IRON METABOLISM IN MACROPHAGES

by

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Abbreviations

Abbreviations and symbols used in the text.

α	anti-
Ab	antibody
Ag	antigen
apoFt	apoferritin (devoid of iron)
apoTf	apotransferrin (devoid of iron)
BSA	bovine serum albumin
^{o}C	degree Celsius
Ci	curie
Con A	concanavalin A
CpM	Corynebacterium parvum-activated macrophage(s)
cpm	counts per minute
C -R	complement receptor
d	dalton
DEAE	diethylaminoethyl
DFO	desferrioxamine
DNA	deoxyribonucleic acid
E1%	extinction coefficient for 1% protein solution
EP	endogenous pyrogen
Fab	fragment obtained by papain hydrolysis of immunoglobulins
Fc	crystallisable fragment of immunoglobulins
FcR	receptor for the Fc portion of immunoglobulins
FCS	fetal calf serum
Fe	iron
FeNTA	ferric nitrilotriacetate
fg	femtogram
Fig.	figure

Ft	ferritin
g	gram or acceleration of gravity
h	hour(s)
Hb	haemoglobin
HBSS	Hanks' balanced salt solution
hRNA	heterogeneous ribonucleic acid
Hs	haemosiderin
Ia	I-region associated antigen
Ig	immunoglobulin
IgG	immunoglobulin "G"
IL-1	interleukin-1
IRMA	immunoradiometric assay
LEM	leucocyte endogenous mediator
leu	leucine
LPS	lipopolysaccharide
M	molar (moles/liter)
MEM	Eagle's minimum essential medium
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MPS	mononuclear phagocytic system
M_r	relative molecular weight
mRNA	messanger ribonucleic acid
mRNPs	ribonucleoprotein particles
MtM	Mycobacterium tuberculosis-activated macrophage
M.W.	molecular weight
nd	not done, not determined
NIH	National Institute of Health
nm	nanometer

NP40	Nonidet P40
NTA	nitrilotriacetate
O.D.	optical density
OTB	orthotoluidine blue
PPb	Perls' Prussian blue
PBS	phospate buffered saline, consisted of NaCl (8 g/l), KCl (0.2 g/l, Na_2HPO_4 (1.15 g/l) and KH_2PO_4 (0.2 g/l).
PEC	peritoneal exudate cells
PFC	plaque forming cells
pg	picogram
pH	reciprocal $\log_{10}$ of hydrogen ion concentration
PHA	phytohaemagglutinin
pI	isoelectric point
PMSF	phenylmethyl sulphonyl fluoride
RBC	red blood cells, erythrocytes
RES	reticuloendothelial system
rM	resident macrophage(s)
RNA	ribonucleic acid
ROI	reactive oxygen intermediates
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute (medium number 1640)
RT	room temperature
S-	Sepharose 4B coupled to- ,immunoadsorbents
S.D.	standard deviation from the mean
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
sFt	serum ferritin

symbols

~	approximately

<	less than
>	greater than
/	per
%	per cent
TBql	tarabequerel
TCA	trichloroacetic acid
TIBC	total iron binding capacity
Tf	transferrin
TfR	transferrin receptor
tM	thioglycollate broth-elicited macrophage(s)
Tx 100	Triton x 100
uCi	microcurie
ug	microgram
ul	microlitre
uM	micromolar
UV	ultraviolet
vs	versus
v/v	volume per volume
w/v	weight per volume

Declaration of joint work.

The table No. 6 presented in chapter 5 forms part of the work performed by Michelle V. Felstein during her Senior Honours B.Sc. Immunology project.

Summary

A study has been made of intracellular events in the iron metabolism of resident, immunologically- and nonimmunologically-provoked mouse peritoneal macrophages, to clarify the relationship between iron release and intracellular iron metabolism of the mononuclear phagocyte system in normal and inflammatory conditions.

The dynamics and magnitude of peritoneal infiltration of those cells populations that are generated by intraperitoneal inoculation of Corynebacterium parvum or thioglycollate broth were characterized and compared with the resident population present in unstimulated mice. The cellular influx differed according to the stimulus inoculated, the major differences being that the neutrophil influx was in a two-wave mode in C. parvum-inoculated mice in contrast to only a one-wave mode in thioglycollate broth-inoculated mice. Macrophage morphological changes and the absolute numbers of macrophages were also observed. After 4 days post-stimulation the total yield per mouse of thioglycollate broth-elicited macrophages (tM) was more than 3 times that from C. parvum-inoculated mice (CpM) and CpM yields were 2.5 times that of resident macrophages (rM). Macrophages from inoculated mice differed morphologically from rM. The stimulated cells were found to be a young population, tM being more immature than CpM. The identification of Ia^{+}ve cells was used to contrast the macrophage populations and it was found that while the rM and CpM contained similar percentages of Ia^{+}ve cells tM had a lower percentage. But when expressed in absolute numbers,

tM and CpM appeared to contain a similar number of Ia^{+}ve cells, which was double that of rM.

These three population of macrophages were pulsed with ^{59}Fe-^{125}I-tranferrin-antitransferrin immune complexes (^{59}Fe-^{125}I-Tf-antiTf) and release of ^{59}Fe and ^{125}I measured. It was found that tM and CpM processed the immune complexes more rapidly than rM, but that there was an impairment of Fe release by tM compared with rM and CpM, which, when release was related to release of ^{125}I (degradation of complexes), were similar to each other.

A sensitive IRMA assay was developed for measuring mouse ferritin (Ft) with which Ft release and intracellular Ft levels were determined. None of the experiments designed to test Ft secretion by macrophages supported an active release process, and it was concluded that if peritoneal macrophages release Ft is mainly due to cell leakeage and not by secretion.

It was found that tM had a lower Ft and Fe content than CpM and rM, but the ratio (Ft:Fe) was higher in tM than in CpM or rM, in both of which it was similar. These results implied the posibility that tM contain Ft molecules more loaded with Fe than rM and CpM.

Synthesis of Ft was measured by ^{3}H-leucine incorporation. tM showed a lower, and CpM a higher capacity to synthesise Ft than rM. Induction of Ft synthesis by Fe showed that all three types of macrophage increased their synthesis of Ft, but the response of tM was 3-4 fold lower than that of rM and CpM. This was not due to a lower iron uptake by tM, as all three types of macrophages took up similar quantities of iron from ^{59}Fe-nitrilotriacetate.

Synthesis of Ft was studied over four days post-stimulation and the depression of Ft synthesis in tM was found to begin at the first day, becoming more pronounced as time elapsed. Degradation of Ft was also studied. Pulse chase experiments showed that CpM have an accelerated degradation of Ft in comparison with rM. In tM it was not possible to demonstrate Ft degradation due to their initial low synthesis of Ft. These results suggested that even with increased Ft synthesis the CpM could release the same proportion of iron as rM, presumably due to accelerated Ft degradation. This finding, in addition to the lack of demonstrable degradation of Ft associated with the high retention of iron of tM, points to a conecction between Ft degradation and iron released by the cells.

Fractionation of intracellular iron by gel chromatography after pulsing with ^{59}Fe-^{125}ITf-antiTf and detergent lysis showed that in all cells ^{59}Fe was mostly associated with Ft, with only small amounts in low molecular weight compounds. However, in the insoluble fraction containing cell debris and undegraded immune complexes the ratio ^{59}Fe:^{125}I in tM was about 4 times higher than in rM and CpM, suggesting increased haemosiderin (Hs) formation. A simple method for determining intracellular partitioning of ^{59}Fe was devised, which involves iron chelation, differential centrifugation, immunoprecipitation and ultrafiltration. With this method it was possible to fractionate the intracellular ^{59}Fe, following pulsing with ^{59}Fe-Tf-antiTf, into fractions such as ^{59}Fe-Hs-like compound, ^{59}Fe-Ft, ^{59}Fe-non-Ft-intermediate M.W molecules (presumably iron in enzymes) and a chelatable

pool. It was found that in all the macrophages the majority of the iron was associated with Ft, and the chelatable fraction was very similar in size in all the macrophages. The ^{59}Fe-non-Ft fraction was increased in tM as also was the ^{59}Fe associated with Hs, whereas the Hs fraction was negligible in CpM and rM. This suggested that the tM need metabolically 'active' iron, while at the same time the incoming iron is being deposited in Hs.

To explain the findings on intracellular iron metabolism in CpM and tM two mechanisms for immunologically activated and nonimmunologically elicited macrophages are proposed and discussed. A mechanism of iron metabolism in normal macrophages is also proposed, which accounts for the biphasic release of iron by macrophages seen either *in vivo* or *in vitro*. The proposed mechanisms could help to explain the alteration of iron release by macrophages leading to increased retention seen in inflammatory processes such as rheumatoid arthritis.

Chapter 1

Introduction

3. INTRODUCTION

Quantitatively it is almost certain that senescent erythrocytes, moribund cells and cell fragments represent the bulk of material phagocytosed by the MPS (Silverstein et al, 1977). The macrophages play a central role in iron recirculation as they are responsible for the phagocytosis and catabolism of the effete erythrocytes and the return of iron to the circulation. In humans the release of iron by the MPS represents 84% of the daily plasma iron turnover (Jacobs, 1980).

Nevertheless, little is known of the mechanisms which could be involved in iron release to Tf by the macrophages (Deiss, 1983). Even less is known about the intracellular iron metabolism of the cells of this system. However, it has been shown that iron release by macrophages is not regulated by the availability of free Tf binding sites (Brock et al, 1984), thus suggesting that the controlling factors of iron release are intracellular.

Until recently there have been few attempts to determine whether iron handling by macrophages can be modulated by inflammation. However, Esparza and Brock (1981b) and Nishisato and Aisen (1983) have shown that peritoneal macrophages stimulated by nonspecific inflammatory agents release less iron than resident macrophages. Neither of the above-mentioned authors studied the intracellular events occurring in the macrophages causing retention of iron.

Apparently the synthesis of Ft is a universal characteristic of cells (Jacobs, 1980; section 1.7). The characteristics of the metabolism of Ft (section 1.5) make it an extremely versatile buffering system in the prevention of noxious effects of excessive intracellular free iron and at the same time the Ft molecule serves as an iron store. It is known that monocytes contain more Ft than other leucocytes and that these precursors of macrophages synthesise Ft in response to iron (Summers et al, 1975). Andreesen et al (1984) showed that during monocyte differentiation their levels of Ft increase, this work apparently being the first to show the high levels of Ft present in human macrophages. Although mouse peritoneal macrophages have been used to study iron release (section 2.11), there are no quantitative data on Ft content for murine macrophages in the literature. Thus, the Ft levels in mouse macrophages were investigated (section 5.1.9).

It has been proposed that the abnormal retention of iron by the liver and spleen which occurs in inflammatory conditions is caused by increased Ft synthesis (Konijn and Hershko, 1977; Konijn et al, 1981). However, these studies did not determine whether hepatocytes, epithelial or Kupffer cells were responsible for the increased Ft synthesis. In section 5.1.12 the synthesis of Ft by different macrophages has been investigated.

It is known that the serum Ft (sFt) is increased in ACD (Lee, 1983) and the sFt is the only iron parameter in serum that distinguishes ACD from iron deficiency. Furthermore, in ACD sFt is apparently not reflecting the iron status. Although it has been postulated that the

origin of sFt is the RES (or MPS) and that it appears in the circulation following "secretion" by these cells (Worwood, 1980a), the "secretion" of Ft by mononuclear phagocytes is still a controversial issue. It is not known if the sFt increase in inflammation is due to an active secretion process or to cellular leakage. Therefore several experiments were performed to investigate factors controlling Ft release (section 5.1.11).

Inflammatory or immunological stimuli give rise to macrophage populations with different properties (Adams and Hamilton, 1984). Therefore with the aim of clarifying and attempting to increase our understanding of the events in the metabolism of iron in macrophages several distinct populations of peritoneal macrophages were studied. Partial characterization of the macrophage populations used in this work is presented in section 4.2. Resident, C. parvum-activated and thioglycollate broth-elicited macrophages have been used as models of normal, immunologically and nonimmunologically provoked macrophages respectively in these investigations, which focus mainly on their intracellular iron metabolism. The comparison of these three macrophage populations has yielded some increase in our knowlege of the foregoing obscure and outstanding points regarding the normal iron metabolism of macrophages that, as a system (MPS or RES), are responsible for the homeostasis of the iron recirculation in the body. It has also been possible to propose how some of the underlying metabolic controls of iron release by macrophages under normal or inflammatory conditions function (section 6).

Chapter 2

Literature review of iron metabolism

2.1 Chemical characteristics of iron

Iron is the most important essential trace element for living organisms. This is not only because it is the most abundant transition metal in biological systems, but also because of its extremely diverse biochemical functions. These functions are based on its chemical properties.

Iron belongs to the transition metals and can exist in two stable oxidation states, Fe(III) and Fe(II). The outer electrons are in the 3 d orbital with maximum unpaired spins, five unpaired electrons for Fe^{3+} and four for Fe^{2+}. This situation leads to high-spin complexes, but if ligands bind strongly they destabilize some of the d orbitals producing paired electrons. This replacement of the valence electrons produces low spin Fe(III) and Fe(II) complexes. The change in spin state has consequences not only on electronic properties, but also on the stereochemistry, enabling low spin complexes to produce strong short bonds, and at high spin complexes long ones, rendering an ion that can change its size. This effect is important in the chemistry of haem proteins, especially in haemoglobin in which there is a displacement of the atom of Fe from the porphyrin ring when oxygen is liberated. It is believed that this displacement is the key of both reversibility and cooperativity in oxygen binding.

Iron forms coordination aquo ions $Fe(H_2O)_6^{2+}$ and $Fe(H_2O)_6^{3+}$ which are only stable at extremly low pH. Neutralization causes the formation of green and brown gelatinous precipitates respectively. The hydrolysis is complex because the OH^- ion has a strong tendency to bridge

polyvalent metals ions, producing large polycations. The ferric hydroxy species shows a greater tendency to polymerization than the ferrous.

The solubility product constant of $Fe(OH)_3$ is about 10^{-39}, wich means that the equilibrium concentration of aqueous Fe^{3+} cannot exceed about 10^{-18} M at pH 7. In contrast the solubility product constant of $Fe(OH)_2$ is 10^{-15}, which allows aqueous concentrations near 0.1M to exist at the same pH. Polymerization can be prevented by chelating agents that bind iron tightly. These can be artificial, like EDTA (ethylenediamine tetraacetate) or NTA (nitrilotriacetate), or natural like porphyrins, citrate, carbonate etc. (May and Williams, 1980).

The complexes that iron can form in biology can be divided in two: High molecular and low molecular weight. Representatives of the former are those formed with macromolecules such as haemoglobin, myoglobin, transferrin, ferritin etc. The latter consist of small chelating agents. Although the low molecular weight complexes have a very limited concentration in biological fluids because of the powerful metal binding properties of the competing macromolecules, they are usually present in higher concentration than the corresponding aquated metal ions. These low molecular weight complexes probably play their most important role in transport across biological menbranes and as intermediate states between binding and release by macromolecules (Saltman and Hegenauer, 1983).

The oxidation-reduction potential of iron ranges from -0.27 to 1.12 Evolts, which is close to the entire range from oxygen to hydrogen, and it is probably to this property that iron owes its place in biology.

2.2 Biological role of iron

Adult women and men contain approximatly 45 and 55 mg of iron per kg of body weight respectively. Haemoglobin normally accounts for 60-70%, myoglobin 8-10%, cytochromes and iron containing enzymes comprise around 1-2%. Transferrin iron accounts for only 0.1-0.2% of total body iron and the rest (20-30%) is distributed between the proteins of storage, ferritin and haemosiderin.

The most extensive group of iron containing proteins are enzymes, which are concerned with oxidation reactions. They can be divided into those that use haem as a prosthetic group and those that do not. The field of iron containing enzymes is very wide (see review by Wrigglesworth and Baum, 1980) and falls outside the scope of this literature survey.

Most of of the proteins responsible for reversible oxygen binding use iron, the few exeptions being found in certain molluscs and arthropods which use copper in haemocyanin as oxygen carrier. The iron in the majority of these proteins is in the haem form. The best known representatives are haemoglobin and myoglobin. In mammals, haemoglobin serves to transport bound oxygen in erythrocytes, and myoglobin acts as an oxygen store in skeletal muscle.

2.3 Proteins of transport and storage of iron

At the pH, ionic composition and oxygen tension of most biological fluids the stable state of iron is the ferric form. Its hydrolytic propensities are such that organisms have been obliged to evolve specific iron-sequestering agents to maintain iron in a soluble form, avaible for biosynthesis of essential iron proteins and enzymes. (Aisen, 1980). Iron-binders may also protect against formation of such potentially noxious compounds as peroxide, superoxide and hydroxyl radicals formed when oxygen reacts with iron (Willson, 1977). In vertebrates these functions are well exemplified by the transferrins (Aisen, 1980; Brock, 1985).

2.4 Iron storage

Iron is stored in two forms, ferritin and haemosiderin. Ferritin, but not haemosiderin, is water soluble. For this reason, ferritin has been rather thoroughly characterized, whereas little information is as yet avaible on the properties of haemosiderin. Numerous histologists of the nineteenth century probably observed granules of iron in liver cells, but Newmann (1888) first distinguished the granular Prussian blue-positive pigment and he coined the term haemosiderin. Laufberg (1937) crystallized a protein containing 20% iron and called it ferritin, recalling Schmiedeberg (1894) who purified a protein with 6% of iron which he called ferratin.

2.4.1 Haemosiderin

Haemosiderin (Hs) is not a specific substance. It is an amorphous intracellular polymer, the composition of which is variable, apparently depending on the site and conditions of its formation. It is formed primarily of aggregates of modified ferritin (Hoy and Jacobs, 1981; Fischbach et al, 1971) and may incorporate fragments of cellular organelles and other cell debris (Harrison et al, 1974). Hs has a high Fe/protein ratio, from 0.4 to 2 (Munro and Linder, 1978).

Hs is best observed by light microscopy in the iron-overloaded cell by the Prussian blue reaction, but it occurs in normal cells as well. Evidence acquired by this means indicates that as far as the liver is concerned, Hs is normally located almost exclusively in Kupffer cells. Hs is located in other tissues as well but principally in cells of the reticuloendothelial system (Sturgeon and Shoden, 1969).

As mentioned above it is convenient, from a biochemical point of view, to distinguish between water-soluble (buffer-soluble) intracellular iron (ferritin and low molecular weight fraction, iron-labile pool, sections 2.4.2 and 2.7) and water-insoluble (buffer-insoluble) haemosiderin (Wixon, et al 1980). However, Hs and ferritin (Ft), when examined by X-ray diffraction, Mössbauer spectroscopy and electron microscopy show a similar atomic structure but a slightly smaller particle size in the case of Hs (Fischbach et al, 1971; Bell et al, 1984). This result suggests that Hs is formed by the denaturation and

proteolytic cleavage of Ft, leaving a slightly smaller iron core, presumably because some of the iron is lost during the process.

The factors controlling Hs formation are unknown, and althought it has been considered as the graveyard of iron within the cells, clinical experience has shown that despite its insolubility, the iron can be mobilized (Jacobs, 1974; 1980).

The transformation of ferritin to haemosiderin can be summarized as follows. Ft appears before Hs when cells (Jacobs et al, 1980) or animals (Sturgeon and Shoden, 1969; Bell et al, 1984) are loaded with iron. Hs retains the immunological properties of Ft (Richter 1983). In cultured cells, surface changes take place on ferritin molecules as they age or when they are grown under conditions of iron overload (Hoy and Jacobs, 1981) and it is suggested that these changes could favour ferritin aggegation and possibly uptake by lysosomes. Fractions of tissue ferritin enriched in polymers have now been shown to exibit similar changes i.e. the molecules are more acidic and have altered immunoreactivity. The coincidence of surface changes with polymer formation in human tissues may indicate that surface properties influence aggregation. Accordingly to Iancu and Neustein (1981) lysosomes preferentially take up iron-loaded molecules suggesting that surface properties may also influence this process. If the high iron to protein ratios of polymers found in tissues are due to proteolysis, this presumably occurs within lysosomes and this is the ultimate site of Hs formation (Jacobs, 1980).

When there is a continuous process of Ft synthesis it appears to be balanced by conversion into Hs. It has been suggested that iron overload is easily produced in the liver because its considerable capacity to synthesize Ft cannot be matched by its ability to process the product in secondary lysosomes prior to excretion. Instead the lysosomal accumulation of Ft behaves as a sump that gradually converts the protein into Hs which then remains in situ (Trump et al, 1975).

Most of the Hs is permanently retained in the cell within solid membranous particles (siderosomes), but some of these may lose their membrane, remaining free in the cytosol (Munro and Linder, 1978). This situation is undesirable because naked iron cores can produce cell dysfunction, damage and necrosis via the generation of toxic radicals (Selden et al, 1980). As will be described in this thesis, such a situation also opens up the possibility of direct iron deposition into Hs from the low molecular-weight pool (section 2.7). In support of this personal view, in vitro studies of ferritin formation have shown that the pH-dependence of Fe^{2+} oxidation was similar whether apoferritin was present in the system or not (Harrison et al, 1980) suggesting that a special oxidation mechanism involving the protein is unnecessary after the initial steps of nucleation (see section 2.5.2.1).

2.4.2 Ferritin

Following the study of Laufberg (1937) and the pioneer work of Granick (1946) a great deal of knowledge has been accumulated about ferritin. Horse spleen has been the favourite source of Ft because of its relative abundance in this organ and the ease with which it crystallizes. In analogy to the terminology of enzymes the term apoferritin (apoFt) is used to denote ferritin which does not contain iron.

There are several methods of isolating Ft which take advantage of some of its specific characteristics, such as being thermostable up to 80 ^{o}C, acidic (pI 4.1-5.8), of high molecular weight (450000 d) which enables it to sediment under ultracentrifugation (110000 g) and crystallizing with salts such as ammonium and cadmium sulphates (Munro and Linder, 1978; Arosio et al, 1976; Drysdale and Ramsay, 1965). For each method there are proponents and detractors who argue the advantages and disadvantages. Some of the methods may select certain types of ferritin; for example, centrifugation at high speed sediments the most iron-laden molecules and oligomers leaving in solution apoferritin and Ft of low iron content (Linder and Munro, 1972). During crystallization with 5% $CdSO_4$ part of the Ft remains soluble (Munro and Linder, 1978; Linder and Munro, 1972). Heat treatment selects the more stable proteins and also may leave partially damaged Ft which will behave anomalously in physiological studies (Frenkel, 1983). Because of these problems the method of isolation employed will be mentioned where pertinent.

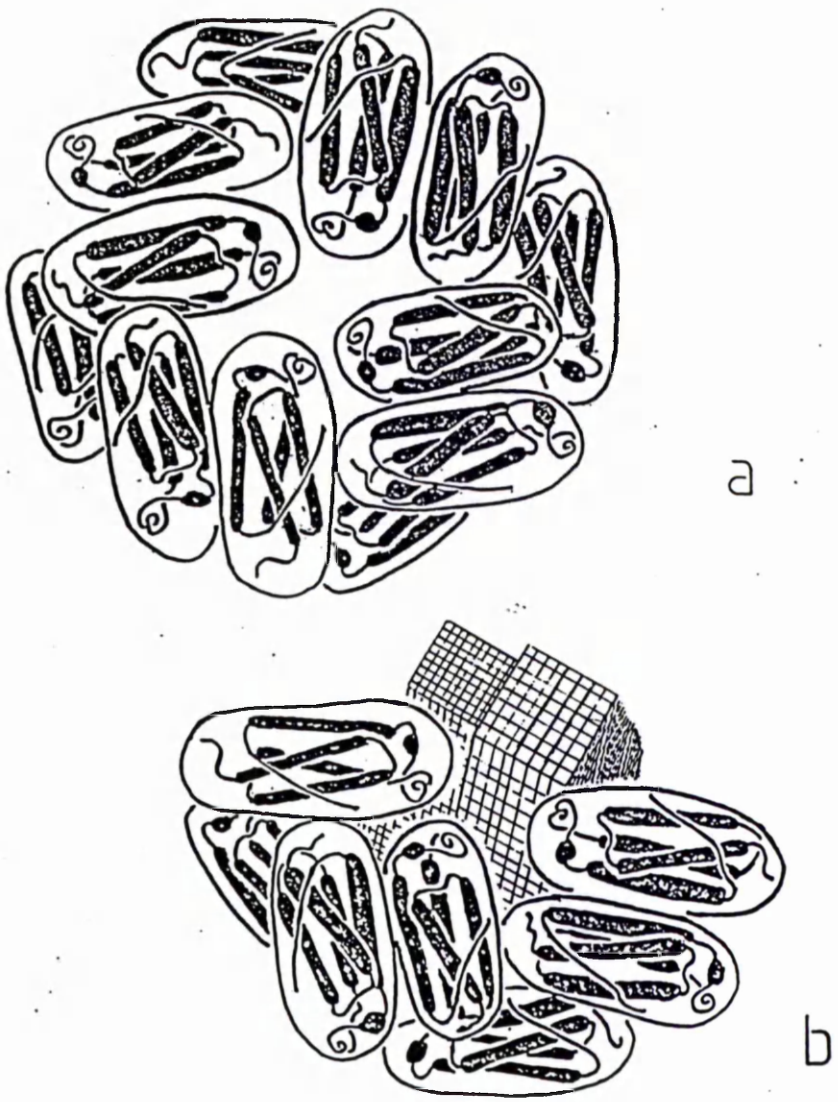
2.4.2.1 Ferritin structure

The ferritin molecule has been described as a roughly spherical compact shell (Fig. 1) with an outside diameter of 124-130 Å, the hollow centre being 70-80 Å across, making the protein shell around 25 Å thick. The molecule consists of 24 subunits arranged in a form that leaves 6 channels, the dimensions of which are in dispute. Harrison et al (1980) reported that they are more than 10 Å across in some places, but in other regions are narrowed to about 7 Å by inward pointing sidechains and Crichton (1984) considers that they are only 3-4 Å in diameter. It is through the channels that iron can pass into or out of the molecule, an essential requirement for its functions, and the dimensions are important because it is believed that iron has to be transported by small molecules. The disagreement in the crystallographic measurements may, however, be irrelevant because proteins behave rather more loosely in solution than in a crystal.

The subunits are of a rod shape, each about 27x27x54 Å (Harrison et al, 1980), and consists of a polypeptide whose amino acids form an α-helix in 5 chains (70%), turns and one loop. The segments of the 4 subunits which form the channels are α-helices and extremely hydrophobic (Fig. 1). Several other ferritins and apoferritins have been found to crystallize in octahedral crystals, which resemble those from horse spleen (Harrison et al, 1980) and whose conformations are very closely related to those of horse ferritin. These include human, dog, rat, jackal, guinea pig, invertebrate (e.g. chiton) and earthworm Ft (Arosio,

Figure 1

A model of the ferritin molecule.



a) Complete molecule showing arrangement of the subunits. One of the channels through which Fe is though to enter and leave can be seen at the centre. In each subunit model the polypeptide chain is represented.
b) A split ferritin model showing the iron core.
Drawing based on models by Harrison (1974, 1980, 1985) and Frenkel (1983).

1983). The similarities of these crystals and of the electron microscopic appearence of these and many other Fts suggest a highly conserved three-dimensional structure.

The interior of the apoferritin molecule may be filled with an iron micelle. X-ray diffraction studies of ferritin have revealed patterns very unlike those of any known inorganic iron oxides or hydroxides. Iron in Ft is present in the form of a crystalline aggregate whose principal component is FeOOH and some phosphate, but apparently the phosphate is not an essential structural component of the core and is normally located on the surface (Harrison et al,1974). The crystalline unit has a hexagonal symmetry and each one is formed by 32 FeOOH molecules. The fully iron-laden ferritin can accommodate 140 of these units to give a total of about 4500 iron atoms per ferritin molecule. The volume of the iron core would thus be 170000 $\mathring{A}^3$ (Crichton, 1973).

2.4.2.2 Ferritin Heterogeneity

2.4.2.2.1 Due to iron content. Ferritin isolated from any source will consist of molecules differing in the amount of iron that they contain, showing a large spectrum of molecular weights on ultracentrifugation. Apparently different average iron contents are maintained in different tissues and in different animals. The major components, from a density point of view, are apoferritin (10-15%), ferritin containing 1800-2100 iron atoms/molecule in horse spleen and 2000-2400 atoms/molecule in rat liver (over 50%), and a very heavy fraction containing over 3500 atoms/molecule (2-4%). The rest (30-35%) are molecules

containing between 50 to 1800 atoms/molecule.

Iron is also not evenly distributed among Ft oligomers (see below), but it is known that their iron/protein ratio is increased compared with the monomers, and it has been suggested that dimer and oligomer formation from iron-laden Fts is the first step of Hs formation. Linder and Munro (1978) have shown the presence of dimers, trimers and higher oligomers in human spleen with high iron contents in their Ft, in contrast to the trace amounts of dimer and no oligomers in the preparations with low iron/protein ratios. The iron/protein ratios of Ft reported in the literature could be affected by the method used for isolation, because it has been shown that polymers are less soluble in $(NH_4)_2SO_4$, less heat stable and possess altered immunological characteristics. The iron/protein ratio of Ft varies according to iron status and also with other conditions where the direct relationship is unclear, such as in regenerating tissue and in tumoral processes (Linder et al, 1975). However, under normal conditions it is believed that the average Ft molecule holds between 1200 and 1400 atoms of iron (Harrison et al, 1974, 1980; Crichton et al, 1977; Crichton, 1984) which represents a third of its total capacity.

2.4.2.2.2 Due to oligomer formation. Oligomeric association has been recognized a long time ago as part of the extensive protein polymorphism of Ft. Electrophoresis of pure ferritin in acrylamide gels is characterized by the separation of a major band, generally accepted as consisting of monomeric Ft molecules, and one or more

minor, slower-migrating bands consisting of dimers, trimers and higher oligomers of Ft (Munro and Linder, 1978; Zamiri and Mason, 1968; Massover, 1983). Although it is generally thought that the second band is due to dimers, there exist some exceptions in which the first two bands consist of monomers of different Fts. It has been shown that the so called "fast" band in rat heart Ft consists of a single molecule which is different from the second band and is formed of 34-38 subunits of the two types described for rat Ft (see below), while the second band consists of a molecule of 24 subunits of the small kind (Linder and Munro, 1978). Massover (1983) presented evidence of a band present in Ft from liver of siderotic mice which consists of a monomer of 24 subunits of mostly 18000 d polypeptides, in contrast to the second band which consists of a monomer of 24 subunits of 22000 d.

2.4.2.2.3 Due to subunit composition. Amino acid analysis and peptide mapping of several mammal, fish, amphibian, fungal and plant Fts have revealed a number of striking similarities throughout evolution. This suggests considerable phylogenetic conservation of Ft structure. However, certain amino acids appear to be susceptible to variation (Linder et al, 1975). Comparative studies of Ft from individual tissues (Linder et al, 1974, 1975; Crichton et al, 1981) have established that there are significant differences in amino acid content. This evidence justified the conclusion that there are multiple forms of Ft in different tissues of the same species. The term "isoferritin" (isoFt) is used to denote a molecular difference between two Fts, analogous to isoenzymes that

are distinct molecular forms with the same catalytic property. To qualify for the status of "isoferritin" (isoFt), the difference in electrophoretic or electrofocusing mobilities must not be due to aggregation or oligomer formation from the basic molecule, nor can it be due to differences in iron content. Normally the aggregates are removed by gel filtration or by ultracentrifugation.

The most revealing technique is isoelectric focusing with which it is posible to see dramatic differences among several types of Ft. The extent of heterogeneity shown on isoelectric focusing revealed that purified Ft from all tissues consists of families of related isoFts which have characteristic banding patterns and a degree of organ specificity. There is general agreement on the pH range occupied by the isoFts from each tissue in different species. In the case of man, liver and spleen show 6-7 isoFts with isoelectric points ranging from 5.2 to 5.8 (Drysdale et al, 1975; Powell et al, 1974; McKeering et al, 1976; Wagstaff et al, 1978; Bomford and Munro, 1980). Human heart Ft contains 4-6 isoFts in with a pI range of 4.8-5.2 and HeLa cells 2-3 isoFts with a range of 4.2-4.6. Ferritin from different human tissues can therefore be resolved into a total of 15 to 20 discrete bands by isoelectric focusing in gels (Drysdale et al, 1977).

Over the years the concept of Ft structure has evolved from accepting that a single type of subunit fitted the data (Hoffmann and Harrison, 1963) to a two-subunit system (Drysdale et al, 1977). Many other papers have reported

several peptides which were later demonstrated to be products of proteolytic cleavege.

Drysdale et al (1977), revised their model of Ft structure to take into account results obtained by electrophoresis in gradient pore gels containing sodium dodecyl sulphate, which separate subunits on the basis of molecular size only. By this method he was able to distinguish two types of subunit, heavy (H) and light (L). Using such gels, both H and L subunits have been identified in Fts of horse, rat and human tissues (Arosio et al, 1978) with the larger H subunit predominating in heart isoFts and the smaller L subunit in liver and spleen isoFts. The molecular weights reported for such subunits are around 19 kd for the L and 21 kd for the H (rat 19.7 and 20.7 kd, horse 18.8 and 21.2 kd, and human 19 and 20.8 kd; Arosio et al, 1978).

The model Drysdale has proposed considers that the isoFts are hybrid molecules composed of varing proportions of H and L subunits. According to this view the Ft in a particular tissue will display a range of isoFts that will depend on the proportion of the two subunits available in the cell. Presumably the exposed surfaces of the H and L subunits in the assembled Ft molecule must differ in charge so that the net charge of the Ft shell will be determined by the proportions of the two subunits. The relative amounts of the two subunits in any particular isoFt will determine the size of the apoFt shell, thus accounting for the molecular weight differences of Fts (Vulimiri et al, 1977a). Since the two subunits differ slightly in aminoacid composition and sequence (Arosio et al, 1978), the differences in peptide mapping and immunological

properties can be accounted for (Linder et al, 1970; Hazard et al, 1977).

Althought this model explains several features of Ft heterogeneity, there are several reports that complicate the picture, such as the presence of a small number of sugar residues (Shinjyo et al, 1975). Since the carbohydrate content represents less than one residue per subunit, it suggests heterogeneity of subunits. Although Ft is mainly located intracellularly, small amounts of Ft are normally found in serum in concentrations between 10 and 300 ug/l (Jacobs, 1974; Worwood, 1980a). Cragg et al (1981) demonstrated the presence of a third type of subunit in serum Ft and called it the G band (for glycosylated). This subunit accounted for 27% and 24% of serum Ft isolated from two haemachromatotic patients.

