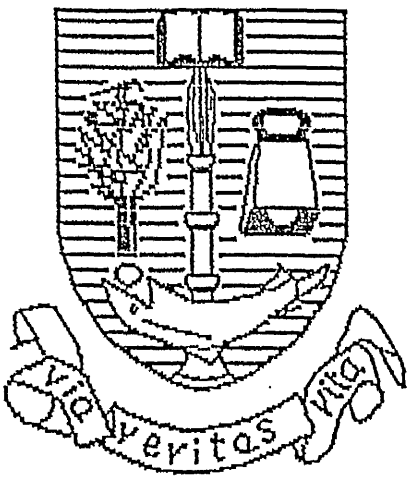

MACROPHAGE FUNCTION IN MILK AND THE FATE OF TRANSFERRED PHAGOCYTES IN THE NEONATAL GUT

by

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PUBLICATIONS

Work described in this thesis is included in the following publications:

1. Hughes, A., Brock, J.H., Parrott, D.M.V. & Cockburn, F. (1985) Effect of human colostrum and infant formula on the phagocytic activity of macrophages. I. Resident and stimulated mouse peritoneal macrophages. Clinical and Experimental Immunology, 61, 169.

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ABBREVIATIONS

Abbreviations used in the text included some of the commonly-used abbreviations listed in the paper "Uniform requirements for manuscripts submitted to biomedical journals", British Medical Journal, 1, (1979), 532. Other abbreviations used in this thesis include:

α	anti- or alpha-
ADCC	antibody-dependent cellular cytoxicity
ALA	α-lactalbumin
ANAE	α-napthyl acetate esterase stain
APC	antigen presenting cell
BALT	bronchial-associated lymphoid tissue
BCG	bacillus Calmette-Guerin
BLG	β-lactoglobulin
BSA	bovine serum albumin
CAPD	continuous ambulatory peritoneal dialysis
CAS	casein
CMPI	cow's milk protein intolerance
conA	concanavalin A
CR	complement receptor
DW	distilled water
Fab	fragment obtained by papain hydrolysis of immunoglobulins
Fc	crystallisable fragment of immunoglobulins
FCR	receptor for the Fc portion of immunoglobulins
FCS	foetal calf serum
Fe	iron

Fe(NTA)	ferric nitrilotriacetate
Fig.	figure
Fn	fibronectin
g	gram or acceleration of gravity
GALT	gut-associated lymphoid tissue
GVH	graft versus host reaction
HBSS	Hank's Buffered Salt Solution
HTf	human transferrin
Ia	I-region associated antigen
Ig	immunoglobulin
IFN	interferon
IL-1	interleukin-1
IL-2	interleukin-2
LK	lymphokine
Lf	lactoferrin
LPS	lipopolysaccharide
MAb	monoclonal antibody
ME	meat extract
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
MPO	myeloperoxidase
M.Wt	molecular weight
NCTC	National Collection of Type Cultures
N/D	not done
NK	natural killer cell
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PMA	phorbol myristate acetate

PMN	polymorphonuclear leucocyte
PPD	purified protein derivative
ROI	reactive oxygen intermediates
sIgA	secretory immunoglobulin A
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TG	thioglycollate
TRIS	Tris-(hydroxymethyl)methylamine

MISCELLANEOUS

Ci	curie
mCi	millicurie
μCi	microcurie
pH	reciprocal $\log_{10}$ hydrogen ion concentration
w/v	weight per volume
v/v	volume per volume
+ve	positive
-ve	negative

SYMBOLS

>	greater than
<	less than

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SUMMARY

To investigate the effect of infant feeding regimes on macrophage function, interaction of infant formula and human breast milk supernatant with macrophages was studied. Phagocytosis and degradation of radiolabelled transferrin-antitransferrin immune complexes by resident and stimulated mouse peritoneal macrophages was inhibited by liquid infant formula, particularly in the case of resident cells. Ingestion and killing of Staphylococcus aureus NCTC 8532 by resident macrophages was also reduced following pre-incubation with formula. In contrast, human milk had little or no effect on either function.

Human milk supernatant and liquid infant formula inhibited phagocytosis but not degradation of radiolabelled immune complexes by human peritoneal macrophages, whereas human breast-milk macrophages appeared to be unaffected by either milk or formula.

Immunofluorescence studies showed that both murine and human macrophages exposed to formula bound casein (CAS) and β-lactoglobulin (BLG) but little, if any, α-lactalbumin (ALA). Comparison of the effect of various artificial milks, cow's milk and purified CAS on the activity of mouse macrophages indicated that both the concentration and the degree of denaturation of CAS may be important in the impairment of macrophage function by milk.

Human peritoneal macrophages and breast-milk

macrophages showed a positive correlation between binding of milk proteins in the liquid formula and expression of HLA-DR antigen. Thus, it is proposed that formula milk might impair macrophage function in the small intestine, and binding of milk proteins by HLA-DR +ve macrophages may be important in sensitisation of infants to cow's milk. Furthermore, in infants on a mixed-feeding regime, ingested milk macrophages might also act to present cow's milk proteins.

To investigate survival of milk macrophages in the gastrointestinal tract of the neonate, newborn mice were fed peritoneal macrophages labelled with fluorescent beads. Intact cells survived in the stomach for at least 4h after feeding. In addition, transferred cells were recovered in the gut and spleen sections from a small number of mice. Thus, milk macrophages may be able to persist in the neonate and may thus contribute toward the immunological development of the infant.

The consequences of infant feeding on the immunological status of the neonate are discussed in the context of the presented work.

CHAPTER 1

LITERATURE REVIEW

1.1. MACROPHAGES

Once regarded simply as scavenger cells, macrophages are now considered to be important accessory and regulatory cells in humoral and cellular immunity. Widely distributed throughout the body, they display remarkable versatility in their physiological and biochemical properties. An extensive range of secretory products and surface receptors confer on the cell diverse functional activity. Examples of the antimicrobial and antineoplastic activities are well-documented, but their involvement in the clotting system (Bianco et al, 1980; Østerud et al, 1980), their role in clearing cellular debris (Maruta and Mizuno, 1971) and their ability to store iron (Lee, 1983) have also been investigated. Furthermore, macrophages are known mediators of inflammation and have the capacity to function as cytotoxic cells. Some of the major properties of macrophages are documented below. First, however, it is necessary to define some of the terms which will be used to describe macrophages. Essentially, the criteria described by van Furth (1980) were adopted:

Normal, resting or resident macrophages: macrophages which have not been exposed to any form of stimulus.

Activated macrophages: macrophages with increased functional activity induced by a given stimulus.

Activation implies a new functional activity or an increase in one or more of the functional activities of the cell. Before activation this cell may have been a resident macrophage.

Elicited macrophages: macrophages attracted to a given site by a given substance. It does not refer to the developmental stage of the cells or their functional state.

Stimulated macrophages: macrophages which are elicited and/or activated by the application of a stimulus.

1.1.1. MACROPHAGE HETEROGENEITY

Considerable physiological, biochemical and functional heterogeneity exists within most macrophage populations and between populations from different anatomical sites. Phenotypic differences among macrophages are usually attributed to differences in the maturational state of the cells (van Furth, 1980) or to local adaptation to environment (Simon et al, 1977; Bar-Eli et al, 1981). However, it is possible that these differences are a result of distinct sub-populations of differentiated cells (Volkman, 1976). Perhaps tissue macrophages and blood monocytes are derived from different bone marrow precursor cells (Daems and de Bakker, 1982).

The experiments of Parwaresch et al (1981) favour a single lineage of cells. Prolonged culturing of blood monocytes on a glass surface results in gradual transition of the monocytes' esterase isoenzyme patterns

into that of resident peritoneal macrophages. Furthermore, bone marrow reconstitution techniques have demonstrated that mouse alveolar macrophages are bone marrow derived (Johnson, K.J et al, 1980).

In general, it is thought that mononuclear phagocytes arise primarily in the bone marrow, circulate in the blood as monocytes and then migrate into the various tissues of the body where they differentiate into resident tissue macrophages (van Furth, 1980). However, as this is by no means certain, it is not possible to explain with certainty the reasons for biochemical and functional heterogeneity.

1.1.2. CELL SURFACE RECEPTORS

Membrane receptors are the means by which macrophages detect and respond to changes in their environment. Although several receptors have been identified and their function elucidated, the most extensively studied are the receptors for complement (CR) and the Fc fragment of immunoglobulin molecules (FCR: McKeever and Spicer, 1980). These recognition structures enable macrophages to distinguish opsonised particles and bind them for ingestion. Furthermore, FCR may allow the cell to transport antibodies.

Specific receptors for chemotactic substances, insulin (McKeever and Spicer, 1980); lactoferrin (van Snick and Masson, 1976); fibronectin (Wright and Meyer, 1985); hormones (Werb, 1980) and lectin (Weir and

Ögmundsdóttir, 1980) are acknowledged. However, studies pertaining to macrophage receptors and documented forthwith will focus on the contribution made by CRs and FCRs in macrophage interactions.

1.1.2.1. THE Fc RECEPTOR (FCR)

Although FCRs for IgM (Uher et al, 1981), IgA (Fanger et al, 1980) and IgE (Melewicz et al, 1982) are known, the most extensively studied is the FCR for IgG. As it has been found on a variety of mononuclear and lymphoid cell types (Unkeless et al, 1981), it seems reasonable to assume that the role of the FCR in the immune response is not restricted to the phagocytic event but may encompass other functions equally as important immunologically.

The FCR is a transmembrane glycoprotein which is synthesised in the rough endoplasmic reticulum as a precursor and then transported intracellularly to the plasma membrane (Green et al, 1985). It moves freely within this membrane and is thought to function independently of the receptors for complement (Griffin et al, 1975).

Binding of ligand to the FCR results in internalisation and degradation of soluble immune complexes, the phagocytosis of antibody-coated particles and secretion of inflammatory agents and oxygen intermediates (Unkeless et al, 1981). In response to FCR stimulation, macrophages also secrete proteases extracellularly (Ragsdale and Arend, 1979).

It is now generally accepted that mouse

macrophages possess three distinct types of FCR which bind specific subclasses of IgG. Receptors for IgG2a, IgG2b/G1 and IgG3 have been demonstrated (Unkeless, 1986) and in response to cell activation by gamma interferon, the number of IgG2a FCR expressed has been reported to increase (Ezekowitz and Gordon, 1986).

Immune complexes are responsible for the induction of inflammation and tissue damage such as that seen in glomerulonephritis (Koffler et al, 1971). The ability of macrophages to recognise and ingest immune complexes may be important in deciding the severity of tissue injury. Kurlander et al (1984a) have shown that IgG-FCR are involved in clearance of immune complexes from the circulation. Whilst monoclonal antibodies directed against IgG2b/G1 receptors or the Fab fragment of IgG were capable of blocking the clearance of the circulating complexes, clearance of monomeric IgG was unaffected.

In a series of experiments using radiolabelled immune complexes, Finbloom (1985) compared binding of complexes to the IgG2b/G1 receptors of resident, thioglycollate-elicited (TG) and BCG (bacillus Calmette-Guerin-elicited) macrophages. The capacity of the different cells to then process these complexes was also examined. Resident macrophages were not as efficient as BCG or TG macrophages at ingestion or degradation of the complexes. Furthermore, resident macrophages cleared surface bound complexes at a slower rate then the elicited macrophages. In addition, it was observed

that down regulation of FCR accompanied internalisation of the complexes.

All subclasses of IgG mediate phagocytosis and lysis of erythrocytes (Ralph et al, 1980). Upon binding and ingestion of multivalent IgG-immune complexes or IgG-coated erythrocytes, there is irreversible loss of the FCRs (Mellman et al, 1983; Mellman and Plutner, 1984). Apparently, this is due to the internalisation and the rapid and selective lysosomal degradation of receptors (Mellman et al, 1983). The experiments of Finbloom (1985) also suggest that the FCRs are not recycled, at least not within 4h. Apparently this does not apply to binding of monomeric ligands. Internalisation of FCRs still occurs but the receptors do not undergo lysosomal degradation. Instead they are rapidly transported to the cell surface where they are re-expressed (Mellman et al, 1984).

Receptors for Fc have also been identified on the surface of human macrophages. These include human blood monocytes (Kurlander et al, 1984b); milk macrophages (see 1.2.1.2.1); intestinal macrophages (Golder and Doe, 1983); alveolar macrophages (Conkling et al,1982) and peritoneal macrophages from laparoscopy patients (Kurlander et al, 1984b) and patients on continuous ambulatory peritoneal dialysis (CAPD: Goldstein et al, 1984).

It appears that human macrophages have at least two FCR for IgG, a high avidity receptor and a low avidity receptor (Kurlander et al, 1984b). The latter, which is not present on blood monocytes, is more

abundant and it may be this receptor which is involved in clearance of immune complexes. Evidence in support of this role comes from the preliminary experiments described by Unkeless (1986). The monoclonal antibody 3G8 (MAb 3G8) is directed against the low avidity IgG-FCR on human cells. Intense MAb 3G8 immunoperoxidase staining was found in sections of human organs which are thought to be the loci of immune complex clearance. Furthermore, it appears that infusion of MAb 3G8 completely blocks clearance of IgG-sensitised erythrocytes in the chimpanzee (Unkeless, 1986).

Human macrophage FCRs also confer on the cell the capacity to mediate antibody-dependent cytotoxicity reactions (ADCC, Conkling et al, 1982; Mandyla et al, 1982) and phagocytose opsonised microorganisms (Weaver et al, 1981) and it has been proposed that the low-avidity receptors may facilitate such functions (Kurlander et al, 1984b).

The high-avidity receptor on human peritoneal macrophages is probably identical to the FCR on peripheral blood monocytes (Kurlander et al, 1984b) especially since these two cell types bind monomeric IgG1 with similar avidity (Kurlander et al, 1984b). It may be this high-avidity receptor which renders the cells capable of transporting immunoglobulin.

Finally, binding at the FCR on human cells induces secretion of some proteins whilst decreasing synthesis and secretion of others (Passwell et al, 1980). Cultured monocytes incubated with Fc fragments

of IgG showed a decreased synthesis of lysosomal enzymes and the second component of complement (C2). However, production of prostaglandins and increased protein synthesis also accompanied receptor binding.

1.1.2.2. RECEPTORS FOR COMPLEMENT (CR)

Complement receptors enhance phagocytosis of opsonised particles. At least six distinct types of receptor for complement have been identified, three of which interact with C3 fragments - CR1, CR3 and C3a-R (Ross and Newman, 1980). This section on CR will deal with the general properties of receptors for C3.

The classic immune adherence receptor CR1, plays a major role in the phagocytosis of C4b-, C3b- and iC3b-coated ligands (Griffin et al, 1975; Ehlenberger and Nussenzweig, 1977; Leijh et al, 1984). Phagocytosis of erythrocytes and bacteria coated with antibody and complement can occur by synergistic interaction between FCR and CR1 (Ehlenberger and Nussenzweig, 1977; Leijh et al, 1984). On monocytes or unstimulated cells, occupation of CR1 alone is not sufficient for endocytosis, the CR1 functioning only to bind ligand. However, the CR1 on TG macrophages (Bianco et al, 1975) or T-lymphokine activated macrophages (Griffin and Griffin, 1979) ingests surface bound ligands independently of FCR. How this change occurs is unknown. Studies by Kaplan et al (1978) indicate that, unlike the FCR, the CR1 on the macrophage normally occupies a relatively fixed membrane distribution. Receptor number does not alter upon cell stimulation

(Ezekowitz and Gordon, 1986). There is, instead, migration of the receptors across the membrane surface to the site of ligand attachment (Griffin and Mullinax, 1981). Cellular activation may mobilize protein phosphorylation by protein kinase C (DiVirgilio et al, 1984). This is one of the mechanisms by which actin filament assembly is 'activated' (Hartwig, 1986) and it has been suggested that ingestion of C3-coated particles occurs in this way (Hartwig, 1986).

Fibronectin (Fn) receptors interact with CR1 to enhance phagocytosis when the Fn receptors are cross-linked by surface-bound ligands (Wright and Meyer, 1985). This suggests that phagocytosis will be promoted at sites of infection or injury where there will be deposition of Fn-bound fibrin, bacteria or denatured collagen.

Thus, CR1 appears to promote binding and ingestion of ligands by FCR by increasing ligand-phagocyte contact. However, extrinsic factors such as lymphokines or Fn contribute further to phagocytosis by inducing endocytosis of CR1-bound ligands. It should not be assumed that functions induced by added C3b are CR1-dependent because C3b complexes added to phagocytic cells are subsequently converted to iC3b-, C3d-g- and C3d- complexes (Ross and Newman, 1980). Furthermore, some C3b-mediated functions may require cell-associated factors and be less dependent upon CR1 (Ross and Newman, 1980).

The CR3 is a lectin which binds to a carbohydrate

expressed only in iC3b and does not bind to other types of C3 fragments (Ross and Newman, 1980). It binds directly to zymosan and is the zymosan-recognition receptor. Finally, the presence of a receptor for C3a on lymphocytes or macrophages has been suggested by the observation that purified C3a suppresses lymphocyte blastogenic responses (Hobbs et al, 1982).

1.1.3. MACROPHAGE FUNCTION

Some macrophage functions have already been touched upon in the preceding sections. This section will present a broader picture of cell function. Three main areas will be discussed (a) defence against microorganisms; (b) accessory function in the immune response; (c) secretory products and their functions.

1.1.3.1. DEFENCE AGAINST MICROORGANISMS

Perhaps the best known of all the functions of the macrophage is its ability to phagocytose and kill microorganisms. The efficiency with which macrophages eliminate microbes is dependent largely, if not entirely, on their state of activation and on their chemotactic function.

In response to suitable agents, macrophages undergo metabolic stimulation - an event termed the respiratory burst. This is associated with increased oxygen consumption and glucose metabolism (Rossi et al, 1986), and is coupled with an increased production of reactive oxygen intermediates (ROI) which may be

antimicrobial (see 1.1.3.3). The production of ROI is dependent on the source of the cell and its state of activation and differentiation (Johnston et al, 1980). Human peritoneal macrophages (CAPD: Goldstein et al, 1984), blood monocytes and breast milk macrophages (Tsuda et al, 1984) generate increased levels of ROI in response to stimulation by phorbol myristate acetate (PMA).

Gamma interferon is the major lymphocyte secretory product which induces macrophage oxidative metabolism (Nathan and Tsunawaki, 1986). This cytokine enhances the cytolytic activity (Dean and Virelizier, 1983) and anti-parasitic activity (Wilson and Westall, 1985) of human cells.

Cell surface receptors are of obvious importance in the interaction between cells and microorganisms. Whilst CR3 facilitates cell binding of unopsonised yeast (Ross and Newman, 1980), other lectin-like receptors recognise sugars in the cell wall of several strains of bacteria (Weir and Ögmundsdóttir, 1980). Furthermore, FCR and CR1 are important in the elimination of antibody coated and complement coated organisms (see preceding section). Ezekowitz and Gordon (1986) have reported that IgG2a FCR are effective triggers of the respiratory burst in activated macrophages. Cell activation increases ligand-receptor interactions (see Section 1.1.2.). Furthermore, there is greater phagosome-lysosome fusion (Kielan and Cohn, 1980). Once this occurs ingested substances are exposed to, and degraded by a battery of lysosomal enzymes. The lysosomal

enzymes and other macrophage secretory products which may be involved in the elimination of microorganisms are discussed in more detail in section 1.1.3.3.

Finally, since macrophages are chemotactic toward complement components, lymphokines and a variety of other substances (Synderman and Mergenhagen, 1976), they may accumulate at sites of infection and inflammation. In so doing, they may contribute further to the defence of the host against invasion of microorganisms.

1.1.3.2. ACCESSORY CELL FUNCTION

It is now generally accepted that an antigen presenting cell (APC) is required for the induction of an immune response. One of the major accessory cell types involved is the macrophage although other cell types such as dendritic cells, B cells, endothelial cells and Langerhans cells have also been implicated.

The antigens are presented by the APC in the context of the products of the major histocompatibility gene complex (MHC)-the class II MHC glycoproteins (Lafuse and David, 1984). It is now known that T and B cells recognise distinct regions of the antigen molecule (Chesnut *et al*, 1980). Whilst antibody production is directed against the three-dimensional conformation of the antigen, T-cell proliferation is triggered by the primary structure. Native or denatured protein will induce T-cell proliferation (Chesnut *et al*, 1980). This provoked the concept that a process of intracellular alteration of antigen by macrophages was a

prerequisite for T cell recognition.

The first direct evidence that antigen degradation may be critical for presentation was provided by Ziegler and Unanue (1981). Uptake of Listeria monocytogenes occurred within 5min but a processing period of 30-60min was required before T cell binding was observed. Diment and Stahl (1985) showed that as early as 10min after ingestion of mannosylated-BSA, rabbit alveolar macrophages released partially catabolized protein into the extracellular medium. Degradation by proteases took place in a prelysosomal compartment prior to fusion with lysosomes. Perhaps early release of partially degraded protein amplifies antigen presentation by recruiting the help of other accessory cell types e.g dendritic cells. Dendritic cells have the capacity to present antigen but are non-phagocytic cells (Lee and Guidos, 1984) and cannot process antigen for presentation. They have a strong constitutive expression of Ia and may present the modified antigen released by the macrophages. The macrophage itself requires additional processing of antigen if it is to participate in presentation. In support of this, Allen and Unanue (1984a) showed that partially catabolized protein released by the macrophage then associates with the cell membrane. These peptides could not stimulate T cells unless they were re-processed by additional macrophages. Dendritic cells might not require an additional processing event.

The importance of macrophage processing in

presentation of soluble antigens such as hen egg white lysozyme (Allan and Unanue, 1984a), ovalbumin and keyhole limpet haemocyanin (Grey et al, 1984) has also been provided. Addition of lysosomotropic agents up to 25 min after addition of antigen prevented triggering of T cells.

Grey et al (1984) were unable to find a role for Ia in the binding of degraded protein at the surface of the accessory cell. They proposed that interaction between the two might only take place following binding to the T cell receptor. It certainly appears that the Ia molecule is not involved in the initial binding and processing of antigen (Ziegler and Unanue, 1981). It plays a role in the latter stages of presentation as a macrophage receptor for the processed antigen and/or as a recognition structure for T cells (Ziegler and Unanue, 1981). However, there is evidence to suggest otherwise. The T cell receptor appears to be a single molecule with the dual role of recognising both antigen and the Class II molecules (Haskins et al, 1984). In addition, Ia positive moieties and processed antigen can be reversibly dissociated without any cellular involvement (Friedman et al, 1983).

Thymocyte binding to macrophages (Agrwal and Thomas, 1984) but not cytotoxic activity (Blumenthal et al, 1983) correlates with Ia expression. Other factors may be involved in determining whether Ia expression accompanies macrophage cytoxicity. Levels of interleukin-1 (IL-1) may also be important. By maintaining constant levels of membrane IL-1, Kurt-

Jones et al (1985) demonstrated that the extent of antigen presentation correlated with the amount of Ia expressed. However, at constant levels of Ia antigen, the extent of presentation was associated with the levels of membrane IL-1 activity. Durum et al (1984) stress the importance of soluble IL-1 in antigen presentation. They showed that activated T cells stimulated IL-1 production by H-2 compatible macrophages and that purified IL-1 triggered T cell responses in the presence of anti-Ia antibodies.

The major lymphokine responsible for Ia induction is gamma interferon (IFN-γ : Steeg et al, 1982). Upon depletion of IFN-γ producing lymphocytes from cultures (Volk et al, 1985) or addition of antibodies to IFN-γ (Steeg et al, 1982) Ia inducing activity is abolished.

Most of the aforementioned studies have investigated the accessory cell function of murine macrophages. Comparable studies on human macrophages are not always possible. However, Class II (HLA-DR) antigen expression has been demonstrated on human cells. Monocytes (Kaplan and Gaudernack, 1982), breast milk macrophages (Leyva-Cobián and Clemente, 1984), peritoneal macrophages (Goldstein et al, 1984) and intestinal macrophages (Golder and Doe, 1983) all show a high proportion of HLA-DR positive cells.

1.1.3.3. SECRETORY PRODUCTS

A host of products released under a variety of conditions enable the macrophage to regulate a large

number of body processes. Secretory products are important in immunoregulation and killing of microorganisms and tumours. Some of the most important secretory products and their functions are outlined below.

Neutral proteinases. Essentially, these enzymes are synthesised and secreted by macrophages in the activated state (Gordon, 1980). There are several neutral proteinases which include collagenase, elastase and plasminogen activator. The last may also activate collagenase activity through the action of plasmin, a fibrinolytic enzyme (Werb et al, 1977). Plasmin is released from plasminogen by plasminogen activator and may then interact with the coagulation and complement systems or generate mediators of macrophage function in the inflammatory response (Gordon, 1980).

Lysosomal enzymes. These consist of a large number of acid hydrolases which are usually contained within the lysosomes. β-glucuronidase and acid phosphatase are well-known examples. Release of these enzymes does not usually require a stimulus but increased secretion occurs upon cell activation. Although release is also linked to phagocytosis it proceeds independently of the intracellular fate of the ingested material. The activity of lysosomal enzymes will depend on the environmental pH, the presence of inhibitors and the proximity of the cells to the substrate (Schnyder and Baggiolini, 1980).

Lysozyme. Lysozyme is a major secretory product of macrophages. It contributes to the antibacterial activity of the cells by digesting the peptidoglycan of bacterial cell walls. Secretion of this enzyme is not affected by the activation state of the cell, phagocytosis, or exposure to LK (Gordon, 1980). Immunocytochemical studies have located the enzyme in granules in the cell cytoplasm and have shown the release of the enzyme into phagosomes (Miyauchi et al, 1985). Constitutive synthesis and secretion of the enzyme is high and a useful cell-specific marker (Gordon, 1980).

Reactive oxygen intermediates (ROI). As mentioned in Section 1.1.3.1., in response to activation, macrophages are capable of a respiratory burst. This produces reactive metabolites of oxygen such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$) and possibly singlet oxygen ($^1O_2$: Rossi et al,1986). Superoxide anion is the major product of oxygen metabolism and is not very microbicidal. It exerts its effect through the products it forms. Upon dismutation, it produces H_2O_2 which is a very potent microbicidal agent (Klebanoff, 1980).

Arachidonic acid products. Macrophages are rich in arachidonic acid. Upon membrane triggering with phagocytic stimuli such as antigen-antibody complexes (Humes et al, 1977), arachidonic acid is released from the membrane phospholipid. Rouzer et al (1982) have shown that macrophages challenged with immune complexes

release large quantities of prostacyclin, prostaglandin E_2 and leukotriene C which are metabolic products of arachidonic acid. Leukotriene C is one of the slow-reacting substances of anaphylaxis whilst prostaglandin E is an important mediator of inflammation.

Complement components. Macrophages produce all the proteins of the complement system with the exception of C6-C9. Thus, macrophages generate all the components necessary for local opsonisation. Production of these components is dependent on the differentiation and activation state of the cells as well as the tissue of cell origin (Colten, 1986).

Interleukin-1 (IL-1). One of the most important regulatory factors released by macrophages is IL-1. Lymphocyte induction of macrophage IL-1 activity and the effect of IL-1 on Ia expression were mentioned earlier (see Section 1.1.3.2.). Macrophage IL-1 is also released in response to stimulation by latex ingestion, antigen-antibody complexes and endotoxin (Calderon et al, 1975). It stimulates the T cell proliferative response to antigens (Durum et al, 1984) and contributes to the liberation of the T cell growth factor IL-2 by lymphocytes (Smith et al, 1980). Endogenous pyrogen and the inducer of serum amyloid A, the major acute phase reactant, also appears to be IL-1 (Sztein et al, 1981).

1.2. PROPERTIES OF HUMAN MILK

Following parturition, the immunologically naive infant is introduced into an unfamiliar and perhaps hostile environment. To command a strong defence against a barrage of foreign substances, the neonate requires supplementary immune protection until its own immune system is more fully developed. Maternal immune factors may bestow transient protection via breast milk.

Human milk contains a variety of antimicrobial substances (Welsh and May, 1979; Hanson et al, 1985) which help protect the human infant against infection (Arnon et al, 1982; Frank et al, 1982; Saarinen, 1982; Cooperstock et al, 1983; Narayanan et al, 1984). It also appears that prolonged breast-feeding reduces the incidence of atopic disease (Saarinen et al, 1979; Fergusson et al, 1982; Gruskay, 1982). However, it is not clear whether this is a consequence of delayed exposure to solid feeding rather than a beneficial effect of breast milk itself. Nevertheless, greater awareness of the protection afforded by breast milk has led to a recent increase in the number of breast-feeding mothers (Coles et al, 1978; Hitchcock et al, 1982; Martinez and Nalezienski, 1980; Martinez and Krieger, 1985) especially among older mothers and those from high socio-economic backgrounds (Clark and Beal, 1982; Hitchcock et al, 1982; Rousseau et al, 1982).

In the more under-developed nations, breast-

feeding is well-established and is often undertaken for one year or more post-partum (Short, 1984). In Bangladesh, the duration of lactation appears generally to decrease with increasing affluence (Huffman et al, 1980).

Although the protection afforded by breast milk is now widely accepted, it is recognised that an alternative source of nutrition must also be available. For a variety of reasons many women are either unable or unwilling to breast-feed. Furthermore, in Third World countries, the poor nutritional status of the mother often necessitates the use of an alternative or supplementary source of food to breast milk. Finally, the infant with special nutritional demands must also be considered. Whilst this substitute should be nutritionally beneficial to the infant, there is an equal need to ensure its safe use. For example, the risk of infection arising from poor sanitary conditions in Third World countries must be minimised.

In all cases, the best equivalent to human milk is infant formula. As medical research highlights the special requirements of the newborn infant, the formulas are adapted and fortified. However, it is desirable that the composition of the final product closely resemble that of human milk.

In the following sections, the composition of human breast milk will be described and a comparison made with cow's milk and infant formula products.

1.2.1. THE PROTECTION OFFERED BY HUMAN MILK

1.2.1.1. NON-CELLULAR COMPONENTS

Human milk contains a wealth of soluble components which have been implicated in the protection of the neonate. It provides non-specific factors such as lysozyme, bifidus factor, lactoferrin, lactoperoxidase, lipid and complement. However, maternal antibodies are regarded as the most important since they are the vehicle by which specific protection is transferred.

1.2.1.1.1. IMMUNOGLOBULINS

The human infant receives maternal antibodies both *in utero* where IgG is transferred across the placenta (Ogra *et al*, 1977) and in breast milk. Although all five immunoglobulin classes have been identified in human milk (Goldman and Smith, 1973), by far the most abundant is secretory IgA (sIgA: Tomasi, 1972). Mickleson and Moriarty (1982) monitored the levels of IgA, IgM and IgG at various times post-partum. Although each immunoglobulin decreased over the lactational period, the relative proportion of each class remained the same.

It is not completely clear whether there is absorption of milk immunoglobulins by the neonatal gut. Ammann and Stiehm (1966) could find no difference in the serum immunoglobulin levels of formula-fed and breast-fed infants. However, using sensitive assay systems Leissring *et al* (1962) detected absorption of small

quantities of maternal antibody up to 15h after a feed. The observations of Ogra et al (1977) indicate that a transient period of absorption takes place up to 24h after birth. Thereafter, a much reduced level of maternal antibody was detected in infants' serum.

It appears, therefore, that the major role of IgA is to provide local protection in the neonatal gastrointestinal tract. It acts as a protective coat which prevents bacterial adhesion to the intestinal mucosa (Fubara and Freter, 1973) and/or it inhibits the activity of bacterial enterotoxins (Stoliar et al, 1976). The fact that sIgA is resistant to enzymatic digestion (Lindh, 1975) and able to pass intact into the faeces of breast-fed infants (Jatsyk et al, 1985) provides additional support for this. Brock et al (1977) have shown that chymotrypsin had little effect upon either the structure or activity of bovine colostral IgG1. Furthermore, although trypsin caused some loss of specific activity, there was relatively little cleavage of the antibody structure. More recently, McClead and Gregory (1984) have shown that specific antibodies in bovine colostrum resist digestion by pepsin and trypsin and remain immunologically and functionally active.

Antibodies directed against a number of microorganisms have been detected in human milk. They include antibodies to Vibrio cholerae, Salmonella, Shigella, E.coli, Clostridia, Streptococci, Corynebacterium diphtheriae, Diplococcus pneumoniae,

Coxsackievirus, polio and echovirus, rotavirus and influenza virus (Cruz and Arévalo, 1985; Cruz et al, 1985; Eggert and Gurner, 1984; Glass et al, 1983; Goldman and Smith, 1973; Kim et al, 1984; Majumdar et al, 1982).

Apparently, the specificity of milk immunoglobulin molecules is determined by the type of microorganisms to which the mother is exposed. A significant proportion of IgA in milk is serum-derived but it may also be synthesised by plasma cells from both local and distal lymphoid tissue. The present consensus of opinion is that IgA-producing plasma cells in milk are derived from sensitised precursor cells in the gut-associated lymphoid tissue (GALT) and bronchial-associated lymphoid tissue (BALT). These migrate to the mucosal surfaces of the mammary gland where they secrete IgA specific for the antigens previously encountered in the GALT or BALT (Goldblum et al, 1975; Goldman et al, 1982; Halsey et al, 1982; Roux et al, 1977). Thus, the infant is provided with a means of protection from enteric and respiratory tract pathogens to which the mother has had prior exposure.

1.2.1.1.2. COMPLEMENT COMPONENTS

To date there have been relatively few studies concerned with complement components in human milk. In 1971, Mata and Wyatt detected functionally active C3 in human colostrum. Support for this and for the presence of active C4 and Factor B was provided by Ballow et al (1974). Then, in 1977 it was shown that at least nine

components of complement (C1-C9) and Factors B and D of the alternative pathway are present, though they appear to have relatively low activity (Nakajima et al, 1977).

