preliminary studies were carried out to check the assay as described in Section 2.2.12.. Figure 2.3.1. shows a typical standard curve showing the change in absorbance (510nm) against peroxidase concentration, which may be seen to be linear within a range of 0.01 to 0.25 units/cm^3 of peroxidase standards.

Triton X-100 was initially used to lyse the cells, but was found to interfere with the assay: the Triton-containing control samples caused a rapid increase in absorbance due to the formation of an insoluble material. The assays were therefore repeated using sonicated aqueous "cell samples" (as described). The control samples

of water and culture medium did not cause any change in the absorbance value. Figure 2.3.2. shows the relationship between the volume of the "cell sample" and the absorbance change. It may be seen that the extent of the reaction is proportional to the amount of enzyme present.

2.2.15. Determination of lactate dehydrogenase activity

2.2.15.1. Sample preparation. The medium was aspirated from the cultures to be assayed, carefully removing as much as possible without disturbing the cells. Serum-free medium ($1.0cm^3$) was then added to each well. After 4 hours incubation at 37°C the medium was carefully removed again and centrifuged at 850g for 7.5 minutes, to remove any cells in suspension. The supernatant and pellet were separated and each kept at 4°C, the supernatant being termed the "medium sample".

Triton X-100 (0.05 percent v/v; $1.0cm^3$) was added to each well in order to dislodge and lyse the adherent cells. After about 7.5 minutes the lysate was sucked out of the well and placed in the centrifuge tube that contained the corresponding pellet derived from the centrifuged culture medium. This solution is termed the "cell sample". Samples were kept at 4°C and assayed immediately for LDH activity.

2.2.15.2. Measurement of lactate dehydrogenase activity. The method described by Lowry et al. (1957) was used to determine lactate dehydrogenase activity. Duplicate 10µl portions of undiluted medium samples and cell samples, and also diluted (1 in 2, to 1 in 20) cell samples, were placed in Luckhams tubes. To start the reaction

the substrate solution 10nmoles/litre NADH in 50mmoles/litre Tris buffer; pH 7.6 containing 10mmoles/litre of sodium pyruvate; 10µl) was added to each sample. After a 37°C incubation period of between 10 and 45 minutes, HCl (1.5moles/litre; 5µl) was added to destroy NADH, which otherwise contributes some fluorescence. After about 30 seconds NaOH (7moles/litre; 250µl) was added to give a concentration of 6 moles/litre, and the sample incubated for a minimum of 30 minutes at 37°C. Water (2.5cm^3) was then added and the fluorescence measured using a Perkin-Elmer spectrofluorimeter with an excitation wavelength of 364nm and an emission wavelength of 465nm. Standard solutions containing up to 100nmoles of NAD in Tris buffer (pH 7.6) were subjected to the assay procedure in order to construct a standard curve. Samples (10µl) of both Triton X-100 and serum-free medium were also included in the assay to ensure that neither contributed greatly to the fluorescence measured.

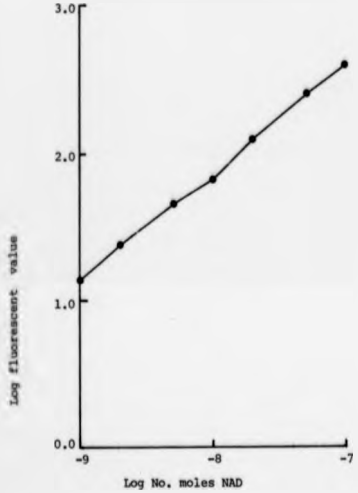
Initial investigations were carried out to

1. Check that the reaction rate was linear with time
2. Determine a suitable reaction time
3. Ensure that the reaction rate was directly proportional to the amount of enzyme included in the assay.
4. Assess the optimal sample dilutions.

For determining the ideal reaction time and sample dilutions, it was important to test medium samples and cell samples from both young (Day-1) and more mature (Day-7) monocyte/macrophage cultures to ensure that the assay would be suitable for both types of sample from different aged cultures.

Figure 2.4.1.

A typical standard curve of fluorescence against NAD concentration

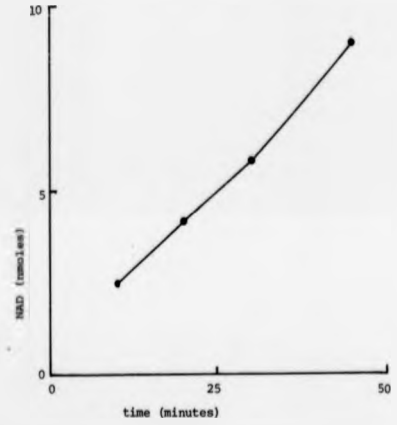


Each point represents the mean of triplicate determinations.

The exact details of this experiment are given in Section 2.2.15.2.

Figure 2.4.2.

Production of NAD with time for the lactate dehydrogenase assay, using medium samples from Day-1 monocyte/macrophages

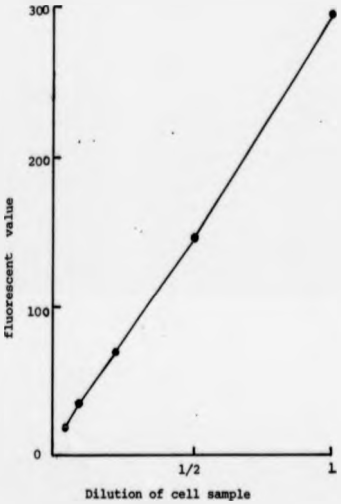


Each point represents the mean of triplicate determinations.

The exact details of this experiment are given in Section 2.2.15.2.

Figure 2.4.3.

Relationship between sample dilution and fluorescence, using cell samples from Day-7 monocyte/macrophages



Each point represents the mean of triplicate determinations.

The exact details of this experiment are given in Section 2.2.15.2.

Figure 2.4.1. shows a standard curve of fluorescence against NAD concentration, which may be seen to be linear within a range of 1-100 nmoles of NAD. The control samples for Triton X-100 and medium were very low and thus these values were not subtracted from sample values. However only sample values giving at least double the fluorescence of the appropriate blank were deemed accurate.

Figure 2.4.2. shows the progress curve for incubation periods up to 45 minutes using medium samples from one day old monocyte/macrophage cultures. The rate of the reaction may be seen to be linear with time. However "cell samples" when incubated for periods of greater than ten minutes were found to have higher fluorescence values than the standard curve; thus in adopting an incubation period of 20 minutes, which would be sufficient to allow for the detection of the lower levels of LDH in the medium samples, it was necessary to assess an appropriate dilution factor for the cell samples.

Figure 2.4.3. shows the relationship between the dilution of a sample and the fluorescence. It may be seen that the extent of the reaction is proportional to the amount of enzyme. In addition to using undiluted samples in the assay, cell samples were diluted 1 in 5 in Tris buffer (pH 7.6) to ensure the detection of NAD within the range of the standards.

2.2.16. Estimation of [^{125}I]iodide in radiolabelled substrates by electrophoresis

The stability of ^{125}I-labelled preparations was examined by estimating the amount of free [^{125}I]iodide present. Paper electrophoresis was used for the estimation of free [^{125}I]iodide, using the method described by Sargent (1969). Using a Shandon model

477 electrophoresis kit, Whatman No. 1 strips of filter paper (30 x 5cms) and barbitone buffer (pH 8.6), the current (400 volts, 2 milliamps) was passed for 10 minutes to allow the system to equilibrate. To each strip, radiolabelled sample (20µl) was carefully applied along the origin line, using a micropipette. The current was passed for a further 20 minutes.

Each strip was cut into 5mm lengths which were numbered from the origin end, firmly pressed into a Luckhams tube, and assayed for radioactivity. The radioactivity, corrected for background, was plotted against the strip number and a comparison of the areas of the peaks allowed the percentage of free [^{125}I]iodide to be determined.

CHAPTER 3

The isolation and culture of human monocytes

3.1. INTRODUCTION

The first objective of my research was to select and evaluate a reliable procedure for obtaining human monocytes from peripheral blood. Two steps are usually used to isolate monocytes from the other cellular components of blood. First, cells are often separated according to their differences in density, by employing a density gradient material. Following fractionation monocytes are found in clearly discernible cell bands in the gradient. The cell bands are collected and termed "mononuclear cell preparations". Other cells (mainly lymphocytes) are found in the preparations, owing to the overlap in cell densities between monocytes and other cells. Thus a second step is employed to remove these "contaminating cells". Fortunately, the monocytes, unlike the lymphocytes (the main "contaminating" cell), are adherent cells; further separation is therefore often achieved by allowing the monocytes to adhere to a surface, then removing any non-adherent cells by washing.

With respect to the "mononuclear cell preparations", there are two main considerations when evaluating a monocyte isolation technique: the percentage purity of monocytes (i.e. the percentage of the cells present that are monocytes) and the monocyte yield (i.e. the percentage of monocytes recovered from the original blood sample; Leb et al., 1983).

The second objective of my research was to choose a reliable method for culturing the isolated monocytes in vitro. A number of methods have been reported and these are outlined shortly. In addition, it was important to monitor a number of features of the cells during culture, to assess whether the cells represented a maturing

mononuclear phagocyte population.

3.1.1. The separation of human monocytes from blood

A few of the methods used to obtain monocytes from blood are very primitive, such as the use of erythrocyte aggregating agents (e.g. dextran), which essentially just produces a leukocyte-rich plasma. However, as just mentioned many of the methods used employ density-gradient materials, which produce "mononuclear cell preparations" containing mainly mononuclear cells (i.e. lymphocytes and monocytes).

The most frequently used density-gradient material is Ficoll-Hypaque, developed by Boyum (1968). Depending on the gradient material employed, the monocyte purity and yield vary. Percoll was used by Bertoft et al. (1980) to isolate monocytes from blood in a simple one-step procedure, giving mean estimated yields of 100 percent and purities of 20 percent. In contrast, Boyum (1983), using a newly developed gradient material Nycodens, reported higher monocyte purities (approximately 95 percent), but low yields (approximately 35 percent).

3.1.2. The purification of monocytes from "mononuclear cell preparations"

Following the various separation methods, lymphocytes are often removed from the "mononuclear cell preparations" by allowing the monocytes to adhere to a glass or plastic surface, then removing the non-adherent cells by washing. Although lymphocytes are usually the main "contaminating" cell-type, granulocytes may also be present.

These are adherent cells and so are not removed with the lymphocytes. However, they only survive in culture for up to 24 hours. Monocytes are cultured and/or analysed on the substrate upon which they were allowed to adhere. To obtain suspensions of monocytes, the cells may be detached by physical or chemical means. The anaesthetic lidocaine, physical scraping or the use of chelators such as ethylene diaminetetraacetic acid (EDTA), are used with varying success to detach monocytes (Ackerman & Douglas, 1978; Rabinovitch & DeStefano 1975; Shreiber et al., 1983). Suspension cultures of monocyte/macrophages have also been grown using teflon vessels, to which monocytes will not adhere (Van der Meer et al., 1978).

3.1.3. Parameters used to evaluate Monocyte isolation methods

As previously stated, the percentage of monocytes in a "mononuclear cell preparation" is one of the main parameters used to determine the usefulness of a separation technique. In order to determine monocyte purity in both "mononuclear cell preparations" and monolayers of cells, the accurate identification of monocytes is necessary. Morphological identification of monocytes in Giemsa-stained preparations is not always reliable, as monocytes can easily be confused with large lymphocytes. Thus, additional methods are often employed such as cytochemical staining for esterase activity (Yam et al., 1971), an enzyme that lymphocytes (the main "contaminating cells") do not possess. This parameter is doubly useful, as by including sodium fluoride in the reaction, one can also distinguish between monocytes and macrophages, for the staining activity is inhibited by fluorides in monocytes but not in macrophages. Since lymphocytes are not phagocytic cells, monocytes are often distinguished from them by their ability to phagocytose materials

such as polystyrene (latex) beads, IgG-coated sheep red blood cells or opsonized bacteria (Chian et al., 1983; Johnson et al., 1977; Van Furth & Diesselhoff-den Dulk, 1970). However, in using this test, it must be remembered that some types of granulocytes are also phagocytic. Other parameters used to evaluate isolation techniques include the monocyte yield (i.e. the percentage recovery of monocytes from blood, as mentioned above), and the viability of the isolated cells. The calculation of monocyte yields involves determining the monocyte purity and the total number of white blood cells in both the original blood sample and the "mononuclear cell preparation". Monocyte viability is often assessed using the dye trypan blue which will only enter damaged cells (Tullis, 1953).

3.1.4. The culture of human monocytes in vitro

The establishment of long-term cultures (up to three months) of monocyte-derived macrophages has only been reported by a few workers (e.g. Johnson et al., 1977; Zuckerman et al., 1979). Studies on "monocyte/macrophages" (a term that will be used to describe cells that may be monocytes or macrophages) are more frequently made on younger cultures (i.e. less than one week). (N.B. during this time the monocytes are thought to transform into macrophages). In man, owing to the difficulty in obtaining normal macrophages (outlined in Section 1.2.5.), this is the main source of macrophages.

Monocytes are usually grown in either plastic tissue culture vessels or the Leighton tube system, in the latter, cells adhere to glass or plastic coverslips immersed in culture medium (Yatsiv et al., 1978; Zuckerman et al., 1979). Cells are usually seeded at an initial density of 1 to 4 x 10^6 mononuclear cells per cm^3.

Cultures of monocyte/macrophages are usually maintained in a basal medium (e.g. medium 199), supplemented with various types of serum (10 to 40 percent v/v). For example Zuckerman et al. (1979), using a mixture of fetal calf and horse serum, reported successful cultures. In contrast, Johnson et al. (1977) recommended fresh human serum, reporting that either fetal calf or horse serum alone did not support cultures for more than 24 hours. The basal medium may be further supplemented with nutrients such as pyruvate, non-essential amino acids, vitamins and ascorbic acid, antibiotics are often included to avoid microbial growth. A pH of 7.4 is usually maintained by the carbon dioxide bicarbonate buffer system, when the cells are cultured in a 5 percent carbon dioxide atmosphere. Cells are maintained at 37°C in a humidified environment. Feeding protocols vary greatly and include renewing the medium on alternate days, replacing half of the medium weekly, and not replenishing the medium at all (Johnson et al., 1977; Seljelid & Pertoft 1981; Zuckerman et al., 1979).

3.1.5. Morphological characteristics of monocyte/macrophage cultures

Many changes occur as monocytes mature into macrophages in vitro, and these have been outlined in Chapter 1. Here, the basic morphological characteristics of monocyte/macrophage cultures are described in detail.

The most obvious indication that monocytes are maturing in vitro is their change in size. Lewis (1925) noted an increase in monocyte size which often occurred between the third and fourth day in culture, in hanging-drop cultures of human blood. Johnson et al.

(1977) growing cells in human serum reported a doubling in cell diameter to (60 to 70μm) which occurred between days 3 and 7, and was preceded by an earlier decrease (1/2 to 2/3) from the initial cell diameter of 30 to 40 μm (i.e. within the first day). Zuckerman et al. (1979), maintaining cells in medium supplemented with fetal calf and horse serum, noted a similar increase in cell size between Days 3 and 6, the diameter of cells after 24 hours in culture being 10 to 12 μm, in agreement with Johnson et al. (1977).

Monocytes when introduced into culture are initially round, but undergo pronounced morphological changes particularly after 3 to 6 days, transforming into the mature cell, the macrophage. The "mature" population is usually very heterogenous, typically containing large flat cells which may be triangular, round, spindle-shaped or epitheloid (Zuckerman et al., 1979). "Mature" cells have very ruffled membranes and sometimes filopodia and long cytoplasmic projections extending from one pole. Multinucleate giant cells are often observed after Day 3 in culture (Zuckerman et al., 1979). In contrast, Parakkal et al. (1974) obtained very homogenous cultures with a symmetrical morphology and extensive filopodial arrangements. Zuckerman et al. (1979) suggested that such differences may be due to differences in the substrate used (i.e. glass v. plastic). This is probable, as Kaplan and Gaudernack (1982) did report differences in morphology when comparing monocytes grown on different substrates. They also noted that monocytes from different donors adopted "macrophage-like" appearances at different time-points in culture, a point discussed further in Chapter 9.

3.1.6. Cell loss during the culture of monocytes/macrophages

Since human monocytes do not proliferate, one would expect the number of adherent cells to remain stable during culture *in vitro*. However as mentioned earlier (briefly in Chapter 1), the number of adherent cells progressively decreases with time. Johnson *et al.* (1977) noted that the monocyte number decreased, so that by Day 5 only 50 percent of the cells initially present remained in the monolayer; the number of cells then decreased very slowly over the next few days. Similar results were obtained by Musson *et al.* (1980), who noted that the loss varied from donor to donor, in most cases a plateau of 50 to 60 percent being reached on Day 4. The rate and extent of cell loss does vary between authors and it appears that an important factor may be the type of substrate on which the cells are grown. Johnson *et al.* (1977) observed that, whilst equal numbers of cells adhered initially to both plastic and glass, cells detached from the glass much more rapidly during culture.

Musson *et al.* (1980) suggested that the cell loss may be due to overcrowding of cells (this is discussed later, Section 3.4). In addition, a varying degree of clumping has been reported in monocyte/macrophage cultures. For example, Johnson *et al.* (1977) when using autologous serum (non-fresh) noted clumping after 6 to 12 hours and reported that these clumps subsequently detached from the culture vessel.

3.1.7. Choice of a method

In order to achieve the objectives outlined in Chapter 1, it was necessary to establish a suitable method for isolating monocytes from

blood. Three density-gradient media were tried: dextran, in the first instance, because it is readily available; Percoll, because of the high monocyte yield reported and its simplicity of use; and Nycodenz (developed as an improvement to Ficoll-Hypaque), due to the high monocyte purities reported. Each method was evaluated according to several parameters, the viability of the isolated cells, the monocyte yield and purity in the "mononuclear cell preparations", and also the purity of the resultant monolayers (assessed on Day 1 and Day 7). Monocytes were identified by morphological, cytochemical and functional criteria (i.e. Giemsa staining, esterase staining and ability to phagocytose polystyrene beads).

Secondly it was important to choose a suitable culture system for the growth of monocyte/macrophages. The Leighton-tube system (with glass coverslips) was tried, since it was already in use in this laboratory for the culture of rat peritoneal macrophages. However, since Johnson et al. (1977) reported that monocyte/macrophages adhere better to plastic surfaces than to glass, both glass and plastic coverslips were tested in this system. In addition, plastic tissue culture wells were also tried, since they are generally easier to use. Cell loss was determined for each culture system in order to make a choice as to the most suitable vessel.

Thirdly, having once established a suitable monocyte isolation and culture system, it was important to ensure that the cells being cultured were monocyte/macrophages. This was achieved by checking the purity of the cells in culture as mentioned above, and also by comparing the morphological characteristics of the cells with previous reports. Also an initial check for monocyte-to-macrophage maturation was made by determining whether sodium fluoride resistance

to the esterase stain developed. Further characteristics of the cultures and checks for cell maturation were made at a later date, and these are detailed in Chapter 5.

3.2. EXPERIMENTAL METHODS

Three methods were used to obtain monocytes from human blood. Two of them were density-gradient centrifugation methods, using Percoll and Nycodens, and the third used the erythrocyte aggregating agent dextran. The three methods are described in Section 2.2.1. to 2.2.3.. Each of the procedures was performed on blood obtained from 6 or more different donors. For the Nycodens method two different density preparations were employed, (as this was a new product for which the optimal physical properties of the material had not yet been determined).

Small samples of (i) the original blood were used to determine the total number of white cells and the percentage of monocytes present as described in Section 2.2.4. and (ii) the "mononuclear cell preparations" were used to determine cell viability, monocyte yield and purity as described in Sections 2.2.5 and 2.2.6..

"Mononuclear cell preparations" from the Percoll method were introduced into Linbro wells or Leighton tubes (containing glass or plastic coverslips) as described in Section 2.2.7.. Non-adherent cells were removed by washing as described in Section 2.2.7.. The remaining cells were examined on Day 1 and Day 7 for monocyte/macrophage characteristics, using morphological cytochemical and functional criteria as described in Section 2.2.8.. Cell size and the degree of cell loss were determined daily over a one-week period, in cultures

Table 3.1.

Number of white blood cells and monocytes in blood

Initial No. of white blood cells/cm^3 ($x10^6$)	Initial No. of monocytes /cm^3 ($x10^5$)	% of white blood cells represented by monocytes
7.0	3.5	5
8.7	3.5	4
5.6	2.8	5
4.6	2.8	6
5.6	2.8	5
7.7	6.9	9
5.6	2.8	5
7.7	6.9	9
6.1	5.5	9
6.1	5.5	9
4.5	4.5	10
5.0	4.5	9
8.2	4.1	5
4.5	1.8	4
4.2	2.1	5
5.3	2.1	4
6.8	4.1	6
6.0	3.0	5
9.6	7.7	8
8.9	6.2	7
6.8	5.4	8
7.7	5.4	7
4.2	2.5	6
6.8	2.7	4
mean 6.4(± 0.32)*	mean 4.1(± 0.35)	mean 6.4(± 0.40)

(* figures in brackets = standard errors)

The exact details of these experiments are given in Section 3.3.1.

Table 3.2.

Viability of "mononuclear cell preparations" from various isolation methods

Method for obtaining "mononuclear cell preparations"	% of cells viable in "mononuclear cell preparations"*	Mean % (± standard error)
Percoll density gradient	98.5	
	96.0	
	99.0	98.4 (± 0.5)
	99.0	
	99.0	
	99.0	
Nycodenz density gradient (Density = 1.076g/cm^3	100.0	
	100.0	
	96.0	99.3 (± 0.7)
	100.0	
	100.0	
	100.0	
Nycodenz density gradient (Density = 1.069/cm^3)	99.0	
	99.0	
	99.5	99.3 (± 0.5)
	99.5	
	99.0	
	99.5	
Dextran sedimentation	99.0	
	99.0	
	99.0	99.0 (± 0.2)
	99.0	
	99.0	
	100.0	

*Each value represents the mean of duplicate determinations.

The results are summarised in Table 3.6.

The exact details of these experiments are given in Section 3.3.2.

which had been isolated on Percoll as described in Sections 2.2.9. and 2.2.10.

3.3. RESULTS

3.3.1. Number of white blood cells and monocytes in blood

Table 3.1. gives the numbers of white blood cells, monocytes, and the monocytes expressed as a percentage of the total white cells, for blood taken from 24 donors. It can be seen that there was an average of 6.4×10^6 white cells per cm^3 of blood, 6.4 percent of which were monocytes (i.e. 4.13×10^5 cells/cm^3). There was considerable inter-donor variation for each of the values. Both the white cell counts, and an assumed value of 5 percent* for the percentage of monocytes present in the original blood sample were used to calculate the yield (or percentage recovery) of monocytes following the various isolation techniques (see Section 2.2.6.) (*the actual value obtained for the percentage of monocytes was not used in this calculation, since it is difficult to assess this percentage accurately, owing to the difficulty in distinguishing monocytes from large lymphocytes in Giemsa-stained preparations).

3.3.2. Viability of "mononuclear cell preparations"

Table 3.2. shows the percentage of viable cells in "mononuclear cell preparations", for six donors, for each isolation method. Each value represents the mean of duplicate determinations. It can be seen that there was no difference between the percentage of viable cells from different donors or from different isolation procedures. In all cases the mean percentage viability was high (i.e. greater than 98

Table 3.3.

Percentage monocyte purity in "mononuclear cell preparations" from various isolation methods

Method for obtaining "mononuclear cell preparations"	% of white blood cells which were monocytes in the "mononuclear cell preparation"	Mean % (± standard error)
Percoll density gradient	26	
	18	
	19	21.8 (± 0.6)
	18	
	23	
	27	
Nycodenz density gradient (Density = 1.076/cm^3)	90	
	80	
	97	90.0 (± 2.4)
	88	
	90	
	95	
Nycodenz density gradient (Density = 1.069/cm^3)	97	
	98	
	98	97.5 (± 0.7)
	97	
	98	
	97	
Dextran sedimentation	7	
	4	
	6	7.7 (± 1.3)
	6	
	12	
	11	

The results are summarized in Table 3.6.

The exact details of these experiments are given in Section 3.3.3.

Table 3.4.

Percentage yield of monocytes from blood, following various isolation methods

Method for obtaining "mononuclear cell preparations"	% yield or % of monocytes recovered from blood, in the "mononuclear cell preparations"	Mean % (± standard error)
Percoll density gradient	53.5	
	117.6	
	51.2	115.4 (± 27.5)
	82.9	
	221.0	
	166.0	
Nycodenz density gradient (Density = 1.076g/cm^3)	15.6	
	11.1	
	28.6	15.9 (± 3.1)
	20.0	
	7.5	
	12.7	
Nycodenz density gradient (Density = 1.069g/cm^3)	29.6	
	64.6	
	53.3	46.0 (± 5.8)
	51.5	
	29.0	
	48.0	
Dextran sedimentation	38.8	
	29.6	
	58.2	42.9 (± 4.8)
	45.5	
	53.8	
	31.2	

These results are summarised in Table 3.6.

The exact details of these experiments are given in Section 3.3.4.

percent).

3.3.3. Monocyte purity in "mononuclear cell preparations"

Table 3.3. shows the percentage of cells that were deemed monocytes in Giemsa-stained smears of "mononuclear cell preparations" for six donors for each isolation procedure. It can be seen that for each procedure there was little inter-donor variation between the purity values. However the different isolation methods gave different values for monocyte purity. Following separation of blood on Nycodenz gradients, high monocyte purities were obtained (mean greater than 90 percent), whereas using Percoll and dextran much lower purities were obtained (mean 21.8 percent and 7.6 percent respectively). The other cells in the "mononuclear cell preparations" were predominantly lymphocytes (less than 1 percent were granulocytes), except following dextran sedimentation where approximately 60 percent were granulocytes.

3.3.4. Monocyte yield in "mononuclear cell preparations"

Table 3.4. shows the yield or percentage recovery of monocytes from blood following the various isolation methods (6 donors for each). It can be seen that for each isolation method, with the exception of the Percoll method, there was little variation between the various donors in the percentage of monocytes recovered. For the Percoll method the yield varies from 51.2 percent to 221.0 percent. (It is explained later (Section 3.4.) why recoveries of greater than 100 percent were obtained.) Comparison of the mean percentage yields for each method shows that the highest mean monocyte recovery was obtained using Percoll (115.4 percent) and the lowest with Nycodenz

Table 3.5.1.

Percentage purity of the monolayer on Day 1, assessed by 3 parameters

Method for obtaining cells	% of monocyte/macrophages determined by:- (i) morphology in Giemsa stained preparations	(ii) esterase activity*	(iii) phagocytic ability (latex beads)
Percoll density gradient	94	97 (8)	92
	93	98 (7)	91
	95	96 (6)	95
	97	95 (9)	99
	96	99 (4)	98
	95	97 (2)	95
mean ± standard error	95 ± 0.6	97 ± 0.6 (6 ± 1.1)	95 ± 1.3
Nycodenz density gradient (Density = 1.07g/cm^3)	94	97 (9)	94
	95	95 (8)	94
	95	97 (7)	96
	93	95 (6)	95
	93	98 (3)	96
	94	94 (3)	94
mean ± standard error	94 ± 0.4	96 ± 0.3 (6 ± 1.0)	95 ± 0.4
Nycodenz density gradient (Density = 1.069g/cm^3)	95	98 (4)	93
	95	98 (9)	96
	99	100 (8)	95
	91	100 (6)	94
	93	96 (8)	96
	96	95 (1)	95
mean ± standard error	95 ± 1.1	98 ± 0.8 (6 ± 1.2)	95 ± 0.5
Dextran sedimentation	76	75 (4)	79
	76	74 (5)	71
	74	73 (4)	75
	78	72 (5)	72
	75	75 (3)	78
	77	74 (2)	75
mean ± standard error	76 ± 0.6	74 ± 0.5 (3.8 ± 0.5)	75 ± 1.3

* The figures in brackets represent esterase activity in the presence of NaF (1.5 mg/cm^3).

These results are summarized in Table 3.6.

The exact details of these experiments are given in Section 3.3.5.

Table 3.5.2.

Percentage purity of the monolayer on Day 7, assessed by 3 parameters

Method for obtaining cells	% of monocyte/macrophages determined by:- (i) morphology in Giemsa stained preparations	(ii) esterase activity*	(iii) phagocytic ability (latex beads)
Percoll density gradient	95	98 (97)	97
	96	97 (98)	97
	93	96 (96)	96
	95	99 (98)	97
	95	100 (99)	99
	95	97 (96)	95
mean ± standard error	95 ± 0.4	98.0 ± 0.6 (97.3 ± 0.5)	97.0 ± 0.5
Nycodenz density gradient (Density = 1.076g/cm^3)	95	99 (96)	97
	93	99 (98)	98
	97	98 (99)	99
	93	99 (99)	96
	96	99 (98)	97
	94	99 (99)	95
mean ± standard error	95 ± 0.7	99 ± 0.2 (98.2 ± 0.5)	97.0 ± 0.6
Nycodenz density gradient (Density = 1.069g/cm^3)	95	99 (100)	99
	94	99 (100)	99
	94	99 (99)	100
	96	100 (100)	100
	95	100 (98)	100
	95	99 (99)	97
mean ± standard error	95 ± 0.3	99 ± 0.2 (99.3 ± 0.3)	99 ± 0.5
Dextran sedimentation	94	99 (99)	98
	92	99 (98)	100
	96	97 (100)	99
	93	97 (99)	99
	95	100 (99)	100
	94	100 (99)	98
mean ± standard error	94 ± 0.6	99 ± 0.6 (99 ± 0.3)	99 ± 0.4

* The figures in brackets represent esterase activity in the presence of NaF (1.5 mg/cm^3).

These results are summarized in Table 3.6.

The exact details of these experiments are given in Section 3.3.5.

Table 3.6.

Summary of results for different isolation methods

	method for obtaining cells:-			
	Percoll Density Gradient	Nycodenz density gradient (Density 1.076gm/cm^3)	(Density 1.069gm/cm^3)	Dextran Sedimentation
% of cells Viable in "mononuclear cell preparations"	98.4 ± 0.5	99.3 ± 0.7	99.3 0.5	98.3 ± 0.9
% of white cells which were monocytes in "mononuclear cell preparations" i.e % purity	21.8 ± 0.6	90.0 ± 2.4	97.5 ± 0.7	7.7 ± 1.3
% yield or recovery of monocytes from blood in "mononuclear cell preparations"	115.4 ± 27.5	15.9 ± 3.1	46.0 ± 5.8	52.9 ± 4.8
% purity of the monolayers on Day 1 and Day 7 (in brackets) as assessed by:-				
(i) Morphology in Giemsa-stained preparations	95 ± 0.6 (95 ± 0.4)	94 ± 0.4 (95 ± 0.7)	95 ± 1.1 (95 ± 0.3)	76 ± 0.6 (94 ± 0.6)
(ii) esterase activity	97 ± 0.6 (98 ± 0.6)	96 ± 0.3 (99 ± 0.2)	98 ± 0.8 (99 ± 0.2)	74 ± 0.5 (99 ± 0.6)
(iii) phagocytic ability (latex beads)	95 ± 1.3 (97 ± 0.5)	95 ± 0.4 (97 ± 0.6)	95 ± 0.5 (99 ± 0.5)	75 ± 1.3 (99 ± 0.4)

Each value represents the mean ± S.E. from 6 donors.

The exact details of these experiments are given in Section 3.3.6.

(Density=1.078g/cm^3) (15.9 percent).

3.3.5. Purity of cells in culture

Tables 3.5.1. and 3.5.2. show the purity of Day-1 and Day-7 cultures of monocyte/macrophages obtained from the various isolation methods for 6 donors, as assessed by morphological, cytochemical and functional criteria. Values are shown after 24 hours (Day 1) and a week (Day 7) in culture. It can be seen that there was little difference in the purity values determined by any of the parameters used (i.e. Giemsa-staining, esterase staining or phagocytic ability). For cells isolated using Percoll or Nycodenz it can be seen that the mean purity values were all high on Day 1 and Day 7 (greater than 94 percent). However following dextran sedimentation, it can be seen that, whilst Day-7 cells showed a similar high degree of monocyte purity (greater than 94 percent), the purity of Day-1 cells was lower (i.e. mean 74 to 76 percent depending on the parameter). The reason for this difference is explained later (Section 3.4.).

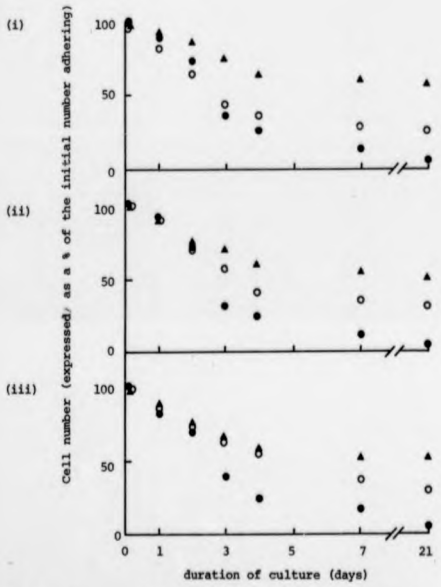
Tables 3.5.1 and 3.5.2. also shows, that the presence of sodium fluoride greatly inhibited the esterase activity of Day-1 cells, yet had no effect on the esterase activity of Day-7 cells. The significance of this is highlighted in the Discussion (Section 3.4.).

3.3.6. Summary of results used to evaluate the isolation methods

Table 3.6. gives a summary of the results described in Sections 3.3.2. to 3.3.5.. The viability of the "mononuclear cell preparations" was similar with each isolation method. Likewise , with the exception of the dextran method, no difference was seen between the methods in the

Figure 3.1.

Effect of culture surface on cell adherence: surfaces employed = Linbro wells (▲), or Leighton tubes with plastic (○) or glass (●) coverslips: cells obtained from three donors (i) S.T. (ii) A.M. (iii) D.F.



Each point represents the mean of duplicate determinations.

The exact details of these experiments are given in Section 3.3.7.

Figure 3.2.1.

Photomicrograph of Day-0 cells (magnification = x 300).

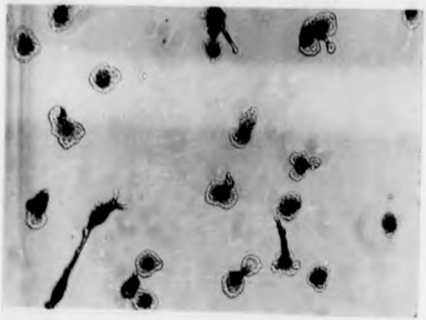


Figure 3.2.2.

Photomicrograph of Day-7 cells (magnification = x 300).

An explanation of these photomicrographs is given in Section 3.3.8.

purity of the monolayers on Day 1 and Day 7. Following dextran sedimentation lower monolayer purity values were obtained initially on Day 1, but by Day 7 the values were equal to those obtained with the other methods. The striking differences were seen in the monocyte yield and purity values of the "mononuclear cell preparations". The greatest monocyte yield was obtained following the Percoll method and the greatest purity following the Nycodenz (Density= 1.069 gm/cm^3) method.

3.3.7. Cell loss during the culture of monocyte/macrophages

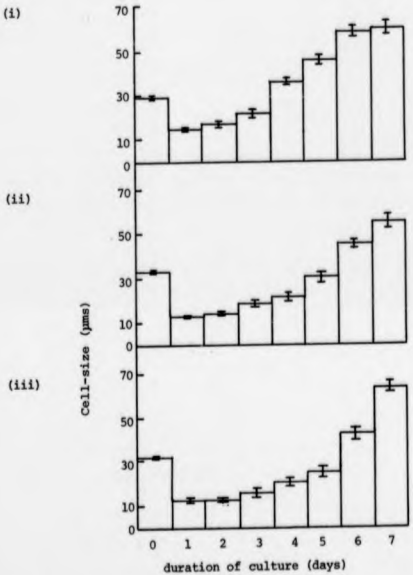
Figure 3.1. shows the effect of the type of culture vessel on cell loss during prolonged incubations. For each of three donors the number of adherent cells decreased with time. After Day 7 the rate of this loss was not so great. For each donor, maximum cell adherence was seen using Linbro wells (i.e. less than 50 percent of the initial cell number adherent on Day 7), and maximum cell loss was seen in Leighton tubes containing glass coverslips (i.e. less than 16 percent of the initial cell number adherent on Day 7).