As can be observed from the foregoing, the final picture of Ft heterogeneity is still unfinished, and provides the stimulus for much current research. Some definitive answers will come from the analysis of the genome, which may resolve current speculation. Information currently at hand is insufficient to deduce the number of structural-genes for Ft protein subunits, but cloning of Ft genes is in progress. Munro et al (1985) have prepared cDNA from the messenger RNA for the smaller subunit of rat Ft. They proposed that the two subunits must be encoded by separate mRNAs and that the light subunit is encoded by a multigene family (30 copies) indicating that gene organization and expression are complex. They found from the derived amino acid sequence that there is a high

homology with the amino acid sequences for horse (88%) and human (84%) spleen Ft subunits. This recent information raises questions of the regulation of the differential expression of the Ft multigene family in different tissues and in individual cells, in which it is known that the patterns of isoFt varies.

2.5 Metabolism of ferritin

Outstanding phenomena in the process of Ft metabolism are that the net content of tissue Fts can be increased by increasing body iron levels and the dichotomy of pathways of Ft subunit synthesis. The content of protein in the cells is determined by the balance of biosynthesis, exportation, uptake and degradation.

2.5.1 Ferritin biosynthesis

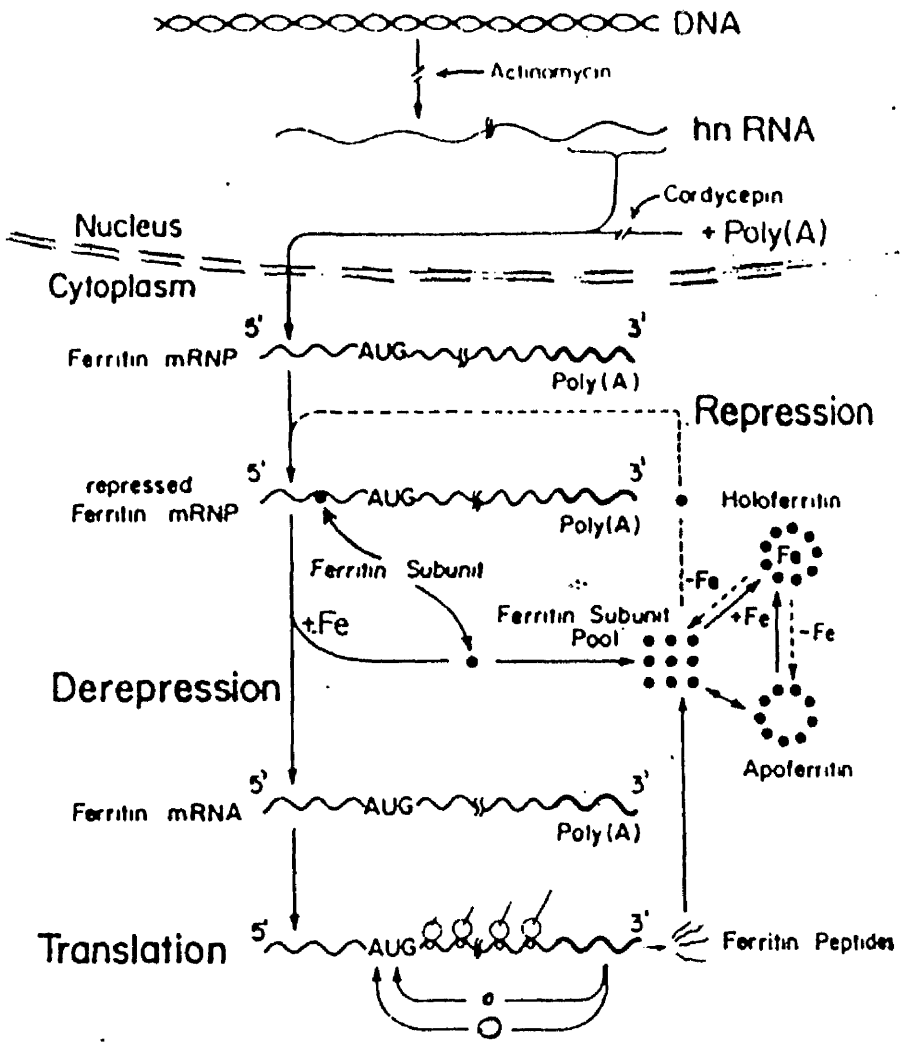
Fineberg and Greenberg (1955a) demnostrated by *in vivo* labelling with ^{14}C-leucine or ^{14}C-glycine that iron administration to guinea pigs rapidly stimulates Ft synthesis in the liver. Drysdale and Munro (1966) showed in rats that iron administration did not alter incorporation into mixed liver proteins, whereas the specific activity of Ft protein increased 6-fold at 5 hours, and that it declined after 12 h to the basal level. Separation of Ft fractions of different iron content by centrifugation in sucrose gradients showed that the initial product was apoFt but as time elapsed more of the ^{14}C passed into iron-rich Ft. This induction has been confirmed for different tissues such as liver (Coleman and Matrone, 1969; Matsuda, 1969), spleen, kidney, duodenum and testis (Yoshino *et al*, 1968),

heart (Vulimiri et al, 1977b), and for cell preparations including blood monocytes (Summers et al, 1974, 1975), reticulocytes (Matiolini and Eylar, 1964) and HeLa cells (Chu and Fineberg, 1969).

The response of Ft synthesis to iron administration is not due to a burst of new messenger RNA (mRNA) formation. Suppression of mRNA synthesis with actinomycin D failed to reduce the induction of synthesis by iron (Drysdale and Munro, 1966, Chu and Fineberg, 1969; Zähringer et al, 1976). Inhibition of polyadenylic acid addition to mRNA with cordycepin also failed to prevent the induction by iron of Ft synthesis (Zähringer et al, 1976). The search for messenger in the cytosol revealed that the mRNA for Ft was, in untreated rats, present in polyribosome form (56%) and in an inactive form (44%) in ribonucleoprotein particles (mRNPs). In contrast, when the rats were stimulated with iron 91% was engaged with polyribosomes and 9% remained unused in mRNPs (Zähringer et al, 1976), suggesting translocation of mRNA from mRNPs (inactive) to the polyribosomes for translation. Zähringer et al (1976) postulated that in tissues of animals not receiving excess iron, a subunit of Ft is normally attached to the initiation site of the mRNA in the free mRNPs pool, thus preventing the messenger from binding to the ribosomes. When iron enters the cells, it shifts the equilibrium towards assembly of completed Ft shells so that these inhibiting subunits are removed and more Ft mRNA becomes available to initiate new chains in the cytosol (Fig. 2).

Figure 2

Proposed model for the translational control mechanism by which iron regulates the synthesis of ferritin.



From Zähringer et al (1976).

This adaptive mechanism refers only to mRNA translated by free polyribosomes. It is known that the Ft translated in the membrane-attached ribosomes is insensitive to iron stimulation (Konijn et al, 1973; Zähringer et al, 1977). This attractive hypothetical mechanism accommodates many previous observations in the ferritin field and has stood for several years. But recently Drysdale (1983) has presented conflicting data, showing no significant increase in Ft mRNA in polyribosomes of iron-treated rats, nor finding any significant decrease in levels of Ft in mRPNs at any point up to 24h after iron treatment, following the same protocol used by Zähringer et al (1977). Other evidence for translational control of Ft subunit synthesis by iron has been found in tadpole reticulocytes (Theil et al, 1983), where iron loading causes a 40-50 fold increase in amino acid incorporation into Ft. However when assayed by translation in wheat germ lysate, the level of mRNA in control and iron-loaded cells seemed to be similar, about 10% of the translation products. These results indicate that iron somehow altered the availability of Ft mRNA for translation, but not only by shifting mRNA from mRNPs to polyribosomes. Whether iron causes a repartition of Ft mRNA or has some other effect on the translatability of the messenger has been studied. Theil et al (1983) reported that Ft mRNA of tadpole reticulocytes is translated more efficiently in wheat germ lysates than is globin mRNA, one of the most active mRNA species known. By contrast, Konijn et al (1973) have shown that Ft mRNA from rat liver is not translated as efficiently as some other liver proteins in reticulocyte lysates. It is not known whether these

differences reflect differences in procedure, in Ft mRNA species, or in intrinsic properties of the translation systems.

There are also indications that iron is not a major controlling factor in Ft subunit synthesis. In translation experiments heat extraction can differentiate between subunits and complete shells. The Ft subunit synthesis was 4% of total Ft synthesis for control and 6% for iron loaded cells but nearly all of these newly synthesized subunits were lost by heating to 75^{o}C indicating that they were still present as free subunits or small oligomers. However more subunits were recovered as heat stable shells from the iron-loaded cells (26% vs 11%), suggesting that the major effect of iron in stimulating Ft synthesis in this system is post-translational (Drysdale, 1983). Similar experiments with pulse labelled rat liver cells also suggest that iron has only a modest effect on the overall synthesis of Ft subunits, but a major effect on the proportion converted into shells.

Some other cases also point to Ft synthesis being controlled by factors other than iron. For example, White et al (1974) showed in normal and leukaemic leucocytes that uptake of amino acids into heat-stable Ft represents about 3% of uptake into cellular protein but that this level is not increased by iron supplementation. Glass et al (1978) using Friend erythroleukaemic cells in which differentiation was induced found that Ft synthesis was not increased in the differentiating cell when iron uptake was maximal, the iron entering the cell being sequestered by

pre-existing Ft molecules.

The increased synthesis of liver Ft in inflammation (Konijn and Hershko, 1977) is another possible example of the dissociation of Ft synthesis from iron availability. In this case they postulated that Ft is acting as an "acute phase protein", possibly regulated by interleukin-I (Lee, 1983), as discused in the section on anaemia of chronic disease (section 2.8).

In rats, iron stimulates Ft synthesis in liver to a greater extent than in heart and kidney (Munro and Linder, 1978). Some of these phenomena may reflect different responses in H and L subunit synthesis to iron (Kohgo et al, 1980). Only certain Ft phenotypes may therefore be responsive to iron.

Caskey et al (1983) have shown that the expression of immunoreactive human Ft by hybridome cell lines required chomosome 19, but the level of Ft depended upon the presence of other chromosomes. Since all the cell lines had the same iron supply this result suggests that factors other than iron may play important roles in Ft gene expression, these factors being coded in different chromosomes. It seems that much of the stimulatory effect of iron on Ft biosynthesis is due to a post-translational effect leading to shell assembly. In such cases, there may be substantial differences in turnover rates of subunits in normal and iron-loaded cells. In some systems, iron seems to have little effect on the rate of synthesis where Ft may be required for functions other than iron storage.

2.5.2 Iron uptake and release by ferritin

Beilig and Bayer (1955) proposed that iron uptake and release involves a reduction-oxidation cycle: ApoFt or partially filled Ft takes up Fe^{2+}, which is oxidized in the Ft interior. To be removed, iron must once again be reduced to the ferrous state. Iron is deposited into Ft in a "first in last out" mode (Hoy et al, 1977). This "first in last out principle" of iron deposition into Ft is followed in vitro and also in vivo (Treffry and Harrison, 1984).

2.5.2.1 Uptake of iron into ferritin

The most widely accepted theory of how iron cores are formed in the Ft cavity was formulated by Harrison and colleagues (Macara et al, 1972; Harrison et al, 1974, 1980, 1985). Iron as Fe^{2+} enters the apoFt cavity through one of the six channels connecting the cavity with the outside. Ferric iron may also be incorporated into Ft, but not to apoFt, at 10 times slower rate (Hoy and Harrison, 1976). In the Ft cavity the Fe^{2+} combines with the appropriate aminoacid side chain (apparently a histidyl residue), which tends to catalyze its oxidation to the ferric state in the presence of an oxidizing agent, which may include O_2 (Treffry et al, 1977). This first stage is known as the nucleation step. As iron acquires the ferric state, it forms the typical iron oxide crystalloid. The iron oxide then acts as a crystal growth nucleus without any further involvement of the amino acid side chains.

When iron is added to apoFt in the proportion of 200 to 1000 iron atoms per molecule, a sigmoidal iron uptake curve is obtained. The initial portion apparently represents the nucleation stage which is slow, whereas the rapid phase (growth stage) represents crystal growth on the surface of the iron micelle. When Ft fractions with variing iron content were incubated with iron or an iron chelator, the iron uptake or release was greatest with Ft containing some 1200 atoms/molecule (Harrison et al, 1974), see Fig. 3.

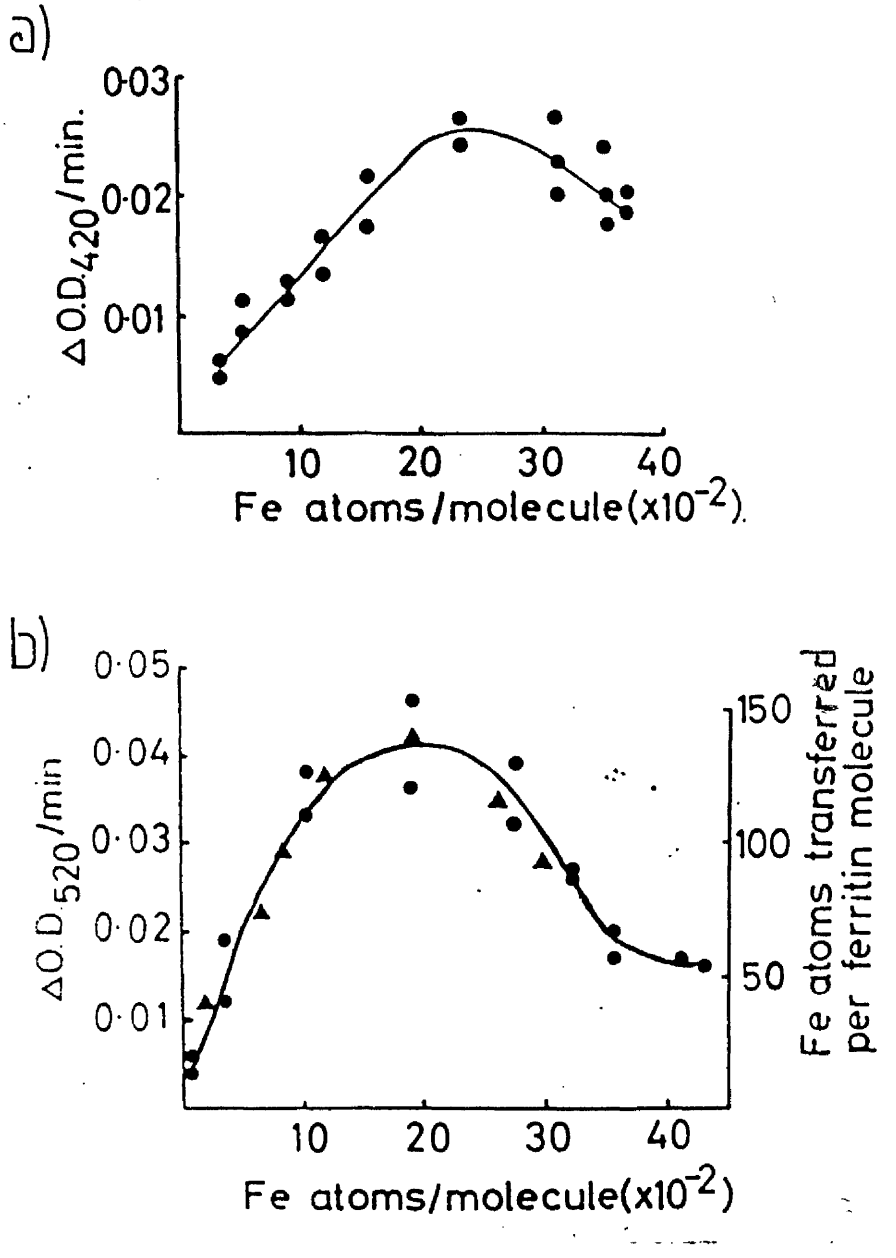
Computer simulation of the system has shown that the iron oxide micelle has the highest "free" surface area available for crystal growth when close to 1200 iron atoms are present. This computer simulation was checked (Hoy and Harrison, 1976) by giving to rats a small dose of iron, insufficient to stimulate increased Ft synthesis, and finding that the maximal incorporation into Ft was in the molecules that had about one-third of their maximum iron content.

2.5.2.2 Iron release from ferritin

Although chelators like NTA, EDTA, phenanthroline and others are very useful and effective in removing Fe from Ft in model studies, they do not represent the physiological mechanism of iron mobilization from Ft. It is believed that in vivo iron is mobilized from Ft by reduction, and the most effective reducing agents appears to be flavinoids, especially reduced riboflavin ($FADH_2$) and reduced flavin mononucleotide ($FMNH_2$) (Sirivach et al, 1974).

Figure 3

Iron uptake and release by ferritin.



a) Initial rates of iron uptake by horse spleen Ft fractions as a function of iron content.
b) Release of iron from horse speen Ft fractions as a function of iron content. Points, represent iron transferred. From Harrison et al (1980).

Dognin and Crichton (1975) found that at constant Ft (protein) concentration, the most rapid iron release using 5mM cysteine occurred when 800-1400 iron atoms were present in each Ft molecule. Jones et al (1978b) subsequently found that flavinoids mobilized Fe from Ft most rapidly when the latter contained 1200 iron atoms/molecule. These resuts are thus consistent with the kinetics of iron uptake presented by Harrison et al (1980) and they indicate that Fe uptake is also controlled by the size of the core surface availability, when unfractioned Ft is studied.

The limited access to the interior of the Ft molecule via the channels has more dramatic consequences for iron mobilization than for iron uptake. The molecular size of the reductants proposed has to be taken into account, i.e. it is believed that the limiting step of Fe reduction by dihydroflavin is its penetration into shells. The supply of reduced flavinoids, proposed by Sirivach et al (1974), apparently comes from a system called ferrireductase, which includes an enzyme that regenerates reduced flavinoids from NADH.

The roles of different isoFts in iron storage has been examined by measuring the iron content of the individual isoFt bands and by testing in vitro uptake of iron by the apoFt produced from these isoFts. Bomford et al (1978) and Wagstaff and Jacobs (1981) found that the most basic species of human Ft (L-rich) had the lowest iron content, while increasing iron content was related to decreasing isoelectric point (Ft rich in H subunits). Wagstaff and Jacobs (1981) in a study of in vitro uptake of iron by

human isoFts showed that isoFts at the end of the spectrum (L-rich) accepted iron most slowly; as the pI of the apoFt fell, iron uptake progressively increased in parallel with H subunit content. This study has provided evidence that in human Ft H-rich isoFts are more efficient in oxidizing iron than the L-rich form. Apparently for horse Ft this is true too, even if the horse H and L-subunit pattern runs in the opposite direction on electrofocussing gradients, so that the most basic isoFts are richest in the largest H subunit. Russell and Harrison (1978) found the highest iron content in the most basic (H-rich) isoFts indicating that subunit composition rather than the surface charge is the determinant of iron uptake *in vivo* in the horse and man. However, in contrast to horse and man Fts the role of H and L subunits in relating shell composition to iron uptake are reversed in the rat (Bomford and Munro, 1980)

Theil *et al* (1983) showed that spleen apoFt prepared from normal and copper-intoxicated lambs displayed different properties of iron uptake and release *in vitro*, which corresponded to the steady state iron content *in vivo*. Thus, apoFt from a holoprotein with high iron content took up iron rapidly and releasead it slowly. The main structural difference detected between the two Fts was the presence of large numbers of subunit crosslinks (distinct from S-S bonds) in the low-iron molecules. The presence of subunit dimers has been observed in other types of Ft, especially when the Fe/protein ratio is low (Theil *et al*, 1983) and the percentage of crosslinked subunits and decreasing iron content correlates quite well (r= 0.94).

Thus it seems that iron release and uptake are regulated by different aspects such as nucleation points, size of crystalloid, subunit composition, crosslinking of subunits, availability of reductants and iron availability in the "labile pool" (see intracellular iron metabolism, section 2.7).

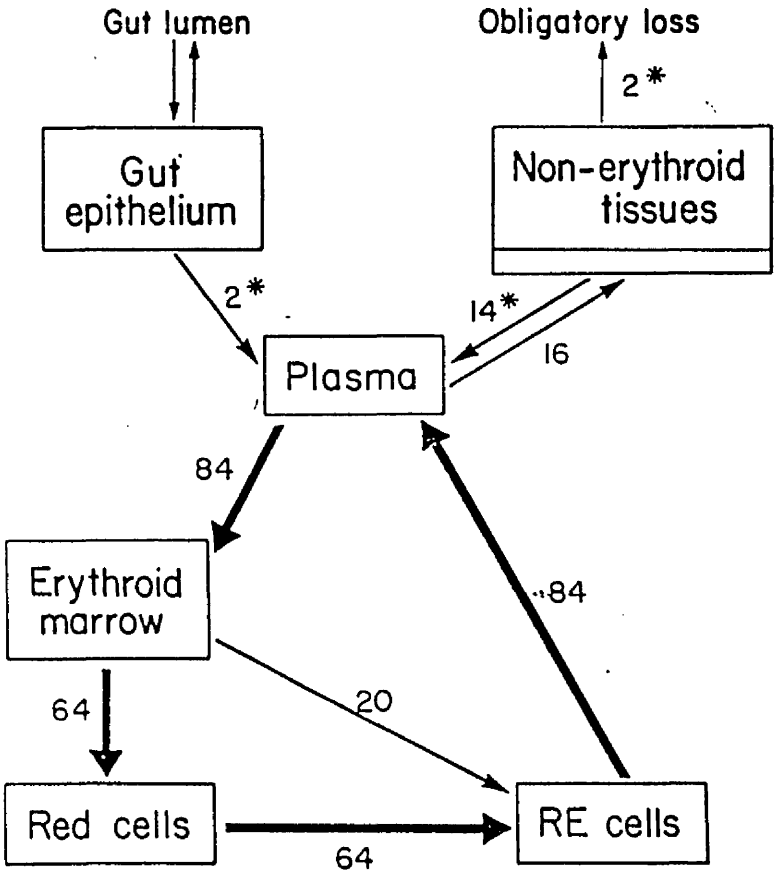
2.6 Normal iron balance

In the steady state iron requirements are largely determined by the amount lost from the body. Normally, the small obligatory loss due to desquamation from epithelial surfaces, gastrointestinal blood loss, and biliary excretion, is in man about 1mg daily or less (Jacobs, 1974). Iron balance is maintained by physiological adjustments of the absorption mechanism so that the amount of iron crossing the small intestinal epithelium barrier is related to internal iron status (iron stores) irrespective of serum iron concentrations. The major internal pathways of iron methabolism are shown in figure 4.

Within the body most of the iron present is found as haemoglobin in circulating red blood cells and most of the remainder as storage compounds such as Ft and Hs in reticuloendothelial cells and other tissues. The most common route of cellular uptake of iron is via transferrin (Tf). Although the erythron has great avidity and normally claims most of the circulating Tf iron, its needs are finite. When the developing red cell has acquired enough iron for its optimal haemoglobin content, it matures with loss of Tf receptors and ceases to take up iron. In contrast, other cells probably maintain an iron flux

Figure 4

Major internal pathways of iron metabolism.



Figures show percentage of plasma iron turnover in man. Asterisks indicate pathways involved in external iron balance. From Jacobs (1980).

throughout their life span, and the amounts involved are presumably related to their metabolic requirements. The mononuclear phagocytes, on the other hand, have an obligatory iron load due to their role in haemoglobin catabolism.

2.7 Intracellular iron metabolism

Intracellular iron exists in a number of forms that can be summarised as iron-containing or iron-dependant enzyme systems (some of which are present in all cells), Ft (apparently universal), Hs and a "labile pool". In the following review macrophage iron metabolism will be stressed.

The central role of the reticuloendothelial (RE) or mononuclear phagocytic system (MNP) in regulating iron flow through the plasma pool is well established. Nevertheless, a great deal of uncertainty still exists in regard to the precise chemical forms of intracellular iron compounds and their movement and interaction. Haemoglobin (Hb) contained in senescent erythrocytes is catabolized in the RE cells of the spleen, bone marrow, liver and other tissues. These cells are, therefore, the major site of haem degradation in the body. Their prominent role in Hb breakdown and iron reutilization is presumably determined by their capacity to phagocytose effete or damaged erythrocytes. After phagosome formation the degradation of the ingested erythrocyte begins due to the protease activity when lysosomes merge and the lytic enzymes are released producing a phago-lysosome (see macrophage biology, section 2.2).

Tenhumen et al (1968) described a microsomal enzyme system, haem oxygenase, which appears to be largely responsible for the catabolism of haem from Hb and other haemoproteins in mammals. Haem oxygenase utilizes molecular oxygen and NADPH to open the porphyrin ring by specific oxidative cleavage of the methene bridge with elimination of the bridge carbon atom as CO, formation of biliverdin and loss of the iron (Jackson 1974). The released iron somehow finds its way across the lysosomal membrane, its mode of transport to the cytosol being uncertain. Despite a vast literature our current concept of how iron crosses membranes is poor and full of controversy. However, despite the uncertanties, transport of iron across biological membranes usually involves a ferric-ferrous transition (Romslo, 1980) and Fe^{3+}-low molecular weight complexes have been postulated as the species which transports iron through membranes (May and Williams,1980).

Once in the cytosol, the multiplicity of possible reactions of iron with low molecular weight molecules such as ascorbic acid, glutathione, cysteine, glucose, fructose and other molecules such as ATP and other nucleotides, and several reported protein ligands (Romslo, 1980), has given rise to the concept of an intracellular transit pool which is the focal point of intracellular iron metabolism. The idea of a "labile iron pool" or transit pool has emerged from difficulties in explaining intracellular iron metabolism without postulating such an intermediate and is supported by a great deal of indirect evidence (Jacobs, 1977a).

Although the labile pool has been compared with "Nessie" for its reticence to be characterized (Crichton, 1984), it seems possible that in many tissues iron which crosses the cell membrane does enter a common cytosolic labile pool from which iron is available for the synthesis of a variety of iron containing proteins, for return to circulation, or for Ft synthesis. The molecules proposed as forming part of the labile pool have to take into account ligand exchange mechanisms, binding constants, and the membrane permeability necessary for intracellular iron mobilization (Saltman and Hegenauer, 1983) in addition to the solubility characteristics of iron at physiological pH.

Lipschitz et al (1971) have suggested that this labile pool in RE cells is the main source of chelatable iron in the body. Mulligan and Linder (1981) have hypothesized that this transit pool might consist principally of non-protein-attached, low molecular weight iron chelates and/or iron loosely bound to proteins in general, all of which should be accessible to chelating agents. Following this hypothesis, assessment of the apparent size of this labile pool was acheived by rapid separation of the iron firmly bound to Ft and haem proteins from that chelatable with desferrioxamine B (DFO) (Linder et al, 1983). Their results showed that tissues (rat liver, kidney, heart, brain, spleen and pig brain) have small but significant amounts of iron in a form available to DFO. Although these tissues did not vary greatly from one another in terms of the size of this pool, spleen had the lowest amount, and iron status did not appreciably influence the pool size.

The relationship beetween Ft and the labile iron pool has also been subject to controversy. However, it now seems clear that Ft synthesis is induced by iron via the enlargement of this pool. As this pool can be tapped by extracellular complexing agents, it is reasonable to assume that such chelators can also derive iron from Ft under physiological conditions. Baker et al (1983) have shown that after pulsing hepatocytes with ^{59}Fe-^{125}I-Tf for 21h, subsequent incubation with pyridoxal isonicotinoyl hydrazone as well as DFO can decrease the ^{59}Fe associated with Ft, shifting up to 60% of it to a Ft-free supernatant after 17h.

The mobilization of iron from Ft to the low molecular weight pool in the face of an increased demand is not a simple mechanism of competitive binding (Crichton, 1973), because the more iron-saturated the Ft is the less readily it releases its metallic content (Harrison et al, 1974; see iron release from Ft section 2.5.2.2). This physiological feature, implying that in conditions of overloaded Ft it is more difficult to mobilise iron is expanded in sections 2.5.2 and 6.

A sensitive iron buffering system must be expected to exist when the cycle of Ft synthesis and degradation (Hs formation) is considered to be in equilibrium with the labile pool. A rapid initial response to demand could occur by depleting the labile pool whereas longer term supplies would come from the biological turnover of Ft and Hs. On the other hand, a surge in the concentration of low molecular weight complexes would first be compensated for by labile pool binding and then, as soon as this uptake had

been saturated, by an induced synthesis of Ft.

The main source of iron in macrophages is erythrocytes, but it can also come from ingested bacteria, lactoferrin (Lf) and transferrin (Tf). A significant proportion of the iron is released to Tf immediatly after phagocytosis, and some of the iron is released more slowly. Fillet et al (1974) proposed that there were two separate phases of release, which would suggest that iron released within RE cells enters a pool from whence it may either pass to plasma Tf or become incorporated in the intracellular stores. A fine balance of the alternative pathways from the common labile pool would allow iron to flow preferentially to one or the other. Iron release from macrophages is discussed in detail in section 2.11.

2.8 Anaemia of chronic disease

The anaemia of chronic disease or disorders (ACD) may be defined as that anaemia associated with chronic infections, inflammatory, traumatic, or neoplastic illness and with a characteristic disturbance of iron metabolism. The latter is manifested by hypoferraemia despite iron stores that range from adequate to increased.

Cartwright and Lee (1971) pointed out that only iron deficiency is a more common cause of anaemia than ACD. Apparently after full development it does not worsen. Most often, ACD takes the form of a normocytic, normochromic anaemia. However, it is not infrequently hypochromic, and sometimes it is microcytic. Westerman et al (1980) have shown by digital image processing that it is possible to

detect a population of hypochromic cells even if the erythrocyte indices fall within normal values in ACD patients.

The abnormality in iron metabolism can be established by finding subnormal values of serum iron (SI) concentrations and Tf saturation with evidence of normal or increased iron stores. Serum Ft concentration usually is increased in ACD and is often the most convenient means for distinguishing it from iron deficiency (Lipschitz et al, 1974; Elin et al 1977).

A number of other biochemical changes occur in association ACD, such as the appearence in plasma of the so called proteins of the "acute phase response", including C-reactive protein (Morley and Kushner, 1982), and amyloid A (Rosenthal and Franklin 1975). Synthesis of other proteins is considerably augmented. Among these are fibrinogen, haptoglobin (Cartwright, 1966), ceruloplasmin (Kushner, 1982), orosomucoid, complement components (Bornstein, 1982; Kampschmidt and Upchurch, 1974) and probably Ft (Konijn and Hershko, 1977).

The paradoxical association of hypoferraemia with normal or increased iron stores is the hallmark of ACD. Impaired flow of iron from tissues to plasma appears to be the major explanation for the hypoferraemia and the macrophages are considered to be responsible for the major defect in iron release (see section 2.11). Lee (1983) has proposed the production and exposure to Interleukin-I as the "final common pathway" that ties together the various diseases associated with ACD. Historically Interleukin-I (IL-I) has received several names such as Leucocyte pyrogen

(LP) (Beeson, 1948), endogenous pyrogen (EP)(Atkins and Wood, 1955), leucocyte endogenous mediator (LEM) (Kampschmidt and Upchurch 1979; Pekarek et al, 1972) and lymphocyte activating factor (LAF). Apparently the best evidence now indicates that the effects of the above named factors are properties of a single substance, IL-I (Murphy et al, 1980; Mizel and Farrar, 1979).

Macrophages appear to be the major source of IL-I. These may be alveolar, peritoneal or Kupffer cells (Bornstein, 1982) or blood monocytes (Bodel, 1974). Macrophages from these sources usually require "activation" before they form and release IL-I (see section 2.8). After being activated there is a latent period before IL-I is released (Dinarello and Wolff, 1978). Synthesis requires new mRNA formation and there appears to be no appreciable storage of IL-I in the cells. IL-I is a molecule of 13-16 kd which presents heterogeneity on isoelectric focusing, giving two or more bands in a pI range of 4.6 to 7.3. It is very hydrophobic and its activity is destroyed by heat ($70^{\circ}C$), by protease digestion and by exposure to a pH greater than 8.5 (Bornstein, 1982).

It has been proposed (Weinberg, 1978) that the hypoferraemia of infection represents a host defence mechanism, an attempt to deny iron to invading microorganisms. The phrase "nutritional immunity" has been applied to mechanisms of this type by means of which the host endeavours to produce famine among the invaders. The bacteria require iron and often they produce siderophores to help combat the iron-sequestering proteins of the host

(Tf, Lf). Fever may be the host counteraction which suppresses siderophore synthesis (Garibaldi, 1972). The antibacterial effect of Tf and Lf is well established (Bullen, 1981) but can be overcome if the proteins are saturated. The importance of nutritional immunity has been supported by a large number of experimental and clinical observations (Weinberg, 1978).

From a teleological point of view the nutritional immunity seems to play an important role in infection and the fact that other situations present similar conditions grouped under ACD could show a limited capacity of the body to react to different stimuli, sometimes with undesirable consequences.

2.9 Iron metabolism in inflammation

Several propositions have been enunciated to explain the abnormalities found in iron metabolism during an inflammatory process.

2.9.1 Reduced erythrocyte survival. Although modest, this reduction is found in patients with rheumatoid arthritis (Cavill and Bentley, 1982). Pathogenic mechanisms probably involve increased aging of the red bood cells with consequent physicochemical alterations of their membrane (Haurani and Ballas, 1985). During inflammation the RES becomes hyperactive in the removal of erythocytes (Cartwright, 1966; Cartwright and Lee, 1971) which is presumably due to the macrophage activation (Mackaness, 1970) that enhances host resistance to infections. The shortened erythrocyte survival may therefore also be a reflection of increased phagocytic activity that, under

activation, could lower the recognition threshold of senescent erythrocytes.

2.9.2. Defective erythropoiesis. In most anaemias there is good correlation between erythropoietin levels and the degree of anaemia, but this correlation is lost in ACD (Douglas and Adamson, 1975; Mahmood et al, 1977), where erythropoietin is clearly lower than that expected for the degree of anaemia. The fact that erythropoiesis in ACD can increase when stimulated by cobalt or hypoxia indicates that the capacity to respond to such stimuli is retained (Lee, 1983). Experimental evidence has shown also that the bone marrow response to the hormone is similar in rats with turpentine abscesses and in normal controls (Gutnisky and Van Dyke, 1963). One possible explanation for an erythropoietic defect could be the competitive proliferative demands placed on the pluripotential myeloid stem cell. Hellman and Grate (1967) have suggested that demand for one cell element might limit the ability of the marrow to produce another. If this is correct, the demand for granulocytes seen in inflammation (Quesenberry et al, 1973; Reissman et al, 1970) might divert the progeny of the pluripotential stem cells into the granulocyte line at the expense of the erythroid.

2.9.3 Release of Lactoferrin. Van Snick et al (1974, 1975) suggested that Lf might shuttle iron from the circulation to the macrophages, where it becomes incorporated into stores. The granules of the neutrophil leucocytes contain Lf which is largely iron free and is released during phagocytosis, and they postulated that it

will bind iron, causing a hypoferraemia by shuttling it to the RES. Experimental work to confirm this hypothesis has been criticised, mainly due to the unphysiological dose of Lf used to induce hypoferraemia (Roeser, 1980).