Since this report research has concentrated on complement synthesis by breast-milk macrophages. Cole et al (1982) have provided evidence that these cells can synthesise and secrete C2, C3 and Factor B. However, it is not known whether the milk macrophages are solely responsible for the complement proteins in breast milk. These proteins may also have been transferred from plasma or produced locally by the cells in the mammary gland.

Ballow et al (1974) were unable to detect any difference in serum levels of complement between formula-fed and breast-fed infants. This suggests that if the complement from milk survives in the infant it is not absorbed in significant quantities. However, it seems more probable that the complement is destroyed by the proteases of the infant gut. Complement from breast milk may function instead to provide transient, local protection in the mammary gland or in the infants' intestinal tract. Certainly, colostrum supernatant does have some opsonising activity (Weaver et al, 1981).

1.2.1.1.3. LYSOZYME

Initially present in high quantities in breast milk, the concentration of lysozyme decreases over the first four weeks of lactation. Thereafter, the concentration rises and by six months a level is reached

which is maintained through the first year and possibly the second year of lactation (Goldman et al, 1982). It should be noted that although the lysozyme concentration drops quite considerably within the first month, the daily volume of milk increases. This ensures that the infant still receives appreciable amounts of the enzyme.

Breast milk lysozyme has been found in concentrations as much as 3000 times that in cow's milk and is a more stable enzyme than its bovine counterpart (Chandan et al, 1964). It has been shown in vitro to promote lysis of E.coli in the presence of IgA and complement (Adinolfi et al, 1966). The action of the complement and the antibody is thought to make the peptidoglycan more easily accessible to the lysozyme and in so doing, render the bacterium more susceptible to lysis (Muschel and Jackson, 1963; Wilson and Spitznagel, 1968).

Rosenthal and Lieberman (1931) were the first to suggest that human milk lysozyme influences the bacterial flora of the infant gut. They also showed that lysozyme appears in the faeces of breast-fed, but not formula-fed, infants. In addition, Lodinová and Jouja (1977) observed a significant increase in faecal sIgA levels in infants fed lysozyme-containing formula. It has been suggested (Namba et al, 1981) that orally-administered lysozyme may produce cell wall fragments from intestinal bacteria which may then behave as adjuvants or immunostimulating substances in a local immune response.

1.2.1.1.4. BIFIDUS FACTOR

Breast feeding is known to increase the number of lactobacilli in the neonatal gut (Goldman and Smith, 1973). Bullen and Tearle (1976) suggested that the high lactose, low protein and low phosphate content of breast milk are contributory factors. Also, Bezkorovainy and Topouzain (1981) identified an oligosaccharide moiety of human CAS which promotes growth of *Bifidobacterium bifidum* var *pennsylvanicus*. However, it is generally recognised that a group of substances, collectively named the "Bifidus factor" and described by Gyǒrgy in 1971, is the growth promoter of lactobacilli. They consist of lactose, fucose galactose and the nitrogen-containing polysaccharides N-acetylneuraminic acid and N-acetylglucosamine.

1.2.1.1.5. LACTOFERRIN

Lactoferrin is an iron binding protein which is thought to protect the newborn infant from disease organisms which have a significant need for iron as a nutrient (Brock, 1980). Additional support for this role has come from the observations of Brines and Brock (1983). They showed that human apo-lactoferrin is relatively resistant to proteolytic digestion *in vitro*. It may therefore survive and function in the neonatal gastrointestinal tract.

1.2.1.1.6. LACTOPEROXIDASE

Lactoperoxidase is derived from milk leucocytes

(Moldoveanu et al, 1982). It acts in conjunction with H_2O_2 and thiocyanate to produce an unstable oxidation product, hypothiocyanite, which is bactericidal. Organisms susceptible to killing by this lactoperoxidase system include E.coli, Pseudomonas aeruginosa, Salmonella typhimurium and Streptococci (Reiter, 1979).

1.2.1.1.7. LIPID

A nonspecific antiviral factor associated with the cream fraction of human milk has been shown to inactivate Japanese B encephalitis virus, Friend leukemia virus and Rauscher leukemia virus (Fieldsteel, 1974). Apparently, this antiviral activity is associated with the monoglyceride and free fatty acid fractions (Welsh et al, 1978). It is thought to act by disrupting the integrity of the virus envelope (Welsh and May, 1979). The high triglyceride, low monoglyceride content of cow's milk may account for the lack of lipid-mediated activity shown by this milk (Welsh et al, 1978).

The antistaphylococcal factor present in human milk and described by György (1971) also seems to be a fatty acid.

1.2.1.2. IMMUNOCOMPETENT CELLS

In addition to the soluble defence factors described in the preceding section, human milk and colostrum have an appreciable cellular component, the total concentration of which varies between mothers and from one day to the next (Crago et al, 1979). Although

the concentration falls as lactation progresses, the total cell numbers remain high because the volume of milk produced increases (Lawton and Shortridge, 1977).

The total cell count in milk is in the range of $0.5-10.0\times10^{6}$ cells and comprises macrophages (30-85%); polymorphonuclear leucocytes (1-80%); lymphocytes (1-15%) and occasionally epithelial cells (Lawton and Shortridge, 1977). As lactation proceeds, the secretory epithelial cell predominates (Brooker, 1980), the proportion of macrophages decreases to 5-25% of the cell population and polymorphs and lymphocytes are scarcely encountered (Lawton and Shortridge, 1977).

During pregnancy and lactation, the total number of lymphocytes and macrophages in the human mammary gland markedly increases (Ferguson, 1985). Similarly, there is an accumulation of IgA-secreting plasma cells in the mammary parenchyma of the mouse (Halsey et al, 1982; Roux et al, 1977). The origin of plasma cells and other elements of immunocompetence in the mammary gland appears to be linked to the lymphoid follicles in the respiratory and intestinal tract. Immunoglobulin producing cells observed in the mammary tissue during lactation and in breast milk are thought to originate from antigen-sensitised precursor cells in the GALT and BALT (see Section 1.2.1.1.1).

Using immunofluorescence and autoradiographic techniques, Roux et al (1977) showed that radiolabelled mesenteric lymph node (MLN) cells homed to the mouse mammary gland during lactation. This migration was

inducible and only occurred during late pregnancy. Manning and Parmely (1980) observed T lymphocyte migration to the mammary gland at the onset of lactation and showed that these cells were derived from both intestinal and peripheral T cell pools. To date the origin of mammary macrophages has not been investigated.

Another line of ongoing research concerns the transfer of milk leucocytes and their products to the neonate. Goldblum et al (1975) reported that as early as 3 days after oral immunisation of mothers with E.coli, cells producing antibody against the O antigen of the organism were detected in the colostrum. Oksenberg et al (1985) have also detected E.coli-sensitised lymphocytes in breast milk. Furthermore, Ogra et al (1977) observed transfer of tuberculin reactivity to neonates who suckled from tuberculin-positive mothers.

The ability of milk cells to survive gastric destruction and elicit an immune response upon adoptive transfer to the neonate is poorly understood. Mason (1962) showed that during the 4h interval between feeds of the breast-fed infant, the pH of the stomach oscillates within the range of pH 3.0-6.0, a range which is apparently not inimical to human milk leucocytes (Paxson and Cress, 1979). It is conceivable, therefore, that milk cells may remain viable in the human infant following ingestion. Certainly, animal studies have shown the presence of cells interdispersed in the milk curd of the infant stomach. Head et al (1977) observed large numbers of macrophages,

lymphocytes, plasma cells and neutrophils in the stomach of 2d-old, well-fed mice and rats. Furthermore, these cells were present in proportions similar to those found in rat milk. Miller (1981) was able to show conclusively using autoradiographic techniques, that virtually all of the cells present in the stomach of the suckling mouse were indeed maternally derived.

It is conceivable that if the milk cells survive long enough to pass from the infant stomach, they may contribute to the immunological defence of the neonate. In support of this, Weiler et al (1983) and Sheldrake and Husband (1985) have shown that orally administered, fluorescent labelled cells have the capacity to infiltrate laboratory rodents and neonatal lambs.

The characteristics and functions of individual milk cell types are outlined below.

1.2.1.2.1. MACROPHAGES

Generally speaking, macrophages constitute the highest proportion of cells in human milk (see Section 1.2.1.2). Investigation of their morphological and functional characteristics suggests that they are similar to macrophages from other sites. Cell size is variable, this being attributed to the often large quantity of milk fat globules present in the cytoplasm (Lascelles et al, 1969; Crago et al, 1979). Typically characteristic of macrophages, these milk cells readily adhere to and spread extensively on glass surfaces and

stain positively for α-napthyl acetate esterase stain (Balkwill and Hogg, 1979; Leyva-Cobián and Clemente, 1984; Lascelles et al, 1969; Pitt, 1979). Furthermore, they are actively phagocytic cells which can ingest carbonyl iron, colloidal carbon, colostral fat globules, latex and zymosan during culturing (Blau et al, 1983; Lascelles et al, 1969).

Although the function of the milk macrophage has not been fully elucidated, a positive role for the cell in the prevention of necrotising enterocolitis in the rat has been postulated (Pitt et al, 1977). The protective effect of the breast milk was lost if the milk was frozen and then thawed to kill the cells. However, addition of blood leucocytes, milk leucocytes or peritoneal exudate cells restored the protective effect. As yet, evidence of a similar role by breast milk cells in the human form of the disease has not been forthcoming. Nevertheless, it does appear that the incidence of the disease in breast-fed infants is reduced (Santulli et al, 1975). Kliegman et al (1979) failed to show such a beneficial effect of breast milk but they suggested that their techniques of freezing and storing the milk may have reduced cell numbers and/or function.

The involvement of milk macrophages in defence of the neonate has been inferred by in vitro studies in the following areas: (a) defence against microorganisms (b) accessory cell function and (c) synthesis of biologically active compounds. An outline of such studies is given in the following sections.

1.2.1.2.1.1. DEFENCE AGAINST MICROORGANISMS

As mentioned earlier (Section 1.1.3.1), the phagocytic activity of macrophages is dependent on their state of activation. Human milk macrophages undergo a vigorous oxidative metabolic response upon activation. In response to zymosan or PMA, they release substantial quantities of O_2^- and H_2O_2 (Tsuda *et al*, 1984). Furthermore, the amount released is similar for late and early phase milk cells (Cummings *et al*, 1985). Overall, the amount of O_2^- released by milk cells is higher than that by blood monocytes (Cummings *et al*, 1985). This, the authors suggest, may reflect prior exposure of the milk cells to contaminating bacteria. Milk macrophages exposed to bacterial lipopolysaccharide (LPS) exhibit the characteristic spreading and pseudopod formation of activated macrophages (Pabst and Johnston, 1980).

Activation of milk macrophages increases phagocytosis of zymosan (Blau *et al*, 1983). However, Cummings *et al* (1985) failed to find any relationship between an enhanced oxidative response and greater candidacidal activity. They suggested that intracellular fat may have affected their results. However, *C.albicans* is killed almost exclusively by the myeloperoxidase (MPO) mediated reaction (Lehrer, 1975) and milk macrophages are MPO-deficient (Ho and Lawton, 1978; Tsuda *et al*, 1984).

There appears to have been only one study which has specifically examined the chemotactic response of

milk macrophages. Blood monocytes and milk cells were compared for their response to a panel of chemoattractants (Clemente et al, 1986). A very weak response was demonstrated by the macrophages. This may be attributable to ingestion of soluble components since monocytes exposed to a cell- and fat-free milk supernatant displayed comparable defects.

Two of the most important macrophage receptors involved in phagocytosis are the FCR and CR (see Section 1.1.2). Milk macrophages possess both IgG-FCR and C3b receptors but not IgM-FCR (Balkwill and Hogg, 1979; Cole et al, 1982). The importance of these receptors in phagocytosis of E.coli and C.albicans was shown by Weaver et al (1981). Only serum-opsonised or colostrum-treated organisms were ingested. Furthermore, the FCR allows the milk macrophages to elicit antibody dependent cellular cytoxicity (Mandyla et al, 1982).

Quantitative studies on the microbicidal activity of milk macrophages have provided conflicting results. Ho and Lawton (1978) examined phagocytosis of E.coli and C.albicans by milk macrophages and showed that although the cells demonstrated powerful phagocytic activity, their microbicidal activity was weak. In contrast, Murphy et al (1983) and Robinson et al (1978) observed good phagocytosis and killing of the organisms tested apart from C.albicans. For the reasons mentioned earlier in this section, poor killing of C.albicans is to be expected. However, the disparity of the other

results warrants explanation. Different killing assays were performed in each study. Of these, the protocol adopted by Ho and Lawton (1978) appears the most scientifically sound. In their method, survival of ingested organisms was measured using a pour-plate colony count. Both Murphy et al (1983) and Robinson et al (1978) used antibiotics in their assay systems to kill uningested bacteria. Poor washing may have given exaggerated results for antimicrobial activity.

Milk macrophages apparently acquire immunoglobulins through ingestion from the milk supernatant (Crago et al, 1979). An interesting observation by Pittard et al (1977) suggests an important role for the milk macrophages in the transport of this immunoglobulin. Passive release of IgA and a little IgM occurs during culturing of the cells. In addition, the studies of Weaver et al (1981) show that during phagocytosis there is enhanced release of the IgA.

1.2.1.2.1.2. ACCESSORY CELL FUNCTION IN THE IMMUNE RESPONSE

Induction and expression of immunity in the neonate may require effective presentation of antigen at the surface of the milk macrophages. Macrophages bearing Ia antigen are important for presenting antigen to T lymphocytes (Yamashita and Shevach, 1977). The first study which investigated this function in milk macrophages reported that these cells could effectively present tetanus toxoid or a purified derivative of

tuberculin (PPD) to T cell blasts (Mori and Hayward, 1982). Furthermore, addition of an anti-HLA-DR-like antibody (OK Ia) inhibited presentation. By immunofluorescence, 23% of the total milk cell population were positive for Ia.

Leyva-Cobián and Clemente (1984) found that 70-85% of adherent milk macrophages expressed HLA-DR antigen and supported the proliferative response of T cells to mitogens. Furthermore, a large proportion of these cells were phagocytic for C. albicans. Further confirmation of the ability of milk macrophages to contribute in the immune response came from a recent study by Oksenberg et al (1985). They reported that these cells had the capacity to present antigen and induce proliferation of blood T cells when pulsed by either PPD, phytohaemagglutin (PHA), a synthetic peptide or allogeneic lymphocytes.

Milk macrophages have also been implicated in the specific differentiation of B cells committed to IgA production. Cell-free supernatant taken from a mixed culture of early milk lymphocytes and macrophages and added to cultures of peripheral blood lymphocytes, stimulated IgA synthesis but had no effect on IgG or IgM synthesis (Pittard et al, 1977).

1.2.1.2.1.3. SYNTHESIS OF BIOLOGICALLY ACTIVE COMPOUNDS

Although Pitt (1979) observed peroxidase staining of milk macrophages, no demonstrable staining was observed by Cole et al (1982). Human milk

myeloperoxidase and lactoperoxidase may be derived from human milk leucocytes (Moldoveanu et al, 1982) but the milk neutrophil is the most likely source (Crago et al, 1979; Ho and Lawton, 1978). Tsuda et al (1984) suggested that the low, almost negligible myeloperoxidase activity in their milk macrophage preparations may have been due to contaminating neutrophils.

Lysosomal fusion with the phagocytic vacuole and the concomitant release of lysosomal enzymes into the vacuole is an important digestive process. The lysosomes of milk macrophages vary in number (Tsuda et al, 1984) but there tends to be greater lysosomal staining of milk macrophages than blood monocytes (Pitt, 1979). Due to large deposits of intracellular fat, migration of lysosomes through the milk cell may be impaired. Nevertheless, the lysosomes from these cells are known to contain a number of enzymes of which esterase is probably the most widely documented (Pittard et al, 1977; Crago et al, 1979; Mandyla et al, 1982; Blau et al, 1983; Leyva-Cobián and Clemente, 1984; Tsuda et al, 1984; Cummings et al, 1985). However, the activities of lysosomal enzymes catalase and superoxide dismutase have also been reported in the homogenates of colostral macrophages (Tsuda et al, 1984). Furthermore, the potent bile salt-stimulated lipase found in human milk (Hernell and Bläckberg, 1982) may also be derived from these cells.

The activity of neutral proteases which are elastinolytic, caseinolytic and esterolytic have been

identified in human milk (Lindberg et al, 1982). Furthermore, the activity of several protease inhibitors has also been detected (Lindberg et al, 1982). Their simultaneous release may help control local protease activity.

Several components of the complement system have also been indentified in breast milk (see 1.2.1.1.2) but the origin and functional activity of these proteins has not been fully explored. The milk macrophage, however, may be the source of the C3, active C2 and Factor B (Cole et al, 1982). Milk macrophages secrete greater quantities of C2 and Factor B than blood monocytes which do not produce either of these components during the first three days culture (Cole et al, 1982). Presumably, complement components in milk facilitate phagocytosis of microorganisms.

Milk macrophages synthesise and secrete lysozyme of similar activity to that produced by blood monocytes and neutrophils (Pitt, 1979). Curiously, Blau et al (1983) observed that milk macrophages treated with Con A, zymosan or bacterial endotoxin released less lysozyme than those cells receiving no stimulus. In general, the level of lysozyme synthesis is not influenced by the state of activation (Gordon et al, 1974). In response to stimulation, there is perhaps a selective decrease in the synthesis of this enzyme in favour of other lysosomal enzymes.

Breast milk macrophages may also be the source of lymphokines, biochemically similar to interferons which

are produced upon stimulation of a mixed suspension of milk leucocytes (Lawton et al, 1979).

Finally, breast milk macrophages have also been shown to produce prostaglandins (Blau et al, 1983), an epidermal growth-stimulating factor (Carpenter, 1980) and a factor cytostatic for human tumour cells (Balkwill and Hogg, 1979).

1.2.1.2.2. LYMPHOCYTES

The lymphocyte population of human breast milk consists primarily of T-cells (Crago et al, 1979). Whilst mammary gland T-cells may be derived from both peripheral and intestinal lymphoid organs, mammary B-cells seem to derived only from the latter (see 1.2.1.2).

All five classes of immunoglobulin have been identified in human milk (Goldman and Smith, 1973). but Bahna et al (1982) consider that the very small quantities of IgE and IgD observed arise from local mammary gland production. Although milk lymphocytes bearing IgM, IgG and IgA have been identified, only small quantities of IgM and IgA are released during in vitro culturing even after stimulation of the cells (Pittard et al, 1977; Crago and Mestecky, 1984). However, mixed cultures of milk lymphocytes and macrophages produce greater release of immunoglobulins (Pittard et al, 1977). Pittard and Bill (1979) have shown that when these mixed cultures are taken from early milk a soluble mediator is released which is capable of specifically inducing IgA release from blood

lymphocytes. Thus, an immunoregulatory factor released from early milk cells may selectively induce milk B-cell differentiation.

Immunofluorescence studies have identified functionally distinct T-cell subsets, the majority of which are fully mature (OKT 3^+) and do not possess the OKT 6 surface markers generally found on immature cells (Richie et al, 1982). Milk T-lymphocytes contain OKT 4^+ and OKT 8^+ subsets which mediate helper-inducer and cytotoxic-suppressor immune functions respectively. More specifically, HNK-1^+ natural killer (NK) cells have been identified in human colostrum and shown to be both functionally and morphologically modified by factors in colostral supernatant (Moro et al, 1985).

Milk T-cells are hyporesponsive to mitogenic stimulation relative to peripheral blood lymphocytes (Parmely et al, 1976; Oksenberg et al, 1985). Hyporeactivity is not due to suppressor factors or cells in the milk nor is it due to defective antigen presentation by milk macrophages. It appears to be affected at the T-cell level (Parmely et al, 1976; Oksenberg et al, 1985). Thus, milk T-lymphocytes respond in a manner that reflects a selected population of immunocompetent cells.

Analysis of cell-mediated immunity to microbial antigens has shown that these cells are restricted in their ability to recognise and respond to certain infectious agents. Whilst milk lymphocytes respond more vigorously to pathogenic E.coli and influenza virus (Parmely et al, 1976; Ruben et al, 1982; Oksenberg et

al, 1985) than blood lymphocytes, they respond less vigorously to Yersinia enterocolitica, C.albicans, tetanus toxoid and streptokinase (Parmely et al, 1977; Oksenberg et al, 1985). Furthermore, proliferative responses to mumps, measles and tuberculin (PPD) antigens were similar for the two sources of lymphocytes (Parmely et al, 1977).

Adoptive transfer of cell-mediated reactivity has been demonstrated by cases in which T-cell reactivity to PPD was transferred to unsensitised infants by suckling from PPD-reactive mothers (Ogra et al, 1977). Immunocompetent milk cells may be responsible for this transfer.

Studies on the transfer of histocompatibility antigens and T-cells to the suckling rodent provide conflicting views on the ability of milk T-lymphocytes to influence survival of skin grafts or to initiate a graft-versus-host (GVH) reaction. However, it appears that milk cells alone are insufficient to induce tolerance to allografts (Head et al, 1977; Silvers and Poole, 1975) unless they have been pre-sensitised to the foreign antigens of the recipient (Beer et al, 1974).

Using total milk cell populations, Lawton et al (1979) showed production of both a heat-stable and a heat-labile interferon. Lymphocyte-enriched cultures of milk cells also produced two lymphokines which were characterised as lymphocyte-derived chemotactic factor and immune (gamma-) interferon (Keller et al, 1981). Kohl et al (1982) have shown that colostral leucocytes

induced by herpes-simplex virus produced a cytokine which mediated NK cytotoxicity. Thus, transfer of T-lymphocytes, or their products, in milk may account for the transfer of immune reactivity and the lower incidence of viral infection in the suckling infant.

1.2.1.2.3. POLYMORPHONUCLEAR LEUCOCYTES (PMN)

Considering that PMN sometimes constitute a major proportion of the cell population in milk (see 1.2.1.2), their morphological and functional characteristics are not well-documented. Furthermore, several studies investigate the properties of the total milk phagocyte population and do not distinguish between neutrophils and macrophages. However, histochemical staining techniques have revealed that milk PMN contain numerous lipid-filled vacuoles and are peroxidase positive (Ho and Lawton, 1978; Crago et al, 1979). Furthermore, immunofluorescence techniques detected the presence of various immunoglobulin classes, secretory component and ALA intracellularly which the cells may have ingested from milk (Crago et al, 1979).

Neutrophils release lactoferrin (Lf) during phagocytosis (Van Snick et al, 1974). Milk PMN may be the source of milk Lf especially as it has been detected within these cells (Crago et al, 1979).

Several authors have investigated the antimicrobial activity of milk phagocytes (Ho and Lawton, 1978; Robinson et al, 1978; Murphy et al, 1983; Pickering et al, 1983) but only two quantitative studies have specifically dealt with milk neutrophils (Ho and

Lawton, 1978; Pickering et al, 1983). Phagocytosis of C.albicans and E.coli by milk PMN is comparable to that by milk macrophages and blood leucocytes. However, milk neutrophils are more active than milk macrophages and less active than blood leucocytes with respect to killing of Candida (Ho and Lawton, 1978). Binding and ingeston of milk fat by blood neutrophils caused a reduction in O_2 metabolism, hexose monophosphate shunt activity and the general metabolic and phagocytic activity of the cells (Pickering et al, 1980; Pickering et al, 1983). However, D.F Johnson et al (1980) have shown that the phagocytic activity is comparable for milk PMN and blood PMN, whilst the metabolic activity of the former is greater than that of the blood cells. Thus, it appears that the milk neutrophil was initially a more metabolically and phagocytically active cell than the blood neutrophil.

Milk neutrophils are also thought to be involved in antibody-dependent cell-mediated cytotoxicity (Mandyla et al, 1982) and they appear to exhibit chemotactic responses albeit lower than those of blood neutrophils (Khan et al, 1980).

1.2.2. THE DISADVANTAGES OF BREAST MILK

Although breast milk is now considered the most suitable food for the normal neonate, several shortcomings of breast milk are acknowledged.

Firstly, breast-feeding may not always be possible because of insuffient milk or infections of the mammary

gland. Furthermore, infectious organisms, chemical contaminants, drugs and antibiotics may sometimes be transmitted to the neonate (Ogra and Greene, 1982). Although the extent to which such transfer affects the neonate has not been well-documented, it is known that food antigens ingested by the mother may cause allergic reactions in susceptible infants (Gerrard, 1979).

It is also known that the breast-fed child does not grow as rapidly as its formula-fed counterpart (Ogra and Greene, 1982) although it is acknowledged that this does not necessarily mean that the weight gain of breast-fed infants is inadequate. Finally, the nutritional quality of breast milk may be insufficient for the premature or low birth weight infant.

1.2.3. COMPOSITIONAL DIFFERENCES BETWEEN HUMAN MILK, COW'S MILK AND INFANT FORMULA

The chemical composition of various milks is shown in Table 1. Several major differences are evident. Of note is the proportion of CAS and whey proteins. CAS is the major protein in cow's milk. It is not a single entity but a group of protein subunits, and whilst human milk contains primarily β-casein and a little κ-casein, cow's milk contains a large proportion of α-caseins (Lönnerdal, 1985). The smaller amount of CAS in human milk allows better precipitation of the milk to form a soft curd more quickly digested in the gastrointestinal tract than the curd from cow's milk (Vorherr, 1978).

TABLE 1

Nutritional comparison of various infant formulae, human milk and cow's milk per 100ml.

		MATURE HUMAN MILK		OSTERFEED[c] BABYMILK (Farley Health Products)	PREMIUM[c] [§] (Cow & Gate)	SMA[c] GOLDCAP (Wyeth)	OSTERMILK[c] COMPLETE FORMULA (Farley Health Products)	OSTERMILK[c] TWO (Farley Health Products)	PLUS[c] (Cow & Gate)	SMA[c] (Wyeth)	MILUMIL[c] (Milupa)	COWS[d] MILK
		DHSS[a]	Macy et al[b]									
Macronutrients												
Protein†	g	1.34	1.45	1.45	1.5	1.5	1.7	1.8	1.9	1.5	1.85	3.4
Casein/Whey ratio		—	32/68	39/61[a]	33/67[a]	40/60[a]	77/23[a]	77/23[a]	77/23[a]	82/18[a]	80/20	77/23[a]
Fat	g	4.2	3.8	3.82 (milk fat + veg oils)	3.8 (milk fat + veg oils)	3.6 (beef oleo + veg oils)	2.6 (milk fat + veg oils)	2.4 (milk fat)	3.5 (milk fat + veg oils)	3.6 (beef oleo + veg oils)	3.1 (milk fat + veg oils)	3.9
Saturated	%	50.1	52	39.5	47.5	46.7	39.5	63.4	47.5	46.7	53.5	63.2[a]
Unsaturated	%	48.5	48	60.5	52.5	53.1	60.5	36.6	52.5	53.1	46.5	36.6[a]
Carbohydrate (as disaccharide)	g	7.0	7.0	7.0	7.2	7.2	8.6	8.3	6.9	7.2	8.4	4.6
Lactose	g	7.0	7.0	7.0	7.2	7.2	2.8	5.3	6.9	7.2	6.0	4.6
Maltodextrin	g	—	—	—	—	—	5.8	3.0	—	—	1.3	—
Amylose	g	—	—	—	—	—	—	—	—	—	1.1	—
Energy												
	kcal	70	68	68	68	65	65	62	65	65	68	67
	kJ	293	285	284	284	275	273	260	272	275	286	280
Minerals												
Sodium	mg	15	15	19	18	15	31	31	28	26	27	52
Potassium	mg	60	65	57	60	56	70	79	89	74	86	155
Chloride	mg	43	43	44	57	40	56	58	57	47	44	98
Calcium	mg	35	33	36	40	44	61	65	66	56	71	124
Magnesium	mg	2.8	4	5.2	4.5	5.3	6.0	6.4	6.0	5.3	7.0	12
Phosphorus	mg	15	15	31	27	33	49	53	53	45	55	98
Trace Elements												
Iron	µg	76	150	650	650	670	650	650	650	670	700	50
Copper	µg	39	40	43	—	50	39	16	—	50	13	20
Manganese	µg	ND	0.7	1.2	10	16	3.3	1.0	10	16	11	ND
Zinc	µg	295	530	350	340	370	330	200	340	370	224	360
Iodine	µg	7	7	4.5	3.4	7	10	11	3.4	7	ND	ND
Renal Solute Load*												
	mOsm/l	88	91	93	99	92	115	122	127	103	120	225
Vitamins												
Vitamin A	µg	60	53	100	80	79	97	95	80	79	61	40
Vitamin D	µg	0.01	0.01	1.0	1.1	1.05	1.0	1.0	1.1	1.05	1.0	0.02
Vitamin E	mg	0.35	0.56	0.48	1.0	0.95	0.46	0.45	1.0	0.95	0.6	0.09
Vitamin K	µg	ND	1.7	2.7	2.8	5.8	2.6	1.5	2.8	5.8	4.0	ND
Vitamin B_1	µg	16	16	42	70	80	39	38	70	80	40	40
Vitamin B_2	µg	31	42.6	55	100	110	53	51	100	110	50	200
Niacin/Niacinamide	µg	230	172	690	850	1000	650	640	850	1000	400	80
Vitamin B_6	µg	6	11	35	80	51	33	32	80	51	30	40
Vitamin B_{12}	µg	0.01	Trace	0.14	0.11	0.11	0.13	0.13	0.11	0.11	0.15	0.3
Folic Acid	µg	5.2	0.18	3.4	3.5	5.3	3.2	3.1	3.5	5.3	10	5
Pantothenic Acid/ Calcium Pantothenate	µg	260	196	230	250	210	220	220	250	210	400	360
Biotin	µg	0.76	0.4	1.0	3.1	1.5	0.97	0.95	3.1	1.5	1.1	2.1
Vitamin C	mg	3.8	4.3	6.9	5.5	5.8	6.4	6.2	5.5	5.8	6.0	1.5

* Method of Ziegler and Fomon, 1971: Calculated values

†Total Nitrogen × 6.38

§ Powder only — Ready-to-Feed per 100ml: protein 1.8g; fat 3.45g; lactose 6.9g; energy 65 kcal (272 kJ).

Sources

a DHSS Reports on Health and Social Subjects Nos 12 (1977), 18 (1980) and 20 (1980).

b Macy, Kelly and Sloan, 1953 and Mettler, 1976

c Manufacturers' information (1980 and 1981)

d Paul and Southgate, 1978 (100g = 103ml)

ND – Not determined

Manufacturers and Parent Companies

Farley Health Products Ltd. (Glaxo Group Ltd. U.K.)

Cow & Gate Ltd. (NV Nutricia, Holland)

John Wyeth and Brother Ltd. (American Home Products, U.S.A.)

Milupa Ltd. (Altana AG, West Germany)

Prepared by:

FARLEY HEALTH PRODUCTS *(A Glaxo Group Company)* (1982)

The major whey protein of cow's milk, BLG, is absent from human milk (Bell and McKenzie, 1964) but concentrations of carbohydrate, lactose ALA, lysozyme, cysteine, taurine and sIgA are significantly higher in human milk (Mata, 1978). Cow's milk compensates for the low levels of IgA with high levels of IgG (Ogra, 1979).

Infant formula can be categorised into three major types-(1) cow's milk based; (2) soy based or (3) protein hydrolysates. The most frequently used are milk-based and contain vegetable oils and lactose and/or corn syrup solids as a carbohydrate source (Table 1; Mead-Johnson, product information, 1981). They are adapted and fortified for infants with special requirements and are commercially available as concentrated liquids, powder or ready-to-feed liquids.

Soy based formulas provide an alternative for infants adversely affected by cow's milk. Early soy based formulas were made from whole soy flour, but to minimise gastrointestinal disorders and prevent malodorous stools, they are now made with isolated soy protein (Wysoy, product information, 1981).

A range of specialised formulae made from protein hydrolysates is available for infants suffering from metabolic disorders such as lactose intolerance and fat malabsorption. CAS and meat are the two most common sources of protein in such formula preparations (Cow and Gate Ltd., product information, 1981).

1.3. IMMUNOLOGICAL CONSEQUENCES OF FORMULA FEEDING

Many foods cause adverse reactions upon ingestion, but cow's milk is considered the most common cause of allergic disease (Bleumink, 1983). The disease manifests itself clinically as gastrointestinal, respiratory or dermatologic disorders and is more prevalent in infants less than 1yr of age (Minford et al, 1982). One proposal suggests that the younger infant is more susceptible because the mucosal barrier is more permeable and allows greater access of intact proteins (Udall and Walker, 1982). Certainly, bovine serum albumin (BSA) and BLG have a greater affinity for microvillus membrane models from immature rats than for those from mature rats (Stern et al, 1984). Furthermore, protein breakdown by mucosa-associated enzymes is decreased in the everted gut sac models from immature animals compared to those from mature animals (Stern et al, 1984).

Various aspects of the immune response to cow's milk proteins have been examined. Adverse reactions to milk may result from a breakdown in function at the mucosal barrier and/or a loss of systemic hyporesponsiveness.

Ingested food is degraded in the gut by proteolytic enzymes and, more importantly, by lysosomal enzymes (Heyman et al, 1982). Antigenic peptides crossing the mucosal barrier elicit an immune response. This leads to secretion of specific

antibodies, predominantly of the IgA class, which prevent further antigen absorption (Kagnoff, 1981). A breakdown in any of these processes can lead to the development of allergic disease.