3.3.8. Morphology of monocyte/macrophage cultures

Figures 3.2.1. and 3.2.2. show typical photomicrographs of monocyte/macrophage cultures on Day 0 and Day 7. Day-0 cells were typically rounded in appearance, in contrast to the heterogenous morphology of Day-7 cells. Day-7 cells displayed a variety of shapes and sizes (i.e. rounded, triangular, epitheloid, fusiform), and a number of the cells produced long thin cytoplasmic projections. The morphology of Day-7 cells often differed slightly from donor to donor, as did the degree of cell clumping and formation of

Figure 3.3.

Cell size (μms) during culture: each value represents the mean of 40 measurements ± standard error. Cells obtained from three donors (i) D.B. (ii) R.T. (iii) S.M.



The exact details of these experiments are given in Section 3.3.9.

multinucleated giant cells. Although the cells appear sparse in the photomicrographs, unfixed cultures were much denser, the reason for this cell loss is given later (Section 3.4.).

3.3.9. The size of monocyte/macrophages during culture

Figure 3.3. shows the variation in cell-size of monocyte/macrophage populations with time. For each donor there was an initial decrease in cell size over the first 24 hours (approximately from 30 to 12 μm). This decrease was followed by a steady increase in cell size, so that by Day 7 the cells were approximately twice their original size (i.e. approximately 60μm).

3.4. DISCUSSION

It is evident from Table 3.1., that there is considerable donor-dependent variation in the number of white cells present in blood samples. However the range of white blood cell counts for the donors (4.2 to 9.6×10^6 cells/cm^3), falls within the normal range of 4 to 11×10^6 cells/cm^3 given by Darcie & Lewis (1975). This indicates that the donors probably did not have inflammatory conditions during which one would expect high white blood cell counts. From Table 3.1. it is also clear that the number and percentage of the white cells that are monocytes also varies between the donors. Again the values obtained (i.e. 4 to 10 percent monocytes) were very close to the range of 2 to 10 percent monocytes reported by Darcie & Lewis (1975). Our donors did not include any with a monocyte population of only 2 or 3 percent. This may simply have been due to the small number of donors, or alternatively large lymphocytes may have been mistaken for monocytes in the Giemsa-stained blood smears, thus

causing over-estimates of the actual monocyte numbers. The latter is quite probable as a number of authors have highlighted the difficulty in distinguishing the two cell-types (e.g. Nichols & Hainton 1973).

One of the parameters used to evaluate the three separation techniques was the viability of the cells in the "mononuclear cell preparations". As is apparent from Table 3.2., there were no differences detected in cell viability: for each method a mean of less than 2 percent of the cells stained with trypan blue. These results are similar to those obtained by Boyum (1983) using Nycodenz, and by Grunberger et al. (1985) using Percoll, for cell separations. Cell viability was therefore not a critical parameter for selecting a method, but it was important to show that none of the methods used caused cell toxicity. This is so because, if toxicity had occurred, then certain subsets of cells might have been selectively lost.

Differences were apparent between the isolation methods in the monocyte purities of the "mononuclear cell preparations" (see Section 3.3.3.). The lowest monocyte purity was seen using dextran (mean = 7.7 percent). This was an expected result, since dextran effectively only sediments erythrocytes, leaving a leukocyte-rich plasma in which one would expect approximately 5 percent monocytes. Consequently, and unlike the other separation methods, a high proportion of granulocytes was observed in the Giemsa-stained "mononuclear cell preparations". Granulocytes are adherent cells and thus one would expect the monolayers from the dextran method to be contaminated with these cells. This was indeed observed and the consequences are discussed shortly. Although the mean monocyte purity value for the Percoll method was also low (ie. 22 percent), less than 1 percent of the contaminating cells were granulocytes (the remainder were

non-adherent lymphocytes, which do not pose a problem in culture as they are removed by the washing procedure). This is evident by the high purity value of the monolayers after 24 hours in culture. The mean monocyte purity for the "mononuclear cell preparations" was very similar to that described by Pertoft et al. (1980), viz. 20 percent. The highest values for monocyte purity in "mononuclear cell preparations" were obtained using the Nycodenz gradients (i.e. greater than 90 percent), which closely agrees with Böyum's (1983) estimates of approximately 95 percent. The reason for the higher purities obtained with this method is that the high osmolarity of Nycodenz causes lymphocytes to increase their density by losing water, more than the less sensitive monocytes, thus leaving mainly monocytes at the top of the gradient.

Differences were also apparent between the isolation methods in the monocyte yields obtained in the "mononuclear cell preparations" (see Section 3.3.4.). In agreement with Böyum (1983) low yields were obtained using Nycodenz, and in agreement with Pertoft et al. (1980) high yields were obtained using Percoll. Both these authors reported a wide variation in the monocyte yields between donors, an observation borne out by our Percoll results. The greater than 100 percent yields obtained with the Percoll method require explanation. Such yields were also reported by Pertoft et al. (1980) using Percoll, and by Van Furth et al. (1979) using Ficoll-Hypaque. Van Furth et al. (1979) suggested that these are attributable to the assumption made, when calculating the yield, that there are 5 percent of monocytes in blood. This assumption must in some cases represent a gross under-estimate of the actual number present (N.B. the normal range is 2 to 10 percent). Nevertheless the yields obtained using Percoll still represent high recoveries of monocytes. By considering

the monocyte yield data, the Percoll method appeared to be the most favourable. However the monocyte purity data indicated the Nycodenz method to be preferable. Before selecting a method, the purity of the monocytes in culture was also considered.

It is clear from Section 3.3.5. that the purity of the monolayers obtained, following each separation method, only differed on Day 1 (all the purity values were greater than 94 percent on Day 7). For both the Percoll and Nycodenz methods, the purities (as assessed by Giemsa-staining, esterase activity and phagocytic ability) were high on Day 1. However, with the dextran method, the initial purity of the monolayer was much lower (i.e. approximately 75 percent). The reason for this was the higher proportion of granulocytes in these "mononuclear cell preparations". These cells are adherent and remain in culture for up to 24 hours, after which they die and are removed by the monocytes (Pertoft et al., 1980). Thus by Day 7 the granulocytes were not present, as was apparent in our Day-7 cultures see Table 3.6.. With the Percoll method, although the monocyte purity in the "mononuclear cell preparation" was initially low (i.e. mean = 22 percent), the purity in the monolayer on Day 1 was high (i.e. mean approximately 96 percent). This is because the "contaminating" cells were mainly non-adherent lymphocytes (less than 1 percent were granulocytes). It is evident therefore that, unless there is gross contamination in the "mononuclear cell preparations" by granulocytes (as with the dextran method), the purity of the monolayer on Day 1 is unaffected by non-adherent cells such as lymphocytes. In view of this, the yield became my paramount consideration, particularly as I was dealing with a cell-type that is low in number. Consequently the Percoll method was chosen for further studies, as it produced the greatest yields.

The Percoll method is also advantageous in that it is simple, and effective without the use of anticoagulants which may affect cell function. For example heparin, a commonly used anticoagulant, has been shown to enhance the phagocytosis of [^{51}Cr]erythrocytes by peritoneal macrophages (Blumenstock et al., 1981). Also by using defibrinated blood, platelet contamination is avoided: these cells, present in anticoagulated blood adhere to monocytes and alter their function and affect their ability to adhere (Pertoft et al., 1960). Some criticism has however been levelled against the use of Percoll. It has been reported that the phagocytic ability of cells isolated using Percoll was diminished (Serio et al., 1979) and that between 20 and 37°C Percoll is ingested by mouse peritoneal macrophages (Wakefield et al., 1982). However, it is generally thought that Percoll does not affect the monocytes' phagocytic ability (Gmelig-Meyling and Waldmann 1980). Also Grunberger et al. (1985), who used Percoll to isolate monocytes, did not report the presence of Percoll in electronmicrographs of monocytes; they observed that the monocytes were better preserved, in comparison to cells isolated on the commonly used gradient material Ficoll-Hypaque. In our studies, to reduce the possible uptake of Percoll, centrifugations were carried out at approximately 15°C. Spinning at even lower temperatures (i.e. 4°C which would completely abolish uptake), tended to cause excessive cell clumping and consequent cell loss, as clumps were difficult to break up and detached from the monolayers.

Percoll-isolated monocytes are reported to undergo normal maturation in vitro (Fluks 1981). It was evident that the cells were maturing in our studies, as by Day 7 the cells had developed resistance, to sodium fluoride inhibition of their esterase activity, (a characteristic of more mature macrophages), in agreement with Musson

et al. (1983). Also, by morphological criteria (examination of living cultures), the cells were deemed to mature into macrophages, as the cells adopted the characteristic forms described in Section 3.1.5. becoming a very heterogenous population. In addition, as documented in Section 3.1.5., the monocyte/macrophage populations underwent characteristic changes in size. The cells initially decreased in diameter to about half the initial value, then increased in size over the next few days, until by day 7 they were approximately double their original diameter. Our results are in close agreement with those of Johnson et al. (1977).

The initial number of cells seeded from the "mononuclear cell preparations" (1×10^6 cells per well) gave monolayers in which cells were neither sparse nor crowded (the problem of overcrowding is discussed later in this Section). However it was sometimes observed, using this inoculum size, that cells were slightly sparse in some areas of the wells. This problem was difficult to overcome, owing to the small-sized blood samples, since increasing the inoculum size would have drastically reduced the number of wells available for experimentation. (N.B. Although the cells appear very sparse in the photomicrographs (see Figure 3.2.1.), this is due to the fixing process, in which many cells detach).

Several types of culture vessel were employed in these initial cultures. As documented in Section 3.3.7., cells were found to adhere better over longer periods, to a plastic surface, in particular the Linbro wells, in preference to glass. This feature has been reported by a number of authors. For example Johnson et al. (1977), reported that equal numbers of cells adhered initially to glass or plastic surfaces, and yet with time in culture cells

detached more readily from glass. Johnson et al. (1977) used Linbro wells for the plastic surface and, for the glass surface, monocyte monolayers were established on coverslips, which were then placed into Linbro wells. In our system, it was surprising that the cells adhered to the plastic Linbro wells in preference to the plastic coverslips. Other authors have also found that cells may adhere to one type of plastic better than to another (Goldblatt et al., 1978). It was thus decided to employ the Linbro wells routinely for further studies.

Although cell loss (i.e. a decrease in the number of adherent cells) occurred during culture, this was not alarming since, as described in Section 3.1.6., similar cell losses have been observed by many authors (e.g. Johnson et al., 1977 and Musson et al., 1980). It has been suggested that Percoll affects the ability of mouse peritoneal macrophages to adhere to plastic (Wakefield et al., 1982). However, as outlined above, the percentage of cells remaining adherent with time in our system did not differ from reports by others. At this stage in the work it was not possible to compare absolute numbers of monocytes adhering with data from other authors, as only the percentage of the initial number of cells present on Day 0 was determined during culture. However, later on absolute numbers of adherent cells were determined, the results are discussed in Section 3.4..

The reason for the cell losses that occur in monocyte/macrophage cultures is not clear. As previously stated, Musson et al. (1983) and Odegaard et al. (1974) suggest that cell loss may be due to overcrowding, i.e. inadequate space to accommodate the cells, owing to their increase in size. Both noted that decreasing the initial

inoculum size reduced the proportion of cells lost. However it is clear from our studies and others, that the increase in cell size does not occur until after Day 1, while the cell loss occurs from the onset of culture. This implies that overcrowding is not the only factor involved in cell loss. In addition, in our system the cells were not obviously overcrowded, yet cell losses still occurred. As mentioned earlier, cell clumping and subsequent cell detachment, a phenomenon reported by many (e.g. Zuckerman et al., 1979), may also contribute to this cell loss. However other factors must also be involved, as cell clumping is often not very marked. In our cultures cell clumping was usually not extensive, but variable. When on occasion it was extensive, cells detached very quickly, so that by Day 4 very few cells remained. Such unsuccessful cultures approximated1 in 10 of our cultures. The reason for these failed cultures was not clear, although it is known that many factors can cause impairment of monocyte maturation, such as underlying donor disease states (see Table 1.2.). Other authors have stated similar difficulties (Knight, 1985; Leoni, 1985).

Using the medium and feeding protocol described in Section 2.2.7. (i.e. medium199 supplemented with fetal calf serum (10 percent) and horse serum (10 percent) and replacing half the medium weekly), we found that cells could be maintained for up to eight weeks (results not shown in detail). Zuckerman et al. (1979) using the same protocol also obtained successful long-term cultures. They reported that the use of animal sera was preferable to autologous sera, being more readily available and helping to standardise the cultures. Johnson et al. (1977) failed to maintain monocyte/macrophages using either fetal calf serum or horse serum alone, and this is consistent with the data of Zuckerman et al. (1979) who found that reproducible

cultures were only obtained when a mixture of the two were used. Zuckerman et al. (1979) pointed out the problem of serum batch-variation and its effects on the cultures, a matter discussed further in Chapter 4.

In summary, the three initial aims of the study have been met. Firstly a method based on Percoll fractionation was found to be very suitable for isolating monocytes from blood. Secondly the culture method of Zuckerman et al. (1979) was found to produce successful cultures of monocyte/macrophages when using Linbro wells. Thirdly the cultured cells were positively identified as belonging to the mononuclear phagocyte system. In addition, by morphological and cytochemical criteria, the cells were deemed to be maturing in culture. This last aspect is further explored in Chapter 5.

CHAPTER 4

The pinocytic activity of monocyte/macrophages: preliminary studies

4.1. INTRODUCTION

Having established a suitable procedure for isolating and culturing human monocytes (detailed in Chapter 3), the next objective was to design a method for quantifying pinocytosis. We needed to know whether pinocytic vesicles were being formed, and at what rate, by cells at various stages of differentiation.

4.1.1. Quantifying pinocytosis

The best way of quantifying pinocytosis is to determine the volume of ambient fluid captured per unit time. Although morphological methods have been used, in which intracellular vesicles are counted, it is difficult to extrapolate to the rate of pinocytosis, and even to be confident that the vesicles counted are not just transverse sections of canaliculi (without studying serial sections). Biochemical methods are more direct. The uptake rates of suitable substrates are determined by measuring their cellular accumulation with time. With a non-degradable substrate the rate of pinocytic capture may be inferred directly from the rate of cellular accumulation (provided its rate of exocytosis is slow). The substrates that have been chosen for such studies are ones where uptake by cells can be readily measured: they are either enzymically active, fluorescein-labelled or radiolabelled. Radiolabelled substrates are particularly useful owing to the greater sensitivity of radiometric assays. It is important that the substrate's only possible mode of entry into the cell is by pinocytosis, and therefore soluble macromolecules are ideally suited for assessing pinocytic activity. Although biodegradable substrates can be used to study pinocytosis, it is obviously not sufficient to measure only the amount of radioactivity retained within the cells;

one must also have some measure of the material that has been captured and degraded, bearing in mind that with some substrates, digestion products may be released back into the medium.

Owing to the phenomenon of adsorptive pinocytosis described in Chapter 1, the rate of pinosome formation is not the only factor that determines the rate of entry of a substrate. The same cell may capture different substrates at different rates, depending on the degree of membrane affinity between the substrate and the cell. Substrates captured entirely in the fluid-phase (i.e. those with no membrane affinity), are captured at similar rates (reviewed by Pratten et al., 1980), and their uptake is proportional to the extracellular substrate concentration. The fluid-phase markers used most frequently, are ^{125}I-labelled polyvinylpyrrolidone, [^{14}C]sucrose and [^{3}H]dextran. In contrast to fluid-phase markers, substrates captured by adsorptive pinocytosis do demonstrate some surface binding, and at very high substrate concentrations the binding sites become saturated. The factors thought to confer increased avidity for plasma membranes are outlined in Chapter 1.

When studying the pinocytic uptake of substrates quantitatively, there are a number of ways to express the results. The simplest and most direct is to record the amount of substrate captured. In the simplest case of a tissue pinocytosing at a steady rate, one would expect the amount captured to increase with time in a linear fashion. Deviations from linearity are difficult to interpret with confidence. For example, if the rate of accumulation falls off with time, this may indicate that the substrate is being released, or degraded then released, or that cell-death is occurring. If linear uptake with time is obtained, the results are easily interpreted and, if one is

measuring the uptake of a fluid-phase marker, then its accumulation is related to the rate of fluid capture. A term frequently used to express rates of uptake is the Endocytic Index, which is defined as the volume of culture medium whose substrate content has been captured by a defined amount of tissue in a given time (Williams et al., 1975). Normalising data in this way is valuable , as one can readily compare the uptake rates of different substrates by the same or even different cells.

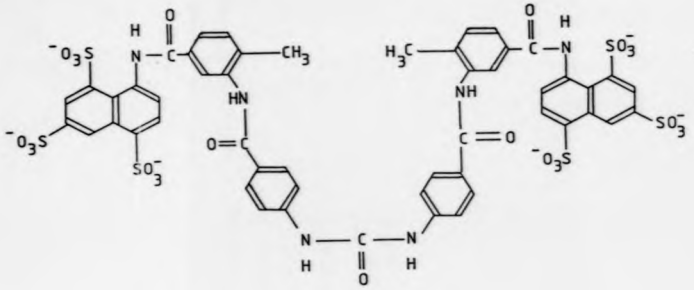
In this type of experiment, it is important to distinguish between pinocytic uptake and surface adsorption. A number of criteria are used to characterise pinocytosis (Lloyd & Williams, 1984; Pratten et al., 1980). A slow progressive cellular accumulation of substrate over a long time-period is characteristic, and this contrasts sharply with surface-binding, where the binding sites rapidly become saturated. Also, there should be no microscopic evidence of surface binding of the substrate. Since in many studies pinocytosis has been shown to be inhibited by low-temperature and metabolic or cytoskeletal inhibitors, these should also all be effective. In addition, when cells are incubated with the substrate and then washed, there should be a low rate of substrate release from the cells into the fresh medium (surface-bound substrate would be rapidly released).

4.1.2. Choice of substrate for preliminary experiments

Initially, it was decided to quantify pinocytosis using a non-degradable, radiolabelled fluid-phase marker. ^{125}I-labelled polyvinylpyrrolidone was chosen, since it was used by Knight & Soutar (1982) to determine pinocytosis in human monocyte/macrophage cultures

Figure 4.1.

Structural formula of suramin



(Section 1.2.7.8.) and also its clearance by rat peritoneal macrophages has been regularly measured in this laboratory (Pratten et al., 1977). However, it was envisaged that the uptake of this substrate might be difficult to detect. Firstly, owing to limited cell availability, there would only be small numbers of cells in each well to capture it (each well would be seeded with 1×10^6 "mononuclear cells", a number which would decrease with time in culture, as described in Chapter 3). Secondly, Knight & Soutar (1982) needed to use very high concentrations of ^{125}I-labelled polyvinylpyrrolidone, in order to detect its uptake by human monocyte/macrophage (3×10^6 "mononuclear cells" seeded/well). Thirdly, Pratten et al. (1977) needed very long incubation periods (up to 54 hours) to measure its uptake by rat peritoneal macrophages (1×10^6 cells). Such approaches (i.e. high substrate concentrations and long incubation periods) were considered either too expensive or inappropriate for this study, in which it was hoped to measure changes in pinocytic activity dependent on cell-age. To overcome this possible problem, it was decided to take advantage of the phenomenon termed "piggy-back" endocytosis (described briefly in Chapter 1), whereby a substance is added that enhances the rate of capture of the radiolabelled substrate by acting as a bivalent ligand between the cell surface and the substrate. Suramin, a polysulphonated polycyclic hydrocarbon was chosen for this purpose (see Figure 4.1. for structure), as explained in Section 1.1.2.4. It enhances the uptake of ^{125}I-labelled polyvinylpyrrolidone by rat peritoneal macrophages (Pratten & Lloyd, 1983).

4.1.3. Properties of suramin

Suramin is thought to enter cells by pinocytosis, and to accumulate in the lysosomes (Buys et al., 1973). It is a trypanocide and more recently has been used with varying success in the treatment of HTLV-III (AIDS virus) (Buchs 1985). Suramin was thought to inhibit selectively, the viral reverse transcriptase enzyme, but Basu and Moduk (1985) reported that the mechanism is non-selective, as suramin also binds to non-enzymic proteins such as albumin. Suramin has a number of other effects on cells, including inhibition of intracellular protein digestion (demonstrated in rat liver lysosomes), and inhibition of lysosome-endosome fusion (shown in macrophages) (Davies et al., 1971; Korolenko et al., 1981).

4.1.4. Effect of suramin on pinocytosis

Pratten & Lloyd, 1983 reported that suramin (100ug/cm^3 or 500ug/cm^3) enhanced the uptake of ^{125}I-labelled polyvinylpyrrolidone by rat peritoneal macrophages, by approximately 1.5 fold and greater than 10-fold respectively. They demonstrated that the substrate was ingested and not just bound, as its capture was abolished in the presence of the metabolic inhibitor sodium fluoride. Since suramin had no effect on the clearance of two other fluid-phase markers ([^{14}C]sucrose and [^{3}H]dextran), it was deduced that suramin was not affecting the rate of pinosome formation. Pratten & Lloyd, (1983) concluded that suramin was acting as a bivalent ligand (as described above), converting a fluid-phase substrate into an adsorptive one.

Similar observations have been reported for another polysulphonated aromatic molecule, trypan blue. Roberts et al. (1980) reported a 4

to 6 fold increase in the uptake of ^{125}I-labelled polyvinylpyrrolidone by rat visceral yolk-sacs (17.5-Day) when cultured with trypan blue (50µg/cm^3). Other polyanions e.g. dextran sulphate, polyglutamic acid and DIVEMA (pyran copolymer) bind avidly to rat peritoneal macrophages, but do not affect the uptake of ^{125}I-labelled polyvinylpyrrolidone (Pratten et al., 1978; Pratten et al., 1981).

4.2. EXPERIMENTAL METHODS

This chapter describes the development of a satisfactory method for measuring pinocytosis in monocyte/macrophages. Modifications and improvements were frequently made during the evolution of the final protocol and in this section, only the method first used is described in detail. The various steps taken whilst developing the procedure are outlined briefly in this Section, and discussed in the results (Sections 4.3.2. and 4.3.3.). The definitive method is described in detail in an Appendix to this Chapter (Section 4.5).

4.2.1. Initial method for measuring pinocytosis

Blood (20cm^3) was withdrawn from donors as described in Section 2.2.1., and "mononuclear cell preparations" isolated as described in Section 2.2.1.. Monolayers of monocyte/macrophages were then prepared in 24-well Linbro plates (1 x 10^6 cells seeded/well) as described in Section 2.2.7..

All incubations with the radiolabelled substrate were in wells in the same plate and were commenced at the same time. Substrate additions were achieved by removing 0.1cm^3 of culture medium and replacing it

with 0.1cm^3 of a warmed solution of ^{125}I-labelled polyvinylpyrrolidone and suramin in culture medium, giving final concentrations in the wells of 10µg/cm^3 and 500µg/cm^3 respectively. In addition, substrate was added to cell-free wells (i.e. wells containing 0.9cm^3 culture medium only) for a period of 6 hours, to determine the degree to which the substrate bound to the well itself.

Incubations were terminated after 0.5, 3 and 6 hours as follows. Plates were temporarily removed from the incubator, and from the appropriate wells, were removed duplicate 200µl (Finnpipette) portions of post-culture medium; to each portion was added 800µl water (a standard counting volume of 1.0cm^3 was chosen to ensure comparability) and their radioactivity measured with a 1282 Compu-gamma gamma counter.

Next, the remaining culture medium was aspirated from the wells using a Pasteur pipette attached to a mechanical pump. The cells were washed by six changes of warm medium (1.0cm^3 portions). After the final aspiration NaOH solution (0.5M; 1.0cm^3) was added to each well. The plate was returned to the incubator for any remaining incubation time. After the final washing procedure, the cells were reincubated for a further hour to ensure dissolution of the cells.

The digests were thoroughly mixed using a Finnpipette. Digest (900µl) was then added to 100µl NaOH solution (0.5M) and its radioactivity counted using a 1282 Compu-gamma gamma counter. The same sample was then used for protein determination using the method described by Lowry *et al.* (1951). The data relating to each well was used to calculate a "clearance value" i.e. the volume of culture medium whose content of radioactivity had become cell-associated,

expressed per mg. of cell protein. The calculation was made using the formula:-

$$\text{Clearance} = \frac{\text{cell-associated radioactivity (cpm per well)}}{\text{Radioactivity of medium (cpm per µl.)} \times \text{cell protein (mg per well)}}$$

The units of clearance are thus µl. per mg. protein.

In an experiment where clearance values increase linearly with time, the rate of increase (Endocytic Index) has the units µl per mg protein per hour.

4.2.2. Modifications to the initial method for measuring pinocytosis

Whilst developing the procedure for measuring pinocytosis a number of changes were made to the initial method described above. In brief these were as follows. A greater volume of blood was taken from each donor, which required the cell-isolation method using Percoll gradients to be scaled up appropriately. Greater numbers of cells were seeded into each well, and therefore it was necessary to replenish the culture medium after several days (to counteract the more rapid build-up of waste metabolites). The incubation periods with the radiolabelled substrates were lengthened, and the amount of substrate also increased. Incubations were all started at different time-points prior to the end of the experiment, but terminated together. The washing procedure at the end of each of the experiments was modified by using a long-form pipette for the aspirations and reducing the suction power of the pump. Finally, cells were digested in less NaOH.

Figures 4.2.1. to 4.2.3.

Typical examples of uptake of ^{125}I-labelled polyvinylpyrrolidone (10 $\mu g/cm^3$; in the presence of suramin, 500 $\mu g/cm^3$) by cells at various stages of maturation, obtained from different donors, Day-9 cells (Figure 4.2.1.); Day-11 cells (Figure 4.2.2.) and Day-15 cells (Figure 4.2.3.)

(i) protein content of cells.
(ii) cell-associated radioactivity. (----) double the amount of radioactivity adsorbing to cell-free wells.
(iii) clearance.

Each point represents the value obtained from a single well.
The exact details of these experiments are given in Section 4.3.1.

Figure 4.2.1.

A typical example of uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-9 cells.

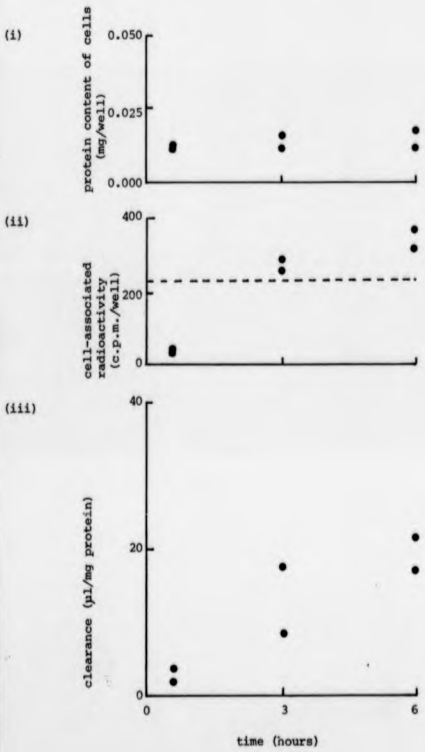


Figure 4.2.2.

A typical example of uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-11 cells

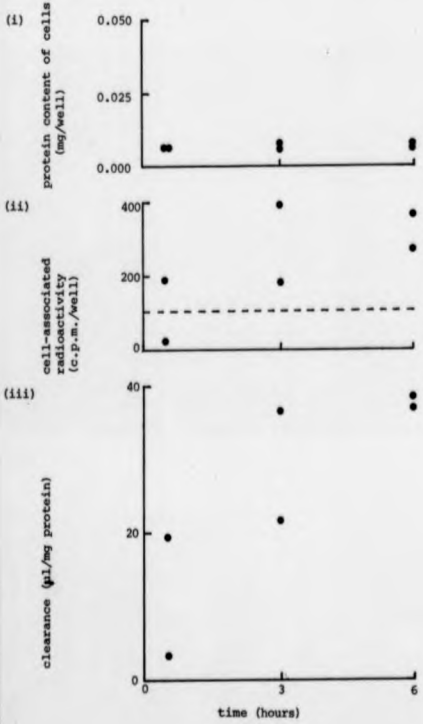
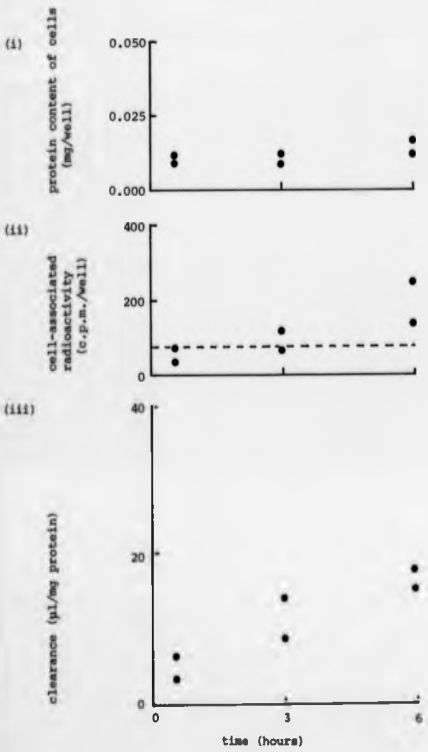


Figure 4.2.3.

A typical example of uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-15 cells.



4.3. RESULTS

This chapter includes only a few of the results of our extensive preliminary experiments, in which ^{125}I-labelled polyvinylpyrrolidone and suramin were used to detect pinocytosis. Many of the results obtained are not included for, as will become apparent, pinocytosis was difficult to detect with many cultures. The results which are given are used to outline the problems encountered during this early work and to justify the various modifications consequently made to the protocol.

4.3.1. Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by monocyte/macrophages

The first experiments were performed as described in Section 4.2.1., using cells obtained from a number of donors and cultured for periods of nine days or more. Figures 4.2.1. to 4.2.3. show some typical results of these experiments. It was encouraging to note that duplicate values were reasonably close. The values for cell-protein were similar for each of the incubation periods, indicating no great cell losses. Furthermore the cell-associated radioactivity values increased with duration of incubation. The clearance values (that is the amount of cell-associated radioactivity normalized to cell-protein) are seen to increase with duration of incubation. It was a matter of concern, however, that most of the uptake values for the shorter incubation periods were less than twice the amount of radioactivity associated with cell-free wells.

When we performed the same experiments but with cells that had been cultured for shorter periods (up to 5 days), we could not reliably

Figures 4.3.1. to 4.3.3.

Typical examples of uptake of ^{125}I-labelled polyvinylpyrrolidone (10 $\mu g/cm^3$; in the presence of suramin, 500 $\mu g/cm^3$) by cells at various stages of maturation obtained from different donors, Day-1 cells (Figure 4.3.1.); Day-3 cells (Figure 4.3.2.) and Day-5 cells (Figure 4.3.3.).

(i) protein content of cells.
(ii) cell-associated radioactivity (----) double the amount of radioactivity adsorbing to cell-free wells.
(iii) clearance

Each point represents the value obtained from a single well.
The exact details of these experiments are given in Section 4.3.1.

Figure 4.3.1.

A typical example of uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-1 cells

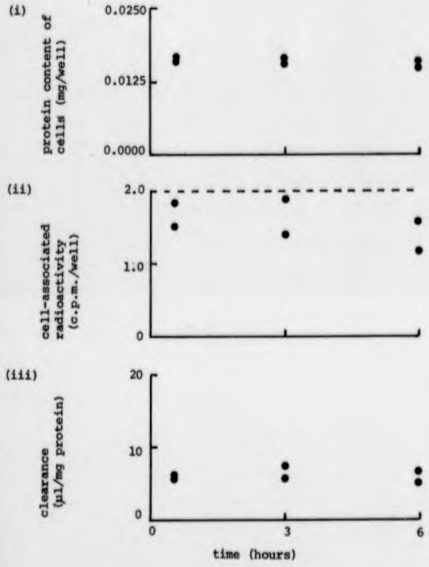


Figure 4.3.2.

A typical example of uptake of ^{125}I-labelled polyvinypyrrolidone (in the presence of suramin) by Day-3 cells

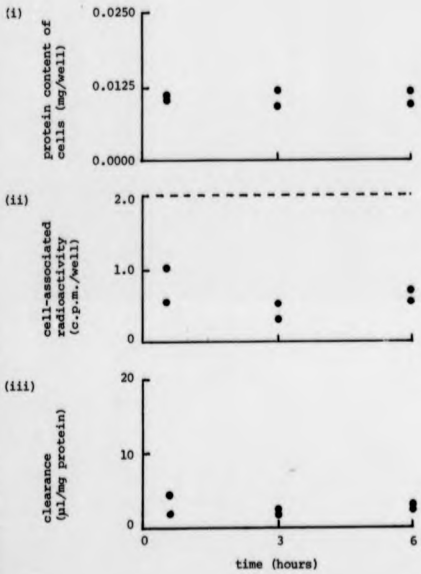
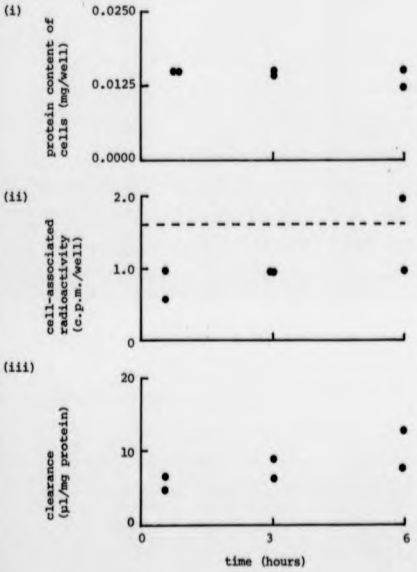


Figure 4.3.3.

A typical example of uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-5 cells



detect uptake of ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin. Figures 4.3.1. to 4.3.3. show typical results for these experiments. It is seen that most of the cell-associated radioactivity values were well below twice the amount of radioactivity associated with cell-free wells. The clearance values did not show any clear progression with duration of incubation. Furthermore, despite the earlier rather more promising results with cells cultured for longer periods (Day-9 or more), further experiments with cells of this age gave similar results to those just described.

It was clear that for the majority of these preliminary experiments, the uptake values were not sufficiently high to be detected above the amount of radioactivity adsorbing to the plastic wells.

Microscopic examination of cells before and after the washing procedure (used to terminate incubations with radiolabelled substrate), indicated that many of the cells detached during this period. It was initially thought that higher and more acceptable uptake values might be obtained by reducing this cell-loss, and therefore it was decided to modify the washing procedure.

4.3.2. Minor improvements to the method

The problem of cell loss during the washing procedure was not easily overcome. Additional care in washing cells was tried, but to no avail. Cell loss was decreased, though, when the mechanical pump was operated at a reduced suction, and even less, when a long-form pipette rather than a short-form was employed for the aspiration procedure. This improvement was probably due to reducing the sheer

force applied to the cells.

Although these changes greatly reduced cell-loss, the cell-associated radioactivity values were still often very close to the radioactivity values obtained in cell-free wells. It was not clear why we could not reproduce the first set of experiments where reasonable uptake values and progressive uptake were seen, and therefore it was decided to see whether different batches of culture plates of sera and of suramin or ^{125}I-labelled polyvinylpyrrolidone were having any effect on the ability of the cells to capture the substrate. Cells grown for 9 days were used for these experiments. None of these investigations clarified the problem, but two changes were adopted during this period. Firstly, we decided to standardize the serum employed by reserving a large quantity from a single batch of each type used (horse serum and fetal calf serum); and secondly, since for some of the cell-protein estimates, the optical density readings were very low, we reduced the volume of NaOH used to digest the cells (from 1.0cm^3 to 0.5cm^3). It was also thought that the approach used to terminate the incubations (at intervals throughout the experiment) could be improved: for while the cells used for the shorter incubation periods were being treated, the remaining wells (for the longer incubation periods) were temporarily away from the incubator and therefore being exposed to additional changes in their environmental conditions. This problem was easily overcome by adopting the policy of adding the substrate at different time-points throughout the experiment and terminating all the incubations together (the former being a much quicker procedure than the latter). An incidental advantage to this, was that cells had all reached exactly the same stage of maturation at the end of the experiment.