2.9.4 Maldistribution of iron stores. Iron is normally stored predominantly in the liver and in the RE cells of the spleen and bone marrow (Jacobs and Worwood, 1978). In contrast, studies of rheumatoid arthritis have revealed substantial deposits in other sites, such as synovial tissue (Mowat and Hothersall, 1968). It has been estimated that in active disease these deposits could amount to 800mg of iron (Mowat et al, 1969). The increased iron deposits are not confined to the synovium. Muirden and Senator (1968) found that the lymph nodes of patients with rheumatoid arthritis also contained substantially increased quantities of iron. In malignant diseases, such as Hodgkin's disease (Cooper et al, 1974), increased iron storage has also been demonstrated. It therefore appears that iron may be deposited in macrophages of tissues which do not normally participate significantly in iron turnover and that this sequestration may coincide with a scarcity of iron in more conventional storage sites.

2.9.5 Impaired release of iron. Although the abnormalities associated with ACD are probably a multifactorial event, or as Hansen (1983) puts it "a bag of unsolved questions", the most probabe cause of the hypoferraemia is defective release of iron from cells, and particularly from macrophages. The molecular events leading to iron retention have not been established, but several hypotheses have been proposed, which are considered in the

next section.

2.10 Cellular mechanisms of iron sequestration

Several potential mechanism have been proposed to explain the retention of iron within the macrophages.

2.10.1 Reduced production of Tf by RES. Haurani et al (1973) presented evidence for Tf synthesis by peritoneal macrophages which were allowed to ingest ^{59}Fe-labelled red blood cells. After culturing the cells with ^{3}H-leucine the lysate was subjected to ion-exchange chromatography. They found a coincident overlapping of ^{3}H and ^{59}Fe activity in a peak which was shown to be Tf. They postulated that Tf holds the iron which appears in the rapid release of iron from the RES (Haurani and Ballas, 1985). Although they did not present evidence, they suggested that the production of Tf might be reduced during activation of the RES, thus encouraging the formation of Ft and Hs (Haurani and Meyer, 1976; Haurani and Ballas, 1985).

2.10.2 Increased turnover of lactoferrin. The main argument, apart from those considered in section 2.9.2, in favour of this hypothesis is the high affinity of Lf, especially Fe-Lf for cells of the RES. Van Snick et al (1975) proposed that the slow pathway of iron release involves the transfer of iron to the RES from Lf during inflammation (i.e. shuttle of iron via Lf), which explains the "blockade" of iron release from the RES. They proposed the following mechanism. Lf is able to remove iron from Tf and has a high affinity for the membrane of macrophages. Thus, the major exchanges of iron between the two proteins

presumably occur at the level of the RES. As soon as iron is taken up by Lf, it is reintroduced into the RES, where it accumulates in the form of Ft and from which it will return to plasma in the late phase of release.

2.10.3 Ferritin synthesis. Konijn and Hershko (1977) put forth the idea that the increased synthesis of apoFt observed in rat liver during inflammation is due to a stimulus other than iron, and that the synthesis of apoFt is like that of other "acute phase" proteins in inflammatory processes. According to their hypothesis the surplus apoFt binds a larger-than-normal proportion of the iron entering the cell, effectively diverting the metal from the rapid-release pathway. They concluded that the increased Ft synthesis in inflammation appears to be the primary event responsible for the increased retention of iron.

2.10.4 Partitioning of RE storage iron. Roeser (1980) proposed that a diversion of the increased RE iron load to Ft and Hs, and especially a shift towards Hs, could well result in making it less available for release. His arguments are based on several facts such as Hs iron not being readily available for tranfer to the plasma iron pool (Hershko, 1977); Ft was reduced and Hs increased in the livers of turpentine-treated rats (Hershko et al, 1974) and in inflammatory states the formation of lysosomes and the lysosomal enzymatic content are increased (Allen, 1969). Hence, Ft degradation could be enhanced.

2.11 Iron release from mononuclear phagocytes

Cruz et al (1942) are considered by Freireich et al (1957) and Haurani and Ballas (1985) to be the pioneers who showed that in normal animals the iron released from destroyed red blood cells is rapidly reutilized for erythrocyte production in preference to storage iron, and who proposed that "last iron to arrive at the RES is the first to leave". Freireich et al (1957) concluded from their study in dogs in which turpentine had been injected that sterile inflammation results in a diminished reutilization of iron from senescent erythrocytes for red cell production. The utilization of Tf-Fe for erythropoiesis is not impaired, implying that there is a defect in the release of iron from the tissues to the plasma Tf during inflammation. The defect was present in animals with normal, deficient and increased iron stores.

Fillet et al (1974) showed, in dogs, that intravenous injection of heat-damaged erythrocytes labeled with ^{59}Fe resulted in the release of radioiron in two phases, early and late, with $t_{1/2}$ release of 34 min and 7 days, respectively. A 10-fold increase of the iron load in the RES did not affect the release phenomenon, neither did the level of plasma Tf-iron. Acute inflammation induced by endotoxin caused a striking decrease in iron release during both phases. The biphasic release pattern has been questioned by Bentley et al (1979) who used a ^{59}Fe colloidal iron complex in men and observed a continuous release of ^{59}Fe into plasma and no evidence of separate early and late phases. Probably uptake and handling is

different for the synthetic colloid and for erythrocytes, and might account for this disagreement (Esparza and Brock, 1981a). The most important criticisms of the studies of iron processing by the RES have been the use of large amounts of erythrocytes with low ^{59}Fe specific activity that could change the iron load of the macrophages (Roeser, 1980), and the use of unphysiological synthetic iron compounds.

Hershko et al (1974) also observed that in rats the in vivo release of iron by the RES was biphasic, and that the early phase was diminished in inflammation produced by turpentine inoculation. Esparza and Brock (1981a) used a quasiphysiological method to study the fate of iron in normal and stimulated (inflammatory) macrophages, which involved loading peritoneal macrophages with insoluble ^{59}Fe-^{125}I-Tf-antiTf immune complexes. They gave direct in vitro evidence of impairament of iron release by inflammatory cells. The cells were not overloaded because the degradation kinetics of the immune complexes followed a first order mode, thus discounting the possibility that the effect on iron release could be due to the loading method. They suggested that the impaired iron release is an intrinsic defect/property of the RES in inflammatory situations. The release of iron was not affected by the presence of apoTf or saturated Tf, therefore suggesting that the iron release is independent of extracellular unsaturated Tf concentration, at least in this system. DFO addition to the culture medium caused a marked increase of iron release both from resident and inflammatory macrophages showing that these cells contain a chelatable

pool. In contrast, the addition of ascorbic acid did not show any effect on iron release as has been shown in other cells (Roeser, 1980; Deiss, 1983).

Brock et al (1984) confirmed and expanded their previous study. They showed that the biphasic release of iron is present in normal and stimulated peritoneal macrophages. The early phase was of shorter duration in the inflammatory cells and the second (late) phase was similar in both cells. Their results support the idea that Tf acts as a passive recipient of released iron. They suggested that the iron probably crosses the cell membrane passively as Fe^{2+}, which is then oxidised on release. This was based on the detection of a high molecular-weight compound (probably polymerised iron) when the Tf in the medium was saturated, the absence of any pronounced inhibition of iron release by metabolic inhibitors and the fact that the release of iron was reduced but not totally inhibited at $0^{o}C$. They also suggested that it seems likely that the early phase represents iron which enters the labile pool and is then released, while the late phase is due to iron which is taken up by Ft and then slowly exchanges with the labile pool, a proportion of which is released.

Chapter 3

Literature review of macrophages

3.1 Macrophages

Cells of the phagocytic reticuloendothelial system (RES) or the mononuclear phagocyte system (MPS) are found largely in the spleen, bone marrow, and in the liver. They remove damaged and senescent red blood cells, convert the haem moiety to bilirubin, and eventually return the iron into circulation making it available to the haemopoietic system and the rest of cells of the organism. The MPS is quantitatively the most important source of plasma iron (see section 2.6) and it plays a central role in the regulation of iron reutilization, which in normal conditions is highly efficient.

In addition, macrophages have a very important role in immunology. In natural immunity, a non-induced form of host resistance, macrophages play a major role, principally due to their phagocytic capacity, by which they deal with environmental threats. In specific, acquired immunity, macrophages came to be regarded by immunologists as an adjunct to the immune response -an accessory cell, "processing" antigen for its presentation to immunocompetent cells. This narrow view has been displaced by a flood of recent developments showing that their essentiality for immune function is also exerted via certain macrophage secretory products which can replace viable macrophages in key stages of the immune response.

The macrophage range of capabilities is so comprehensive as to raise the question of how a single cell could possibly fulfill all these demands. Though unlikely, this possibility is not yet ruled out; but some

specialization would seem inevitable. (From Förster and Landy, 1981).

3.1.1 The mononuclear phagocyte system

The cells included in the mononuclear phagocyte system are found in bone marrow (fixed macrophage), connective tissue (histiocyte), liver (Kupffer cell), lung (alveolar macrophage), lymph nodes (free and fixed macrophage, interdigitating cell?), spleen (free and fixed macrophage), serous cavities (pleural and peritoneal macrophage), bone (osteoclast), nervous tissue (microglial cell), skin (histiocyte, Langerhans cell?), synovia (type A cell?) and other organs (tissue macrophage). The cells labeled "?" lack some of the characteristics found in classic macrophages from other sites (see section 3.4).

3.2 Origin of macrophages

The bone marrow origin of these cells has been established many years ago. They come from the stem cell, which then becomes a committed stem cell, monoblast, promonocyte, monocyte and then macrophage. Their pattern of division has also been determined. In the mouse, the cell-cycle time of the monoblast is 11.9 h, and division of one cell gives rise to two promonocytes; the promonocytes divide only once, after 16.2 h, and give rise to two monocytes. Thus, from monoblast to promonocyte, there is a fourfold amplification. After monocytes are formed, they randomly leave the bone marrow within 24 h. Monocytes remain in circulation with a $t_{1/2}$ of 17.4 h (van Furth, 1981a, 1981b). Quantitative kinetic studies in normal mice

have shown that of the monocytes leaving the circulation, 56% become Kupffer cells (Crofton et al, 1978), 15% other macrophages (Blusse et al, 1981), and 8% become peritoneal macrophages (van Furth, 1970b). The monocytes do not divide further to become macrophages.

The alternative concept of the origin of macrophages, i.e. that this population of cells is renewed by the division of mature macrophages, has been criticized on the following grounds. Firstly the fate of monocytes; in normal mice, it is known that the turnover in the circulation amounts to 3-5 $\times 10^4$ monocytes per hour with cells leaving the circulation randomly. Hence if mature macrophages divide there should not be a high turnover of monocytes (van Furth, 1980). Secondly, there are the common characteristics (morphological and cytochemical) of monoblast, promonocyte, monocyte and macrophages such as Fc and $C3_b$ receptors in their membrane and their ability to phagocytose and pinocytose. Finally, there is the very low labelling in vivo and in vitro of macrophages with DNA precursors, this low labelling apparently is reflecting incorporation by a few recently arrived cells (van Furth, 1981b).

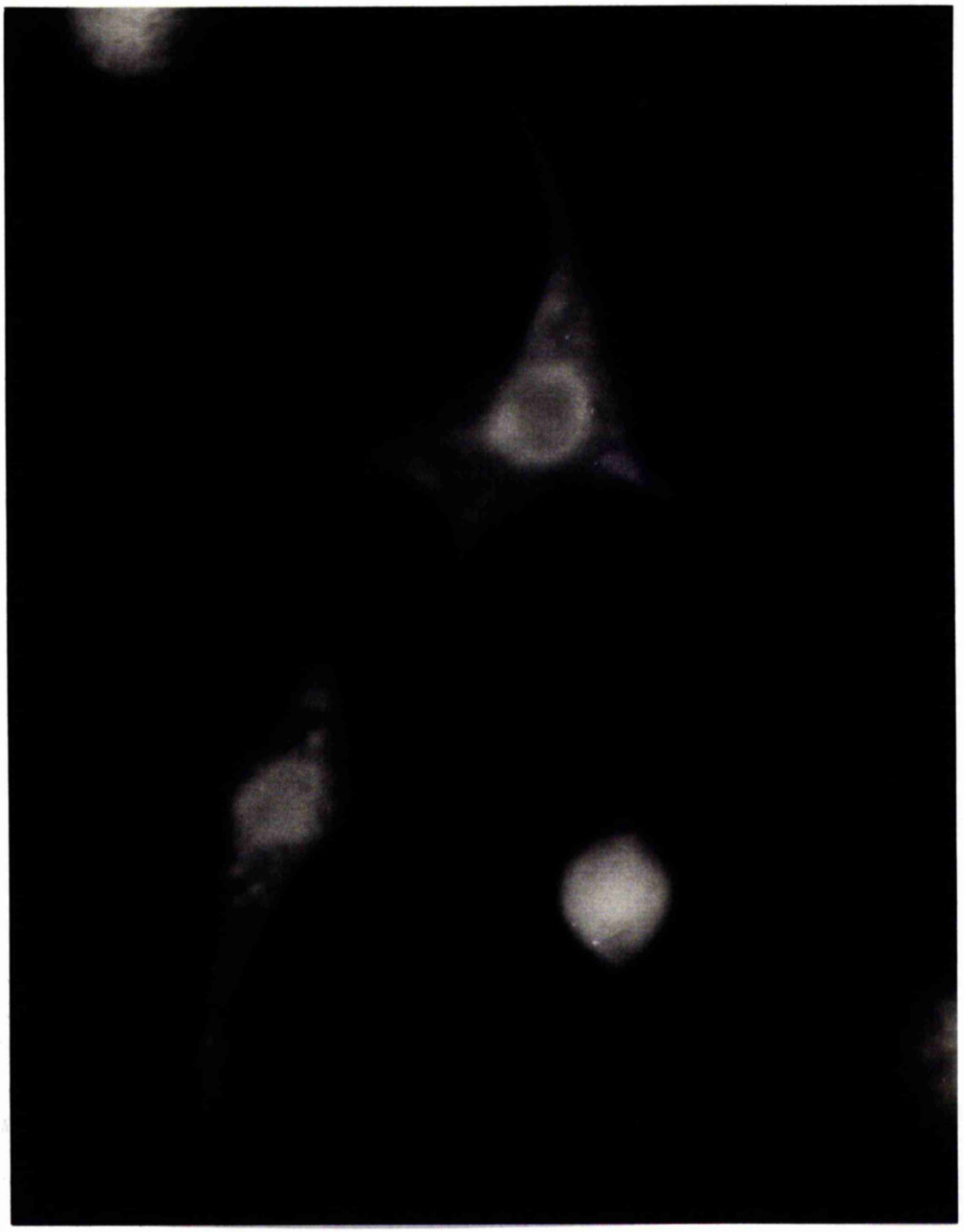
3.3 Morphological characteristics of mononuclear phagocytes

The macrophage populations are heteregeneous in several aspects and their morphology is one of them. Romanowsky-stained smears or cytopreparations are the commonest techniques used to evaluate cell morphology,

hence the following description is based on Leishman and Wright's stains. The morphology can vary depending upon the type of phagocyte and state of differention. Generally, the cell size ranges from 12 to 20 um in diameter, sometimes even larger. The nucleus is round or oval or slightly folded (horseshoe shape), with cerebriform appearence, blue-purple in colour and occupying a central position. The nuclear-cytoplasmic ratio, in precursors, varies according to the maturation state from 4:1 to 2:1 and it is 1:1 at the monocyte stage, after which it changes depending on the macrophage to which the cell transforms. The cytoplasm in the mature cell is abundant, with bleb-like pseudopodia, pale grey-blue in colour, and with numerous fine pale red dust-like particles which are evenly dispersed and vacuolated (O'Connor, 1984). Under culture conditions the peritoneal macrophages adhere to surfaces, presenting a characteristic well spread and elongated morphology (Fig. 5; see figures in chapter 4). The main qualitative difference between the monocyte and the macrophage is the appearance of a variety of phagocytic vacuoles, since the monocyte is a much less effective phagocyte. The macrophage phagolysosomes may be small and multiple or large and single and contain a variety of partially digested particles. Lysosomes may be seen fusing with the phagosomes (see section 3.4.2).

Figure 5

Cultured resident peritoneal macrophages.



Note the morphological heterogeneity. The macrophages were cultured for 4 days, stained with safranin and observed under UV light. This staining shows more clearly the spreading morphology than other methods. Magnification x 5000.

3.4 Biology of macrophages

3.4.1 Membrane, phagocytosis, receptors and antigens. The aspects considered here are principally those concerned with mouse macrophages. The membranes of the macrophage, both surface and intracellular, are of central interest because so many of the specialized functions of macrophages are membrane mediated. These include all types of endocytosis, associated delivery of vesicles to the lysosomal compartment by specific intracellular membrane fusion, and possible retrieval and redeployment of any receptors involved. Phagocytosis is the act by which phagocytes transport particulated material from the extracellular milieu into vacuoles formed by invagination of the cell membrane. Phagocytosis involves several stages. The first consists of recognition/adhesion of the phagocytosable material, which can be specific (mediated by receptors e.g. for Fc or complement) or nonspecific, e.g. ingestion of latex beads (Silverstein et al, 1977). Secondly, adhesion is followed by the emission of pseudopodia and, thirdly, by the engulfment stage. The engulfment stage follows a closing-zipper mode, when the recognition is mediated by receptors and, probably, by hydrophobic interactions of the nonspecific adhesion of particles (Rossi, 1980). During the course of phagocytosis there is a profound modification of oxidative metabolism. This oxidative phenomenon is referred to as respiratory burst and it is discussed in section 3.7. Exocytosis is another important membrane-dependant event and intracellular membranes are important in the complex

oxidative killing systems delivering high-energy oxygen metabolites to the lumen of the phagosome without causing damage to the cell (Bianco and Pytowski, 1981).

The composition of the various membrane compartments of the macrophage remains largely unknown, although a wide variety of plasma membrane components have been described and some differences have been noted between plasma membrane and phagolysosomal membrane. Quantitative stereological measurements indicate that peritoneal macrophages interiorize large amounts of plasma membrane continuously during pinocytosis (Silverstein et al, 1977). Steinman et al (1976) have determined that an area of plasma membrane equivalent to the entire macrophage cell surface is interiorized twice every hour. Since plasma membrane components are neither degraded nor synthesized in a correspondingly rapid fashion, it seems likely that the majority of membrane is recycled to the cell surface (Silverstein et al, (1977). Among the components described on the plasma membrane of macrophages are a variety of receptors of which a small number are considered here (for review see Morahan, 1980). The Fc receptor mediates dramatic cellular events upon interaction with immune complexes. These include the release of prostaglandins and leucotrienes, the secretion of neutral proteases such as plasminogen activator and elastase, release of activated oxygen species, cell mediated cytotoxicity, and phagocytosis (Unkeless et al, 1981). The receptor for C3b mediates phagocytosis of IgM-coated particles in the presence of complement (Silverstein et al, 1977), opsonized bacteria by the alternate pathway, etc. The

mannosyl/fucosyl terminal receptor mediates pinocytosis via binding of mannose or fucose terminated glycoproteins, including various lysosomal glycosidases. It may function as a retrieval pathway for lysosomal glycoproteins and may also be involved in clearance of antigen-IgM complexes (Stahl et al, 1980). The presence on the macrophage plasma membrane of receptors for Lf and Tf also has been reported (van Snick et al, 1975; Hamilton et al, 1984b), see section 2.10.2.

MHC antigens coded by the major histocompatibility complex such as Ia antigen are present on the macrophage (Gordon et al, 1981). The presence and possible functional significance of Ia antigen on the macrophage membrane is considered in section 3.6. Several monoclonal antibodies are now being used to probe the macrophage membrane. Although few functions have been assigned to the antigens recognized by these antibodies, their almost complete restriction to the cells of the mononuclear phagocyte system suggest the possibility of interesting specialized functions (Morahan, 1980).

3.4.2 Lysosomes

The macrophages contain a large quantity of these vesicles. The lysosome is a bag of hydrolytic enzymes used for the controlled intracellular digestion of macromolecules (de Duve, 1963). This controlled digestion can be achieved because most of the enzymes are synthesised as zymogens (precursors) which require modification to be activated, and also because sometimes the enzymes form

enzyme-inhibitor complexes. This controlled digestion by lysosomal enzymes is primary due to their enclosure within a membrane which when merged with other vesicles becomes a secondary lysosome. Some 40 enzymes are known to be contained in lysosomes. They are all hydrolytic enzymes, including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulphatases. They are acid hydrolases, optimally active near to the pH of 5 which is maintained within this organelle. The lysosome has a unique surrounding membrane which holds the enzymes but permits the final products of the digestion to escape so that they can be either excreted or reutilized by the cell (Alberts et al, 1983).

Two general classes of lysosomes are usefully distinguished. There are primary lysosomes, which are newly formed and therefore have not yet encountered substrate for digestion, and secondary lysosomes, that contain substrates and enzymes. The primary lysosomes are small and generally round. The secondary lysosomes are of diverse morphology which reflects variations in the amount and nature of the ingested material that they contain. Generally the secondary lysosomes formed from endocytotic vesicles are smaller than those of phagocytic origin. The secondary lysosomes are often given special names according to their content, such as digestive vacuoles (with bacteria), multivesicular bodies (containing numerous vesicles), or autophagic vacuoles (partially digested intracellular membranes or organelles) (Holtzman, 1976).

3.5 Secretory products of macrophages

A large variety of products are secreted by the macrophages, some of them needing to be triggered and only appearing during certain stages. It is now apparent that the spectrum of released products depends on the source of the cells as well as the kind of stimuli to which they are exposed in vivo or in tissue culture. These variations depend on complex cellular regulatory mechanisms by which mononuclear phagocytes respond to their pericellular enviroment. Such modulation is triggered in part through their receptors. As it is not intended to provide an extensive account of these products here, just those which are connected directly with this work are considered.

3.5.1 Enzymes.

Lysosomal hydrolases. The release of lysosomal acid hydrolases occurs in response to exogenous stimuli such as phagocytosis of particles or lymphokines (Page et al, 1978). Lysozyme. This enzyme mediates the digestion of bacterial cell walls. It is secreted continuously (Gordon, 1975).

Neutral proteases. These include collagenase, elastase, plasminogen activator and a cytolytic proteinase. Their secretion is regulated during macrophage activation (Gordon et al, 1981). Among the molecules that can regulate the neutral protease secretion are IL-1 and α_2 macroglobulin-trypsin complexes.

3.5.2 Reactive oxygen intermediates. Secretion of reactive oxygen species (e.g. O_2^-, H_2O_2, $OH^\cdot$), appears to depend upon the number or affinity of receptors for the

triggering ligand. The level of the oxidase complex needed to produce reactive oxygen intermediates and the efficiency of coupling between the receptors and the oxidases are also important (Adams and Hamilton, 1984), see section 3.7).

3.5.3 Interleukin-I and Interferon. Macrophages are a major source of these, and they can be released in response to a wide variety of stimuli including viruses, bacterial cells, fungi and fungal products, lymphokines and other agents (Lucas and Epstein, 1985; Mitchell and Kampschmidt, 1985) The association between interleukin-I and ACD is considered in section 2.8.

3.6 Antigen-presentation by macrophages

The requirements for a cell to function optimally in antigen presentation are, firstly, the capacity to express the Class II MHC glycoproteins on its surface and, secondly, the capacity to process antigen.

The I region of the murine MHC codes for polymorphic cell surface molecules, the Ia antigens, which play an important role in cell collaboration in the immune response and in the recognition of allogeneic cells. Ia antigens are expressed on only a limited number of cell types, including a portion of the MPS. The capacity of macrophages to synthesize and express Ia antigens on their surface is not constitutive (Nussenzwieg et al, 1980; Beller et al, 1980) but varies depending on the state of maturation of the macrophage, culture conditions (Steinman et al, 1981), the age of the individual, and the balance of stimulatory and inhibitory conditions (Unanue, 1984). The percentage of Ia-positive ($Ia^{+}ve$) cells varies from tissue to tissue and

also according to different stimuli, and Ia expression in macrophages correlates with changes in antigen presentation (Beller and Ho, 1982; Steeg et al, 1981). The extent of Ia expression can be regulated by the interaction of Ia^{+}ve macrophages with some agents that trigger phagocytosis, maintaining Ia expression and retarding the transition from Ia^{+}ve to Ia-negative (Ia^{-}ve), such as ingestion of BCG, Listeria monocytogenes, Trypanosoma cruzi (Adams and Hamilton, 1984; Unanue et al, 1984). The products of antigen-stimulated T cells preferentially induce Ia expression in previously immature Ia^{-}ve macrophages (Unanue, 1984), such as various lymphokines of which gamma-interferon is the major one resposible for Ia induction (Steeg et al, 1981). A number of inhibitory molecules diminish Ia expression, such as prostaglandins of the E class, alpha-fetoprotein (Lu et al, 1980) and glucocorticoids (Unanue, 1984).

The macrophages and other antigen presenting cells take up proteins or other antigens and apart from complete degradation to amino acids or basic components, they undergo a series of changes that enable to this cell to present antigens to T cells. This handling of the antigen is referred to as "antigen processing" (Unanue, 1984).

3.7 Respiratory burst of macrophages

Phagocytic cells show marked alterations in oxidative metabolism when stimulated with a suitable particle or membrane perturbant. This phenomenon is collectively referred to as the "respiratory burst". It results in marked increases in oxygen consumption, oxidation of

glucose via the hexose monophosphate shunt, generation of hydrogen peroxide, superoxide anion, and chemiluminescence. Most, if not all, of the oxygen undergoes enzyme-catalyzed univalent reduction to form superoxide anion (O_2^-; oxygen plus another electron) and oxygen (Babior et al, 1978). Thus, in the presence of protons, H_2O_2 is formed. Superoxide anion and H_2O_2 interact with each other in a cycle of reactions involving iron to form the potent oxidant, hydroxyl radical, perhaps the most important microbicidal oxygen species (Johnston et al, 1981). The production of the hydroxyl radical ($OH^{\cdot}$) is catalysed by Fe the overall reaction being known as the Haber-Weiss reaction. Firstly it involves a reduction in the oxidation state of Fe (Fe^{3+} to Fe^{2+}) by the superoxide anion. Secondly, the Fe^{2+} is oxidised by H_2O_2 (Fenton reaction) generating Fe^{3+} plus the $OH^{\cdot}$ radical (Blake, et al, 1985a). Superoxide and H_2O_2 are produced within most cells in microsomes, mitochondria, peroxisomes or elsewhere by the action of certain cytochromes, amino acid oxidases, and other enzymes. Although small amounts of reactive oxygen intermediates may not reach the outside of the cells, they could influence intracellular antimicrobial events (Nathan et al, 1980) or cause damage to their own membranes by lipoperoxidation (Blake et al, 1985a).

Schnyder and Baggiolini (1981) have proposed that the initiation of the respiratory burst may itself lead to sustained macrophage activation, as monitored by the subsequent secretion of hydrolases and proteases.

3.8 Relationship between resident, elicited and activated macrophages.

The terminology applied to the cells in an inflammatory exudate is generally unclear. In order to avoid this situation the definitions proposed by van Furth (1981a) are followed. Such definitions of the macrophages used in the present work are:

Resident macrophage. Macrophage present at any given site in the absence of an exogenous or endogenous inflammatory stimulus. These cells are sometimes called normal macrophages; they can also occur in a an inflammatory exudate as small subpopulation already present before the stimulus was applied.

Elicited macrophage. Macrophage attracted to a given site by a given substance. This term refers only to the mononuclear phagocytes accumulating at a particular site and does not indicate the developmental stage of the cell or their functional state.

Activated macrophage. Macrophage with increased functional activity induced by a given stimulus. Activation thus 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. Explicit mention of how it was activated should be made.

In the peritoneum there are abundant macrophages lying free or attached to the walls of this cavity. This type of macrophage population is refered as resident (rM) and is considered as "normal". This cell population superficially resembles monocytes in many ways such as morphology (but

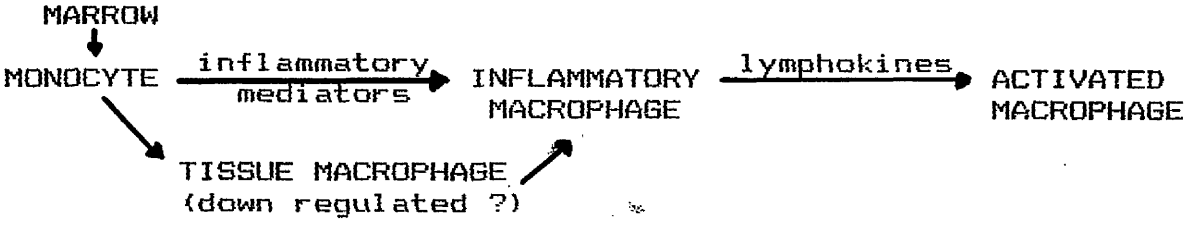
slightly bigger), degree of spreading, phagocytic competence and diminished capacity to respond to lymphokines (Vernon-Roberts, 1972). Despite these similarities to the monocyte the rM is a mature cell. The rM do not produce H_2O_2 following phagocytosis (Nathan and Root, 1977) nor respond to lymphokines or endotoxin by becoming tumour-cytotoxic (Blumenthal et al, 1983).

Macrophages taken from sites of nonimmunologically mediated inflammation (elicited macrophages) are very distinct from rM. The alterations that distinguish rM from elicited macrophages comprise increase in size and content of acid hydrolases, secretion of neutral proteases and a high capacity to phagocytose (Adams and Hamilton, 1984). Such macrophages have been elicited by intra-peritoneal inoculation of inducers of inflammation such as thioglycollate broth (Cohn, 1978), newborn calf serum (van Furth, 1980), carrageenan (Spector, 1980) etc. The elicited macrophages are, like rM, poor producers of H_2O_2 but in contrast to rM they can respond to lymphokines to become tumour-cytolytic (Blumenthal et al, 1983). The elicited macrophages, like the rM, have a lower percentage of Ia^+ve bearing cells than "activated" macrophages and neither population is effective as "antigen presenting" cells.

A different macrophage population is that taken from sites of immunologically mediated inflammation. In addition to possessing characteristics borne by elicited macrophages, namely, size and enhanced phagocytic activity, this immunologically "activated" macrophage population can perform other tasks such as presentation of antigen to T lymphocytes (Unanue, 1984) and the killing of tumoral cells

(Adams and Hamilton, 1984). These immunologically activated macrophage populations are good producers of H_2O_2 (Nathan and Cohn, 1980; Nathan, 1981). They generally have a high percentage of Ia^+ve cells (Unanue, 1984).

Adams and Hamilton (1984) have proposed the following relationship among resident, elicited and activated macrophages.



They suggested that exogenous inducers of inflammation such as thioglycollate broth lead to an intermediate cell (responsive or inflammatory macrophage) which under appropriate stimulus (lymphokines) gives an activated macrophage.

Traditionally macrophages have been elicited with bacteriological media like thioglycollate broth (Morahan, 1980; Hamilton et al, 1984a; Adams and Hamilton, 1984; Weiel et al, 1984; Tsuro et al, 1984), proteose-peptone (Morahan, 1980), casein or methaemalbumin (Kleber et al, 1978), etc, and activated with peritoneal inoculation of BCG, Trypanosoma cruzi, Toxoplasma gondii (Nathan and Cohn, 1980; Beelen and Walker, 1983; Adams and Hamilton, 1984; Freedman et al, 1984) or with C. parvum (Wilkinson et al, 1973; Wilkinson, 1982; Morahan, 1980; Hamilton et al, 1984a; Chapes and Haskill, 1984; Tsuro et al, 1984).

Chapter 4

Macrophage populations

4.1 Methods

4.1.1 Cell cultures (Adherent macrophages)

Peritoneal exudate cells (PEC) were obtained from adult CF-NIH, CBA or Balb C mice of either sex by lavage with 5 ml of PBS. Resident macrophages (rM) were withdrawn from unstimulated mice. Thioglycollate-elicited macrophages (tM) were obtained from mice which had been inoculated intraperitoneally with 2ml of Brewer thioglycollate broth (Difco Co., Surrey, England), Corynebacterium parvum-activated macrophages (CpM) from mice which had received 2mg intraperitoneally of freeze-dried C. parvum strain 4982 and Mycobacterium tubertulosis-activated macrophages (MtM) from mice which were inoculalated intraperitoneally with 2mg of heat inactivated and freeze-dried M. tuberculosis strain H37 RV (both bacteria were kindly provided by P.C. Wilkinson, Glasgow University). Except where otherwise stated the stimulant was inoculated 4 days previously.

The PEC were centifuged 400g for 2 minutes, the supernatant was discarded and replaced with RPMI 1640 (Flow Laboratories, Irvine, Scotland) + 10% fetal calf serum (FCS; Gibco, Paisley, Scotland). The cells were counted in a haemocytometer, their viability determined by eosin dye exclusion, and plated. Normally 4 times as many rM and 2 times as many CpM were plated than tM in order to obtain the maximum number of adherent cells and approximatly the same quantity of cell protein. After 1.5- 2 h incubation in a 10% CO_2-90% air humidified incubator (New Brunswich Scientific) the loose cells were removed. The removal of

loose cells was performed by repetitive suction and expulsion of the culture media from a Pasteur pipette (3 cycles) to ensure that all loose and loosely attached cells become resuspended. Special care was taken to remove cells at the Petri dish edges, where they tended to aggregate. After removal of these loose cells, the plates were further washed 3 times (by suction-expulsion cycles) with with Hank's balance salt solution (HBSS; Gibco), and fresh medium added. The composition of this medium varied according to the experiment. The state of the adherent cells was monitored throughout by phase-contrast microscopy (Nikon). These cultures will be referred to as adherent cultures or adherent macrophages.

Adherent cultures were established in either multiwell plates (Flow) or in 35 mm Petri dishes (Miles Laboratories, Napeville, Illin., USA) depending on the number of cells needed, and for staining purposes the adherent macrophages were established on circular glass 16mm cover slips (Chance Propper, Smethwick, Warley, England) incubated in square multiwell plates (Sterilin, Teddington, Middlesex, England).

4.1.2 Cytopreparations and stains

Cytopreparations were made from total PEC from normal mice, mice injected with either C. parvum or thioglycollate broth and also from separated mononuclear cells from thioglycollate-inoculated mice. About 10^5 cells in 1 ml of PBS were deposited on to slides in a Shandon cytocentrifuge at 500 rpm for 1 min. These cytopreparations, blood films

and adherent cultures of macrophages (made on round coverslips; section 4.1.1), from all three types of mice were treated in a similar manner for staining with Leishman stain.