Apparently, immature animals have decreased trypsin-like activity (Udall et al, 1984) and breakdown less protein at the mucosal barrier (Stern et al, 1984) than older animals. Hirata et al (1965) have shown that the human infant's ability to handle milk proteins also increases with age. However, the duodenal juice from infants, children and adults digests CAS equally well (Lindberg, 1974). Thus, the limited ability to digest proteins in infancy may not necessarily be due to a lower proteolytic activity in the duodenal juice per se, but to its smaller volume. In support of this, Jakobsson et al (1982) found that the duodenal juice from infants with CMPI could rapidly hydrolyse CAS.

The nature of the local immune response to cow's milk is not completely clear. Shiner et al (1975) observed an increase in mucosal IgE-containing plasma cells in two infants with CMPI, following milk challenge. This was accompanied by an increase in mucosal IgM, IgE and C3. After a feed of cow's milk, children with CMPI showed an increase in plasma cells containing antibody to BLG and BSA (Pearson et al, 1983). Furthermore, the majority of such cells were of the IgA class although IgE-cells were also observed (Pearson et al, 1983). In cases of cow's milk enteropathy, the number of IgM-, IgG- and IgA-containing cells increases following a milk challenge

(Stern et al, 1982). It may be as Shiner et al (1975) suggest, that a reaginic IgE-mediated reaction occurs in the early stages of CMPI and is replaced by other reactions involving IgG, IgM and IgA.

At the cellular level, Juto et al (1982) reported that the lymphocyte blastogenic response to mitogens is greater in children fed cow's milk than in those breast-fed. The authors suggest that early weaning to cow's milk alters T-cell reactivity and effects the interaction between different subpopulations of T-cells and /or macrophages. Immune responses to food proteins are subsequently altered.

Since they are deprived of milk IgA, formula-fed infants will have lower levels of IgA at the mucosal surface than breast-fed infants. Cunningham-Rundles et al (1978) have shown that in cases of IgA deficiency, ingestion of food results in circulating immune complexes and/or food antigen. Certainly, the major allergen of cow's milk, BLG (Bleumink and Young, 1968) has been detected antigenically intact in the sera of term infants fed a cow's milk based formula (Roberton et al, 1982). Furthermore, circulating immune complexes containing BLG have also been found in the sera of milk-allergic infants following milk challenge (Paganelli et al, 1983). Both atopic and non-atopic children formed IgA complexes but increased levels of IgG, IgE or C1q complexes were observed in the sera of atopics (Paganelli et al, 1983).

An IgE-mediated response is more likely in

genetically predisposed infants (Croner et al, 1982) and in cases of low antigen dose (Firer et al, 1981). The reductive cleavage studies of Huang et al (1985) suggest that restricted parts of the BLG molecule are involved in reactivity with IgE whereas conformational determinants are reactive with IgG. Fragments of less than 10,000 molecular weight are non-antigenic. The duodenal juice from infants with CMPI hydrolyses BLG and ALA at a slower rate than it hydrolyses CAS (Jakobsson et al, 1982). Thus, both the rate and the degree of protein degradation may be important.

Serum antibody levels to cow's milk proteins are lower in infants after a feed of heat-treated cow's milk or a milk based formula of low protein content than after a feed of pasteurised cow's milk (May et al, 1982). Decreased antibody levels to cow's milk proteins were also found in the sera of rabbits fed formula milk compared with those fed cow's milk (Eastham et al, 1982). Collectively, these studies suggest that the milk proteins are modified during heating and processing of formula. Both the type of formula and the method of processing may therefore determine the sensitising capacity.

Soy formulas are as antigenic as cow's milk based formulas with respect to antibody responses in rabbits but more antigenic than protein hydrolysate formulas (Eastham et al, 1982). However, although partial tolerance to cow's milk proteins was achieved by feeding a cow's milk based formula, a more prolonged tolerance was observed after feeding soy formula. In human

infants, Zoppi et al (1982) observed lower levels of immunoglobulin, transferrin and complement components in the sera of soy-fed infants compared with infants fed a cow's milk based formula. Moreover, T-cell markers and reactivity were impaired in soy fed individuals. All these effects exemplify the clinical hypoallergenicity of soy formula.

McLaughlan et al (1981) have shown that heat treatment reduces the sensitising capacity of milk proteins CAS and BLG. Severe heating reduces the sensitising capacity of CAS and abolishes that of BLG and ALA (Kilshaw et al, 1982). Heat-denatured whey proteins fail to sensitise guinea pigs for anaphylaxis and produce little, if any, antibody response (Heppell et al, 1984). The three-dimensional conformation of the whey proteins is altered by heating whereas the irregular structure of CAS is changed little (McLaughlan et al, 1981). Formulas of equivalent chemical composition had different sensitising capacities when processed differently. With less heat treatment, there was greater sensitisation (McLaughlan et al, 1981). To produce hypoallergenic cow's milk based formulas, it has been suggested that the antigenicity of whey proteins be reduced by heating before addition of essential nutrients (Kilshaw et al, 1982; Heppell et al, 1984).

CHAPTER 2

EFFECT OF MILK ON THE FUNCTION OF MOUSE PERITONEAL MACROPHAGES

2.1. INTRODUCTION

Susceptibility to allergic disease and infection in the newborn infant diminishes with prolonged breast-feeding (see Section 1.2). It is not known whether this is a consequence of delayed exposure to foreign antigens and/or a protective effect of the breast milk. Although a variety of soluble breast milk components such as IgA may contribute to this protection (see 1.2.1.1), immuno-competent cells may be equally important.

Macrophages constitute a significant proportion of the cells in breast milk (Lawton and Shortridge, 1977). Pitt et al (1977) reported that these milk cells appear to play a key role in prevention of necrotising enterocolitis in rats. They also showed that leucocytes, in general, were more bactericidal in rat milk than in formula. More specifically, Russell et al (1976) have shown that CAS from cow's milk interferes with the phagocytic activity of polymorphonuclear leucocytes. These findings, if applicable to the human infant, would not only indicate an important protective role for breast milk macrophages, but also suggest that their effect might be impaired by mixed feeding. This is particularly important considering that milk macrophages may already exhibit reduced activity.

Comparisons with blood cells has revealed that milk cells have a reduced ability to phagocytose and/or kill microorganisms (Ho and Lawton, 1978; Pickering et

al, 1983), to mediate antibody dependent cellular cytotoxicity reactions (Mandyla et al, 1982), and to exhibit chemotaxis (Clemente et al, 1986). Incubation of the blood cells with milk supernatant produced comparable defects. Collectively, these studies suggest that the activity of macrophages associated with the gut mucosa might also be affected by cow's milk and/or human milk.

Many foods have been reported as a cause of allergic reactions (Bleumink, 1983) but cow's milk appears to be the most sensitising, especially in children. Several mechanisms may be involved in the development of such reactions, which may result from a breakdown at the mucosal barrier and/or loss of systemic hyporesponsiveness.

Both intact cow's milk proteins and specific immune complexes containing these proteins have been detected in the sera of bottle-fed infants (see 1.3). Furthermore, BLG which is often implicated in CMPI (Devey et al, 1976) binds to rat microvillus membranes and can be absorbed as large peptides and/or small fragments (Stern and Walker, 1984). Most of the protein digestion in the gut is done by lysosomal enzymes rather than proteolytic enzymes such as pepsin and trypsin (Heyman et al, 1982). Thus, intestinal macrophages may be crucial in the degradation and transport of foreign food antigens. Interference with their ability to clear and degrade immune complexes and/or release lysosomal enzymes or complement

components may lead to inflammatory conditions similar to those of CMPI. Certainly, intestinal macrophages play a prominent role in many inflammatory conditions of the gastrointestinal tract (Territo and Cline, 1976). In the case of macrophages in the Peyer's patches, interaction with components of cow's milk might represent a first step in sensitisation of the infant to cow's milk protein.

The aim of the present study was to determine whether or not human milk or infant formula preparations interfere with the phagocytic activity of macrophages. If either milk does exert any effect on cell function, be it stimulatory or inhibitory, it would also be interesting to see if this was dependent on the concentration of milk used.

Mouse peritoneal macrophages were chosen for the in vitro assay system because they are easily obtainable, and it was possible to examine the effect of milk exposure on a phagocytically more active cell, the thioglycollate-elicited macrophage. There appears to have been no previous study of the effect of breast-milk on the antimicrobial activity of macrophages or on the cells' ability to phagocytose and degrade immune complexes.

The extent to which cells bind and ingest cow's milk proteins after exposure to formula may be important in the initiation of CMPI. The cow's milk proteins CAS, BLG and ALA are the most well-known, and commonly used substitutes for human milk proteins. The ability of these proteins to interact with mouse macrophages was

therefore investigated using an indirect immunofluorescence technique.

2.2. MATERIALS

2.2.1. Animals. Male and female CF/NIH mice, not younger than 4 weeks of age were used throughout. Mice were obtained from stock animals bred in the departmental animal unit.

2.2.2. Reagents

2.2.2.1. Phosphate buffered saline (PBS). This consisted of NaCl (8g/l), KCl (0.2g/l), Na_2HPO_4 (1.15g/l) and KH_2PO_4 (0.2g/l) (Analar grade, BDH Chemicals Ltd., Poole, Dorset, England). When required, aliquots were sterilised by filtration.

2.2.2.2. Thioglycollate broth. Dehydrated Brewer Thioglycollate Medium (Difco, Surrey, England) was prepared acccording to manufacturers' instructions and stored in the dark until required.

2.2.2.3. Culture medium. This was prepared by supplementing RPMI 1640 culture medium (without Hepes: Flow, Irvine, Ayrshire, Scotland) with 0.3mg/ml glutamine (BDH), 100IU/ml penicillin and 100µg/ml streptomycin (Flow). Finally, 5% (v/v) heat-inactivated (56°C, 30min) foetal calf serum (FCS: Gibco, Paisley, Scotland) was added. Antibiotics were omitted from the medium used in the bactericidal assays (see 2.3.7.2.).

2.2.2.4. Ferric nitrilotriacetate solution (FeNTA). One volume of a freshly prepared solution of $FeCl_3$ (20mM: BDH) was added dropwise, with stirring, to an equal volume of NaNTA (80mM, pH 7: Sigma Chemical Co., Poole, Dorset, England) and the pH adjusted to 5 with 1M NaOH. The resulting concentration of FeNTA was 10mM.

2.2.2.5. Tris-glycerol buffer. A solution containing 0.2M Tris (25 parts: Hopkins and Williams, Essex, England) 0.1M HCl (5.3 parts) and distilled water (DW: 100 parts), was added to glycerol until the pH of the solution was 8-9.

2.2.2.6. PBS-glucose. This consisted of glucose (1mg/ml) in PBS (see: 2.2.2.1.). Aliquots were sterilised by filtration and stored at $4^{o}C$.

2.2.2.7. Casein solutions. These were prepared in PBS. To ensure proper dissolution of the protein in the PBS, 0.1M NaOH was added, a little at a time, with stirring. When the CAS had dissolved, 0.1M HCl was added in small aliquots until the pH of the solution was 7. A concentrated stock solution (60mg/ml CAS) was made fresh and diluted as required in PBS.

2.2.3. Formula milks. Powdered formulas: Ostermilk Complete Formula (Farley Health Products, Plymouth, England) and Wysoy (Wyeth, Taplow, England), a cow's milk free soya formula, were reconstituted in tap-water according to the manufacturers' instructions. Pasteurised cow's milk and Ostermilk Complete Formula

(Farley), a ready-to-feed liquid, were diluted as required in culture medium (see 2.2.2.3.).

2.2.4. Normal mouse serum. CF/NIH mice were anaesthetised in ether and exsanguinated by withdrawal of blood from the heart. Pooled mouse blood was allowed to clot at room temperature. The serum was removed to plastic, conical bottomed test-tubes (Sterilin, Teddington, Middlesex, England), centrifuged twice at 1500g for 5min to pellet any erythrocytes, and stored at -20°C in 2ml aliquots until required.

2.2.5. Normal rabbit serum. Approximately 20ml blood was collected from the ear vein of a rabbit into a glass universal and allowed to clot at room temperature. The serum was then pooled, transferred to plastic conical-bottomed test-tubes (Sterilin) and centrifuged twice at 1500g for 5min to remove any erythrocytes. Aliquots of 2ml were dispensed into glass bijoux and stored at -20°C.

2.2.6. Antisera. Antisera to CAS and BLG were raised in rabbits from the departmental animal unit. The animals were first given a subcutaneous injection (0.5ml) into each flank such that each animal received a total of 1mg of protein with Freunds Complete Adjuvant (Difco). This was followed one month later with similar booster injections containing a total of 2mg. One week later, blood was collected from the ear vein of each rabbit, allowed to clot and the serum collected

by centrifugation. The antibody titre was checked by the Ring Precipitin Test as follows: a small volume of antigen (0.01mg/ml, 0.1mg/ml or 1mg/ml) was gently layered on top of a similar volume of antiserum in a glass Durham tube. The formation of strong precipitin bands with all three concentrations of CAS and BLG indicated a very good titre.

Lyophilized rabbit antiserum to bovine ALA was kindly provided by Dr. A. Pinheiro, Department of Biochemistry, University of Zaragoza, Spain and reconstituted in 1ml of DW. This antiserum was checked by the Ring Precipitin Test as above and found to give strong bands with the 0.1mg/ml and 1mg/ml concentrations of protein. All antisera were stored in glass bijoux bottles at $-20^{o}C$ until needed. With prolonged use, insoluble debris and aggregates formed, and were removed by centrifugation in a haematocrit centrifuge (Hawksley, England) for 2min.

2.2.7. Reagents for α-napthyl acetate esterase staining.

2.2.7.1. Fixative. Formol calcium solution, containing 10% formaldehyde and 1% $CaCl_2$ in DW was used.

2.2.7.2. Phosphate buffer (0.06M, pH5). This consisted of 98.5ml of KH_2PO_4 (9.08g/l) and 1.5ml of $Na_2HPO_4.2H_2O$ (11.88g/l). The buffer was sterilised by autoclaving.

2.2.7.3. Hexazotised pararosaniline. This contained equal volumes of two solutions which were prepared as

follows:

Solution A: 5ml of concentrated HCl was added to 20ml of DW to which had been previously added 1g of pararosaniline (Sigma). This solution was gently warmed to dissolve the pararosaniline, allowed to stand at room temperature to cool and finally filtered. The filtrate was then stored in the dark at $4^{o}C$.

Solution B: this was a freshly prepared solution of 4%$NaNO_2$ in DW.

2.2.7.4. <u>α-napthyl acetate esterase (ANAE) stain</u>.

Hexazotised pararosaniline (2.4ml) was added to 40ml of the phosphate buffer. Next, a freshly prepared solution of 10mg of α-napthyl acetate (Sigma) in 0.4ml acetone was added and the pH raised to 5.8 with 2N NaOH. The stain was filtered before use.

2.3. METHODS

2.3.1. Preparation of macrophage cultures

2.3.1.1. Collection of mouse peritoneal macrophages

Resident macrophages were obtained by washing the peritoneal cavity of unstimulated CF/NIH mice (see 2.2.1) with 2ml sterile PBS (see 2.2.2.1). Stimulated murine peritoneal cells were similarly obtained using mice which had been injected intraperitoneally 4 days earlier with 2ml of thioglycollate broth (see 2.2.2.2).

Peritoneal washings were centrifuged at 170g for 2min, the supernatant discarded and the cells resuspended in culture medium (see 2.2.2.3). Cell viability was determined by eosin exclusion and was generally in the range of 85-95%. Only cells showing viability >90% were used.

2.3.1.2. Preparation of cell monolayers

Replicate cultures containing approximately 1.5×10^6 cells in 1ml of culture medium (see 2.2.2.3) were incubated in a 24-well sterile plate (Flow) in the presence of 5% CO_2. After 2h, non-adherent cells were removed and the adherent population washed twice with HBSS. Approximately 60% of resident cells and 75% of stimulated mouse peritoneal cells adhered. Thus, each well contained approximately 1×10^6 adherent cells. More than 90% of these cells had the morphological and staining characteristics of macrophages (see 2.3.2 and Fig.1).

2.3.2. Differential staining

2.3.2.1. α-napthyl acetate esterase (ANAE) stain

Peritoneal cells were collected and counted as described in section 2.3.1.1. Approximately $5x10^5$ were deposited on clean glass slides in a Shandon cytocentrifuge at 1000 r.p.m. for 90sec. and then air-dried. Adherent macrophages were obtained by allowing peritoneal exudate cells to adhere to round, sterile glass coverslips (16mm diameter: MacFarlane Robson, Glasgow, Scotland) placed in the wells of a square-well plastic culture dish (Sterilin). Culture wells were seeded with approximately $5x10^6$ cells and incubated for 2h to allow attachment of the macrophages to the coverslips. After washing twice in PBS to remove non-adherent cells, these preparations were also air-dried.

Preparations were then fixed in formol calcium (pH 6.7: see 2.2.7.1) at 4^oC for 10min and washed in tap water for 20min at room temperature. ANAE activity was demonstrated by incubating with the staining reagent (see 2.2.7.4) for 2h at 37^oC. Cell preparations were then washed gently in running tap water for 10min, left to dry at room temperature and then counterstained with 2% methyl green for about 30sec. Finally, cells were washed in tap water, air-dried and mounted with DPX. Slides were examined using the light microscope (x400) and the different cell types scored. Macrophages show a cytoplasm filled with diffuse brown staining material (Fig.1).

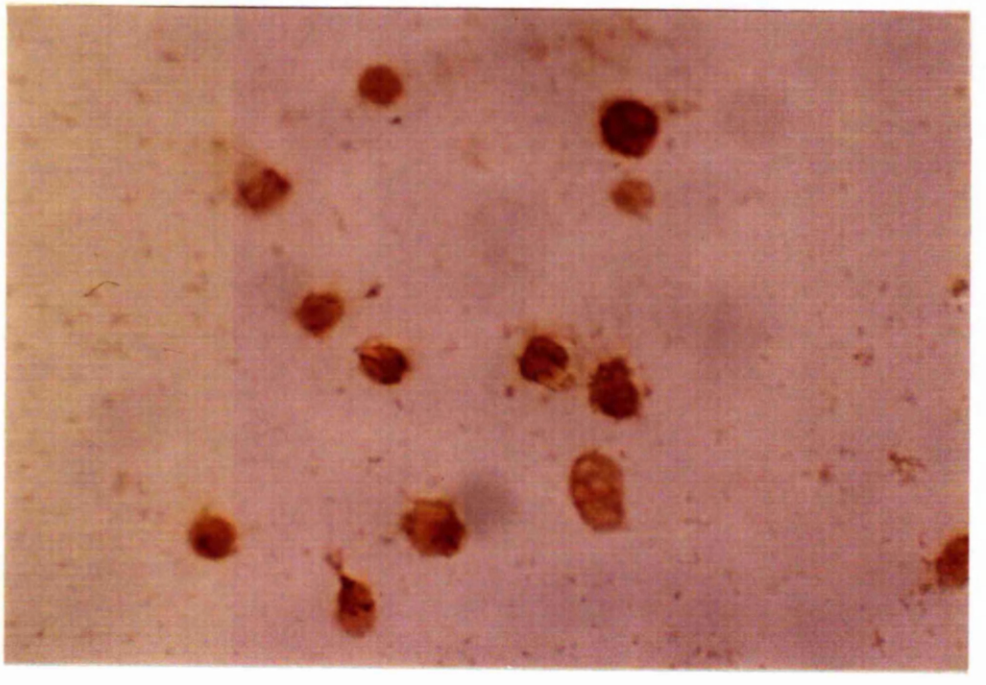


FIGURE 1 : An adherent culture of resident mouse peritoneal cells stained with α-napthyl acetate esterase stain (x800).

2.3.2.2. Leishman stain

Cytospin preparations of peritoneal exudate cells and adherent peritoneal cell populations were prepared and air-dried as described above (see 2.3.2.1). All cell cultures were then fixed and stained by first adding undiluted Leishman stain (Exogen Ltd., Clydebank, Glasgow, Scotland) for 2min and then diluted stain (1 in 3 in DW) for 10min. Preparations were then washed in tap water, air-dried, and mounted in DPX. A differential cell count of the cytospin preparations was obtained by examining different fields of view in a Leitz-Wetzlar light microscope (x400). The percentage of each cell type was then calculated. Examination of several fields of view also ensured that the majority of the cells present were macrophages.

Differential counts for resident mouse peritoneal cells showed that the majority of cells were macrophages (56-80%). However, lymphocytes (10-31%); neutrophils (0-8%); eosinophils (0-1%) and occasionally basophils (4-9%) were also observed. In comparison, stimulated cells contained macrophages (61-88%); lymphocytes (9-12%) and neutrophils (24-27%).

2.3.3. Processing of human milk specimens.

Human breast milk or colostrum was collected from healthy mothers at the Queen Mother's Maternity Hospital, Yorkhill, Glasgow. Samples were generally obtained up to 8 days postpartum by manual expression, or occasionally by breast pump expression, into sterile plastic universals (Sterilin) and processed within 2h of

collection. Centrifugation at 200g for 10min separated the milk into 3 components; a cellular pellet, an aqueous supernatant and a top layer of lipid. Acellular fractions were removed and thoroughly vortexed in order to disperse fat globules uniformly throughout.

2.3.4. Preparation of ^{125}I-labelled transferrin-antitransferrin immune complexes.

2.3.4.1. Iodination of human transferrin (HTf).

Each molecule of human transferrin (MWt 80,000) binds two iron atoms (MWt 56). A 4mg/ml solution of human apotransferrin (Hoechst Ltd., Hounslow, England) was prepared in 0.05M PBS and the iron binding sites saturated by adding excess iron in the form of FeNTA(see 2.2.2.4). Addition of 0.5ml of the transferrin solution to 1mCi of ^{125}I (carrier free: Radiochemical Centre, Amersham, England) was followed immediately afterward with 25µl of a 1mg/ml solution of chloramine T (BDH) in PBS. After a 5min time lapse with occasional shaking, 25µl of a 1mg/ml solution of sodium metabisulphite (Analar) in PBS was added, thoroughly mixed and followed immediately by addition of 25µl of a 5mg/ml solution of KI (Analar) in PBS.

Labelled protein was separated from unreacted iodide by passing the solution through a Sephadex G25 column (Pharmacia, Uppsala, Sweden) eluted with PBS. Aliquots of approximately 2ml were collected and the activity checked with a hand monitor (Mini-Instruments Ltd., Essex, England). The column was run until both the protein peak and then the unreacted iodide peak had

been eluted. To further ensure separation of iodinated transferrin from free iodide in solution the fraction(s) which constituted the protein peak were dialyzed against PBS for 24h at 4°C.

2.3.4.2. Formation of antigen-antibody complexes in optimal proportions.

A series of tubes containing 20µg of ^{125}I-HTf and different amounts (20-400µl) of rabbit anti-HTf antiserum (Table 2) were made up to volumes of 0.4ml with PBS. Solutions were incubated at 50°C for 10min and then at 4°C for a further 5min. Insoluble immune complexes were then collected by centrifugation for 7min in a microhaematocrit centrifuge (Hawksley). The ^{125}I-activity in the supernatants and pellets was counted in an LKB Compugamma Model 1282 (LKB Instruments, Croydon, England). Activity of each precipitate was calculated as a percentage of the total radioactivity added. A ratio of 100µl antiserum to 20µg ^{125}I-HTf was found to be optimal (Table 2).

2.3.5. Determination of the mid-log phase growth of Staphylococcus aureus NCTC 8532

2.3.5.1. Maintenance of stock cultures of Staph aureus

A freeze-dried ampoule of Staph aureus 8532 was obtained from the National Collection of Type Cultures, London and subcultured on to nutrient agar slopes (Oxoid, Basingstoke, Hampshire, England) which were stored at room temperature until required. Sub-culturing of these bacteria on to fresh slopes was carried out every 3-4 weeks.

TABLE 2

Determination of optimal proportions of rabbit anti-human transferrin (RαHTf) and ^{125}I-human transferrin (^{125}I-HTf) for formation of immune complexes.

µl RαHTf	µg ^{125}I-HTf	µl PBS	% radioactivity precipitated
400	20	0	71.2
200	20	200	73.6
100	20	300	72.5
50	20	350	49.5
20	20	380	2.1

2.3.5.2. Growth curve of Staph. aureus

A 5ml volume from an overnight culture of Staph. aureus in Meat Extract (ME) broth (Oxoid) was subcultured into 100ml of fresh warm broth in a stoppered flask and placed in a shaking water bath at 37°C. Immediately, and at 30min intervals thereafter, aliquots of 2ml were removed and used to determine the growth of the bacteria. From these 2ml aliquots, 1ml was used to make serial dilutions in sterile saline. The number of viable bacteria was then determined by the plate counting method of Miles and Misra (1938). Plots of the number of viable bacteria with time were made and the mid-log phase determined (Fig.2). Bacteria present in the remaining 1ml volume were harvested by centrifugation at 1600g for 5min, washed twice and resuspended in 1ml of sterile PBS-glucose (see 2.2.2.6). The E_{550} reading of this suspension was measured against a PBS-glucose blank on an LKB Ultrospec 4050 Spectrophotometer. For each time interval during the log-phase, a plot of the E_{550} reading against number of viable bacteria was made (Fig.3). This standard curve was used to estimate the number of viable bacteria at the beginning of each individual experiment.

2.3.6. Preincubation of mouse peritoneal macrophages with milk or casein.

Monolayers of resident and/or stimulated mouse peritoneal macrophages were prepared as described previously (see 2.3.1.2). The macrophages were then incubated for 1h with 50% (v/v) or 75% (v/v) human milk

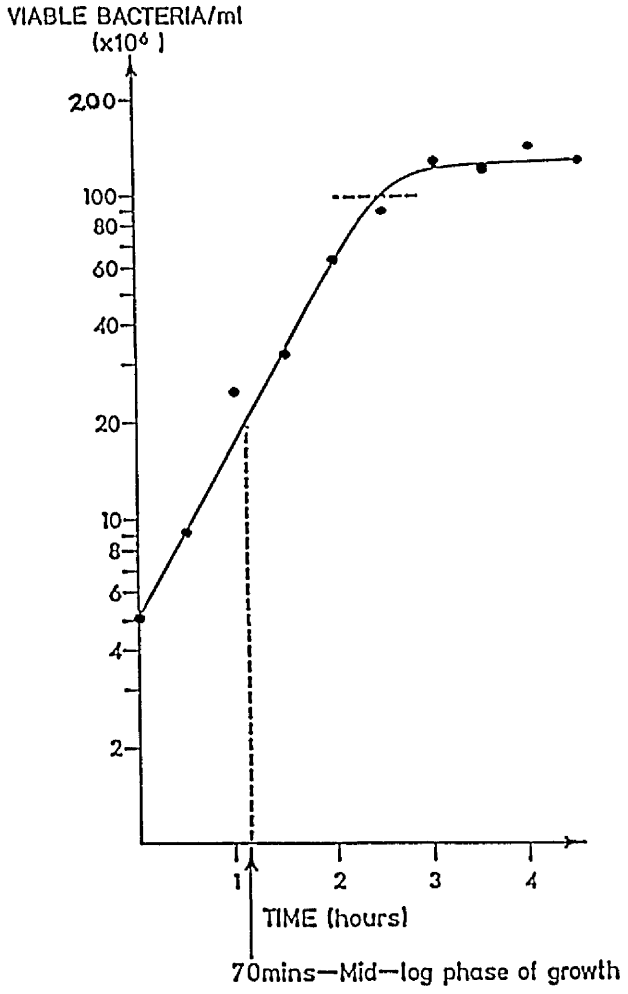


FIGURE 2 : Growth curve of Staph.aureus 8532.

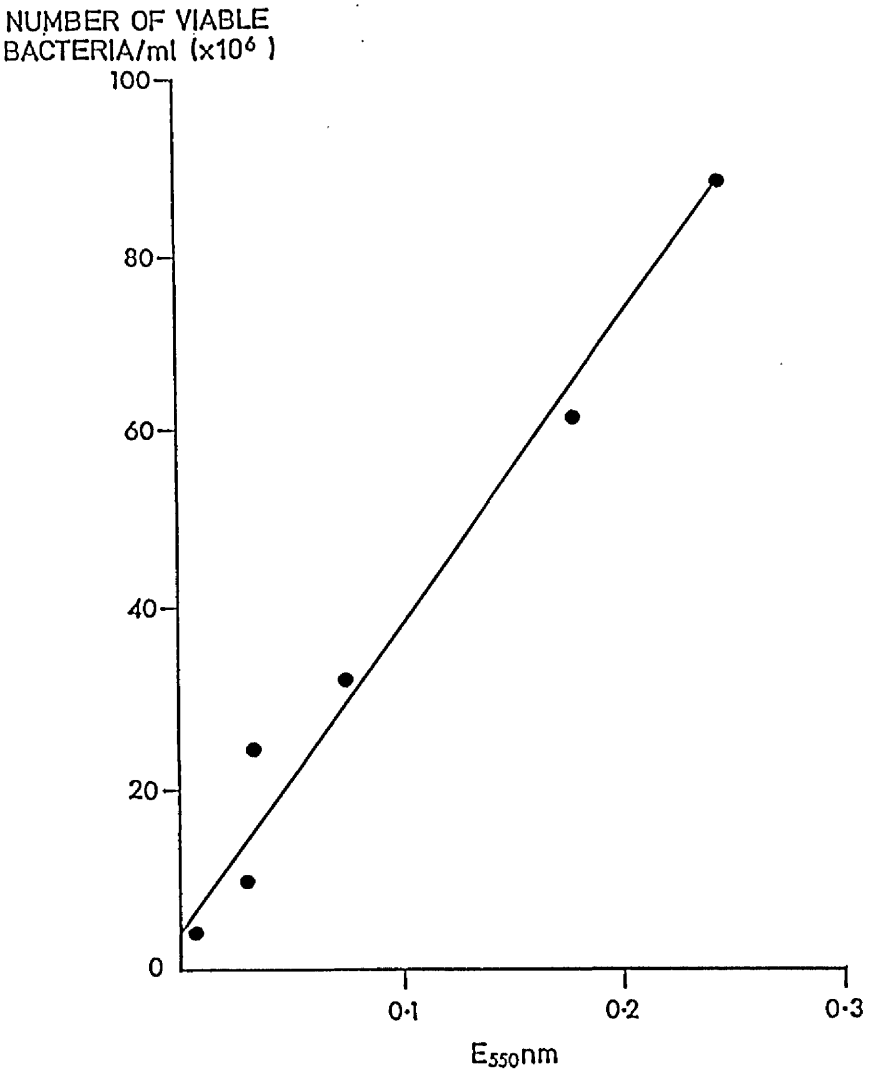


FIGURE 3 : Plot of number of viable bacteria per ml of bacterial culture against turbidity at E_{550}nm.

supernatant, pasteurised cow's milk or formula milk in culture medium (see 2.2.3). In controls, Hank's Buffered Salt Solution (HBSS: Gibco) was substituted for milk or formula. Various concentrations of CAS (see 2.2.2.7) were also tested for their effect on macrophage function. After incubation, all solutions were removed and the cells washed twice in HBSS before addition of fresh culture medium.

2.3.7. The phagocytic activity of mouse macrophages

2.3.7.1. Handling of immune complexes by mouse macrophages

The capacity of resident and stimulated macrophages to ingest and degrade ^{125}I-labelled immune complexes (see 2.3.4.2) was investigated using the method described by Esparza and Brock (1981). Following exposure to milk, adherent macrophages in each well were cultured for 2h with labelled complexes containing 10µg of Tf in a 50µl volume. Uningested complexes were then removed and the cells washed twice with 1ml of HBSS. The uningested complexes and washings from each well were pooled and retained. Meanwhile, cells were reincubated with fresh culture medium. After 18h, culture supernatants were removed and centrifuged at 170g for 2min to remove any cells which had become non-adherent. To recover any undegraded immune complexes, each supernatant was mixed with an equal volume of 20% (w/v) trichloroacetic acid (TCA) to precipitate the protein and then centrifuged at 500g for 15min.

The ^{125}I-activity of the TCA soluble and insoluble fractions, uningested complexes, and non-adherent cells was measured in an LKB Compugamma gammacounter. The remaining ^{125}I-activity of the adherent macrophages was determined after dissolving the cells in 1ml of 2% (w/v) sodium dodecylsulphate (SDS: BDH) for 5min. To ensure dissolution of the cells, the SDS was passed several times through a pasteur pipette before removal from the wells. Percentage ingestion and digestion of immune complexes was calculated as follows:

$$\%\text{Ingestion} = \frac{T-U}{T} \times 100$$

$$\%\text{Digestion} = \frac{S}{T-U} \times 100$$

where T= total radioactivity added; U= radioactivity in uningested complexes and S= TCA-soluble radioactivity in the culture supernatant.

2.3.7.2. Bactericidal activity of mouse macrophages

Resident peritoneal macrophages were allowed to adhere to culture plates (see 2.3.1.2), exposed to milk as described earlier (see 2.3.6) and their ability to then ingest and kill Staph aureus investigated using one of two methods.

2.3.7.2.1. METHOD 1

A modification of the combined radiometric assay and colony-counting method described by Peterson et al (1977) was used, as follows:

Staph aureus 8532 was radiolabelled by growing overnight in 10ml of Mueller-Hinton broth (Oxoid)

containing 20μCi of [methyl-^{3}H]-thymidine (specific activity of 40-60 Ci/mMol: Amersham). After centrifugation at 1600g for 10min, the bacterial pellet was washed twice and resuspended in 10ml PBS. A total count of the bacteria present in the suspension was estimated visually using MacFarlane tubes (Difco, Michigan, U.S.A). Although MacFarlane tubes give a rapid estimate of the total count, the number of viable organisms present is unkown. When calculating the percentage killing by the macrophages it is necessary to know what proportion of the original suspension were live bacteria. Thus, serial dilutions of the culture were made in PBS and a viable count determined by a pour plate method. Approximately 10^{8} bacteria (total count) were opsonised in 25%(v/v) normal mouse serum (see 2.2.4) for 30min at 37^{o}C, re-centrifuged for 5min and finally washed and resuspended in 1ml of culture medium.