4.3.3. Major improvements to the method

It was evident up to this point that we could not consistently obtain reasonable uptake values: we therefore decided to increase the number of cells seeded into each well (from 1 x 10^6 to 3-4 x 10^6). In order to do this, it was necessary to take larger volumes of blood (180cm^3) from individuals. It was not possible to take such large volumes of blood from hospital out-patients, since often they were already giving considerable amounts for routine laboratory tests, and therefore we started to take blood from fellow workers. Although collection of large volumes was straightforward (using a butterfly needle and a series of 60cm^3 syringes), defibrinating the blood proved difficult. After a number of attempts it was found preferable to defibrinate the blood in Duran bottles (500cm^3), containing bent paperclips. Owing to the larger amounts of blood, it was necessary to "scale-up" the cell-isolation procedure. This step was relatively straightforward, simply involving an increase in the amount of gradient material and in the size of vessels used (31.5cm^3 portions of blood were layered onto 18cm^3 of Percoll solution in 50cm^3 conical tubes).

In most of the uptake experiments using the considerably increased numbers of cells, a marked improvement was seen, but still in a number of cases the uptake values were near to the values obtained in cell-free wells. A further change was therefore made to the incubation periods, in that they were lengthened considerably (up to 48 hours). Again some improvement was found in the reliability of the uptake values, though we were still obtaining results near to the limits of detection with cells from some donors. The final change we made was to increase the amount of ^{125}I-labelled polyvinylpyrrolidone

Figures 4.4.1. to 4.4.3.

Uptake of various concentrations of ^{125}I-labelled polyvinylpyrrolidone ((▲), 10μg/cm^3; (o) 50μg/cm^3; (●) 100μg/cm^3; in the presence of suramin, 500μg/cm^3) by Day-7 cells obtained from the three donors L.S. (Figure 4.4.1.); C.C. (Figure 4.4.2.) and A.S. (Figure 4.4.3.)

(i) protein content of cells.

(ii) cell-assocaited radioactivity. (— — —) double the amount of radioactivity associated to cell-free wells (10μg/cm^3); (·····, 50μg/cm^3) and (— · — · —, 100μg/cm^3).

(iii) clearance.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 4.3.4.

Figure 4.4.1.

Uptake of various concentrations of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-7 cells

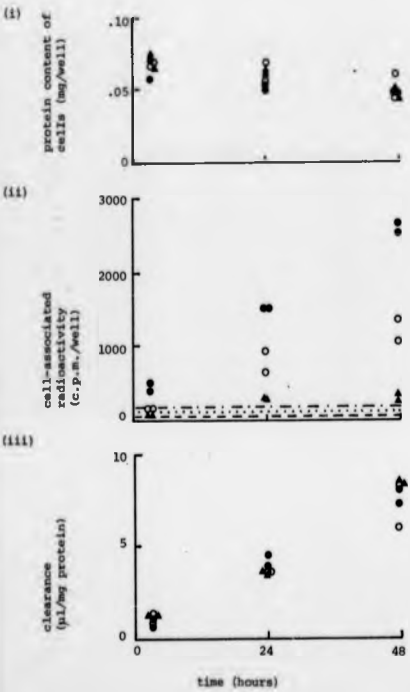


Figure 4.4.2.

Uptake of various concentrations of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-7 cells

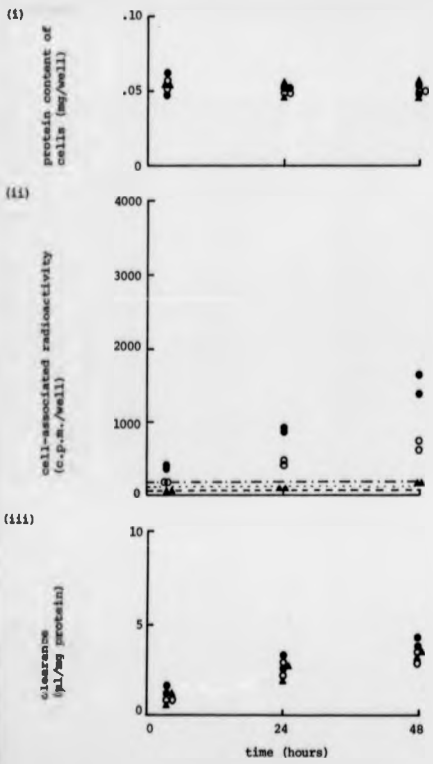
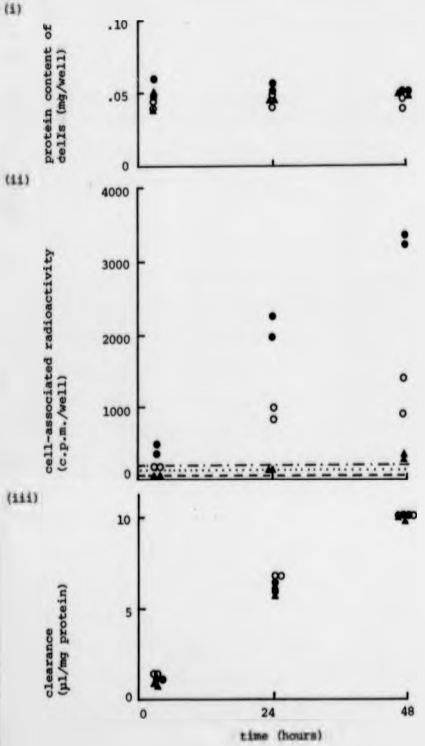


Figure 4.4.3.

Uptake of various concentrations of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-7 cells



in the incubations by a factor of 5. Although this was an expensive step to take, it proved beneficial, as reliable uptake results were then obtained for all donors' cells. The first of these successful results where cells were incubated with various concentrations of ^{125}I-labelled polyvinylpyrrolidone, are presented in the next Section.

4.3.4. Uptake of different concentrations of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by monocyte/macrophages

The definitive procedure used for these experiments is described in Section 4.5. Cells from three donors (L.S.; C.C.; A.S.) were obtained and cultured for seven days, at which point incubations with radiolabelled substrate were commenced. At various times (48, 24 and 3 hours) prior to the end of the experiment, cells were incubated with various concentrations of ^{125}I-labelled polyvinylpyrrolidone (10, 50, 100$\mu g/cm^3$) plus suramin (500$\mu g/cm^3$). Substrate was also added to cell-free wells (wells containing only 0.9cm^3 of medium). Incubations were all ended at the same time by which time 9 days had elapsed since the cell isolation, Figures 4.4.1. to 4.4.3. show the results. It is seen that for each of the donors the cell-protein values were similar and not dependent on duration of incubation. Also, as might be expected because of the greater number of cells present in the wells (3 to 4 x 10^6 seeded per well), protein values were higher than those for the earlier experiments, where only 1 x 10^6 cells were seeded. Duplicate cell-associated radioactivity values were close and, apart from when the lowest concentration of ^{125}I-labelled polyvinylpyrrolidone was used (10 $\mu g/cm^3$), all the radioactivity values were well above double the value for the

Table 4.1.

Endocytic Indices for the clearance of various concentrations of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin, 500$\mu g/cm^3$) by Day-7 cells

Donors	Concentration of ^{125}I-labelled polyvinylpyrrolidone ($\mu g/cm^3$)		
	10	50	100
L.R.	0.154	0.128	0.151
C.C.	0.068	0.064	0.067
A.S.	0.201	0.194	0.198

The exact details of these experiments are given in Section 4.3.4.

The units of Endocytic Index (see Section 4.2.1.) are μl per mg. protein per hour.

radioactivity associated with cell-free wells. The cell-associated radioactivity values increased with time, and were also proportional to the concentration of ^{125}I-labelled polyvinylpyrrolidone in the medium. The radioactivity adhering to the plastic wells also increased slightly as the concentration of ^{125}I-labelled polyvinylpyrrolidone in the medium increased. When the radioactivity data were expressed as clearance values (which account for the extracellular radioactivity and the cell protein), there was an increase with time, but there was no difference in the clearance rates for the different concentrations of ^{125}I-labelled polyvinylpyrrolidone. Table 4.1. shows the Endocytic Indices. It is seen that the rates vary between cells isolated from different donors.

4.4. DISCUSSION

The main goal of the work described in this Chapter was to establish a suitable method for assessing pinocytosis in monocyte/macrophages. It was considered important that the method adopted would be reproducible and suitable for cells at various stages of maturation, and that the amount of radioactivity associating with the cells would be well above that adsorbing to cell-free plastic wells.

The method initially used did not meet these criteria: most of the cell-associated radioactivity values obtained were only slightly above the radioactivity adsorbing to cell-free wells. In consequence results were not reproducible.

Although the very first experiments, using mature cells (cultured for 9-days or more), did appear acceptable, in that clearance values were

well above the blank-values, later experiments using cells of this age (and also less mature cells) gave unnacceptably low values. It is not clear why the first experiments with Day-9 cells were successful, but the later ones were not. A possible explanation is that the batch of serum initially employed may have yielded more pinocytically active cell-populations. Other workers have commented on differences in cell-behaviour with different batches of serum (e.g. Jessup, 1985).

A number of changes were made to the procedure initially used (one of which was standardising the serum used), before reliable and reproducible results were obtained consistently. These changes are summarised in Section 4.2.2.. The reasons for making the alterations have already been discussed in the results Section (4.3.). It is enough to add here that, although it was not possible to say that any single modification contributed the most to the success of the definitive method, several of the changes were thought to play a prominent role. Briefly, these changes included increasing:-

1. the volume of blood collected, (this required the cell-isolation procedure to be scaled up) which allowed greater numbers of cells to be seeded into individual wells,

2. the amount of radiolabelled substrate added to the cells,

3. the duration of the incubation periods with the radiolabelled substrate.

Using Day-7 cells the uptake values found for ^{125}I-labelled polyvinylpyrrolidone (10 to 50$\mu g/cm^3$; in the presence of suramin; 500$\mu g/cm^3$) when using the definitive method, were generally well

above the values for cell-free wells. Since the clearance values increased progressively over a prolonged period, we could be reasonably confident that we were detecting uptake of the substrate rather than just surface-binding. The rate of uptake was found to be proportional to the concentration of ^{125}I-labelled polyvinylpyrrolidone used (in the presence of suramin 500µg/cm^3); the Endocytic Indices, being normalized to the amount of extracellular radioactivity present, were therefore similar.

The Endocytic Indices did vary between cells from different donors. For ^{125}I-labelled polyvinylpyrrolidone (50µg/cm^3) in the presence of suramin (500µg/cm^3) the values ranged from 0.064 to 0.194 µl/mg. protein/hour. This was not considered surprising in view of the enormous genetic variation found in human subjects. This point is discussed extensively in Chapter 9. It was possible to compare these results with data for similar experiments but using rat peritoneal macrophages. Pratten & Lloyd (1983), reported a mean Endocytic Index of 4.31 ± 0.84 µl/mg. protein /hour which was notably higher than the values we obtained.

Prior to further studies on the endocytic behaviour of monocyte/macrophages using the definitive method, it was essential to determine conclusively whether the cells were differentiating *in vitro*, using several different maturation markers. These experiments are described in the next Chapter.

4.5. A SATISFACTORY METHOD FOR STUDYING PINOCYTOSIS IN MONOCYTE/MACROPHAGES

Blood (100 cm^3) was collected from volunteer male donors, serially into three 60cm^3 syringes with the aid of a butterfly needle to allow

the syringes to be changed easily. The blood was transferred directly into a Duran bottle (500cm^3) containing approximately 10 bent metal paperclips, and defibrinated by gentle rotation of the bottle for approximately 15 minutes.

Into duplicate conical tubes (50cm^3 polystyrene), Percoll (18cm^3; adjusted to a density of 1.076 gm/cm^3 in 0.15m NaCl) was placed. Defibrinated blood (31.5cm^3 aliquots) was carefully layered over the Percoll and the tubes centrifuged (15°C; 800g; 30 minutes). The mononuclear cell band was collected using a Pasteur pipette and washed (100g; 10 minutes) in medium 199 (prewarmed to 37°C). Then the cells were suspended in culture medium (medium 199 containing heat inactivated, fetal calf (10 percent) v/v) and horse serum (10 percent v/v) and antibiotics; see Section 2.2.1.) to a density of 3 to 4 x 10^6 cells per cm^3, and 1cm^3 portions seeded into wells of 24-well Linbro plates. Monolayers were established as described in Section 2.2.7.. Any cells remaining in culture beyond 96 hours had half of their culture medium replaced by 0.5cm^3 of prewarmed fresh culture medium, at this point. Incubations with the radiolabelled substrate were commenced at various times (usually 48, 24, 3 hours) prior to the end of the experiment. Substrate additions were achieved by removing 0.1cm^3 of culture medium and replacing it with 0.1cm^3 of a warmed solution of the radiolabelled substrate made up in culture medium. In addition, substrate was added to cell-free wells (containing 0.9cm^3 of culture medium only), for a period of 48 hours; to determine the degree to which the substrate bound to the well itself.

Incubations were terminated at the same time as follows. From each well were removed duplicate 200µl (Finnpipette) portions of

post-culture medium; to each portion was added 300µl water (a standard counting volume of 0.5cm^3 was chosen to ensure comparability) and their radioactivity measured with a 1282 Compu-gamma gamma counter.

Next, the remaining culture medium was aspirated from the wells using a long-form Pasteur pipette attached to a mechanical pump, which had been adjusted to reduce the suction. The cells were washed by six changes of warm medium (1.0cm^3 portions). After the final aspiration NaOH solution (0.5 moles/litre; 0.5cm^3) was added to each well, and the cells reincubated for a further hour to ensure solubilisation of the cells.

The digests were thoroughly mixed using a Finnpipette. Digest (450µl) was collected and made up to 500µl with NaOH (0.5moles/litre), and used for radioactivity and protein determination. The values for cell-protein and radioactivity per well were calculated by multiplying the measured values (on 900 or 450ul) by a factor of 1.11 (1000 ÷ 900 or 500 ÷ 450).

The data relating to each well were used to calculate a "clearance" value, i.e. the volume of culture medium whose content of radioactivity had become cell-associated, expressed per mg of cell protein. The calculation was made using the formula:-

$$\text{Clearance} = \frac{\text{cell-associated radioactivity (cpm per well)}}{\text{Radioactivity of medium (cpm per µl)} \times \text{Cell protein (mg per well)}}$$

The units of clearance are thus µl per mg protein.

CHAPTER 5

Confirming monocyte-to-macrophage differentiation

5.1. INTRODUCTION

The importance of ascertaining that, in the cell-system finally established in Chapter 4, monocytes were maturing into macrophages was emphasized in Section 4.4. The necessity for using several different markers to check this maturation was also stressed. In this section, we describe several biochemical maturation markers, which we have used to supplement the morphological and cytochemical criteria used in the initial work described in Chapter 3.

5.1.1. Relationship between cell number, cell-protein and cell-DNA in monocyte/macrophages

One parameter often used to help verify monocyte-to-macrophage differentiation is an increase in the protein content per cell. As detailed in Section 1.2.6.4., this increase usually occurs around the fourth day of culture, and is made evident by a continuing decrease in cell number with time, whilst the amount of cell protein starts to increase. Methods for determining these two parameters have already been used in Sections 3.3.7 and 4.2.1. However the microscopic method previously used to assess cell number, although adequate for the earlier experiments (where we were looking for major differences in the numbers of adherent cells isolated by various methods), was not considered accurate enough for the present studies. The problem with the technique is that monolayers are not always evenly distributed, and therefore the determination could mis-represent the actual cell number. To avoid this problem, it was decided to detach cells from the culture vessel and to count cells in the resultant suspensions.

Several methods are used to detach cells, many of which are detailed in Section 3.1.3.. However, with many detachment methods (e.g. using trypsin or versene), the loosed cells often clump and are not easily dispersed. Because of this it was decided to use the method of Unkeless and Eisen (1975), which overcomes the problem by lysing cells in Triton containing ethylene-diaminotetraacetic acid and counting the nuclei dispersed in the lysates. Although Unkeless and Eisen (1975) used a haemocytometer for nuclei counts, we had the facility for Coulter-counting; therefore this was employed, being a quicker method.

Since the protein-to-DNA ratio has been reported to increase as monocytes mature into macrophages (Knight & Soutar, 1982), the DNA content was also assessed in cells of various ages. It was envisaged, owing to the small numbers of cells in each well, that the values obtained for cell-DNA would be low, and therefore a fluorimetric assay was chosen. The method selected was very sensitive, the lower detection limit being 1ng DNA, and was useful because it was possible (unlike other methods) to determine cell-protein for cells in the same sample.

5.1.2. Stimulated lysosomal enzyme release from monocyte/macrophages

As described in Section 1.2.8, another parameter frequently used as an indicator of monocyte-to-macrophage differentiation is a marked increase in the secretion of lysosomal enzymes in response to certain phagocytic stimuli, particularly those with chronic inflammatory potential such as opsonized particles, e.g. zymosan. This secretion by the differentiated cells is not as marked if unopsonized particles

(e.g. polystyrene) are used.

This response has been reported in detail by Knight & Soutar (1982) and Musson et al. (1980); both described only a small increase in hexosaminidase secretion with unstimulated cells, as cells were maintained in culture, but with cells stimulated with opsonized zymosan a moderate increase in hexosaminidase secretion up to the fifth day in culture was seen, followed by a very sharp increase beyond Day 5. The secretory response of the cells to unopsonized particles (polystyrene) differed: Musson et al. (1980) reported the pattern of secretion to be the same as for zymosan-stimulated cells up to Day 5, after which (rather than the sharp increase) only a steady increase was seen; whilst Knight & Soutar (1982) reported the pattern to be very similar to that seen for the unstimulated cells.

5.1.3. Peroxidase activity in monocyte/macrophages

A further marker of monocyte-to-macrophage maturation is the loss of peroxidase activity over the first few days of culture (see Section 1.2.8.). Peroxidase catalyses the oxidation by hydrogen peroxide of several organic substrates. In monocytes, the main role for peroxidase is well established as being involved in antimicrobial activity (Klebanoff & Clark, 1978). The enzyme reacts with hydrogen peroxide and halide ions to form potent microbicidal agents such as hypochlorous acid (which oxidises cytochromes and nucleotides, therefore destroying electron-transport chains and depleting nucleotide pools, leading to cell-death).

It is believed that monocytes do not synthesize peroxidase, acquiring the enzyme from the precursor cells the promonocytes. The

monocyte-derived macrophage rapidly loses all peroxidase activity (Daems et al., 1979). The fate of peroxidase is not established, although it is known that peroxidase-containing granules fuse with phagosomes as the monocytes mature. It is possible that the enzyme is then degraded by lysosomal hydrolases as described by Steinman & Cohn (1972) for the fate of interiorized horseradish peroxidase by mouse peritoneal macrophages.

Detection of peroxidase activity may be achieved cytochemically, since the enzyme oxidizes several dyes in the presence of hydrogen peroxide. Biochemical assays are usually based on either the production of coloured oxidation products or on the disappearance of the hydrogen peroxide. A wide variety of hydrogen donors have been used in such assay systems, but many are carcinogenic, for example o-dianisidine.

For our purposes a qualitative cytochemical assay was considered inadequate and therefore a recently developed quantitative peroxidase assay (Worthington Enzymes, 1982) was chosen, which employs a non-carcinogenic hydrogen donor.

5.1.4. Lactate dehydrogenase activity in monocyte/macrophages

Lactate dehydrogenase is a ubiquitous metabolic enzyme. It is found in the cytoplasm of cells, and is responsible for the interconversion of pyruvate and lactate. As described in Section 1.2.6.4., an increase in the specific activity of lactate dehydrogenase has been reported as monocytes are cultured in vitro (O'Dorisio et al., 1984). Additionally the release of lactate dehydrogenase is used as an indicator of cell lysis (Haneberg et al., 1984). It was decided to

Figure 5.1.1. to 5.1.3.

Changes in (i) cell number

(ii) protein content of cells

and (iii) DNA content of cells

during the in vitro culture of monocyte/macrophages. Cells obtained from three donors S.J. (Figure 5.1.1.); D.O. (Figure 5.1.2.) and J.H. (Figure 5.1.3.).

Each value represents the mean of two determinations and is expressed as a percentage of the mean value obtained on Day 0.

The exact details of these experiments are given in Section 5.3.1.

Figure 5.1.1.

Changes in cell number, protein content and DNA content of monocyte/macrophages

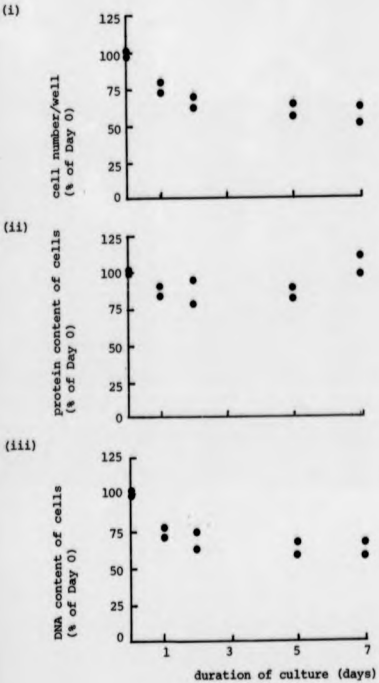


Figure 5.1.2.

Changes in cell number, protein content and DNA content of monocyte/macrophages

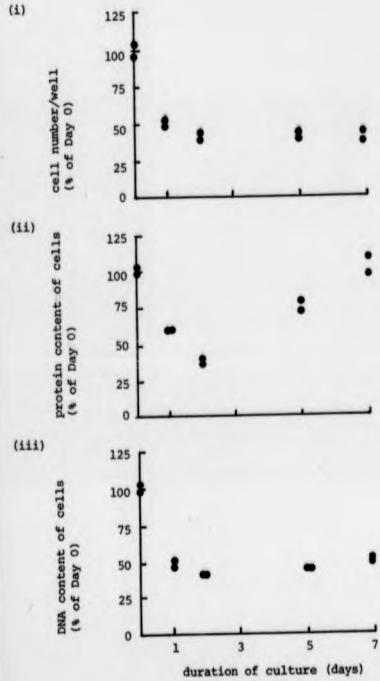
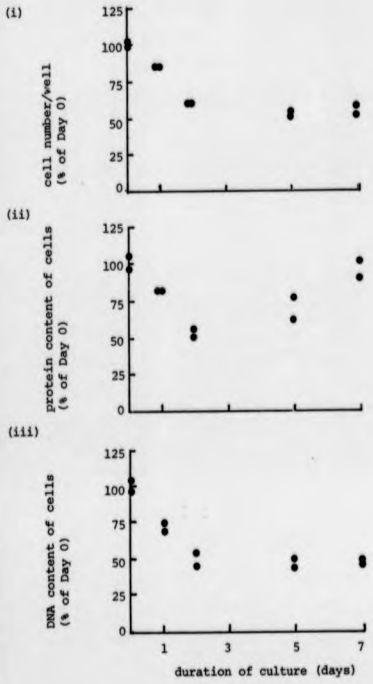


Figure 5.1.3.

Changes in cell number, protein content and DNA content of monocyte/macrophages



determine both cell-associated and released lactate dehydrogenase, both to contribute to the data on cell maturation and also to give an indication of cell death. For this purpose, owing to the small numbers in each well, a highly sensitive fluorimetric assay was chosen.

5.2. EXPERIMENTAL METHODS

All the procedures adopted in this Section are described in Chapter 2. Further details of the experiments are described in the following results Section.

5.3. RESULTS

5.3.1. Cell-protein, DNA and cell number in cells of various ages

Cells were obtained from three donors (S.J.; D.O.; J.M.) and cultured by the definitive method as described in Section 4.5.. After cells had been in culture for various periods (0, 1, 2, 5 and 7 days), cells in duplicate wells were used to determine cell numbers as described in Section 2.2.11.; and cells from two further wells, for both DNA and protein determinations, as described in Section 2.2.12.. Figures 5.1.1. and 5.1.3. show the values obtained for cell number, protein and DNA for cells after various periods in culture. Each parameter is expressed as a percentage of the initial mean value obtained for Day-0 cells. In most cases duplicate values were close. With all three donors the cell numbers decreased sharply over the first two days in culture to about fifty percent of the initial number. After the second day in culture a further but only slight decrease was seen with two of the donors (S.J.; J.M.), with the third

Table 5.1.

Changes in cell number, protein content and DNA content of monocyte/macrophages at various stages of maturation

Duration of culture (days)	Cell number x 10^5	Protein mg/well	DNA µg/well
0	7.1 ± 0.1	0.039 ± 0.001	7.1 ± 0.4
1	5.0 ± 0.5	0.028 ± 0.002	4.8 ± 0.4
2	3.9 ± 0.4	0.022 ± 0.003	2.9 ± 0.3
5	3.6 ± 0.3	0.028 ± 0.001	3.8 ± 0.2
7	3.6 ± 0.3	0.037 ± 0.001	3.8 ± 0.2

Each value represents the mean ± standard error of three determinations (duplicate wells for each of three donors).

The exact details of these experiments are given in Section 5.3.1.

Table 5.2.

Protein-to-DNA ratios for monocyte/macrophages at various stages of maturation: protein mgm-to-DNA mgm

Duration of culture (days)	Donors. S.J.	D.O.	J.H.	Mean (± standard error)
0	4.5	5.5	6.1	5.3 ± 0.3
	5.4	4.7	5.8	
1	5.6	5.9	5.3	5.8 ± 0.2
	6.1	6.4	5.7	
2	6.5	4.7	4.8	5.6 ± 0.3
	6.4	5.3	5.6	
5	7.7	8.3	8.5	7.6 ± 0.5
	6.2	8.9	6.2	
7	8.5	10.6	10.0	9.9 ± 0.5
	8.2	11.1	10.9	

The exact details of these experiments are given in Section 5.3.1.

(D.O.) cell numbers remained stable. In each case the amount of DNA per well closely paralleled the cell numbers. The protein values all followed the same pattern, with a decrease over the first two days in culture followed by an increase. The size of the decrease varied: with two donors (D.O.; J.M.) the decrease was to approximately half the initial value, and with the third donor (S.J.) the decrease was much less. With all three donors the protein values after a week in culture were similar to the initial value on Day 0. Table 5.1. shows the mean absolute values for each parameter, and Table 5.2. the mean protein/DNA ratios for each age of cell. From these results it was calculated that a Day-0 cell contained on average approximately 53pg protein and 10pg DNA; and a Day-7 cell 103pg protein and 11pg DNA. The protein/DNA ratio almost doubles during the first week of culture.

5.3.2. Hexosaminidase release from monocyte/macrophages

Blood was obtained from three donors (G.S.E.; M.R.; J.C.) and monolayers prepared as described in Section 4.5.. Cells were maintained in culture for up to 12 days in the usual way (Section 4.5.). Cells at various stages of maturation (Day 2, 5, 9 and 12) were used to determine hexosaminidase release upon phagocytic challenge, using the procedure detailed in Section 2.2.13.. Briefly this procedure involved prewashing the cells, then incubating cells in duplicate sets of wells for 4 hours in either "experimental medium", or in "experimental medium" with polystyrene beads (4×10^8 beads/cm^3) or opsonized zymosan (0.38mg/cm^3) added. Medium samples were then collected and the amount of hexosaminidase released over this period determined using the assay described in Section 2.2.13.4.. The cells themselves were either digested in NaOH or Triton X-100 to provide samples for protein and intracellular

Figures 5.2.1. to 5.2.3.

Intracellular content and release of hexosaminidase from cells of various ages, obtained from three donors G.S.E. (Figure 5.2.1.); M.R. (Figure 5.2.2.) and J.C. (Figure 5.2.3.). Cells incubated (4 hours) in the absence (○) or presence of either polystyrene beads (●; 4 x 10^8 beads per cm^3) or opsonized zymosan (▲; 0.38mg per cm^3).

(i) protein content of cells.

(ii) hexosaminidase released.

(iii) specific activity of hexosaminidase.

Each value represents the mean of two determinations.

The exact details of these experiments are given in Section 5.3.2.

*1 unit = the release of 1μmol of 4-methylumbelliferone per hour at 37°C; pH 4.3.

Figure 5.2.1.

Intracellular content and release of hexosaminidase from cells of various ages

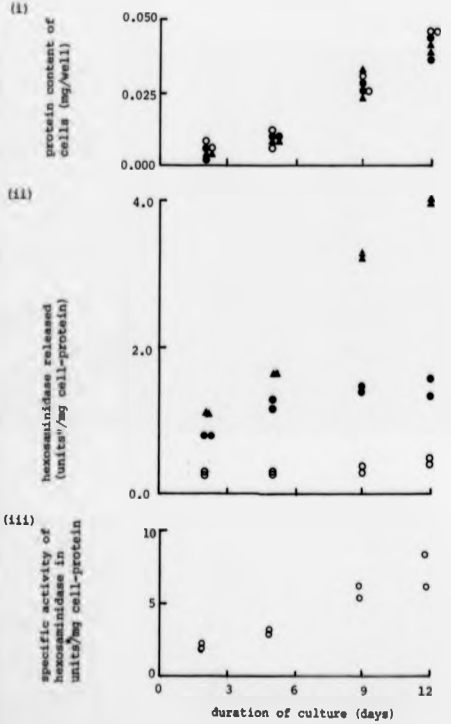


Figure 5.2.2.

Intracellular content and release of hexosaminidase from cells of various ages

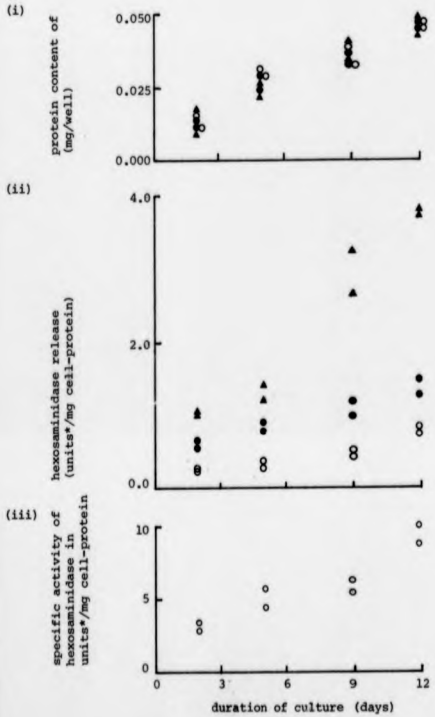
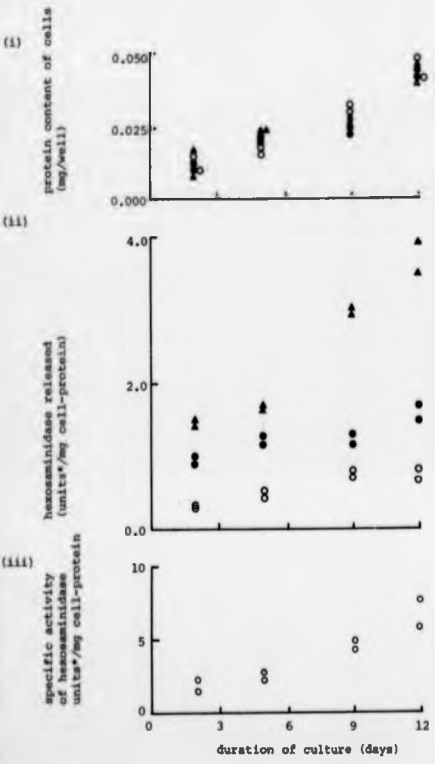


Figure 5.2.3.

Intracellular content and release of hexosaminidase from cells of various ages



Figures 5.3.1. and 5.3.2.

Intracellular content of peroxidase in monocyte/macrophages of various ages. Values expressed in units* per well (Figures 5.3.1.) or Units* per mgm cell-protein (Figure 5.3.2.). Cells obtained from three donors:-

(i) M.S.

(ii) R.D.

(iii) P.Q.

Each value represents the mean of duplicate determinations.

The exact details of these experiments are given in Section 5.3.3.

*1 unit = the decomposition of 1μmol hydrogen peroxide per minute at 25°C; pH 7.0.

Figure 5.3.1.

Intracellular content of peroxidase (units per well) of cells in various ages

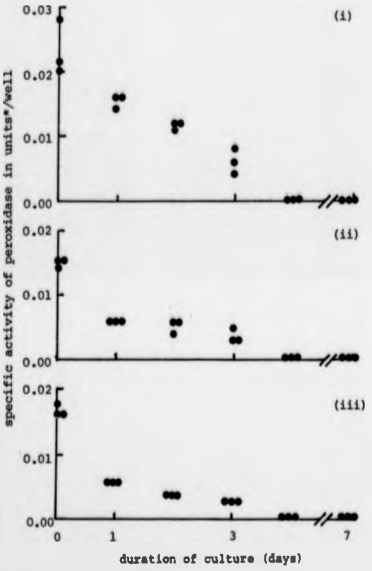
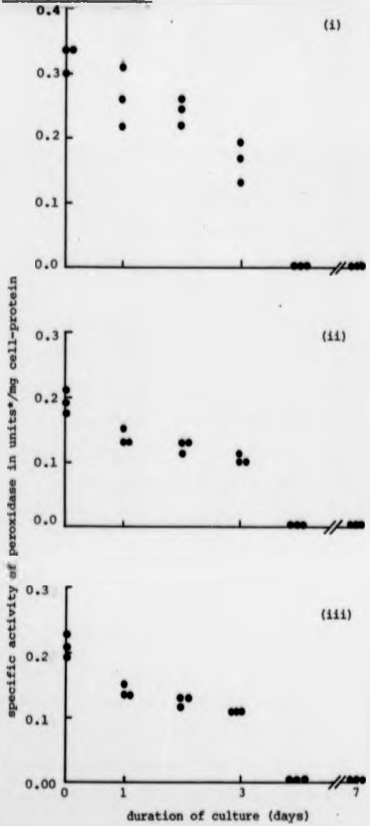


Figure 5.3.2.

Intracellular content of peroxidase (units per mg cell-protein) in cells of various ages



hexosaminidase determinations as described in Section 2.2.13.3.

Figures 5.2.1. to 5.2.3. show the results of these experiments. With all three donors it is seen that the cell-protein values progressively increased as the cells were maintained in culture, and that the presence of either opsonized zymosan or polystyrene beads did not affect the cell-protein values. In the studies presented above (Section 5.3.1.) we demonstrated a similar increase in cell protein as the cells were cultured for up to 7 days. Here it is seen that this increase continues as cells are cultured for longer periods (up to 12 days). The intracellular hexosaminidase values of unchallenged cells are seen to increase between the second and twelfth day of culture. The amount of enzyme released into the medium during the four hour incubation period slowly increased, even in the absence of particles. However, with cells incubated with either polystyrene or zymosan particles, a greater increase in the amount of enzyme released was observed between the second and fifth day of culture, followed by a very sharp increase in cells incubated with opsonized zymosan, but rather little change with those incubated with polystyrene beads.

5.3.3. Peroxidase activity in monocyte/macrophages

Blood was obtained from three donors (M.S.; R.D.; P.Q.) and cell monolayers obtained and cultured as described in Section 4.5.. At various stages of culture (Day 0, 1, 2, 3, 4 and 7) cell and medium samples were obtained as described in Section 2.2.14.1., and their peroxidase activity determined as described in Section 2.2.14.2.. Figure (5.3.1.) shows the results of these experiments. It may be seen that, for each donor, there was a decrease in peroxidase

Table 5.3.