4.1.2.1 Leishman stain

The preparations were air-dried and then fixed with methanol for 5 min. After fixation undiluted Leishman stain was poured on to the preparations and left undisturbed for 2 min, and then distilled water was added (about 2 volumes, finishing with a ~1:3 dilution), and left in contact for 5 min more. The preparations were then washed with tap water, dehydrated with alcohol-xylene and mounted either on to glass slides (the round cover slips) or covered with appropriate cover slips (cytospin preparations on glass slides). The preparations were always made in duplicate or quadruplicate. Examination for differential cell counts was made in a Leitz-Wetzlar light microscope at a x200 magnification, and 400 or more cells were counted in each slide.

4.1.2.2 Perls' Prussian blue stain

The Perls' Prussian blue (PPb) reaction is used to demonstrate aggregates of Fe^{3+} which react with acid-ferrocyanide giving a blue colour (Culling, 1963). The reagents used were 4% (w/v) potassium ferrocyanide and 4% (v/v) hydrochloric acid, in deionised water. The reagents were always freshly prepared and only mixed before used. The macrophages were stained for PPb either immediatly after the loose cells were eliminated (section 4.1.1) or after 22h of incubation in RPMI + 10% FCS containing 250 mM

FeNTA (section 5.1.12.2). The same PPb reagent was used to demonstrate iron in Ft when testing the purity of the mouse Fts by disc electrophoresis (section 5.1.1.2).

4.1.2.3 Immunofluorescent staining

The anti-Ia^k monoclonal antibody used to detect the presence of Ia^k antigen in macrophages was an IgG_{2bk} antibody that does not cross react with mouse haplotypes b, d, p or q (Jones et al, 1978a), The anti-Ia^k was isolated from cell culture supernatant (kindly provided by A. McI. Mowat, Glasgow University), of the mouse hybridoma cell line 10-2-16 from CWB mouse (isolated by R. W. Steinman, The Rockefeller University), by affinity chromatoghaphy. The immunoadsorbent used was prepared as described in (section 5.1.3), consisted of Sepharose 4B coupled to the gamma globulin fraction of antiserum raised in sheep against mouse IgG (SAPU, Lanarkshire, Scotland). A total of 500 ml of cell culture supernatant was passed (in four runs) through the antimouse IgG column (1.2 x 18 cm) at a flow rate of 35 ml/h and at 4^oC. Desorption of the anti-Ia^k from the column was performed with a chaotropic ion (3M KSCN in 0.1M sodium borate buffer pH 8). After collection of the fractions those containing SCN^- ion (detected with $FeCl_3$; section 5.1.5), which also contain the Ia^k, were pooled and immediately passed through a 60 ml column of Sephadex G-25, to eliminate the SCN^-. The high M.W.fraction was tested for SCN^- and was found to be negative. The fractions from the different runs were pooled, aliquoted and stored frozen at -20^oC.

This isolated anti-Ia^k was then labelled with

fluorescein isothiocyanate (Sigma) by the method of Wofsy et al (1980). This fluorescein-labelled antibody was used initially for direct staining of the macrophages, but the fluorescent cells appeared extremely faint. Thus an indirect method was tried in parallel, consisting of the detection of the anti-Ia^k (used as first antibody) with fluorescent sheep IgG Fab fragments (New England Nuclear, Boston Ma., USA). After optimization of both methods, direct and indirect, the latter showed brighter staining of the cells than the former, though both methods give the same percentage of fluorescent cells. Thus, the direct method was abandoned.

The final optimized indirect method adopted was as follows. The adherent macrophages on round cover slips were fixed in methanol for 5 min, immediately after removal of the loose cells (section 4.1.1). The macrophages were then washed 6 times with 1.5 ml of PBS, the first 3 times immediately after fixation and the other washes at intervals of 10 min. The PBS was then removed and 3-4 drops (enough to totally cover the round cover slip) of a 1:20 dilution of the isolated anti-Ia^k antibody in PBS containing 1% BSA were dropped on to the macrophages. They were then incubated at RT in a moist chamber for 1.5h, after which the preparations were washed as described above (6 washes in half an hour). After the PBS had been removed a 1:25 dilution of the fluorescent antimouse Fab fragments in PBS containing 1% BSA was added dropwise. The preparations were incubated in the dark in a moist chamber at RT for 1h. After this incubation the macrophages were

washed as described above. Both antibody dilutions were prepared in excess and centifuged for 10 min at 10000g, to eliminate possible aggregates, ~100ul always being left in the bottom of the tube. After the final wash with PBS the cover slips were mounted upside down on glass slides with a drop of PBS containining 10% glycerol (w/v), and sealed with transparent nail varnish. The fluorescent cells were observed in a Leitz-Wetzlar UV light microscope which also allows the observation of cells in phase contrast, thus enabling the fluorescent cells to be counted under UV light and the total macrophages under phase contrast. 400 to 1200 cells were counted in each preparation, and all preparations were performed in duplicate. The peritoneal macrophages tested for Ia^k positive cells were from CBA mice (haplotype K), either unstimulated (rM), or inoculated with C. parvum or thioglycollate broth. As a positive control peritoneal macrophages from CBA mice inoculated with M. tuberculosis were included and as negative control resident macrophages from NIH mice (haplotype q) were used.

4.1.2.4 Safranin stain

The adherent macrophages, plated on round cover slips (section 4.1.1), were fixed as described (section 4.1.2.1) and stained in a specially built rack with a 0.05% aqueous solution of safranin (w/v) for 2 min at RT. After washing off the stain with running tap water for 1 min the macrophages were dehydrated and mounted as described (section 4.1.2.1). Examination of the cells was performed under UV light in a Leitz-Wetzlar microscope.

3.1.3 Separation of mononuclear leucocytes

The separation of mononuclear leucocytes was performed by isopycnic centrifugation on Ficoll-Isopaque. A Ficoll solution was made by dissolving 9.504 g of Ficoll 400 (Pharmacia) in distilled water up to a volume of 105.6 ml at 56°C. the solution of Isopaque was made by diluting 20 ml of Isopaque 440 (Nyegaard and Co., Norway) with 24 ml of distilled water. The Ficoll and Isopaque solutions were mixed in a 21.5 to 10 parts ratio. The specific gravity of the solution (1.085 g/ml) was checked with a hydrometer and it was sterilised by steaming for 90 min.

The mice were bled by cardiac puncture with a heparinized syringe fitted with a 26 gauge needle. The blood (0.5-0.9 ml) was carefully laid on to the top of 2ml of the Ficoll-Isopaque solution and centrifuged at 400g for 30 min. The mononuclear leucocytes that remain on top of the Ficoll-Isopaque were carefully removed and washed 3 times with PBS.

4.1.4 Determination of lactate dehydrogenase

Lactate dehydrogenase (LDH) activity was measured with a commercial kit (kit 226-UV; Sigma). The test is based on measuring the formation of NADH at 340 nm from NAD by the enzymatic transformation of lactate into pyruvate. The reagents were prepared according to the manufacturer's recommendations and the optical density changes were detected in a recording spectrophotometer (Unicam SP 1800; Pye Unicam Ltd., Cambridge, England) at 340 nm and 22°C. LDH was measured in 0.1ml of the supernatant of the

adherent cultures (section 4.1.1) and in 0.1ml of a macrophage lysate made by adding 1 ml of distilled water to the adherent cultures at RT. Complete lysis was achieved after 0.5h (monitored by phase contrast microscopy). The culture medium was centrifuged at 400g for 10 min to remove the unattached cells, and the lysate at 10000g for 3 min prior taking the 0.1 ml aliquot for LDH activity measurement.

4.2 R e s u l t s.

4.2.1 Characterization of the peritoneal cell populations

The aim of these experiments was to characterize the dynamics and magnitude of peritoneal infiltration by those cell populations that are generated by intraperitoneal inoculation of C. parvum or thioglycollate broth, and to compare some characteristics of these cells with the resident cells of this serous cavity. In addition, these experiments provided an estimate of the purity of the adherent cells used in the cultures throughout this work.

In order to make differential leucocyte counts smears and cytopreparations were made from the peritoneal exudate cells (PEC) of mice which had received either no stimulation, or inoculation at different times with C. parvum or thioglycollate broth (see section 4.1.1). The adherent macrophages were stained with Leishman, safranin and Perls' Prussian Blue reaction and Ia^{k+}ve cells were identified by monoclonal antibody. Blood smears were only stained with Leishman stain (section 4.1.2).

Results of the differential counts of the PEC are shown in table 1a and 1b, the typical spreading morphology can be seen in figures 6, 7 and 8, morphology of rM and the morphological changes that the macrophages showed during stimulation can be seen in figures 9, 10 and 11.

In normal conditions (resident cells) the PEC are composed almost entirely of lymphocytes and macrophages (Tables 1a and 1b). On the other hand, after inoculation of the stimulant there are also other cells. In order to obtain a more reliable figure than the percentage, which is

Table 1

Differential counts of peritoneal cells from unstimulated mice, C. parvum-inoculated mice and thioglycollate broth-inoculate mice.

a) counts expressed as percentage of total leucocytes.

Mice inoculated with:	Days after inoculation	Percentage of: Neutro	Lymph	Eosin	Macro	
Nothing (resident)	0	< 1	65	2	33	
C. parvum	1	44	14	7	34	
"	2	52	6	5	38	*
"	3	12	7	7	74	*
"	4	38	6	5	51	**
Thioglycollate broth	1	49	4	1	55	*
"	2	23	13	7	57	*
"	3	14	4	9	73	***
"	4	3	5	9	84	***

Neutro= Neutrophils, Lymph= Lymphocytes, Eosin= Eosinophils, Macro= Macrophages.

* Bigger cells than resident macrophages with large vacuoles.
** Big cells but less vacuolated.
*** Big cells, highly vacuolated, and presence of "granules", i.e. dense basophilic vacuoles (see figures 10 and 11)
No basophils were seen in any population.
Figures are mean of duplicate cytopreparations from 3-4 pooled peritoneal exudates : 400 cells counted.

Table 1 (cont.)

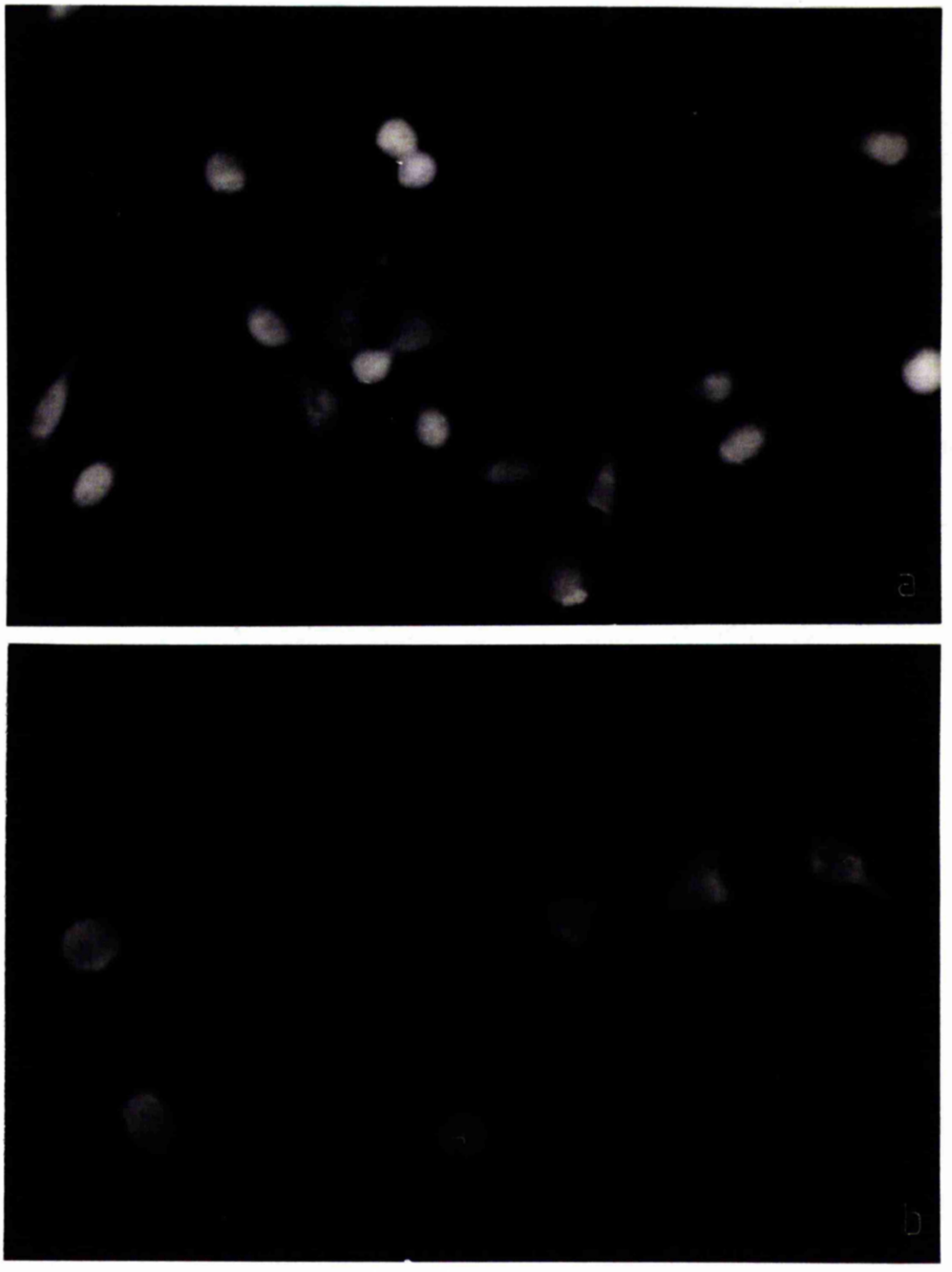
b) Counts expressed as absolute number of cells, peritoneal yield and ratio neutrophils/macrophages.

Mice inoculated with:	DaI	Total PEC	Absolute numbers of: Neutro	Lymph	Eosin	Macro	Neutro Macro
Nothing (resident)	0	3.75	0.02	2.44	0.06	1.23	0.01
C. parvum	1	3.60	1.58	0.50	0.25	1.22	1.29
"	2	3.75	1.95	0.22	0.19	1.42	1.36
"	3	4.64	0.55	0.32	0.32	3.52	0.16
"	4	5.94	2.26	0.35	0.29	3.03	0.74
Thioglycollate broth	1	7.70	3.77	0.35	0.08	3.50	1.07
"	2	11.90	2.79	1.55	0.78	6.81	0.41
"	3	14.40	2.01	0.50	1.37	10.51	0.19
"	4	12.20	0.36	0.55	1.04	10.25	0.03

PEC= Peritoneal exudate cells, DaI= Days after inoculation, Neutro/Macro= Ratio neutrophils/macrophages. Figures are millions of cells. Total PECs are mean of 4 pooled peritoneal exudates. All other figures were calculated from table 1 'a'.

Figure 6

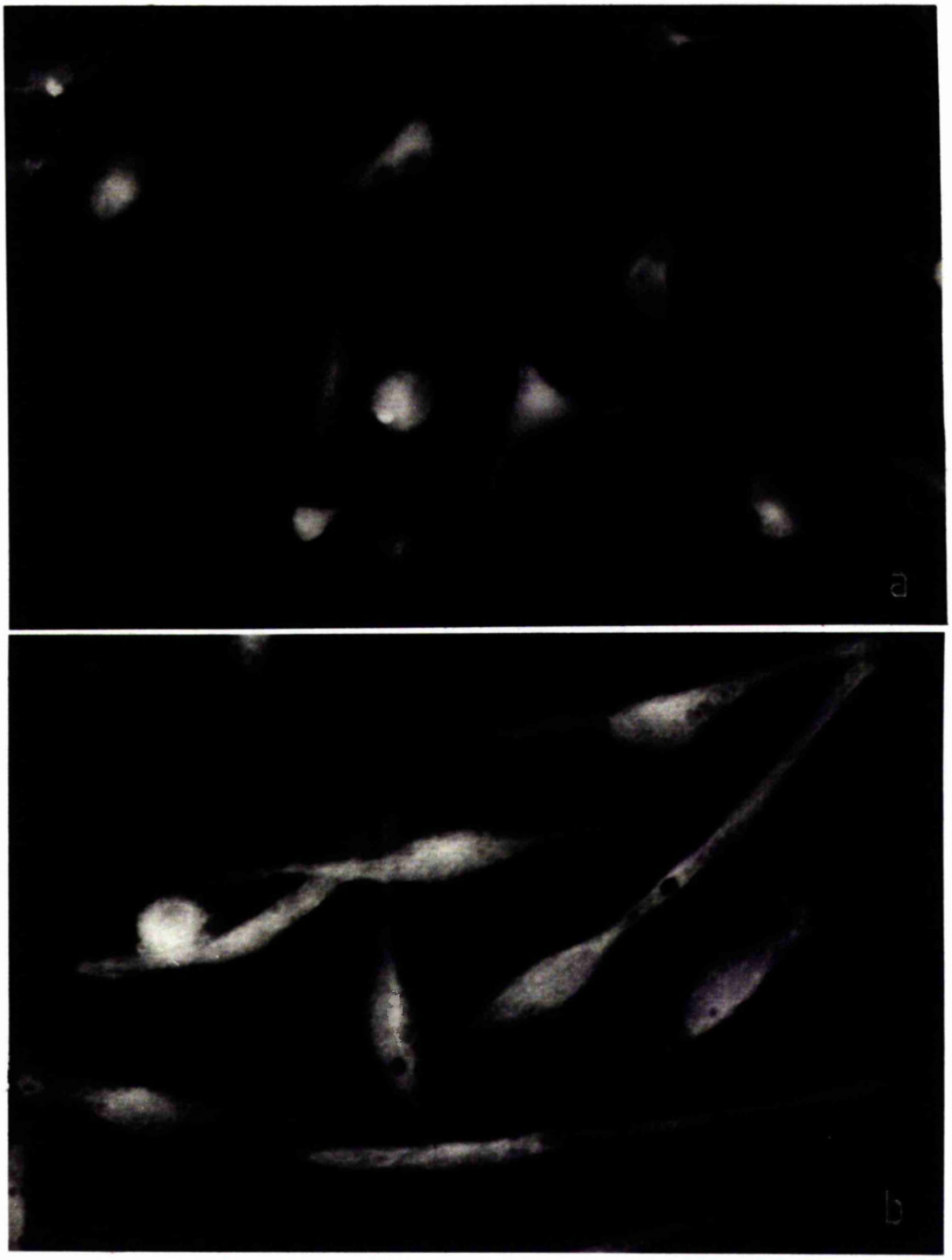
Adherent culture of resident peritoneal macrophages stained with safranin.



a) After removal of nonadherent cells (1.5 h of incubation).
b) After 14 h of incubation.
Note the typical spreading changes with time (a vs b).
Magnification x 1300. Compare with the other type of macrophages.

Figure 7

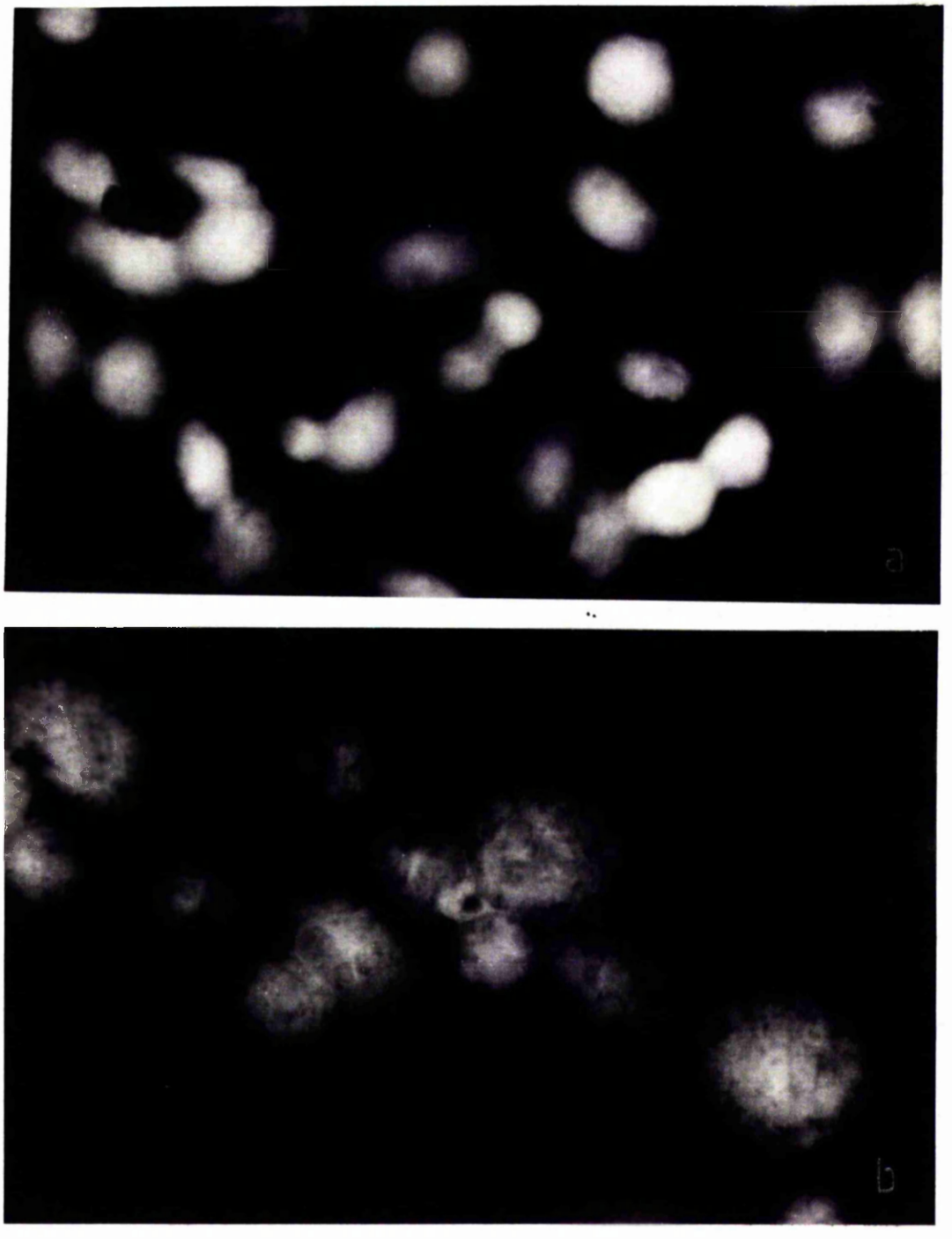
Adherent culture of C. parvum activated peritoneal macrophages stained with safranin (4th day post-inoculation).



a) After removal of nonadherent cells (1.5 h of incubation).
b) After 14 h of incubation.
Note the typical spreading changes with time (a vs b).
Magnification x 1300. Compare with the other type of macrophages.

Figure 8

Adherent culture of thioglycollate broth elicited peritoneal macrophages stained with safranin (4th day post-inoculation).



a) After removal of nonadherent cells (1.5 h of incubation).
b) After 14 h of incubation.
Note spreading changes with time (a vs b). Photograph a) is diffuse due to spherical morphology. Magnification x 1300. Compare with the other type of macrophages.

Figure 9

Adherent culture of resident peritoneal macrophages stained with Leishman stain.

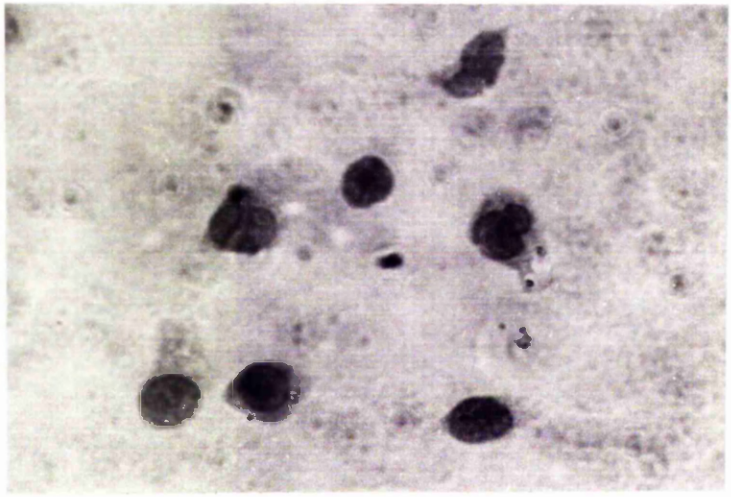
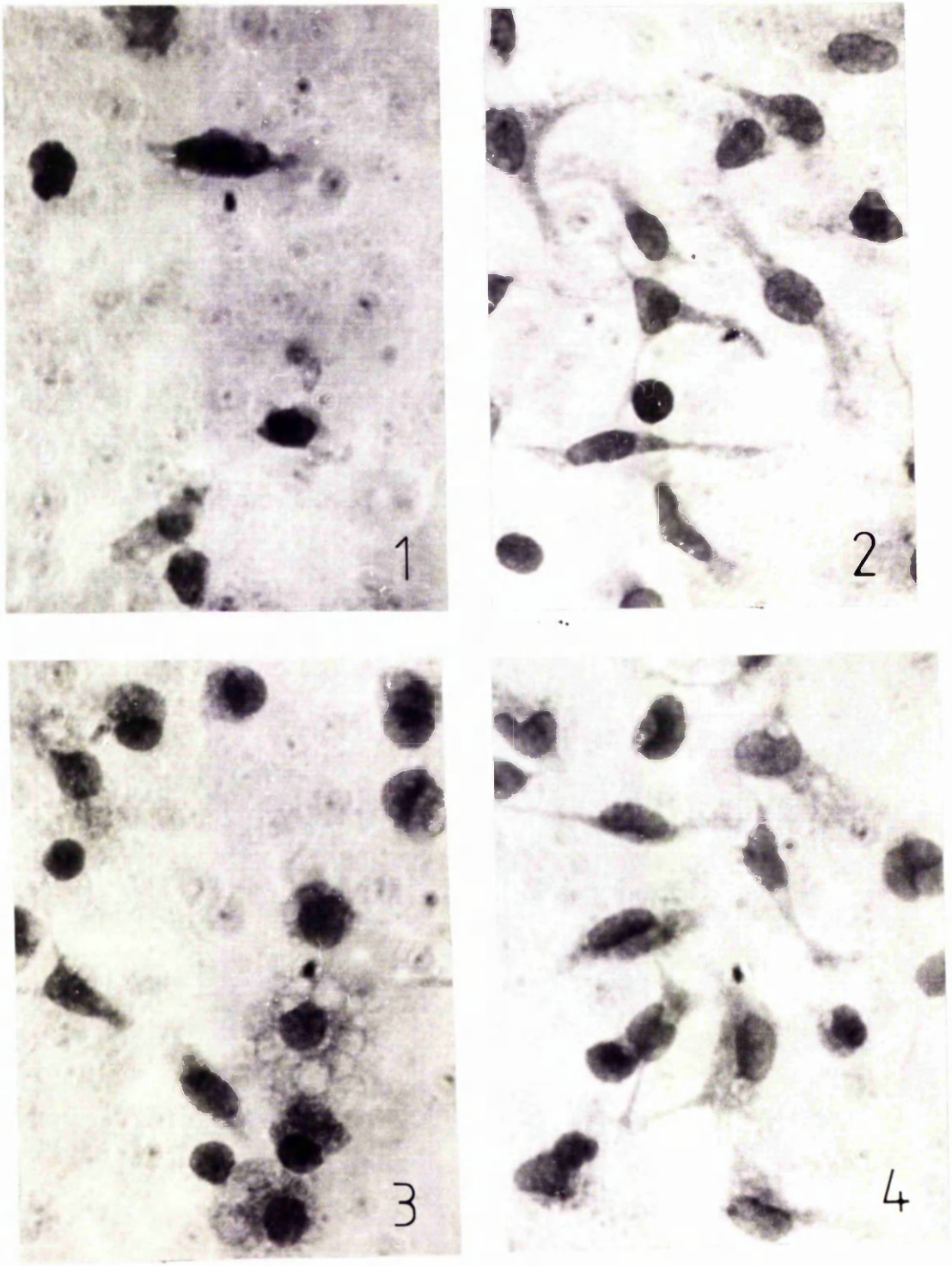


Figure 10

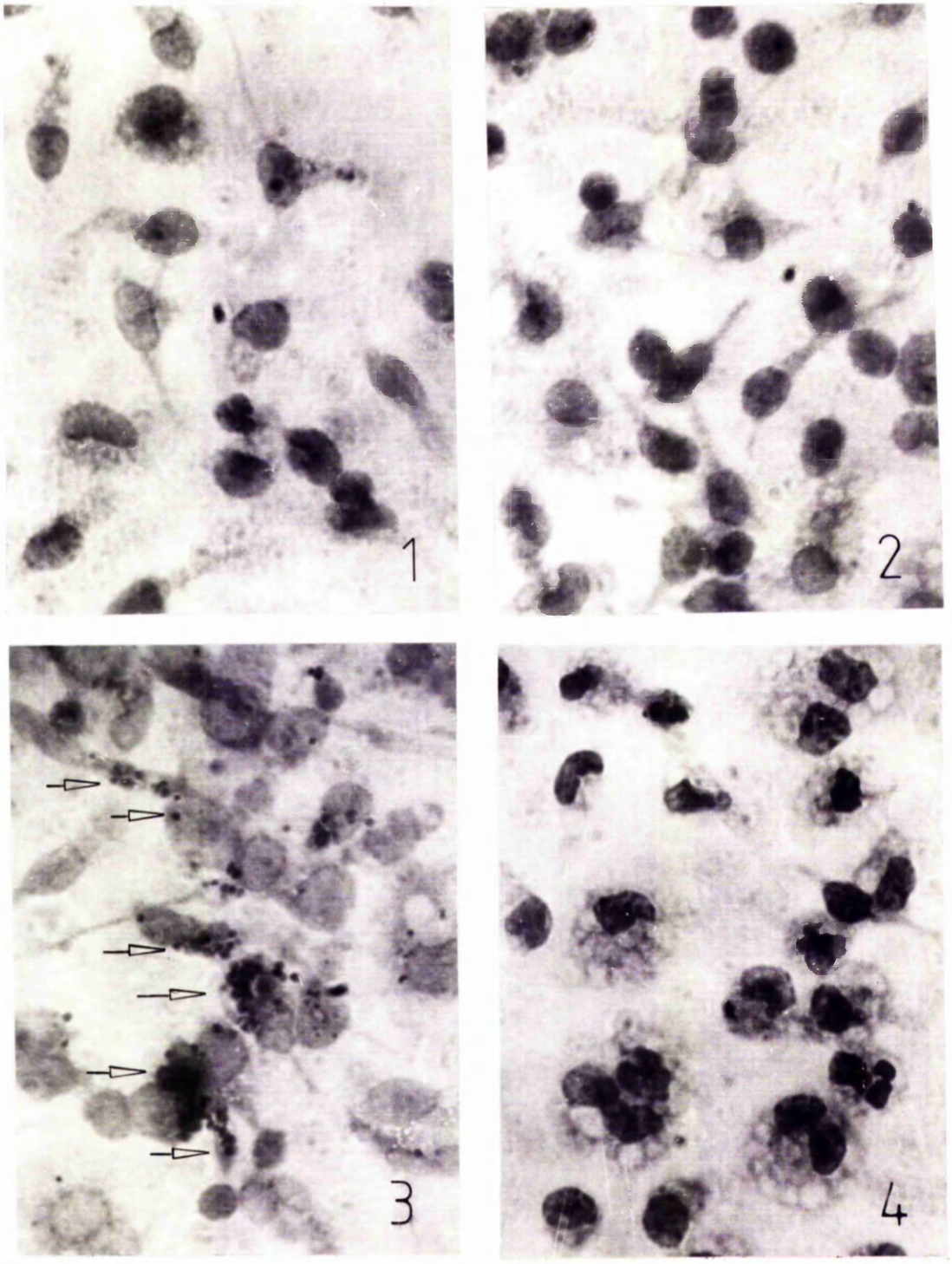
Adherent cultures of C. parvum activated peritoneal macrophages stained with Leishman stain.



The numbers indicate the day after peritoneal inoculation of C. parvum.

Figure 11

Adherent cultures of thioglycollate broth elicited peritoneal macrophages stained with Leishman stain.



The numbers indicate day after peritoneal inoculation of thioglycollate broth. Arrows point to dense basophilic "granules"

a relative estimation and does not show the extent of the cellular infiltration that is taking place, the quantities of peritoneal cells are also expressed as absolute numbers in table 1b.

The cell populations observed in the inoculated mice differed from each other in several features. The influx of neutrophils in the peritoneum of mice which have received C. parvum showed a double-wave pattern, there being two peaks during the time studied, one on the first and another on fourth day after inoculation. The second neutrophil influx appeared to be slightly bigger than the first one but it should be pointed out that during the second wave there were already some neutrophils in the cavity. In contrast the cellular infiltration of neutrophils in the mice inoculated with thioglycollate broth followed a different mode. The number of neutrophils in the peritoneum of these mice was nearly double that in the mice inoculated with C. parvum, and the accumulation was greatest on the first day, after which their numbers decreased sharply, almost disappearing by the fourth day. This was in contrast to the mice inoculated with C. parvum which at this time had the largest neutrophil accumulation. It was noticed that the tM showed at the third day certain "granules" (Fig. 11) which were very similar to those present in granulocytes. These granules were dense, basophilic, and of similar size to those seen in neutrophils.

The ratio of neutrophils to macrophages (Table 1b) changed dramatically during the different days and these variations are reflected in the numbers of adherent

macrophages, expressed as total protein, present in the cell cultures (Table 2). These variations could be explained on a competitive basis, i.e. the more nonadherent cells that are present in the PEC the fewer the macrophages that will find a free surface on the dish.

The total cell yield per mouse (Table 1b) showed that the cell influx into the peritoneum of the thioglycollate broth-inoculated mice was greater than that observed in the peritoneal cavity of the C. parvum-inoculated mice; the total number of cells in the cavity was double on the first day, and this difference was maintained throughout the time studied. With both agents, the accumulation of macrophages in the peritoneum showed a constant increase up to the third day after inoculation, when they reached a maximum, and on the fourth day there was a small drop. However, the number of macrophages accumulating in the peritoneal cavity of the thioglycollate broth-inoculated mice was 3 times that observed in the C. parvum-inoculated mice, which also showed, as well as lower cell numbers, a less steep increase than the former.

The differential counts of the blood smears (Table 3) revealed that in the blood of C. parvum-inoculated mice the neutrophil percentage decreased steadily throughout the first 3 days of the stimulation and on the fourth day there was an increase. This pattern correlates with the cellular infiltration seen in the peritoneum of these mice. On the other hand the percentage of neutrophils seen in the blood of thioglycollate-inoculated mice showed a change only on the first day, and thereafter the percentage remained

Table 2

Cell protein in adherent cultures of peritoneal macrophages from unstimulated mice, C. parvum-inoculated mice and thioglycollate broth-inoculated mice.