Opsonised bacteria were co-cultured with macrophage monolayers which had been preincubated with milk or formula (see 2.3.6) such that the ratio of bacteria to cells was approximately 10:1 and the total volume of culture supernatant per well was 1ml. After 1h, culture supernatants were removed and the cells washed twice with HBSS. Supernatants plus washings were added to Ultrafluor Liquid Scintillation fluid (National Diagnostics, Aston Clinton, Bucks, England) and the ^{3}H-activity measured in a Packard 3320 Scintillation Spectrophotometer (London, England).

Lysis of Staph aureus bound but not internalized by the cells was achieved by reincubating the monolayers for 30min with 1ml fresh RPMI medium containing 1µg/ml lysostaphin (Schwartz-Mann, New York, U.S.A). Upon removal of this incubation medium, the macrophages were washed twice with HBSS before addition of fresh medium for a further hour. Intracellular bacteria were released by lysis of the cells with 1ml sterile DW passed several times through a Pasteur pipette. Serial dilutions of the lysate (to a final dilution of 10^{-4}) were made in DW and the number of live bacteria present determined by the colony-counting method of Miles and Misra (1938). The ^{3}H-activity of the supernatants, washings and lysate was then measured as before and used to calculate the percentage phagocytosis of bacteria as follows:

$$\%\text{Phagocytosis} = \frac{\text{cpm in macrophage lysate}}{\text{total cpm added to cell culture}} \times 100$$

Since the number of viable bacteria added to each well was already known, the number of bacteria ingested by each cell culture could then be calculated. From the plate counts of live, intracellular bacteria the percentage survival was then determined.

2.3.7.2.2. METHOD 2

A radiometric assay designed for neutrophils in suspension (Bridges et al, 1980) was adapted for use with adherent macrophage cultures.

A 0.5ml inoculum from an overnight culture of

Staph aureus in ME broth was added to 10ml of fresh broth and incubated in a shaking water bath at 37°C for a further 70min i.e until the mid-log growth phase of the organism (see 2.3.5.2). Bacteria were harvested by centifugation at 1600g for 10min, washed and resuspended in sterile PBS-glucose (see 2.2.2.6). Organisms were opsonised in the presence of 25%(v/v) normal mouse serum (see 2.2.4) for 20min at 37°C, washed twice, resuspended in PBS-glucose and viability determined spectrophotometrically (see 2.3.5.2).

The optimum ratio of bacteria:macrophages was determined as follows:

Monolayers were prepared (see 2.3.1.2) using five different concentrations of cells and then co-cultured with 2×10^5 bacteria. Ratios of 1:1, 1:2, 2:1, 1:5 and 5:1 for cells:bacteria were used. Control wells containing bacteria only and cells only were also included. After 1h, culture supernatants were first passed gently (x2) through a pasteur pipette to dislodge bacteria bound to but not ingested by the macrophages and then removed into fresh wells. To these wells and controls were added 20µl of 1 in 50 dilution of [5,6]^{3}H-uridine (specific activity of 35-50 Ci/mMol: Amersham). After 30min, the bacterial suspensions were removed from each well, the wells washed twice with HBSS and the washings plus supernatants pooled in plastic test-tubes (Sarstedt Ltd., Leicester, England) standing in ice. Bacteria were recovered by centrifugation at 1600g for 5min, washed 3 times in cold DW and finally resuspended in 200µl of DW. The suspensions were then

mixed with 2ml of Ultrafluor liquid scintillation solution and the ^{3}H-activity measured. The percentage ingestion of bacteria was calculated from the formula:

$$\frac{(B+C)-N}{(B+C)} \times 100$$

where N= radioactivity in non-ingested bacteria; B= activity in bacteria from control wells and C= activity in cells only.

The ratio of bacteria:cells which resulted in 50-70% ingestion of bacteria was considered the most appropriate since this degree of ingestion would permit any effect of milk or formula to be observed. For this reason, the 1:1 ratio was chosen (Table 3).

In the bactericidal assay, two series of experiments were performed in parallel. One set of wells was used for measuring ingestion and the other for killing of the bacteria. Replicate cultures of adherent resident mouse peritoneal macrophages were prepared (see 2.3.1) and pre-incubated in the presence of milk (see 2.3.6). Cell cultures in both the phagocytosis assay and killing assay received the same treatment as far as possible. The only difference in procedure followed the removal of uningested organisms. The order in which the culture medium and DW were added was crucial since it determined whether only extracellular organisms or both intracellular and extracellular organisms incorporated labelled uridine.

TABLE 3

Determination of the optimal proportions of bacteria and macrophages to use in bactericidal assays - Method 2.

Ratio of cells:bacteria	Percentage phagocytosis of bacteria:
1:5	44.4±2.2 (3)
1:2	63.2±19.2 (3)
1:1	64.5±5.0 (3)
2:1	80.1±10.3 (3)
5:1	87.8 (1)

Figures represent mean±standard deviation. Numbers in parentheses indicate the number of experiments carried out.

2.3.7.2.2.1. Phagocytosis of Staph. aureus by macrophages

In one set of wells, culture supernatants containing uningested bacteria were removed as before and retained. Fresh culture medium (200µl) was added to cell cultures before the addition of 200µl of sterile DW so that the macrophages were not lysed. Non-ingested bacteria were returned to the appropriate wells and 20µl of tritiated uridine added. Consequently, only viable, extracellular bacteria became labelled. After 30 min, the incubation mixture was removed, the wells washed with sterile HBSS (2x1ml) and the washings plus supernatants pooled in plastic test-tubes (Sarstedt) standing in ice. All samples were centrifuged at 1600g for 5min to harvest the bacteria. Pellets were washed three times in cold DW, resuspended in 200µl DW and finally mixed with 2ml of Ultrafluor liquid scintillation solution. The ^{3}H-activity and the percentage ingestion of bacteria was measured as before (see 2.3.7.2.2).

2.3.7.2.2.2. Killing of Staph. aureus by macrophages

The remaining set of wells were treated in the same way as the above except that after removal of uningested bacteria, the sterile DW was added before the fresh culture medium. Also, the DW was passed several times through a Pasteur pipette to ensure lysis of the cells and release of internalised bacteria. Upon addition of labelled uridine, therefore, both extracellular bacteria and intracellular bacteria which

had remained viable incorporated the label. From this step onwards all the wells in the killing assay were treated in the same way as the wells in the phagocytosis assay (see 2.3.7.2.2.1). The percentage killing of ingested bacteria was calculated using the formula:

$$\frac{(B+C)-V}{(B+C)} \times 100$$

where B= activity of bacteria in control wells; C= activity in cells only and V= activity of viable bacteria from macrophage cultures.

2.3.8. Staining of cow's milk proteins bound by macrophages exposed to formula.

2.3.8.1. Exposure of adherent macrophages to formula milk.

Resident mouse peritoneal macrophages were allowed to adhere to glass coverslips placed in a square-well plastic culture dish (see 2.3.2.1). Following removal of non-adherent cells, monolayers were washed twice with HBSS and reincubated for a further hour in the presence of 50%(v/v) Ostermilk Ready-to-Feed liquid formula (Farley) in culture medium. The formula milk was then discarded and the cells washed thoroughly (x2) in HBSS.

2.3.8.2. Detection of membrane-bound milk proteins using immunofluorescence.

Binding of cow's milk proteins was tested using an indirect double layer immunofluorescence technique. Adherent macrophages were fixed for 10min at room temperature in formalin (2.5% in saline) and washed in PBS for 5min. The PBS was removed from the wells and

the area around the coverslip dried. One drop of rabbit antiserum to CAS, BLG or ALA, used diluted in PBS at 1:50, 1:50 and 1:20 respectively, was placed on the coverslip. For controls, cells were incubated with either a 1:50 or 1:20 dilution of normal rabbit serum (see 2.2.5). A piece of damp blotting paper placed in the lid of the culture dish ensured a moist environment. The cultures were then incubated for 30 min and washed thoroughly in PBS for 5min. The area surrounding the coverslip was dried and the preparation reincubated in the dark with one drop of FITC-labelled sheep anti-rabbit IgG (Miles-Yeda, Israel). Three different batches of this antiserum were used at dilutions of 1/5, 1/10 and 1/20 respectively during these experiments.

After half an hour, the coverslips were washed thoroughly with PBS and counterstained with 0.05% methyl green for 45sec. Coverslips were again washed thoroughly in PBS, removed from the wells, then mounted with 1-2 drops of Tris-glycerol buffer (see 2.2.2.5) and the edges sealed with nail varnish. Cells were examined under oil in a Leitz Ortholux fluorescence microscope with a Fluorotar objective and an excitation wavelength of 490nm. This objective permitted examination of the field using both fluorescent and phase-contrast microscopy. Several fields of view, chosen at random, were examined. A minimum of 100 cells were observed and scored as positive or negative for binding of proteins when compared with control cells.

2.3.9. Statistical Methods

Analysis of variance and the Wilcoxon stratified sum of ranks were used as appropriate.

2.4. RESULTS

2.4.1. Effect of human milk and liquid infant formula on phagocytosis of immune complexes by mouse peritoneal macrophages.

To investigate the effect of milk on macrophage function, monolayers of resident and stimulated mouse peritoneal macrophages were preincubated with human milk or liquid Ostermilk Complete Formula for 1h (see 2.3.6) and then cultured in the presence of radiolabelled transferrin-antitransferrin immune complexes (see 2.3.7.1). Control cultures which had not been exposed to milk were also included.

Resident mouse peritoneal macrophages showed a moderate ability to phagocytose radiolabelled immune complexes. However, this ability was significantly inhibited when the cells were pre-incubated with either 50% or 75% concentrations of liquid infant formula (Table 4). The effect was more pronounced at the higher concentration. Stimulated cells had a greater ability to phagocytose complexes than resident cells and were less affected by exposure to liquid formula. Only the higher concentration of 75% inhibited phagocytosis.

In contrast, although human milk supernatant did cause some decrease in phagocytosis of immune complexes by resident cells, this did not reach statistical significance at either of the concentrations tested. However, as shown with formula the decrease was more

TABLE 4

Phagocytosis of ^{125}I-labelled transferrin-anti-transferrin immune complexes by mouse peritoneal macrophages following a 1h incubation with human milk supernatant or liquid infant formula.

Macrophages pre-incubated with:	Percentage of immune complexes ingested: Resident macrophages	Stimulated macrophages
50% medium (control)	10.1±0.4 *	67.7±1.1
50% human milk	9.7±1.6	69.8±1.2
50% formula milk	6.0±1.2 **	66.2±3.9
75% medium (control)	10.1±2.2	69.1±1.8
75% human milk	7.4±2.1	70.2±2.8
75% formula milk	2.8±0.8 **	56.8±2.0 **

Figures are the results from one representative experiment.

* Mean±standard deviation. ($n=3$)

** $P<0.01$ compared to appropriate control.

Statistical analysis: analysis of variance was used.

noticable at the higher concentration of milk. Once again the more active cells were less affected by milk. Although phagocytosis was very slightly enhanced by breast milk at 50% concentration an increased concentration did not highlight this further. It should be noted that microscopic examination of monolayers did not reveal any loss of cells after culturing with either formula or colostrum thus any differences observed were not simply a result of differences in the number of adherent macrophages.

Resident peritoneal macrophages were capable of efficiently degrading internalised complexes (Table 5). However, this capacity was severely depressed when the cells were incubated with either 50% or 75% concentrations of the infant formula preparation. Once again, the stimulated cells showed some resistance to the inhibitory effect of the formula. Pre-incubation with human milk had some inhibitory effect on resident cells. However, this reached statistical significance only at 75% concentrations.

Since there was no difference between the activity of control cells incubated in 50% HBSS and those incubated in 75% HBSS, the different effects observed with the two concentrations of milk was not simply a reflection of differences in the amount of culture medium present. The results indicate that liquid infant formula milk interferes with the ability of mouse peritoneal macrophages, in particular resident cells, to phagocytose and degrade immune complexes. The addition of human milk to cell cultures had a less pronounced

TABLE 5

Degradation of phagocytosed radiolabelled immune complexes by mouse peritoneal macrophages following a 1h incubation with human milk supernatant or liquid infant formula.

	Percentage of ingested complexes degraded :	
Macrophages pre-incubated with:	Resident macrophages	Stimulated macrophages
50% medium (control)	31.9±2.6 *	50.5±1.4
50% human milk	28.4±4.5	49.7±5.9
50% formula milk	9.5±2.5 **	45.8±3.6
75% medium (control)	35.3±2.2	47.2±10.5
75% human milk	25.1±1.2 **	52.1±3.7
75% formula milk	9.2±2.4 **	41.8±3.3

Figures are the results obtained from one representative experiments.

* Mean±standard deviation. ($n=3$)

** $P<0.01$ compared to appropriate control.

Statistical analysis: analysis of variance was used.

effect. Degradation of complexes was reduced only at 75% concentrations of breast milk.

2.4.2. Effect of human milk and infant formula on the antimicrobial activity of resident mouse peritoneal macrophages.

Two different experimental protocols were employed to investigate the antimicrobial activity of resident mouse macrophages following exposure to liquid formula.

Tables 6 and 7 show the results obtained with the method of Peterson et al (1977). The results in Table 6 indicate that liquid formula significantly inhibited ingestion of opsonised bacteria. The ability of the macrophages to kill internalised organisms was somewhat variable and the results from three individual experiments are shown (Table 7). Despite this variability, formula milk only slightly inhibited killing of internalised bacteria. Cells exposed to breast milk showed no significant difference in either ingestion or killing when compared to control cells.

Van den Broek et al (1982) questioned the ability of lysostaphin to remain extracellular in neutrophil suspensions. The method of Peterson et al (1977) necessitated the use of lysostaphin to kill bacteria which had remained bound to, but had not been ingested by, macrophages. If lysostaphin were indeed able to penetrate the macrophages one would not expect the high percentage survival of intracellular bacteria shown in the killing assay of experiment B (Table 7).

TABLE 6

Effect of human milk and infant formula on the phagocytosis of Staph.aureus 8532 by resident mouse peritoneal macrophages - Method 1.

	Percentage bacteria ingested		
Macrophages pre-incubated with:	50% HBSS (control)	50% Human milk	50% Formula milk
Resident macrophages	20.1±2.8	22.0±3.0	14.9±2.6 [a]

Figures represent the mean±standard deviation (n=3)

a : P<0.01 compared to control.

Statistical analysis: analysis of variance was used.

TABLE 7

Effect of human milk and infant formula on the survival of Staph.aureus 8532 within resident macrophages.

	Percentage survival		
Macrophages preincubated with:	50% HBSS (control)	50% Human milk	50% Formula milk
Experiment A	27.6±14.7	20.7±1.7 *	32.8±4.8 *
Experiment B	86.9±3.2	83.4±0.7 *	107.0±25.2 *
Experiment C	19.5±0.2	<0.01	16.7±11.0 *

Figures represent the mean±standard deviation. (n43)

* P>0.1 compared to corresponding control.

Statistical analysis: analysis of variance was used.

Nevertheless, an alternative bactericidal assay was required to consolidate or refute the results already obtained.

A modification of the method described by Bridges et al (1980) provided an alternative approach. Results are shown in Table 8. From these data, it is clear that liquid formula, but not human milk, caused a significant reduction in phagocytosis of Staph. aureus when compared to controls. Furthermore, this formula also interfered with the macrophages' killing capacity. Although human milk supernatant caused some reduction in the killing capacity of mouse macrophages, this was not a statistically significant effect (Table 8).

As expected, the second assay supported the findings of the earlier method with regard to ingestion of bacteria, since lysostaphin was not required at this stage in the experimental procedure. Interestingly, different conclusions were drawn from the two procedures when antimicrobial activities were compared. Although neither assay showed breast milk to significantally influence the cells' killing capacity, this was not the case with formula milk. Using the first experimental protocol, macrophages exposed to formula behaved no differently from control cells. However, the second assay showed that formula milk significantally reduced the macrophages' ability to kill ingested organisms. Moreover, the variability in killing observed using the second assay was less pronounced than that of the first. It is possible that the procedure used for washing the

TABLE 8

Effect of formula or human milk on ingestion and killing of S.aureus NCTC 8532 by resident mouse peritoneal macrophages - Method 2.

Cells pre-incubated with:	HBSS (control)	Human milk	Formula milk
% Bacteria ingested	51.9 (33.6-61.3)	48.3 (35.5-57.7)	25.0[a] (15.2-39.3)
% Killing	36.7 (19.0-50.8)	28.7 (7.8-44.0)	16.4[a] (6.4-34.8)

Figures represent median with range in parentheses

a : P<0.01 compared to control.

Statistical analysis : Wilcoxon's stratified sum of ranks was used.

macrophages in the first series of experiments was insufficient to ensure complete removal of lysostaphin.

Taking all things into consideration, it appears that the second bactericidal assay is the more scientifically sound. Consequently, the results obtained using this assay were considered more valid. In conclusion, resident mouse peritoneal macrophages exposed to liquid formula suffer a significant and severe reduction in their ability to ingest and kill opsonised bacteria. Cells exposed to human milk suffer no such detrimental effect.

2.4.3. Effect of casein on phagocytosis and degradation of immune complexes by resident mouse macrophages.

To see whether CAS contributes to the inhibitory effect of liquid formula milk, different concentrations were tested for their effect on the phagocytosis of radiolabelled transferrin-antitransferrin immune complexes by resident mouse peritoneal macrophages. Adherent cells were exposed to a range of CAS concentrations which included those found in cow's milk and infant formula (Farley Health Products, 1982).

The results shown in Table 9 indicate that concentrations of CAS as low as 7.5 mg/ml significantly inhibited the phagocytic activity of resident mouse macrophages and reduced the ability of these cells to degrade ingested immune complexes.

TABLE 9

Effect of pre-incubation with casein on the phagocytosis of immune complexes by resident mouse peritoneal macrophages.

	Casein concentration (mg/ml):			
	0	7.5	15	30
% phagocytosis	31.6±4.2	22.8±1.5*	16.2±2.8*	18.0±2.1*
% degradation	30.4±7.9	22.0±1.6*	17.5±0.8*	16.9±3.6*

Figures represent the mean±standard deviation. n=3.

* $P<0.01$ compared to corresponding control.

Statistical analysis: analysis of variance was used.

2.4.4. Effect of various milks on the phagocytic activity of mouse peritoneal macrophages.

To see if the effect of different milks correlated with their CAS content, several formula milks, pasteurised cow's milk, and human milk were compared for their effect on the activity of resident mouse macrophages. A liquid and a powdered formula of comparable chemical composition were tested to see if the method of processing was also an important determinant. A further comparison was made with soy formula which contains no cow's milk proteins. All the milks tested and the HBSS control were used at 50% (v/v) concentration in culture medium. After exposure to milk, macrophage monolayers were assessed for their capacity to phagocytose and degrade radiolabelled immune complexes.

All the milks tested exhibited some degree of inhibition with regard to ingestion of immune complexes (Table 10) but only with the liquid formula and soya milk was the effect significant. The soy milk caused by far the greatest decrease in uptake. Degradation of internalised material was also greatly reduced by the liquid formula and the soy milk. In contrast to ingestion, the liquid formula caused a more severe decrease in the cells' ability to handle internalised complexes than did the soy milk. As expected, the human milk supernatant had little effect on either ingestion or digestion but, interestingly, neither did

TABLE 10

Effect of various formula milks, human milk and cow's milk on phagocytosis and degradation of immune complexes by resident mouse peritoneal macrophages.

Cells pre-incubated with:	Percentage ingestion of complexes	Percentage digestion of complexes	Casein content (mg/ml)	Processing/ treatment
50% medium control	38.1±0.9	42.2±1.0	-	-
50% human milk	35.5±2.3	43.3±0.4	6.0***	None
50% cow's milk	30.3±1.1	45.3±0.2	25.5**	Pasteurised
50% liquid formula	24.8±0.5*	18.5±1.1*	12.8**	Heat sterilised liquid
50% powdered formula	35.8±0.7	42.9±1.0	12.5**	Spray-dried powder
50% soy formula	11.1±1.1*	32.3±2.7*	-	Spray-dried powder

Figures represent mean±standard deviation. n=3

* P<0.01 compared to control.

** Farley Health Products (1982)

*** Schultze and Heremans (1966).

Statistical analysis: analysis of variance was used.

pasteurised cow's milk or powdered formula.

From previous experiments (see 2.4.3), it appeared that the CAS content of the milk might determine, in part, the extent to which the functional activity of the cells was affected. However, no direct correlation was found between CAS concentration and the inhibitory effect of the different milks. Cow's milk, which has the greatest CAS content, did not cause any significant effect whilst soy milk, which is CAS-free, caused a marked reduction in both phagocytosis and degradation of complexes. Furthermore, although the liquid and powdered formula contain virtually the same amount of CAS, the latter had little effect on the phagocytic activity of macrophages whilst the former produced a significant inhibition of activity. It is therefore clear that other factors are important in determining the extent to which infant formula inhibits the phagocytic activity of macrophages.

2.4.5. Binding of cow's milk proteins by mouse peritoneal macrophages.

A double layer indirect immunofluorescence technique (see 2.3.8) was employed to establish whether bovine milk proteins could survive processing of the liquid infant feed and subsequently bind to the macrophage cell surface during incubation.

The results from several individual experiments are shown in Fig.4. About 80% of murine macrophages exposed to the formula stained positively for CAS. The proportion positive for BLG was only slightly lower. In

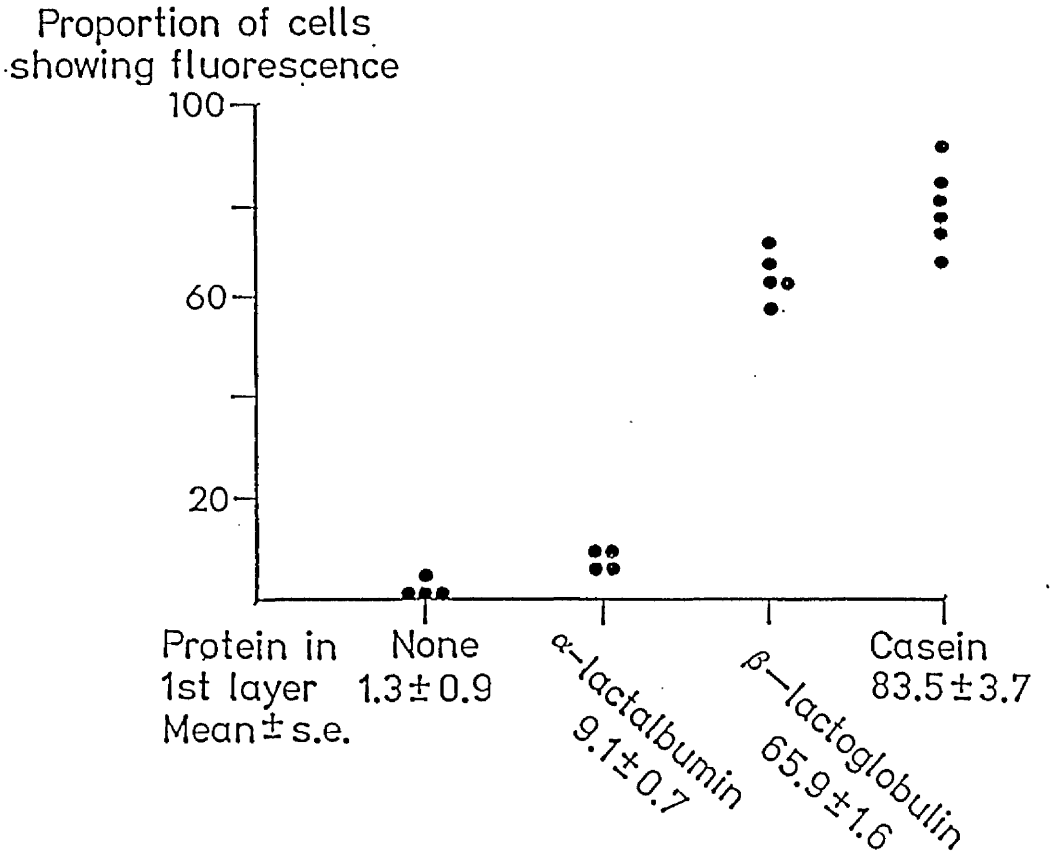
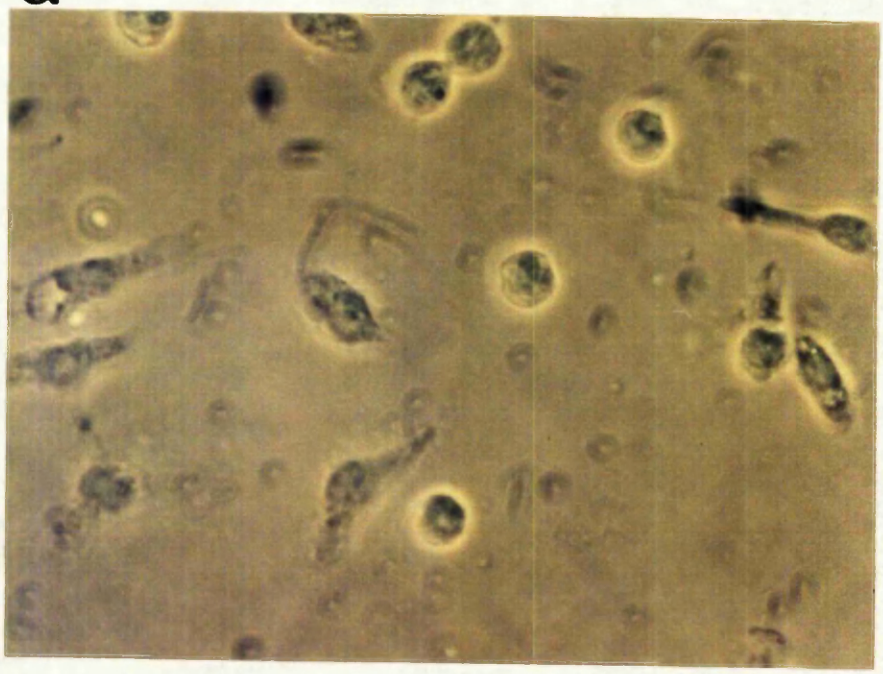


FIGURE 4 : Binding of cow's milk proteins, α-lactalbumin, β-lactoglobulin and casein, by mouse peritoneal macrophages following a 1h incubation with liquid formula milk.

contrast, only 10% or less of the cells stained positively for ALA. The staining was judged as specific since only about 4% of the cells stained positively when normal rabbit serum was substituted for antisera to milk proteins. Furthermore no positive cells were seen with any antiserum in control cultures that had not been exposed to formula (Fig. 5). Examples of the binding of CAS, BLG and ALA by adherent mouse macrophages, are shown in Figures 6, 7, 8. From these results, it is apparent that milk proteins resist extensive alteration during the manufacture of the liquid formula and are capable of binding to the macrophage cell surface.

a



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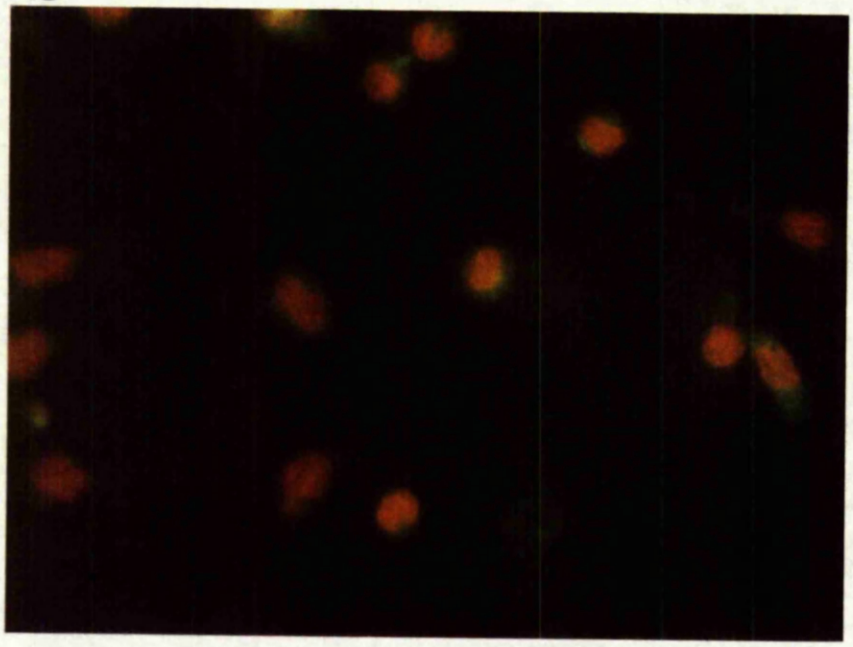


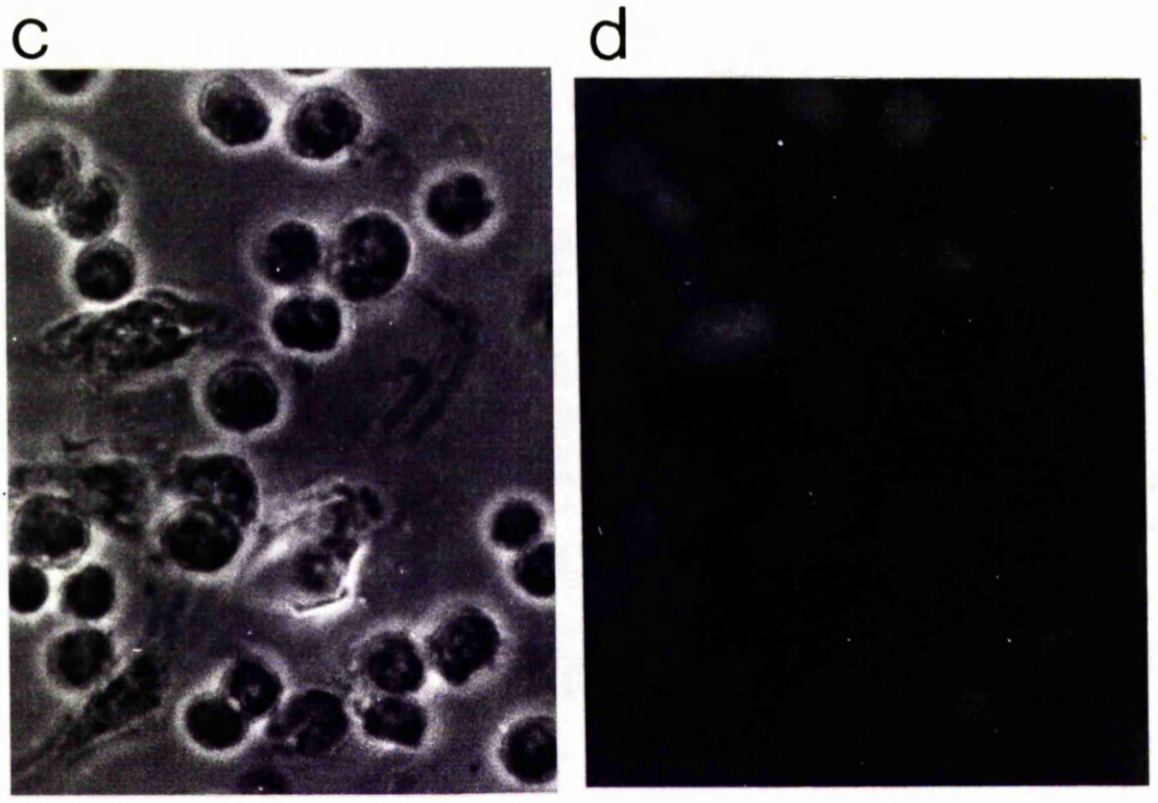


FIGURE 5 : Control cultures of adherent mouse peritoneal cells preincubated with liquid infant formula and then stained with fluorescent antibody.

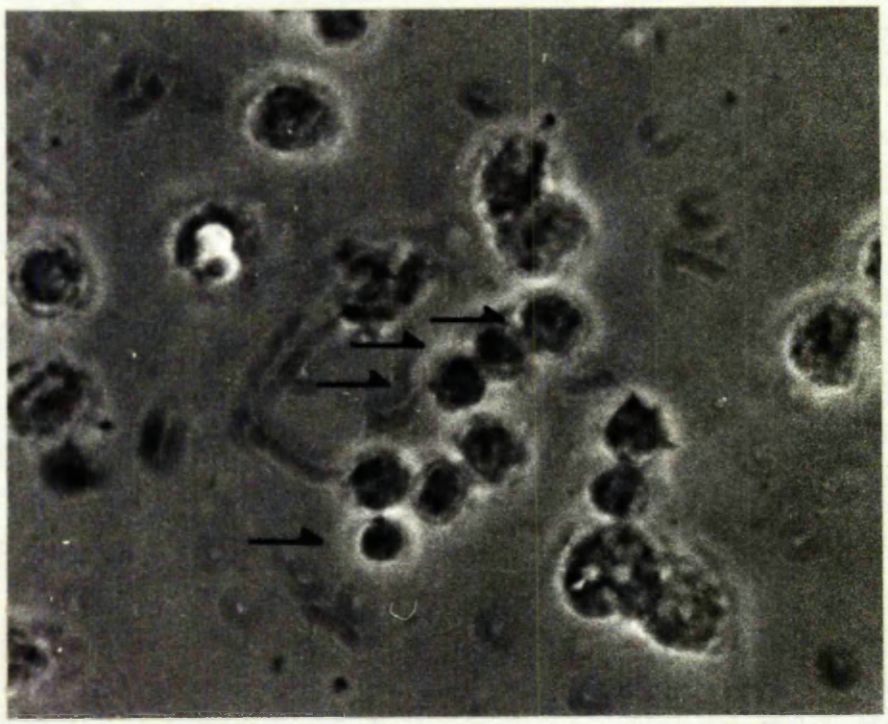
(a) Cells as seen under phase contrast (x1750).