Intracellular content of peroxidase of cells of various ages

Duration in culture (days)	specific activity of peroxidase in:- Units*/well	Units/mg cell-protein
0	0.018 ± 0.001	0.24 ± 0.02
1	0.009 ± 0.001	0.18 ± 0.02
2	0.007 ± 0.001	0.16 ± 0.02
3	0.004 ± 0.005	0.13 ± 0.01
4	0.000	0.00
7	0.000	0.00

Each value represents the mean ± standard error of nine determinations (triplicate wells for each of three donors).

The exact details of these experiments are given in Section 5.3.3.

*1 unit = the decomposition of 1μmol hydrogen peroxide per minute at 25°C; pH 7.0.

activity per well as the cells matured. The enzyme was undetectable by Day 4. There is seen to be little variation between triplicate wells from the same donor on any particular day; also there is little donor-to-donor variation, donor M.S. giving slightly higher enzyme values over the first few days.

In view of the decrease in cell-protein content per well previously observed over the first few days in culture (Section 5.3.1.), it is particularly important to express these data as peroxidase activity per mg protein, and this is shown in Figure 5.3.2.. It may be seen that there is not only a decrease in the activity per well but also in the specific activity. Table 5.3. shows the mean (± S.E.) peroxidase values for the three donors. No peroxidase activity was found in any of the medium samples.

5.3.4. Lactate dehydrogenase activity in, and release from, monocyte/macrophages

For these experiments monolayers of cells were obtained in the usual way from 6 donors (G.S.; R.D.; L.S.; J.B.L.; P.Q.; P.R.). With cells cultured for various periods up to seven days, samples of both medium and cells were obtained as described in Section 2.2.15.1.. Samples were assayed for lactate dehydrogenase activity as described in Section 2.2.15.2.. In these experiments, although duplicate determinations were performed, samples were taken only from single rather than duplicate wells; the reason was that some of the cells obtained from these donors were used for uptake experiments, the results of which are presented below (Section 6.3.4.).

Figures 5.4.1. to 5.4.6.

Intracellular content and release of lactate dehydrogenase from cells of various ages, obtained from six donors G.S. (Figure 5.4.1.); R.D. (Figure 5.4.2.); L.S. (Figure 5.4.3.); J.B.L. (Figure 5.4.4.); P.Q. (Figure 5.4.5.) and P.R. (Figure 5.4.6.).

(i) protein content of cells.
(ii) specific activity of lactate dehydrogenase.
(iii) lactate dehydrogenase released (over 4 hours) into the medium.

Each value represents the mean of two determinations.

The exact details of these experiments are given in Section 5.3.4.

*1 unit = the release of 1μmol of NAD per hour at 37°C; pH 7.6.

Figure 5.4.1.

Intracellular content and release of lactate dehydrogenase from cells of various ages

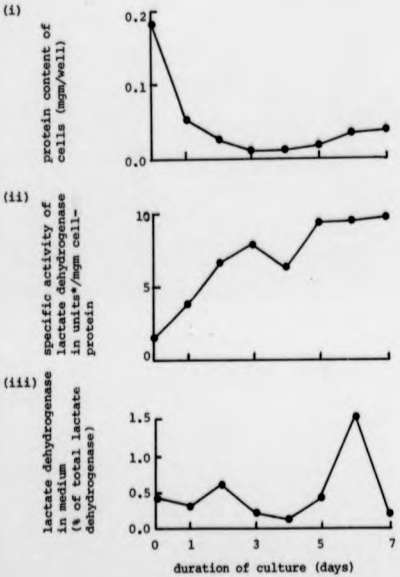


Figure 5.4.2.

Intracellular content and release of lactate dehydrogenase from cells of various ages

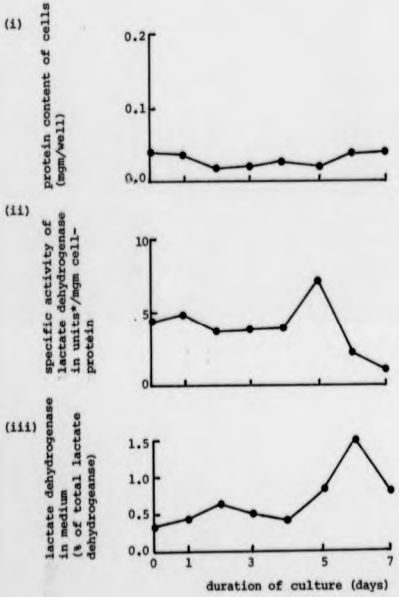


Figure 5.4.3.

Intracellular content and release of lactate dehydrogenase from cells of various ages

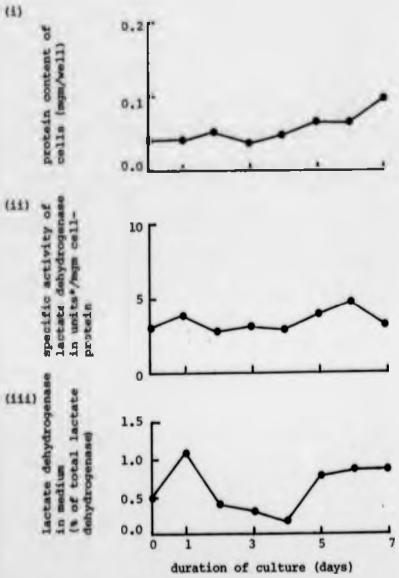


Figure 5.4.4.

Intracellular content and release of lactate dehydrogenase from cells of various ages

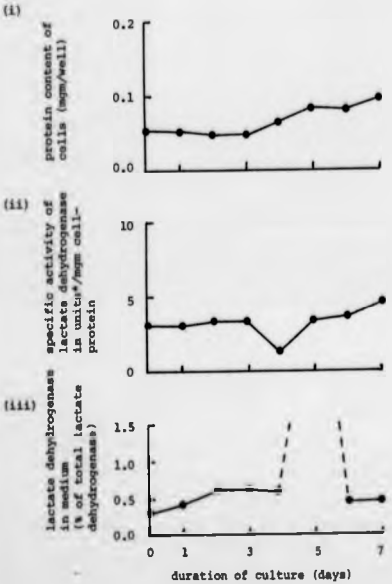


Figure 5.4.5.

Intracellular content and release of lactate dehydrogenase from cells of various ages

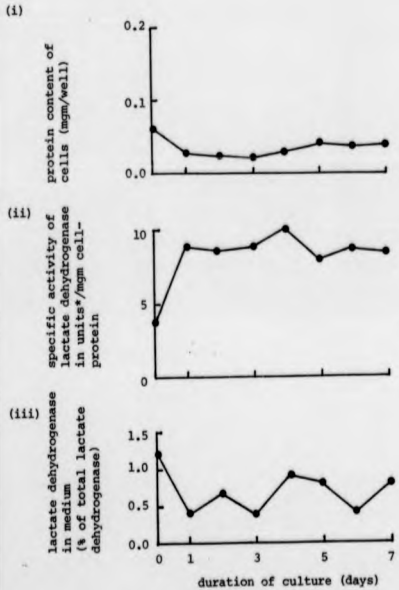
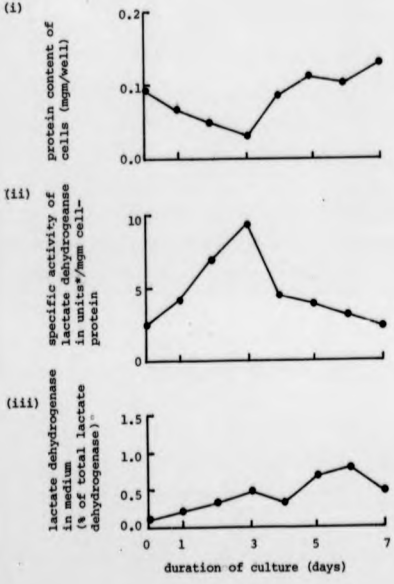


Figure 5.4.6.

Intracellular content and release of lactate dehydrogenase from cells of various ages



Figures (5.4.1. to 5.4.6.) show the results of these experiments. Firstly, it is seen that generally, the cell-protein values initially fell, then rose as the cells were maintained in culture. The specfic activity of the ensyme is seen to rise quite dramatically up to the third or fourth day in culture for three of the donors (G.S.; R.D.; L.S.), after which the pattern was variable. With the other three donors no clear pattern was found. The amount of lactate dehydrogenase released into serum-free medium over the four-hour period is seen to be usually less than one percent of the total ensyme activity. In one well though the value was very high (approximately 11 percent); this rather odd result is thought to be due to a pipetting error.

5.4. DISCUSSION

In this section we discuss the value of the various "maturation markers" we employed to ascertain monocyte-to-macrophage transformation, and also the importance and reasons for having to use several of these parameters.

5.4.1. Relationship between cell-protein, DNA and cell number for monocyte/macrophages

One of the most widely used "maturation markers" is an increase in the protein content per cell (Section 5.1.1.). In monocyte/macrophages cultured using the definitive cell-culture system described in Section 4.5., we often found a close correlation between cell number and cell-protein over the first few days of growth. Both parameters decreased usually by the second day in culture to approximately half their initial values, after which the

cell numbers remained fairly constant, whilst the protein values started to increase. This represents an increase in the protein content per cell beyond Day 3 to 4. Our results are in close agreement with those of Johnson et al. (1977), who reported the increase in protein per cell to occur around the fourth day in culture.

Several authors do not report an initial lag-period before the increase in the protein content of cells, but in several of these studies the cells were not examined between the first and third day of culture (Nakagawara et al., 1981; O'Dorisio et al., 1984). However Knight & Soutar (1982) and a few other workers, do positively report an early increase in the amount of protein per cell. Differences in the time of onset of this parameter are probably due to differences in culture conditions: for example in the study by Knight & Soutar (1982), cells were maintained in autologous serum, and in our culture system horse serum and fetal calf serum were employed. We calculated that monocytes (Day-0 cells) contained approximately 50pg of protein per cell and the older Day-7 cells more than double this amount. Johnson et al. (1977) reported that the young (Day-0) cells contained slightly higher amounts of cell-protein (92pg per cell) than we found. Although they used the same assay as we used, egg lysozyme was used as the protein standard, rather than bovine serum albumin, and it was not clear how well the cells were washed (to remove serum protein) prior to the assay, (in our system cells were washed six times). Both of these factors may have contributed to the slightly higher protein values reported by Johnson et al. (1977). In the study by Knight & Soutar (1982) the DNA content of cells was used to indicate the number of cells. It is generally accepted that with monocyte/macrophages there is a close

correlation between cell number and DNA content. In our cell system we were able to demonstrate this, for the pattern of change seen for the amount of DNA per well during cell culture was very similar to the change observed in the cell numbers. Because of the close relationship between cell number and DNA, we also find an increase in the protein-to-DNA ratio, as cells mature in culture: this ratio doubled over the first week in culture. Knight & Soutar (1982) also reported a similar increase in protein-to-DNA ratio over this period (approximately 3-fold).

In conclusion, monocytes cultured in our cell-system, were found to have increased amounts of protein per cell with time in culture (usually occurring around the fourth day) a phenomenon recognised as one of the commonly used markers of cell maturation. In addition, cells were also found to demonstrate an increase in their protein-to-DNA ratio during culture, a further but less commonly used "maturation marker".

The first of these parameters is very valuable for monitoring cell maturation, as the procedures employed for protein and cell number determinations are technically easy and are often already in use in the standard laboratory. Where Coulter machines are not available, counts can be made using a standard counting chamber. It is important to remember that one cannot simply measure cell-protein and use this as a "maturation marker" without assessing cell numbers, for it is highly probable that in some cases where excessive cell-loss occurs a decrease in cell-protein could be found as cells matured, even though the amount of protein per cell was increasing. It is known that cells from some donors are not as adherent as others, and also the procedure for changing half of the medium on Day 4 could

easily result in cell loss as a number of cells are known to be in suspension. Nevertheless wherever an increase in cell-protein is seen, this is an indication of cell maturation. The second marker, i.e. an increase in protein/DNA ratio, although giving similar information to assessing protein per cell, involves a very time-consuming assay for DNA, and therefore the former marker was considered more valuable. The next "maturation marker" discussed is also very time-consuming but, as we shall see, provides a good piece of evidence for cell differentiation.

5.4.2. Stimulated and unstimulated release of lysosomal enzymes from monocyte/macrophages

As described in Section 5.1.2., one commonly used marker which helps to demonstrate monocyte-to-macrophage maturation is the greatly stimulated release of lysosomal enzymes in response to opsonized particles, which is observed in macrophages but not monocytes. In our cell-system we demonstrated this for cells cultured beyond the fifth day. These "older" cells released far more of the lysosomal enzyme hexosaminidase than the "younger" cells, in response to opsonized zymosan. In addition, we showed that whilst the "younger" cells released similar amounts of enzyme when challenged with unopsonized polystyrene, the older cells did not show the marked increase in secretion seen with opsonized zymosan. It still remains unclear why cells respond differently to the two types of particle. Since similar increases were not seen when no phagocytic challenge was present, it was concluded that the observed increases were not merely due to the concomitant increase in intracellular enzyme activity that we also observed as the cells matured.

This marked increase in degranulation observed after the fifth day in culture when cells were challenged with opsonized zymosan (and not when cells were challenged with polystyrene or unchallenged) occurred at the same time as in previously reported studies (Knight & Soutar, 1982; Musson et al., 1980). The extent of the increased secretion observed (in response to opsonized zymosan) between the second and twelfth day of culture, approximately two-fold, was the same as the increase observed by Knight & Soutar (1982). In contrast, Musson et al. (1980) reported a greater than ten-fold increase over this period. It was not clear why Musson et al. (1979) reported greater increases for in all these experiments; very similar concentrations of zymosan and latex were employed, and similar numbers of cells, excluding the possibility that the particle-to-cell ratio was causing the differences. It seems probable that the differences were due to variation in the donors used, the cell isolation methods, and the culture conditions.

We were confident that the assay used to estimate hexosaminidase was trustworthy, for not only were duplicate values close, but the intracellular enzyme values we obtained (Day-2 cells 2.23 ± 0.32 units/mg protein and Day-9 cells 5.48 ± 0.27), compared very favourably with those previously reported. Epstein et al. (1981) using a similar fluorimetric assay reported for Day-3 cells 1.7 ± 0.09 units/mg protein, and for Day-7 cells 4.7 ± 7.4, (Day-9 cells were not studied); and Knight & Soutar (1982), using a spectrophotometric assay reported for Day-2 cells approximately 2 units/mg protein and for Day-9 cells approximately 5. Musson et al. (1980) using a spectrophotometric assay did not report intracellular levels directly, but rather the total culture content of the enzyme. However, since the proportion of enzyme release was also given, it is

possible to estimate the intracellular levels. If expressed in terms of cell-protein for Day-2 and -7 cells, these values are approximately 4000 and 48;000 units/mg protein. Although these values are only rough approximations, they are vastly greater than the values we obtained and those reported by Epstein et al. (1981) and Knight & Scutar (1982). It was not clear why these values were so high, but it is worth noting that Musson et al. (1980) have also reported much higher protein values for their cultures, a factor they attributed to differences in culture conditions.

In conclusion, this experiment proved useful, for we were able to demonstrate cell maturation on two grounds: firstly by the observed dramatic increase in lysosomal enzyme secretion in response to opsonized zymosan observed after the fifth day in culture, and secondly by the increase in intracellular levels of the enzyme observed during cell-culture. In addition we were also able to add to the previous evidence for an increase in cell-protein reporting here a continued increase when cells were cultured beyond seven days. Although this procedure is rather time-consuming, it proved to be useful as a maturation marker.

5.4.3. Peroxidase activity in monocyte/macrophages

In addition to the markers of cell maturation just described, we were able to demonstrate a loss in peroxidase activity. This enzyme was progressively lost as the cells were maintained in culture, so that cells cultured beyond the third day contained no peroxidase activity. It was clear that the enzyme was not being secreted by the cells for we could not detect its presence in medium samples. Our findings were in close agreement with those described by Johnson et al.

(1977) who reported a progressive decrease in peroxidase activity, but an absence of peroxidase in the media, the disappearance of the enzyme on Day 4 (detected by a biochemical procedure) paralleling the disappearance of cytochemically discernible peroxidase-positive granules in the cells. A quantitative comparison of results was not possible, for Johnson et al. (1977) expressed their results in relation to a standard, rather than in units of activity. Unlike myself and Johnson et al. (1977), Haneberg et al. (1984) noted no change in peroxidase activity in human monocyte suspension cultures over their first 24 hours in culture. These results were simply expressed in terms of the change in absorbance per minute, therefore excluding a direct comparison with my results. Haneberg et al. (1984) also reported peroxidase activity in the "medium samples" (up to 10 percent of the total activity). This seems to be due to a high incidence of cell death, as indicated by an observed high release of LDH (10 percent of the total activity) observed at the end of the 24 hour culture period. The high cell death rate indicated may have been due to the lack of serum in these cultures. Peroxidase may have been present in small quantities in our "medium samples", but was not within the detection limits of the assay procedure.

In conclusion, peroxidase was not detected in cultures over 4 days old, strong evidence that the cells were maturing into macrophages. The loss of peroxidase activity in maturing monocytes is therefore a simple and useful marker of cell maturation.

Goren (1977) thought it odd that the macrophage, generally thought to be the major functional phagocytic cell in the mononuclear phagocyte system, does not usually possess peroxidase, a major antimicrobial component. This he termed the "peroxidase paradox". Although it is

generally accepted that the mature macrophage loses its peroxidase activity, a few exceptions exist. These include resident macrophages (in species other than human) and also some tumoricidal macrophages. Goren (1977) in his review discusses evidence indicating that these "peroxidase-positive" resident macrophages may reacquire peroxidase from short-lived granulocytes, which they scavenge at sites of inflammation, and also points out that the peroxidase-positive tumoricidal macrophages may simply be recently elicited monocytes (i.e. cells which have not already lost their peroxidase activity).

5.4.4. Lactate dehydrogenase activity and release from monocyte/macrophages

Unlike the frequently reported decreases in peroxidase activity during monocyte-to-macrophage maturation, the change in intracellular lactate dehydrogenase activity over this period, has to my knowledge only been reported by one group. O'Dorisio et al. (1984) reported an increase in this enzyme's activity over a 14-day culture period from 0.015 ± 0.007 to 0.318 ± 0.097 units per 10^6 cells (where one unit is equivalent to one μmol NADH reduced per minute). By expressing these values in terms of cell-protein (based on our earlier data for cell-protein and cell numbers in Section 5.3.1.), it is clear that the intracellular activity we found in freshly isolated cells was of the same order of magnitude. However, unlike O'Dorisio et al. (1984), we saw no clear pattern in the enzyme's activity as the cells were maintained in culture. The reason for this difference was not obvious, but it may have been that the dramatic increase reported by O'Dorisio et al. (1984) occurred with cells cultured for longer periods than 7 days, for they only gave values for Day-0 and Day-14 cells.

In our studies, very little enzyme was released into the culture medium over a four-hour period (usually less than 1 percent); this indicates little cell lysis. The small amount of enzyme released over four-hour periods was in agreement with the results of Haneberg et al. (1984), who reported that less than 2 percent of the total enzyme was released by Day-0 cells over a similar period.

Although measuring lactate dehydrogenase activity in monocyte/macrophages did not prove useful as an aid in demonstrating cell maturation, we were not perturbed, for the previous study of O'Dorisio et al. (1984) in which an increase was apparent, was not a thorough study, with no intermediate time-points between Day 0 and Day 14 investigated. It also has to be said that our results would have been more valuable if we had been able to study cells in duplicate rather than single wells. However, this work did prove useful for checking cell viability, for we could now be confident, from the low levels of lactate dehydrogenase in the medium, that the majority of the adherent cells were viable.

5.4.5. Concluding comments

Cells maintained in the culture system described in Section 4.5., demonstrated a number of properties that indicated that they were differentiating in vitro. These included an increase in cell-protein, in the protein-to-DNA ratio, in intracellular hexosaminidase activity, and a dramatic increase in hexosaminidase secretion in response to opsonized zymosan; also a decrease in peroxidase activity. In addition our preliminary studies (Chapter 3) included evidence of cell-maturation from morphological and cytochemical (non-specific esterase) criteria. We were therefore

confident that the culture conditions were favourable for the transformation of monocytes into macrophages.

This extensive work was considered very necessary and valuable for, in a study of this nature, where cells are being investigated at various stages of maturation, it is obviously of great importance to establish that the cells are indeed differentiating. Ideally during the course of the study we would have checked cell-maturation for every donor's cells used, but clearly this was impracticable because of limited cell numbers and the need to check more than one maturation marker. Therefore, to give an indication of cell-differentiation, we always examined cells for morphological evidence of maturation.

A further consideration, when using differentiating cells, is the rate at which cells mature. In our studies the disappearance of peroxidase activity occurred at exactly the same time in culture for each of the donors' cells. This was also the case with three further donors' cells for the occurrence of the dramatic increase in hexosaminidase secretion in response to opsonized zymosan. The onset of the increase in protein-per-cell differed between the donors' cells, and this was thought to indicate that these cells were differentiating at different rates. It was also apparent that (unlike the other markers), the extent of the change in cell-protein differed. This may have either been due to donor-dependent variation or due to different ratios of subpopulations being present in the populations, a point discussed in Chapter 9.

In conclusion, although we would not be able to guarantee cell-differentiation, we have shown that the culture conditions to be

used reliably lead to cell-differentiation, as made evident by a number of accepted "maturation markers".

CHAPTER 6

Definitive experiments on the uptake of macromolecules by maturing monocytes

6.1. INTRODUCTION

Having established a reliable *in vitro* culture system suitable for the quantitative study of pinocytosis in monocyte/macrophages (Chapter 4), and having demonstrated that in this system monocytes differentiate into macrophages (Chapter 5), we could now proceed to investigate the uptake of various macromolecules during this maturation period. In the next section, the reasons for choosing the different substrates are given.

6.1.1. ^{125}I-Labelled polyvinylpyrrolidone

The first experiment planned was to reproduce the experiment reported by Knight & Soutar (1982), described in Section 1.1.4., in which monocyte/macrophages of various ages were incubated with very high concentrations of ^{125}I-labelled polyvinylpyrrolidone (without suramin) for short periods. This would allow a direct comparison to be made with their study.

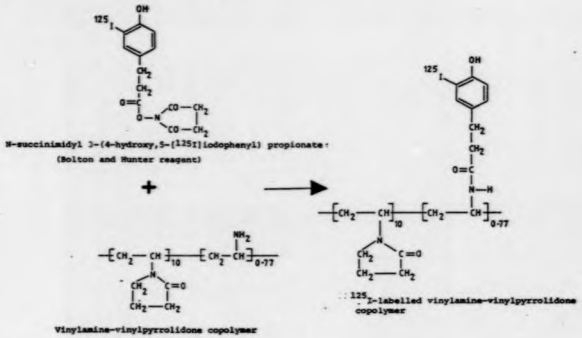
6.1.2. ^{125}I-Labelled polyvinylpyrrolidone (with suramin)

The reasons for studying the uptake of ^{125}I-labelled polyvinylpyrrolidone enhanced by the inclusion of the anion suramin (via piggy-back-endocytosis) have already been outlined in Section 4.1.2..

It was also decided to investigate whether varying the concentration of suramin affected the clearance of ^{125}I-labelled polyvinylpyrrolidone or the protein content of the cells. In the *in vitro* study described in Section 4.1.4., Pratten & Lloyd (1983),

Figure 6.1.

The probable reaction of the copolymer with the Bolton and Hunter reagent



using rat peritoneal macrophages, reported a suramin concentration-dependant increase in the rate of uptake of ^{125}I-labelled polyvinylpyrrolidone: a steady increase was seen up to 250$\mu g/cm^3$, with a more rapid increase above this concentration. With other tissues no stimulation of uptake was seen. For example, Freeman & Lloyd (1986) showed that with yolk-sacs of rat conceptuses (harvested at 9.5 days of gestation), suramin below 100$\mu g/cm^3$ had no effect on the uptake of ^{125}I-labelled polyvinylpyrrolidone, and at higher concentrations (above 500$\mu g/cm^3$) a reduction was found. In addition, suramin above 5mg/cm^3 decreased the total protein content of these yolk-sacs, an effect attributed to inhibition of protein uptake.

6.1.3. ^{125}I-Labelled vinylamine-vinylpyrrolidone copolymer

As described in Section 1.1.2.2., various cations (e.g. the basic H_4 isoenzyme of lactate dehydrogenase, and vinylamine-vinylpyrrolidone copolymer), are cleared by rat peritoneal macrophages more effectively than their negatively or uncharged counterparts (Kooistra et al., 1981; Pratten et al., 1982). The vinylamine vinylpyrrolidone copolymer has the structure shown in Figure 6.1.. It was chosen for our study, since it is very similar in structure to polyvinylpyrrolidone, but with some cationic character.

In the study of Pratten et al. (1982), two sizes of the copolymer were used (average mol. wt. 46,000 and 120,000), which were each radiolabelled by two methods, the Bolton and Hunter reagent, which eliminates some of the positive charge of the copolymer, and methyl 3,5-di [^{125}I]iodohydroxybensimidate, which preserves all the positive charge. The labelling efficiencies achieved with the former reagent were greater than the latter (i.e. approximately 15 percent v 3

percent). Both sizes of copolymer were taken up by rat peritoneal macrophages at higher rates than ^{125}I-labelled polyvinylpyrrolidone itself, the polymers labelled using methyl 3,5-di [^{125}I]iodohydroxybenzimidate at rather higher rates than those labelled using the Bolton & Hunter reagent. Also, the larger polymers were captured at slightly higher rates than the smaller ones.

In the present experiments the larger copolymer was chosen and it was labelled using the Bolton & Hunter reagent.

6.1.4. Formaldehyde-treated ^{125}I-labelled bovine serum albumin

It is thought that hydrophobicity in macromolecules increases their affinity for various cell membranes. As described in Section 1.1.2.2., Kooistra et al. (1981) reported that rat peritoneal macrophages in vitro pinocytose formaldehyde-denatured bovine serum albumin (fBSA) at much higher rates (i.e. approximately 5x) than native BSA, and similar findings have been reported for other cells, such as the 17.5 day rat visceral yolk-sac (Moore et al., 1977). These increases are generally thought to be due to the formaldehyde treatment changing the tertiary structure of the protein to expose more hydrophobic sites. It was therefore decided to investigate the clearance of fBSA by human monocyte/macrophages. However, since fBSA is rapidly degraded in the lysosomal system of cells, it would be necessary, as described in Section 4.1.1., to measure degradation products released back into the medium as well as cell accumulation, in order to determine the Endocytic Index of this substrate.

6.1.5. Aims

The main aim of these experiments was to examine the rates at which a number of substrates (differing in their physical properties) were captured by human monocytes at various stages of their differentiation.

6.2. EXPERIMENTAL METHODS

6.2.1. Preparation of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer

N-Succinimidyl 3-(4-hydroxy 5-[^{125}I]iodophenyl)propionate (Bolton & Hunter reagent; supplied by Amersham Radiochemical Centre, Bucks., U.K.) was used to radiolabel the vinylpyrollidone-vinylamine copolymer (M.W. 120,000; donated by Professor H. Ringsdorf, University of Mainz), as described by Pratten et al. (1982). This conjugation labelling method had been developed by Bolton & Hunter (1972) for the iodination of polypeptides. Figure 6.1. shows the probable reaction of the copolymer with the Bolton & Hunter reagent, based on the known mechanism of reaction with proteins. The N-succinimidyl group is displaced by free amino groups to form a conjugate in which a radioiodinated phenyl group is covalently linked via an amide bond to the substrate.

For the reaction, Bolton and Hunter reagent (100µl; 0.5mCi) was placed in a 25cm^3 universal container, and the benzene solvent was completely evaporated by applying a very gentle stream of nitrogen over the surface. The copolymer (2.0mg) in cold (4°C) sodium borate buffer (1.0cm^3; 0.1 moles/litre; pH 8.5) was added and the mixture

was agitated gently (30 minutes at 4°C). Glycine (0.2 moles/litre) in borate buffer (4.0cm^3) was added, to conjugate any unreacted ester, and the mixture agitated for a further 5 minutes. The solution was then dialysed at room temperature against sodium chloride (1 percent w/v; 5 litres) for 3 to 4 days with twice-daily changes of the sodium chloride solution.

The preparations were stored at 4°C. The amount of free [^{125}I]iodide in the samples was determined by electrophoresis as described in Section 2.2.16.. The test was performed immediately after the dialysis and prior to use. In addition, to ensure that the experimental conditions did not affect the stability of the radiolabelled copolymer, the test for free [^{125}I]iodide was performed following incubation of the sample in culture medium (24 hours, 37°C).

6.2.2. Uptake of radiolabelled substrates by monocyte/macrophages

Usually the general procedure described in Section 4.5. was used to detect the uptake of the radiolabelled substrates into the cells. The exact details of the experiments are given with the results.

In the experiment to repeat that of Knight & Soutar (1982), prior to incubations with the radiolabelled substrate, cells were washed twice with medium 199 (prewarmed; 1cm^3) and, after the final wash 0.5cm^3 of lipoprotein-free medium (prewarmed medium199 containing bovine serum albumin; 5mg/cm^3) was added to each well. ^{125}I-Labelled polyvinylpyrrolidone was added in the standard way. Incubations were terminated by the usual procedure, but with seven 1.0cm^3 changes of sodium chloride (0.15 moles/litre in Tris/HCl buffer 50

mmoles/litre; pH 7.4; containing albumin 2mg $/cm^3$, followed by a further wash in albumin-free buffer. Cells were solubilized in NaOH (0.5 moles/litre; 0.5cm^3) and assayed for protein and radioactivity in the usual way (see Section 4.5.).

6.2.3. Uptake of ^{125}I-labelled fBSA

With the digestible substrate ^{125}I-labelled fBSA, in addition to determining the radioactivity in the medium and associated with the cells in the usual way (Section 4.5.), it was also necessary to quantify the trichloro-acetic acid(TCA)-soluble radioactive degradation products released back into the medium. The sum of the latter and the cell-associated radioactivity provide a measure of the total uptake.

For this additional step, serum (0.1cm^3) and TCA (20 percent; 0.5cm^3) were added to the medium samples (for which the radioactivity had already been determined), the solutions were then mixed and centrifuged (600g; 30 minutes). Then the supernatant was carefully poured into a fresh Luckhams tube and its radioactivity determined. Counts were corrected by a factor evaluated to account for the loss of acid-soluble radioactivity retained within the pellet after the centrifugation and decantation procedures. This acid precipitation procedure was also carried out for medium samples from cell-free wells (wells containing just culture medium only) to see whether the culture medium itself contained any proteolytic components.

6.3. RESULTS

6.3.1. Characteristics and stability of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer

The vinylamine-vinylpyrrolidone copolymer was labelled on two occasions: on the first the labelling efficiency was 32 percent and on the second 60 percent. After dialysis the preparations contained less than 1 percent free [^{125}I]iodide. The preparations were stable: on storage at 4°C (up to 3 months) or incubation in culture medium (37°C; 24 hours), no increase in the percent of free [^{125}I]iodide was seen.

6.3.2. Uptake of ^{125}I-labelled polyvinylpyrrolidone by cells of various ages

In these experiments the procedure adopted was as similar as possible to that described by Knight & Soutar (1982). This procedure is described in Section 6.2.2., and differed from the definitive uptake method established in Chapter 4 in that cells were pre-washed in medium 199 prior to the incubation periods; during the incubations, cells were maintained in a smaller volume of lipoprotein-free culture medium containing ^{125}I-labelled polyvinylpyrrolidone (rather than the usual 1.0cm^3 of serum-containing medium); and at the end of the experiment cells were washed more extensively in ice-cold albumin-containing buffer. In addition a parallel experiment was performed in which cells were treated in the standard way, as described in Section 4.5, except that, during the incubations, cells were maintained in 0.5cm^3 of standard culture medium. With both protocols, owing to limited cell numbers, incubations with

Figures 6.2.1. to 6.2.3.

Uptake of ^{125}I-labelled polyvinypyrrolidone (220µg/cm^3) over 3 hours by cells at various stages of maturation, obtained from three donors G.W. (Figure 6.2.1.); R.D. (Figure 6.2.2.) and G.S. (Figure 6.2.3.). Cells washed as described by Knight & Soutar (1982) (●), cells washed in the standard way (○).

(i) protein content of cells.

(ii) cell-associated radioactivity.

(----) double the amount of radioactivity adsorbing to cell-free wells.

(iii) clearance.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 6.3.2.

Figure 6.2.1.

Uptake of ^{125}I-labelled polyvinylpyrrolidone by cells at various stages of maturation

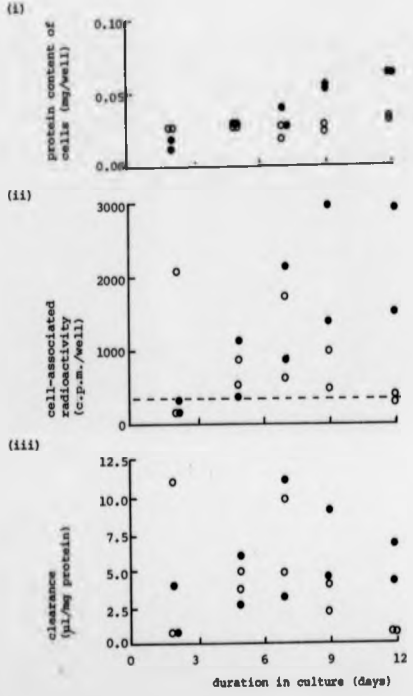


Figure 6.2.2.

Uptake of ^{125}I-labelled polyvinylpyrrolidone by cells at various stages of maturation

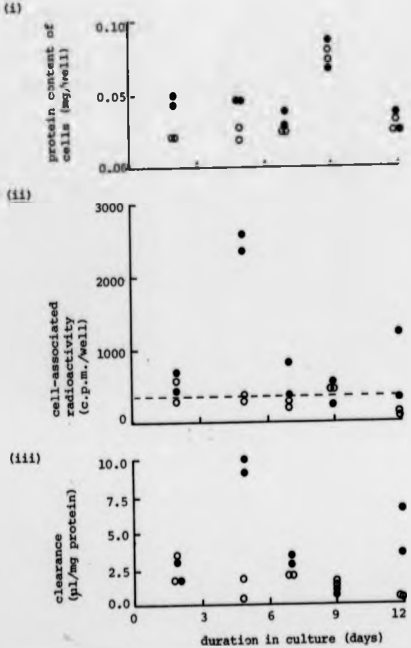
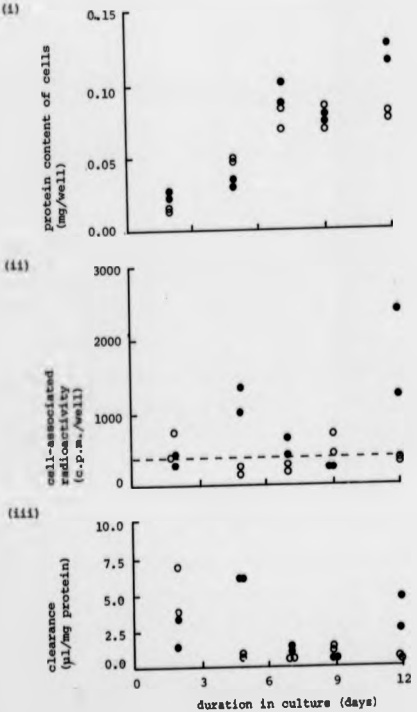


Figure 6.2.3.

Uptake of ^{125}I-labelled polyvinylpyrrolidone by cells at various stages of maturation



^{125}I-labelled polyvinylpyrrolidone were for 3-hour periods only.

For these experiments cells were isolated from the blood of three donors (G.W., R.D., G.S.) and monolayers prepared in 24-well Linbro plates ($3x10^6$ cells seeded/well) as described in Section 4.5. Cells were used for the uptake experiments described above on Days 2, 5, 7, 9 and 12 of culture, each age of cell being maintained in an individual plate.