	Total cell protein (ug/35 mm dish)		
		Mice inoculated with:	
Days after inoculation	Nothing (resident)	C. parvum	Thioglycollate broth
0	64	--	--
1	--	48	45
2	--	93	120
3	--	108	167
4	--	85	168

Figures are mean of duplicates : The difference between the duplicates averaged 6.3% for rM, 3.9% for CpM and 2.2% for tM of their respective mean.

Table 3

Differential counts of blood smears from unstimulated mice, C. parvum-inoculated mice and thioglycollate broth-inoculated mice.

Mice inoculated with:	Days after inoculation	Percentage of: Neutro	Lymph	Eosin	Mono
Nothing (resident)	0	31	55	2	12
C. parvum	1	30	50	2	18
"	2	20	55	3	22
"	3	14	67	8	11
"	4	43	40	--	17
Thioglycollate broth	1	50	45	--	5
"	2	29	64	--	7
"	3	32	56	--	12 *
"	4	35	50	--	15 *

Neutro= Neutrophils, Lymph= Lymphocytes, Eosin= Eosinophils, Mono= Monocyte.
* Cells with young/immature appearence and presence of macrophage-like cells with large vacuoles.
Figures are mean of duplicated blood smears made from pooled blood from 4 mice: 400 cells counted.

similar to that found in normal mice. The percentage of lymphocytes apparently remained constant in both types of mice, apart from a small rise in the thioglycollate-inoculated mice on the second day. This lymphocyte increase was seen also in the peritoneum on the same day (Table 1a).

The observation of smears from thioglycollate-inoculated mice revealed unusual cells in the blood stream with the appearence of macrophages. This finding was checked by making cytopreparations of blood mononuclear cells separated by a Ficoll-Isopaque step-gradient (section 4.1.4). The differential counts of these mononuclear cells revealed that there is a large percentage of cells with macrophage morphology after 3 days of inoculation (Table 4).

The adherent cells were also stained with Perls' Prussian blue (PPb) reaction (section 4.1.2.1) in order to stain Hs. None of the types of macrophages showed the characteristic blue colour indicative of Hs when they were stained after the adherence step (section 4.1.1). In initial experiments where cells were stained for PPb after being incubated for 22 h in medium containing 250 ug of FeNTA it was possible to demonstrate a blue reaction, but later this was found to be an artifact due to use of an old FeNTA preparation which may have polymerised.

Examination of the adherent cultures stained with Leishman stain revealed that they were almost entirely composed of macrophages, >98% for rM, 99% for CpM and >99% for tM. Throughout the iron metabolism experiments the viability and state of the adherent cultures were monitored

Table 4

Differential counts of blood mononuclear cells, thioglycollate-inoculated mice.

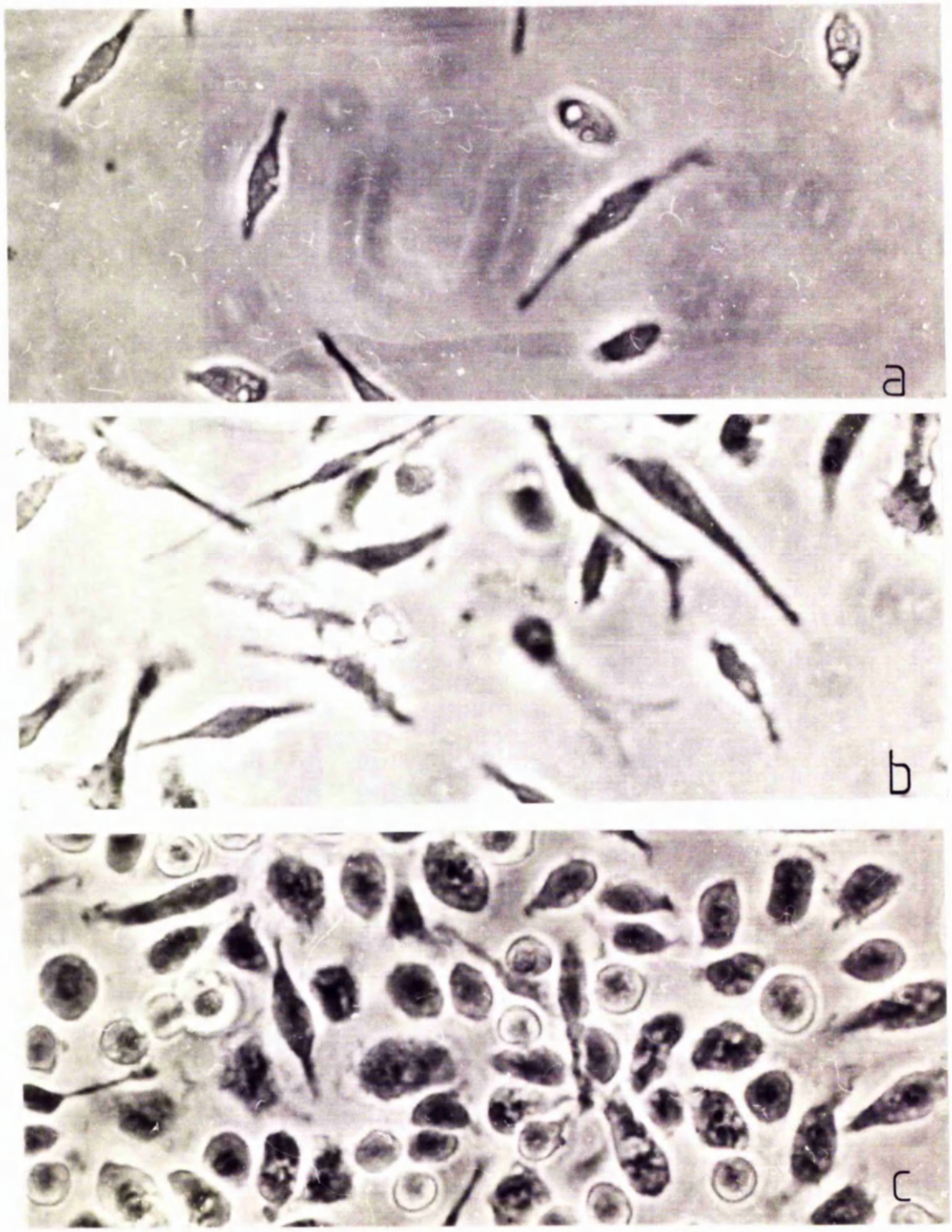
Day after inoculation	Percentage of: Lymph	Mono	Macro
1	76	24	--
2	92	8	--
3	58	20	28
4	32	20	48

Lymph= Lymphocytes, Mono= Monocytes, Macro= Macrophages
Figures are percentage of total mononuclear leucocytes: mean of duplicate cytopreparations from 4 pooled mice bloods.

by phase-contrast microscophy (Fig. 12). In the event of bacterial contamination or changes in the characteristic spreading morphology, the macrophage cultures were discarded.

Figure 12

Adherent cultures of resident, C. parvum-activated and thioglycollate broth-elicited peritoneal macrophages observed under phase-contrast microscopy.



a) resident macrophages.
b) C. parvum-activated macrophages.
c) Thioglycollate broth-elicited macrophages.
Photographs were taken after removal of nonadherent cells.
Magnification x 1300.

4.2.2 Ia antigen on peritoneal macrophages

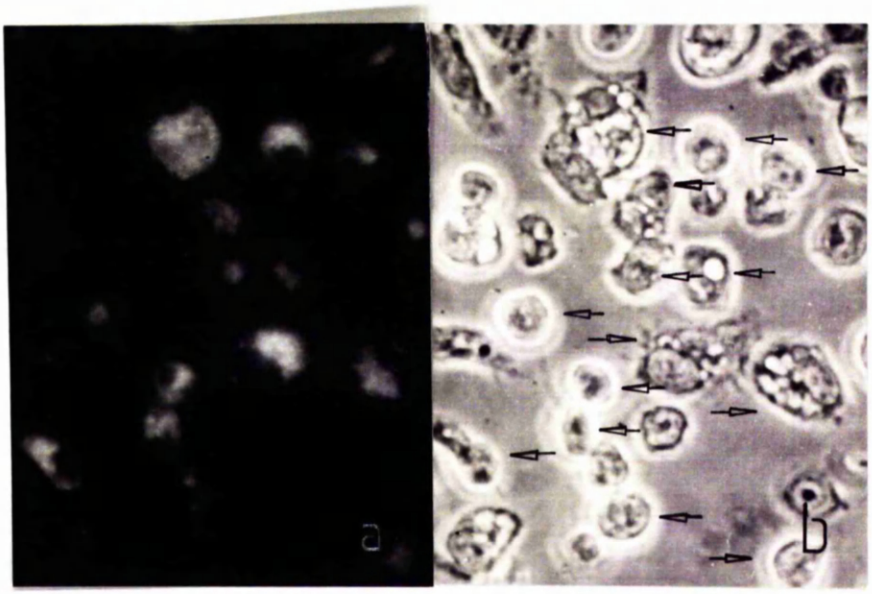
CBA mice (haplotype k) were used to obtain the peritoneal exudates, the negative control consisting of resident cells from NIH mice (haplotype q). As well as the usual macrophages (rM, CpM and tM), mice were also inoculated intraperitonelly with 2mg of *M. tuberculosis* in 2 ml and these macrophages (MtM) were used as a positive control for the presence of Ia antigen. Typical results of the four types of macrophages (adherent cells) stained by indirect immunofluorescence method are shown in figures 13 and 14. Results of Ia expression are given either as a percentage or as absolute numbers in table 5. The peritoneal cell yield per mouse, from which the absolute numbers were calculated can be seen in table 1.b.

The Ia expression by the different macrophages showed that in the *C. parvum*-inoculated mice the percentage of positive cells (23%) was similar to the percentage found in the resident cells from unstimulated mice (29%), but in the thioglycollate broth-inoculated mice the percentage of Ia^{+}ve cells was only 10%. However, if the quantities of Ia^{+}ve cells are calculated in absolute numbers the picture changes. There are 2.74 times more Ia^{+}ve cells in the peritoneum of the thioglycollate broth-inoculated mice, than in the resident population, and the peritoneum of *C. parvum*-inoculated mice contains nearly double the quantity of Ia^{+}ve cells present in the resident population (Table 5), but there are slightly less Ia^{+}ve cells present than in the peritoneum of thioglycollate broth-inoculated mice.

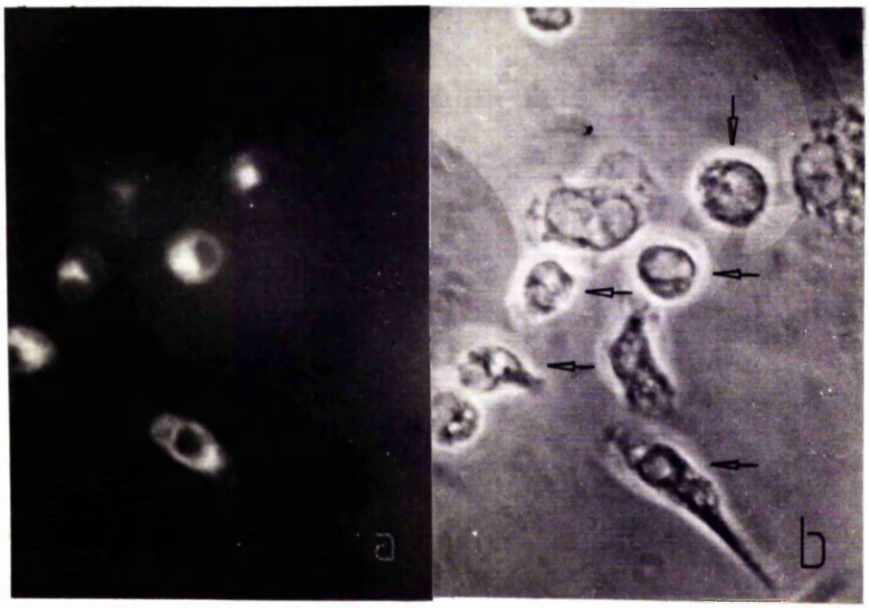
Figure 13

Adherent culture of M. tuberculosis and C. parvum activated peritoneal macrophages stained for Ia antigen by indirect immunofluorscent method.

M. tuberculosis-activated macrophages



C. parvum-activated macrophages

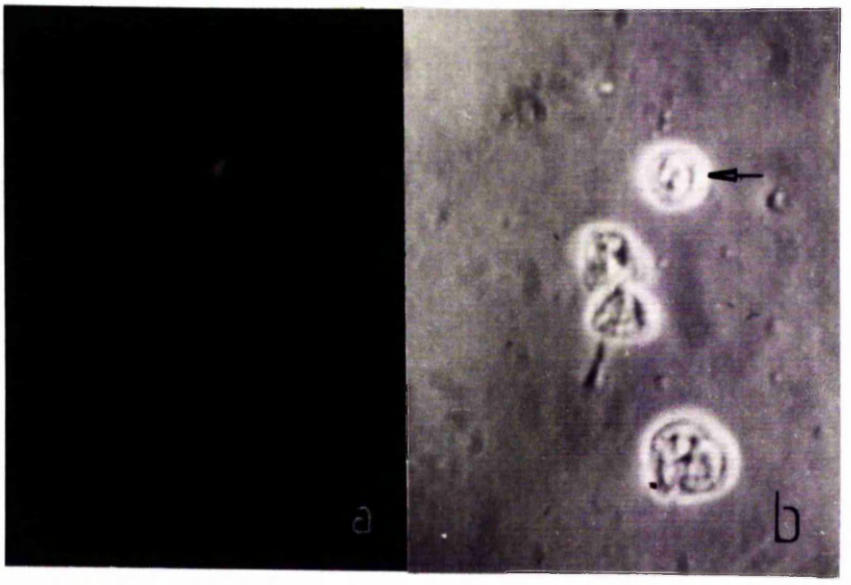


a) Fluorescent cells under UV light.
b) Phase-contrast microscophy of the same cells as in a). Arrows indicate those cells showing immunofluorescence. Magnification x 1300.

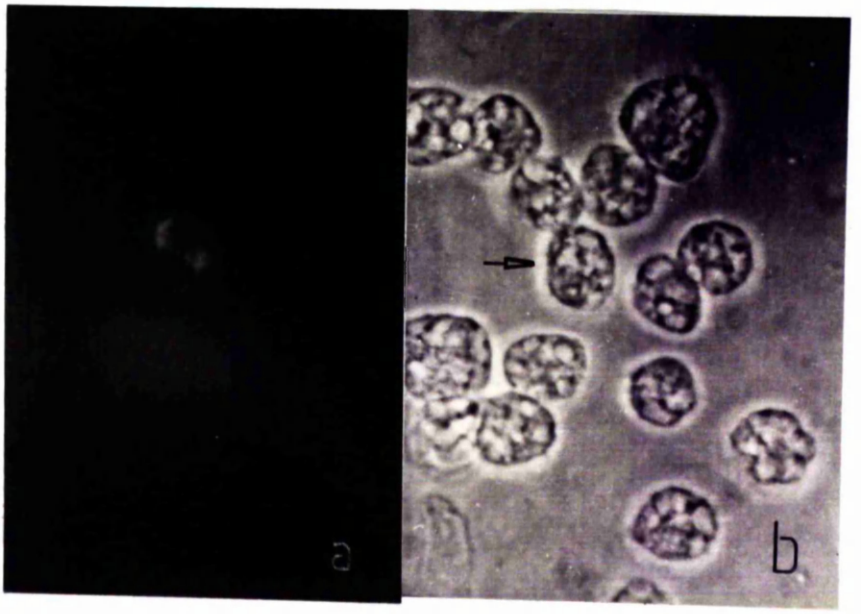
Figure 14

Adherent culture of resident and thioglycollate broth elicited peritoneal macrophages stained by indirect immunofluorscent method.

Resident macrophages



Thioglicollate broth-elicited macrophages



a) Fluorescent cells under UV light.
b) Phase-contrast microscophy of the same cells as in a). Arrows indicate those cells showing immunofluorescence. Magnification x 1300.

Table 5

Expression of Ia^k antigen on macrophages from unstimulated mice, *C. parvum*-inoculated mice and thioglycollate broth-inoculated mice.

	Macrophages from mice inoculated with:			
	Nothing (resident)	*C. parvum*	Thiogly broth	*M. tuberculosis*
Ia^+ve cells (percentage)	29.0±8.4	23.2±6.0	9.7±3.9	54.6±6.2
Ia^+ve cells (absolute numbers $x10^5$)	3.58	7.02	9.81	nd
Ratio* Ia^+ve cells	1	1.96	2.74	nd
Ratio* total macrophages	1	2.46	8.33	nd

Data of 4th day after inoculation. nd= not done.
Figures of percentage of I^+ve cells are from 4 independent replicated experiments in which 400-1000 cells were counted: mean ± S.D.
Figures of absolute numbers were calculated with data from table 1 'a' and 'b'.
* Ratios calculated on resident= 1.

4.3 Discussion

The normal cell population of the peritoneum, resident cells, is mainly formed by lymphocytes and macrophages but this situation changes when a foreign substance is introduced into the cavity. In such a situation the cells seen in the peritoneal cavity have accumulated there in response to the stimulant inoculated. The infiltration of neutrophils precedes that of the macrophages. The cells enter the cavity having been attracted by several chemotactic factors (for review see Wilkinson, 1982). The presence of chemoattractants for different cell populations in the peritoneum varies with the type of stimulant used. Wilkinson et al (1973) showed in guinea pigs that after the inoculation of glycogen (elicitation similar to that produced with thiogycollate broth) there is almost immediately chemotactic activity for macrophages and neutrophils. The chemotactic activity of the peritoneal exudate for neutrophils declined to undetectable levels after 3-4 days, whereas the attractant activity for macrophages was present and high for all four days. On the other hand, the peritoneum inoculated with coryneform bacteria (*C. liquefaciens*) showed the presence of chemotactic factors for neutrophils in waves, one on the first day and the other on the third, in contrast to the chemoattractants for macrophages which were present throughout and did not decrease (Wilkinson and O'Neill, unpublished).

Although the stimulants used differed (different Coryneform bacteria : *C. liquefaciens* vs *Propionibacterium*

acnes; glycogen vs thioglycollate broth) the respective patterns of peritoneal cell infiltrations studied here showed remarkable similarity with those patterns of cellular infiltration observed in the studies of chemotactic factors mentioned above. (N.B. Both C. liquefaciens and P. acnes are referred to as Corynebacterium parvum (Wilkinson, personal comunication).

The fact that neutrophils are attracted into inflammatory lesions in large numbers during the first few hours whereas there is a delay in the accumulation of macrophages does not require an explanation based on special chemotactic factors (Wilkinson, 1982). Neutrophils are present in large numbers in blood, there is a large marginated pool and a large marrow reserve of mature forms which can be released rapidly. Monocytes are present in smaller numbers in the blood and there is little marrow reserve of mature forms, so that differentiation from immature forms is required to increase the release of marrow monocytes into blood.

The yield of inflammatory macrophages is dependent upon the irritant injected and the time elapsed after treatment (Table 1b). The yields obtained in this work were less than those reported by Meltzer (1981) who, working with C3H/HeN mice, reported a yield per mouse of 4 $\times 10^6$ for resident cells and 30 $\times 10^6$ cells for mice inoculated with thioglycollate broth 7 days previously. These differences could reflect variation among mouse strains and methodological differences, since both methods of elicitation of the cells are similar but the time was different (4 vs 7 days). Tsuro et al (1984) reported

macrophage yields for C57BL/6 mice which, although harvested after 4 days, were less than half of those reported here for all three types of macrophages. Regarding CpM and tM, these differences were partially due to the amount of stimulant introduced. Tsuro's experiments were performed with half the quantities of stimulant used in the present study and there is also the alternative explanation of possible strain difference in response to the stimulants.

Comparison of the macrophages from the stimulated mice with the resident cells clearly showed a divergence in their morphological appearence. The cytocentrifugation was carefully controlled to avoid artifacts, leaving cells well spread and displaying fine nuclear and cytoplasmic detail. From the morphological pattern, i.e. a less dense nucleus with finer chromatin, it is apparent that the stimulated cells are a young population, the thioglycollate-elicited macrophages being more immature than those activated with C. parvum. This difference can be explained if the magnitude of cell proliferation is taken into acount; i.e. the macrophage production in the thioglycollate-inoculated mice has to be larger to provide the number of cells seen in the peritoneum. Therefore, the cells observed should have recently arrived from the blood stream and also recently produced in the bone marrow. In addition, there is the abnormal finding of macrophage-like cells in the blood of the thioglycollate-inoculated mice. This could be interpreted as indicating that the over-production of mononuclear phagocytes is such that accelerated

differentiation of immature cells was taking place to cope with the demand of the inflammatory stimulus.

Examination of cytopreparations stained with Leishman stain showed that in the elicited macrophages there were different vacuoles; i.e. basophilic 'granules' which in appearence and size are much like than those seen in granulocytes. It is possible that they had been ingested by the macrophages. This idea is based on the scavenging behaviour of the macrophages and the fate of the neutrophils in the early influx. The neutrophils are the first cells to arrive at the peritoneal cavity and even though present in large numbers, after a time they disappear. This disappearence could be interpreted as death of the neutrophils, hence the macrophages would take up their cellular debris and 'granules'. That these 'granules' remained in the macrophages undegraded or, more likely, partially degraded is speculative, but the fact that there exist methods for isolation of neutrophil granules (Baggiolini, 1972; Cohn and Hirsch, 1960), implies that the plasma membrane is less resistant than the membrane of the granules. Thus it is possible, if some of the neutrophils are lysed in the peritoneum without prior release of their granule contents, that some of these granules could remain as such in the peritoneum. Since neutrophil granules contain iron free lactoferrin (Baggiolini, 1972), it is possible that these granules seen in macrophages could influence the iron metabolism of the cells during inflammation. This aspect is discussed in section 5.3.

The identification of Ia^+ve cells in macrophages was selected in order to contrast the populations of cells generated by the immune-stimulant C. parvum against the noninmmune-stimulant thioglycollate broth, as a large number of reports implicate Ia antigen with immune processes, such as antigen presentation. Despite the fact that the molecular mechanism of antigen presentation is still in dispute, there is a general agreement of the essentially of the Ia molecule in antigen presentation (section 3.6, Unanue , 1984; Steinman et al, 1981). The macrophages elicited with oil, thioglycollate broth or peptone broth, which have low percentages (<10%) of Ia^+ve cells, could not generate T-cell activation in assays for 'antigen presenting cells'. In contrast, those cells activated with L. monocytogenes (Beller et al 1980) or BCG or T. cruzi are 50 to 100% Ia^+ve (Beller et al , 1980), and can cause T-cell activation, functioning as antigen presenting cells.

There is general agreement that the estimation of macrophages by inoculation of thioglycollate broth gives a low proportion of Ia^+ve cells (Beelen and Walker, 1983; Beller et al, 1980; Unanue, 1984). In agreement with those studies the percentage of Ia^+ve cells in the tM observed in the present work was low (~10%). But the finding of a large absolute number of Ia^+ve cells in tM could appear contradictory. These large values of absolute numbers in thioglycollate-injected mice are not in disagreement with other workers, because it is well established that this type of elicitation gives a large yield of cells. Thus it

is understandable that the tM studied in this work rendered a large number of Ia^+ve cells because the low percentage is multiplied by a large yield. This calculation of absolute numbers has not been made by other workers and, probably, this form of expression gives a better picture of the cell population.

On the other hand when the Ia^+ve cells found in CpM are expressed in absolute numbers it is apparent that both types of macrophages, CpM and tM, contain similar numbers of Ia^+ve cells (CpM $\sim 7 \times 10^5$, tM $\sim 10^6$; Table 5). This could be interpreted as indicative that the major difference in cellular production of macrophages between C. parvum- and thioglycollate broth-injected mice is in the number of Ia^- ve cells. The total yield of tM was more than three times that of CpM (Table 5).

Apparently the values presented here for CpM of Ia^+ve cells are the first reported, so it is not possible to compare with other authors. Nevertheless the comparison with rM, on a percentage basis, showed an unexpected similarity (29% in rM, 23%in CpM; Table 5). This similarity was unexpected due to the generalized assumption that immune stimuli give rise to a large proportion of Ia^+ve cells (Unanue, 1984). The explanation, probably, is that this type of stimulus (inoculation of C. parvum) gives different results to those stimuli (L. monocytogenes, BCG and T. cruzi) from which the generalization was made.

The term "activated" has been widely used to denote several types of macrophages, but the loose use of this term has lead to problems (Van Furth, 1970a; Karnovsky and Lazdins, 1978; Morahan, 1980; Wilkinson, 1983). These

problems might have arisen by the broad use of the term "activation" for macrophages that have been provoked to appear into the peritoneal cavity, historically mainly with the aim of increasing cell yields. Therefore the use of 'activated macrophage' will be restricted in this work to the macrophages evoked with C. parvum inoculation, in analogy to those activated with other bacteria (Mackaness, 1962, 1969; Hamilton et al 1984; Wilkinson, 1983). This is in keeping with the recomendations of Van Furth (1970a, 1981b) and Morahan (1980). The use of Ia antigen as a marker of activation is very controversial. Discussion of this controversy goes beyond the scope of this work, but one can exemplify the extreme views by the following statements: Unanue, 1984 has stated that "Two cardinal elements of macrophage activation --Ia expression and cytocidal function --follows a similar, if not common, activation pathway". In contrast to this Blumenthal et al (1983) have presented evidence showing dissociation of cytotoxic activity from Ia antigen expression, from which they conclude that "the presence of Ia on macrophages has nothing to do with activation". The lack of a high percentages of Ia^{+}ve cells in the adherent cells from C. parvum-inoculated mice, reported here, could also indicate that the direct association between activation and presence of Ia antigen is erroneous.

The studies presented and discussed in this section have permitted the characterization of the dynamics and magnitude of the cellular infiltration observed in the peritoneum of mice that have been inoculated either with C.

parvum or thioglycollate broth. The infiltrating cell populations were compared with the resident population present in unstimulated mice. The adherent cells from these three cell populations --macrophages-- were used for all the following experiments of iron metabolism in macrophages. The purity of all these macrophages is very high and it is evident that both activated and elicited populations of macrophages (CpM and tM) are formed by younger cells than resident macrophages (rM), tM being the most immature. These three cell populations offered the possibility of studing the events of iron metabolism in three type of peritoneal macrophages, in different differentiation state. Since the exudate cells appeared in the peritoneal cavity as a result of different stimuli they also permit a comparison between the behaviour of immunologically activated cells (CpM) and nonimmunologically elicited cells (tM) to be made, and thus an investigation of the underlying factors of iron retention during inflammatory processes.

Chapter 5

Iron metabolism in macrophages

5.1 Methods

5.1.1 Isolation of ferritin

The isolation of this protein was performed following the method reported by Linder and Munro (1972), with some variations. After collecting 100 g of livers or 30g of hearts from NIH mice, the tissue was minced with scissors and a homogenate was made in four volumes of water using a MSE (Crawley, Sussex, England) homogenizer. All materials were kept in an ice bath. The homogenate was heated to 70^{o} C for 10 minutes then cooled on ice and centrifuged for 15 minutes at 2000g to remove heat-inactivated proteins. The pH of the supernatant was adjusted to 4.8 with concentrated acetic acid, left for 4h, and the further precipitate removed by centrifugation. The pH was adjusted to 7.2 with 1M K_2HPO_4, solid ammonium sulphate was added to give 50% saturation and the solution was left overnight at 4^{o}C. The precipitate was removed by centrifugation at 2000g, dissolved in the minimum amount of PBS and dialysed against six changes of the same buffer until the reaction for sulphate with 1% $BaCl_2$ was negative. The sample was fractionated by gel filtration on AcA 22 (LKB; Bromma, Sweden) in a column (1.2 x 90 cm) eluted with PBS, and 1.5 ml fractions collected. The tubes with red colour, indicative of ferritin, were pooled and centrifuged at 110000g for 4 h in a L2-65 Beckman centrifuge to sediment the ferritin. After redissolving, the purity of the ferritin was checked by disc electrophoresis in polyacrylamide gel. For those preparations that showed impurities the steps from gel filtration onwards were

repeated. Protein content of the ferritin preparations was measured by the Lowry et al method (1951) using BSA (Sigma Chemical Co., Poole, Dorset, England) as standard.

5.1.1.2 Disc electrophoresis in polyacrylamide gels

The gels were prepared by the technique of Davis (1964), and run in the Davis buffer system. The resolving gel consisted of 10% polyacrylamide in 35uM Tris-10uM glycine buffer pH 8.8 and the stacking gel of 4% polyacrylamide in 1uM Tris adjusted whith 1M HCl to a pH of 6.8. The running buffer was 0.25M Tris added with 2M glycine up to reaching a pH of 8.5. Gels were run at 2mA per tube using bromophenol blue as a tracking dye, usually for 4 h. Gels were stained for protein with Coomassie blue and for iron with Perls' Prussian blue reagent (8 % ferricyanide w/v mixed 1:1 with 8% HCl v/v, both freshly prepared in iron free water). The criterion of purity used was that all the bands of protein coincided with bands staining positively for iron.

5.1.2 Production of antibodies against ferritin

The inoculum was prepared by emulsifying complete Freund's adjuvant and a solution of Ft (100 ug/ml) to give a final volume of 1 ml in a syringe of the same capacity. To make a stable emulsion the syringe was vibrated on a vortex for 20 minutes, with the plunger and needle secured with tape. The stability of the emulsion was tested by dropping a small part into a beaker with water.

The scheme for immunization of rabbits consisted of multiple subcutaneus inoculations in various sites, followed by 10 days rest, and then a similar repeat inoculation. After another 10 days a booster was given (50 ug of Ft in 1ml of PBS). The rabbits were bled after a week and the antisera tested by Ouchterlony gel diffusion. These antisera were salt fractionated 3 times with 33% $(NH_4)_2SO_4$ (by adding saturated $(NH_4)_2SO_4$ solution e.i. 50% of the original antisera volume), aliquoted, and stored at -20 C.

5.1.3 Affinity chromatograpy

The following Sepharose 4B congugates were prepared. Sepharose-Rabbit antimouse liver ferritin (S-R-antiMLFt), Sepharose-normal Rabbit IgG (S-RNIgG), Sepharose-mouse liver ferritin (S-MLFt) and Sepharose-Sheep antimouse IgG (S-S-anti-MIgG). The same procedure was used in each case, as follows (Esparza 1979; Pharmacia). The required amount of CNBr-activated Sepharose 4B (Pharmacia Ltd., Milton Keynes, England) was swollen for 15 minutes in 1mM HCl and washed on a sintered glass filter with 200 ml/g of gel of the same solution. The gel was washed and suspended in the coupling buffer (0.1M $NaHCO_3$ pH 8.3 containing 0.5M NaCl). The gel was centrifuged for 3 minutes at 500g, the supernatant discarded, and the gel immediatly mixed with a solution (2 x the volume of gel) of the protein to be coupled in same buffer. The antibody to conjugate (IgG fraction) was extensively dialysed against the coupling buffer, prior to adjusting the concentration to 5-10 mg/ml as determined by the absorption at 280 nm. For ferritin an

appropiate dilution was made in the coupling buffer.

The mixture was left rotating in an end-over-end mixer for 2 h at room temperature, after which it was centrifuged and the supernatant saved to calculate by difference the amount of protein coupled. The remaining active groups were blocked by addition of 2 volumes of 1M ethanolamine and rotating for 2h more at RT. Excess protein and blocking agent were removed by washing the gel alternated with 0.1M sodium acetate buffer pH 4 containing 0.5M NaCl followed by coupling buffer. This procedure was repeated three times. The conjugates were stored at $4^{o}C$ in PBS containing 1% BSA + 0.02% sodium azide. The binding capacity of S-R-antiMLFt was measured with ^{125}I-Ft labelled by the chloramine T method in order to find the minimun gel volume which could bind 1 ug of ferritin. Desorption of the ligand from the immunoadsorbents was achieved with a chaotropic ion (3M KSCN in 0.1M $NaHCO_3$ pH 8.5) followed by gel filtration on Sephadex G-25 (Pharmacia).

5.1.4 Optimal proportions for antibody-antigen reactions

The equivalence point of antigen-antibody reaction was determinated by technique of Sewell (1968). The antiserum and antigen in opposite serial dilutions in Eppendorf tubes were allowed to react and after 30 minutes incubation at $37^{o}C$ the tubes were centrifuged at 10000g. The supernatant of each tube was tested in a double immunodiffusion plate against undiluted antiserum and antigen placed in alternate wells. After 1 day of incubation in a humified chamber at RT, excess protein was removed by overnight

washing in PBS, and the gels were air dried and stained with Coomassie blue.

5.1.5. Labelling of rabbit antiferritin with iodine 125

The anti ferritin (R-antiMLFt) was labelled with the Bolton & Hunter (Bolton and Hunter, 1973) reagent (Amersham International plc., Buckinghamshire, England), attached to a Sepharose-Ft complex (S-Ft) protect its binding site, using the method of Alvarez-Hernandez and Loria (1980), as follows. An excess of R-antiMLFt (0.5 ml) was incubated overnight with 50 ul of packed S-Ft complex in an end over end rotator. The S-Ft-R-antiMLFt complex was washed 5 times with 0.1M borate buffer pH 8.2 and left with the minimun volume of liquid. The benzene in which the ^{125}I-Bolton and Hunter reagent is supplied was dried under a gentle stream of nitrogen which was passed through a dessicant tube packed with $CaCl_2$ and silica gel in order to remove any trace of moisture. When the ^{125}I-Bolton and Hunter reagent was completely dry the S-Ft-R-antiMLFt complex was added to the vial which was placed on ice for 15 minutes, stirring periodically. Glycine (10mM; 50ul) was then added to the reaction mixture to block remaining reagent and the complex was removed from the vial and passed through a column of Sephadex G 25 (Pharmacia) in a disposable 10 ml pipette (Sterilin), equilibrated with PBS. One ml of potassium thiocyanate (3M in 0.1M borate buffer pH 9.0) was added to the top of the column. After 5 minutes elution was started, firstly allowing the KSCN to enter the gel bed, then eluting with PBS. Fractions of 0.5ml were collected by hand, and an aliquot of each (10ul)

was counted in a gamma counter (Compugamma 1282, LKB Wallac, Croydon, Surrey, England). The presence of SCN^- was tested with a drop of 10% $FeCl_3$ which gives a dark red colour. The high-molecular weight fractions were pooled, aliquoted and stored at $-20^{o}C$.

5.1.6.1 Immunorariometric assay for ferritin (IRMA)

The assay for ferritin was based on a 2 site-IRMA (Miles et al, 1974) with some modifications (Alvarez-Hernandez et al, 1981), the principle of which is the binding of a cold antibody to a surface, allowing the antigen to react, and detecting how much is present by a second labelled antibody. This gives a direct dose-response curve.