(b) The same field as in (a) under U.V., showing that control cells incubated with normal rabbit serum have not stained with the fluorescent antibody.

(c) Another population of adherent cells as seen under phase contrast (x2500).

(d) The same field as in (c) under U.V., showing the proportion of control cells which have not stained with the fluorescent antibody.

a



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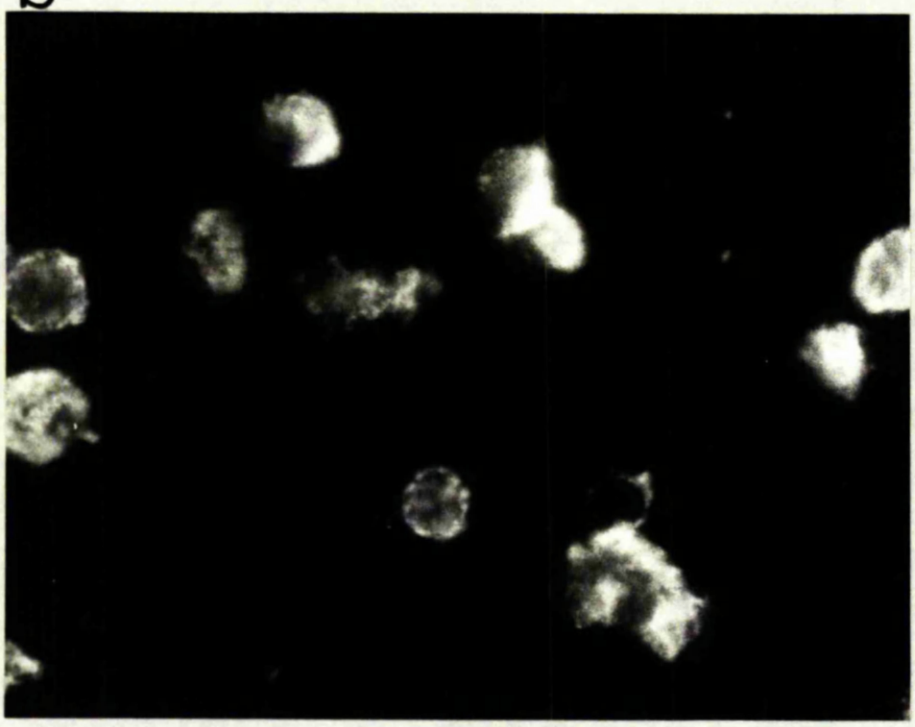


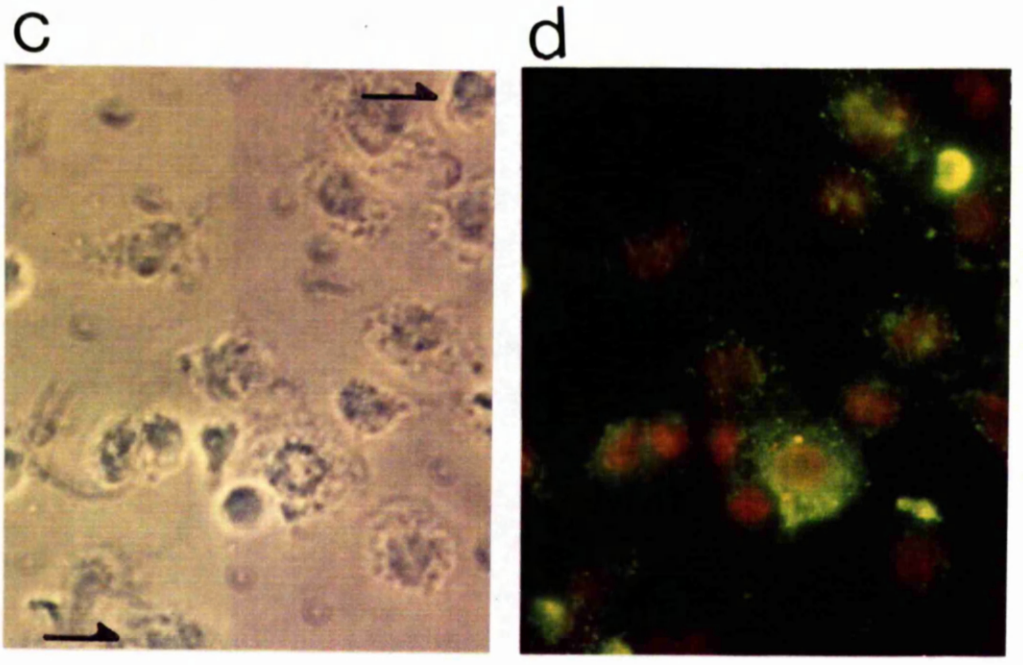


FIGURE 6 : Staining of mouse peritoneal macrophages which bind casein following exposure to liquid infant formula.

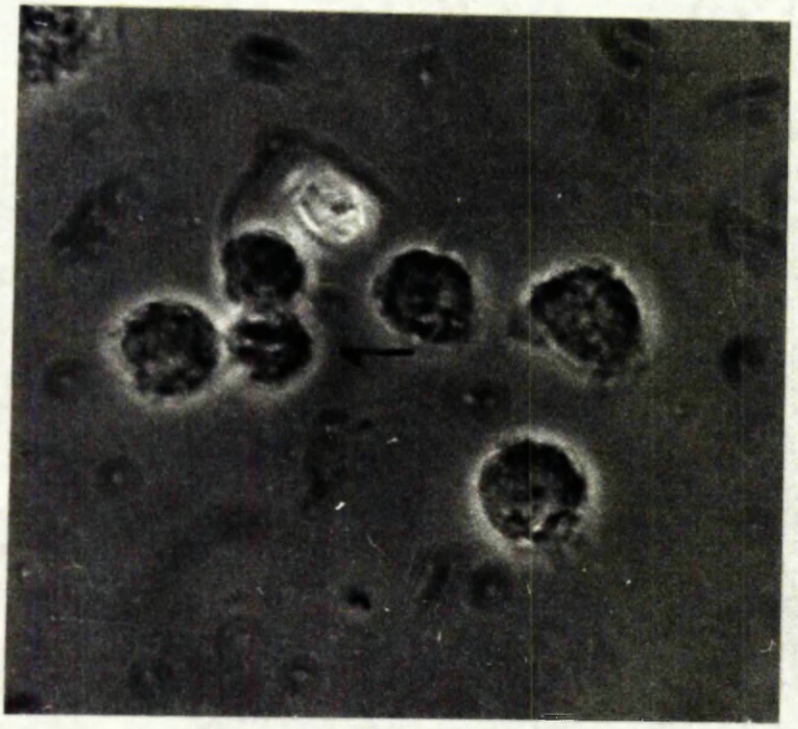
(a) Cells as seen under phase contrast (x2500).

(b) The same field as in (a) under U.V., showing cells which have bound bovine casein at their surface. The arrows in (a) point to cells which have not bound casein.

(c) Phase-contrast microscopy of a second field of view (x1750).

(d) The same cells as in (c) under U.V., showing the proportion of cells which bind casein. The arrows in (c) indicate negatively-stained cells.

a



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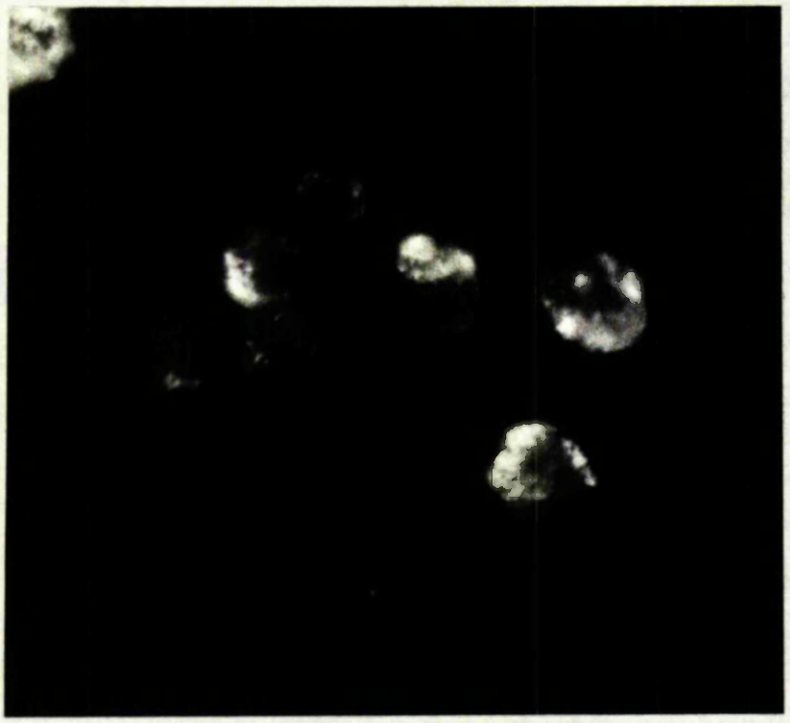


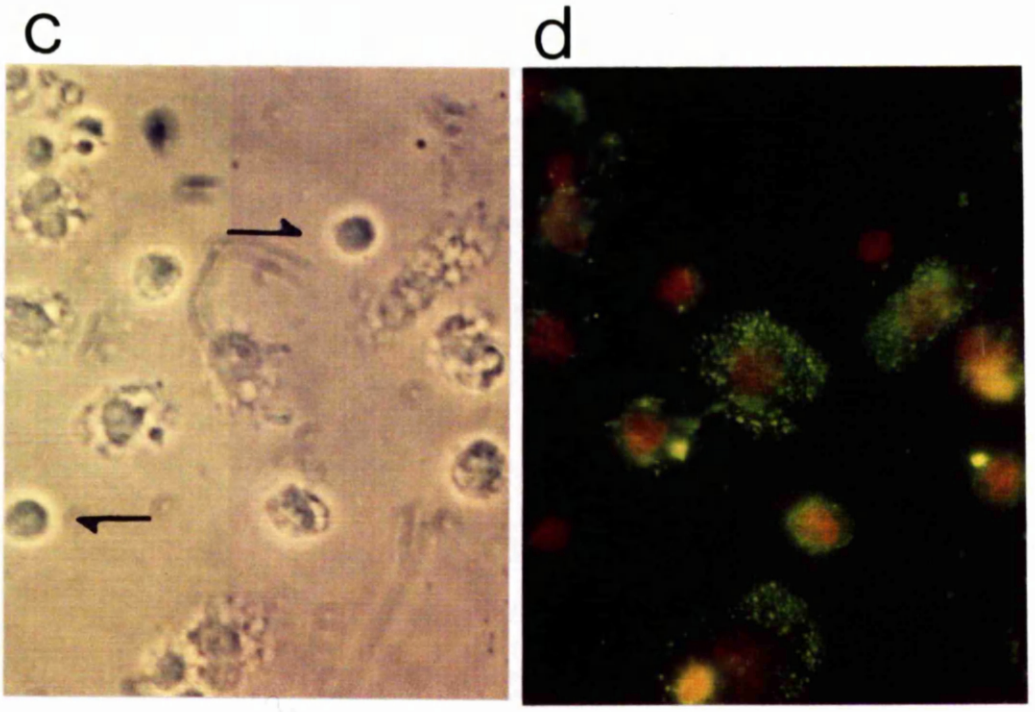


FIGURE 7 : Staining of mouse peritoneal macrophages which bind β-lactoglobulin after exposure to liquid infant formula.

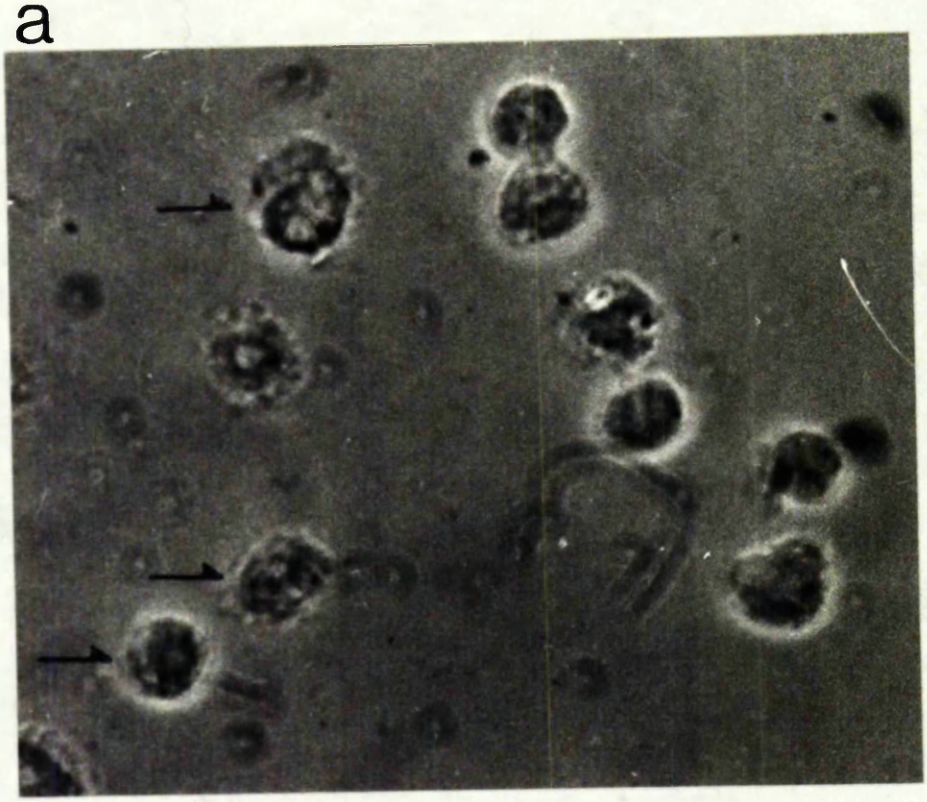
(a) Phase-contrast microscopy of cells (x2500).

(b) The same cells as in (a) under U.V., showing cells which have bound β-lactoglobulin.
The arrows in (a) indicate cells which have not bound bovine β-lactoglobulin.

(c) Phase-contrast microscopy of a second field of view (x1750).

(d) The same cells as in (c) under U.V., showing the proportion of cells which bind β-lactoglobulin.
The arrows in (c) point to negatively-stained cells.

a



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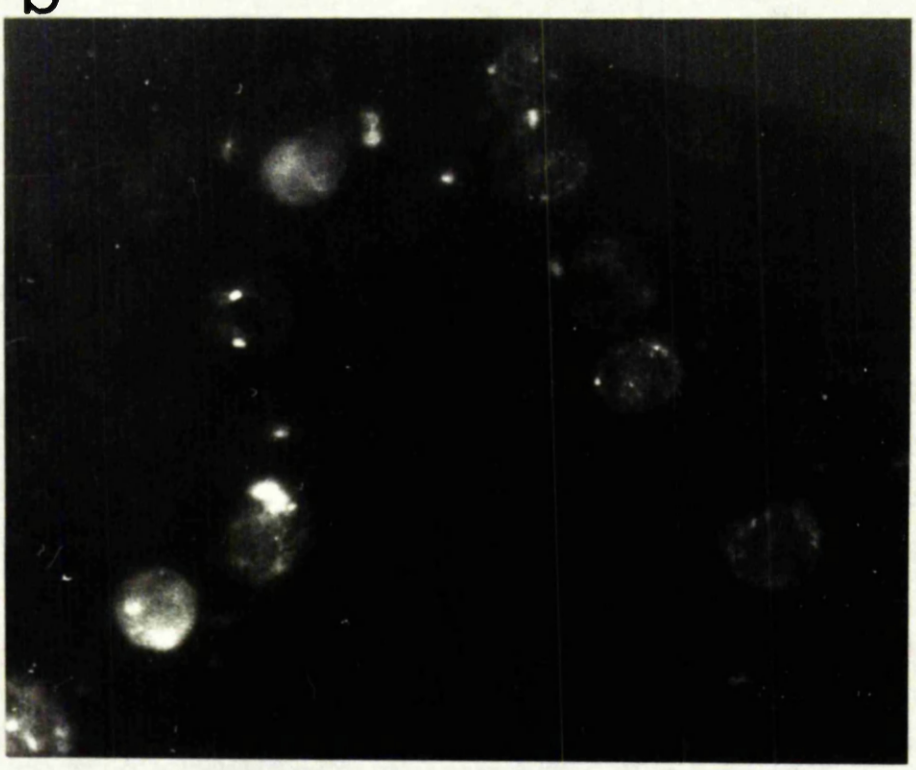


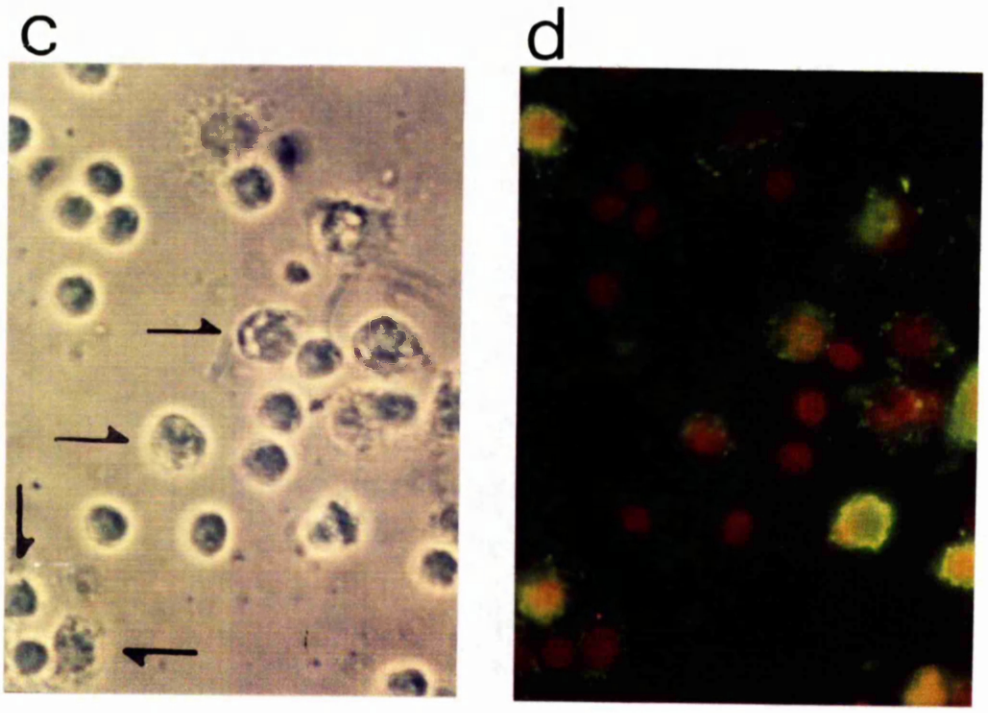


FIGURE 8 : Staining of mouse peritoneal macrophages which bind α-lactalbumin following exposure to liquid infant formula.

(a) Cells as seen under phase contrast (x2500).

(b) The same field as in (a) under U.V., showing cells which bind bovine α-lactalbumin.
The arrows in (a) point to positively-stained cells.

(c) Phase-contrast microscopy of a second field of view (x1750).

(d) The same field as in (c) under U.V., showing the proportion of cells which bind α-lactalbumin.
The arrows in (c) point to positively-stained cells.

2.5. DISCUSSION

Exposure of mouse peritoneal macrophages to a cow's milk based infant formula preparation caused a reduction in phagocytosis of transferrin-anti-transferrin immune complexes, particularly in the case of resident cells. The efficiency with which the cells processed the ingested complexes was also inhibited. Human milk supernatant was less inhibitory. It caused a slight but insignificant reduction in the phagocytic activity of resident cells and only inhibited degradation of complexes at high concentration. Stimulated cells were unaffected. The antimicrobial activity of resident cells was also examined and found to markedly decrease following preincubation with liquid formula. Once again, human milk supernatant had little effect.

Since CAS is the major cow's milk protein and it was shown by Russell *et al* (1976) to inhibit the ability of bovine neutrophils to kill *S.aureus*, purified CAS was also tested for its effect on macrophage function. Like the formula milk, it significantly inhibited both ingestion and digestion of immune complexes by resident macrophages. Consequently, it is not suprising that the liquid formula preparation used with a casein:whey ratio of 77:23 was a successful inhibitor of phagocyte function when compared with human milk which has a casein:whey ratio of, on average, 32:68 (Farley Health

Products, 1982).

The state of the CAS may also be an important consideration since experiments comparing different formula milks and bovine milk did not find a direct relationship between CAS concentration and inhibitory activity. Since the liquid and powdered cow's milk based formulae differed only in their method of processing, it is feasible that the milk proteins present were denatured to varying degrees in the two milks. Certainly, formula milks with identical chemical composition have different anaphylactic-sensitising capacities if processed differently (McLaughlan et al, 1981). Greater modification and denaturation of the milk proteins occurs with greater heat treatment. The extent of protein denaturation during treatment of the milk may determine the accessibility of the protein to the macrophage, and thus the effect the milk has on cell function. Moderate heating such as in pasteurisation or ultra heat treatment produces little change in the CAS of cow's milk (McLaughlan et al, 1981). Partial denaturation of CAS during the more severe processing of the liquid artificial milk may account for the adverse effect of this milk on macrophage function.

It is not clear how CAS might exert its inhibitory effect. Ingestion of CAS micelles has no effect on the formation of acid phagosomes during phagocytosis (Reinitz et al, 1982). However, one hypothesis suggests that binding of CAS at the surface membrane may limit phagocyte-particle interaction (Russell et al,

1976). Furthermore, lysosomes may discharge their contents into phagocytic vacuoles containing CAS. This would divert and thus reduce the number of lysosomes available to interact with other phagocytic vacuoles and subsequently impair the cell's ability to handle ingested bacteria or immune complexes. Stimulated cells might be more resistant to this effect because of their enhanced content of lysosomal enzymes.

It is possible that the presence of emulsifying agents in the Ostermilk complete formula liquid preparation might have had some effect on macrophage function, as this formula contains 0.1% glycerol monostearate and 0.04% lecithin (Farley Health Products, personal communication). However, this would not explain the inhibitory effect of CAS alone.

Following exposure to formula, a fluorescent antibody technique was used to detect cell surface binding of CAS and the major whey proteins BLG and ALA. The majority of resident macrophages bound CAS and BLG but little ALA. Unlike CAS, BLG is partly denatured during moderate heat treatment (Lyster, 1970). However, components present in skimmed milk but not present in diafiltered whey may protect the BLG molecule to some extent by precipitating the protein during heat treatment (Kilshaw et al, 1982). Certainly, antigenically intact BLG has been detected in the sera of infants fed a cow's milk based formula (Roberton et al, 1982). Moreover, the work of Kilshaw et al (1982) showed that autoclaved milk (121^{o}C, 20min) could still

sensitise guinea pigs for anaphylaxis and stimulate production of antibodies to CAS and BLG.

In the light of these studies, it is not surprising that antigenically intact BLG could be detected on the surface of macrophages following exposure to heat sterilised liquid formula. Like BLG, ALA is partly denatured during moderate heat treatment (Lyster, 1970) but is not known whether ALA is similarly protected by factors present in skimmed milk.

The effect of soy milk on macrophage function was unusual in that it had a very profound inhibitory effect on ingestion of immune complexes without causing a comparable reduction in digestion of the phagocytosed material. The reason for this is not clear, but it may be due to blockage of the macrophage Fc receptors by lectins present in the soy milk. It might be possible to test this proposal by adding an appropriate blocking monosaccharide to the incubation medium.

Soy-formula is made from 90% purified protein (Wysoy, product information, 1981) and is more antigenic than crude soy powder (Eastham et al, 1982). In the human infant, soy protein induces a lower antibody response than cow's milk protein (May et al, 1982; Zoppi et al, 1982) and impairs T lymphocyte cell markers and reactivity (Zoppi et al, 1982). Taken together, these studies suggest that a diet of soy may impair cellular immune function in vivo.

Overall, human breast milk supernatant had no effect on stimulated macrophages and caused only a slight reduction in the phagocytic activity of resident

macrophages. However, the supernatant caused a marked reduction in the ability of resident cells to digest intracellular immune complexes. Individual samples of milk supernatant also significantly inhibited the cells' ability to kill S. aureus. Thus, the inhibitory effect of the breast milk is apparently exerted during processing of ingested material. Phagocytosis of milk fat may impair cell function (Pickering et al, 1983) by causing lysosome disruption and depletion of the lysosomal enzymes available to digest intracellular material (Mandyla et al, 1982). Overloading of the cells with ingested lipid may also restrict lysosome-phagosome fusion or divert lysosomes away from phagocytic vacuoles thereby squandering their lysosomal enzymes (Russell et al, 1976). Confirmation of the latter was provided by the cytochemical studies of Paape et al (1977) who found peroxidase-positive material in phagosomes containing fat. It was suggested that azurophil granules may be migrating to and fusing with phagosomes containing fat, thereby reducing the concentration of pH depressing substances that reach non-fat containing phagosomes. This may explain why phagocytosis of fat globules inhibits the depression of intraphagosomal pH (Reinitz et al, 1982).

The fatty acid composition of the lipid fraction may be an important factor in determining the extent of inhibition for Pickering et al (1983) have shown that early human milk, with a high proportion of fatty acids is a more potent inhibitor of neutrophil function than

mature milk. It is also noteworthy that ingestion of milk fat from early milk may be greater than that from late milk because there are more small fat globules (Rueegg and Blanc, 1981). Taken together, these studies offer an explanation of why some samples of human milk strongly inhibited antimicrobial activity of mouse macrophages while other samples had no effect.

It is also possible that uptake of fat may contribute to the inhibitory effect of the formula milk. However, the amount present in this milk is less than that in breast milk (Farley Health Products, 1982) as is the number of small fat globules (Rueegg and Blanc, 1981).

Collectively, these studies offer some explanation for the inhibitory effect of milk, particularly liquid infant formula and soy formula, on the phagocytic capacity of mouse macrophages. Immunofluorescence showed that antigenically intact proteins survived processing of the heat-sterilised liquid formula. Similar interaction of human intestinal macrophages with cow's milk proteins may have important immunological consequences for the formula-fed infant. These infants may be more susceptible to infection and allergic disease than their wholly breast-fed counterparts. From the results in the present study, it appears that infant formula may exert an inhibitory effect at the cellular level. Comparable studies on human macrophages would further our understanding of the immunological consequences of infant feeding. To this end, the work reported in the

following chapter examines the effect of liquid infant formula and human breast milk supernatant on human milk and peritoneal macrophages.

CHAPTER 3

EFFECT OF MILK ON THE FUNCTION OF HUMAN MACROPHAGES

3.1. INTRODUCTION

The preceding chapter reported evidence for an interaction between murine macrophages and intact cow's milk proteins present in formula milk. Furthermore, it was shown that the phagocytic activity of murine macrophages was impaired by exposure to infant formula but breast milk had little or no effect.

If the inhibitory effect of formula is applicable to human milk cells and gut-associated macrophages, the immunological status of the human infant may be seriously impaired by early weaning or mixed feeding. The formula may reduce the cells' ability to clear immune complexes and/or kill potential pathogens and initiate an immune response through the cells' potential to present antigen. Under such circumstances, babies may be more susceptible to infection and more likely to develop sensitivity to cow's milk proteins. Furthermore, in the mixed-fed infant any potential beneficial effect of breast milk cells may be severely reduced. However, comparable studies on human macrophages have not been performed.

Ideally, the effect of formula on the function of breast milk macrophages and human intestinal macrophages would have been the next objective. However, due to the problems associated with isolation of the latter cell type, human peritoneal macrophages were used as an alernative source of human macrophages. These can be

readily obtained in good numbers from the spent dialysis fluid of patients undergoing continuous ambulatory peritoneal dialysis (CAPD).

The ability of macrophages in the gastrointestinal tract of the neonate to ingest and degrade immune complexes is important in the elimination of antigenic food proteins. Consequently, phagocytosis and degradation of radiolabelled immune complexes by human breast milk macrophages and peritoneal macrophages pre-exposed to formula was examined. Immunofluorescence studies were also performed to see whether these cells bind intact cow's milk proteins present in the formula. Furthermore, a high proportion of human breast milk macrophages (Leyva-Cobián and Clemente, 1984), intestinal macrophages (Golder and Doe, 1983) and peritoneal macrophages (Goldstein et al, 1984) bear Class II (HLA-DR) antigen at their cell surface. Since macrophages bearing the HLA-DR phenotype act as antigen presenting cells, it was also considered of interest to determine whether there was any correlation between expression of this antigen and binding of cow's milk proteins.

3.2. MATERIALS

3.2.1. Culture medium. Unless otherwise stated, cells were cultured at 37^{o}C in a humidified 5% CO_2 incubator (New Brunswick Scientific). The culture medium consisted of RPMI 1640 medium (Flow) supplemented with 0.3mg/ml glutamine (BDH), 100IU/ml penicillin, 100µg/ml streptomycin (Flow) and 5% heat inactivated FCS (56^{o}C, 30min; Gibco).

3.2.2. Human milk samples. These were collected and processed as detailed elsewhere (see 2.3.3).

3.2.3. Spent peritoneal dialysate fluids. Peritoneal effluent was collected from uninfected patients undergoing continuous ambulatory peritoneal dialysis (CAPD) at the Renal Unit, Western Infirmary, Glasgow. Approximately 2 litres of spent dialysate fluid was collected after an overnight dwell and processed within an hour of collection. The CAPD solutions used were either Travenol or Dianeal.

3.2.4. Cell lines. B-JAB: a human lymphoma cell line which expresses surface HLA-DR antigen was kindly provided by Dr. W. Cushley, Department of Biochemistry, University of Glasgow.

K562: a transformed human erythroid precursor cell line which lacks surface HLA-DR, was kindly donated by Dr. M.J Lesko, Department of Bacteriology and Immunology, Western Infirmary, Glasgow.

3.3. METHODS

3.3.1. Preparation of human leucocyte suspensions from breast milk and dialysis fluid

3.3.1.1. Isolation of human breast milk leucocytes

Human breast milk or colostrum was collected as detailed in Section 2.3.3. Following centrifugation at 200g for 10min, the milk separated into three layers: a cellular pellet, an aqueous phase and an upper lipid layer. After the middle and fat layers had been removed and mixed thoroughly to disperse fat globules, the sedimented cells were washed twice in 5ml of HBSS and resuspended in culture medium (see 3.2.1). Viability of the cells was determined by eosin exclusion. Total viable cell counts were in the range of 4×10^5 to 2×10^7 cells (mean: 6.9×10^6), and only specimens which gave >85% viability were used. Milk supernatants were retained for use as pre-incubating media in phagocytosis assays.

3.3.1.2. Isolation of human peritoneal leucocytes

The cells from peritoneal effluent (see 3.2.3) were harvested by centrifugation at 900g for 15min in plastic 1 litre buckets. After the bulk of the supernatant was removed, the cells were resuspended in the remaining fluid and transferred to sterile plastic universals. The dialysate was then centrifuged at 300g for 5min and the cell pellets washed in PBS and pooled. If a large number of erythrocytes was evident, these

were removed by hypotonic lysis for 45sec in 10ml of sterile distilled water; isotonicity was restored by the addition of 10ml of sterile saline (x2 strength) and the peritoneal leucocytes recovered by an additional centrifugation step.

Cells were resuspended in culture medium and the percentage viability determined by eosin exclusion. Total viable cell numbers were in the range $2x10^5$ to $4x10^7$ cells with a mean of $8.5x10^6$ cells. Samples with <85% viability were discarded.

3.3.2. Differential staining of cell suspensions

Suspensions of breast milk cells (see 3.3.1.1) or human peritoneal cells (see 3.3.1.2) were used to make cytocentrifuged preparations and/or adherent cultures.

Approximately $2x10^5$ cells were deposited on to clean glass slides by centrifugation at 1000 r.p.m for 90sec in a Shandon cytocentrifuge. Preparations were then air-dried before staining. Between $0.5-1.0x10^6$ cells were used to make adherent cultures on sterile round glass coverslips (see 2.3.2.1). Enumeration of non-adherent cells in a haemocytometer revealed that approximately $2.0-3.0x10^5$ cells had attached to the coverslips in each well. Monolayers were allowed to dry at room temperature before staining.

Differential cell counts were determined using both ANAE and Leishman stain (see 2.3.2.1 and 2.3.2.2).

3.3.3. Effect of milk on the phagocytic activity of human macrophages

3.3.3.1. Exposure of cell monolayers to milk

Monolayers of human macrophages were established in the wells of a 48-well plastic culture dish (Costar). Each well was seeded with 0.5-1.0x10^6 human peritoneal or breast milk cells, suspended in 0.5ml of culture medium (see 3.2.1). Cells were incubated for 2h. The culture supernatant was then discarded and any remaining non-adherent cells removed by washing with HBSS (2x0.5ml). Approximately 2.0-3.0x10^5 cells adhered to the plastic. Of these, >95% had the staining characteristics of macrophages (Figs 9 and 10). Adherent cells were pre-incubated with 0.5ml of 50% breast milk or liquid infant formula (see 2.3.6). Upon removal of these solutions, the cells were washed twice with HBSS and reincubated with 0.5ml of fresh culture medium.