Cells were incubated for three-hour periods, with ^{125}I-labelled polyvinylpyrrolidone ($0.22mg/cm^3$) in either $0.5cm^3$ of lipoprotein-free medium or $0.5cm^3$ of standard culture medium. Incubations were terminated either in the standard way, or using the procedure adopted by Knight & Soutar (1982) described in Section 6.2.2..

The results of these experiments are shown in Figures 6.2.1. to 6.2.3.. For two of the donors (G.W., G.S.) the protein content of the cells increased along with the age of the cell, but with the third donor (R.D.) no progressive increase was seen (see Section 5.4.1. for a discussion of this phenomenon). No clear age-related pattern can be seen in the cell-associated radioactivity values, whether expressed as radioactivity per well or as clearances; moreover many of the values for radioactivity per well were below double the mean amount of radioactivity associated with cell-free wells. The range of values for this cell-free well radioactivity was quite broad, varying from well to well, and therefore it was considered inappropriate, in this and other experiments, to subtract this value from the cell-associated radioactivity values. No consistent differences were seen between the results using cells treated in the

standard way and those handled according to the method used by Knight and Soutar (1982).

Concluding that uptake of ^{125}I-labelled polyvinylpyrrolidone in the absence of suramin is technically difficult over a short time-interval, owing to low uptake, we turned next to experiments conducted in its presence. We also decided initially to study cells of one age only but to conduct incubations over several time-periods, so as to be able to detect progressive uptake of radiolabel.

6.3.3. Uptake of ^{125}I-labelled polyvinylpyrrolidone (with various concentrations of suramin) by Day-9 cells

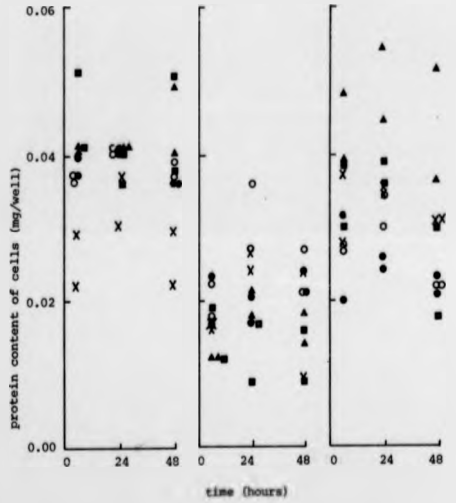
As described in Section 6.1.2., it was of interest to see what effect different concentrations of suramin had on the uptake of ^{125}I-labelled polyvinylpyrrolidone. For these experiments, it was, decided to use Day-9 cells, as in the initial investigations described in Chapter 4, the most reliable uptake data had been obtained using older cells. We also decided to measure uptake over a longer (48h) period, and to use donors (G.S.; A.B.; P.M.) whose cells had previously yielded high uptake values.

Cells were obtained and monolayers established in the usual way (Section 4.5.). Commencing on the ninth day of culture, incubations with ^{125}I-labelled polyvinylpyrrolidone in the presence of different concentrations of suramin were started as described in Section 4.5.. This involved the removal of medium (0.1cm^3) and the addition of ^{125}I-labelled polyvinylpyrrolidone (prewarmed, 0.1cm^3) made up in culture medium containing suramin, to give final concentrations in the wells of 50μg/cm^3 and 0 to 1000μg/cm^3 respectively. Substrate was

Figure 6.3.1.

Protein content of Day-9 cells obtained from three donors (i) G.S.; (ii) A.B. and (iii) P.M., following incubations with ^{125}I-labelled polyvinylpyrrolidone in the absence (O) or presence of suramin at various concentrations (●) 50μg/cm^3; (▲) 250μg/cm^3; (■) 500μg/cm^3; (✕) 1000μg/cm^3.

Each point represents the value obtained from a single well details of these experiments are given in Section 6.3.3.



Figures 6.3.2. to 6.3.4.

Clearance of ^{125}I-labelled polyvinylpyrrolidone (50μg/cm^3) by Day-9 cells obtained from three donors G.S. (Figure 6.3.2.); A.B. (Figure 6.3.3.) and P.H. (Figure 6.3.4.) in the absence (O) or presence of suramin at various concentrations (●) 50μg/cm^3; (▲) 250μg/cm^3; (■) 500μg/cm^3; (X) 1000μg/cm^3.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 6.3.3.

Figure 6.3.2.

Clearance of ^{125}I-labelled polyvinylpyrrolidone by Day-9 cells in the absence or presence of various concentrations of suramin

Figure 6.3.3.

Clearance of ^{125}I-labelled polyvinylpyrrolidone by Day-9 cells in the absence or presence of various concentrations of suramin

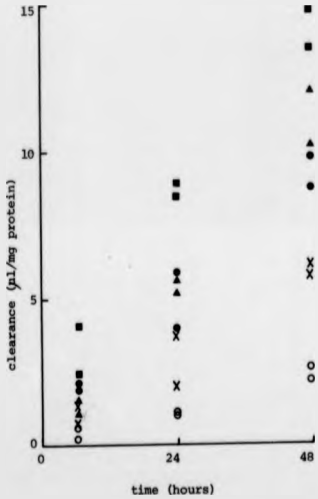


Figure 6.3.4.

Clearance of ^{125}I-labelled polyvinylpyrrolidone by Day-9 cells in the absence or presence of various concentrations of suramin

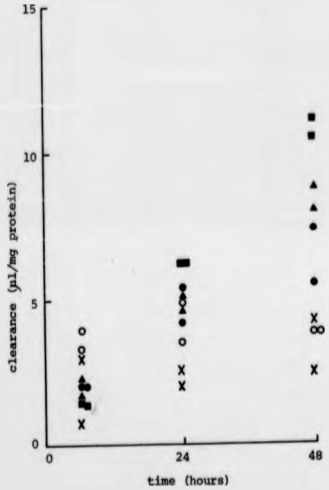


Table 6.1.

Endocytic Indices for the clearance of ^{125}I-labelled polyvinylpyrrolidone by Day-9 cells in the absence or presence of various concentrations of suramin

Donors	concentrations of suramin(μg/cm^3)				
	No suramin (control)	50	250	500	1000
G.S.	0.242 (100%)	0.436 (180%)	0.468 (193%)	0.561 (232%)	0.113 (47%)
A.B.	0.047 (100%)	0.171 (364%)	0.231 (491%)	0.257 (547%)	0.118 (251%)
P.H.	*	*	0.150	0.221	*

The figures in brackets are the values expressed as a percentage of the matched control (no suramin).
The units of Endocytic Index (see Section 4.2.1.) are μl per mg. protein per hour.

* = uptake non-linear

therefore calculation of Endocytic Index not valid.

The exact details of these experiments are given in Section 6.3.3.

added in this way at 48, 24 or 3 hours prior to the termination of all incubations on Day 11. Incubations were terminated as described in Section 4.5..

Figures 6.3.1. to 6.3.4. show the results of these experiments. It is seen that duplicate values were usually in reasonable agreement. For two donors (A.B., P.H.), no correlation was seen between the cell-protein content of the wells and the amount of suramin present. However, with donor G.S. most of the wells incubated with the highest concentration of suramin (1000$\mu g/cm^3$), had the lowest cell-protein values. In all cases the cell-associated radioactivity values (not shown), were greater than double the amount associated to cell free wells. In most experiments the clearance values progressively increased with incubation time usually in an apparently linear fashion. It was therefore possible to calculate Endocytic Indices (Table 6.1.) in all but a few cases.

With each donor's cells, the rate of clearance of ^{125}I-labelled polyvinylpyrrolidone increased as the suramin concentration increased, up to 500$\mu g/cm^3$. For the two donors where it was possible to calculate Endocytic Indices for ^{125}I-labelled polyvinylpyrrolidone (in the absence of suramin) it was seen that the magnitude of the increase was 2 to 6 fold when suramin (500$\mu g/cm^3$) was added. With the highest concentration of suramin (1000$\mu g/cm^3$), no further increase was observed in the uptake rate, but a decrease that was most marked for cells from donor G.S., the clearance values falling below that of untreated cells. Considerable donor-dependent variation was observed in the absolute values for Endocytic Index (Table 6.1.), ^{125}I-labelled polyvinylpyrrolidone being cleared by G.S. cells at almost five times the rate of A.B. cells.

6.3.4. Uptake of ^{125}I-labelled polyvinylpyrrolidone (with suramin) by cells of various ages

Having established in the previous experiments that the uptake of ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin was readily measurable and clearly progressive with time, we were confident that we could proceed to study uptake using cells of different ages. Ideally uptake at several time-points up to 48 hours would have been measured (as in 6.3.3), but this was not possible owing to limited cell availability and our wish to study cells from the same donation at all the different cell-ages and thus eliminate the effects of inter-donor variation.

For these experiments blood was obtained in the usual way (Section 4.5.) from 6 donors (G.S.; R.D.; L.S.; J.B.L.; P.Q.; P.R.). Mononuclear cells were isolated and monolayers prepared as described in Section 4.5.. Uptake experiments were performed daily (Day 0 to 7), according to the method in Section 4.5.. Individual plates were used for each of the cell ages, but in some cases cells from more than one donor would be found on a plate.

Substrate additions were made in the usual way (Section 4.5.). For these experiments ^{125}I-labelled polyvinylpyrrolidone with suramin (0.1cm^3) was added to the cells to give a final concentration in the well of 50μg/cm^3 and 500μg/cm^3 respectively. After 24 hour incubation periods the incubations were terminated and protein and radioactivity determinations carried out as described in Sections 4.5..

Figures 6.4.1. to 6.4.6.

Uptake of ^{125}I-labelled polyvinyl pyrrolidone (50µg/cm^3, in the presence of suramin, 500µg/cm^3) over 24 hours by cells at various stages of maturation obtained from six donors G.S. (Figure 6.4.1.); R.D. (Figure 6.4.2.); L.S. (Figure 6.4.3.); J.L. (Figure 6.4.4.); P.Q. (Figure 6.4.5.) and P.R. (Figure 6.4.6.). Medium replenished on Day-4 (●) or Day-5 (○).

(i) protein content of cells.

(ii) cell-associated radioactivity.

(— — — —) double the amount of radioactivity adsorbing to cell-free wells.

(iii) clearance.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 6.3.4.

Figure 6.4.1.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) over 24 hours by cells at various stages of maturation

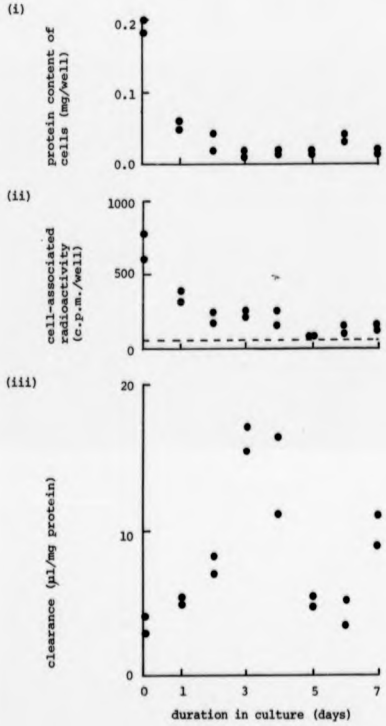


Figure 6.4.2.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) over 24 hours by cells at various stages of maturation

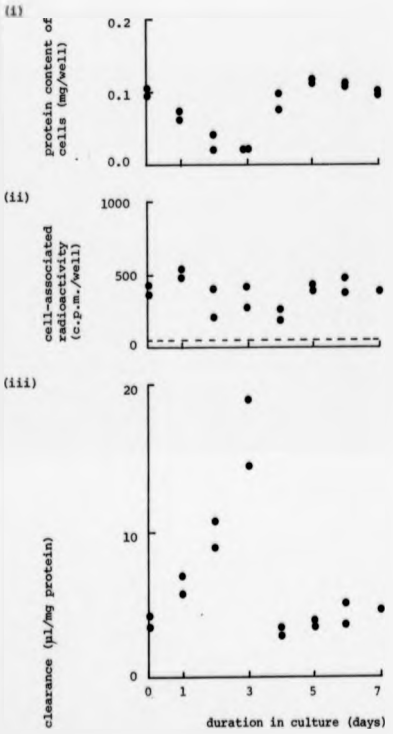


Figure 6.4.3.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin over 24 hours by cells at various stages of maturation

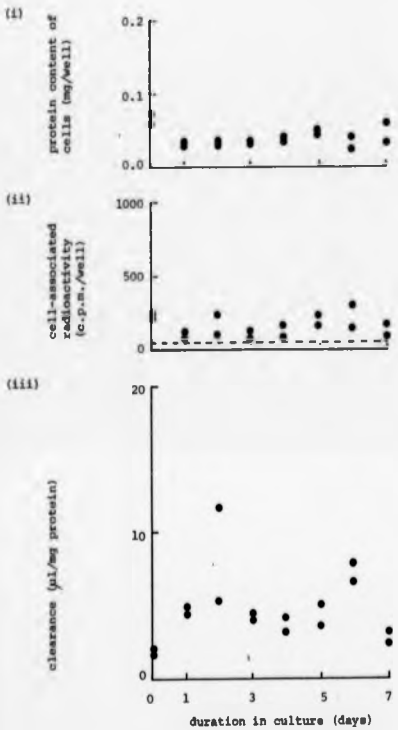


Figure 6.4.4.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) over 24 hours by cells at various stages of maturation

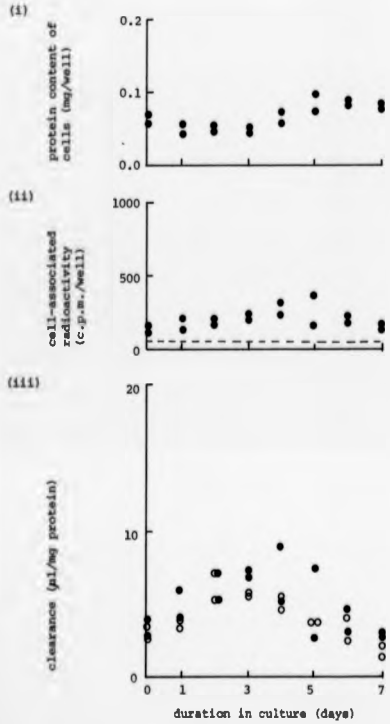


Figure 6.4.5.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) over 24 hours by cells at various stages of maturation

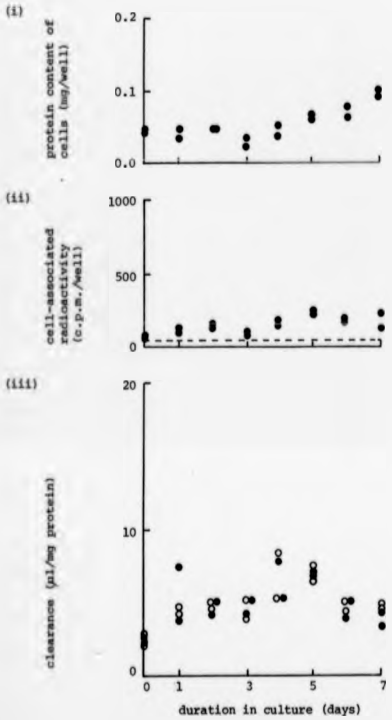


Figure 6.4.6.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) over 24 hours by cells at various stages of maturation

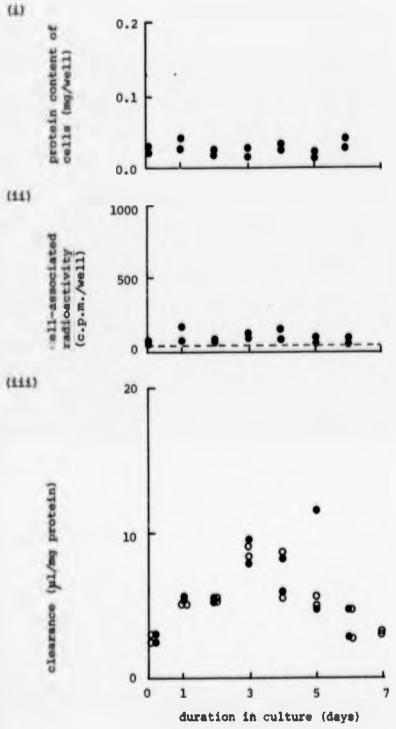
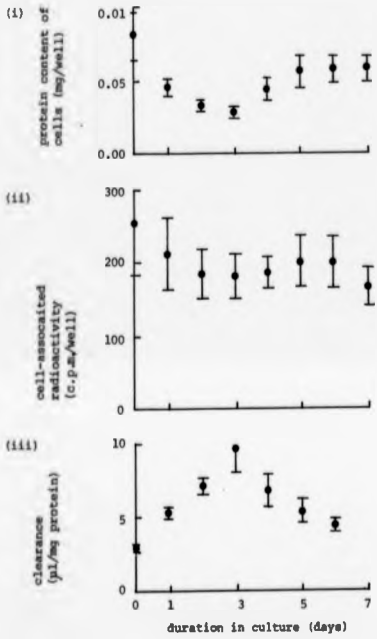


Figure 6.4.7.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) after 24 hours by cells at various stages of maturation.

Each point represents the mean ± S.E. of the values expressed in Figures 6.4.1. to 6.4.6. The exact details of these experiments are given in Section 6.3.4.



In addition, to determine whether the routine practice of replenishing the medium on Day 4 (Section 4.5.) affected the results, an experiment was made in which, for some of the donors, extra wells were set up and their medium replenished on Day 5 instead.

The amount of radioactivity adsorbing to the well itself was determined by adding ^{125}I-labelled polyvinylpyrrolidone, with suramin, to cell-free wells (i.e. wells containing 0.9cm^3 of culture medium only).

The results of these experiments are given in Figures 6.4.1. to 6.4.6.. On the whole, duplicate values were close. It is seen, with most of the donors, that the cell-protein content of the wells decreased over the first few days of culture, then on Day 3 to 4 started to increase. The radioactivity associated with the cells often followed a similar pattern to the corresponding protein values. Nearly all of the cell-associated radioactivity values were higher than double the value found to be associated with the cell-free wells. For each of the donors the clearance values steadily increased (approximately three fold) upto Day 2 to 4 after which there was often a steady or sharp decrease. The clearance values were similar whether the medium was replenished on Day 4 or 5.

Figure 6.4.7. shows the mean results for duplicate wells from all six donors. It is seen that, over the first 5 days of culture, the values for protein and for cell-associated radioactivity followed a similar pattern - first a fall and then a slight rise. However, the magnitude of the initial decrease in each parameter differs. There is approximately a 3-fold decrease in protein over the first 3 days, while the mean decrease in cell-associated radioactivity over this

Figures 6.5.1. to 6.5.3.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer ($10\ \mu g/cm^3$) by Day-7 cells obtained from three donors C.C. (Figure 6.5.1.); T.S. (Figure 6.5.2.) and P.R. (Figure 6.5.3.)

(i) protein content of cells.

(ii) cell-associated radioactivity,

(— — — —) double the amount of radioactivity adsorbing to cell-free wells.

(iii) clearance.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 6.3.5.

Figure 6.5.1.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells

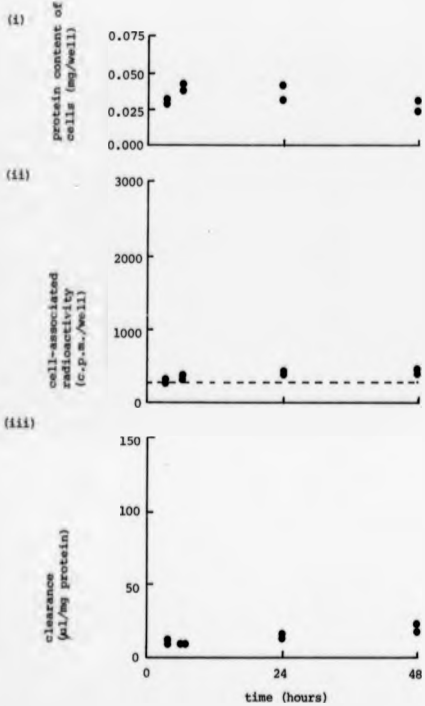


Figure 6.5.2.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells

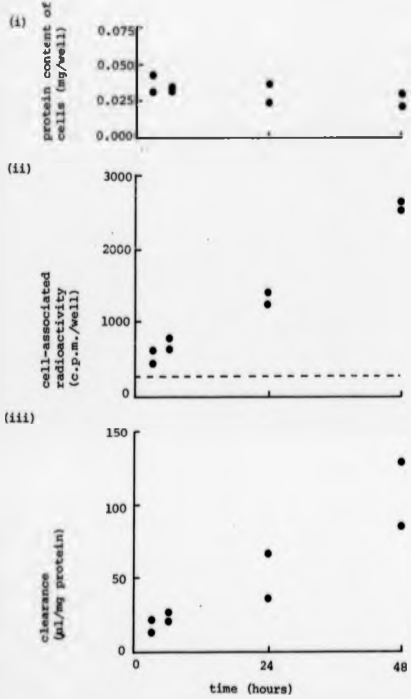


Figure 6.5.3.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells

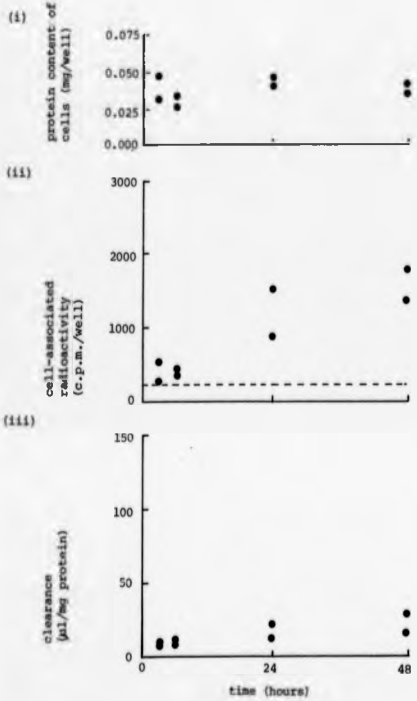


Table 6.2.

Endocytic Indices for the clearance of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer (10 μg/cm^3) by Day-7 cells

Donors	Endocytic Index
C.C.	0.175
T.S.	1.934
P.R.	0.328

The exact details of these experiments are given in Section 6.3.5.

The units of Endocytic Index (see Section 4.2.1.) are μl per mg. protein per hour.

period is only half as much (i.e. approximately 1.5 fold). In consequence the mean clearance values peak on Day 3, at the same time as the lowest protein values were observed.

6.3.5. Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells

Since these were our first experiments using the ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer, older cells were used for the same reason as given in Section 6.3.3.; and uptakes were measured at several time-points over 48 hours, to see whether uptake was progressive over a lengthy period. Blood was withdrawn from three donors (C.C.; T.S.; P.R.), and mononuclear cells isolated and monolayers prepared as described in Section 4.5.. Commencing on the seventh day of culture, incubations with ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer were started as described in Section 4.5.. This involved the removal of medium (0.1cm^3) and the addition of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer in culture medium (prewarmed; 10μg/cm^3; 0.1cm^3). Substrate was added in this way at 48, 24, 6 or 3 hours prior to termination of all incubations on Day-9. Substrate was also added to six cell-free wells as described in Section 4.5., to determine whether it adsorbed to the well itself.

Figures 6.5.1. to 6.5.3. show the results of these experiments. It is seen that duplicate values are reasonably close. The cell-associated radioactivity values generally increased with incubation time, and most of them were greater than double the amount of radioactivity associated with cell-free wells. The clearance values progressively increased with the incubation time in a linear

Figures 6.6.1. to 6.6.3.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer ($10\mu g/cm^3$) over 24 hours by cells at various stages of maturation, obtained from three donors T.A. (Figure 6.6.1.); J.C. (Figure 6.6.2.) and C.C. (Figure 6.6.3.)

(i) protein content of cells.

(ii) cell-associated radioactivity.

(— — — —) double the amount of radioactivity adsorbing to cell-free wells.

(iii) clearance

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 6.3.6.

Figure 6.6.1.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer over 24 hours by cells at various stages of maturation

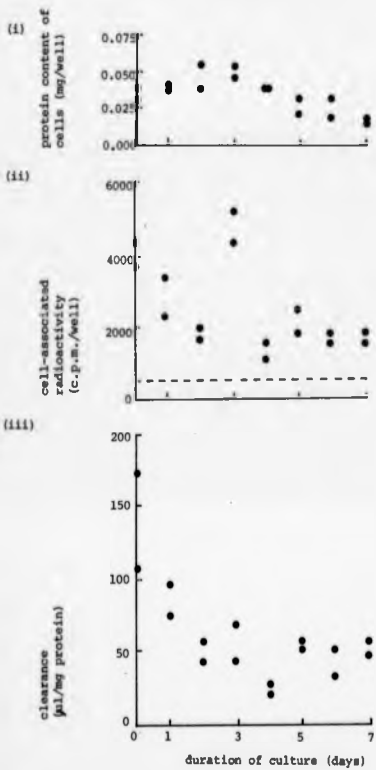


Figure 6.6.2.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer over 24 hours by cells at various stages of maturation

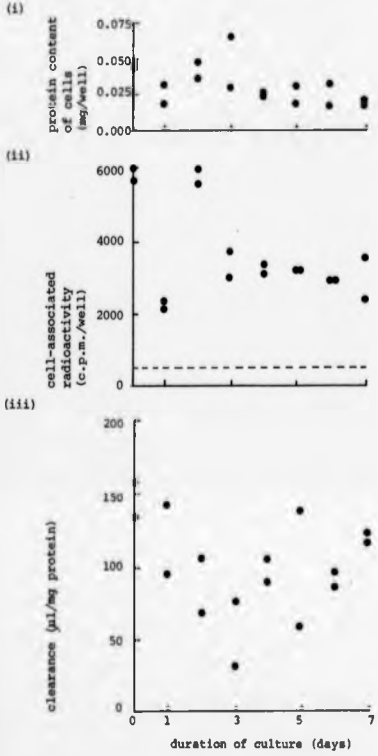
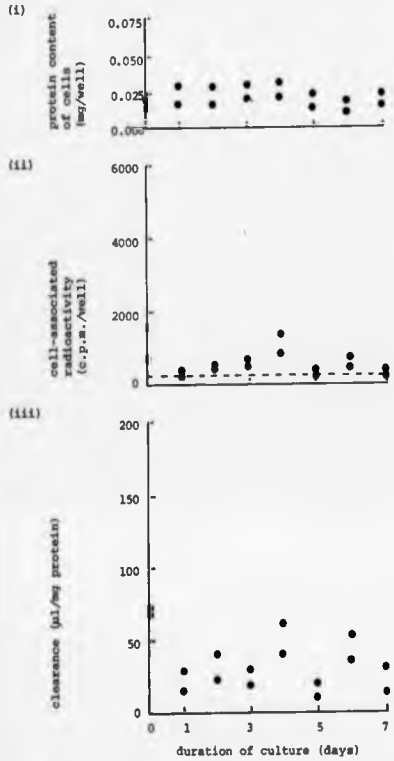


Figure 6.6.3.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer over 24 hours by cells at various stages of maturation



fashion, permitting the calculation of Endocytic Indices (Table 6.2.). There was considerable inter-donor variation in the Endocytic Indices for the copolymer, a greater than ten-fold difference being seen between the extreme values.

6.3.6. Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by cells of various ages

Having established in the previous experiments that the uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer was progressive with time, we could proceed to investigate uptake by cells of different ages. As in the corresponding experiments with ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin (Section 6.3.4.), uptake was measured by a single sampling after 24 hours, so as to permit cells from the same donation to be studied at different stages of maturation.

The procedure for these experiments was the same as for the experiments described in Section 6.3.4., except that the substrate used here was the ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer at a final concentration of (20 $\mu g/cm^3$). The three donors were T.A., J.C. and C.C..

Figures 6.6.1. to 6.6.3. show the data from these experiments. For each of the donors, an increase was seen in the cell-protein content per well between the first and third day in culture, after this point a decrease was seen. In most of our previous studies an increase or stable amount of cell-protein was detected after Day 3. This phenomenon is discussed in Section 5.4.1..

Duplicate values for cell-associated radioactivity were reasonably close and in all but a few cases were higher than twice the mean amount of radioactivity found to be associated with cell-free wells. Cells from two of the donors (T.A.; J.C.) showed a decrease in clearance as the cells aged, up to Day 3 or 4, and then a slow increase. Cells from the third donor (C.C.) showed little change with age, although there was a sharp drop from Day 0 to Day 1.

6.3.7. Uptake of ^{125}I-labelled fBSA by cells of various ages

The ^{125}I-labelled fBSA used in these experiments was donated by Glyn Steventon and Dr. Ken Williams (University of Keele), and prepared as described by Moore et al. (1977). Briefly, BSA had been labelled by the chloramine T method and then exposed to equivolume formaldehyde (10 percent in 0.5 moles/litre bicarbonate buffer; pH 10; 72 hours).

Although several preliminary experiments were undertaken with various ages of cells incubated with fBSA (25 $\mu g/cm^3$) for up to 24 hours, we could not reliably quantify the released degradation products in the medium. The values we obtained were too close to the values obtained in cell-free wells. It was therefore concluded that factors in the culture medium were causing degradation of the fBSA. It was not possible to detect cell-associated radioactivity in monocyte/macrophages, this is perhaps not surprising, for in rat peritoneal macrophages cell accumulation is low, the bulk of the fBSA captured being rapidly digested and released into the medium (Pratten et al., 1977).

It was decided to discontinue this aspect of the work, as it would not be possible to determine clearance rates for fBSA in the culture system we were using.

6.4. DISCUSSION

6.4.1. Uptake of ^{125}I-labelled polyvinylpyrrolidone by cells of various ages

When uptake of ^{125}I-labelled polyvinylpyrrolidone in the absence of suramin was examined over a short (3 hour) period, it was observed that many of the cell-associated radioactivity values were less than double the amount of radioactivity adsorbing to cell-free wells. It was concluded that uptake of this fluid-phase marker over a short period could not reliably be detected in our culture-system. Furthermore, for each of the three donors we could detect no clear differences between the ability of cells of different ages to clear the substrate from the medium; this was true whether cells were washed at the end of the experiment by our standard procedure or by the procedure adopted by Knight & Soutar (1982).

In contrast to our results, as described in Section 1.1.4., Knight & Soutar (1982) reported, in an almost identical experiment, an increase in the pinocytic activity between the second and fifth day of culture, with no further increase thereafter. The clearance values published by Knight & Soutar (1982) were lower than those reported here ranging from about 0.1 to 0.5µl/mg protein (ours ranged from about 0.5 to 12.5). Knight & Soutar's values had been corrected for the amount of radioactivity associated with cell-free wells, which was stated to be less than 10 percent of the radioactivity

associated with the cells, and therefore represented a much lower proportion of the total radioactivity than in our system. This major difference in the amount of radioactivity associating with cell-free wells may have been due to the different batches of culture vessels employed (like Knight & Soutar (1982) we used Linbro plates, but with smaller sizes of wells) or alternatively the batch of polyvinylpyrrolidone used by Knight & Soutar (1982) may have differed in its ability to adsorb to plastic.

If our clearance values are corrected for the radioactivity adsorbing to the wells (results not given), our values are still slightly higher than those of Knight & Soutar (1982). A number of factors may have contributed to this difference, such as differences in the donors' cells used, the methods used to isolate the cells (Knight & Soutar (1982) used a Ficoll gradient, we used Percoll), or the type of serum employed to maintain the cells. As outlined earlier (Section 1.2.) many authors have reported isolation procedure-, donor-, and serum-dependent differences in various cell functions.

In the experiment of Knight & Soutar (1982) both the source of cells and the serum employed can be criticized. Firstly, cells from two of the four donors had been isolated from buffy-coats (rather than from fresh blood), and a problem with this cell source is that the initial monolayers tend to contain many neutrophils (Leoni, 1985). Secondly, in contrast to our experiments, where a standard mixture of horse serum (10 percent w/v) and fetal calf serum (10 percent w/v) (obtained from a frozen stock) was used throughout the culture period, Knight & Soutar (1982), by using autologous serum, used fresh serum initially and then on Day 3, when the medium was replaced, autologous serum which had presumably needed to be stored frozen up

to that point. It is of interest that the observed increase in pinocytic activity occurred between the second and fifth day of culture, and therefore one cannot know whether this increase may have been brought about by a change in the culture environment, rather than as a direct consequence of cell maturation.

As highlighted previously in Section 1.1.4., the experiment reported by Knight & Soutar (1982) was only a small part of a major study on low-density lipoprotein metabolism and, apart from a statement that uptake of ^{125}I-labelled polyvinylpyrrolidone was constant for at least four hours, in an experiment in which only two uptake time-points were used, no further details were given. Clearly in their main experiment, in whichuptake was only measured over a single time period, no information on the kinetics of uptake was obtained. In order to make any firm conclusions concerning pinocytosis, it is not only important to gain adequate kinetic data, by measuring uptake over a lengthy period at several time-points, but also to perform studies using inhibitors of pinocytosis. To my knowledge, these parameters were not investigated in the experiment described by Knight & Soutar (1982).

6.4.2. Uptake of ^{125}I-labelled polyvinylpyrrolidone (with various concentrations of suramin) by Day-9 cells

In contrast to the experiments just discussed (Section 6.4.1), in which uptake of ^{125}I-labelled polyvinylpyrrolidone (in the absence of suramin) was measured over a short (3-hour) period, in these experiments suramin(0 to 1000μg/cm^3) was included with ^{125}I-labelled polyvinylpyrrolidone , and incubations were performed for various times over a 48 hour period. As a consequence the uptake values were

generally well above double the amount of radioactivity adsorbing to cell-free wells. Also, by demonstrating progressive accumulation of ^{125}I-labelled polyvinylpyrrolidone (with suramin), we could be confident that this was genuine uptake and not merely adsorption to the surface of the cells. In the wells containing no suramin, despite having chosen donors whose cells had previously demonstrated high endocytic abilities, it was only possible to calculate Endocytic Indices for ^{125}I-labelled polyvinylpyrrolidone for two of the donors, (G.S. cells 0.242 and A.B. cells 0.047µl/mg protein/hour). However, the addition of suramin (500 µg/cm^3) to cells incubated with ^{125}I-labelled polyvinylpyrrolidone, had the desired effect of increasing its uptake (2 to 6 fold), so that with all three donors it was possible to determine Endocytic Indices (G.S. cells 0.561; A.B. cells 0.257; P.H. 0.221µl/mg protein/hour). The Endocytic Indices we obtained in these experiments were slightly higher than our previous values (0.128, 0.064, 0.194µl/mg protein/hour) (Chapter 4). This was not surprising though, because the donors used in this present study had been selected because their cells had been shown previously to be very endocytic. The present results were slightly lower than the values reported by Pratten & Lloyd (1983) using rat peritoneal macrophages, which when normalized to cell protein were 0.35 ± 0.04µl/mg protein/hour for ^{125}I-labelled polyvinylpyrrolidone and 4.31 ± 0.84 for ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin (500µg/cm^3) (representing a 12 fold increase). The two to six-fold increase we observed in the uptake of ^{125}I-labelled polyvinylpyrrolidone was of the same order of magnitude as that reported by Pratten & Lloyd (1983). It was therefore highly probable that the increase in the uptake of this fluid-phase marker in the presence of suramin was due to the phenomenon of piggy-back endocytosis.

Owing to donor-dependent variation in uptake values, it was usually difficult to make direct comparisons between results for different experiments but, since cells from the donor G.S. had also been used in the previous experiments where ^{125}I-labelled polyvinylpyrrolidone was measured over a short period, it was possible to make comparisons. In this earlier study the uptake values for ^{125}I-labelled polyvinylpyrrolidone obtained over a three hour period ranged from 0.5 to 4.5µl/mg protein/hour for 9 and 12 day cells. In the present study, although an uptake value was not measured over three hours, the predicted value would be approximately 1.0, which is in close agreement with our earlier study.