During the optimization of the method several kinds of tubes (polystyrene and others) were initially tested in order to find which type adsorbs most protein. Testing was caried out using the ^{125}I anti-Ft and counting the radioactivity that remained after washing. However it was finally decided that the best results were obtained with a multiplate (Cat. No. 76-364-05, S-MRC-96-Clear, U-shaped wells-clear; Linbro Chemical Co., New Haven, Conn., USA). Initially the wells were cut off for counting with a horizontal hot wire, but this practice was abandoned, because the well to well variation was higher than if the plate was cut up with scissors.

5.1.6.2 Titration of first and second antibodies

The titration of the first antibody was done by maintaining constant the amount of the second antibody (10000 cpm/well), and using known Ft concentrations. The titration of the second antibody was carried out by maintaining the first antibody constant. The aim of these experiments was to find the lowest concentration giving a linear response with an adequate working range.

The conditions and the working protocol finally chosen were as follows:

1) A 1:500 dilution of the first Ab in 0.01 M sodium carbonate buffer pH 9.2 was freshly made (both buffer and dilution) and 50 ul were incubated overnight to allow adsorption to wells.

2) Unbound antibody was removed by washing 3 times with 100 ul of PBS.

3) The plate was incubated for 4 h with 100 ul of 0.5 % BSA (RIA grade; Sigma) in PBS, to prevent nonspecific binding of antigen or of the second antibody. It has also been shown that this improves the linearity of the standard curve (Alvarez-Hernandez and Loria, 1980).

4) Unbound albumin was removed as before (step 2).

5) Different concentrations of standard Ft (0, 5, 10, 15, 20, 25, 50, 100, 500 and 1000 ng/ml) or the unknown samples in at least two different dilutions (normally 1:20 and 1:75), were incubated overnight (50ul), each one in quadruplicate. All dilutions were made in PBS containing 1% BSA to avoid a matrix effect.

6) Unbound antigen was removed as before (step 2).

7) The plate was incubated overnight with 50 ul of a dilution containing approximatly 30-35000 cpm of the labelled R-antiMLFt.

8) Unbound antibody was removed as before (step 2).

9) The wells were cut up and counted in a gamma counter for 5 minutes.

If any of the samples gave a value above the upper limit of the standard curve, they were reassayed at a higher dilution.

5.1.7 Double labelling of transferrin

Human tranferrin (Behringwerke, Hoechst, Hounslow, Middlesex, England) was labelled first with ^{59}Fe by overnight incubation of 1mg of transferrin, dissolved in PBS containing 1% $NaHCO_3$ with 1 ug of ^{59}Fe-citrate (11.7 uCi/ug, Amersham). Iodination was carried out by the Iodogen method (Fraker and Speck, 1978), as follows. The Iodogen (Sigma) (200 ul; 100 ug/ml in benzene) was dried under a gentle nitrogen stream at the bottom of a glass tube. The protein was added and then 500 uCi of carrier-free $^{125}Iodine$ (Amersham). The reaction was allowed to proceed for 15 minutes after which 100ul of KI(1M) was added and the mixture removed from the tube. Removal of free iodine was performed on a Sephadex G-25 (Pharmacia) column in a disposable 10 ml pipette (Sterilin).

Radioactivity was counted in a Compugamma counter (LKB) and the fractions of high molecular weight containing both ^{59}Fe and ^{125}I were pooled. Optical density at 280 nm was measured in a spectrophotometer (LKB) and the protein concentration was calculated using the extintion coeficient

for a cell of 1 cm of 1.4 (Sober, 1973).

5.1.8 Release of iron by peritoneal macrophages

The iron released by peritoneal macrophages was determined by the method reported by Esparza and Brock (1981b), which consists in measuring the release of ^{59}Fe and ^{125}I following the ingestion of ^{59}Fe-^{125}I-transferrin-anti transferrin immune complexes (^{59}Fe-^{125}I-Tf-anti- Tf). The equivalent point of Tf-anti-Tf was found following the method described in section 5.1.4. Normally, 75ug of human Tf precipitated with 100 ul of anti-human Tf (SAPU, Lanarkshire, Scotland). After sterilization of the labelled ^{59}Fe-^{125}I -Tf (section 5.1.7) by passage through a 0.45um Millipore filter (Millipore S.A., Molsheim, France). Immune complexes were prepared by adding sterile antiTf up to the equivalence point. This antigen-antibody mixture was incubated at 50°C for 15 min in a water bath and then placed at 4°C for 5 min. The resulting insoluble immune complexes were centrifuged at 2000g for 5 min, washed twice with PBS and resuspended in RPMI + 10% FCS medium. The adherent macrophage cultures (section 4.1.1) were incubated with 1 ml of the resuspended immune complexes, containing usually 50ug of insoluble Tf per well, at 37°C for 2-2.5h to allow ingestion. After this time the cultured macrophages were washed twice with 2ml of HBSS to remove the non-phagocytosed immune complexes. Fresh medium was added and the cells incubated overnight (18h) at 37°C. All wash supernatants and medium containing the non-phagocytosed immune complexes were retained. After

overnight incubation the culture medium was removed and the adherent macrophages washed twice with 2ml of HBSS. In order to lyse the macrophages 1 ml of 2% SDS in water (w/v) was added to the adherent cultures. This macrophage lysate contains the radioactivity that remained intracellularly. The culture medium was centrifuged at 400g for 4 min immediately after removal to spin down cells that become unattached during the incubation period. Culture supernatants and washes were treated with equal volumes of 20% trichloroacetic acid (TCA) to precipitate ^{125}I present in nondegraded or partially degraded Tf and to freed the iron released by the macrophages which at this time is in the culture medium Tf. After centrifugation at 2000g for 30 min the supernatants were collected and the precipitates washed with 10% TCA. The TCA supernatants contain free ^{59}Fe and ^{125}I. The ^{59}Fe and ^{125}I radioactivity in the supernatants, washings, precipitates and in the cell lysates were counted simultaneosly in a gamma counter (1282-Compugamma, LKB, Croydon, Surrey, England). This counter of radioactivity gamma has dual channels and corrects the spillover for both radioisotopes. The release of ^{59}Fe and ^{125}I were calculated according to Esparza and Brock (1981b) in total radioactivity-ingested and a release factor (^{59}Fe:^{125}I), therefore, can also be calculated.

5.1.9 Ferritin levels in different types of macrophages

The level of ferritin in macrophages was measured by the IRMA in a lysate of adherent cultures (section 4.1.1) made by adding 1 ml of distilled water to the wells and

measured in the same lysate, to relate Ft to protein. The three different type of macrophages (CpM, rM, tM) were always measured in the same assay.

5.1.10 Iron content of macrophages

Adherent macrophage cultures were washed once in HBSS and three times with PBS, and lysed with 2% sodium dodecyl sulphate (SDS; BDH). The SDS and PBS were prepared with deionised distilled water, and plastic-ware known to be iron-free (Mainou-Fowler, 1985) was used throughout. The iron content of the lysates was assayed by carbon furnace atomic absorption spectroscopy carried out by D. Halls (Royal Infirmary, Glasgow), and protein by the Lowry et al 1951.

5.1.11 Release of ferritin by macrophages

Ferritin in the culture media after overnight incubation (14h) of the adherent macrophages (section 4.1.1) was measured by IRMA. To determine if the ferritin measured in the supernatant was coming from actual secretion or from leakeage from dead cells, three strategies were adopted. Firstly, viability of the loose cells that become unattached after the incubation period was assayed by exclusion of 0.2% eosin (section 4.1.1). Secondly release of lactate dehydrogenase (LDH; Sigma; section 4.1.3) was measured as a parameter of cell lysis, and thirdly glycosylation was inhibited with 3ug/ml of tunicamycin (Sigma).

5.1.12.1 Synthesis and degradation of ferritin

The adherent cells were incubated for 14 h in Minimal Eagles Medium (MEM) without glutamine or leucine (Gibco) supplemented with 5% FCS, 0.4 MBq/ml of ^{3}H-leucine (Amersham, specific activity 537 TBq/nM), 300 ug/ml glutamine (Analar grade, BDH), 10 mM mercaptoethanol (BDH), 50 uM cold leucine (BDH), penicillin (100 Iu/Ml; Flow) and 100 ug/ml of streptomycin (Flow). After incubation the medium was removed and the cells were washed 3 times in HBSS and lysed with 1 ml of 1% Triton X-100 (Tx; BDH) in PBS. The lysate was centrifuged for 5 minutes at 10000 g in a microcentifuge (Hawksley, Northampton, England).

Initially, for evaluating specific radioactivity incorporated into Ft, direct precipitation, a double antibody technique was attempted by firstly incubating the lysate for 3h with 25 ul of R-antiMLFt, or with normal RIgG as a control for nonspecific binding of radioactivity, and secondly adding Sα-RIgG at equivalence. A very high background, masking all specific radioactivity, was found even after 6 washes with 1 ml of Tx-PBS. Testing a series of different buffers for lysing and washing yielded no improvement, so it was decided to use a S-R-anti-MLFt complex, with a binding capacity of at least 1 ug of Ft, and as a control S-RIgG, both with the same content of protein (6mg/ml of gel). The best results for washing the complexes were obtained using a buffer consisting of PBS containing 0.5% BSA, 1% Triton x-100 and 1% Nonidet P40 (NP40; BDH). The minimum number of washes needed to remove unbound radioactivity was found by counting 100 ul of the

supernatant.

The following method was then adopted:

After the incubation period the radioactive medium was removed and an aliquot of 20 ul was taken to count unutilised radioactivity. Cells were washed three times with HBSS, and 1 ml of the PBS-BSA-Tx-NP40 buffer was added to lyse the cells. The plates were left at room temperature, shaking them several times. The lysate was transferred to Eppendorf tubes (Anderman and Co. Ltd., East Molesey, Surrey, England) and centrifuged for 5 minutes at 10000g. The supernatant was then added to another tube which contained 75 ul of 50 % (V/V) of either S-R-antiMLFt or S-RIgG, and rotated at RT for 3 h to allow binding to occur. An aliquot of the supernatant was saved for determining total remaining intracellular radioactivity. The immune complexes were washed 5 times with 1.5 ml of PBS-BSA-Tx-NP40. The complexes were drained and quantitatively transferred to vials with 2 ml of scintillation fluid (Ultrafluor; National Diagnostics, Somerville, New Jersey, USA). Radioactivity was counted in a Packard 3320 scintillation counter. Parallel cultures without ^{3}H-Leucine were run in order to determine total protein content.

The rate of degradation was measured by pulse-chase experiments. The radioactive medium was changed, and after the cells had been washed with HBSS, RPMI + 10% FCS added, and the incubation was continued for different times after which the ^{3}H-Leu incorporation into Ft was determined as above.

5.1.12.2 Induction of ferritin synthesis by iron

Induction of ferritin synthesis was performed on 35mm Petri dishes by overnight incubation (14h) of the adherent macrophages in MEM-^{3}H-leucine, as described above, in the presence of different concentrations of iron nitrilotriacetate (FeNTA).

5.1.12.3.1 Iron uptake by macrophages

Uptake of iron was determined by incubating the adherent cultures for 14h in RPMI + 10% FCS containing various concentrations of iron nitrilotriacetate (FeNTA) trace-labelled with ^{59}Fe (11.7 uCi/ug; Amersham). Precautions were taken to ensure homogeneity of the iron species (section 5.1.12.3.2). At the end of the incubation, an aliquot of media was saved, the cells were washed three times with HBSS and 1ml of 2% SDS in H_2O was added to lyse the cells. ^{59}Fe activity was measured in media and lysates in a gamma counter.

5.1.12.3.2 Homogeneity of iron nitrilotriacetate

Homogeneity of ^{59}Fe-FeNTA was tested after mixing FeNTA with ^{59}Fe-citrate (Amersham) up to a ratio 10:1 (w/w). An ascending chromatography was performed in Whatman 3MM paper, the mobile phase consisting of glacial acetic acid:ethanol:deionised water (3:2:5). After running and air drying, the chromatograph was sprayed with acid-ferric thiocyanate (Perls' Prussian blue reaction) to detect the presence of iron, and radioactivity was counted in evenly cut 2 x 5 mm strips of the paper in a gamma counter (LKB).

5.1.12.4 Synthesis of ferritin subunits

Adherent macrophages were incubated in MEM+^{3}H-leucine as described before. After lysis and centrifugation the supernatant was divided in to two equal parts and one was placed in a water bath at 75^{o}C for 10 minutes to precipitate free ferritin subunits leaving heat-stable shells in solution (Drysdale, 1983). After this procedure specific radioactivity incorporated into ferritin was determined and synthesis of ferritin subunits was obtained by subtraction from the figure given by an unheated control.

5.1.12.5 Membrane associated ferritin

The ^{3}H-Leucine incorporation was carried out as described but the adherent macrophage cultures were established from mice which had been inoculated 1, 2, 3 and 4 days beforehand.

Cells were lysed by addition of 1ml of PBS to the adherent culture and freeze-thawing twice, disruption being checked by phase-contrast microscopy. This PBS-lysate was transferred to an Eppendorf tube and centrifuged at 10000g for 3 min, the supernatant was kept aside and the precipitate washed 3 times with 1 ml of PBS. The Petri dish was washed 3 times with 1 ml of PBS, after which 1.5 ml of PBS-BSA-Tx-NP40 buffer (section 5.1.12.1) was added to solubilize the macrophage membrane that remained attached to the dish bottom. These solubilized membranes from the Petri dish were combined with the precipitate of the PBS-lysate, which contains all cell debris, and thus

more membrane. After resuspension of the cell debris to allow solubilization of the protein associated with membranes, the tube was centrifuged at 10000g for 3 min to eliminate PBS-BSA-Tx-NP40 buffer-insoluble debris. The supernatant of this centrifugation contains all the protein associated with membranes, and was called membrane fraction. The cytosol fraction (supernatant of PBS-lysate, 1ml) was added to 0.5 ml of PBS-BSA-Tx-NP40 of triple concentration to give similar conditions to the membrane fraction . Determination in both fractions of specific ^{3}H-leucine incorporation into Ft was carried out as described before.

5.1.13 Intracellular distribution of iron

5.1.13.1 Gel filtration. Intracellular distribution of iron was investigated in adherent macrophages that had been incubated with ^{59}Fe-^{125}I-transferrin-antitransferrin immune complexes (^{59}Fe-^{125}I-Tf-anti-Tf) for 1.5h. After removal of uningested immune complexes (section 5.1.8) the adherent cultures were incubated in RPMI + 10% FCS for 3 or 22h. The cells were then lysed with 1 ml of 1% Tx-PBS and after 5 minutes centrifugation at 10000g the cell lysate and the supernatant from the incubation period were subjected to gel filtration in AcA-22 (LKB) equilibrated and eluted with 1% Tx-PBS (Tx-PBS) in a 90 x 1.2 cm column. The column was calibrated with blue dextran (Sigma), purified mouse liver ferritin (section 5.1.1.1), ^{59}Fe-^{125}I-transferrin (section 5.1.7) and orthotoluidine blue (BDH) run under the same conditions.

^{59}Fe and ^{125}I were counted in the fractions from the column and in the precipitate from the lysate which contains cells debris, partially degraded immune complexes and posibly haemosiderin.

5.1.13.2 Distribution of intracellular iron using desferrioxamine. Intracellular distribution of iron was investigated in adherent macrophages that had been incubated with ^{59}Fe-Tf-anti-Tf immune complexes for 1.5h. After removal of uningested immune complexes the adherent cells were lysed with 1 ml of 1% Tx 100-PBS containing 1mM DFO (Sigma) and the lysates were centrifuged for 5 min at 10000g. The lysate precipitates were resuspended in 1.5 ml of 0.1M sodium acetate pH 4.3 containing 2% (w/v) SDS and immediatly centrifuged for 5 min at 10000g. These supernatants, which contained iron liberated from any undegraded immune complexes, were counted and then discarded. The precipitates, after a further washing with PBS, were kept for counting radioactivity. The acetate-SDS-buffer was selected from several other buffers tested due to its ability to solubilise iron from undegragaded Tf-anti-Tf immune complexes. Thus, the residual insoluble ^{59}Fe left in the cell debris of the lysates after this acetate-SDS buffer treatment could be taken as representing iron in Hs. The Tx 100-PBS lysate supernatants were transferred to Eppendorf tubes containing 50 ul of packed S-R-antiMLFt immunoadsorbent, and rotated at RT for 3h to allow binding to occur. The immunoadsorbents were sedimented by centrifugation for 1 min at 10000g, washed 3 times with Tx 100-PBS and kept for counting radioactivity. This fraction

represents Ft-bound iron (^{59}Fe-Ft). The supernatants from the immunoadsorption were further fractionated in Amicon Ultrafiltration cells (Amicon Co., Lexington, Mass., USA) fitted with PM 10 membranes. This ultrafiltration was performed by centrifugation of the Amicon-U-cells in a fixed angle rotor centrifuge (Super-Minor,MK2;MSE) at RT at 2000 rpm for 1h. It had been previously shown that the PM 10 membrane did not bind DFO-chelated iron. The volume of the retentate after 1h of centrifugation, is, apparently, the minimum achievable, because it was not possible to reduce this volume with longer centrifugation. The volume of the retentates was always less than 75ul. The retentate contains ^{59}Fe bound to soluble proteins (other than Ft) of M.W. >10000 d. This fraction was called 'intermediate M.W.-^{59}Fe' and it probably consists mainly of iron in enzymes. The filtrate contains low M.W.-^{59}Fe (<10000 d), essentially all of which is bound to DFO. This fraction was called "chelatable-^{59}Fe". The ^{59}Fe in all 4 fractions was counted in a gamma counter (LKB).

This procedure thus allows fractionation of intracellular ^{59}Fe into fractions consisting broadly of Hs, Ft, other protein-bound Fe and a low M.W. chelatable pool.

R e s u l t s

5.2.1 Release of iron by peritoneal macrophages.

The proportion of ingested iron released following incubation with ^{59}Fe, ^{125}I-transferrin-anti-transferrin immune complexes was greater from rM than from tM (Table 6). However, even more iron was released by CpM. The rate of degradation of immune complexes, as measured by the release of TCA-soluble ^{125}I was, however, lower in rM than in CpM and tM, which were similar to each other. Comparison of the rates of release of ^{59}Fe and ^{125}I revealed that rM and CpM released iron at about the same rate in relation to the rate of degradation of the complexes, but this 'release factor' was 3-4 fold lower for tM.

5.2.2 Ferritin and iron content of peritoneal macrophages.

The Ft content of macrophages was measured by an immunoradiometric assay (IRMA), described in section 5.1.6. The results of preliminary experiments carried out to develop an IRMA for Ft are presented first.

5.2.2.1 Isolation of Ferritin. Since Ft is a family of isoproteins with immunological differences (Bomford and Munro, 1980), it was of interest to isolate Ft from organs such as liver and heart which are known to have different characteristic isoFt isoelectrofocussing patterns (Arosio et al, 1978). The elution pattern of mouse Ft in ACA 22 (Fig. 15) showed a large peak for liver Ft with the typical

Table 6

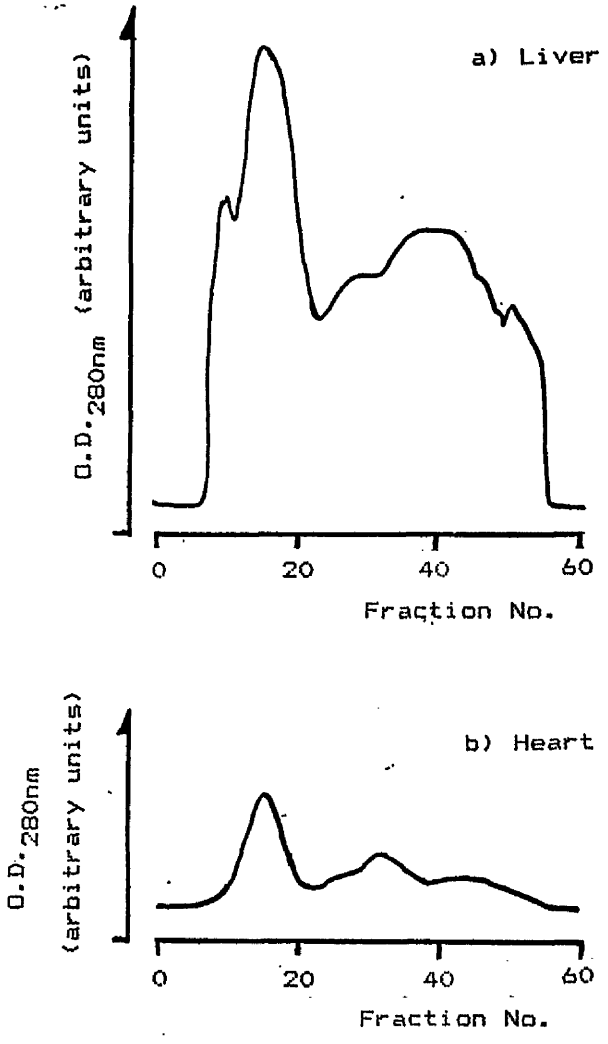
Handling of ^{59}Fe-^{125}I-transferrin-antitransferrin immune complexes by mouse peritoneal macrophages.

	Type of macrophage:		
	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
Ingestion of complexes (%)	41 ± 1	54 ± 1	56 ± 1
Release* of ^{125}I TCA-soluble	33 ± 1	58 ± 1	61 ± 1
Release* of ^{59}Fe	21 ± 0	30 ± 0	10 ± 1
Release factor $^{59}Fe/^{125}I$	0.62	0.52	0.16

* percentage of isotope ingested
Figures are mean ± standard deviation n = 3

Figure 15

Gel filtration on AcA 34 of redissolved and dialysed $(NH_4)_2SO_4$ precipitate of mouse liver and heart homogenates.



O.D. = optical density. Eluting buffer PBS : Column 1.2 x 45 cm : Fraction 3 ml (60 drops).

brown-red colour. The peak eluting at the M.W. of Ft from the sample from heart showed less colour. After ultracentrifugation of the peaks, their purity was tested by disc electrophoresis which showed that there was concordance between bands that stained for protein and those stained for iron, and that there were no extra protein bands. The yields obtained of the purified proteins were very low, 4.15 mg of liver Ft and 250 ug of heart Ft. These Fts were used to raise antibodies and as standard for the IRMA. The liver Ft was also used to produce an immunoadsorbent coupled to Sepharose 4B.

5.2.2.2 Antisera against mouse Ft. The Ouchterlony gel diffusion of salt-fractionated antisera against liver Ft and heart Ft revealed that the antiliver Ft gave a strong precipitin band against liver Ft, a less strong line towards heart Ft and none against normal mouse serum. The antibody against heart Ft gave a faint reaction towards both types of Ft. It was concluded that the antiliver Ft was sufficiently strong to recognize liver Ft, that it also could recognize heart Ft, and that the antiheart Ft was so weak that the rabbit would need to be boosted, but there was not enough antigen to perform this. It was found in subsequent experiments (section 5.2.2.3) that it was possible to use only the antiliver ferritin to measure total Ft content and detect the synthesis of Ft in macrophages. The rabbit antimouse liver Ft (RαMLFt) was therefore used for the IRMA as first and second antibodies and to prepare an immunoadsorbent.

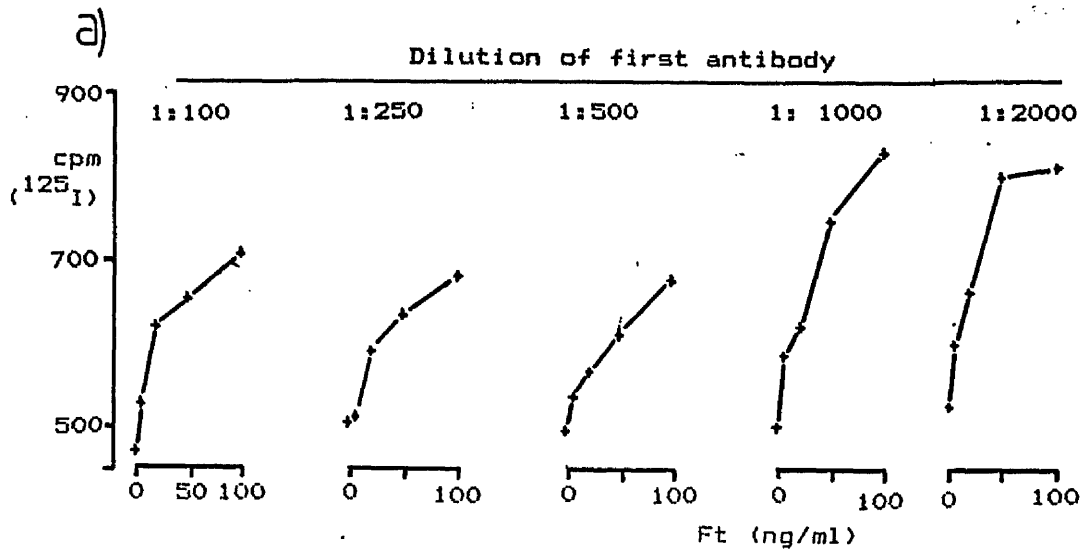
5.2.2.3 Immunoradiometric assay for Ft (IRMA). The R-antiMLFt was labelled with the Bolton & Hunter reagent as described in section 5.1.5. During the optimization of the method the dilutions of the two Abs were chosen on the basis of finding the lowest concentration giving a linear response with an adequate working range (see Fig.16). Under these conditions, the IRMA, showed good linearity (Table 7). The assay did not present the so called hook effect, which can give rise to false low responses at high concentrations of the antigen, up to at least 1 ug/ml (Fig. 17). The sensitivity of the assay was high, as low as 2.5 ng/ml (0.125 ng in the system), but it was decided that if the cpm of the unknown were less than those of the 5ng/ml standard the sample would be reassayed.

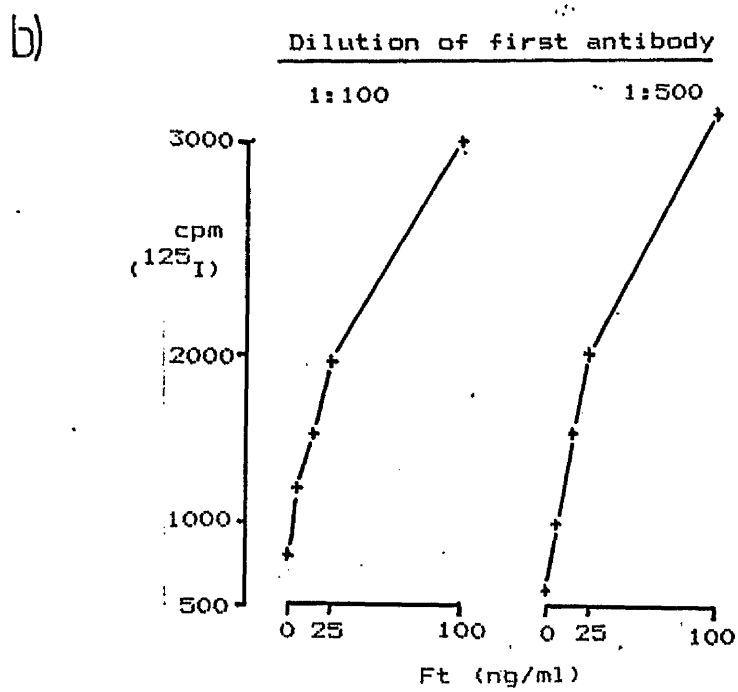
The antiheart Ft was tested in the IRMA as first Ab and found to be very weak, giving a low dose response curve. On the other hand the antiliver Ft gave a good dose response with heart Ft as standard (Fig 18), hence it was decided to carry out the experiments with only the antiliver Ft.

The effect on sample dilution was tested using macrophage lysates. A high degree of variability was found at low dilutions (Fig. 19) and there was also a matrix effect at dilutions up to 1:20. Further dilutions were made from lysates previously shown to contain high concentrations of Ft, and it was found that from a 1:20 dilution up to 1:75 the variation was acceptable, the coefficient of variation (CV) range being 7.1-12.8%, for this assay (Table 8).

Figure 16

Dilutions of first and second antibodies for standard curve of IRMA for mouse ferritin.





a) Second antibody dilution contained 10000 cpm of [125]-I.
b) Second antibody dilution contained 35000 cpm of [125]-I.
Liver Ft was used as antigen and anti-LFt as antibodies.

Table 7

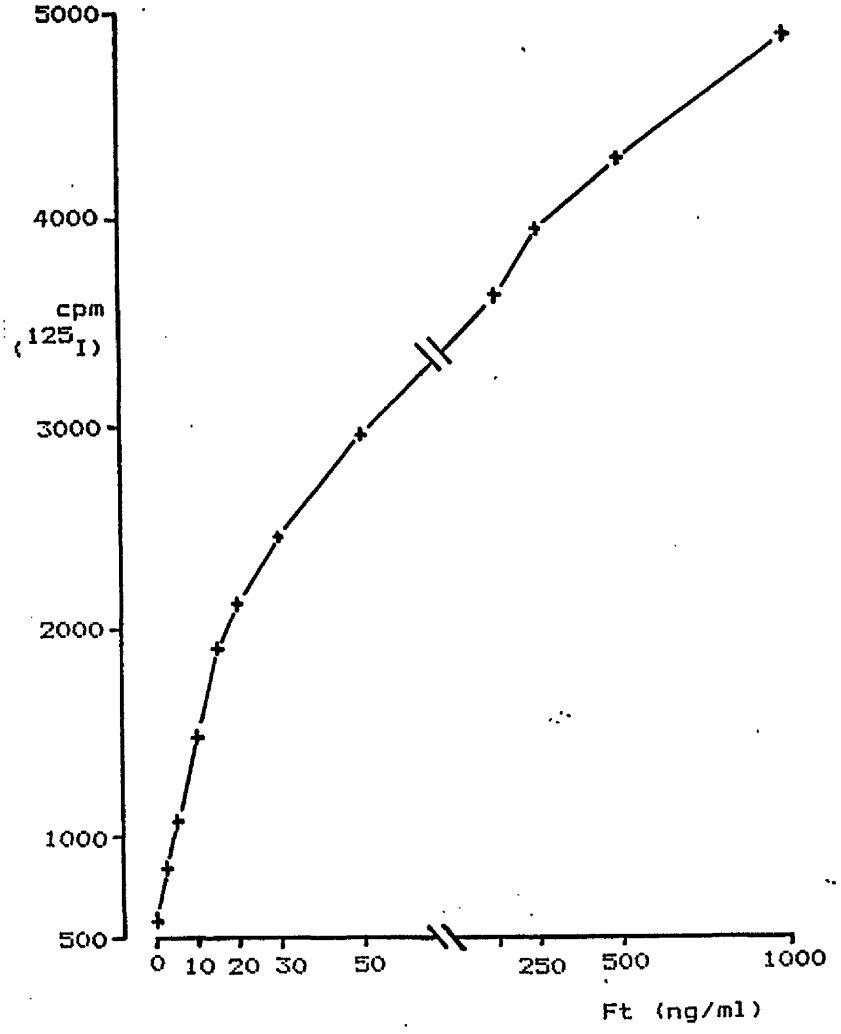
Correlation coefficients of IRMA for mouse ferritin.

Standard range (ng/ml)	n	Mean ± S.D.	CV	Range
0-25*	8	0.986 ± 0.009	0.94	0.967-0.999
0-30	4	0.984 ± 0.009	0.98	0.974-0.997

CV = coefficient of variation.
* Two batches of labeled Ab

Figure 17

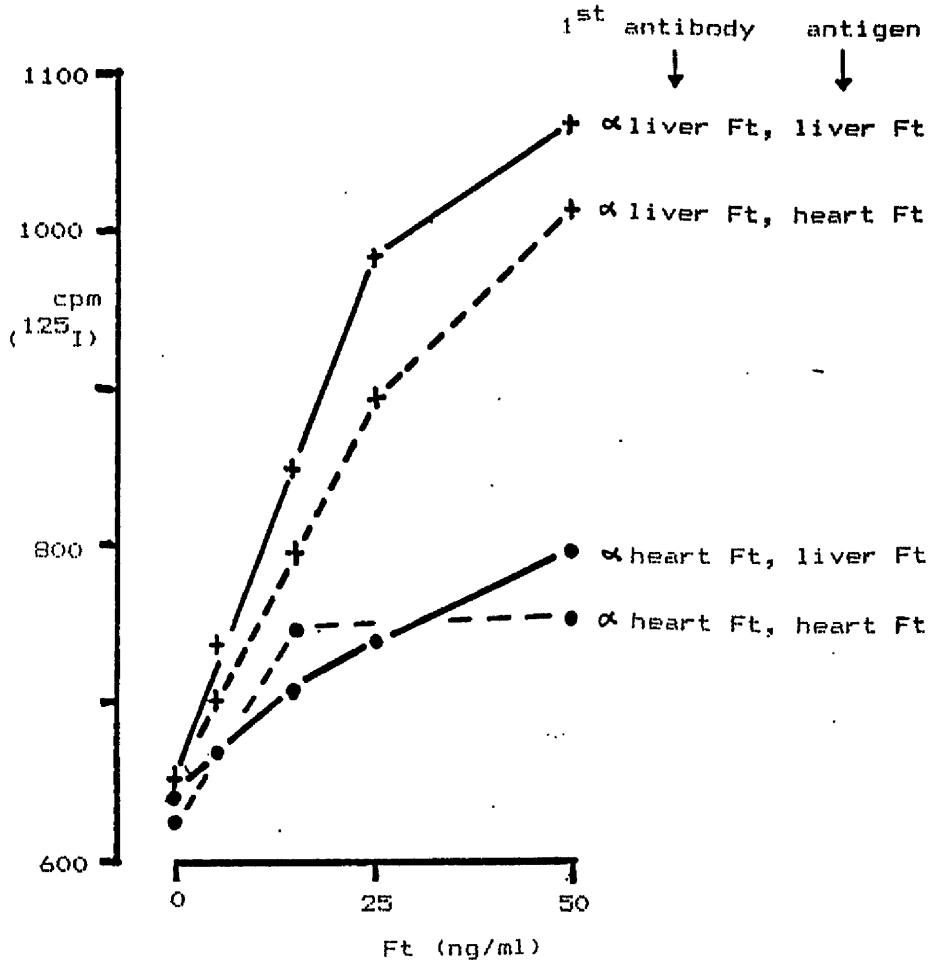
Working standard curve of IRMA for mouse ferritin.



Note absence of "hook effect" up to 1000 ng/ml.

Figure 18

Standard curve of IRMA for mouse liver and heart ferritin.



The second antibody was always labelled anti-LFt.

Figure 19

Effect of sample dilution on the IRMA for mouse ferritin in lysates of peritoneal macrophages at low dilutions.