3.3.3.2. Uptake and degradation of immune complexes by human macrophages

Phagocytosis of ^{125}I-labelled immune complexes by breast milk macrophages or human peritoneal macrophages following exposure to milk was assayed as for mouse peritoneal cells (see 2.3.7.1). However, in the present study the adherent cell population was smaller so the cells in each well were incubated with immune complexes containing 5µg of transferrin instead of 10µg.

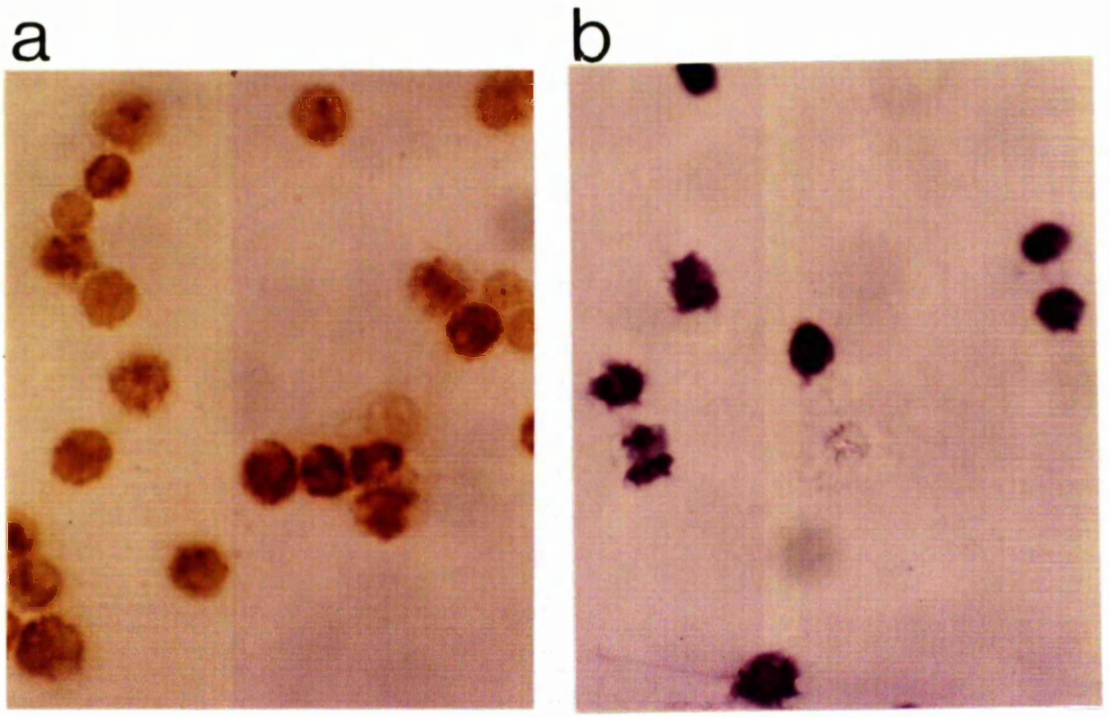


FIGURE 9 : An adherent culture of human peritoneal cells stained with α-napthyl acetate esterase stain and Leishman stain.

(a) ANAE stain (x1600).

(b) Leishman stain (x1600).

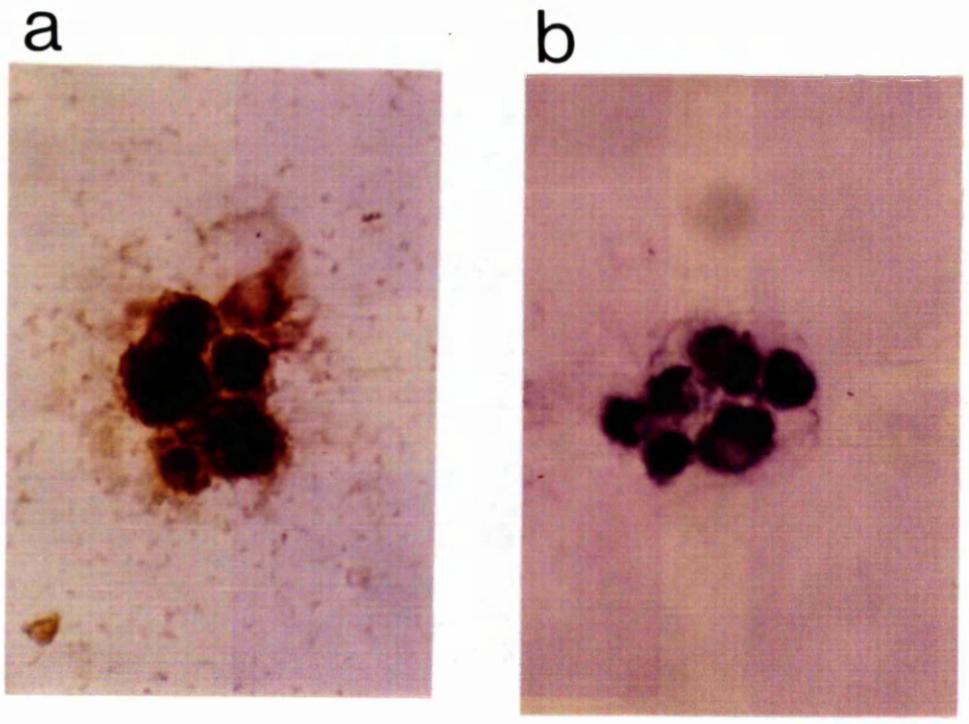


FIGURE 10 : An adherent population of human breast milk cells stained with α-napthyl acetate esterase stain and Leishman stain.

(a) ANAE stain (x1600).

(b) Leishman stain (x1600).

3.3.4. Binding of cow's milk proteins and expression of HLA-DR antigen by human macrophages exposed to formula

Human macrophages isolated from breast milk or CAPD fluid were established as monolayers on circular glass coverslips (see 2.3.2.1). After adherence, cell monolayers were exposed for 1h to Ostermilk Ready-to-Feed liquid formula (Farley) which had been diluted 1:1 in culture medium. Upon removal of the formula, the cultures were washed thoroughly with PBS to minimise background fluorescence by residual traces of milk. Binding of milk proteins CAS, BLG and ALA was detected by an indirect fluorescent antibody technique. The experimental procedure was essentially the same as that outlined earlier using mouse macrophages (see 2.3.8.2). However, following incubation with FITC-conjugated sheep anti-rabbit IgG and the subsequent washing with PBS, the cells were further stained with one drop of a 1:3 dilution of a phycoerythrin-conjugated monoclonal antibody to human HLA-DR antigen (Becton Dickinson, Oxford, England).

After 30min in the dark, cultures were washed for 10min in PBS. Coverslips were then removed from the wells and mounted with PBS-glycerol (4 parts PBS: 1 part glycerol) and the edges sealed with nail varnish. As before, cells incubated with either a 1:20 or a 1:50 dilution of normal rabbit serum (see 2.2.5) in PBS instead of antiserum to milk proteins served as negative controls and showed <1% positive staining. The cells were examined in a Leitz Ortholux fluorescence

microscope. Staining of HLA-DR was standardised using the HLA-DR -ve and +ve cell lines K562 and B-JAB respectively (see 3.2.4). To ensure correct identification of cells staining positively with both fluorochromes, the cells were observed using excitation wavelengths of both 490nm and 550nm.

A minimum of 100 cells were counted and scored as positive or negative for binding of milk protein and then as positive or negative for expression of HLA-DR antigen. The number of cells in each category (HLA-DR +ve, milk protein +ve; HLA-DR -ve, milk protein +ve; HLA-DR -ve, milk protein -ve; HLA-DR +ve, milk protein -ve) was expressed as a percentage of the total number of cells.

3.3.5. Statistical Analysis. Analysis of variance, Wilcoxon stratified sum of ranks and the X^2-test were used as appropriate.

3.4. RESULTS

3.4.1. Differential staining of human leucocytes

3.4.1.1. Human peritoneal leucocytes

The peritoneal cells from ten patients on CAPD were stained and examined. Generally, the peritoneal exudate consisted mainly of macrophages (41-85%) and usually equal numbers of lymphocytes (3-31%) and neutrophils (4-44%). Sometimes, however, macrophages and neutrophils were present in similar proportions. Very occasionally, small numbers of eosinophils (0-7%) were evident. One patient had eosinophilia (>95% eosinophils) and was excluded from the study. Significant morphological diversity of the macrophages both within and between each preparation was observed (Figs. 9, 11 & 12). Esterase staining of these cells (Fig.12) was not as intense as that of breast milk cells (Fig.14).

3.4.1.2. Human breast milk leucocytes

Breast milk samples from ten mothers were used to make cytopreparations for staining. Identification of the different cell types present in the samples was often difficult because of the large fat content. Nevertheless, examination of Leishman-stained preparations (Fig.13) revealed the presence of predominantly macrophages (43-86%); neutrophils (8-31%); lymphocytes (7-35%) and very occasionally, a small number of eosinophils (0-3%). A very small number of epithelial cells were observed in some of the more

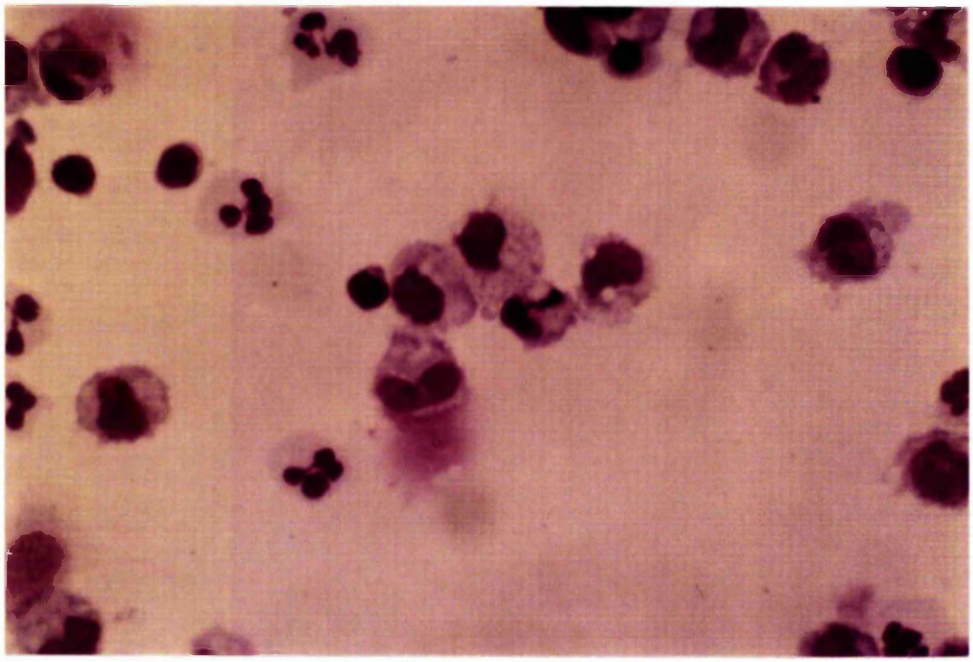


FIGURE 11 : A population of human peritoneal cells stained with Leishman stain (×1600).

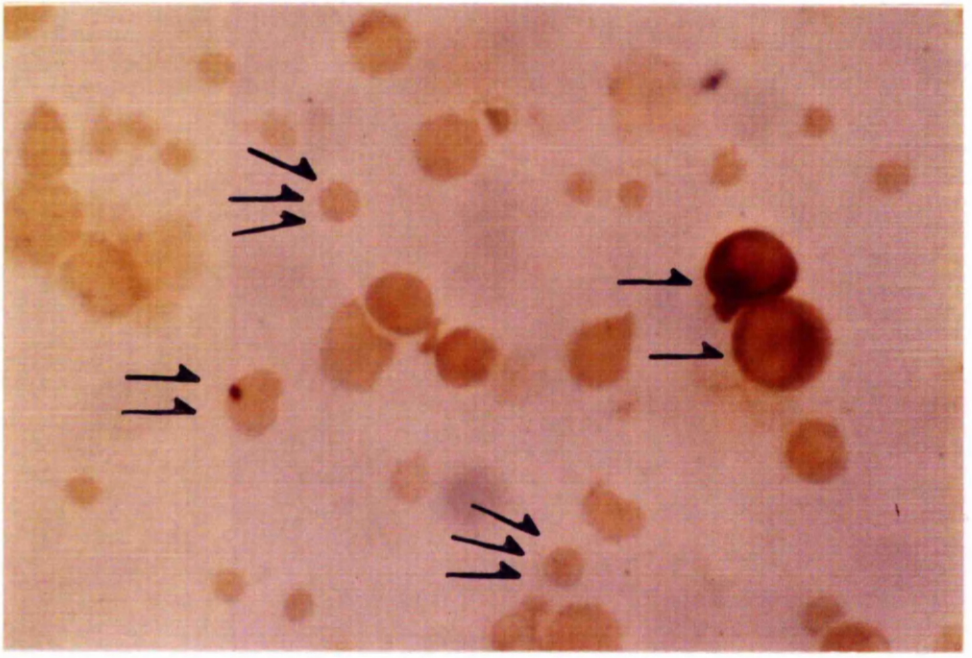


FIGURE 12 : A population of human peritoneal cells stained with α-napthyl acetate esterase stain.

Cells indicated by one arrow are macrophages, double arrows point to a T-lymphocyte and triple arrowsto ANAE-stain negative cells (x1600).

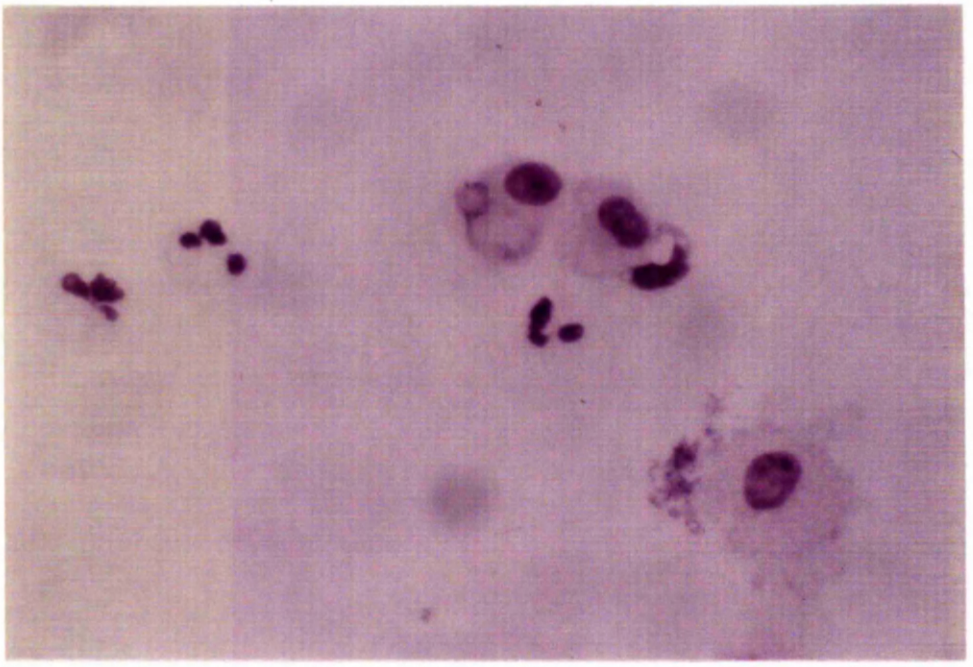


FIGURE 13 : A population of cells from human breast milk stained with Leishman stain (x1600).

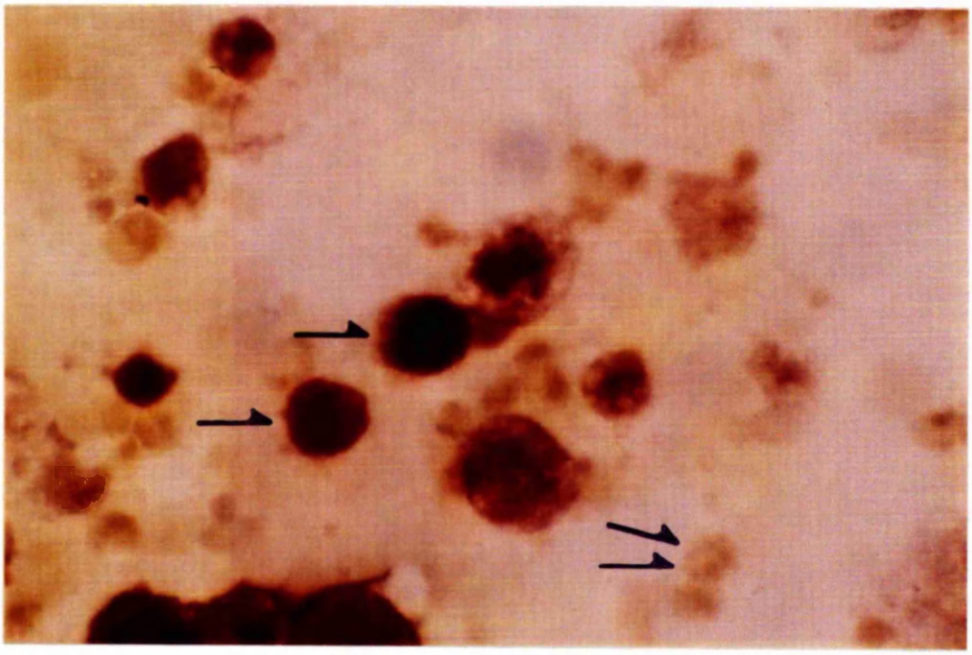


FIGURE 14 : A population of cells from human breast milk stained with α-napthyl acetate esterase stain.

Cell indicated with one arrow are macrophages. Double arrows point to ANAE-stain negative cells (x1600).

mature samples of milk but they were not included in the cell counts.

Identification of macrophages was confirmed using ANAE stain (Fig.14). These esterase positive cells exhibited considerable variation in size and were usually very heavily stained.

3.4.2. Effect of human milk and infant formula on phagocytosis and digestion of immune complexes by human peritoneal macrophages

Human peritoneal macrophages were exposed to either liquid infant formula or human breast milk supernatant and then examined for their ability to phagocytose and degrade radiolabelled transferrin-anti-transferrin immune complexes. Results obtained from 5 representative experiments are shown in Table 11. The phagocytic activity of the macrophages was found to vary between individual samples and was sometimes very low. However, ingested complexes appeared to be efficiently degraded.

Analysis of results from all the experiments carried out showed that the figures were not normally distributed. For this reason, Wilcoxon's Stratified Sum of Ranks Test was used to compare the overall effect of milk and formula on cell activity. The results show that exposure to either formula or breast milk led to a significant decrease in the cells' ability to phagocytose immune complexes (Table 12). The formula also exerted an inhibitory effect on the digestive capacity of the macrophages. In contrast, the breast

TABLE 11

Effect of human milk supernatant and liquid infant formula on the phagocytosis of ^{125}I-labelled immune complexes by individual samples of human peritoneal macrophages.

	Cells preincubated with 50% (v/v):	PATIENT 1	2	3	4	5
% INGESTION OF IMMUNE COMPLEXES	HBSS (control)	16.6 ±1.0	13.3 ±1.0	9.5 ±1.2	1.4 ±0.3	6.3 ±0.5
	Human milk	10.3* ±0.9*	10.2* ±1.4*	6.5* ±0.8*	1.2 ±0.2	5.3 ±1.0
	Formula milk	13.1* ±1.7*	10.2* ±1.5*	7.5 ±1.4	1.1 ±0.4	6.0 ±1.5
% DIGESTION OF INGESTED IMMUNE COMPLEXES	HBSS (control)	40.5 ±0.9	74.7 ±6.1	79.7 ±10.7	67.6 ±6.3	76.5 ±4.3
	Human milk	36.2 ±5.3	74.3 ±2.8	79.9 ±1.7	55.5 ±7.6	69.5 ±9.6
	Formula milk	33.4 ±1.5	68.4* ±3.2*	82.9 ±1.8	60.7 ±8.5	70.9 ±4.8

Figures represent the mean±standard deviation. (n43)

* $P<0.01$ compared with appropriate control.

Statistical analysis : analysis of variance was used.

TABLE 12

Effect of human milk supernatant and liquid infant formula on the phagocytosis of ^{125}I-labelled immune complexes by human peritoneal macrophages.

Macrophages pre-incubated with:	50% medium control	50% human milk	50% formula milk
% phagocytosis of immune complexes	11.5 (6.8-13.8)	9.7[a] (6.4-10.9)	8.4[a] (5.3-10.6)
% of ingested complexes degraded	70.1 (61.5-76.8)	72.8 (55.8-77.5)	65.7[a] (55.8-80.0)

Figures represent median with range in parentheses.

a : $P<0.01$ compared to control.

Statistical analysis: Wilcoxon's stratified sum of ranks was used.

milk supernatant did not greatly affect this function (Table 12).

Thus, both formula and breast milk supernatant are potential inhibitors of the phagocytic activity of human peritoneal macrophages.

3.4.3. Effect of human milk and infant formula on phagocytosis and digestion of immune complexes by human milk macrophages.

Monolayers of breast milk macrophages were cultured in the presence of human milk or liquid formula which had been diluted 50% in culture medium. The ability of these cells to handle labelled transferrin-antitransferrin complexes was then examined and compared with that of control milk cells not exposed to milk. For the same reasons indicated above (see 3.4.2), Wilcoxon's Stratified Sum of Ranks Test was used to analyise the results.The data show that breast milk macrophages exhibited a moderate ability to ingest immune complexes but appeared, on the whole, to be unaffected by preincubation with infant formula (Table 13). Cells exposed to breast milk supernatant showed a reduction in their ability in ingest complexes but this was not statistically significant. Examination of the cultures microscopically before and after incubation with milk did not reveal any loss of cells. In general, milk macrophages were also capable of efficiently degrading internalised material and were not greatly affected by pre-culturing in milk (Table 13).

The results suggest, therefore, that neither

TABLE 13

Effect of human milk supernatant and liquid infant formula on the phagocytosis of ^{125}I-labelled immune complexes by breast milk macrophages.

Macrophages pre-incubated with:	50% medium (control)	50% human milk	50% formula milk
% phagocytosis of immune complexes	11.2 (7.3-14.3)	6.3 (2.0-14.9)	11.1 (7.1-14.0)
% of ingested complexes degraded	29.6 (6.6-50.6)	18.9 (5.0-73.1)	32.9 (14.7-50.5)

Figures represent median with range in parentheses.

Statistical analysis: Wilcoxon's stratified sum of ranks was used.

milk nor formula interferes with the function of breast milk macrophages with regard to uptake and intracellular digestion of immune complexes.

3.4.4. Binding of cow's milk proteins and expression of HLA-DR antigen by human peritoneal macrophages.

Binding of bovine milk proteins present in liquid formula might contribute to its inhibitory effect on human peritoneal macrophages. To investigate this, a double layer indirect fluorescent antibody technique was used to detect milk proteins at the cell surface following a 1h incubation with the formula.

Macrophages seeded on glass coverslips were exposed to liquid formula for 1h and examined for binding of cow's milk proteins CAS, BLG and ALA. Cells incubated with normal rabbit serum instead of antiserum to milk proteins served as negative controls and showed <1% staining. Human peritoneal macrophages pre-incubated with formula stained positively for binding of CAS (29.9%, n=11) and BLG (17.3%, n=10) and little, if any, ALA (0.7%, n=11)(Fig.15). Examples of such binding are shown in Fig.16.

Macrophages bearing Ia (HLA-DR) antigen are known to be able to act as antigen presenting cells. Thus, it was considered of interest to see whether human peritoneal macrophages which bind cow's milk proteins at their surface might bear the HLA-DR +ve phenotype. Consequently, cell monolayers were further stained with a fluorescent monoclonal antibody directed against

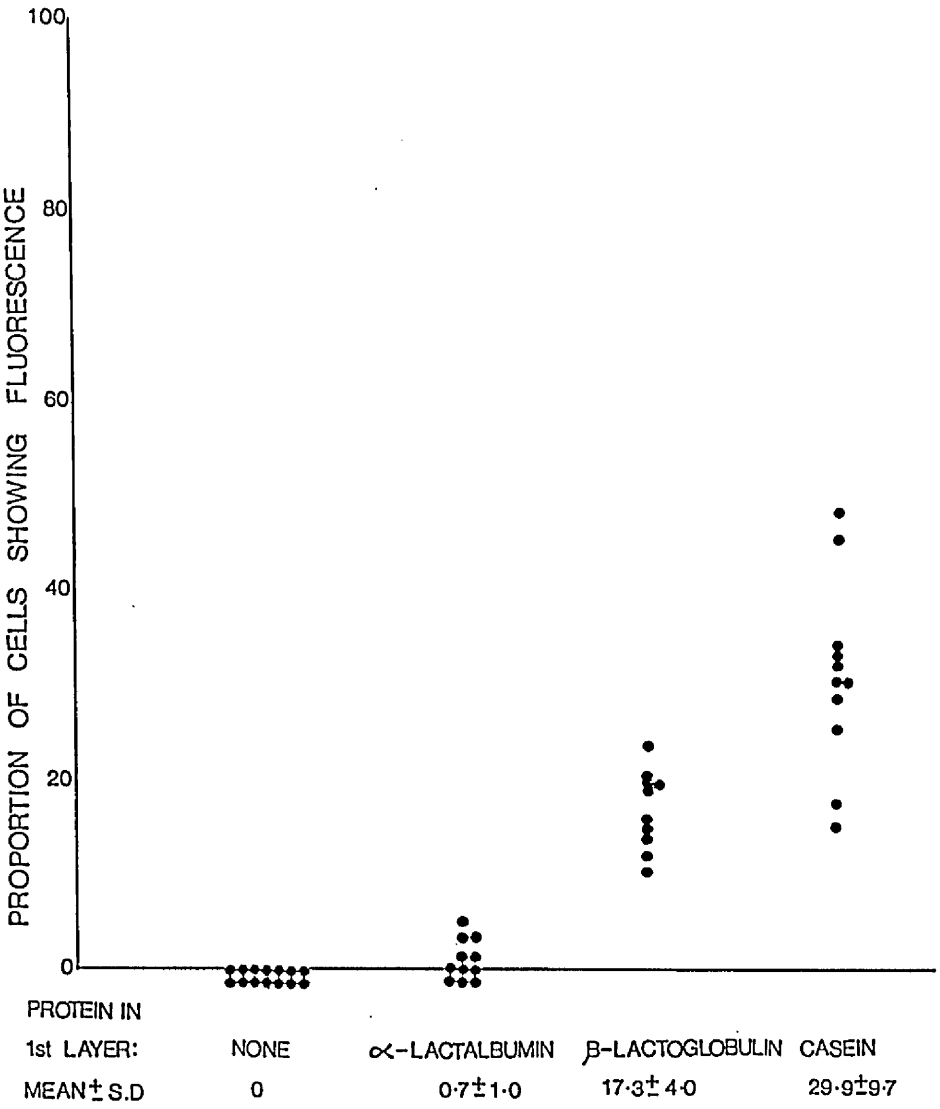
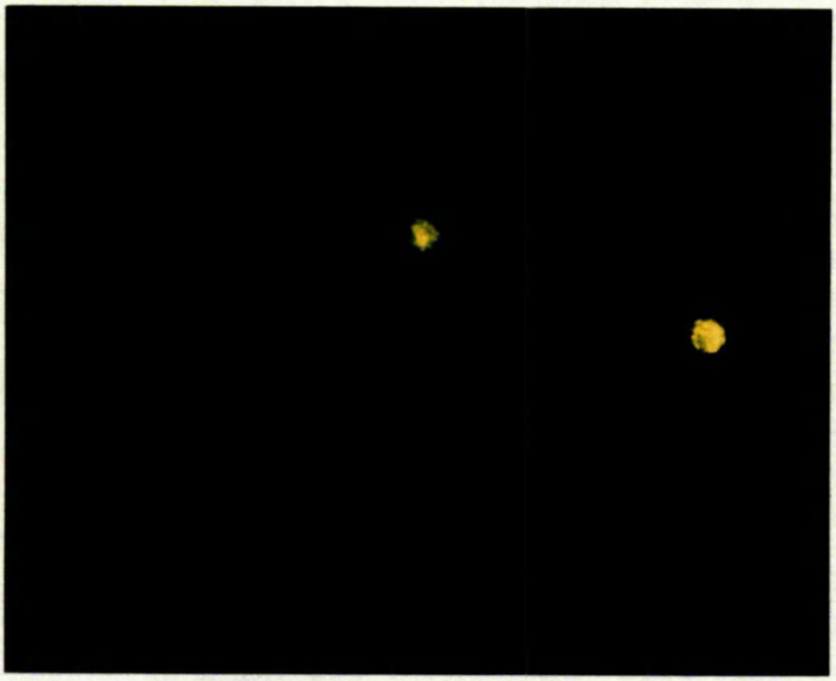
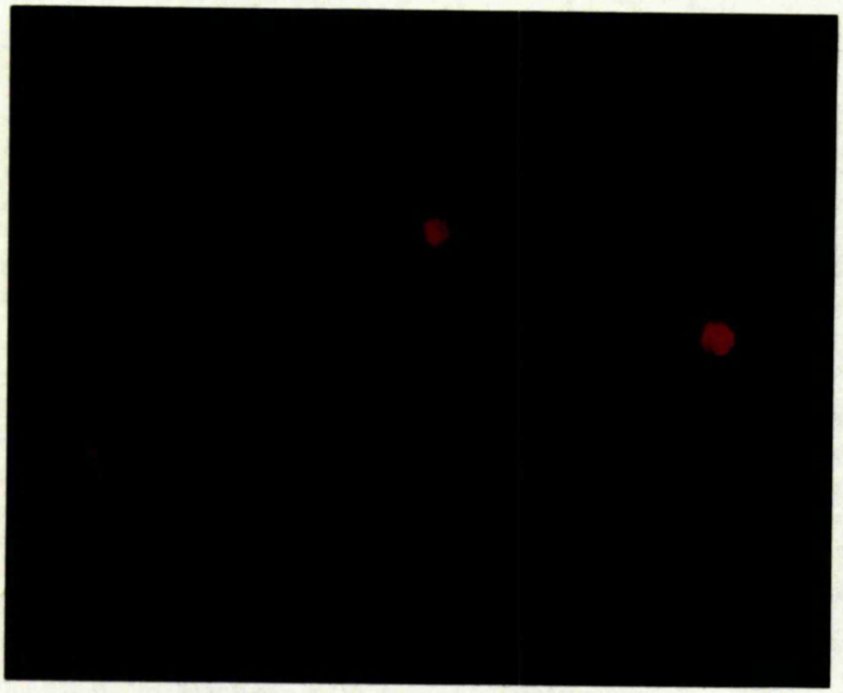


FIGURE 15 : Binding of α-lactalbumin, β-lactoglobulin and casein, by human peritoneal macrophages following a 1h incubation with liquid infant formula.

a



b



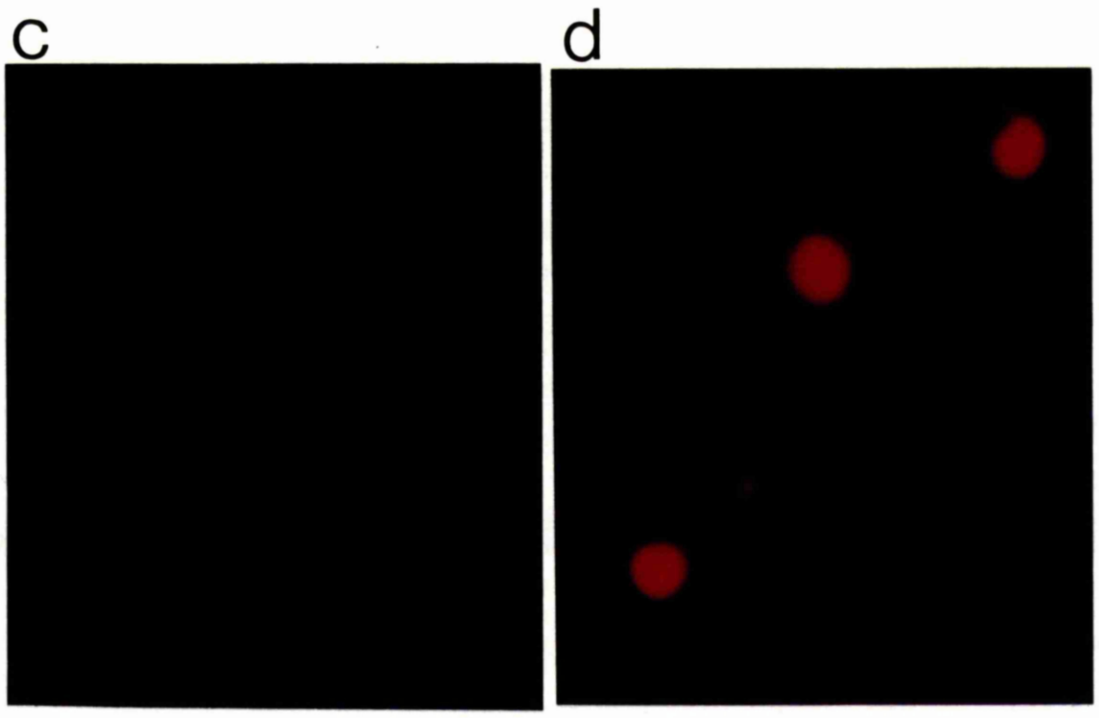


FIGURE 16 : Staining of surface-bound casein and HLA-DR antigen on human peritoneal macrophages by fluorescent antibodies.

(a) Cells as seen under U.V., showing binding of bovine casein (x1600).

(b) The same cells as in (a) examined using an excitation wavelength of 550nm and showing that the cells in (a) also stain positively with the monoclonal antibody to HLA-DR antigen.

(c) Cells which are not fluorescent when examined under U.V. and therefore do not have casein bound at their cell surface (x1600).