The concentration-dependence of the suramin effect was found to be biphasic. A progressive increase in the rate of uptake of ^{125}I-labelled polyvinylpyrrolidone was observed up to 500µg/cm^3, suramin at 1000µg/cm^3 causing a drop in the rate. Pratten & Lloyd (1983), using isolated rat peritoneal macrophages, reported a progressive increase in the uptake of ^{125}I-labelled polyvinylpyrrolidone up to and including a concentration of 1000µg/cm^3. To my knowledge, the only other tissues in which the effect of suramin on endocytosis has been studied is the rat embryo and visceral yolk-sac (9.5 day). Freeman & Lloyd (1986) reported that with both tissues, suramin up to 1000µg/cm^3 had no effect on the uptake of ^{125}I-labelled polyvinylpyrrolidone: with the yolk-sacs a concentration-dependent decrease was observed at higher concentrations (2 to 10mg/cm^3).

Freeman & Lloyd (1986) further reported that suramin (greater than 2mg/cm^3) caused a decrease in the protein content of rat yolk-sacs, which was suggested to be due to suramin affecting protein uptake.

With human monocyte-derived macrophages, we found that suramin up to 1000μg/cm^3 did not generally affect cell-protein values. It may be that at higher concentrations suramin could have an effect on the protein content of human monocyte/macrophages. In conclusion, suramin (500μg/cm^3) increased the uptake of ^{125}I-labelled polyvinylpyrrolidone by human monocyte-derived macrophages, presumably by piggy-back endocytosis, to a level which could easily and reliably be detected in our culture system.

6.4.3. Uptake of ^{125}I-labelled polyvinylpyrrolidone (with suramin) by cells of various ages

Having demonstrated progressive uptake of ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin (500 μg/cm^3) it was now possible to measure uptake as the cells matured, using single but longer incubation periods. Ideally, we would have determined uptakes at several time-points over this period but owing to limited cell numbers this was not possible.

As the cells differentiated, the cell-protein values usually followed the same pattern as previously described (Chapter 5), an initial decrease followed by an increase. The cell-associated radioactivity values were higher than the mean amount of radioactivity associated with the well itself, and therefore these results were considered trustworthy. The clearance values expressed in terms of cell protein increased up to around the third day in culture. Since it was shown earlier (Chapter 5) that the protein content in the initial culture period closely paralleled the cell number, it was clear that the cells were progressively taking up slightly more of the substrate up to the third day in culture. This slight increase (approximately 3

fold) may have been related to cell size, for after the first day in culture cell-size is known to increase: this increase in uptake may therefore be a reflection of the concomitant increase in the surface area of the cell, possibly allowing the formation of more pinosomes. After around the third day in culture, the ability of the cells to capture the substrate was seen to decrease. However, it must be remembered that over this latter culture period, although the protein values often increased, the cell numbers continued to decrease with time (Section 5.3.1.). Therefore, if these results were to have been expressed in terms of cell numbers, one would probably not have seen this decrease but a constant rate of uptake over this period. Although preferable, it is not practicable to express results in terms of cell numbers, since the main methods used to determine this parameter (i.e. using a Coulter counter or haemocytometer) are not suited for routine determinations with adherent cells. The possible alternatives would either be to determine cell numbers in other representative wells, i.e. not the cells used for the uptake studies (using one of the above methods) or to estimate cellular DNA (which correlates well with cell number). However, the first solution is not ideal owing to the well-to-well variability often observed in cell numbers, and secondly estimating DNA would be very time-consuming.

In conclusion, as monocytes differentiated *in vitro*, over the first few days in culture (up to around Day 3) their ability to clear ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin (500μg/cm^3) increased slightly; after this point, although the clearance values decreased (when expressed in terms of cell-protein), it was highly probably that the individual cells were not losing their capacity acquired up to Day 3.

6.4.4. Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells

In these experiments the uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer was measured at several time-points over a 48 hour period. For each donor the amount of radioactivity per well was above double the amount adsorbing to cell-free wells, and the clearance values increased progressively with time. We were therefore confident that we were measuring genuine uptake, and that the variation seen in the absolute Endocytic Index values (i.e. 0.175, 0.328, 1.934µl/mg protein/hour), reflected variation between donors. Because of this variation it was difficult to make a direct comparison between the uptake of this copolymer with the values we obtained previously for ^{125}I-labelled polyvinylpyrrolidone (0.242,0.047 µl/mg protein/hour). However, by comparing the ranges of the values, it was clear that the radiolabelled copolymer was generally entering the cells at a faster rate than the ^{125}I-labelled polyvinylpyrrolidone. Pratten et al. (1982) using rat peritoneal macrophages reported a mean Endocytic Index value for the ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer, which when expressed in terms of cell protein, is 1.86 ± 0.45µl/mg protein/hour. This value falls within the range we observed using monocyte-derived macrophages. The only other tissue in which the uptake of this copolymer has been investigated is the rat yolk-sac, in which a much higher Endocytic Index was reported 8.15 ± 1.95µl/mg protein/hour. It was concluded that it is highly likely that the plasma membranes of human monocyte-derived macrophages have a high affinity for positively charged substrates, in common with several other cells, e.g. rat peritoneal macrophages.

6.4.5. Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by cells of various ages

Since, as described in the previous Section, we had established that ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer was progressively cleared from the medium by monocyte/macrophages, we felt justified in measuring uptake over a single time-period, using cells at different stages in their development. In contrast to the results for ^{125}I-labelled polyvinylpyrrolidone, the clearance values obtained for the copolymer were very high on Day 0, after which uptake values dropped over the next few days, remaining fairly constant thereafter.

It is therefore conceivable that, as the monocytes matured into macrophages various modifications of the plasma membrane took place which initially caused a decrease in their ability to capture cationic molecules. Many changes in the properties of the plasma membrane are already known to occur as the cells mature (see Sections 1.2.6. and 1.2.7.), such as changes in the numbers and types of antigenic determinants and receptors.

6.4.6. Comparison of the endocytic capacity of cells of various ages with that reported by other authors

As outlined in Section 1.1.4., only three authors have reported studies in which pinocytosis has been investigated as monocytes mature *in vitro*, two using human cells and the other horse cells. Although Bennett & Cohn (1966) reported an increase in the pinocytic activity of horse monocytes over the first few days in culture, the technique used, viz. counting vesicles visible by light microscopy, has been much criticised by several authors (e.g. Steinman *et al.*,

1976) since many vesicles are not large enough to be resolved by the light microscope. Because of the reported unreliability of the technique used by Bennett & Cohn (1966), it was decided to not consider their results further.

The study by Knight & Soutar (1982) using human monocyte/macrophages of various ages, in which the uptake of ^{125}I-labelled polyvinylpyrrolidone was examined over three-hour periods (described in Section 1.1.4.), has already been discussed in relationship to the similar experiment we performed. As described earlier in Section 6.4.1., our results for the uptake of this fluid-phase marker were considered unreliable and we were unable to confirm the result of Knight & Soutar. Using a method for measuring pinocytosis that we considered to be trustworthy, viz. uptake of ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin , we again found no evidence for the sharp rise reported by Knight & Soutar (1982) between Days 2 and 5. Although in both experiments, an increase in pinocytic activity was observed over the early culture period (i.e. up to Day 4 or 5), the increase was not as marked in our experiments: only 3-fold versus 5-fold reported by Knight & Soutar (1982). It was difficult to compare our results with the microscopic study in which Van Furth *et al.* (1979) reported very little increase in the ability of differentiating monocytes to capture the fluid-phase substrate dextran, since their results were expressed in terms of the percentage of cells capturing the substrate, and therefore any increase in the total amount of substrate captured may have remained undetected.

The initial high clearance values we observed for the cationic copolymer (vinylamine-vinylpyrrolidone) for Day-0 cells, which

sharply decreased as the cells matured, did not conform with the study referred to above (Knight & Soutar 1982), the probable reasons for the high uptake values observed on Day 0 have been discussed in Section 6.4.5..

Further investigations concerning the mode of uptake of these macromolecules are detailed in the next Chapter.

CHAPTER 7

Inhibitor and release studies

7.1. INTRODUCTION

In Chapter 6 the uptake of two radiolabelled macromolecules by monocyte/macrophages was demonstrated. The significance of these results depends crucially on whether the cellular accumulation seen is due to pinocytosis or merely to surface binding. The various possible approaches to this question have already been described in Chapter 4: these include demonstrating that substrate capture is progressive over a prolonged period; that the capture of the substrate can be inhibited by metabolic or cytoskeletal inhibitors; and that release of substrate is not rapid when loaded cells are introduced into substrate-free medium.

We have already shown (Chapter 6), that the uptake of ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin (500$\mu g/cm^3$) and of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer is progressive over a 48 hour period, and therefore provided strong evidence for pinocytic uptake. In this Chapter, further investigations were made by studying (i) the effect of various known inhibitors of pinocytosis on the uptake of the radiolabelled substrates and (ii) the kinetics of release of previously captured substrate from the cells.

7.1.1. Low temperature, metabolic and cytoskeletal inhibitors

Low temperature, metabolic and cytoskeletal inhibitors have all been shown to inhibit the uptake of macromolecules by various cell-types. Although these results may often be correctly interpreted as indicating inhibition of pinocytosis, care is necessary, since some of the modifiers may be cytotoxic, particularly at high concentrations. The degree of inhibition frequently varies between

different cell-types and occasionally the uptake of one substrate is reduced to a greater extent than that of another (these points are highlighted below).

Two extensive studies in this area have been performed with mononuclear phagocytes: one using rat peritoneal macrophages (Pratten & Lloyd, 1979), and examining the effect of various inhibitors on the uptake of both colloidal ^{198}Au and ^{125}I-labelled polyvinylpyrrolidone; and the other using both mouse peritoneal macrophages and rat kupffer cells (Munthe-Kaas, 1977) , and the one substrate colloidal ^{198}Au.

In both studies low temperatures decreased pinocytosis, but to different extents. Using rat peritoneal macrophages Pratten & Lloyd (1979) reported almost complete inhibition of the uptake of both substrates at 20°C, whereas Munthe-Kaas (1977) using mouse peritoneal macrophages and rat kupffer cells reported only partial inhibition of the uptake of colloid ^{198}Au at 20°C, but complete inhibition at 4°C. Low temperature is thought to inhibit pinocytosis both by reducing membrane fluidity and by lowering the metabolic activity of cells.

The metabolic inhibitor sodium fluoride (which inhibits glycolysis) at a concentration of approximately 50$\mu g/cm^3$ was reported to inhibit by varying extents (50 to 90 percent) the uptake of colloidal ^{198}Au by all three types of cell and also the uptake of ^{125}I-labelled polyvinylpyrrolidone by rat peritoneal macrophages (Munthe-Kaas, 1977; Pratten & Lloyd, 1979). At higher concentrations (above 50$\mu g/cm^3$) sodium fluoride was cytotoxic. Similarly, the inhibitor of oxidative phosphorylation, 2,4-dinitrophenol, reduced the pinocytic activity of the three cell-types, but at still higher concentrations (greater

than around 200$\mu g/cm^3$) proved toxic to the cells. Monoiodoacetate, another inhibitor of glycolysis, was reported to be cytotoxic to rat peritoneal macrophages at concentrations as low as 10$\mu g/cm^3$ (Pratten & Lloyd 1979).

It is generally believed that the cytoskeletal system (microtubules and microfilaments) are involved in endocytic and secretory processes. Although cytochalasin B (an inhibitor of microfilament function) strongly inhibits the phagocytic ability of mouse peritoneal macrophages (Allison et al., 1971), its inhibitory effect on pinocytosis is only partial. At a concentration of 10 $\mu g/cm^3$ it was reported to cause only a little inhibition of the uptake of colloidal ^{198}Au by both mouse peritoneal macrophages and rat kupffer cells (Munthe-Kaas, 1977). Similarly Pratten & Lloyd (1979) reported only upto 35 percent inhibition of the uptake of colloidal ^{198}Au and ^{125}I-labelled polyvinylpyrrolidone by rat peritoneal macrophages. Cytochalasin B is generally thought of as cytotoxic at higher concentrations. Colchicine, an agent that binds to tubulin (and interferes with its polymerization to form microtubules) does effectively inhibit pinocytosis by rat peritoneal macrophages (Pratten & Lloyd, 1979) and mouse peritoneal macrophages and rat kupffer cells (Munthe-Kaas, 1977). The extent of this inhibition varies greatly depending on the substrate used. Pratten & Lloyd (1979) reported that colchicine at a concentration of 10$\mu g/cm^3$ greatly inhibited (by 80 percent) the uptake of colloidal ^{198}Au, but had very little effect on the uptake of ^{125}I-labelled polyvinylpyrrolidone; colchicine (100$\mu g/cm^3$) though did inhibit the uptake of the latter by about 90 percent. Variation in the degree of inhibition was also seen between different cell-types. Munthe-Kaas (1977) reported that very high concentrations of colchicine

($4000\mu g/cm^3$) markedly inhibited (by about 80 percent) the uptake of colloidal ^{198}Au by rat kupffer cells, but only caused a 40 percent reduction in pinocytosis with mouse peritoneal macrophages. High concentrations of inhibitor caused the cells to round up, although the cells were reported to be still viable.

Changes in intracellular calcium levels are thought to be important metabolic signals and, as detailed in Section 1.1.4., it is possible that there is a requirement for calcium ions during pinocytosis. In the study by Pratten & Lloyd (1979), the calcium chelator EGTA (5mmoles/litre) was seen to inhibit greatly the uptake of ^{125}I-labelled polyvinylpyrrolidone, an effect that could be reversed by adding $CaCl_2$ (5mmoles/litre) to the cells along with the EGTA.

Very little information is available on the effects of the modifiers (detailed above) on the uptake of the two substrates we have employed. The uptake of ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin ($500\mu g/cm^3$) by rat peritoneal macrophages is reported to be reduced by sodium fluoride ($50\mu g/cm^3$) (Pratten & Lloyd, 1983). The uptake of the vinylamine-vinylpyrrolide copolymer (m.w. 120,000) by the same cells is reported to be inhibited by low temperature and, although 2, 4-dinitrophenol inhibited the uptake of the smaller copolymer (m.w. 46,000), it had little effect on the capture of the larger (Pratten & Lloyd, 1982). It was therefore decided to investigate the effect of a wide range of modifiers on the uptake of the two substrates. Owing to the reported cytotoxicity of many of the modifiers, we initially examined the effect of the modifiers on cell protein, to give us an idea of the extent of cell death, prior to studying their effects on pinocytosis.

7.1.2. Release studies

A further way of confirming that uptake is by pinocytosis is to demonstrate that, once captured, there is only a slow rate of substrate release from cells when they are introduced into substrate-free medium. This type of experiment has been performed with various tissues/and cells. Williams et al. (1975) incubated rat visceral yolk-sacs with ^{125}I-labelled polyvinylpyrrolidone for a 6 hour period, then washed the tissues in medium and reincubated them in either ^{125}I-labelled polyvinylpyrrolidone-free culture medium for a futher 6 hours, or medium containing non-iodinated polyvinylpyrrolidone. In both instances they observed that only 2 to 3 percent of the radioactivity in the tissue was released within the first 30 minutes, followed by a further 2-3 percent being released progressively over the remaining time. With mononuclear phagocytes, this type of experiment was reported by Pratten et al. 1977 using rat peritoneal macrophages. Cells were incubated with either ^{125}I-labelled polyvinylpyrrolidone or colloidal ^{198}Au for a three hour period, then washed, and reincubated in radiolabelled substrate-free medium for a further 3 hours. The amount of radioactivity released into the medium over this period was determined. Initially (over the first 15 minutes) both colloidal ^{198}Au and ^{125}I-labelled polyvinylpyrrolidone were rapidly lost from the cells (up to around 20 percent of the total radioactivity present in the cells at the commencement of incubation). This rate of release was then progressively slower with the remaining time in culture. In both studies it was concluded that the initial rapid loss was probably due to some adsorbed extracellular radioactivity that survived the washing procedure, and that the following slow progressive release represented mainly either exocytosed

radioactivity or radioactivity released from dying cells.

It was decided to undertake this type of experiment using cells that had captured either ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin or ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer.

7.2. EXPERIMENTAL METHODS

All the experiments described in this Chapter were carried out using monocyte/macrophages at Day-0 and at Day-7.

7.2.1. Determining the effect of modifiers on cell-protein and on pinocytosis

The effect of various modifiers on cell-protein was determined simply by incubating cells (37°C; 24 hours) in duplicate wells either with or without the modifier, or at different temperatures. Cells were then washed using the washing procedure described in Section 4.5., and digested in NaOH (0.5 moles/litre; 0.5cm^3), and cell-protein determined as described by Lowry et al. (1951). The effect of the modifiers on pinocytosis was determined by measuring the rate of capture of radiolabelled substrates (using the definitive method described in Section 4.5.), for cells incubated in the presence or absence of the modifiers. Modifiers were usually added at the same time as the radiolabelled substrate, by removing from the wells an additional 0.1cm^3 of medium (in one case this occurred 20 minutes prior to the addition of the radiolabelled substrate) and replacing it with 0.1cm^3 of the solution of modifier.

In all cases the pH of the solutions of modifiers was checked and adjusted to 7.4 if necessary. When the effect of temperature was being examined, and since only one humidified CO_2 incubator was available, it was decided to simulate the standard incubation conditions by placing Linbro plates into plastic boxes containing a moistened wad of tissue paper and gassing the boxes with air/CO_2 (95/5 percent) prior to sealing them. Cells were equilibrated at the appropriate temperature in the afore-mentioned containers for a period of one hour prior to the experiment. The exact details of these experiments are given in the results Section 7.3.1..

7.2.2 Release experiments

Cell incubations with radiolabelled substrates, either ^{125}I-labelled polyvinylpyrrolidone (50$\mu g/cm^3$) (in the presence of suramin, 500$\mu g/cm^3$) or ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer (10 $\mu g/cm^3$), were carried out in the standard way as described in Section 4.5.. Incubations were for periods of 0.5 or 24 hours at 37°C. Cells were then washed in the standard way, then reincubated in medium containing either no substrate, radiolabelled substrate, or non-iodinated substrate in excess (ten fold the initial concentration of radiolabelled substrate used). After a further hour, incubations were terminated by the standard procedure described in Section 4.5., and the amounts of radioactivity still associated with the cells (expressed per mg cell-protein) compared .

Table 7.1.

Cell protein values (μg/well) for Day-0 or Day-7 cells incubated (24 hours) in the presence or absence of modifier

Modifiers	Day-0 cells from donors			Day-7 cells from donors		
	P.S.	P.T.	R.D.	M.S.	L.S.	M.P.
Control at 37°C (no modifier)	53 (100%)	58 (100%)	45 (100%)	78 (100%)	65 (100%)	73 (100%)
Low temperature 20°C	10 (19%)	20 (34%)	13 (29%)	59 (76%)	39 (60%)	40 (55%)
Low temperature 4°C	5 (9%)	4 (7%)	3 (7%)	20 (26%)	27 (42%)	31 (42%)
2,4-Dinitrophenol 10μg/cm^3	25 (47%)	29 (50%)	30 (67%)	40 (51%)	38 (58%)	39 (53%)
2,4-Dinitrophenol 250μg/cm^3	20 (38%)	21 (36%)	26 (58%)	36 (46%)	31 (48%)	38 (52%)
Sodium fluoride 10μg/cm^3	28 (53%)	27 (47%)	20 (44%)	39 (50%)	38 (58%)	44 (60%)
Sodium fluoride 100μg/cm^3	21 (40%)	25 (43%)	20 (44%)	28 (36%)	31 (48%)	41 (56%)
Cytochalasin B 5μg/cm^3	5 (9%)	4 (7%)	6 (13%)	13 (17%)	16 (25%)	20 (27%)
Cytochalasin B 10 μg/cm^3	6 (11%)	6 (10%)	5 (11%)	10 (13%)	15 (23%)	18 (25%)
Colchicine 10μg/cm^3	54 (102%)	57 (98%)	46 (102%)	72 (92%)	68 (105%)	70 (96%)
Colchicine 100μg/cm^3	52 (98%)	59 (102%)	46 (102%)	74 (95%)	71 (109%)	65 (89%)
EGTA 5mmoles/litre	51 (96%)	56 (97%)	44 (98%)	74 (95%)	60 (92%)	78 (107%)
EGTA 5mmoles/litre +$CaCl_2$ 5mmoles/litre	50 (94%)	54 (93%)	45 (100%)	78 (100%)	64 (98%)	79 (108%)

Each value is the mean from two duplicate wells. Figures in brackets = percentage of matched control (no modifier).

The exact details of these experiments are given in Section 7.3.1.

7.3. RESULTS

7.3.1. Effect of modifiers on cell-protein

Cells were obtained from several donors and monolayers established as described in Section 4.5.. Day-0 cells (from donors P.S.; R.T.; R.D.) and Day-7 cells (from donors M.S.; L.S.; M.P.) were used for determining the effects of incubation in the presence of modifiers on cell-protein, as described in Section 7.2.1.. The final concentrations of the modifiers tested were: 2,4-dinitrophenol (10 or 250$\mu g/cm^3$); sodium fluoride (10 or 100$\mu g/cm^3$); cytochalasin B (5 or 10$\mu g/cm^3$); colchicine (10 or 100$\mu g/cm^3$); EGTA (5 mmoles/litre, with or without equimolar calcium chloride). Where the effect of low temperatures was being examined, cells were incubated (for up to 24 hours) as described in Section 7.2.1. at 37°C, 20°C or 4°C; all other incubations were at 37°C.

The results of these experiments are summarised in Table 7.1.. It may be seen for both Day-0 and Day-7 cells, that there is little donor-dependent variation in the results. In addition, the effect of the modifiers (except low temperature) was similar on both freshly isolated and differentiated cells. Both of the metabolic inhibitors 2,4-dinitrophenol and sodium fluoride caused a significant decrease in cell-protein (approximately 50 percent reduction), even at concentrations as low as 10$\mu g/cm^3$. Cytochalasin B, one of the cytoskeletal inhibitors, drastically reduced cell-protein, even at 5$\mu g/cm^3$. Colchicine (up to 100$\mu g/cm^3$), though, had no discernible effect on the cell-protein values, neither did the chelating agent EGTA (5mmoles/litre), in the presence or absence of equimolar calcium chloride. Low temperature caused a drastic reduction in the cell-protein values of Day-0 cells (20°C and 4°C caused reductions to approximately 25 and 10 percent), but with Day-7 cells, the decrease

Figures 7.1.1. to 7.1.3.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (50$\mu g/cm^3$; in the presence of suramin, 500$\mu g/cm^3$) by Day-0 cells obtained from three donors K.W. (Figure 7.1.1.); G.S. (Figure 7.1.2.) and C.C. (Figure 7.1.3.). Uptake in the presence of colchicine (▲, 100$\mu g/cm^3$) or EGTA (■, 5mmoles/litre), or in the absence (O) of modifiers.

(i) protein content of cells.

(ii) clearance.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 7.3.2.1.

Figure 7.1.1.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-0 cells

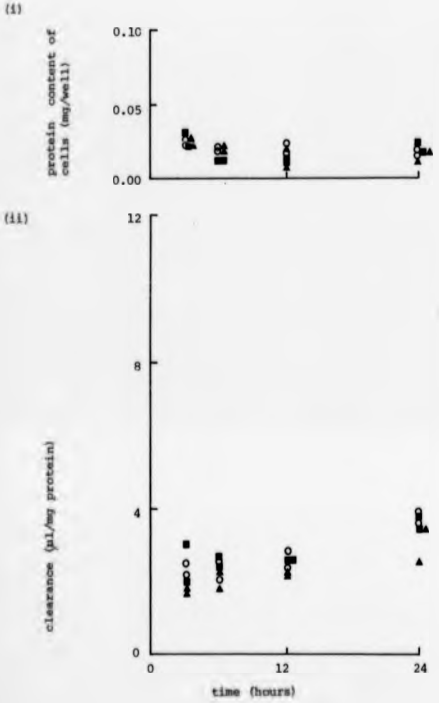


Figure 7.1.2.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-0 cells

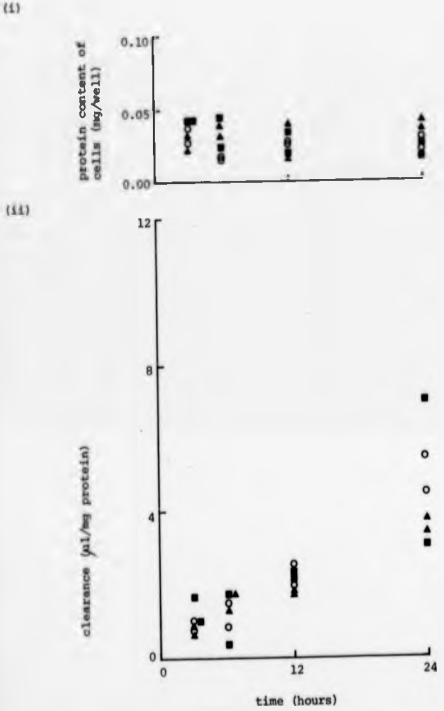


Figure 7.1.3.

Uptake of ^{125}I-labelled polvyinylpyrrolidone (in the presence of suramin) by Day-0 cells

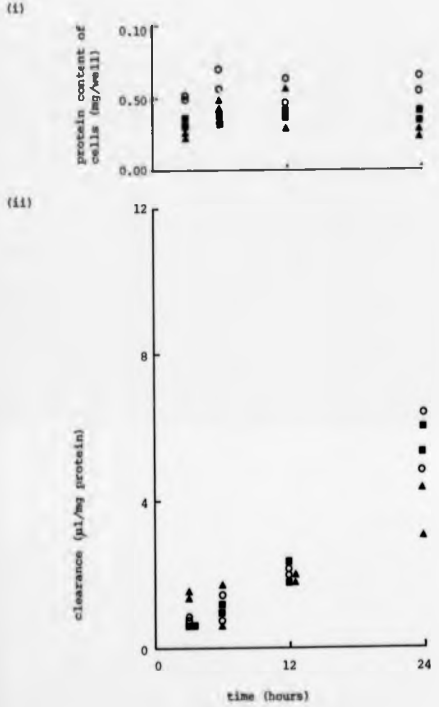


Table 7.2.

Endocytic Indices for the clearance of ^{125}I-labelled polyvinylpyrrolidone (50$\mu g/cm^3$; in the presence of suramin, 500$\mu g/cm^3$) by Day-0 cells in the presence or absence of modifier

Donors	No modifier (control)	Colchicine (100$\mu g/cm^3$)	EGTA (5mmoles/litre)
K.W.	0.084 (100%)	0.054 (64%)	0.064 (77%)
G.S.	0.215 (100%)	0.126 (58%)	0.240 (111%)
C.C.	0.247 (100%)	0.126 (51%)	0.259 (105%)

The figures in brackets are the values expressed as a percentage of the matched control (no modifier).

The exact details of these experiments are given in Section 7.3.2.1.

The units of Endocytic Index (see Section 4.2.1.) are µl per mg protein per hour.

was less marked (to approximately 70 and 30 percent respectively).

Morphological examination of the cells, following the incubation and washing procedures, revealed that for all the modifiers except colchicine and EGTA (where cell-protein values were unaffected), excessive cell loss had occurred and the remaining cells were often atypical, being rounded rather than displaying the heterogenous morphology described in Section 3.3.8..

Because of these adverse effects, it was decided to use only colchicine and EGTA in the remaining studies.

7.3.2. Effect of colchicine and EGTA on pinocytosis

7.3.2.1. Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-0 cells. Blood was obtained from three donors (K.W.; G.S.; C.C.) and cell monolayers established as described in Section 4.5.. Using Day 0 to 1 cells, incubations were commenced at various times (24, 12, 6 and 3 hours) prior to the end of the experiment (on Day 1) as described in Section 7.2.1.. Briefly, this involved removing 0.2cm^3 of culture medium and replacing it with 0.1cm^3 of ^{125}I-labelled polyvinylpyrrolidone in the presence of suramin (giving final concentrations in the wells of 50 and 500 $\mu g/cm^3$ respectively); and also either 0.1cm^3 colchicine or EGTA (giving final concentrations in the wells of 100$\mu g/cm^3$ or 5moles/litre respectively). Incubations were all terminated together on Day 1 as described in Section 4.5..

Figures 7.1.1. to 7.1.3. and Table 7.2. show the results. It is seen that cell-protein values were broadly constant with duration of

Figures 7.2.1. to 7.2.3.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (50µg/cm^3; in the presence of suramin, 500µg/cm^3) by Day-7 cells obtained from three donors R.D. (Figure 7.2.1.); G.S. (Figures 7.2.2.) and J.C. (Figure 7.2.3.). Uptake in the presence of colchicine (▲, 100µg/cm^3) or EGTA (■, 5mmoles/litre), or in the absence (O) of modifiers.

(i) protein content of cells.

(ii) clearance.

Each point represents the value obtained from a single well. The exact details of these experiments are given in Section 7.3.2.2.

Figure 7.2.1.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-7 cells

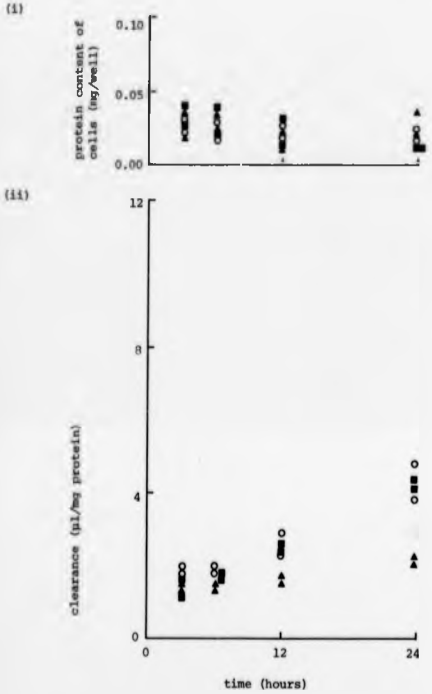


Figure 7.2.2.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-7 cells

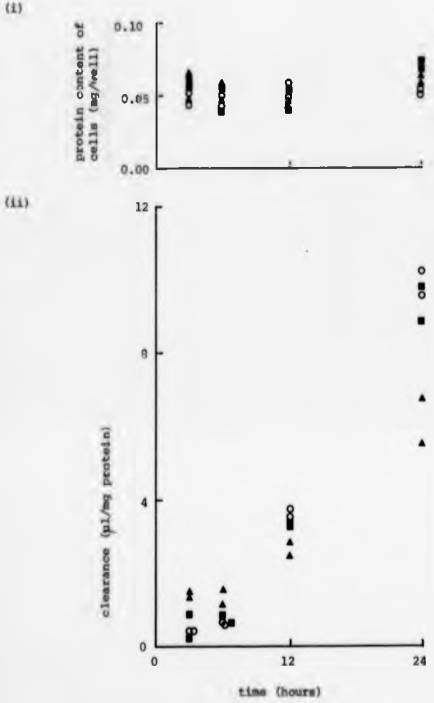


Figure 7.2.3.

Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-7 cells

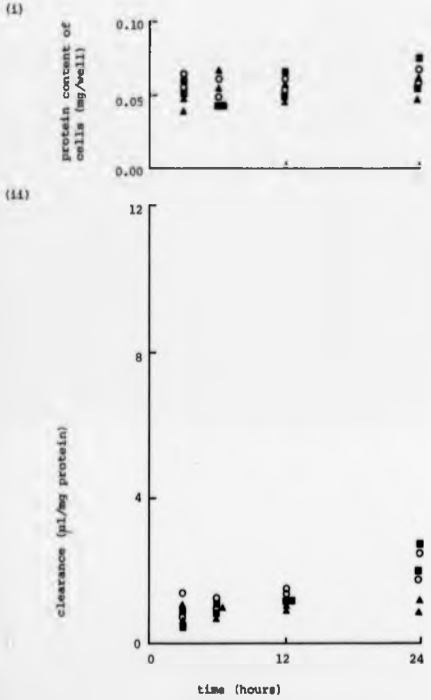


Table 7.3.

Endocytic Indices for the clearance of ^{125}I-labelled polyvinylpyrrolidone (50μg/cm^3; in the presence of suramin, 500μg/cm^3) by Day-7 cells in the presence or absence of modifier.

Donors	No modifier (control)	Colchicine (100μg/cm^3)	EGTA (5mmoles/litre)
R.D.	0.168 (100%)	0.075 (45%)	0.146 (87%)
G.S.	0.515 (100%)	0.272 (53%)	0.479 (93%)
J.C.	0.058 (100%)	0.011 (19%)	0.048 (83%)

The figures in brackets are the values expressed as a percentage of the matched control (no modifier).

The exact details of these experiments are given in Section 7.3.2.2.

The units of Endocytic Index (see Section 4.2.1.) are μl per mg protein per hour.

incubation. For two of the donors (K.W.; G.S.) the cell-protein values were unaffected by the modifiers, but with the third donor (C.C.) the values were lower in the presence of modifiers. With all three donors the clearance values for ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) increased linearly, with or without inhibitors. No increase is seen between the 3 and 6 hour incubations, probably because the cell-associated radioactivity values at the three-hour point were often lower than the amount of radioactivity associating with cell-free wells (results not shown). Therefore, in these and further experiments described in this Section, the 3-hour clearance values were not used when Endocytic Indices were calculated. The Endocytic Indices varied somewhat between the donors (0.084 to 0.247µl/mg protein/hour. Colchicine caused a reduction in the rate by 35-50 percent. EGTA on the other hand had no consistent effect on the clearance rates.

7.3.2.2. Uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin) by Day-7 cells. The method used for these experiments was similar to that just described (7.3.2.1). However, differentiated (Day-7) cells were used (donors R.D.; G.S.; J.C.).

Figures 7.2.1. to 7.2.3. and Table 7.3. show the results. In these experiments the cell-protein values were stable with duration of incubation and unaffected by the presence of either modifier. Again the clearance values increased linearly between 6 and 24 hours and therefore Endocytic Indices were calculated over this period (Table 7.3.). It is seen that in the absence of modifiers the Endocytic Indices varied greatly between the donors (0.058 to 0.575µl/mg protein /hour). Yet, with all three donors, the inhibitors had similar effects on the clearance rates of the substrate; EGTA caused only a

Figures 7.3.1. to 7.3.3.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer ($10\mu g/cm^3$) by Day-0 cells obtained from three donors P.R. (Figure 7.3.1.); R.D. (Figure 7.3.2.) and M.S. (Figure 7.3.3.). Uptake in the presence of colchicine (▲, $100\mu g/cm^3$; △, $100\mu g/cm^3$ added 20 minutes prior to radiolabel), or EGTA (■, 5mmoles/litre; □ 5mmoles/litre added to cells 20 minutes prior to radiolabel), or in the absence (O) of modifiers.

(i) protein content of cells.

(ii) clearance.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 7.3.2.3.