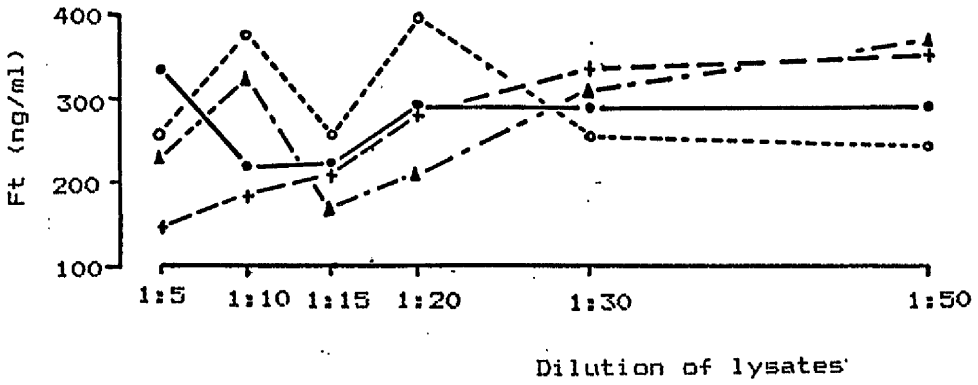


Table 8

Sample dilution effect on the IRMA for mouse ferritin. Lysates of peritoneal macrophages.

Lysate No.	Ferritin concentration (ng/ml) with sample diluted: 1:20	1:30	1:50	1:75	Mean ± S.D.	CV
1	1000	1125	1055	1240	1105 ± 103	9.2
2	850	801	716	750	779 ± 59	7.5
3	1000	838	854	1091	945 ± 121	12.8
4	1100	1050	959	952	1015 ± 72	7.1
5	2100	1800	2000	1712	1903 ± 178	9.4
6	900	900	762	1046	902 ± 116	12.8

CV = coefficient of variation.
Each dilution point was performed in quadruplicate.

5.2.2.4 Ferritin and iron levels in macrophages. The lowest content of Ft was found in tM and the highest in rM (Table 9). In order to obtain a reliable parameter for comparison among different types of macrophages the results are expressed as a proportion of total protein. Scraping the adherent cells from the Petri dish and then counting them could give false high values because the results would be related to only those cells that survived such treatment. However, it is also possible to express as ng of Ft per million cells, the cell numbers being calculated from the protein values using the conversion factors reported by Hamilton *et al* (1984a), these being (in $mg/10^7$ cells), 0.85 for rM, 1.17 for CpM and 1.48 for tM.

The iron content of macrophages showed that the tM, as well as having low Ft levels, also have a low content of iron. However, the Fe:Ft ratio was similar in rM and CpM but considerably higher in tM.

5.2.3 Ferritin release by peritoneal macrophages.

5.2.3.1 Comparison of ferritin release by resident, activated and elicited macrophages. The Ft level in culture supernatants of macrophages after 1 and 14 h of incubation was very similar in all types of macrophages (Table 10), showing no increase with time. Their intracellular Ft content, in ng/ug of total protein was 24.2±0 for rM, 12.4±2.4 for CpM and 4.14±0.3 for tM (mean±SEM) which suggested some correlation with the Ft levels detected in the supernatant. However, it seems likely that this Ft detected in the supernatant came from cells that became unattached during the incubation (section

Table 9

Ferritin and iron content of murine peritoneal macrophages and ratio iron:ferritin (Fe:Ft).

	Type of macrophage:		
	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
Ferritin			
(ng/ug total protein)	21.4 ± 6.4	11.9 ± 4.0	4.3 ± 1.0
(ng/10^6 cells)	1824 ± 544	1394 ± 468	645 ± 148
Iron			
(ng/ug total protein)	3.4 ± 1.0	2.0 ± 0.6	1.2 ± 0.1
(ng/10^6 cells)	289 ± 85	234 ± 70	177 ± 15
Fe:Ft ratio	0.158	0.167	0.274

Figures are mean ± standard deviation n = 7 for Ft n = 3 for Fe

Table 10

Ferritin detected in the culture supernatant of murine peritoneal macrophages.

	Type of macrophage:		
Incubation time (h)	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
1	4.4 ± 0.7	1.3 ± 0.3	2.1 ± 1.7
14	4.0 ± 0.3	1.6 ± 0.6	1.3 ± 0.7

Figures are ng of Ft in supernatant/ug of total protein in adherent cells: mean ± SEM : n = 3 independent experiments.

4.1.1). Although the unattached cells were always removed by centrifugation prior to Ft quantification in the culture media, these unattached cells always contained a proportion of dead cells (data not shown). Thus, those cells that did not exclude eosin were probably leaking intracellular components, among them Ft, before they became unattached. Furthermore, the medium (RPMI + 10% FCS) gave a high background (6 ng/ml when diluted 1:10; 5ng/ml at 1:25), which was due to the FCS, hence the values obtained for Ft in the culture supernatants of macrophages were reduced by the figure for the medium at the same dilution. Attempts were made to reduce the nonspecific high background of the media by culturing the macrophages without FCS, but it was found that the cells soon became granulated and after 14h of incubation nearly all the cells were dead, as judged by phase contrast microscopy and eosin exclusion. The FCS was also passed through an antiFt-Sepharose 4B column, which reduced the background due to the FCS in the RPMI from 6 ng/ml at 1:10 dilution and 5ng/ml at 1:25 dilution to 0.3 ng/ml at both dilutions. Hence the following experiments were performed with medium containing immuno adsorbed FCS.

5.2.3.2 Effect of metabolic inhibitors and measurements of lactate dehydrogenase. The metabolic inhibitors used were tunicamycin (3ug/ml), NaN_3 (10^{-3} M) and NaF (10^{-3} M). Glycosylation is considered as one of the steps for secretion of proteins (Alberts et al, 1983) and its inhibition with tunicamycin abrogates this active process (Bjorkman and Ragnar, 1982; Besquier and Gleckman, 1982). Thus, tunicamycin (3ug/ml) was used to inhibit

protein glycosylation in the macrophages. The NaN_3 and NaF were used as inhibitors of oxidative phosphorylation (Alberts et al, 1983). Although the concentrations of NaN_3 and NaF used apparently do not kill the cells durring short incubation periods, i.e. up to 5h (MacDonald et al, 1969; Wyllie, 1977; Bjorkman and Ragnar, 1982; Besquier and Gleckmam, 1982) the NaF killed all the macrophages at 14h and the cells incubated with NaN_3 were unhealthly after the same period. The cells incubated with tunicamycin were in apparently better condition, as judged by phase contrast microscopy and the amount of adherent cell protein which, although reduced, was not absent as in the Petri dishes containing NaF.

The Ft measured in the supernatant of macrophage cultures incubated with the inhibitors for long periods was clearly from dead cells (Table 11), as the total protein remaining in the adherent cells was reduced, in the case of NaF to undetectable levels. Hence, it was decided to leave out the use of NaF and NaN_3, and also to attempt to measure the LDH activity of supernatants in order to exclude cytoplasm leakage.

The extracellular presence of LDH activity has been used as an indicator of tissue injury and in cell culture as an indicator of viability or cellular leakage. Hence, the aim of LDH measurements was to correlate its presence with that of the Ft detected in the culture supernatants and thus determine whether this Ft was coming from dead cells rather than being secreted or released by healthly cells. The LDH activity present in the lysates of

Table 11

Ferritin release by CpM, in the presence of metabolic inhibitors.

Ft measured in:	Medium containing nothing	tunicamycin	NaN_3	NaF
Supernatant (1 h incubation)	23	9	55	45
Supernatant (14 h Incubation)	35	71	44	265
Lysate	336	340	322	BD
Total Ft measured	394	410	421	310

Figures are ng of Ft detected in culture supernatants and lysates: mean of duplicates: The differences of duplicates averaged 73% for measurements in supernatants and 18% for measurements in lysates of their respective mean. BD = Below limit of detection.

macrophages was measured, and was found to be very low (Table 12). The LDH activity detected in the culture supernatants of the different types of macrophages did not change between 1 and 14h of culture incubation. The activity, due to FCS, found in the medium alone (2 to 4 IU/l) was subtracted. Although this figure is low compared with values in human and mouse serum it represents a high proportion of the amount found in the lysate. In an attempt to eliminate the possibility that proteolysis was destroying LDH activity the macrophages were incubated with the protease inhibitors pepstatin (0.7 pg/ml) and PMSF (1 uM). Unfortunately the viability of the cells was decreased by the inhibitors and the remaining live cells showed morphological changes. The LDH activity present in the culture supernatants did not change during short incubations and at long incubations the viability was very low, hence this practice was abandoned.

It was concluded that due to the low intracellular activities found in peritoneal macrophages, which ranged from 5 to 16 IU/l per culture dish, measurement of LDH activity is not a good indicator of cytoplasm leakage. Furthermore the presence of LDH activity in the FCS masks the low activity that could come from moribund cells.

Despite the problems found when using metabolic inhibitors, a further attempt was made to inhibit glycosylation, as the adherent cultures containing tunicamycin showed better viability than those containing the other inhibitors. In these experiments only CpM were tested due to higher total Ft levels in these cultures. The Ft detected in the culture supernatants at different

Table 12

Lactate dehydrogenase activity detected in the culture supernatant and lysates of murine peritoneal macrophages.

	Type of macrophage:		
LDH activity in:	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
Supernatant (1h)	3.9	3.6	8.4
Supernatant (14h)	3.9	5.7	9.7
Lysate	5.2	14.3	16.0

Figures are LDH IU/l : Average of 2 experiments: The differences between duplicates averaged 27% of their mean.

times did not change during the first 6 hours of incubation (Table 13). At 23 h of incubation the Ft measured in the supernatant rose from 15 to 124 ng/ml in the cultures with tunicamycin. However, this increase was probably due to the lysis of adherent cells, because the total protein measured in the adherent cells at the end of the incubation period was 25% lower than in the control cultures. Attempts were made to measure DNA at 254 nm in the culture supernatants as another indicator of cell lysis, but this was impossible because the medium gave a very high absorption. Since it was therefore, not possible to detect limited cellular death the measurements by IRMA of Ft in culture supernatants were abandoned.

5.2.3.3 Release of radioactive ferritin. Since there were perfomed several experiments that envolved incubation of the macrophages with radioactive metabolites (sections 5.1.12; 5.1.13), further attemps were made to detect release of Ft by peritoneal macrophages. Radioactive ^{3}H-Ft or ^{59}Fe-Ft was detected in culture supernatants by immunoabsorption of the culture medium with antiFt-Sepharose.

Release of ^{3}H-Ft. The macrophages were incubated for 14h in media containing ^{3}H-Leucine (see section 5.1.12.1). It was not possible to detect ^{3}H-Leu-Ft in the culture supernatant due to the low incorporation of label into the Ft present which, after subtraction of the nonspecific radioactivity (bound to normal rabbit gamma globulins-Sepharose complex) gave values around zero.

Table 13

Ferritin release by CpM, in the presence of tunicamycin.

Incubation time (h)	Medium containing nothing (control)	tunicamycin (3 ug/ml)
1	16	15
3	22	17
6	21	21

Figures are ng of Ft detected in culture supernatants: average of duplicates: The differences of duplicates averaged 14.3% for control and 25.5% for tunicamycin of their respective mean.

Release of ^{59}Fe-Ft. The ^{59}Fe released following the ingestion of ^{59}Fe-Tf-anti -Tf immune complexes was fractionated into ^{59}Fe-Ft and non-Ft-^{59}Fe. The percentage of ^{59}Fe released associated with Ft was very similar after 3h of incubation in all macrophages but at 22h there was a rise in the percentage in the supernatants of the tM and rM (Table 14). However the possibility cannot be excluded that this fraction (^{59}Fe-Ft) of released iron came from dying cells (see section 5.2.3.2). These percentages should be treated with caution because the total release of ^{59}Fe in the different macrophages is not the same, it being in tM a lower percentage (see section 5.2.1).

Thus, despite certain technical problems none of the experiments designed to detect Ft release presented in this work supported the idea of active secretion of Ft by cells of the MPS, at least by murine peritoneal macrophages. In some cases the Ft detected in culture supernatants only rose when the adherent macrophages were unhealthy, and in none of the others can this cause be excluded.

5.2.4 Synthesis and degradation of ferritin in peritoneal macrophages

5.2.4.1 Comparison of synthesis and degradation of ferritin in resident, activated and elicited macrophages. The rM and CpM both synthesised considerable amounts of Ft, the synthesis by CpM being a little higher in terms of total protein. When the synthesis is related to cell number this difference is more evident. In the tM only very low levels could be detected (Table 15). Ferritin

Table 14

^{59}Fe-Ferritin release by murine peritoneal macrophages.

	Type of macrophage:		
	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
Incubation time (h)			
3	3.7	3.5	7.5
22	36.8	6.2	23.0
Total ^{59}Fe release (cpm) (% of ingested)	2288 (20.8)	16314 (20.1)	2029 (10.2)

Figures are ^{59}Fe detected in Ft as percentage of total ^{59}Fe release: Average of duplicates: The differences between duplicates averaged 34% of their mean.

Table 15

Synthesis of ferritin by murine peritoneal macrophages.

	Type of macrophage:		
	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
cpm/ug total protein	147 ± 64	175 ± 24	8 ± 0
cpm/10^6 cells	12495 ± 5425	20561 ± 2811	1248 ± 10

Figures represent total incorporation of ^{3}H-leucine over 14h pulse : mean ± standard deviation; n = 8

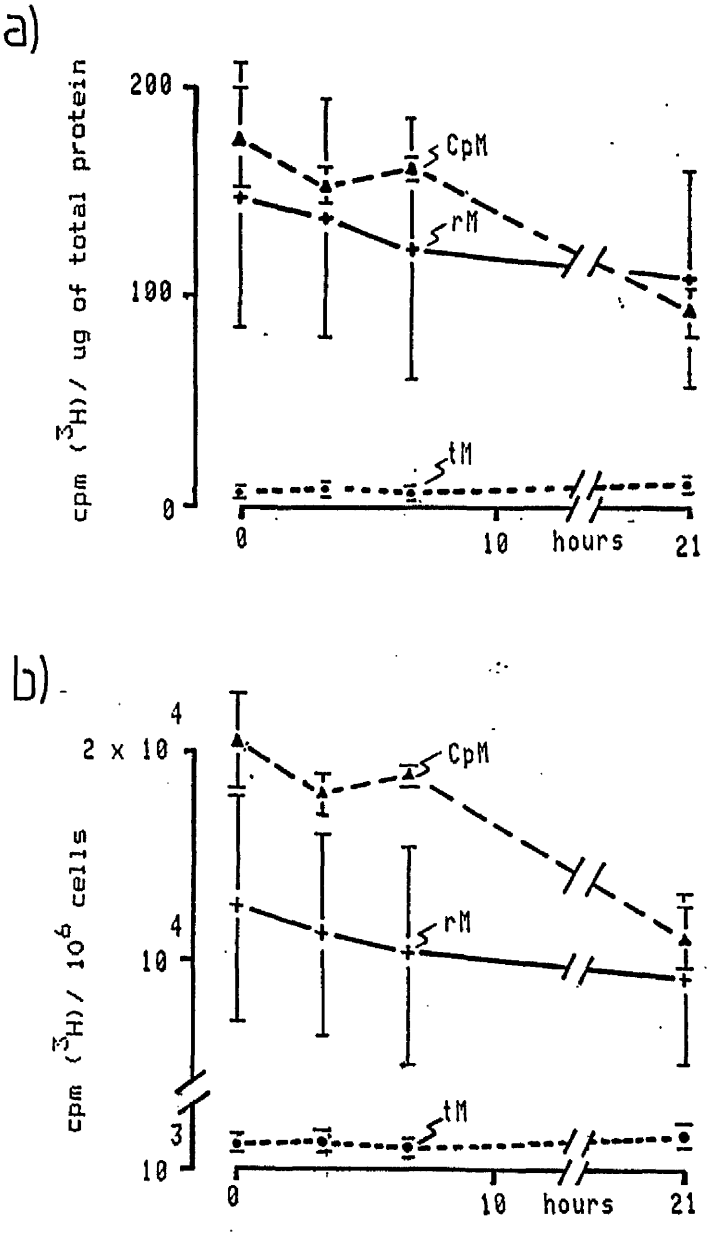
synthesis represented 2.1-3.7% and 3.4-8.8% of total protein synthesis by rM and CpM respectively but only 0.3-0.5% in tM. Thus the depression of Ft synthesis in tM was not part of pleiotropic protein synthesis reduction.

In order to exclude the possibility that the low level of Ft in tM with, their high Fe:Ft ratio (see Table 9) and apparent depression of Ft synthesis, was due to accelerated degradation of the protein, pulse-chase experiments were performed. These showed, in terms of total protein, that rM and CpM catabolised Ft at a similar rate, whereas the much lower level of labelled Ft initially present in tM did not change during the chase period (Fig. 20). On the other hand, when the loss of label incorporated into Ft is looked at in terms of cell number the CpM are apparently degrading Ft faster that the rM, the picture for tM still being the same. The low levels of Ft in tM thus appear to be due primarily to depressed synthesis. Although the experiments were not specifically designed to find the half life ($t_{1/2}$) of Ft in macrophages, if it is assumed that there is no reutilization of the label and bearing in mind the low number of measurements, the apparent $t_{1/2}$ of Ft is 41 h and 25 h for rM and CpM respectively. For tM it is impossible to calculate the $t_{1/2}$.

5.2.4.2 Induction of ferritin synthesis by iron. Since de novo ferritin synthesis is known to be stimulated by iron in all tissues tested and especially in liver, it was of interest to determine the effect of iron on Ft synthesis in macrophages. Adherent cultures were therefore incubated in media containing different concentrations of

Figure 20

Synthesis and degradation of ferritin in peritoneal macrophages.

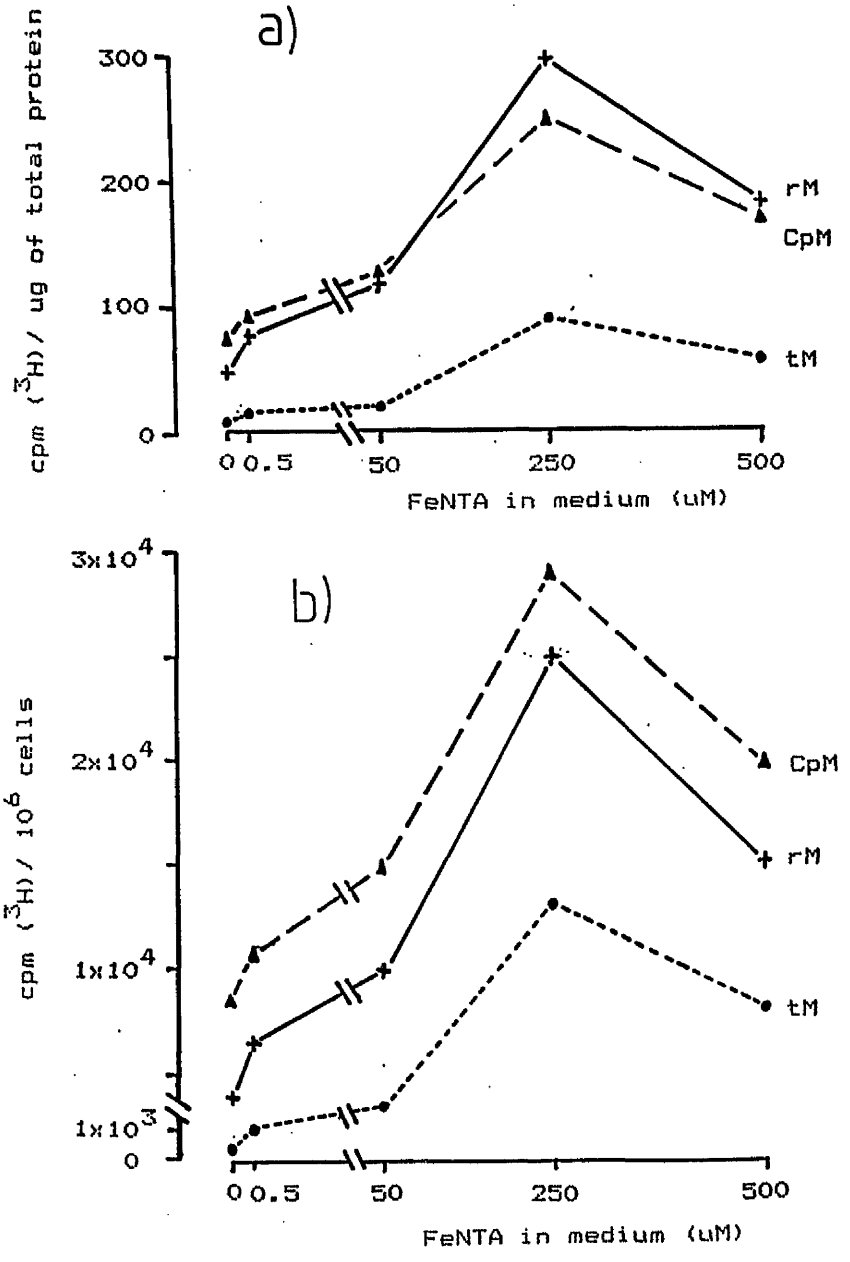


a) Induction of Ft expressed per ug of total protein.
b) Induction of Ft expressed per million cells.
Each point represents ^{3}H-leucine remaining in Ft after 'cool' leucine chase : mean of 4 experiments bars are S.D.

FeNTA. The induction of Ft synthesis by iron was similar in rM and CpM, in terms of total protein (Fig. 21), which apparently were responding in a similar way to the concentrations of FeNTA. There was a sharp increase in Ft synthesis with increasing Fe concentration up to 250uM, after which the synthesis of FT declined, presumably due to toxic effects on cellular metabolism. Although in the tM the stimulation of Ft followed the same pattern as rM and CpM, their Ft synthesis was lower. When the results are related to cell numbers, CpM always produced more Ft than the other types of macrophages at all Fe concentrations, but the tM still showed a depressed synthesis of Ft. The extent of induction in relative terms (comparing no iron to maximum induction, see below) was similar in rM and tM, i.e. 8.2 and 8.5 times respectively whereas for CpM it was only 3.8 times. This was noted in all the experiments that involved the comparison of media with added iron to normal media, the pooled values of six experiment being 6.2±0.9 for rM, 3.3±0.3 for CpM and 10.0±1.3 for tM (mean±SEM). It seems clear that the extent of induction is lower in CpM than the other macrophages. The different responses of Ft synthesis to iron by the macrophages were not due to different abilities to take up iron, as this was similar in all three types of macrophages (Fig. 22, see also Fig. 23) and, as with Ft synthesis, increased with the concentration in the medium up to 250 uM, and decreased thereafter. On the other hand, even though the synthesis of Ft is largely depressed in tM these cells had not lost their capacity to respond to iron, as judged by the extent of induction.

Figure 21

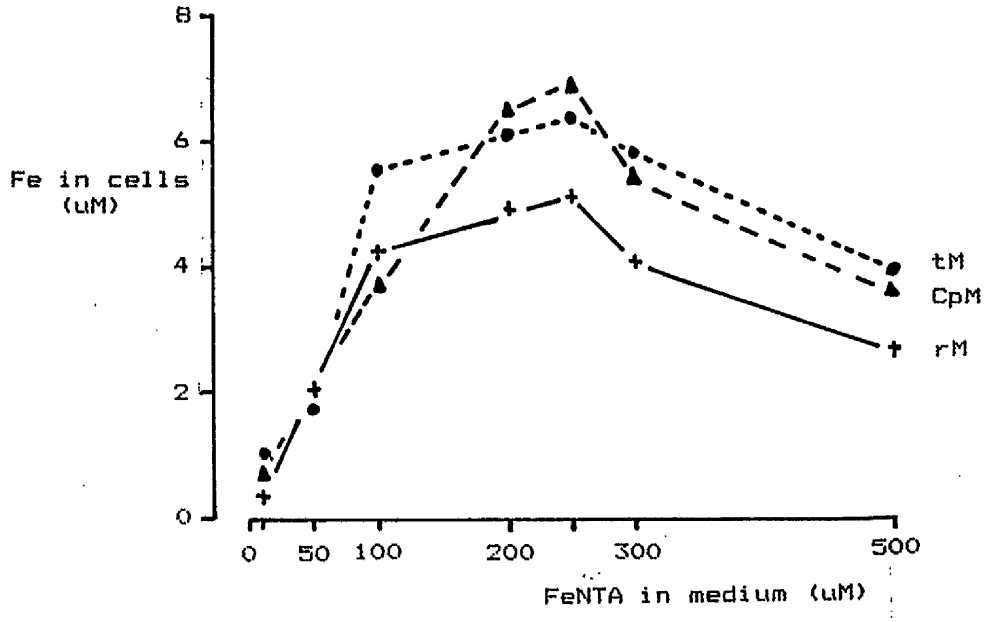
Induction of ferritin synthesis by iron in peritoneal macrophages.



a) Induction of Ft expressed per ug of total protein.
b) Induction of Ft expressed per million cells.

Figure 22

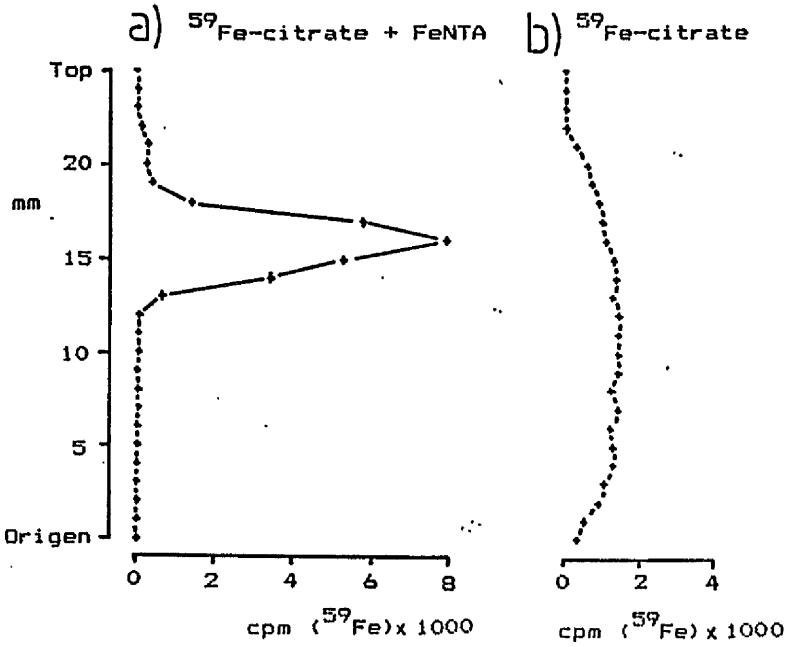
Iron uptake by peritoneal macrophages.



FeNTA in medium was trace labelled with ^{59}Fe-citrate, thus, Fe uptake by cells was calculated by the radiactivity present in cell lysates. The adherent cultures contained similar amounts of cells.

Figure 23

Ascending paper chromatography of ^{59}Fe complexes, ^{59}Fe-FeNTA homogeneity.



mm= millimetres. Mobile phase glacial acetic acid:ethanol:deionised water (3:2:5, v/v). Paper, Whatman 3MM. The peak of radioactivity in a) also showed to be positive for Perls' Prussian blue reaction.

5.2.4.3 Synthesis of ferritin subunits. Since the synthesis of Ft begins with the synthesis of subunits it was of interest to investigate if the stimulated macrophages were synthesising more Ft subunits and if their amount of this synthesis of subunits differed from that of subunits induced by iron. Experiments were therefore performed to compare the incorporation of label into the heat-stable Ft shell and the heat-labile free subunits. It is known that, in rat liver, the subunits which do not form polymeric Ft molecules are more susceptible to thermal denaturation. The proportion of labelled Ft lost by heat treatment was greater in rM and CpM than in tM, although at 3h incorporation of ^{3}H into Ft by tM was too low for a realiable figure to be obtained. The addition of Fe reduced this loss only in rM and had no effect on the other macrophages (Table 16). Drysdale (1983) proposed that the principal effect of iron is to drive the subunits to form shells. Apparently this is the case with rM, but with CpM, in which the loss of label was high both with or without iron it could be that the rate of subunit synthesis was always greater than that of polymerisation to stable shells. Therefore apparently the net effect of iron in rM, is, as predicted, to drive the Ft subunits to form stable shells. In contrast in CpM which did not show any change, it could be that there was already an increase of Ft subunits as total Ft synthesis was already increased (section 5.2.4). Although the tM showed very little incorporation of label into total Ft, apparently a larger proportion of the Ft subunits that are being synthesised always finish as thermo-stable Ft shells.

Table 16

Proportion of ferritin subunits synthesised by mouse peritoneal macrophages and effect of iron.

	Type of macrophage:					
	Resident		Activated (*C. parvum*)		Elicited (Thioglycollate)	
Incubated with:	na	Fe	na	Fe	na	Fe
3h	23	17	24	24	VLI	VLI
14h	31	12	21	23	10	11

Figures are percentage of labeled Ft lost by heat treatment : mean of duplicates : The differences between duplicates for 'na' averaged 52% and for 'Fe' 48% of their mean: na= no addition, Fe= iron added (250 uM FeNTA), VLI= very low incorporation.

5.2.4.4 Ferritin synthesis during macrophage stimulation. The synthesis of Ft at different days after inoculation of the stimulant showed that the Ft synthesis in tM decreased steadily during the 4 days studied. In the CpM the synthesis was maintained at a similar level to the rM during the first three days but rose on the fourth day (Table 17). Ft radioactivity incorporation to total protein incorporation showed a decrease for tM (from 2% to 0.5% over the 4 day period) whereas CpM showed an increase (2.1% to 6.5%). The values at 4 days were in agreement with previous results (see section 5.2.4.1). These results show that in tM the depression of Ft synthesis is not due to a general decrease in protein synthesis, and it becomes more pronounced during the 4 days, whereas in CpM Ft synthesis was similar in proportion to that of other proteins for 3 days, after which it rose.

5.2.4.5 Synthesis of membrane-associated ferritin. Since it is known that Ft is synthesised in free and attached polyribosomes and that macrophages have a large membrane, it was of interest to test if there was label incorporation into Ft which remains associated with cellular membranes. Because of the cellular changes occurring during the stimulation period it was decided to perform these experiments with cells which had been stimulated at different times.

All three types of macrophages produced a similar proportion of membrane proteins, i.e. about 30% of the total protein synthesis during the four days of stimulation (Table 18). The incorporation into membrane-

Table 17

Ferritin synthesis by mouse peritoneal macrophages at different days poststimulation.

Day	Type of macrophage: Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
	cpm/ug total protein		
0	53	--	--
1	--	56	46
2	--	55	20
3	--	46	14
4	--	116	6
	$cpm/10^6$ cells		
0	4505	--	--
1	--	6552	6808
2	--	6435	2960
3	--	5383	2072
4	--	13572	888

Figures are mean of duplicates: The difference between duplicates of cpm/ug total protein averaged 21% of their mean.

Table 18

Synthesis of membrane associated proteins of mouse peritoneal macrophages at different days poststimulation.

	Type of macrophage:		
Day	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
0	33	--	--
1	--	26	31
2	--	26	29
3	--	29	30
4	--	28	29

Figures are percentage of solubilised label from membrane to total protein incorporation : mean of duplicates : The differences between duplicates averaged 9.1% of their mean

associated Ft was similar in rM (0.6% of total cell protein) and CpM (0.5%, for all days after stimulation), but for tM it decreased from 0.5 to 0.2% at the second day and it remained at that level up to 4 days. The ratio of labeled Ft found associated with membranes to that in cytosol showed a decrease for CpM and an increase in tM with time of stimulation (Table 19). The results with tM have to be taken with caution because, as mentioned previously (section 5.2.4.1), the incorporation into total Ft in these macrophages is low and the Ft in membranes represents only a fraction of that.

Although in the stimulated macrophages the ratio of synthesis of membrane proteins to total protein is similar to that in the rM, it is not the case for Ft. The results indicate that for Ft the changes seen during stimulation are more pronounced in the intracellular events than in the membrane.

5.2.5 Intracellular distribution of iron in macrophages.

In order to determine the intracellular fate of the iron phagocytosed via immune complexes, fractionation of intracellular iron of macrophage lysates, by gel filtration using detergent and a study of the intracellular distribution using desferrioxamine, were carried out.

5.2.5.1 Gel filtration. Gel filtration of macrophage lysates was performed following the ingestion of ^{59}Fe-^{125}-Tf-antiTf immune complexes at different times (section 5.1.13.1). Gel filtration of the lysates of all three types of macrophages showed only two

Table 19

Proportion of synthesis of membrane associated ferritin to total synthesis of ferritin in mouse peritoneal macrophages at different days poststimulation.