(d) The same cells as in (c) examined using an excitation wavelength of 550nm and showing that the cells in (c) express HLA-DR at the cell surface.

human HLA-DR.

Approximately 55% of peritoneal macrophages expressed HLA-DR antigen but there was considerable variation between different samples (7-82%). The data in Fig.17 show the association between expression of this antigen and binding of cow's milk proteins. Cells which bound CAS and expressed HLA-DR antigen (23.2±8.1% of the adherent cells) constituted a significant proportion ($p<0.01$) of the total cells binding CAS (29%, Fig.15). Similarly, there was a strong correlation between binding of BLG and expression of HLA-DR (12.9±4.7, $p<0.01$).

3.4.5. Binding of cow's milk proteins and expression of HLA-DR antigen by human milk macrophages

Since liquid formula does not exert any inhibitory effect on the phagocytic activity of human breast milk macrophages, the question arises as to whether this resistance to formula is a result of an inability to bind cow's milk proteins at the cell surface. To investigate whether this is indeed the case, breast milk macrophages were cultured in the presence of liquid formula and subsequently examined for their ability to bind bovine CAS, BLG or ALA using the fluorescent antibody technique described above (see 3.4.4).

Breast milk macrophages pre-incubated with liquid formula stained positively for CAS and BLG. When tested for binding of CAS, 19.5% (n=7) of the cell population were positive (Fig.18). Macrophages bound BLG equally well (19.6%, n=7). In contrast to peritoneal

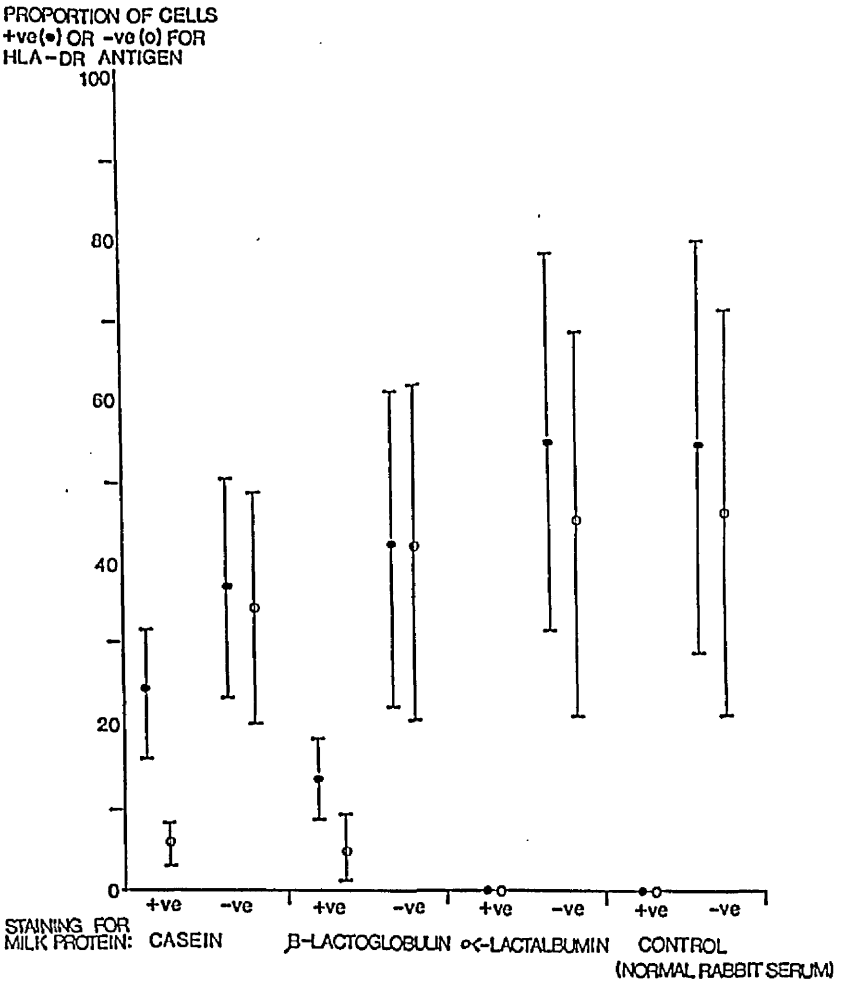


FIGURE 17 : Binding of cow's milk proteins and expression of HLA-DR antigen by human peritoneal macrophages exposed to liquid infant formula.

For each milk protein, their are four categories. The two left hand columns are the cells which bind the milk protein whilst the two right columns represents the cells which do not bind the protein.
Within each pair, the closed circles (•) are the HLA-DR +ve cells and the open circles (o), the HLA-DR -ve cells.
Each circle represents the mean for each category and the upper and lower bars the standard deviation.

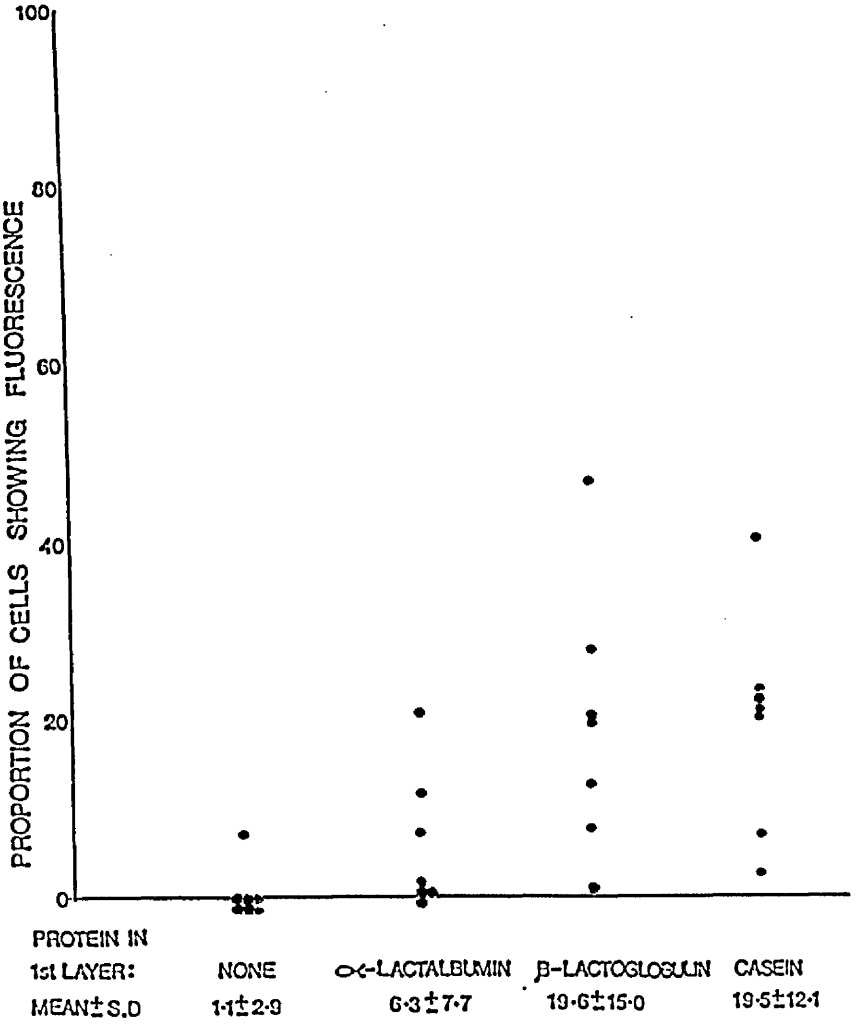


FIGURE 18 : Binding of α-lactalbumin, β-lactoglobulin and casein, by human breast milk macrophages following a 1h incubation with liquid infant formula.

macrophages, a small proportion of breast milk cells from 4 of the 7 donors interacted with ALA (Fig.18). The number of control cells which showed fluorescence was very small (approx. 1.1%).

Characterisation of the milk macrophages as HLA-DR positive or negative revealed that, as with peritoneal macrophages, there was considerable variation with individual samples. The proportion of milk cells which stained positively for HLA-DR varied between 18% and 97% with a mean value of 65%. Figure 19 shows the relationship between binding of cow's milk proteins and expression of this antigen. A significant proportion of the cells which bound CAS also stained positively for HLA-DR (16.3$\pm$9.8% of total cells). Similarly, macrophages which bound BLG (19.6%) were almost all HLA-DR positive (12.6$\pm$9.4% were BLG +ve, HLA-DR +ve). Although only a few of the breast milk macrophages bound ALA (6.3%) almost half of the them also expressed HLA-DR (3.8$\pm$4.4%). However, this association was not significant. Thus, binding of cow's milk proteins CAS and BLG correlated with HLA-DR expression ($p<0.01$ for CAS; $p<0.05$ for BLG).

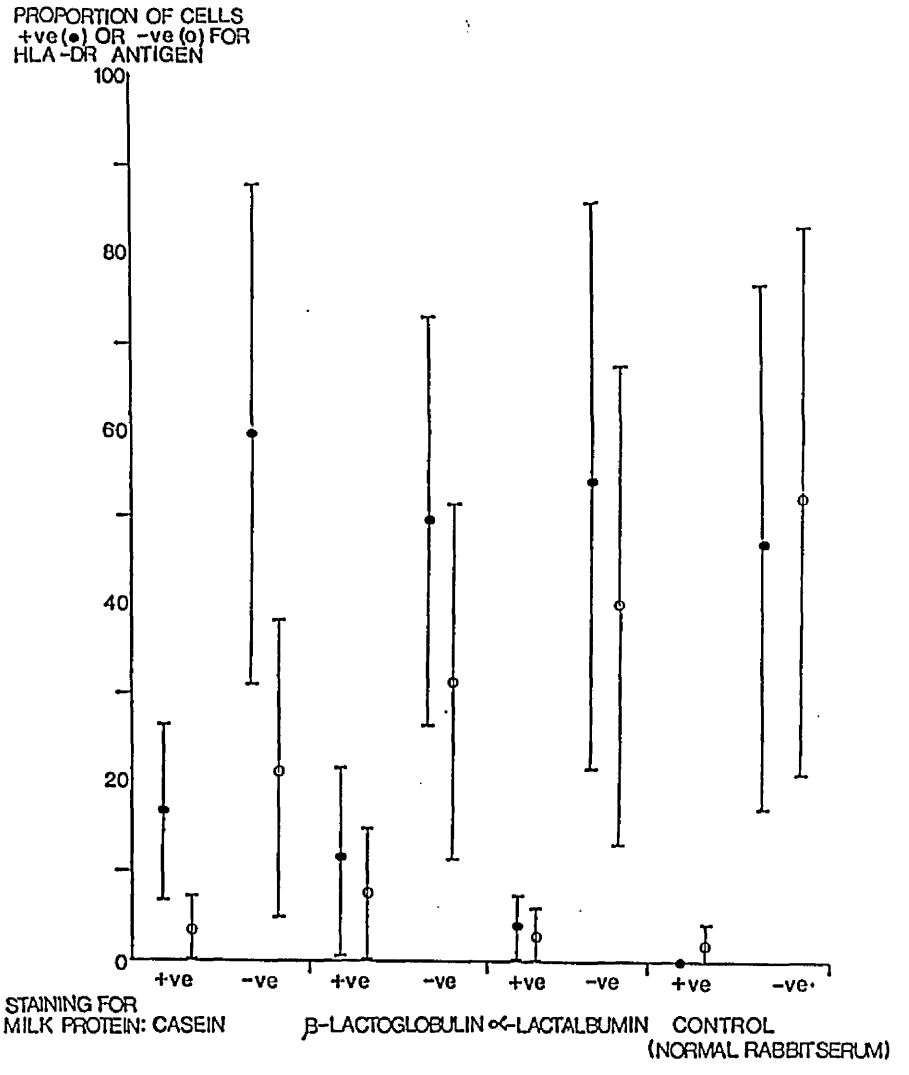


FIGURE 19 : Binding of cow's milk proteins and expression of HLA-DR antigen by human breast milk macrophages exposed to liquid infant formula.

For each milk protein, the two left hand columns represent cells which bind the milk protein and the two right columns represent those cells which do not bind the protein.
Within each pair the closed circle (•) represents the cells which are HLA-DR +ve and the open circles (o), HLA-DR -ve cells.
Each circle represents the mean value for each category and the upper and lower bars, the standard deviation.

3.5. DISCUSSION

In vivo, macrophages in the intestinal mucosa may come in contact with food proteins such as cow's milk proteins. This is increasingly likely in the gut of the newborn infant whose mucosal barrier is relatively immature and pervious to antigen (Udall and Walker, 1982). In this study, peritoneal macrophages from patients on CAPD were chosen instead of intestinal macrophages because they are much less difficult to obtain, yet are nevertheless close to the intestinal serosa. In mixed-fed infants, breast milk macrophages may also be exposed to cow's milk proteins. Consequently, the present study dealt with the effect of milk on phagocytosis of immune complexes by these two cell types, and interaction of milk proteins with the cells.

The work reported herein indicates that the phagocytic activity of human peritoneal macrophages is adversely affected by exposure to human milk supernatant. Initial recognition and/or endocytosis of immune complexes was inhibited but subsequent digestion of the complexes was unaffected. Defects in the activity of human peripheral blood cells exposed to breast milk supernatant have been reported by Pickering *et al* (1983) and Mandyla *et al* (1982). It has been postulated that milk fat globules attached to the cell surface reduce the number of FCRs available to interact

with ligands (Pickering et al, 1983; Mandyla et al, 1982). This may explain why peritoneal macrophages exposed to breast milk supernatant exhibited lower phagocytic activity than control cells. A similar effect on the intestinal macrophages of the neonate may be mitigated in vivo by the potent bile salt-stimulated lipase of human milk which is thought to supplement pancreatic lipase (Hernell and Blackberg, 1982).

Exposure of peritoneal macrophages to liquid infant formula reduced both ingesion and digestion of immune complexes. Immunofluorescence studies were performed to ascertain whether the human peritoneal macrophages, like murine macrophages (see Chapter 2), bound cow's milk proteins present in the formula. As before, a significant proportion of the cells bound intact CAS(29.9%) and BLG(17.3%) but few of the cells bound ALA(0.7%). As discussed previously (Chapter 2), processing of the liquid formula may result in partial denaturation of the proteins, making them more accessible to the macrophages and thus capable of interfering with cell function. The finding that a significant proportion of the cells bound CAS and BLG suggests that binding of bovine milk proteins by mucosal macrophages might contribute towards the development of cow's milk allergies. In particular, it is of interest that CAS and BLG have both been implicated in severe allergic responses whereas ALA which was bound by only a few macrophages has not (Devey et al, 1976).

Binding of cow's milk proteins by gut-associated macrophages may seriously affect processes of the infant

gut especially since the gut of the newborn infant allows greater uptake of macromolecules than that of adults (Walker, 1981). Although proteases in duodenal juice have the potential to digest the cow's milk proteins in formula (Jakobsson et al, 1982), their ability to do so in vivo may be restricted by their small volume (Lindberg, 1974).

Infants fed formula preparations will have lower IgA levels than their breast-fed counterparts. Cunningham-Rundles et al (1978) have shown that under such conditions, ingestion of milk results in formation of circulating immune complexes and/or food proteins. Gut-associated macrophages may be instrumental in mini-mising such events. Le Fevre et al (1985) observed carbon and iron oxide particles in Peyer's patch macrophages when these substances were fed to mice in their drinking water. Following oral administration of BSA to guinea pigs, Bienenstock and Dolezel (1971) detected considerable numbers of macrophage-like cells containing antigen in the intestinal lamina propria. Furthermore, Abraham et al (1974) observed acid phosphatase-rich macrophage-like cells loaded with carageenan in the caecal lamina propria of guinea pigs given carageenan in drinking water. The authors postulated that lysosomal enzyme released by the macrophage-like cells caused a caecal ulceration. Interestingly, Komine et al (1985) found that ALA from murine and bovine milk induced the release of lysosomal enzymes β-glucuronidase and acid phosphatase from mouse

mammary epithelial cells and that release was increased by the addition of mouse or cow whey. Furthermore, Wilkinson (1976) has reported that incubation of human blood monocytes or guinea pig peritoneal macrophages with CAS, causes a rise in the activity of acid phosphatase and β-glucuronidase. Davies and Allison (1976) have postulated that inflammation of the intestine may be due to release of lysosomal enzymes from intestinal macrophages. In view of all these reports and the results of the present study, it is possible to postulate that binding of cow's milk proteins such as CAS and BLG by gut-associated macrophages might contribute to the aetiology of CMPI by impairing cell function and perhaps causing injury to gut tissue.

Since an immune reaction to cow's milk proteins will also require presentation of the proteins by HLA-DR positive cells, the expression of this marker by human macrophages and its correlation with binding of CAS, BLG, and ALA was investigated. With peritoneal macrophages, there was a wide variation in expression of HLA-DR (8-82% of the macrophages). The mean value of 55%, although high, was lower than the figure of 75% reported by Goldstein et al (1984) and may simply reflect the different fluorescent antibody techniques used. According to Goldstein et al (1984), peritoneal macrophages from CAPD patients are not activated macrophages but resting, relatively immature cells recently arrived in the peritoneum. Dialysate fluid may induce an influx of these immature cells into the

peritoneum, and in so doing alter the proportion of cells in different stages of maturation. This may explain the wide variation in the incidence and intensity of HLA-DR staining and the morphological differences observed within the adherent population.

The finding of a positive correlation between binding of CAS and BLG with Class II antigen expression suggests that cells which are capable of acting as antigen presenting cells are also more likely to bind cow's milk proteins. A significant percentage (94%) of adherent human intestinal macrophages bear an Ia-like antigen (Golder and Doe, 1983). Moreover, the adherent phagocytes from mouse Peyer's patches are known to present antigen (MacDonald and Carter, 1982). Similar binding of bovine milk proteins to these cells in the neonatal gut might constitute a first step in the development to an immune response and consequent hypersensitivity to these proteins.

In vitro studies have suggested that breast milk macrophages may persist in the intestinal milieu of the neonate (Paxson and Cress, 1979) and thus contribute to the defence of the neonate against infection. Human milk curd supplies the intestine with a continuous flow of nutrients. It is soft and flocculent and is more easily digested than the tough and rubbery curd of cow's milk (Vorherr, 1978). Moreover, the gastric half-emptying times for breast-fed infants are considerably shorter and follow a biphasic emptying pattern more often than those of formula-fed infants (Cavell, 1981).

The slower emptying of meals of formula increases the likelihood of an interaction between milk cells and formula milk components in mixed-fed infants. Pitt et al (1977) reported that milk leucocytes were more bactericidal in rat milk than in infant formula. This suggests that infant formula may inhibit the activity of milk cells. The present study was undertaken in an attempt to establish whether exposure to liquid infant formula interferes with the phagocytic activity of human breast milk macrophages. Comparable experiments to those previously described for human peritoneal macrophages were carried out.

Ingestion and degradation of immune complexes by breast milk macrophages was, in contrast to human peritoneal macrophages, apparently unaffected by exposure to infant formula. However, the breast milk cells bound CAS and BLG to a similar degree to human peritoneal cells. Furthermore, a small proportion were positive for ALA. Thus, the inability of formula to interfere with the phagocytic activity of milk macrophages is not due to a reduced binding of cow's milk proteins at the cell surface. Binding and ingestion of fat globules from the breast milk may render the milk macrophages resistant to the inhibitory action of the formula. Intracellular fat globules are visible in stained preparations (Fig.14). Assuming that ingestion of milk lipid has already occurred in the mammary gland and interfered with cell function, it is understandable that the formula may cause no further reduction in phagocytic activity. As previously

suggested, binding of milk lipid may inhibit interaction between complexes and Fc receptors. However, the breast milk macrophages were still as efficient as peritoneal macrophages at handling immune complexes, which would then imply that the milk cells were potentially more active.

Since the percentage of adherent milk cells binding CAS was not dissimilar to that of peritoneal macrophages, it would appear that prior occupancy of CAS binding sites on the milk macrophages by human CAS does not occur, or if it does, that human CAS is sufficiently different from bovine CAS to cause no interference. Certainly, it is known that human CAS contains mainly β-casein and a little κ-casein whereas cow's milk contains a large proportion of α-caseins, a subunit class not found in human milk (Lönnerdal, 1985). The proportion of cells binding BLG was also similar for the milk macrophages and the peritoneal cells. The explanation for this is probably more straightforward; as human milk contains little or no BLG (Bell and McKenzie, 1964), one would expect the two types of human macrophages to bind this protein equally well.

A small proportion of the breast milk macrophages stained positively for binding of ALA whereas the peritoneal cells were negative. Hanson and Johansson (1971) reported an immunological relationship between human milk ALA and bovine milk ALA. The immunofluorescence studies may therefore have detected trace amounts of human milk ALA which were still bound

to the surface of the milk cells, especially since Komine et al (1985) have shown that ALA binds tightly to lysosomal membranes. If this is indeed the case, it is not known why bovine ALA in the formula did not bind to the cell surface. It could be argued that this difference might reflect the lower levels of ALA in bovine milk compared to breast milk. Whilst human milk contains 2-3mg/ml of ALA (Lönnerdal, 1985), bovine milk contains 1000-1500ug/ml (Hanson and Johansson, 1971). Alternatively, processing of the formula may have reduced the affinity between the protein and the cell membrane.

Approximately 55% of breast milk macrophages expressed HLA-DR antigen but there was considerable individual variation (18-97%). A higher value of 80% was obtained by Leyva-Cobián and Clemente (1984) in the only comparable previous study; a lower figure of 23% reported by Mori and Hayward (1982) referred to total breast milk mononuclear cells rather than to macrophages. Like the peritoneal macrophages, there was also a correlation between expression of HLA-DR antigen and binding of cow's milk proteins by milk macrophages. This suggests that in infants on a mixed-feeding regime, milk macrophages might act to present cow's milk proteins. However, no firm conclusions can be drawn on the role of breast milk macrophages in preventing/aiding allergic reactions in mixed-fed infants. Although these cells may facilitate an immune reaction to cow's milk proteins by acting as antigen presenting cells in the local immune response of the

gut, they were still able to efficiently ingest and degrade immune complexes even following exposure to infant formula. This suggests that processing of immune complexes would not be affected by mixed feeding. Indeed, it is possible that this phagocytic activity might even be enhanced *in vivo* for Parker *et al* (1984) have shown that human milk CAS produces a hexapeptide which stimulates the phagocytic activity of macrophages.

In conclusion, the results from the present study demonstrate an interaction between infant formula and human macrophages, and suggest that early weaning to bovine milk products may favour development of CMPI. It is postulated that such an interaction induces presentation of intact bovine milk proteins and reduces clearance of immune complexes. Binding of such proteins may cause release of lysosomal enzymes which would otherwise have been used in the degradation of complexes and/or killing of intracellular microorganisms. Breast milk macrophages had a moderate phagocytic activity which was unaffected by exposure to formula and from these studies it is not possible to assess the role of these cells in the prevention of allergic disease.

Comparable studies on mucosal macrophages themselves, if these could be obtained in adequate numbers, and investigation of their ability to act as antigen presenting cells would help to shed more light on their function *in vivo*. The activity of milk macrophages in a milieu comparable to that of the infant

gut also warrants investigation. In order to afford any protection to the neonate, milk macrophages must be relatively resistant to gastric destruction. The following chapter attempts to shed more light on this by observing the fate of orally administered macrophages in the gastrointestinal tract of newborn mice.

CHAPTER 4

FATE OF TRANSFERRED PHAGOCYTES IN THE NEWBORN MOUSE

4.1. INTRODUCTION

In Chapter 3, it was shown that substantial numbers of viable leucocytes are present in human breast milk. The adherent milk cells were shown to function as competent phagocytic cells *in vitro* and it was suggested that they might also function as antigen presenting cells. However, these observations have no relevance if viable milk cells are not transmitted to the newborn or if upon transmission the cells are immediately destroyed in the intestinal tract.

The transfer of maternal cells via milk and their subsequent contribution to the immunocompetence of the neonate is still a matter of much controversy. It has previously been reported that breast-fed infants are protected against organisms to which the mother has had prior exposure (see 1.2.1.1.1). The acquisition of immunoglobulin and cells from the milk may confer specific immunity to the breast-fed infant providing they are resistant to *in vivo* proteolysis.

Evidence of cellular transfer via milk came from a series of fostering experiments in which Beer *et al* (1974) showed that immunological reactivity to skin grafts in newborn rats was influenced by the compatibility of milk cells received during the suckling period. However, Silvers and Poole (1975) failed to show any such effect by milk cells when they repeated these experiments using the same rat strains.

Nevertheless, Ogra et al (1977) provided further indirect evidence of the transfer of both immunoglobulin and antigen-specific lymphocytes from mother to infant. They detected poliovirus IgA-antibody in the circulation of formula-fed infants who had been given colostrum rich in this antibody 18-72h after birth and demonstrated tuberculin-reactivity in the peripheral blood lymphocytes of infants breast-fed by tuberculin-positive mothers. However this transfer of reactivity may be brought about in one of two ways. There may be absorption of secretory products released from the milk cells as they pass through the gastrointestinal tract of the infant and/or there may be uptake of milk cells themselves across the mucosal barrier. In more recent studies more emphasis has been placed upon the latter possibility.

Breast milk cells function as immuno-competent cells in a variety of assay systems (see 1.2.1.2). However, the extent to which these functions are mimicked in vivo is uncertain. If milk cells are to elicit and effect immune responses upon transmission to the neonate they, like immunoglobulin, must not be immediately destroyed within the intestinal tract. Mason (1962) showed that during the 4h interval between feeds in the breast-fed infant, the pH of the stomach is in the range of about pH 3.0-6.0, a range which is not detrimental to the survival of human milk leucocytes (Paxson and Cress, 1979). Studies in animal models have provided conflicting reports on the ability of milk cells to persist in the neonatal stomach. Although

Head et al (1977) consistently found intact, nucleated lymphoid cells within the milk-filled stomachs of 2 day old rats and mice. Silvers and Poole (1975) were unable to find any viable cells in the stomach smears of 12h old neonatal rats.

In the human infant the gastric half-emptying time for meals of breast milk is 48mins (Cavell, 1981). Many of the milk cells 'trapped' in the milk curd may therefore be in the stomach for up to 2h before passage into the duodenum. If however the cells survive this stage, is their primary function to afford local protection or are they capable of penetrating the mucosal barrier and effecting an immune response in other body tissues? Lyscom and Brueton (1983) suggested that uptake of maternal milk cells may explain the increased numbers of intraepithelial lymphocytes evident in the gut mucosa of newborn rats during the first week of life. These workers deduced that the infiltrating lymphocytes were either maternal cells or cells which were under the influence of milk lymphocytes, rather than a response to milk proteins, since the increase in cell number was only observed during the early suckling period.

Evidence of cellular transport across the gut epithelium came from the work of Seelig and Billingham (1981). Isolated ileal segments were infused with lymph node cells via an ileostomy opening. Within 24-48h, infused cells were observed in the intestinal epithelium, laminia propria, Peyer's patches and

mesenteric lymph nodes. Similarly, Sheldrake and Husband (1985) showed that cells injected into the lumen of the duodenum of neonatal rats and lambs were absorbed by the intestine and, in the lamb, transported via the lacteal lymph nodes to the mesenteric lymph nodes. Although both these studies showed that if cells penetrate the mucosal barrier they may enter other host tissues, both involved the introduction of donor cells directly into the lumen of the gut. However, it is still not clear whether initial penetration of the mucosal barrier occurs. Kmetz et al (1970) provided early visual evidence that this is indeed possible. Bovine leucocytes labelled with trypan blue dye and fed to neonatal rabbits were subsequently observed in the intestinal epithelium. Furthermore, Weiler et al (1983) showed that a small percentage of fluorescently labelled milk cells fed to neonatal mice appeared to cross the gut mucosa and infiltrate peripheral tissues. However, in a series of fostering experiments in which unlabelled neonatal mice suckled from fully radiolabelled foster mothers and radiolabelled infants suckled from unlabelled mothers, Miller (1981) failed to show such uptake of milk cells. Clearly, further investigation is needed in order to resolve whether or not cellular absorption by the gut takes place.

The studies cited above used lymph node cells or mixed cell populations. Since the work reported earlier in this thesis is concerned primarily with the function of macrophages in milk, the aim of the experiments described forthwith was to specifically

address the question of survival and transepithelial uptake of transferred macrophages in the neonatal gut and thus provide further evidence for or against an *in vivo* function for milk macrophages in the neonate. A relatively simple approach was adopted to examine the survival of orally administered cells in the stomach and early duodenum. Syngeneic peritoneal cells which had previously ingested fluorescent latex beads were orally administered by intubation to newborn mice. Resident peritoneal cells were chosen as donor cells because macrophages constitute by far the greatest percentage of phagocytic cells present in this population. Thus the experiments were loaded in favour of donor macrophages being easily studied in the recipient. Smears and washings from the stomach contents and the duodenum respectively were subsequently examined for the presence of intact fluorescent cells. If the donor cells persist in the gastric milieu they may then cross the epithelial layer of the infant's intestinal tract and traffic to other host tissues. To see if this was indeed the case, frozen sections of early duodenum, liver and spleen were also examined for the presence of fluorescent cells.

4.2. MATERIALS

4.2.1. Animals. Adult (not younger than 6 weeks) and newborn CF/NIH mice of both sexes were bred from stocks in the departmental animal unit. Breeding pairs were checked daily at 9 a.m and 5 p.m. Mice born after 5 p.m were assigned a birth date corresponding to the following day. Mice fed at day 1 were thus aged 19-43h.

4.2.2. Culture medium. This consisted of RPMI 1640 medium (Flow) which contained 5% heat-inactivated FCS (Gibco).

4.2.3. Fluorescent bead suspension. Fluoresbrite fluorescent monodisperse carboxylated microspheres (2.5% solids latex) were obtained from Polysciences Ltd., Northampton, England. A stock suspension of beads was prepared by diluting the bead concentrate 1:20 in culture medium. To ensure sterility of this suspension during storage, sodium azide (final concentration 0.01%) was also added. Examination of the bead suspension under the electron microscope revealed large clusters of beads and so the suspension was sonicated to disperse the microspheres evenly throughout the medium. The electron microscopy work was kindly performed by Mr. Tom Downey, Department of Pathology, Western Infirmary and sonication of the bead suspension by Dr. D.A.R. Simmons, Department of Bacteriology and Immunology, Western Infirmary. The stock bead suspension was then

stored at 4^{o}C until required.

4.3. METHODS

4.3.1. Isolation of mouse peritoneal macrophages.

This essentially followed the protocol already described in Chapter 2. Peritoneal cells were collected from adult CF/NIH mice by lavage with 2ml of sterile PBS. The mice had received no prior stimulation of any kind. The cells were harvested by centrifugation at 170g for 2min and resuspended in culture medium (see 4.2.2). Percentage viability was determined by eosin exclusion. Since it was previously shown that the predominant phagocytic cell type was the macrophage (see 2.3.2.2), peritoneal cells will henceforth be referred to as peritoneal macrophages although it is accepted that a small percentage of other phagocytic cells was also present (<8%, see 2.3.2.2).

4.3.2. Fluorescent labelling of peritoneal macrophages from adult mice.

4.3.2.1. Titration of fluorescent latex beads for labelling mouse peritoneal macrophages.

Five dilutions of the original bead suspension, ranging from 1 in 10 to 1 in 200, were made in culture medium and sonicated to disperse the beads evenly throughout the medium. The macrophage suspension was adjusted to a concentration of 2×10^7 cells/ml in culture medium. Of this, 500µl volumes were mixed with 250µl of bead suspension and incubated with shaking at 37°C. After 45min, cells were recovered by centrifugation at

170g for 3min. To ensure complete removal of non-phagocytosed beads, the cells were washed twice and resuspended in 2ml of PBS. Cytopreparations of this suspension were made by centrifuging 100µl volumes at 1000 r.p.m for 90sec. Slides were allowed to dry before examination under a Leitz Ortholux microscope with incident U.V (excitation wavelength 490nm) and transmitted phase-contrast optics. A minimum of 100 cells was counted and identified as positive or negative for ingestion of fluorescent beads (>6 beads per cell and <6 beads per cell respectively).

4.3.3. The fate of cells orally-administered to newborn mice.

4.3.3.1. Preparation of fluorescent cells.

Cells were collected as before (see 4.3.1) and finally suspended in 1ml of culture medium. An appropriate volume (250µl per 10^7 cells) of fluorescent beads was introduced into the cell suspension and incubated, with shaking, for 45min at 37^oC. Following centrifugation at 170g for 3min, the cell pellet was washed thoroughly with PBS and resuspended in a volume of culture medium sufficient to feed 50µl aliquots containing $1-5x10^6$ cells to each mouse pup.

4.3.3.2. Oral administration of fluorescent cells to neonatal mice.

Neonatal animals were fed fluorescently labelled macrophages from adult mice via a 1.5cm length of 00 flexible nylon intravenous tubing (Portex Ltd., Hythe,

Kent, England) which was attached to a 25 gauge needle. To prevent unnecessary injury and distress, the tip of the tube was rounded by gentle heating and examined under the light microscope before insertion into the oral cavity. Labelled peritoneal cells contained in 50µl of culture medium were administered to each pup using the feeding tube. Each pup was fed approximately 10^6 cells except in experiments studying cell invasion of host tissues, when each mouse was fed approximately $5x10^6$ cells. Animals which received 50µl of fluorescent beads only (1 in 200 dilution) served as controls. Injured or bleeding mice were excluded from the experiments. After feeding, the litter was returned to its mother.