Figure 7.3.1.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-0 cells

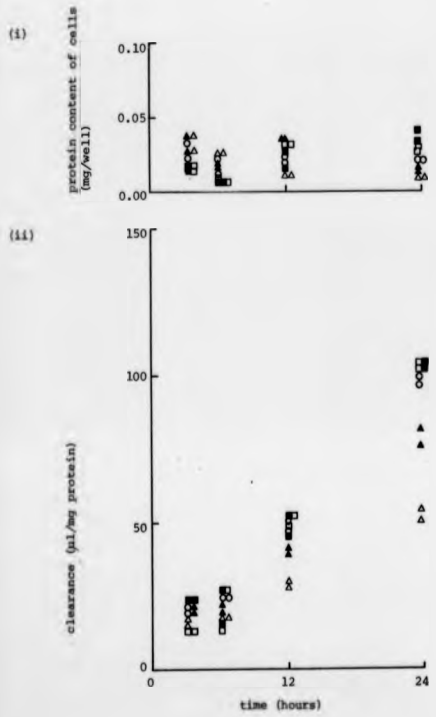


Figure 7.3.2.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-0 cells

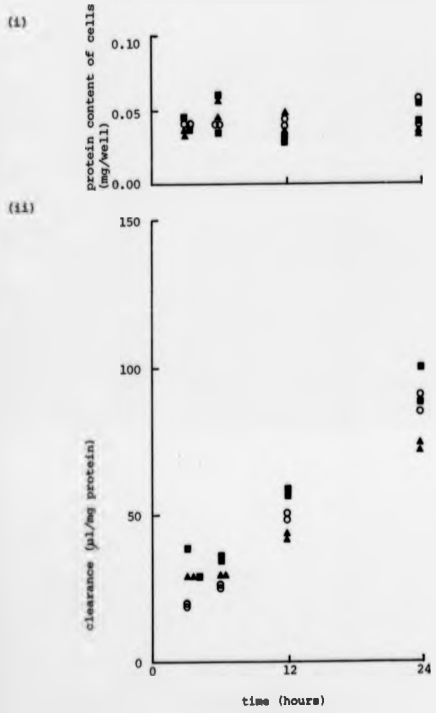


Figure 7.3.3.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-0 cells

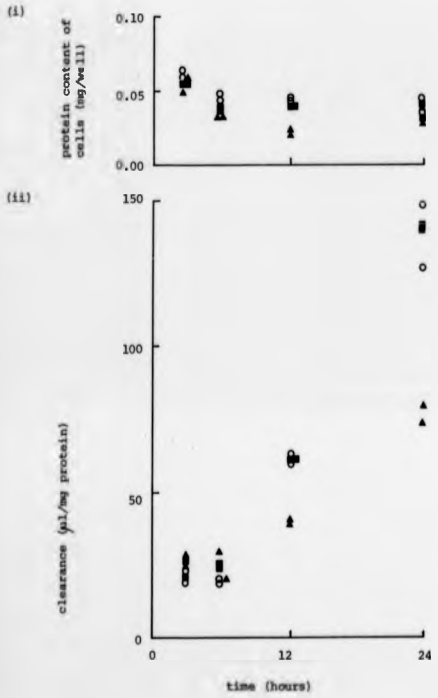


Table 7.4.

Endocytic Indices for the clearance of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer (10μg/cm^3) by Dsy-0 cells in the presence or absence of modifier

Donors	No modifier (control)	Colchicine (100μg/cm^3)	EGTA (5mmoles/litre)
P.R.	3.87 (100%)	2.94 (75%)	4.43 (114%)
		2.11* (55%)	4.48* (115%)
R.D.	3.42 (100%)	2.45 (72%)	3.14 (92%)
M.S.	6.50 (100%)	2.82 (43%)	6.55 (101%)

The figures in brackets are the values expressed as a percentage of the matched control (no modifier).

The exact details of these experiments are given in Section 7.3.2.3.

The units of Endocytic Index (see Section 4.2.1.) are μl per mg protein per hour.

*Cells preincubated with the modifier for 20 minutes.

very slight reduction (7 to 13 percent) and colchicine caused a greater decrease (47 to 83 percent). These results were similar to those described for the Day-0 cells.

7.3.2.3. Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-0 cells. The method used for this experiment was similar to that described above (Section 7.3.2.2.), except that the radiolabelled substrate used was ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer at a final concentration of 10μg/cm^3. Day-0 cells were used, obtained from three donors (P.R., R.D., M.S.). In addition to the normal procedure, with cells from donor P.R. (owing to a high yield), it was possible, in additional wells, to add the inhibitors to cells 20 minutes prior to adding the substrate.

Figures 7.3.1. to 7.3.3. and Table 7.4. show the results. It may be seen that the cell-protein values were reasonably constant with duration of incubation, and were not altered in the presence of EGTA or colchicine. All the clearance values progressively increased in a linear fashion between 6 and 24 hours. From the Endocytic Indices shown in Table 7.4., it can be seen that for the control cells the values varied between the donors (3.42, 3.87 and 6.50μl/mg protein/hour). Colchicine considerably reduced the Endocytic Indices, by (25 to 67 percent, and where a preincubation period (20 minutes) with colchicine was carried out a further reduction (20 percent) was seen. EGTA had no clear effect on the Endocytic Indices, even when cells were preincubated with the inhibitor.

Figures 7.4.1. to 7.4.3.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer ($10\mu g/cm^3$) by Day-7 cells obtained from three donors M.R. (Figure 7.4.1.); G.S.E. (Figure 7.4.2.) and M.A. (Figure 7.4.3.). Uptake in the presence of colchicine (▲, $100\mu g/cm^3$), or EGTA (■, 5mmoles/litre) or in the absence (O) of modifiers.

(i) protein content of cells.

(ii) clearance

Each point represents the value obtained from a single cell.

The exact details of these experiments are given in Section 7.3.2.4.

Figure 7.4.1.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells

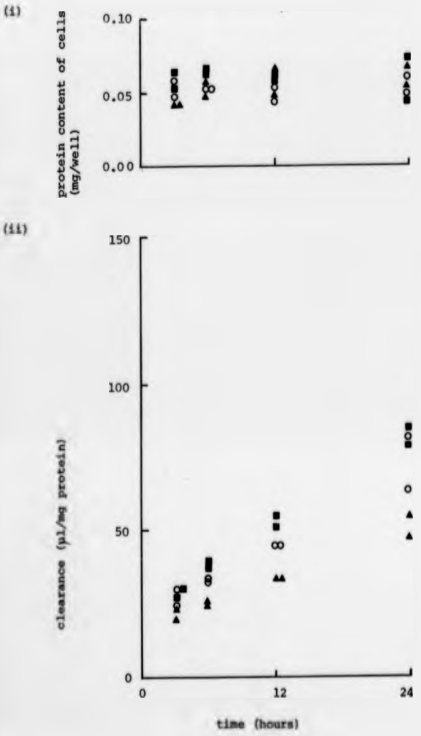


Figure 7.4.2.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells

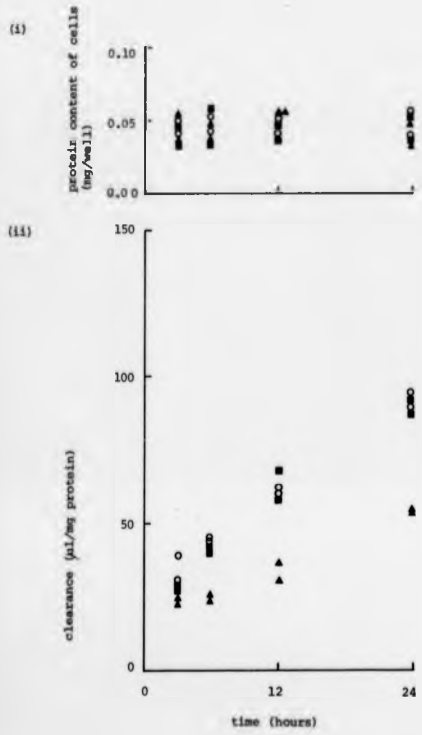


Figure 7.4.3.

Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells

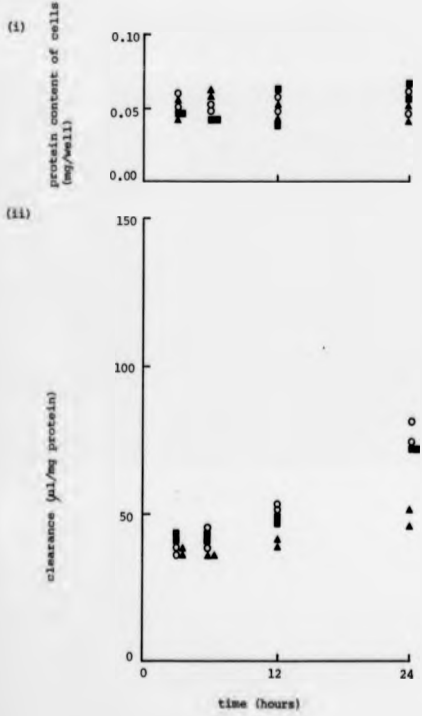


Table 7.5.

Endocytic Indices for the clearance of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer (10μg/cm^3) by Day-7 cells in the presence or absence of modifier

Donors	No modifier (control)	Colchicine (100μg/cm^3)	EGTA (5mmoles/litre)
M.R.	2.36 (100%)	1.44 (61%)	2.49 (105%)
G.S.E.	2.64 (100%)	1.73 (65%)	2.52 (95%)
M.A.	2.09 (100%)	0.73 (35%)	1.83 (88%)

The figures in brackets are the values expressed as a percentage of the matched control (no modifier).

The exact details of these experiments are given in Section 7.3.2.4.

The units of Endocytic Index (see Section 4.2.1.) are μl per mg protein per hour.

7.3.2.4 Uptake of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by Day-7 cells. The procedure used here was similar to that described above (Section 7.3.2.3.), except that Day-7 cells were used (isolated from donors M.R.; G.S.E.; M.A.), and cells were not preincubated with the inhibitors. Figures 7.4.1. to 7.4.3., show the results. Again cell-protein values were constant with duration of incubation and not affected by either EGTA or colchicine. Also, the clearance values increased linearly with duration of incubation between 6 and 24 hours, allowing the calculation of Endocytic Indices (Table 7.5.). The Endocytic Indices (no modifiers) were similar for each of the donors (2.09 to 2.36µl/mg protein/hour); these were slightly lower values than for Day-0 cells. Again colchicine reduced the Endocytic Indices by varying degrees (35 to 65 percent). EGTA had no clear effect on the uptake of the copolymer. The effect of the inhibitors was similar to their effect on the uptake of this substrate by Day-0 cells.

7.3.3. Release studies

7.3.3.1. ^{125}I-labelled polyvinylpyrrolidone. Cells were obtained from three donors (R.G.; J.B.L.; G.W.), and monolayers established as described in Section 4.5.. The release experiment described in Section 7.2.2. was carried out on both Day-0 and Day-7 cells from each donor. First, cells were incubated (0.5 or 24 hours) with ^{125}I-labelled polyvinylpyrrolidone (50 $\mu g/cm^3$) in the presence of suramin (500 $\mu g/cm^3$). Then cells were washed and either harvested or reincubated for a further hour as described in Section 7.2.2., then washed and harvested. It should be noted that the re-incubated cells underwent an extra washing procedure.

Table 7.6.

Release experiments, cells incubated with ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin)

(i) Day-0 cells obtained from donor R.G.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	333 339	0.093 0.092	3,581 3,685
Incubation (24hr) with hot substrate*	1,598 1,161	0.095 0.080	16,821 14,513
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	2,193 1,921	0.135 0.132	16,244 14,553
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	1,844 1,883	0.137 0.119	13,460 15,824
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	1,490 1,792	0.093 0.096	16,022 18,666

(ii) Day-7 cells obtained from donor R.G.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	410 366	0.042 0.041	9,762 8,900
Incubation (24hr) with hot substrate*	555 436	0.052 0.052	10,673 8,385
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	424 550	0.045 0.051	9,089 10,385
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	575 458	0.054 0.054	10,777 8,778
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	541 432	0.052 0.050	10,914 8,690

* ^{125}I-labelled polyvinylpyrrolidone (50$\mu g/cm^3$) in the presence of suramin (500$\mu g/cm^3$)

** polyvinylpyrrolidone (500$\mu g/cm^3$) in the presence of suramin (500$\mu g/cm^3$)

t Background = 25 c.p.m.

The exact details of these experiments are given in Section 7.3.3.1.

Table 7.7.

Release experiments, cells incubated with ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin)

(i) Day-0 cells obtained from donor J.B.L.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	171 177	0.091 0.092	1,879 1,837
Incubation (24hr) with hot substrate*	1,594 1,714	0.117 0.107	13,624 16,019
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	1,183 1,036	0.077 0.076	15,364 13,632
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	1,020 1,050	0.094 0.085	10,851 12,353
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	1,211 1,639	0.069 0.101	17,550 16,228

(ii) Day-7 cells obtained from donor J.B.L.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	501 416	0.049 0.043	10,224 9,674
Incubation (24hr) with hot substrate*	1,613 950	0.056 0.028	28,804 33,929
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	1,340 1,613	0.046 0.056	29,130 28,804
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	1,449 978	0.047 0.028	27,213 34,929
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	2,204 1,044	0.056 0.025	38,000 41,760

* ^{125}I-labelled polyvinylpyrrolidone (50μg/cm^3) in the presence of suramin (500μg/cm^3)

** Polyvinylpyrrolidone (500μg/cm^3) in the presence of suramin (500μg/cm^3)

t Background = 25 c.p.m.

The exact details of these experiments are given in Section 7.3.3.1.

Table 7.8.

Release experiments, cells incubated with ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin)

(i) Day-0 cells obtained from donor G.W.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	187 196	0.084 0.086	2,036 2,279
Incubation (24hr) with hot substrate*	1,441 1,534	0.113 0.119	12,752 12,891
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	920 1,153	0.093 0.098	9,892 11,765
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	1,334 940	0.105 0.081	12,705 11,605
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	1,815 1,541	0.094 0.095	19,309 16,221

(ii) Day-7 cells obtained from donor G.W.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	360 353	0.041 0.043	8,780 8,209
Incubation (24hr) with hot substrate*	1,025 1,108	0.051 0.050	20,008 22,160
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	1,015 1,106	0.052 0.051	19,519 21,686
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	940 1,091	0.050 0.051	18,800 21,392
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	1,047 957	0.045 0.044	23,266 21,750

* ^{125}I-labelled polyvinylpyrrolidone (50µg/ml) in the presence of suramin (500µg/ml)

** Polyvinylpyrrolidone (500µg/ml) in the presence of suramin (500µg/ml)

t Background = 25 c.p.m.

The exact details of these experiments are given in Section 7.3.3.1.

Tables 7.6. to 7.8. show the results. It is seen that mostly duplicate values were reasonably close. The cell-protein values were not lower in those wells that had been subjected to two washing protocols, rather than (the usual) one, indicating that all the loosely adherent cells were removed during the first washings.

When cells (Day-0 or Day-7) were incubated (for 0.5 hours) with ^{125}I-labelled polyvinylpyrrolidone (50$\mu g/cm^3$) in the presence of suramin (500$\mu g/cm^3$) the values obtained sometimes differed between cells at different stages of maturation, and were mostly not very much higher than double the amount of radioactivity adhering to cell-free wells. The amount of cell-associated radioactivity (normalized to cell-protein) increased between the 0.5 and 24 hour incubation periods, in all but one case (Day-7 cells; donor R.G.). The increase was 4 to 8 fold in Day-0 cells and 2 to 3 fold in Day-7 cells. These increases indicated that in most cases the substrate was being captured during this initial incubation period. The very slow rate of capture seen with Day-7 cells (donor R.G.) is difficult to explain as we were reasonably confident that the cells had differentiated (by examination of cell morphology microscopically). When cells that had been incubated with radiolabelled substrate for 24 hours were reincubated in substrate-free medium, there were virtually no differences in the cell-associated radioactivity values. Even when an excess of cold substrate was included in the reincubation medium, to compete with any surface-bound substrate, little difference was seen in the values. Since there was no rapid loss of substrate, we conclude that little (if any) substrate was just adhering to the cell membrane. In addition, if cells were reincubated with the radiolabelled substrate for a further hour, no difference was seen between the values. Although one would not

Table 7.9.

Release experiments, cells incubated with ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer

(i) Day-0 cells obtained from donor C.C.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	16 20	0.052 0.028	308 714
Incubation (24hr) with hot substrate*	147 194	0.038 0.048	3,868 2,958
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	142 305	0.045 0.077	3,156 3,961
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	192 155	0.050 0.051	3,840 3,039
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	180 168	0.050 0.050	3,600 3,160

(ii) Day-7 cells obtained from donor C.C.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	10 11	0.058 0.052	172 211
Incubation (24hr) with hot substrate*	162 78	0.050 0.029	3,240 2,690
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	173 230	0.074 0.068	2,338 3,382
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	180 218	0.064 0.068	2,873 2,735
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	248 184	0.072 0.060	3,444 2,900

* ^{125}I-labelled vinylamine-polyvinylpyrrolidone copolymer (10µg/ml)
** Cold vinylamine-polyvinylpyrrolidone copolymer (100µg/ml)
t Background = 25 c.p.m.

The exact details of these experiments are given in Section 7.3.3.2.

Table 7.10.

Release experiments, cells incubated with ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer

(i) Day-0 cells obtained from donor N.M.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	7 10	0.058 0.062	121 161
Incubation (24hr) with hot substrate*	151 48	0.049 0.014	2,857 3,429
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	169 93	0.054 0.025	2,864 3,720
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	142 143	0.039 0.054	3,641 2,648
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	119 157	0.055 0.035	2,164 4,486

(ii) Day-7 cells obtained from donor N.M.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	20 16	0.058 0.069	344 232
Incubation (24hr) with hot substrate*	93 93	0.027 0.050	3,444 1,860
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	252 161	0.070 0.065	3,600 2,477
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	171 185	0.071 0.072	2,408 2,569
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	201 149	0.054 0.072	3,722 2,069

* ^{125}I-labelled vinylamine-polyvinylpyrrolidone copolymer (10µg/ml)
** Cold vinylamine-polyvinylpyrrolidone copolymer (100µg/ml)
t Background = 25 c.p.m.

The exact details of these experiments are given in Section 7.3.3.2.

Table 7.11.

Release experiments, cells incubated with ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer

(i) Day-0 cells obtained from donor M.O.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	11 10	0.069 0.044	159 227
Incubation (24hr) with hot substrate*	49 62	0.016 0.013	3,062 4,769
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	142 160	0.027 0.029	3,471 3,069
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	135 120	0.037 0.040	3,649 3,000
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	138 148	0.059 0.033	3,538 4,485

(ii) Day-7 cells obtained from donor M.O.

	Radioactivity of cells (c.p.m. per well; corrected for background)[t]	Protein content of cells (mg per well)	Radioactivity of cells expressed c.p.m. per mg protein
Incubation (0.5hr) with hot substrate*	11 11	0.069 0.062	159 177
Incubation (24hr) with hot substrate*	157 169	0.064 0.064	2,453 2,640
Incubation (24hr) with hot substrate*, then replaced with medium (1hr)	186 207	0.094 0.084	1,979 2,464
Incubation (24hr) with hot substrate*, then replaced with cold substrate** (1hr)	201 132	0.069 0.082	2,913 1,610
Incubation (24hr) with hot substrate*, then replaced with hot substrate* (1hr)	164 142	0.055 0.050	2,982 2,841

* ^{125}I-labelled vinylamine-polyvinylpyrrolidone copolymer (10µg/ml)
** Cold vinylamine-polyvinylpyrrolidone copolymer (100µg/ml)
t Background = 25 c.p.m.

The exact details of these experiments are given in Section 7.3.3.2.

expect to see a further increase over such a short period, this step demonstrated that the extra washing procedure itself could not be affecting the values.

7.3.3.2. ^{125}I-Labelled vinylamine-vinylpyrrolidone copolymer.

These experiments were similar to those just described, except that the incubations with substrate were with ^{125}I-labelled or non-iodinated vinylamine-vinylpyrrolidone copolymer (10 and 100mg/cm^3 respectively). The cells (Day-0 and Day-7) were obtained from three donors (C.C.; N.M.; and M.O.).

Tables 7.9. to 7.11. show the results. As with the experiments just described, duplicate values were reasonably close, and for the most part cell-protein values were constant throughout the duration of each experiment. The cell-associated radioactivity values obtained for the short (0.5 hour) incubations, were close to double the amount of radioactivity adhering to cell-free wells, and in all cases the radioactivity values (expressed per mg protein) increased, between the 0.5 and 24 hour incubation periods (Day - 0 cells 5 to 12 fold; Day-7 cells 7 to 16 fold). This indicates that the substrate was being captured during the initial incubation period. When cells (previously incubated with the substrate for 24 hours) were reincubated in either medium alone or with an excess of non-iodinated substrate, although the values were very scattered, there was no obvious difference between the values and the 24-hour uptake values. This indicated that most of the substrate had been genuinely internalized by the cells over the 24-hour period. Again, when cells were reincubated in hot substrate the cell-associated radioactivity values were similar to the 24-hour uptake values.

7.4. DISCUSSION

As stated at the beginning of this chapter, we had already demonstrated that each of the two radiolabelled substrates accumulated progressively over a prolonged time-period. This result is itself indicative of uptake by pinocytosis, but in this chapter other relevant parameters have been studied.

7.4.1. Effect of modifiers on cell-protein

It was planned to study the effect on pinocytosis of a wide range of modifiers: low temperature, calcium chelating agents, and metabolic and cytoskeletal inhibitors. Preliminary studies showed however that most of the modifiers (apart from EGTA and colchicine) caused significant and sometimes drastic reductions in the cell-protein values, indicating cell-loss. Low temperatures, the cytoskeletal inhibitor cytochalasinB (5±10$\mu g/cm^3$), and the metabolic inhibitors 2,4-dinitrophenol (10±250$\mu g/cm^3$) and sodium fluoride (10±100$\mu g/cm^3$) all resulted in lower cell-protein values. This effect was highly consistent with cells from different donors (see Section 7.3.1.). Both freshly isolated cells (Day-0) and differentiated cells (Day-7) were similarly affected by the modifiers except by low temperatures, where cell-protein values were much lower when Day-0 cells were subjected to either 20°C or 4°C. This was not surprising, for not only are monocyte-derived macrophages thought to have a greater capacity than monocytes to adhere to substrates, but also in many monocyte isolation procedures low temperatures are used to stop the cells adhering.

It was not clear from these experiments whether the cell losses (inferred by the reduced protein values) were due to cell death, which would lead to cell detachment, or to viable cells simply losing their adherent properties. This question could easily be resolved by using trypan blue to test the viability of the detached cells. However, it was decided not to pursue this further because, whatever the outcome, many of the modifiers were considered unsuitable for use in the planned experiments on pinocytosis, for the following reasons. First, when the treated cells were examined (microscopically), they were often seen to display atypical morphology: all the cells were very rounded and often very refractile. The population may therefore behave atypically. Secondly, it would be highly probable that the remaining cells would be a selected subpopulation, resistant to the effects of the inhibitor, and may behave differently (different pinocytic activity) to the population as a whole. For these reasons it was decided to use colchicine and EGTA only in the studies on pinocytosis. With both of these agents cell-protein values were not affected, and cells when examined microscopically displayed typical morphology, similar to that described in Section 3.3.8..

It was difficult to compare these results with previous reports because, although all the inhibitors we used (except EGTA) are known to be cytotoxic (at slightly higher concentrations than we used) to rat and mouse peritoneal macrophages and rat kupffer cells (Munthe-Kaas, 1977; Pratten & Lloyd, 1979), the criteria used to determine cytotoxicity were not clear. Cell-protein values were certainly not determined and so cell loss may have been undetected at lower concentrations of the modifiers. This point is discussed further in the next Section 7.4.2..

7.4.2. Effect of modifiers on pinocytosis

For the reasons given in the previous section we only studied the effects of the two modifiers colchicine and EGTA on the pinocytic activity of human monocytes/macrophages. The Endocytic Indices obtained for ^{125}I-labelled polyvinylpyrrolidone and ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer in the absence of either agent were similar to those previously reported (Chapter 6) using similar-aged cells. Therefore we were confident that our measurements were reproducible.

The cytoskeletal inhibitor colchicine (100 $\mu g/cm^3$), consistently reduced the rates at which both ^{125}I-labelled polyvinylpyrrolidone and ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer were captured by both Day-0 and Day-7 cells. Although the extent of inhibition varied between cells from different donors (25 to 83 percent reduction), there was no indication that it depended on the stage of differentation of the cells, or on the substrate being captured. Since this inhibitor did not affect the cell-protein values or the morphology of the cells, we were confident that this was genuine inhibition of uptake, and that we were not simply measuring the pinocytic activity of a selected subpopulation of cells. Although the effect of colchicine on the uptake of these two substrates by mononuclear phagocytes has not previously been reported, Pratten & Lloyd (1979) using rat peritoneal macrophages showed that colchicine (100 $\mu g/cm^3$) greatly inhibited the capture of ^{125}I-labelled polyvinylpyrrolidone and colloidal ^{198}Au (by about 90 and 50 percent respectively). Interestingly, when a lower concentration of colchicine (10 $\mu g/cm^3$) was used, although the uptake of colloidal ^{198}Au was still greatly inhibited, that of ^{125}I-labelled

polyvinylpyrrolidone was not, indicating that the inhibition is substrate-dependent. These results are difficult to understand for, even when substrates are captured adsorptively or in the fluid-phase, the same vesicles are involved. Munthe-Kaas (1977) also reported that colchicine inhibited the capture of colloidal ^{198}Au by mononuclear phagocytes (rat kupffer cells and mouse peritoneal macrophages). However in the latter study, inhibition was only achieved at very high colchicine concentrations (4000$\mu g/cm^3$). Such concentrations were reported to cause atypical morphology (i.e. rounded cells) and, although cells were still viable (determined by trypan blue), it is unlikely that colchicine at this concentration is affecting only the microtubules. As stated in the Introduction, care must be taken when interpreting inhibition studies such as these since, if cell loss is not monitored, it is possible that false conclusions may be drawn. In many studies (e.g. that of Munthe-Kaas, 1977), the results can be criticised, since the amount of radiolabelled substrate captured is expressed in terms of the number of cells initially present, and any cell-loss caused by the inhibitor is not accounted for; one could therefore simply be measuring the effect of the inhibitor on cell-lose and not on pinocytic activity.

On the one occasion when it was also possible to preincubate the cells with colchicine 20 mintues prior to adding the radiolabelled substrate, a greater degree of inhibition was found (approximately 20 percent more). This result was compatible with the report by Starling et al. (1983), in which adelay (2.3 hours) was reported in the inhibitory effect of colchicine on the uptake of ^{125}I-labelled polyvinylpyrrolidone by rat visceral yolk-sacs. Starling et al. (1983) further showed that colchicine entered the cells very rapidly and therefore concluded that the delay was not due to poor drug

permeation into the cell, but to the way in which it interacted with tubulin to bring about the dysfunction of the microtubules.

In contrast to colchicine, we found that EGTA (5mmoles/litre) caused little if any reduction in the clearance of ^{125}I-labelled polyvinylpyrrolidone and ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer by monocyte/macrophages even if cells were preincubated with the inhibitor.

It was difficult to understand why EGTA did not inhibit the capture of the radiolabelled substrates by monocyte/macrophages, while colchicine did. We were confident that the concentration of EGTA used was sufficient to chelate the calcium in the culture medium. Therefore we concluded that either, EGTA does not inhibit pinocytosis in human monocyte/macrophages or, that the substrates were not being captured pinocytically. The latter conclusion was less probable though for, as summarized below (Section 7.4.4.), our other results pointed to uptake by pinocytosis.

Our results contrasted sharply with another study using mononuclear phagocytes: Pratten & Lloyd (1979), using rat peritoneal macrophages, reported that EGTA (5 mmoles/litre) greatly inhibited the uptake of the fluid-phase substrate ^{125}I-labelled polyvinylpyrrolidone, the effect being abolished in the presence of equimolar calcium chloride.

7.4.3. Release studies

The results described in Section 7.3.3.1. indicate that the cell-associated radioactivity of cells (Day-0 or Day-7) incubated with ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin)

represented genuine internalization of the substrate. If the cell-associated radioactivity was just surface-bound substrate, we would have expected rapid and drastic reductions in the amounts of radioactivity remaining associated with the cells when they were reincubated in either substrate-free medium or in medium containing non-iodinated substrate. Results of similar studies carried out with the ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer showed that this substrate too was being internalized by the cells (Day-0 and Day-7).

7.4.4. Concluding remarks

In summary, we have shown that colchicine (100μg/cm^3) significantly inhibited the uptake of ^{125}I-labelled polyvinylpyrrolidone (in the presence of suramin), and of ^{125}I-labelled vinylamine-vinylpyrrolidone copolymer, by monocyte/macrophages. This is consistent with capture being by pinocytosis. Secondly, we demonstrated that EGTA (5 mmoles/litre) had no marked effect on the capture of these substrates. This suggests that either pinocytosis was not responsible for the uptake or, if it was, then pinocytosis in these cells is not affected by this modifier. Thirdly, the release experiments show that both substrates were truly internalized by the cells and are not simply adsorbed to the cell surface (to any appreciable extent). This again implies that pinocytic capture was occurring. Taken as a whole, the results just described, and also the previous evidence for prolonged progressive capture of each substrate by monocyte/macrophages, point to the conclusion that the uptake of radiolabelled macromolecules described in Chapter 6 was by pinocytosis. However the failure to inhibit this capture by EGTA surrounds this conclusion with some element of doubt.

CHAPTER 8

Uptake of polystyrene beads

8.1. INTRODUCTION

In the previous chapters (6 and 7), we have been concerned with the uptake into monocyte/macrophages of two soluble macromolecules, polyvinylpyrrolidone in the presence of suramin and the cationic copolymer vinylamine-vinylpyrrolidone. In order to consider the results in a broader context, we considered it important to study also the capture of particles by cells cultured in the same conditions as used for the studies of pinocytosis.

Phagocytosis is the capture of micro-particles. Most of the methods used to study the phenomenon are morphological. For example, capture of particulate substrates (commonly polystyrene (latex) beads, sheep red blood cells or microorganisms) is usually determined by light-microscopy, and data expressed in terms of the percentage of cells ingesting the particle. However, there are two main drawbacks to this approach. First, for accurate results, large numbers of cells need to be assessed, and consequently the procedure is time-consuming, particularly if an attempt is made to estimate the average number of particles ingested by each cell. Also, it is often difficult to assess whether the substrate has truly been ingested, or is merely bound to the cell surface. The use of radiolabelled substrates is a more convenient way of measuring phagocytosis. In 1984 Pratten and Lloyd reported a simple, highly-sensitive assay for phagocytosis using ^{125}I-labelled polystyrene beads. Using rat peritoneal macrophages, they made a parallel study in which the new radiometric and the more conventional microscopic assay were compared. They showed that both methods gave quantitatively very similar data on phagocytosis; the radioactive assay was however very much less time-consuming. The only drawback to the new method is

that, since net uptake is determined, one cannot assess differential activities of cells within the population. Using this new technique, Pratten & Lloyd (1986) recently reported the uptake by rat peritoneal macrophages of polystyrene beads (100nm to 1100nm diameter). They found that the largest beads were captured at seven-times the rate of the smallest. It was reported that colchicine (a known inhibitor of pinocytosis in their cell-system), greatly inhibits the capture of the smallest beads, but not of the largest. For the intermediate sizes, the degree of inhibition by colchicine decreased as the particle-size increased. It was therefore concluded that the smallest beads are captured solely by pinocytosis, and the largest by phagocytosis. The intermediate sizes of beads appeared to be captured by both processes, the contribution of phagocytosis steadily increasing with increasing particle size.

In the present work the use of radiolabelled polystyrene beads was considered to be particularly valuable. By studying the capture of both 1100nm and 100nm diameter beads, it would be possible to measure phagocytosis and at the same time, using an almost identical substrate, supplement the previous data on pinocytosis.

We have therefore measured the uptake of 100nm and 1100nm diameter polystyrene beads by cells at various stages in their development.

8.2. EXPERIMENTAL METHODS

8.2.1. Preparation of ^{125}I-labelled polystyrene beads

Polystyrene beads (100nm and 1100nm diameter) were obtained from Sigma Chemical Co. , Dorset, U.K. and radiolabelled as described by

Pratten & Lloyd (1984) using the Chloramine T method developed by Hunter et al. (1963). A suspension (10 percent w/v) of beads (100nm or 1100nm diameter; 100µl) was placed in a 25cm^3 universal container, which was maintained on ice throughout the reaction. Cold (4°C) phosphate buffer (4.5cm^3; pH 8.0; 0.05 moles/litre) was added to the beads and the solution mixed. Then [^{125}I]iodide (0.5 µCi in 5µl) was added to the reaction vessel using a Hamilton syringe. Chloramine T (1 mg/cm^3; 2.0 cm^3) was added and the mixture agitated gently (8 mins; 4°C). Sodium metabisulphite (2mg/cm^3; 1.5cm^3) and a small crystal of potassium iodide was added, and the solution mixed. The solution was then dialysed at 4°C against NaCl (1 percent w/v; 5 litres) for 3 to 4 days with twice-daily changes of the sodium chloride solution. The samples were stored at 4°C.

The amount of free [^{125}I]iodide in the radiolabelled samples was determined by electrophoresis as described in Section 2.2.16.. This test was carried out immediately following the dialysis and immediately prior to use. An experiment was also conducted to determine whether the radiolabel remained attached in experimental conditions, i.e. in culture medium at 37°C for 6 hours.

8.2.2. Measuring uptake of polystyrene beads.

The procedure used to measure the uptake of polystyrene beads was essentially the same as the method used for the previous uptake studies, described in Section 4.5.. The exact details of the experiments are given with the results.

8.3. RESULTS

8.3.1. Characteristics and stability of ^{125}I-labelled polystyrene beads.

The labelling efficiency of the small (100nm diameter) beads was 52 percent, and of the large (1100nm) beads 13 percent. After dialysis both radiolabelled preparations contained less than 1 percent [^{125}I]iodide. The preparations were considered stable, because storage at 4°C (up to 2 months) or incubation in culture medium (37°C; 12 hours) did not alter the percent of free [^{125}I]iodide.

8.3.2. Uptake by Day-4 cells.

In this initial experiment monocytes were cultured for 4 days in standard conditions (Section 4.5.). Radiolabelled polystyrene beads were added at various times, by removing 0.1cm^3 of culture medium and replacing it with 0.1cm^3 of a warmed solution containing either small (100nm diameter) or large (1100nm diameter) beads, to a final concentration of 50 µg/cm^3. Addition of the beads was at 2, 4 or 6 hours before the termination of the incubation. These were slightly longer incubation periods (i.e. up to 6 hours rather than 2.5 hours), and higher (x5) concentrations of polystyrene beads than those of Pratten et al. (1986). These modifications were made to increase the possibility of detecting capture of beads. Additionally beads were incubated for 6 hours in cell-free wells containing culture medium only, to determine whether there was any adsorption to the well itself.

Figures 8.1.1. and 8.1.2.

Uptake of polystyrene beads 100nm diameter (Figure 8.1.1.) or 1100nm diameter (Figure 8.1.2.) by Day-4 monocyte/macrophages obtained from two donors G.S. (●) and A.S. (△).

(i) protein content of cells.

(ii) cell-associated radioactivity.

(iii) clearance.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 8.3.2.

Figure 8.1.1.

Uptake of 100nm diameter polystyrene beads by Day-4 cells

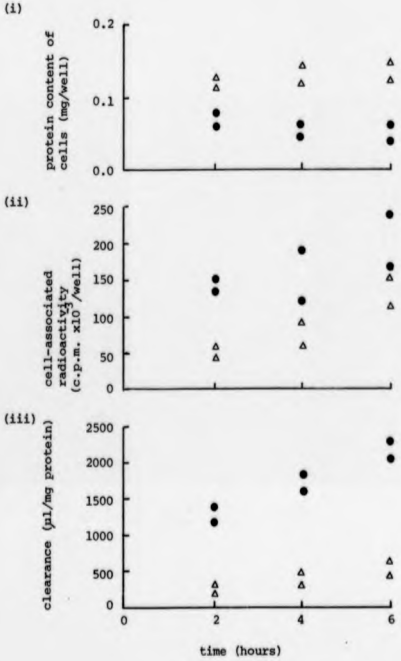


Figure 8.1.2.

Uptake of 1100nm diameter polystyrene beads by Day-4 cells

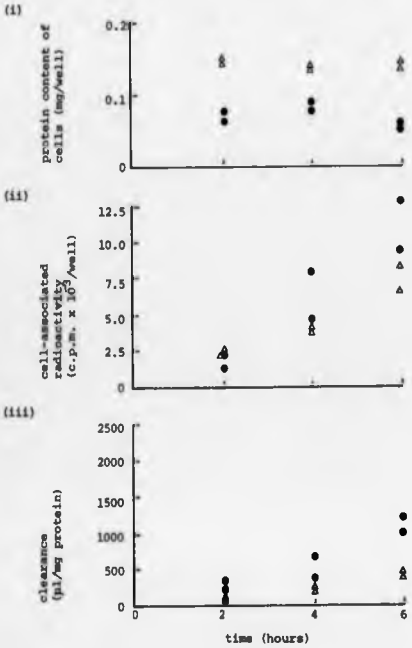


Table 8.1.