4.3.3.3. Examination of intestinal contents following feeding.

At 2h, 4h or 21h after intubation, the neonatal mice were sacrificed by decapitation. Following dissection, the whole stomach of each pup was removed on to a clean glass slide, punctured, and a smear of its contents made using a small piece of flexible plastic. Large deposits of milk curd were removed and the smears air-dried before microscopic examination in a Leitz Ortholux U.V microscope with a Fluorotar objective.

To assess whether fluorescent cells had survived passage through the stomach and into the upper gastrointestinal tract, sections of gut 1-2cm in length were dissected from the anterior end of the duodenum and placed in small plastic tissue culture dishes (Lux,

Miles Laboratories, Slough, England) containing 1ml of HBSS prewarmed at 37^{o}C. The section of gut was cut open longitudinally and the intestinal contents were released into the HBSS by gentle teasing of the tissue. Milk curd was disaggregated by passage several times through a Pasteur pipette. The HBSS was withdrawn and dispensed into plastic bijoux (Sterilin). Care was taken to prevent withdrawal of debris and large milk aggregates.

Any cells present in the HBSS were retrieved by centrifugation at 170g for 3min, washed twice with 1ml of HBSS and finally resuspended in 200µl of culture medium (see 4.2.2). This suspension was used to make two smears by cytocentrifugation (1000 r.p.m, 90sec). Preparations were air-dried prior to examination under U.V. Comparisons were made between smears/washings from control mice and mice fed labelled cells. Cells containing >6 beads were considered as transferred cells and scored accordingly.

4.3.3.4. Preparation of frozen tissue specimens.

The liver, spleen and first 1-2cm of small intestine were removed at the relevant times after feeding (2h, 4h or 21h) and placed in glass universals containing isopentane. Rapid freezing of tissue was achieved by placing the universals in acetone containing dry ice. Once frozen, specimens were stored at -70^{o}C. When required, the specimens were removed from the isopentane and, still frozen, placed in small plastic moulds containing O.C.T embedding compound (Raymond A.

Lamb Ltd., London, England). Blocks of tissue were prepared by gently lowering the moulds into acetone containing dry ice. After freezing, blocks were removed from the moulds and trimmed with a sharp knife to remove excess embedding medium. Semi-serial sections (8μm) were cut in a Pearse-Slee cryostat (South London Electrical Co. Ltd., London, England) operating at $-20^{o}C$. Sections were attached directly to glass slides which had been kept at room temperature, and then air-dried before examination under incident U.V in a fluorescence microscope.

4.4. RESULTS

4.4.1. Labelling of mouse peritoneal macrophages with fluorescent latex microspheres

During this study, two different bead suspensions were employed. Concentrate A was used in the experiments which examined survival of transferred cells in the stomach and upper duodenum while Concentrate B was used in experiments which analysed tissue sections for absorption of transferred cells. The number of phagocytosed fluorescent microspheres per cell was variable. Cells containing more than 6 beads were easily seen and considered as positive for ingestion of fluorescent beads. Cells containing less than 6 beads were classed as negative. A 1/200 dilution of either Concentrate A or B clearly labelled at least 80% of the peritoneal cells (Table 14). This dilution of the original bead concentrate was consequently used to label the mouse macrophages in subsequent experiments.

4.4.2. Survival of orally-administered cells in the stomach of newborn mice

After feeding to newborn mice, labelled cells were regularly observed intact in stomach smears for up to 4h (Table 15). Often the labelled cells were too numerous to count. By 21h, there were no cells and few, if any, extracellular free beads remaining. A typical example of labelled cells as seen in a smear is shown in Fig.20.

TABLE 14

Titration of fluorescent latex beads for labelling mouse peritoneal macrophages.

Dilution of original bead concentrate*	% of macrophages containing >6 beads	
	Concentrate A	Concentrate B
1/50	95.0	92.3
1/100	73.5	91.1
1/200	82.4	86.9
1/500	55.8	53.8
1/1000	45.9	36.7

* Dilutions of the bead concentrate were made in culture medium (see 4.2.2).

TABLE 15

Presence of orally administered cells labelled with fluorescent beads in the stomach smears of newborn mice.

Hours after feeding	No.of mice	% of smears containing labelled cells[a]	No. of smears containing: No labelled cells	<10 cells	10-20 cells	>20 cells
2	46	41.3	27	12	3	4
4	35	68.6	11	6	9	9
21	22	0	22	0	0	0

a : cells containing >6 beads were considered to be +ve.

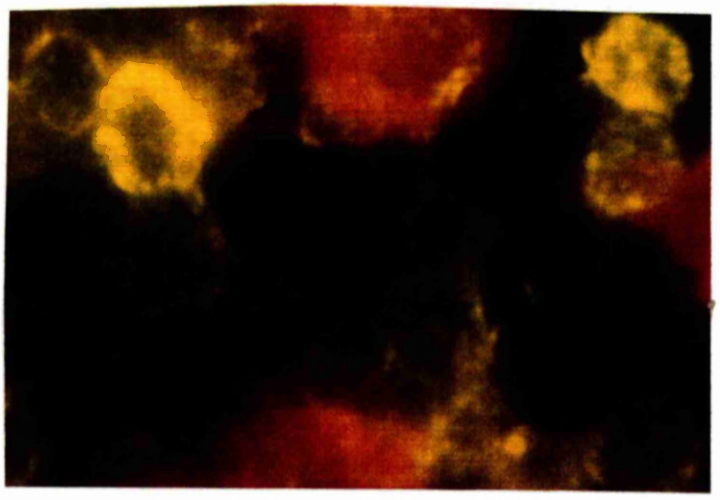


FIGURE 20 : The presence of orally-administered cells in the stomach smear of a newborn mouse 4h after feeding (x3000).

Note: Orange staining is an artifact and should be ignored.

Neonates were returned to their mothers in the interim between feeding and killing. During this period there was probably an influx of fresh maternal milk cells into the stomach. To discount the possibility that the labelled cells observed after 2h and 4h were maternal cells or shed mucosal cells which had become inadvertently labelled by ingesting free fluorescent beads *in vivo*, mice were fed beads alone as controls. Fluorescent cells were never observed in control animals even when many beads were present.

About 60% of the stomach smears made 2h after feeding did not contain labelled cells (Table 15). Of these, a high proportion (19/27 smears) were also found to be negative for free beads (Table 16). Unsuccessful intubation may have been responsible for this. Total elimination or destruction of the beads and/or cells after 2h seems unlikely because after 4h, almost 70% of the smears contained labelled cells and >90% contained beads.

4.4.3. Survival of orally-administered cells in the duodenum of newborn mice

An investigation of the capacity of the transferred macrophages to survive passage into the duodenum was also carried out. In this case, duodenal washings were obtained from the first 1-2cm of the small intestine. Although this was by no means a quantitative study, it was hoped that this might provide a simple method for detecting transferred cells. However, labelled cells were visible in duodenal

TABLE 16

Presence of fluorescent beads in the stomach smears of newborn mice after oral administration of cells labelled with fluorescent beads.

Hours after feeding	No.of mice	% of smears containing free beads	No. of smears containing: No beads	<20 beads	20-40 beads	>40 beads
2	46	52.2	22	17	3	4
4	35	91.4	3	5	11	16
21	22	4.5	21	1	0	0

washings as early as 2h after feeding (Table 17). Furthermore, passage of the cells into the duodenum continued for a minimum of 4h after feeding. However, using this method, only a few transferred cells would be recovered. Thus, if any of the washings were negative it would not necessarily follow that there were no labelled cells present. Table 18 shows that many of the washings contained only a few beads.

4.4.4. Absorption of orally-administered cells across the gut of newborn mice

Frozen sections of the upper duodenum, spleen and liver of newborn mice were prepared 2h, 4h and 21h after oral administration of labelled cells. Examination of the sections demonstrated that these orally-administered cells could, in some animals at least, invade the host and be transported to other tissues of the body. By 4h, labelled cells had invaded the gut (Fig.21) of one newborn mouse and then migrated to the spleen (Fig.22). Since fluorescent cells were conspicuous in the stomach smear from this mouse (Table 19), oral administration of the cells had clearly been successful and gastric digestion had been minimal (only a few free beads were in evidence: Table 20). One fluorescent cell was also visible in a section of gut taken 21h after feeding. Free beads were visible in many of the liver sections examined, but no cells were found (Fig.23).

To ensure that indigenous macrophages did not ingest sufficient numbers of free beads to be scored as

TABLE 17

Presence of orally-administered cells in the duodenum of newborn mice.

Hours after feeding	No. of mice	% of washings containing labelled cells[a]	No. of washings containing: No cells	1 cell	2 cells	3 cells	4 cells	5 cells
2	41	7.3	38	0	2	1	0	0
4	29	34.5	19	4	0	4	1	1
21	21	0	21	0	0	0	0	0

a : cells containing >6 beads were considered to be +ve.

TABLE 18

Presence of fluorescent beads in the duodenum of newborn mice following oral administration of labelled cells.

Hours after feeding	No. of mice	% of washings containing free beads	No. of washings containing: No beads	<20 beads	20–40 beads	>40 beads
2	41	9.8	37	3	1	0
4	29	62.1	11	13	2	3
21	21	0	21	0	0	0

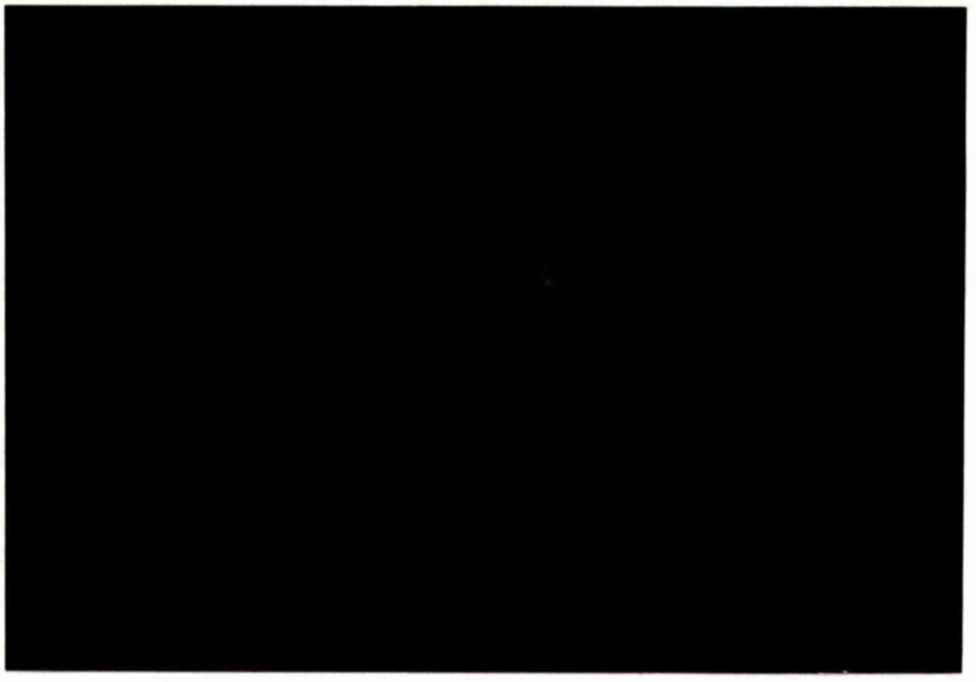


FIGURE 21 : The presence of an orally-administered cell in a section of gut taken from a newborn mouse 4h after feeding (x1600).

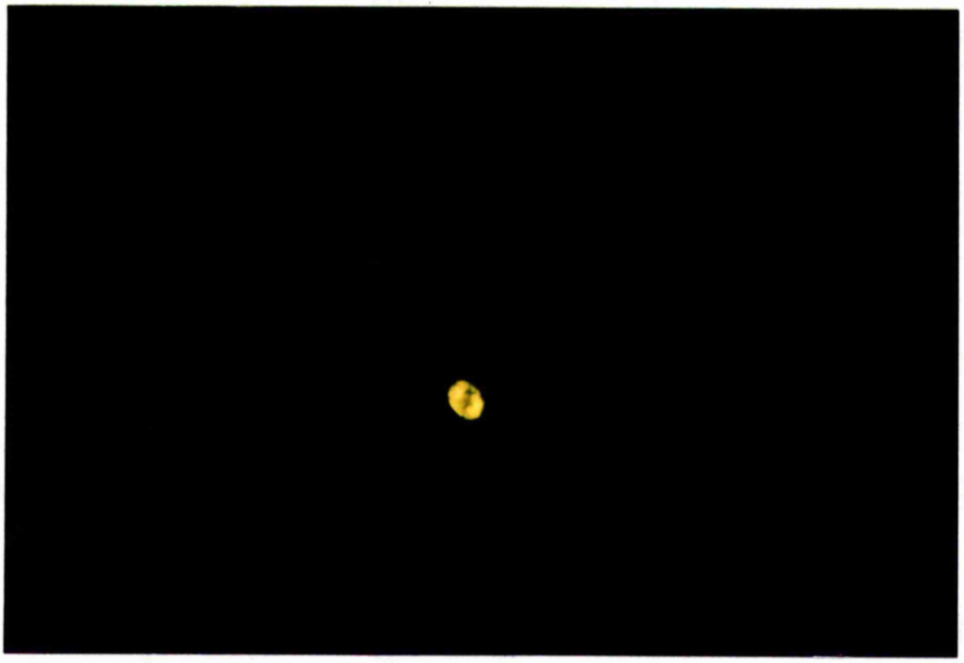


FIGURE 22 : The presence of an orally-administered cell in a section of spleen taken from a newborn mouse 4h after feeding (x1600).

TABLE 19

Numbers of orally-administered cells in the tissues of newborn mice.

Hours after feeding	Mouse number	No. of labelled cells observed in: Stomach smear[a]	Gut[b]	Liver[b]	Spleen[b]
2	1	++	–	N/D	–
4	1	+++	++	–	+
	2	++	–	–	–
	3	+	–	–	N/D
	4	+	–	–	–
21	1	–	–	–	–
	2	–	+	–	–

a : The number of labelled cells observed in a smear of contents was scored as follows:

–	:	no cells
+	:	<10 cells
++	:	10-20 cells
+++	:	>20 cells

b : Total number of labelled cells found in an analysis of 6 sections (8μm) of the duodenum (1-2cm length), liver and spleen of newborn mice. Each '+' represents one cell and '–' no cells.

N/D : not determined.

TABLE 20

Numbers of fluorescent beads in the tissues of newborn mice following oral administration of labelled cells.

Hours after feeding	Mouse number	No. of beads observed in: Stomach smear[a]	Gut[a]	Liver[a]	Spleen[a]
2	1	++	–	N/D	–
4	1	+	++	++	–
	2	–	+	+	–
	3	+	++	–	N/D
	4	+	+	+	+
21	1	+	–	+	+
	2	+	+	+	+

a : Total number of beads found in stomach smears or in 6 sections (8μm) of duodenum (1-2cm length), liver and spleen:

– : no beads
\+ : <20 beads
++ : 20-40 beads
+++ : >40 beads

N/D : not determined.

FIGURE 23 : The presence of free fluorescent beads in the liver of a newborn mouse, 4h after feeding labelled peritoneal cells (x1600).

labelled, additional experiments were performed beforehand in which animals were fed suspensions of beads alone. Although beads were frequently detected in the sections examined, no labelled cells were ever found even when the presence of many beads indicated a fully successful intubation.

4.5. DISCUSSION

The fate of milk cells and their influence on the immune competence of the neonate is at present a matter of conjecture. The work reported in this chapter attempted to shed more light on this topic by specifically examining the fate of orally-admininstered phagocytic cells in the gastro-intestinal tract of newborn mice. Unless cells resist immediate destruction in the neonatal intestine, they are unlikely to afford any immunological protection.

To date, the few studies which have investigated transfer of milk cells to the neonate present conflicting views on the cells' contribution to host defence and, as far as I am aware, there have been no studies which have specifically examined the fate of transferred macrophages. The capacity of cells to survive in the neonatal intestine is central to our understanding of their participation, if any, in the immune response of the neonate. In this study, actively phagocytic macrophages were labelled using a novel and relatively simple technique which permitted easy identification of the cells in host tissues.

In the human infant, much of an ingested meal passes into the duodenum with little or no contact with gastric secretions (Mason, 1962). It follows, therefore, that much of the cellular component will also be immediately transported here. Where possible,

the present study therefore examined survival of transferred cells in both stomach smears and duodenal washings.

At 2h and 4h after feeding, fluorescent cells were recovered in approximately 7% and 35% of duodenal washings respectively. The lower recovery of cells after 2h could mean that passage of cells into the duodenum of the newborn mouse does not follow the same time course as that of the human infant. However, it is also possible that these figures were a result of unsuccessful feeding (see 4.4.2). Neither intact cells nor fluorescent beads were detected after 21h. Since labelled cells were evident in washings obtained up to 4h after feeding, it is possible that these cells had been retained transiently in the stomach before passage into the small intestine. Examination of stomach smears revealed that fluorescent macrophages remained viable in the stomach for at least 4h after feeding. The presence of beads in one smear after 21h suggests that passage from the stomach may continue for some time. Taken together these observations indicate that the milieu within the digestive tract of the newborn mouse is not inimical to cell survival. Furthermore, transport of the cells through the stomach and into the small intestine occurs within 2h and continues for at least 4h after feeding.

These findings concur with those of Head _et al_ (1977). They consistently found intact cells in the stomach contents of young rats and mice up to 2 weeks post-partum. Although the origin of these cells was

not investigated, the different types of cell present were found in proportions similar to those in rat milk. The recent experiments of Miller (1981) provided more direct evidence that such cells are of maternal origin. By nursing normal newborn mice from [^{3}H]-thymidine infused surrogate mothers and radiolabelled infants from normal mothers, Miller showed that virtually all the cells present in the neonatal stomach up to 7d post-partum were maternally derived.

Clinical studies suggest that conditions in the stomach of the human infant are not detrimental to cell survival. Mason (1962) reported that human milk considerably alters the pH of the stomach contents of the newborn. In the period immediately after a feed, there is a sharp rise in the gastric pH from 3.5 to 6.3-6.5. This value is maintained for 60mins after which time the pH gradually falls. The normal prefeed level is not reached until almost 4h after feeding. Paxson and Cress (1979) studying the in vitro response of milk leucocytes to extremes of pH and temperature, noted that alterations in the pH through the range from 3.0-7.0 had no effect on the survival of human milk leucocytes. Of course other factors such as osmolality and peristalsis may be important but, taken together, these two studies suggest that human milk leucocytes may also remain viable in the stomach for some time after feeding. Considering the body of data suggesting that this is possible, it is difficult to understand why Silvers and Poole (1975) failed to find any viable cells

in the stomach of 12h newborn rats. The only suggestion offered is that at such a young age both the concentration of milk in the stomach and its buffering capacity might be lower.

If human milk leucocytes persist in the gut of the human infant for as long as the macrophages in the newborn mouse, it is conceivable that they offer some protection during the period in which the infant's own immune system is developing. However, maternal milk cells may not offer the same protection to those infants on a mixed feeding regime. Cavell (1981) has shown that a meal of formula milk takes longer to leave the stomach of the human infant than a feed of human milk. Moreover, Maffei and Nóbrega (1975) showed that the gastric contents of bottle-fed infants are generally of a lower pH than those of breast-fed infants. Thus, in the mixed fed infant, milk cells may be 'trapped' for prolonged periods in a stomach more acidic than that of the wholly breast-fed infant. These cells may therefore be less resilient to destruction between feeds and consequently less capable of contributing further to the immune status of the infant.

The participation of milk cells in the immune response of the neonate has not yet been proved. Although adoptive transfer of immune reactivity has been demonstrated in breast-fed infants (Ogra et al, 1977), the mode of transfer remains speculative. Soluble milk components and/or milk cells may be responsible. If transfer is initiated by the latter, it follows that the maternal milk cells must gain access to the tissues

of the suckling neonate.

Electron microscopy studies of gut tissue taken from patients with gastritis revealed mobilisation of neutrophils and lymphocytes from blood vessels and lymphoid follicles in the subepithelial regions, through intercellular spaces of the gastric mucosa and into the gastric lumen (Steer, 1975). Furthermore, this cellular migration is apparently unimpeded by tissue boundaries. It may be possible, therefore, for absorption and migration of cells in the opposite direction to occur especially in the relatively immature infant gut.

Kmetz et al (1970) showed that bovine blood leucocytes labelled with trypan blue dye penetrated the gut epithelium of neonatal rabbits following oral administration. However, the validity of these results is open to scepticism. The authors admit the relative instability of the dye used and the reduced activity of the bovine leucocytes during manipulation. Furthermore, control animals fed comparable aliquots of dye alone were not included. Considering these criticisms, it is conceivable that the relative stability of the labelled cells was reduced to such an extent during manipulation that they did not remain viable in the intestinal tract of the young rabbit. Loss of cell viability is even more probable considering that the rabbits were not fed syngeneic cells but a suspension of heterologous bovine leucocytes. Consequently, mucosal cells would become labelled as

dye was absorbed by the gut. This would explain the large number of positive cells visible and their faintness.

Having shown that mouse macrophages could be transported from the stomach to the small intestine without loss of cell integrity, it was of interest to determine whether these cells were then absorbed by the host tissues. Despite the limited number of mice studied, the present report provides evidence for the first time that macrophages are transported across the gut of the newborn mouse. Fluorescent macrophages, orally-administered to neonates, were able to penetrate the gut wall in 2 of the 7 mice studied. It is unknown exactly how soon after feeding absorption begins, but it apparently does so within 4h. Interestingly, the subsequent migration through the gut mucosa was maintained for up to 21h in one mouse. The recovery of a fluorescent macrophage in a section of spleen, 4h after feeding is of great importance since it indicates that cells which have traversed the gut wall may then traffic to other tissues. The results from the present study are encouraging but clearly a more intense and stringent follow-up is warranted.

Similar reports on this topic have used either lymph node cells or mixed cell populations. Attempts to study transmission of milk-borne T-lymphocytes to newborn animals have provided conflicting views of their subsequent participation in the immune response of the neonate.

In a series of fostering experiments, Beer et al

(1974) showed that the immune response to test skin allografts in the newborn rat was influenced by the compatibility of maternal milk cells. To consolidate their findings, these studies were extended to the mouse (Head et al, 1977). However, Sivers and Poole (1975) failed to show that maternal milk cells altered graft survival in neonates, even when they used the same rat strains as the aforementioned studies. Most of the differences observed in these papers can be reconciled if one considers that only a small percentage of the effector T-lymphocytes actually penetrate the neonatal intestine. Slobodian et al (1979) and Trentin et al (1977) also examined the fate of T-lymphocytes in the newborn mouse. However, no firm conclusions could be drawn from their results since absorption of small numbers of lymphocytes would not be detected using their experimental procedures.

The study of Miller (1981) considered survival and transepithelial migration of milk cells in general. Using an autoradiographic technique, maternal milk cells were detected in stomach contents of newborn mice but none were observed in the host tissues. However, only a limited number of cells (400-1200) were examined in each section of tissue.

Studies which have successfully shown intestinal absorption of maternal cells looked for the presence of these cells in either large numbers of neonatal cells or in the neonate as a whole. In so doing, small numbers of maternal cells were detected.

Weiler et al (1983) provided direct visual evidence that FITC-labelled milk cells penetrate the gastric mucosa and infiltrate the newborn mouse. However, they estimated that only 0.1% of the total cell count (i.e about 10^4 cells) received during the first week post-partum have this invasive capacity. The results of Schnorr and Pearson (1984) are in keeping with this. They showed that even when as many as $3x10^8$ FITC-labelled maternal milk cells or peripheral blood leucocytes were fed to newborn lambs, the maximum number of cells recovered per $2x10^5$ blood leucocytes, was 8.

The experiments reported here enlarged upon these earlier studies by specifically examining the fate of macrophages in the neonate. Since the number of maternal milk cells which gain access to host tissues may be small, the present study did not examine milk macrophages themselves. By using peritoneal cells it was possible to feed each newborn mouse a large single dose of cells ($5x10^6$ cells) of which about 80% were labelled (see 4.4.1). This is probably slightly in excess of the total daily intake of milk cells by newborn mice (Weiler et al, 1983). Although the number of sections subsequently examined was small, each section was examined in its entirety for labelled cells. Therefore, small numbers of fluorescent macrophages were detected in host tissues.

The experiments of Seelig and Billingham (1981) lend further credence to the possibilty of cellular migration through tissues. Isolated ileal segments of

rats were injected with radiolabelled lymph node cells via an ileostomy opening. Labelled cells were detected in the intestinal epithelium, lamina propria, Peyer's patches and mesenteric lymph nodes. To confirm these findings, workers from the same laboratory repeated the experiments using a simpler technique for labelling the cells (Gunning et al, 1984). They also showed that cell migration commenced within 2h of injection into tissue. By 24h, labelled cells had recirculated to the wall of the bowel. The main criticism of these two studies concerned the use of adult animals. However, similar experiments conducted by Sheldrake and Husband (1985) using suckling rats (aged 1-3d) and newborn lambs (aged 6-14h) gave similar results. They observed maternal cells in the duodenum, lacteal lymph and mesenteric lymph nodes of newborn lambs 2h after injection. By 4h, infused cells had invaded the duodenum and the jejunum of the newborn rat. Taken together, these reports suggest that cells which penetrate the mucosal barrier are rapidly transported through the tissues to other sites. Furthermore, this process may continue for an extended period following absorption.

From the literature cited hitherto and the observations reported here, it is concluded that mature cells from adult animals may resist destruction in the neonate for a considerable period after ingestion. Passage of the cells into the duodenum occurs quickly and continues for at least 4h after feeding. Once in

the small intestine the cells could either participate in the local immune response or invade the gastric mucosa and be transported by the blood or lymph to reside in other host tissues. This is an acceptable proposal considering that the neonate is immunologically unresponsive. Since its immune apparatus is relatively immature, it is incapable of rejecting any maternal cells which might infiltrate its tissues. Although only a small percentage of ingested cells may be absorbed, it does not appear to be restricted to one cell type. Thus, it is advocated that immunocompetent cells of maternal origin may be transported in milk to the suckling infant where they may afford some protection at a time when the infant is otherwise defenceless.

GENERAL DISCUSSION AND CONCLUSIONS

It is difficult to assess precisely why the breast-fed infant is less susceptible to infection or hypersensitivity reactions than the formula-fed infant. However, the work presented in this thesis suggests that this may be a consequence of both delayed exposure to foreign food antigens and of transient protection by breast milk cells.

It is suggested that cow's milk proteins in formula milk preparations may cause cellular dysfunction of gut-associated macrophages and thus reduce the cells' ability to eliminate potential pathogens and antigen-antibody complexes. This proposal follows the observation that liquid infant formula inhibits the phagocytic activity of both murine and human peritoneal macrophages *in vitro*. Studies on human peritoneal macrophages supported the earlier and more extensive studies on mouse peritoneal macrophages. Cellular ingestion and digestion of radiolabelled immune complexes and *S.aureus* was significantly decreased in macrophages pre-cultured with infant formula in comparison to non-exposed cells. This inhibitory effect may be multifactorial but it is proposed that intact cow's milk proteins present in the formula contribute to cellular dysfunction, especially as purified bovine CAS produced comparable defects.

The inhibitory effect of different formula preparations did not correlate with their CAS content.

It therefore appears that the method of processing is also an important factor in determining the extent to which cell function is affected by infant formula. The results support the view that a hypoallergenic cow's milk-based formula might be produced by first reducing the antigenicity of milk proteins by heat treatment and then adding essential nutrients (Kilshaw et al, 1982; Heppell et al, 1984).

Bovine CAS, BLG and ALA are the most important substitutes for human milk protein. Immunofluorescence studies showed that CAS and BLG retain their antibody binding capacity after processing of the liquid formula and interact with the macrophage membrane. It is suggested that further interaction with macrophages in vivo may occur through immune complex formation. It is of particular interest that cellular binding of ALA was rarely detected, especially since CAS and BLG are regarded as more sensitising than ALA (Devey et al, 1976). Studies on human macrophages showed an association between binding of cow's milk proteins and expression of HLA-DR antigen. A similar association in vivo would mean that antigen presenting cells would be likely to bind milk proteins and initiate a hypersensitivity reaction.

The mechanisms by which bovine milk proteins alter the macrophage response to immune complexes and opsonised microorganisms remains a matter of conjecture. The macrophage FCR moves freely within the plasma membrane (Griffin et al, 1975), so perhaps binding of

cow's milk proteins at the cell surface blocks cell-ligand interaction and/or restricts the mobility of the FCRs, thereby reducing the phagocytic activity. Subsequent digestion of internalised material may be reduced because digestive enzymes are squandered extracellularly upon binding of the milk proteins. Such an event could explain the gut damage which sometimes occurs in cases of CMPI especially since Davies and Allison (1976) have suggested that inflammation of the intestine may be due to release of lysosomal enzymes from intestinal macrophages. A measure of lysosomal enzyme release following exposure to infant formula may consolidate this hypothesis.

Studies of the effect of milk or formula on gut-associated macrophages themselves are required to give a clearer picture of the *in vivo* situation. This may be possible as isolation techniques improve. Meanwhile, it would be interesting to see if bovine milk proteins bind to specific cells in the gut mucosa following exposure to formula. This could be tested using a specific staining or immunofluorescence technique.

As mentioned earlier, it is also proposed that the suckling infant receives transient immunological protection from transferred breast milk macrophages. Previous studies have questioned a role for milk cells in the defence of the neonate on the grounds that milk cells display depressed cellular function when compared to their counterparts in peripheral blood (Mandyla *et al*, 1982; Pickering *et al*, 1983; Clemente *et al*, 1986). The present study supports the hypothesis that a soluble

component(s) in breast milk is responsible for such defects. Breast milk supernatant inhibited the ability of human peritoneal macrophages, and occasionally mouse peritoneal macrophages, to phagocytose and sometimes degrade immune complexes. However, the present study contradicts those cited above by showing that breast milk macrophages were as efficient as human peritoneal macrophages at clearing immune complexes. This suggests that they are potentially more active than the latter cells and may indeed confer some protection on the neonate.

Effective binding and ingestion of immune complexes by breast milk macrophages confirms the presence of FCRs at the cell surface and suggests that effective clearance of antibody-coated particles may occur *in vivo*. Previous studies on the phagocytic activity of breast milk phagocytes have used mixed cell populations and have provided conflicting views on the ability of these cells to eliminate microorganisms (Ho and Lawton, 1978; Robinson *et al*, 1978; Murphy *et al*, 1983). This is basically a consequence of the type of assays employed. This thesis reports a modified bactericidal assay for adherent macrophages cultures which could be used to study the *in vitro* antimicrobial activity of breast milk macrophages.

Formula milk did not affect the ability of milk macrophages to phagocytose or degrade immune complexes. However, no firm conclusions can be drawn on the overall contribution of the milk macrophage to the

immune status of the mixed-fed infant. Breast milk macrophages bound CAS, BLG, and occasionally ALA after exposure to liquid formula. Furthermore, there was a positive association between binding of these proteins and Class II antigen expression. Thus, milk macrophages may initiate hypersensitivity reactions in the mixed-fed infant by presenting cow's milk proteins at their surface. To further test the accessory function of milk macrophages, the capacity of these cells to bind and process radiolabelled milk proteins or bacteria and then present them to T-cells could be examined using the method of Allen and Unanue (1984a).

In vivo studies which examined the fate of transferred peritoneal macrophages in the newborn mouse strengthens the view that milk macrophages play a positive role in the neonate. Orally-administered phagocytes which had previously ingested fluorescent latex beads were observed intact in the stomach contents of newborns up to 4h after ingestion. Furthermore, a few cells were also detected in spleen and gut sections. However, to prove without any doubt that transferred cells survive in the host and migrate through host tissues, more extensive histological studies are required. It would also be of interest to see if pre-exposure of transferred cells to milk or formula alters their survival and transport in the host. Furthermore, these cells might also be tested for their effect on immunological priming in newborn mice by examining the antibody response to bovine milk proteins following adoptive transfer.

Jakobsson et al (1985) detected immunoactive bovine BLG in human milk samples and postulated that this may be the cause of colic in suckling infants. When mothers began a cow's milk free diet, the amount of BLG in the milk decreased and the infantile colic disappeared. These results suggest that breast milk cells may be already exposed to bovine milk proteins in the breast milk itself. The work reported in this thesis shows that the phagocytic activity of the cells does not suffer unduly as a result. However, since breast milk macrophages which bind cow's milk proteins tend to be HLA-DR positive, these cells may be responsible for onset of such conditions as infantile colic and allergic disease. The study of Gerrard (1979) which reported an association between allergic reactions and the maternal diet would support this assumption.

The significance of an interaction between bovine milk proteins and macrophages is discussed in the context of breast milk macrophages and gut-associated macrophages. However, an interaction between cow's milk proteins and macrophages in the mammary gland is equally important. Interestingly, Komine et al (1985) have shown that ALA from mouse milk induces release of lysosomal enzymes from mouse mammary epithelial cells. The authors postulate that such an event is important in the early stages of mammary gland involution. Ferguson (1985) has shown that during involution, there is an increase in the number of macrophages in the mammary

gland. Binding of bovine milk proteins by these cells *in vivo* may therefore interfere with mammary involution.

In conclusion, this thesis supports the view that immune responses to food antigens in the neonate may be related to the age at which these antigens are first encountered. Early weaning to cow's milk products may have serious immunological implications. The importance of having an alternative food source to breast milk is acknowledged. However, it is stressed that the type of formula used should minimise hypersensitivity reactions. The quality of the formulae may be improved by reducing the allergenicity with strong heat treatment (Heppell *et al*, 1984) and then fortifying the preparation with the required nutrients, suitable concentrations of bovine immunoglobulins with specific activity (Yolken *et al*, 1985) and protective substances such as lysozyme and lactoferrin.

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