Endocytic Indices for the clearance of small (100nm diameter) and large (1100nm diameter) polystyrene beads by Day-4 monocyte/macrophages.

Donors	diameter of beads	
	100nm	1100nm
G.S.	223.0	222.0
A.S.	81.8	66.8

The exact details of these experiments are given in Section 8.3.2. The units of Endocytic Index (see Section 4.2.1.) are µl per mg protein per hour.

Figures 8.2.1. to 8.2.3.

Uptake of 100nm diameter polystyrene beads by Day-7 monocyte/macrophages obtained from three donors G.S. (Figure 8.2.1.); A.S. (Figure 8.2.2.) and L.S. (Figure 8.2.3.). Uptake in the presence (O) or absence (●) of colchicine ($50\mu g/cm^3$; 3 hour pre-incubation period) is shown.

(i) protein content of cells.

(ii) cell-associated radioactivity.

(iii) clearance.

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 8.3.3.

Figure 8.2.1.

Uptake of 100nm diameter polystyrene beads by Day-7 cells

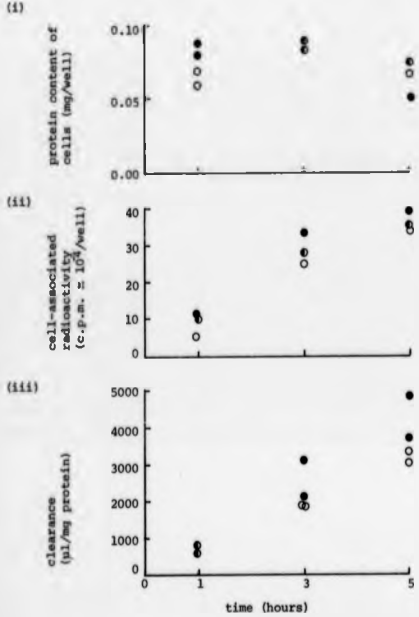


Figure 8.2.2.

Uptake of 100nm diameter polystyrene beads by Day-7 cells

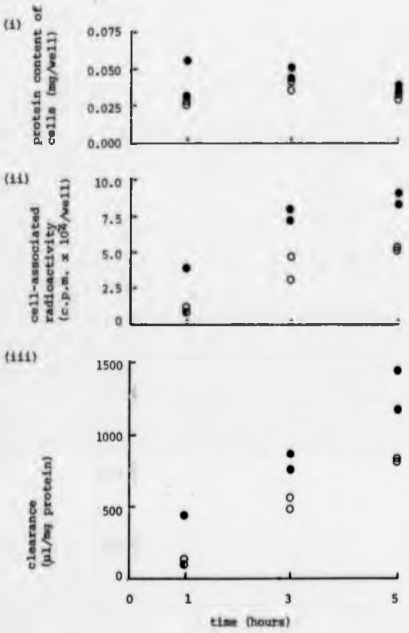
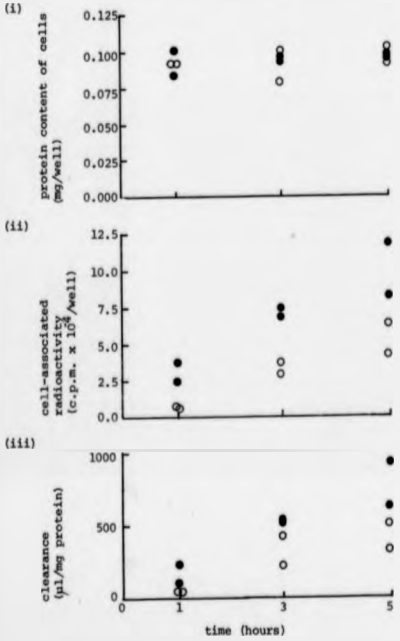


Figure 8.2.3.

Uptake of 100nm diameter polystyrene beads by Day-7 cells



Figures 8.2.4. to 8.2.6.

Uptake of 1100nm diameter polystyrene beads by Day-7 monocyte/macrophages obtained from three donors G.S. (Figure 8.2.4.); A.S. (Figure 8.2.5.) and L.S. (Figure 8.2.6.). Uptake in the presence (O) or absence (●) of colchicine (50 $\mu g/cm^3$; 3 hour pre-incubation period) is shown.

(i) protein content of cells.

(ii) cell-associated radioactivity.

(iii) clearance

Each point represents the value obtained from a single well.

The exact details of these experiments are given in section 8.3.3.

Figure 8.2.4.

Uptake of 1100nm diameter polystyrene beads by Day-7 cells

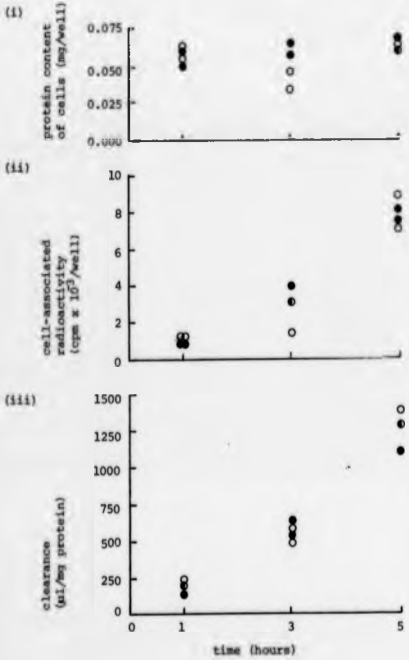


Figure 8.2.5.

Uptake of 1100nm diameter polystyrene beads by Day-7 cells

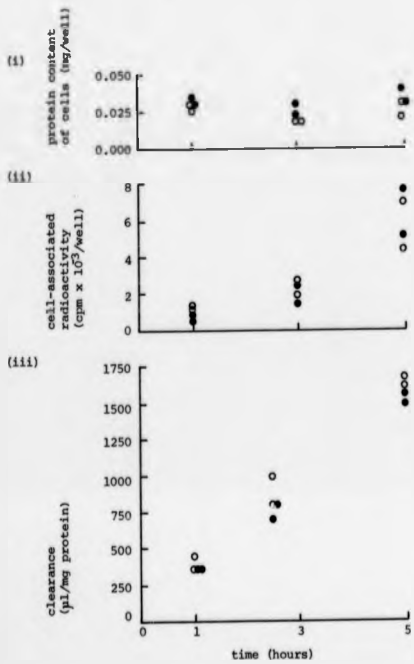


Figure 8.2.6.

Uptake of 1100nm diameter polystyrene beads by Day-7 cells

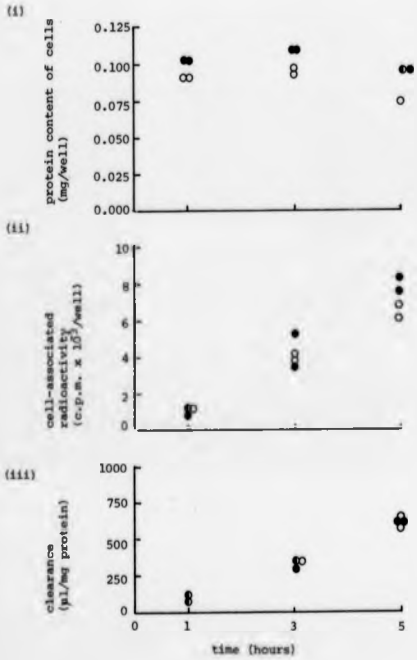


Table 8.2.

Endocytic Indices for the clearance of (i) small (100nm diameter) and (ii) large (1100nm diameter) polystyrene beads, by Day-7 monocyte/macrophages in the presence and absence of colchicine (50 µg/well)

	Donors	colchicine absent	colchicine present	
(i)	G.S.	890	623	(70%)
	A.S.	254	168	(66%)
	L.S.	151	95	(61%)
(ii)	G.S.	259	276	(107%)
	A.S.	302	339	(112%)
	L.S.	138	132	(96%)

The figures in brackets are the values expressed as a percentage of the matched control (colchicine absent). The exact details of these experiments are given in Section 8.3.3.

The units of Endocytic Index (see Section 4.2.1.) are µl per mg protein per hour.

Two donors (G.S., A.S.) were used for this experiment. Figures 8.1.1. and 8.1.2. show the data obtained, expressed as uptake per well and as uptake per mg cell protein. In most cases the values for duplicate wells were close. In all cases the amount of radioactivity found in cells increased with duration of incubation and, when normalized to cell-protein, the uptake was reasonably linear with time, permitting the calculation of an Endocytic Index (Table 8.1.). Although the absolute values for Endocytic Index were 2 to 3 times higher with G.S. cells than with A.S. cells, there was with both donors little difference in the rate of uptake of the two sizes of bead. For each size of particle, the amount of radioactivity associated with cell-free wells was found to be very low, i.e. nearly always less than ten percent of the amount associated with the cells.

8.3.3. Uptake by Day-7 cells.

There were sufficient monocytes from the two donors G.S. and A.S. to undertake a study of uptake after a further three days in culture. Day-7 cells were also available from a third donor (L.S.). In these experiments the protocol was as described above for Day-4 cells, with two differences. The incubations with polystyrene beads were for shorter, but more convenient periods of 1, 3, or 5 hours, and in some cases the microtubule-assembly inhibitor colchicine ($50\mu g/cm^3$) was added 3 hours prior to adding the radiolabelled beads. Figures 8.2.1. to 8.2.6. show the results. Again for each donor values for duplicate wells were reasonably close. In all cases the amount of radioactivity associated with the cells increased over the five-hour incubation period. When the uptake values were normalised to cell-protein, all the clearance values rose linearly with time,

allowing the calculation of Endocytic Indices (Table 8.2.). For both sizes of bead, there was some variation between donors in the values for Endocytic Index. The clearance rates of small (100nm diameter) beads by cells from two of the donors (A.S. and L.S.) were not too dissimilar, whereas the rate for cells from G.S. was several times greater. With the large beads (1100nm diameter) the donor-dependence of Endocytic Index was not so great: all three values lay between 130 and 310 µl/mg protein/hour.

In most cases, colchicine had little or no effect on the protein content of cells (Fig. 8.2.1. to 8.2.6.). With the small beads as substrate, the Endocytic Index was reduced by the presence of colchicine to 60-70 percent of the matched control value. However, with the large beads, colchicine did not reduce the Endocytic Indices (Table 8.2.).

Due to donor-dependent variations it was not possible to make a simple comparison between data on cells of different ages. However with two donors (G.S. and A.S.) Day-4 cells from the same blood sample had been studied, permitting an assessment of whether there were any changes in Endocytic Index with age of cells. Comparison of Table 8.1. and Table 8.2. shows that over the extra three days in culture, approximately a three-fold increase in the capture of the small beads occurred. With the larger beads a significant increase was only observed with cells from one donor (A.S.).

It was also of interest to study the uptake of polystyrene beads in younger cells, and therefore in the experiments described next, Day-1 cells were employed.

Figures 8.3.1. to 8.3.3.

Uptake of polystyrene beads 100nm diameter by Day-1 monocyte/macrophages obtained from three donors M.R. (Figure 8.3.1.); I.S. (Figure 8.3.2.); and R.D. (Figure 8.3.3.). Uptake in the presence (O) or absence (●) of colchicine (50$\mu g/cm^3$; 3 hour pre-incubation period) is shown.

(i) protein content of cells.

(ii) cell-associated radioactivity.

(iii) clearance.

Each point represents the value obtained from a single well. The exact details of these experiments are given in Section 8.3.4.

Figure 8.3.1.

Uptake of 100nm diameter polystyrene beads by Day-1 cells

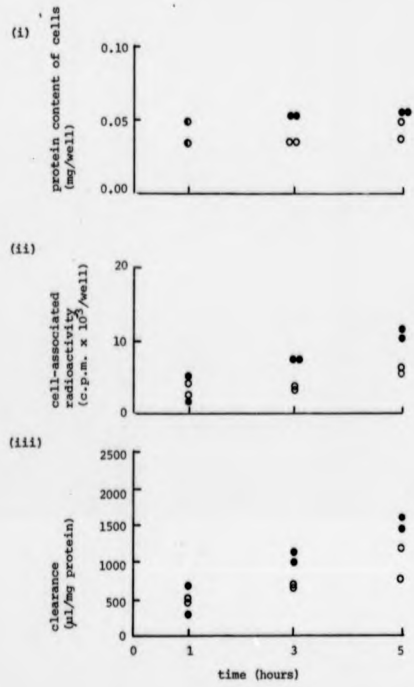


Figure 8.3.2.

Uptake of 100nm polystyrene beads by Day-1 cells

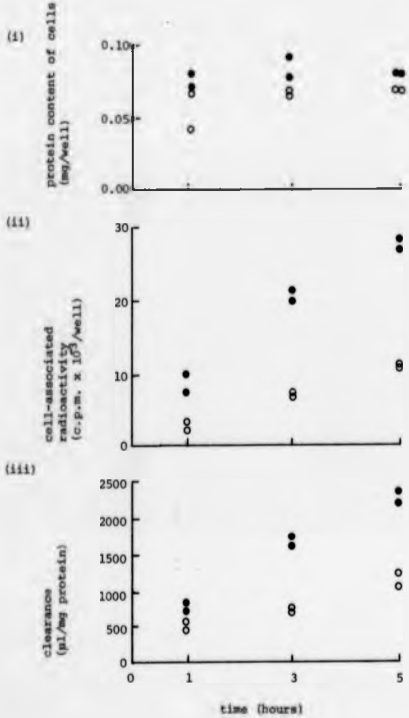
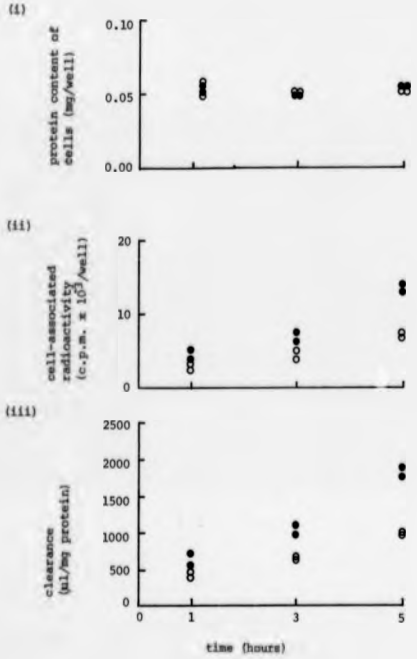


Figure 8.3.3.

Uptake of 100nm diameter polystyrene beads by Day-1 cells



Figures 8.3.4. to 8.3.6.

Uptake of polystyrene beads 1100nm diameter by Day-1 monocyte/macrophages obtained from three donors M.R. (Figure 8.3.4.); I.S. (Figure 8.3.5.) and R.D. (Figure 8.3.6.). Uptake in the presence (O) or absence (●) of colchicine (50$\mu g/cm^3$; 3 hour pre-incubation period) is shown.

(i) protein content of cells.
(ii) cell-associated radioactivity.
(iii) clearance.

Each point represents the value obtained from a single well. The exact details of these experiments are given in Section 8.3.4.

Figure 8.3.4.

Uptake of 1100nm diameter beads by Day-1 cells

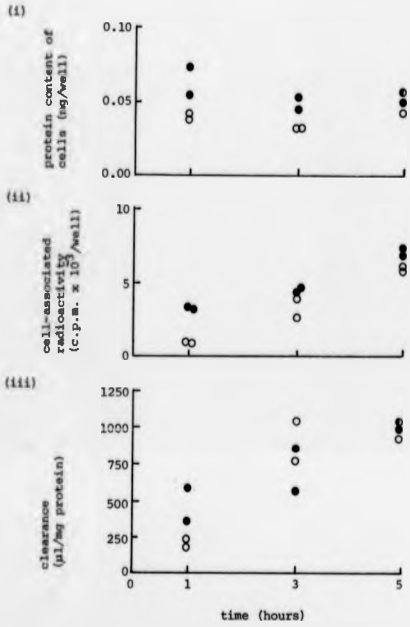


Figure 8.3.5.

Uptake of 1100nm diameter polystyrene beads by Day-1 cells

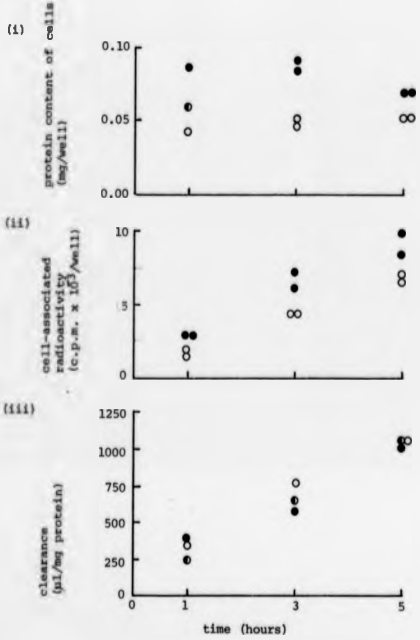


Figure 8.3.6.

Uptake of 1100nm diameter polystyrene beads by Day-1 cells

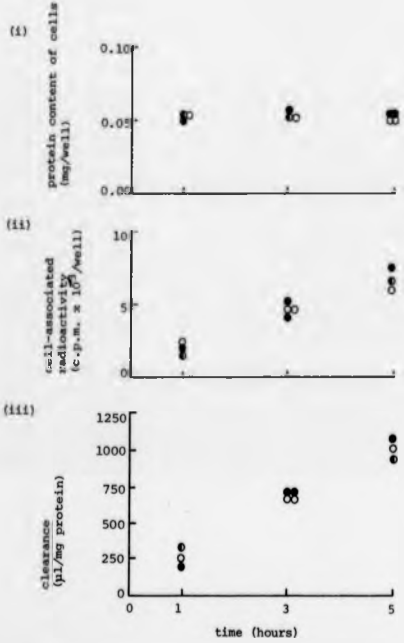


Table 8.3.

Endocytic Indices for the clearance of (i) small (100nm diameter) and (ii) large (1100nm diameter) polystyrene beads by Day-1 monocyte/macrophages in the presence or absence of colchicine (50pg/cm^3)

	Donors	colchicine absent	colchicine present	
(i)	M.R.	262	127	(48%)
	I.S.	384	155	(40%)
	R.D.	301	120	(40%)
(ii)	M.R.	153	190	(124%)
	I.S.	185	203	(110%)
	R.D.	186	168	(90%)

The figures in brackets are the values expressed as a percentage of the matched control (colchicine absent). The exact details of these experiments are given in Section 8.3.4.

The units of Endocytic Index (see Section 4.2.1.) are µl per mg protein per hour.

8.3.4. Uptake by Day-1 cells.

In this experiment, Day-1 cells from three donors (M.R.; I.S.; R.D.) were used. The protocol was the same as that used for Day-7 cells. Figures 8.3.1. to 8.3.6. show the results. In all cases values for duplicate wells were reasonably close. The cell-associated radioactivity increased with time. When uptakes were normalized to cell-protein, graphs of clearance values against time were linear, allowing calculation of Endocytic Indices (Table 8.3.). In this series of experiments there was little donor-dependent variation in the Endocytic Indices for either size of bead. However, the rate of uptake of the smaller beads (100nm diameter) was consistently approximately twice that of the larger beads (1100nm diameter).

Colchicine's effect on cell protein was variable. With donors M.R. and I.S. the protein content of cells was generally slightly less than that measured for the untreated cells, but with R.D.-cells no difference was seen. The effects of colchicine on bead uptake were more consistent. With all three donors the Endocytic Index of the small beads (100nm diameter) was reduced by colchicine to 40-48 percent that of the matched controls. With the large beads (1100nm diameter), colchicine did not much affect the Endocytic Indices.

8.3.5. Uptake of 100nm beads by Day-1 and Day-7 cells obtained from the same donor.

To allow comparisons to be made between young cells (monocytes) and old cells (macrophages), blood was obtained from three further donors (P.M.; G.W.; P.R.) and studied at both 1 and 7 days. Incubations were performed in the same way as the previous experiment but, owing

Figures 8.4.1. to 8.4.3.

Uptake of 100nm diameter polystyrene beads by Day-1 (Δ) and Day-7 (●) monocyte/macrophages obtained from three donors P.H. (Figure 8.4.1.); G.W. (Figure 8.4.2.); and P.R. (Figure 8.4.3.)

(i) protein content of cells

(ii) cell-associated radioactivity

(iii) clearance

Each point represents the value obtained from a single well.

The exact details of these experiments are given in Section 8.3.5.

Figure 8.4.1.

Uptake of 100nm diameter polystyrene beads by Day-1 and Day-7 cells

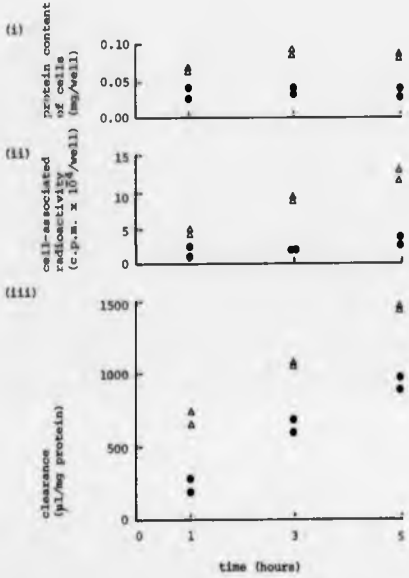


Figure 8.4.2.

Uptake of 100nm diameter polystyrene beads by Day-1 and Day-7 cells

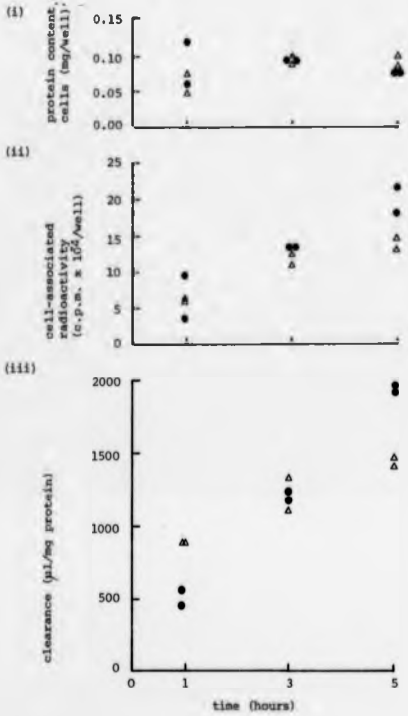


Figure 8.4.3.

Uptake of 100nm diameter polystyrene beads by Day-1 and Day-7 cells

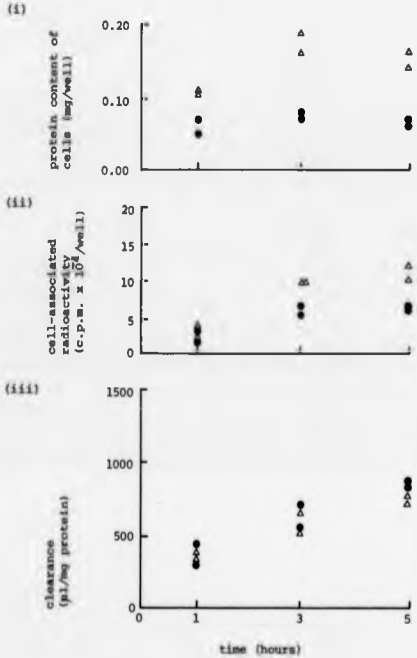


Table 8.4.

Endocytic Indices for the clearance of small (100nm diameter) polystyrene beads by Day-1 and Day-7 monocyte/macrophages

Donors	Duration of cells in culture	
	1 Day	7 Days
P.H.	186	167
G.W.	143	358
P.R.	95	118

The units of Endocytic Index (see Section 4.2.1.) are µl per mg protein per hour.

to cell availability, only one substrate could be used. The small (100nm) beads were chosen in preference to the large, since they had radiolabelled more efficiently (Section 8.3.1.).

Figures 8.4.1. to 8.4.3. show the results. It is seen that duplicate values were reasonably close. In all cases the amount of cell-associated radioactivity increased with the incubation time. When the data were normalised to cell-protein, the clearance values increased linearly with time, allowing the calculation of Endocytic Indices (Table 8.4.). The data show that with donors P.H. and P.R., the absolute values for the Endocytic Index do not differ greatly between the young and old cells, but with donor G.W. a 2 to 3 fold increase occurred between Day 1 and Day 7. As with the previous work with Day-1 and Day-7 cells (see Tables 8.2. and 8.3.), donor-dependent variation in Endocytic Indices was seen with the older cells, but not with the younger.

8.4. DISCUSSION

8.4.1. Validity of results

With all the experiments described in this chapter, the values obtained for cells in duplicate wells were reasonably close. This is a very important observation, because it indicates that when identical experiments carried out on cells from different donors give different results, the variation is a true donor-dependent variation in cell behaviour and is not due to inadequacies in the experimental techniques.

8.4.2. Modes of uptake

With both sizes of bead, the clearance values progressively increased in a linear fashion, over a reasonably long time-period. This type of clearance is indicative of the substrate being ingested by the cells and not merely bound to the cell surface, since binding is thought to be a rapidly saturable process. If we had been measuring random binding the results would have been very scattered. We were therefore confident that we were measuring genuine endocytic uptake.

Pinocytosis and phagocytosis are not easily distinguished. Most inhibitors have similar effects on both processes. However, as mentioned earlier (Section 8.1.), colchicine is known to inhibit greatly the pinocytic capture of substrates by rat peritoneal macrophages, but not phagocytic capture. Therefore we used colchicine to elucidate whether the beads were captured by pinocytosis or phagocytosis. In the previous Chapter(7), in which colchicine had been added to the cells along with the substrate or 20 minutes prior to substrate addition, the extent of inhibition was not very marked. Therefore in these latter experiments a large preincubation period was favoured. It was found that colchicine caused a reduction in the Endocytic Indices of the small beads (100nm diameter), but not the large (1100nm diameter), indicating that the former were captured pinocytically and the latter by phagocytosis. In addition, owing to the relatively high Endocytic Indices for the small beads (82-890µl/mg protein/hour) when compared to our previous uptake data for ^{125}I -labelled polyvinylpyrrolidone alone (0.047-0.242 µl/mg protein/hour) it was thought that these were entering cells by an adsorptive process. These findings are consistent with those reported by Pratten and Lloyd (1986) for uptake of polystyrene

beads by rat peritoneal macrophages.

Interestingly, we found that the inhibitory effect of colchicine was somewhat greater with the younger cells (monocytes) than the older (macrophages), i.e. 52-60 percent v. 30-39 percent inhibition.

8.4.3. Rates of endocytosis.

With the older cells (i.e. Day-4 and 7), considerable donor-dependent variation in the Endocytic Indices was seen with both sizes of bead. Since this phenomenon did not occur with Day-1 cells, it must reflect innate differences in ability to mature or in response to culture in vitro. Owing to these often extensive variations found with the older cells, comparisons with the results of other workers, such as those reported by Pratten & Lloyd (1986) for rat peritoneal macrophages, is only possible for the data for Day-1 cells. The rate of pinocytosis for the small beads was approximately 2-10 times greater for our monocytes than for rat peritoneal macrophages, but the phagocytic uptake of the large beads was lower by approximately a half. Our results further differed from those of Pratten & Lloyd (1986), who reported that the larger beads were cleared almost 10-times more rapidly than the smaller, whereas we observed that the smaller beads were cleared more quickly (2-3 times) by Day-1 cells.

Our experimental approach was very similar to that of Pratten & Lloyd (1986), the only major difference being that we used 5 times the concentration of beads. However, changing the substrate concentration would not have affected the Endocytic Indices, unless binding sites were becoming saturated. We consider it more probable

that the differences are due to the differences in species and cell-types.

8.4.4. Culture/maturation-dependent changes.

It was possible, with some of the experiments (in which cells of different ages were taken from one donation), to compare maturation/culture-dependent changes in endocytosis. The phagocytic capture of the large beads was seen to increase between the fourth and seventh days of culture; only a small increase was noted with one donor, but a large (up to 4fold) increase with the second. Obviously it is difficult to draw conclusions from results obtained from only 2 donors, especially in view of the donor-dependent variations observed with these experiments. Had time allowed it would have been interesting to take this aspect of the work further, in light of the present uncertainty over whether or not non-specific phagocytosis increases with the transformation of monocytes into macrophages (see Section 1.2.7.6.). However, our results do seem to agree with the generally held belief that, with adherent cultures, there is an increase in non-specific phagocytosis. With pinocytosis (i.e. capture of small beads), no clear pattern was observed. Comparison between monocytes (Day-1 cells) and macrophages (Day-7 cells) obtained from the same donations, revealed no difference in pinocytic activity for two of the donors, yet with the third, the macrophages captured the beads at twice the rate; where Day-4 and Day-7 cells were compared (from two further donors), the pinocytic activity was greater (2 to 3 fold) for the older cells. Thus in some but not all cases an increase is observed in adsorptive pinocytic activity during time in culture. Since donor-dependent variation in uptake was also observed in our earlier work (Chapter 6), we were confident that this

variation was genuine, an aspect of our studies which is discussed further in Chapter 9.

CHAPTER 9

Discussion

9.1. DISCUSSION

An important paradox concerning the nature of pinocytosis was presented in the Introduction (Section 1.1.2.3.): pinocytosis is believed to be a constitutive process (with no apparent trigger), and yet it can be inhibited by various modifiers. This enigma prompts the question of the nature of the intracellular events that maintain the "constitutive" membrane internalization characteristic of pinocytosis. In an attempt to answer this question, we sought to use the approach of studying cells in which pinocytosis "starts up", and investigating events surrounding this onset period (Section 1.1.4.). Only one cell-type was known in which a dramatic increase in pinocytosis had been reported: the human monocyte during its differentiation into the macrophage (Knight & Boutar, 1982).

In order to use this model it was first necessary to confirm that pinocytosis was indeed greatly accelerated in macrophages, as compared to the monocytes from which they developed. This in turn required the establishment of a reliable method for isolating a pure population of monocytes and an *in vitro* culture system in which these cells consistently matured into macrophages.

After assessing a number of cell-isolation methods we chose (for reasons given in Chapter 3) the density-gradient material Percoll for obtaining monocytes from human blood. We maintained these cells in adherent culture in Linbro wells and confirmed that the monocytes transformed into macrophages in this culture system. The cells, in addition to adopting the typical macrophage-like appearance within a week of the onset of the culture, were shown to display a number of characteristics of maturing cells. These included increases in

cell-protein, protein-to-DNA ratio, intracellular hexosaminidase activity and hexosamindase secretion (in response to opsonized zymosan), the development of sodium fluoride-resistant esterase activity and the loss of peroxidase activity (see Chapter 5).

To measure the pinocytic activity of cells, cells at various stages of maturation were incubated with various ^{125}I-labelled macromolecules: polyvinylpyrrolidone (in the presence of suramin), vinylamine-vinylpyrrolidone copolymer, and polystyrene beads (100nm diameter). We found no consistent or dramatic increase in the pinocytic activity of monocytes as they matured into macrophages (see Chapters 6 and 8). We are therefore unable to confirm the report by Knight & Soutar (1982), who indicated a five-fold increase in pinocytosis between the second and fifth day of culture. It is fair to point out that this particular experiment by Knight & Soutar (1982) was only a minor part of a major study on lipoprotein metabolism: as discussed previously (Chapter 6), very few kinetics data were presented. We attempted in our studies to obtain more rigorous kinetic data: the uptake of substrate was examined at several time-points, and in addition more repeats were performed. Additional experiments, in which we incubated cells with the larger (1100nm diameter) polystyrene beads, suggested that there was some increase (up to 4 fold) in the phagocytic ability of cells as they matured, an observation consistent with previous reports (see Chapter 8).

The failure to detect a dramatic increase in the pinocytic activity of maturing cells was disappointing, as it meant that we were no longer able to consider using the developing monocyte as a model system for studying events surrounding the onset of pinocytosis. It

is therefore clear that a different approach would be needed to answer the underlying question of the cellular trigger for pinocytosis. One such approach that has already been described in detail is that used by Koenig (see Section 1.1.4.). In brief, various intracellular changes in rat kidney cortex slices were studied, during the stimulation of pinocytosis (of horseradish peroxidase) by various agents. This work indicated that a rise in intracellular calcium ions triggered the rapid increase in pinocytosis. Although various criticisms were made concerning this work (see Section 1.1.4.), I consider that the approach he used has great potential. Similar principles could easily be applied to study the involvement of various secondary messengers in pinocytosis, but using a more suitable cell-type (not slices of tissue) and a pinocytic marker that would not be degraded in the lysosomes.

Although we were not able to use the developing monocyte as a model cell-system for studying events surrounding the onset of pinocytosis, this study was nevertheless valuable. We were able to establish a reliable cell-isolation method for dealing with relatively large volumes of blood. We also demonstrated (by a number of criteria) that monocytes developed into macrophages in the cell-system employed. Furthermore we developed a method for measuring the pinocytic activity of these cells, a method that will be valuable for future studies relating to endocytosis.

In the course of this study several problems became apparent, which directly related to the use of human cells for the investigation. In the first instance, it is often difficult to find a sufficient number of donors willing to donate the relatively large volumes of blood required (large volumes being necessary owing to the small number of

monocytes in blood). A more serious problem is the impracticability of obtaining subsequent donations from the same donor; this was frustrating as it meant that experiments could not easily be repeated using the same donors' cells, desirable because of inter-donor variation (see below). In addition, since we were dealing with limited cell numbers (despite collecting relatively large volumes of blood), it was often not possible to perform what might be considered an ideal experiment, for example with a good number of incubation periods and enough duplicates and controls.

A major problem with these cells was that, because they were not very active pinocytically, the cell-associated radioactivity values obtained were sometimes little higher than the amount of radioactivity adsorbing to the wells themselves. This was particularly evident when cells were incubated with ^{125}I-labelled polyvinylpyrrolidone alone, and in the earlier experiments (Chapter 4) where fewer cells were seeded into the wells. In addition, as described in Chapter 1, the behaviour of monocyte/macrophages is easily modified by only slight changes in their culture environment, for example changing the batch of serum employed.

A further problem was the high degree of variation seen between results from cells isolated from different donors. This inter-donor variation makes it impossible to detect differences in the pinocytic activity of cells, unless either the differences are large or the cells being studied are isolated from the same donor. Even if cells from the same donor are used, they would preferably need to be isolated at the same time, to avoid possible variation relating to the state of health of the donor.

There are a number of possible reasons for inter-donor variation. The differences may simply be due to innate genetic variation. Some donors may have higher proportions of promonocytes (monocyte precursor cells) in their blood, cells that would be isolated along with the monocytes but could have different functional/biochemical properties from the monocytes. A wide variety of common inflammatory conditions are known to increase the number of promonocytes in the blood (see Section 1.2.3.). In addition, the cells may have differing maturation rates: again a number of factors are known to affect the maturation rate of monocyte/macrophages (see Section 1.2.3.). Since the cell-isolation procedure is known to be related to the density of the blood being separated, a further consideration is the initial density of the donor's blood (blood densities are known to vary between individuals). So where cell yields are not 100 percent, it may be that different subpopulations of cells are selected for (high density or low density fractions). It is known that the various monocyte subpopulations can exhibit different properties (see Section 1.2.4.).

This investigation highlights the innate desirability and the innate difficulties of studies on human material. On the one hand it is preferable to use human samples, rather than animal, since in most investigations the ultimate goal is to gain knowledge relating to man (it is not possible to extrapolate results confidently from one species to another). On the other hand, using human material, the high degree of genetic variation found in man often leads to considerable inter-subject variation in physiological and cellular parameters. A consequence of this is that only gross differences can be detected. In spite of the large inter-donor variations, I am confident however that, as human monocytes mature <u>in vitro</u>, there is

no consistent or dramatic change in their ability to pinocytose. A further problem with the use of human blood samples is the risk of the workers contracting various human pathogens, a problem recently highlighted by the increasing number of carriers for the blood-borne HTLV-III (AIDS) virus.

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