	Type of macrophage:		
Day	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
0	7.1	--	--
1	--	7.4	11.6
2	--	5.4	10.2
3	--	7.9	14.2
4	--	3.0	20.9

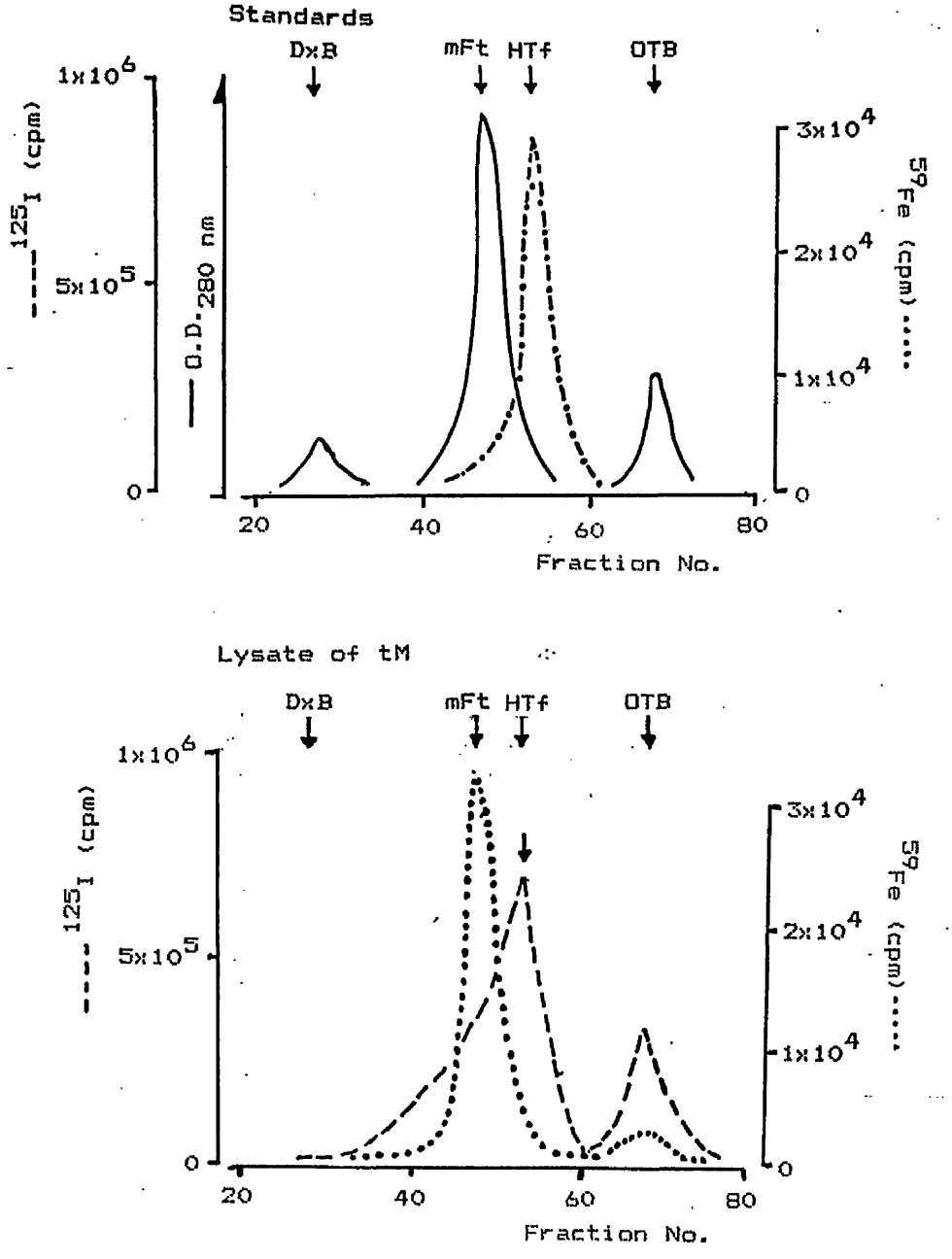
Figures are membrane associated ferritin incorporation as percentage of total ferritin incorporation: mean of duplicates: The difference between duplicates averaged 57% of their mean.

peaks of intracellular ^{59}Fe, one at the elution point of Ft and the other out of the fractionation range of the gel, presumably of very low molecular weight (Figs. 24 and 25). The pattern of ^{125}I revealed that at 3 hours after the ingestion of the immune complexes there were complexes of different sizes present, ranging in elution point from the exclusion volume down to the position of Tf. Although there was always a prominent ^{125}I peak at the elution position of Tf in all types of macrophages, there was no corresponding peak of iron. Apparently this exogenous Tf remained nondegraded after 22h, at least as judged by its elution point on the gel, but had released its iron. These experiments showed that the majority of intracellular iron passed to Ft in all three types of macrophages. Prior to gel filtration the lysates were centrifuged to sediment the immune complexes which remained insoluble and cellular debris. Although this Tx-PBS-insoluble part of the macrophage lysates contained almost the same proportion of ingested iron in all macrophages, the ^{59}Fe:^{125}I ratio was higher in the tM, probably indicating that some of this iron was present not as insoluble immune complexes but in other insoluble forms (Table 20). The original ^{59}Fe:^{125}I count ratio in the immune complexes was 0.072 versus 0.54 in the insouble fraction of tM.

5.2.5.2 Distribution of intracellular iron. Another approach was adopted to investigate the distribution of ^{59}Fe after phagocytosis of ^{59}Fe labeled immune complexes. The cells were lysed in the presence of DFO and fractions were separated as described in section 5.1.13.2. The

Figure 24

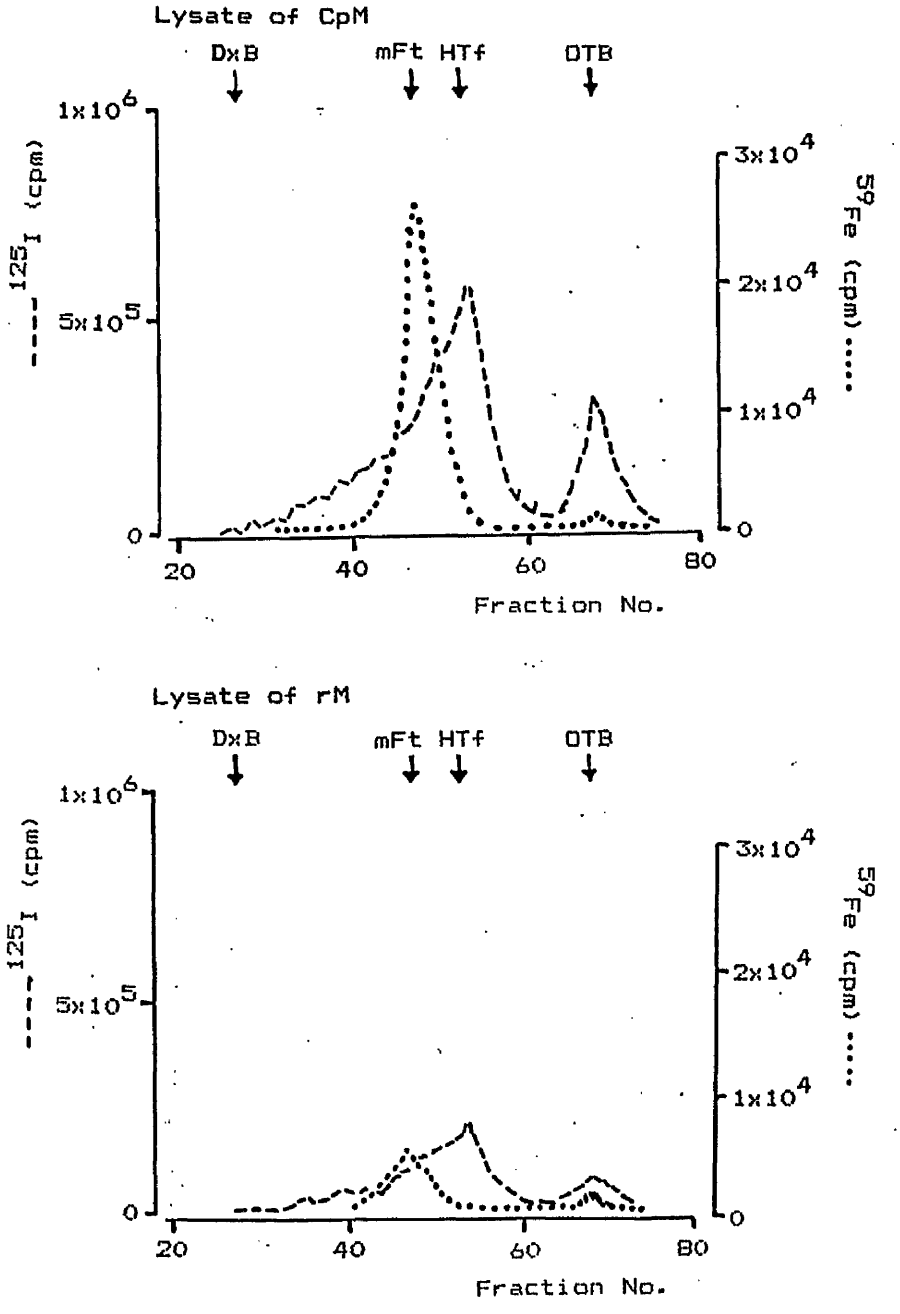
Intracellular iron distribution in peritoneal macrophages, gel filtration of lysates.



DxB = blue dextran : mFt = mouse ferritin.
HTf = human transferrin : OTB = orthotoluidine blue.
Eluting buffer PBS containing 1% of Triton x-100.
Column 1.2 x 60 cm : Fraction 1.5 ml (30 drops).

Figure 25

Intracellular iron distribution in peritoneal macrophages, gel filtration of lysates.



DxB = blue dextran : mFt = mouse ferritin.
HTf = human transferrin : OTB = orthotoluidine blue.
Eluting buffer PBS containing 1% of Triton x-100.
Column 1.2 x 60 cm : Fraction 1.5 ml (30 drops).

Table 20

PBS-Triton-insoluble ^{59}Fe and the ratio $^{59}Fe/^{125}I$ in macrophage lysates following ingestion of ^{59}Fe, ^{125}I-transferrin-αtransferrin immune complexes.

	Type of macrophage:		
	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
Insoluble ^{59}Fe (% of ingested)	3.9	4.7	3.5
$^{59}Fe/^{125}I$ ratio	0.046	0.074	0.54

Original ratio of $^{59}Fe/^{125}I$ immune complexes = 0.072
Figures represent independent measurements.

acetate buffer-insoluble fraction which contains cellular debris and probably Hs was larger in the tM than in rM and CpM. There was more iron in the Ft fraction of the CpM than in the other cells. In agreement with previous results, it was observed that most of the ingested iron entered Ft in all three types of macrophages. The chelatable iron gave fairly similar figures, though tM showed the lowest proportion. The intermediate molecular weight-associated Fe fraction, defined as soluble, nonFt and nonchelatable, was largest in the tM (Table 21). This fraction probably represents the iron which is being incorporated into enzymes.

Table 21

Intracellular iron partition in murine peritoneal macrophages following the ingestion of ^{59}Fe-transferrin-α transferrin immune complexes.

	Type of macrophage:		
Fraction	Resident	Activated (*C. parvum*)	Elicited (Thioglycollate)
Insoluble ^{59}Fe	1.2	0.3	5.1
Ferritin ^{59}Fe	59.7	73.8	51.2
Chelatable ^{59}Fe	6.7	4.5	3.8
Intermediate M.W. ^{59}Fe	18.7	11.5	29.0

Figures are percentage of ^{59}Fe ingested : mean of duplicates of two independent experiments : The difference between duplicates averaged 46% of their mean. See text for explanation of the fractions.

5.3 Discussion

Relatively few studies have been made on iron metabolism in macrophages, despite the central role played by liver and spleen macrophages in iron recirculation. However, previous studies aimed at clarifying the role of macrophages in the anaemia of chronic disease at the point of the so called iron release "blockade" have shown that peritoneal macrophages stimulated by nonspecific inflammatory agents such as thioglycollate broth (Esparza and Brock 1981a) or peptone or methaemoglobin (Nishisato and Aisen, 1983) release less iron than rM. The results presented here confirmed these earlier observations and in addition data on immunologically activated macrophages (CpM) are presented.

In the above mentioned studies the intracellular events occurring in the macrophages were not looked at. However, it was proposed that the impaired release of iron is an intrinsic characteristic of macrophages as it is not regulated by the extracellular availablility of free Tf binding sites (Brock et al, 1984). Although several mechanisms for the iron retention observed in inflammation by the MPS have been proposed, as described in section 2.9, the reason remains unclear, due probably to the multitude of events (section 2.8) such as cellular proliferation/differentiation that are taking place at the same time. Hence, a better dissection was needed.

5.3.1 Iron release by peritoneal macrophages.

The iron release following ingestion of Tf-αTf immune complexes was shown to be different in CpM from that observed in tM (Table 6). The tM retained an increased proportion of the ^{59}Fe. Although these results support the observation that inflammatory macrophages display properties associated with a "blockade" of the RES (Esparza and Brock, 1981a), they also showed that this property is not shared by CpM, which, despite their enhanced phagocytic and degradative activities (similar to tM), they did not show abnormal retention of the pulsed iron. These results suggested the possibility of working with two different types of inflammatory/activated macrophages and comparing them with the resident (normal) cells, in order to clarify the events leading to iron retention during inflammation. This model shows clear differences between immunologically or nonimmunologically stimulated macrophages in their way of handling iron (Table 6). The results of this work, which has investigated intracellular events leading to the withholding of iron by peritoneal macrophages, will therefore be discussed in the following sections.

5.3.2 Ferritin and iron content

Summers et al (1974) reported that among the human leucocytes, monocytes have the highest intracellular Ft content (7 fold higher than lymphocytes). The reported Ft content in human monocytes ranges from 10 to 54 ng/10^6 cells (Summers et al, 1974; Summers et al, 1975; Summers and Jacobs, 1976; McLaren et al, 1981; Bassett et al, 1981;

Hourahane et al, 1981; Worwood et al, 1984; Andreesen et al, 1984). Andreesen et al (1984) found that human monocytes during differentiation to macrophages over 12 days increased their Ft content to 350-1500 ng/10^6 cells. They reported also that human alveolar macrophages contain 2126±1990 ng /10^6 cells . Hence, the macrophages have a larger Ft content than their precursors -- around 200 fold more. The IRMA for mouse Ft developed in this work was found to be a reliable means of measuring the macrophage content of this protein. As shown, the linearity of the standard curve was good and reproducible (Table 7). The intra-assay variation achieved was also good, indicating that small differences could be detected (Table 8). Although the inter-assay variation was not calculated, lysates of all macrophages studied were included in each assay in order to ensure a valid comparison among the different macrophages.

In the present work, which apparently is the first in which the Ft content of murine peritoneal macrophages is reported, the value obtained for rM, 1824±544 ng/ 10^6, cells is in agreement with the findings of Andreseen et al (1984) in human macrophages. The high content of Ft in macrophages would confer certain advantages on these cells in their role of degrading RBC with the concomitant sudden increase of intracellular iron. To prevent noxious effects due to free iron a buffering effect against the exessive entrance of iron into cells would be necessary (May and Williams, 1980). This effect could be achieved by binding of the exess iron, mainly by Ft (see section 1.5). Hence a cell which is liable to find itself with a sudden iron

overload, like the macrophages engulfing erythrocytes, would be better equipped if it contained a large concentration of intracellular Ft. Although the peritoneal macrophages are not normally involved in the removal of effete erythrocytes, their high level of Ft is probably a consequence of their lineage as members of the MPS. On the other hand, even though there is no direct evidence, it could be that the high level of Ft seen in macrophages is a consequence of unnecessary iron uptake via Tf during differentiation when an increase of Tf receptors (TfR) is observed on the macrophage membrane (Andreseen et al, 1984; Hamilton et al, 1984a). The fact that CpM and tM showed an intermediate value of Ft content (Table 9), between those of human monocytes and rM could be interpreted as indicating that these cells are immature, which is in keeping with this theory. In particular, it is known that tM come from recently differentiated monocytes (see section 4.2.1).

Whether the high levels of Ft seen in normal macrophages is an essential feature for, or a consequence of, their function is not known, but in either case it would result in sequestration of iron. It is known that Fe confers stability to the Ft protein (Drysdale and Munro 1966; ; Munro and Linder 1978). Thus to maintain more or less stable the high levels of Ft observed in the macrophages it will be necessary for these cells to retain Fe. The quantity of iron measured in the macrophages studied here was high even when compared with human erythrocytes. The rM contains 290 fg/cell (Table 9) and the

human erythrocyte between 70-123 fg/cell (data calculated from: 450ml of blood= 200 to 250mg of Fe; Hoffbrand, 1980; and normal human male value 5.4 $\pm$ 0.9 x 10^{12} erythrocytes/l; Coulter Counter, Coulter Electronics Ltd., Luton, England). This withholding of iron by the macrophages would be correlated with the demands for these cells and could be exacerbated depending on the extent of differentiation of monocytes to meet demands for macrophages during an inflammatory process. Therefore, part of the iron sequestered in inflammation could be due to cell proliferation of monocytes and differentiation into macrophages. Although this is probably not the only mechanism, it could contribute substantially. Even though the tM have less Ft than the other types of macrophages tested, they contain about 50 times more than human monocytes.

5.3.3 Ferritin release

Although serum Ft (sFt) concentrations are closely related to levels of iron in the RES at storage levels ranging from deficiency to overload (Jacobs et al, 1972), in several diseases it is increased without apparently reflecting iron stores, especially in those conditions grouped under ACD (Section 1.8; Bentley and Williams, 1974; Elin et al, 1977; Birgegård et al, 1978). It has been proposed from diverse clinical evidence that the sFt originates from reticuloendothelial cells, where any movement of iron from RBC to the storage compartment or vice versa is reflected by a change in the sFt (Worwood, 1980). Siimes and Dallman (1974) found ^{59}Fe-Ft entering the

plasma 20-40 minutes following the inoculation of ^{59}Fe-RBC into rats. Thus this study in rats strongly suggests that the circulating Ft comes from the RES. The circulating Ft is taken up by hepatocytes (Cook et al, 1972), and the uptake is mediated by a specific receptor (Halliday et al, 1983). Hershko et al (1974) have suggested that the sFt is Ft released from the RES which is in transit to parenchymal cells.

The source of sFt during inflammation is uncertain. Although sFT is increased in such condition, it is an extremely small fraction compared with the large amounts of intracellular Ft. Several mechanisms could give an elevation of sFt levels, such as changes in the rate of synthesis, secretion, or release, or in the elimination rate, or by leakage from damaged cells. In addition several funtions have been proposed for monocyte-macrophage-derived Ft, such as modulation of granulocyte-macrophage progenitor cells (Broxmeyer et al 1981; 1985). All such putative functions presume that the Ft is actively secreted.

Nevertheless, there is little direct evidence of Ft release or secretion by isolated cells of the MPS. Two techniques have been used to investigate release of Ft from cells, one qualitative, the haemolitic plaque technique, by de Sousa et al (1981) and the other quantitative (direct measurement of Ft) by Worwood et al (1984).

Qualitative approach. The main drawbacks of this approach are that it does not identify which type of cells are releasing Ft when nonpurified populations are used, and it does not show if the cell is viable, or the state of

differentiation of cells which are incubated for long periods with mitogens.

De Sousa et al (1981) reported a haemolytic plaque technique to enumerate cells which are "actively secreting" Ft. This technique, was originally developed to detect antibody secreting cells (Fauci and Pratt, 1976). The cells tested by de Sousa et al (1981) were human blood mononuclear cells which were incubated for 5 days in the presence of phytohaemaggutinin (PHA), with or without Fe-citrate. The cells were then mixed with agar, antibody, complement and erythrocytes and plated. After 6h of incubation the plaque-forming cells (PFC) for Ft (Ft-PFC) were counted. These authors reported ~2 Ft-PFC per 1000 mononuclear cells for cells incubated without iron and the number rose for those incubated with iron to ~6 PFC/1000 cells. They reported also that when the cells were not incubated with PHA there were no Ft-PFC, or only negligible numbers ($1/10^5$ cells). Hence apparently the mononuclear cells are not secreting/releasing Ft in normal conditions, and the PFC seen could probably be produced by an effect of time or incubation with PHA. It might also be that the cells were developing into macrophages which when exposed to agar-RBC would be triggered to phagocytose, with subsequent release of different products. It must be borne in mind that monocytes-macrophages contain more Ft than other mononuclear cells, and if they lyse their intracellular Ft will be released. An attempt was made to carry out the PFC assay with the peritoneal macrophages used in the present work (data not shown). Some modifications were made to the above mentioned method of de

Sousa et al (1981) such as omitting pretreatment with mitogens, and making the plaques in solution without agar, in chambers of 0.1mm where the macrophages were attached to the top of the chamber and with the erythrocytes at the bottom, to avoid artifacts due to ingestion of agar-RBC and effect of mitogens. Unfortunately it was not possible to detect any PFC, due probably to secretory products (probably complement and other diverse enzymes) released by the macrophages used, as these were shown to lyse all the erythocytes even in the absence of anti-Ft or exogenous complement.

Shimizu and Ohnishi (1983) using lymphocytes and monocytes from patients with non-Hodgkin lymphoma, enumerated Ft-PFC with a reverse haemolytic plaque technique. They found that the monocyte population contained more Ft-PFC than the lymphocytes, their number being around 10 PFC/1000 cells. Unfortunaly they did not present data for normal subjects, nor for 'spontaneous' PFC, and the degree of cell purity was not given, all of which are necessary to proprerly evaluate their conclusions.

Ohnishi et al (1984), again with a reverse PFC assay, showed that purified fractions of human lymphocytes (>95%) contained 1.2 Ft-PFC/1000 cells in male and 0.5/1000 in female subjects. For monocyte fractions (>95%) the figures were 9.3 and 6.7/1000 cells respectively. They also showed that in states where increased iron storage is observed, the numbers of Ft-PFC were increased 11-20 times in lymphocytes and 3-7 times in the monocyte fraction. Therefore the monocytes apparently contained 8-13 times

more Ft-PFC than lymphocytes and the lymphocytes were more affected in iron overload conditions. Although this was apparently a well controlled study, the reverse hemolytic technique could not give information of cellular state, such as leakage from moribund cells.

Quantitative approach. Worwood et al (1984) examined the release of Ft from cells by incubation of human mononuclear cells in culture medium. They monitored membrane damage with release of LDH activity into the culture medium, to exclude the possibility that the Ft measured in the medium was coming from damaged cells. It can be inferred, from the methods part of their report, that these authors found several problems with the Ft measurements in the incubation medium (MEM + 10% FCS + other additions) by IRMA such as low concentration and probably matrix effect. They solved the problems by dilution of the standards into culture medium diluted (1:5) with assay buffer and it is assumed that the samples were assayed at the same dilution, though it is not stated. Although their cell preparation of monocytes were contaminated with other cells (67% monocytes, 20% lymphocytes and 13% other cells), they proposed that the release of Ft from mononuclear cells is mainly due to monocytes because their enriched lymphocyte population (74% lymphocytes) showed less Ft in the culture media. They found that the rate of release of Ft by mononuclear cells is very slow, from 2 to 8 ng/ml/10^6 cells during 3 to 44 h of incubation with FeNTA (100 uM). In both this and in an earlier publication (Hourahane et al, 198/), it was also reported that the pattern of Ft released appears to be

different from that of LDH. As with Worwood et al(1984) several problems were encountered and solved in the measurement Ft release, see section 5.2.3, but with these modifications the accuracy of the method decreased, mainly due to the matrix effect that all IRMA assays for Ft generally show, thus losing part of its reliability. As Worwood et al (1984) found for monocytes, the level measured in the culture medium of macrophages was very low. The LDH activity appears to be an unsuitable indicator of cell lysis for macrophages due to the low intracellular activity detected in cell lysates and the high activity found in the FCS used in the culture medium. Worwood et al (1984) made no mention of this high level of LDH activity due to FCS. These workers also fractionated the Ft in culture medium into Ft which binds to Con A-sepharose and that which does not, to detect glycosylated Ft (Worwood et al, 1979; Cragg et al, 1980). They found that 8% of the Ft released binds to Con A and that this increased to 24% after 44 h of incubation, but there was also a parallel increase in LDH activity. The attempts made in this work to inhibit glycosylation with tunicamycin failed to show a reduction of the Ft measured in the medium; indeed after 22 h it rose due to cell death.

Hence, it appears that the evidence available points to the conclusion that if there is an active secretion of Ft from cells of the MPS it is very low, and could be masked by cell death. The lack of a good indicator of cellular leakage makes it difficult to exclude release from dead or moribund cells when dealing with a protein such as

Ft that is normally present intracellularly in large quantities. Furthermore the failure to detect ^{3}H-labeled Ft in the medium, section 5.2.3.3, suggest that healthly cells, at least in the case of peritoneal macrophages, do not release Ft.

Thus the origin of sFt remains unsettled. Although it was impossible to demonstrate active secretion by peritoneal macrophages in the experiments presented here, it could be that other cells contribute to circulating Ft, such as other members of the MPS. In inflammatory processes where infiltration of cells is seen the most probable explanation is that circulating Ft would appear from cell injury. The main feature to suggest that sFt comes from active secretion is the fact that a part of sFt binds to Con A. Thus the assumption that the glycosylated Ft was the product of endoplasmic reticulum (Worwood et al, 1979) was made. The portion of sFt that binds Con A represents aproximately 60% of the total sFt in contrast to almost no binding of tissue Ft (Worwood et al, 1979). But Birgegård (1980) showed that 20% of tissue Ft binds to Con A, and Worwood et al, (1984) later showed that after incubation of tissue Ft with serum the amount of tissue Ft which binds to Con A rose to 16%. Thus the binding of Ft to Con A-Sepharose as an indicator of active secretion could be misleading. Recently Covell et al (1984) have shown that serum interacts with spleen and heart Ft but not with plasma Ft. Although no studies with Con A were made, it opens up the possibility that the binding of sFt to Con A could be mediated through "binders" of Ft present in serum.

5.3.4 Ferritin synthesis

The presence of Ft has been described in all types of cells (Harrison et al, 1974; Bomford and Munro, 1980) and the capacity to synthesize Ft appears to be universal (Jacobs, 1980). It has been proposed that the abnormal retention of iron by the liver and spleen which occurs in inflammatory conditions is caused by increased Ft (Konijn and Hershko, 1977; Konijn et al, 1981). This increased Ft synthesis occurred prior to reduction of serum iron levels, and was considered to result in a diversion of iron into Ft from the so-called labile or pre-release pool. However, these studies did not determine whether hepatocytes, epithelial or Kupffer cells were responsible for the increased Ft synthesis. The results presented here suggest that in macrophages several different mechanisms may operate, as the reduced iron release by tM (Table 9) was accompanied by decreased Ft synthesis (Table 15) and normal release of iron by CpM was accompanied by increased Ft synthesis.

Although the level of Ft in the three types of macrophages studied here differed, Ft synthesis could in all cases be increased from the basal level (Fig. 21) by the addition of Fe to the incubation medium, up to a certain level, above which Ft synthesis declined (Fig 21). This dose response relationship implies that the intracellular iron pool which drives Ft synthesis had equilibrated with the extracellular medium. The decline of Ft synthesis in the presence of excess iron probably indicates that Ft synthesis can be increased until a "threshold" is reached above which the incoming excess iron

cannot be handled by the cells.

The fact that tM showed depressed synthesis of Ft in the absence of additional iron and only a modest increase in total Ft synthesis in response to excess iron could be interpreted as showing that these cells are diverting some iron into enzymatic needs, or to a metabolically inert pool. These alternatives are not mutually exclusive (section 5.3.5. Although the increase of Ft synthesis observed in tM in response to iron was modest in absolute terms, they have not lost their capacity to synthesise Ft, because if the proportional increase in response to iron is considered then tM respond similarly to rM. (Extent of increase in Ft synthesis = ratio of incorporation of ^{3}H-leucine into Ft between macrophages that have been cultured in media with added Fe to that by macrophages incubated without added Fe; section 5.2.4.2).

On the other hand, although the incorporation of labelled leucine into Ft found at basal level (without extra iron) in CpM at the fourth day appeared normal (Table 15), strong indirect evidence indicates that these macrophages already have an increased synthesis of Ft. Such indirect evidence comes from the following findings. The turnover of Ft was found to be faster in CpM ($t_{1/2}$ = 25h) than in rM ($t_{1/2}$ = 41h) (section 5.2.4.1). Thus the apparently normal incorporation of ^{3}H into Ft could be underestimating Ft synthesis due to accelerated degradation. The proportional increase in Ft synthesis was relatively low, 3.3 times compared with 6.6 in rM (section 5.2.4.2). This relatively low increase in Ft synthesis

could be interpreted as indicating that CpM had already increased their Ft synthesis at basal level (without added iron). The synthesis of Ft expressed on a per cell basis also indicates that CpM at the fourth day after injection synthesise more Ft than rM (20560 cpm/10^6 cells in CpM and 12500 cpm/^{6} cells in rM; Table 15; section 5.2.4). When Ft synthesis in CpM was studied over the four days after stimulation it appeared to remain unchanged during the first three days and rise on the fourth day in (Table 17). There are two possibile explanations, one being that the Ft synthesis in CpM remains the same as in rM during the first three days, and the other that the degradation of Ft changes during the stimulation period. There is no evidence to exclude either possibility.

Therefore, the bulk of this indirect evidence suggests that both synthesis and degradation of Ft are increased in CpM. When the results of Ft synthesis are considered with those of iron release by both CpM and tM, several contradictions with the current knowledge of the events occuring during inflammation become evident. These will be discussed in chapter 6.

Birgegård and Caro (1984) have presented data indicating, in contrast to the results reported here, that tM synthesised Ft but that no synthesis could be detected by rM. Part of the disagreement could be explained by the fact that their elicited cells were harvested only 1 day after the injection of the broth and the cells were incubated with added iron. Under such conditions the results of both reports are not dissimilar with respect to tM. In the present work it was found that the tM, on the

first day after stimulation, were synthesising Ft in similar amounts to rM (both cultured without extra iron). But tM showed a steady decrease in their capacity to synthesise Ft from the second to the fourth day post-stimulation (Table 17; section 5.2.4.4). Thus it is understandable that Birgegård and Caro (1984) detected Ft synthesis in tM after 1 day of stimulation and after preincubation of the macrophages with Fe in the form of ^{59}Fe-Tf that could induce Ft synthesis. However it is difficult to explain their failure to detect Ft synthesis by rM, especially not only failed to detect synthesis of Ft by rM, but also they showed that *these* cells synthesised no protein at all during the pulse period (their chromatographs did not show a standard pattern for fractionation of a cell lysate). This seems highly improbable given that rM are known to synthesise a variety of proteins (Adams and Hamilton, 1984).

Konijn and Hershko (1977) have proposed that the increased Ft synthesis in liver during experimental inflammation induced by turpentine inoculation is due to Ft acting as an acute phase protein. The evidence presented here indicates that the increase in Ft synthesis seen in their study could be part of differentiation of macrophages rather than anomalous behaviour of the existing well differentiated liver cells. It is known that the Ft content of human monocytes is greater than that of other leucocytes (section 5.3.2), and that they synthesize more Ft *in vitro* in response to incubation with iron (Summers *et al* 1975). In addition there is the fact that rM have a

higher Ft content that their human counterpart precursors (section 5.2.2.4). Thus it is reasonable to suggest that during normal development of monocytes to macrophages there will be an accumulation of Ft in macrophages.

5.3.5 Intracellular distribution of iron

The the gel filtration of lysates of macrophages on AcA following the ingestion of ^{59}Fe-^{125}I-Tf-αTf immune complexes (section 5.2.5.2) showed only two peaks of ^{59}Fe in all three types of macrophages. The first peak was at the elution position of Ft and the other at that of low molecular weight material. A major proportion of ^{59}Fe was associated with the first peak, and was precipitable with antiFt. The proportion of ^{59}Fe of low molecular weight was similarly small in all types of macrophages. The small size of the peaks could be due to nonespecific retention of this fraction by the gel, as Bøkkeren et al (1985) have shown that Sephadex retained some of the iron of a low molecular weight fraction from rat reticulocytes. Haurani and O'Brien (1972) found that the lysate of murine peritoneal macrophages showed two peaks of ^{59}Fe on DEAE chromatograpy after phagocytosis of ^{59}Fe-RBC, one assocciated with haemoglobin (Hb) and the other with a Tf-like protein. Haurani and O'Brien (1972, Haurani & Ballas 1985). have also postulated that the peritoneal macrophages synthesise this Tf-like protein. The presence of this Tf-like protein could be an artifact due to the addition of 2 mg of Tf to the lysate prior to applying the sample to the column. The absence of the ^{59}Fe-Ft and low M.W. peaks found here also is in disagreement with other authors (Custer et al, 1982; Wyllie, 1979). In

contrast to the findings of Haurani and O'Brien (1972) regarding ^{59}Fe-Tf peak, in the present work the ^{125}I-Tf peak observed in the chromatograpy was always devoid of ^{59}Fe in all three types of macrophages. Wyllie (1979) reported only two peaks of ^{59}Fe in the lysate of alveolar macrophages after incubation in medium 199 with added ^{59}Fe (no mention at all is made of the type of iron compound used), one associated with Ft and the other of low M.W. Custer et al (1982) found four ^{59}Fe peaks on DEAE chromatography in the lysate of human alveolar macrophages following phagocytosis of ^{59}Fe-RBC. The ^{59}Fe peaks were associated with Hb, Tf, Ft and low M.W. The peak of ^{59}Fe-Hb was part of the nondegraded ^{59}Fe-Hb from the ^{59}Fe-RBC and can be discounted. The peak of ^{59}Fe-Tf probably was an artifact of the procedure, as they followed that used by Haurani and O'Brien (1972), i.e. adding Tf, Hb and Ft (2mg of each) to the lysate prior to chromatography. Alternatively, as pointed out by them (Custer et al, 1982) the Tf-like protein could come from the media and be taken up during RBC phagocytosis. Despite this remark, it seems more likely to be from the 2 mg of Tf added prior to the chromatography. Therefore it appears that in the buffer-soluble lysate of macrophages there are only two endogenous fractions demonstrable by chomatography in DEAE or AcA, one being Ft and the other of low M.W.

In the lysate of the macrophages studied here there was exogenous ^{125}I-Tf from the ^{59}Fe-^{125}I-Tf-αTf immune complexes. It is pertinent to point out that even 22h after being phagocytosed a part of this ^{125}I-Tf remained

nondegraded, at least as judged by its elution point. This could indicate that during the degradation of the ^{59}Fe-^{125}I-Tf-αTf immune complexes, not all of the Tf is degraded. But despite the likely presence of nondegraded Tf in all the macrophage lysates, this Tf was always devoid of Fe (section 5.2.5.1).

The high ratio ^{59}Fe:^{125}I in the PBS-Tx-insoluble cellular debris in the tM indicated the possibility of incorporation of ^{59}Fe into a Hs-like compound during the degradation of phagocytosed ^{59}Fe-^{125}I-Tf-αTf immune complexes. Thus, this iron deposition into a Hs-like compound could partially explain the low release of ^{59}Fe observed in these macrophages (section 5.2.1). Although Hs is an ill-defined iron compound, it is well known to be buffer-insoluble and that it is highly inert regarding its release of iron (Jacobs, 1980; Wixon, et al 1980; section 1.4.1). Although the acetate-buffer-insoluble-^{59}Fe fraction detected after ingestion of ^{59}Fe-Tf-αTf immune complexes was larger in the tM (5.1% of ^{59}Fe ingested) than rM and CpM (1.2% and 0.3% respectively), this increase could not account for all the retention of Fe seen in tM. However it does confirm that part of the Fe ingested by tM is being deposited into a Hs-like inert pool.

In agreement with the results of gel fitration of lysates it was found by intracellular distribution of ^{59}Fe (section 5.2.5.1) that the major proportion of ingested Fe was incorporated into Ft in all three types of macrophages, though this incorporation of Fe into Ft was larger in CpM. The DFO-chelatable pool gave similar figures for the three types of macrophages, though tM showed a slightly lower

proportion of ^{59}Fe in this fraction. Although the size of the labile pool of the cells can not be extrapolated from these experiments, they suggest that it is similarly small in the three types of macrophages. The intermediate M.W. fraction, which probably represents iron being used to form enzymes, was largest in the tM, probably indicating that these cells have an increased need for metabolically active iron.

It has to be borne in mind that the ^{59}Fe introduced to the macrophages via labelled immune complexes represents a trace amount. These experiments have shown that even this small amount of Fe is distributed among several intracellular compartments in the macrophages after phagocytosis, but mainly into Ft.

Chapter 6

General discussion: Mechanisms of iron metabolism in resident, activated and elicited macrophages

6 Mechanisms of iron metabolism in resident, activated and elicited macrophages

To explain the dissimilar behaviour observed in immunologically stimulated (CpM) and nonimmunologically stimulated macrophages (tM), two mechanisms of intracellular iron metabolism, could be proposed. A conceptual mechanism of normal iron metabolism in macrophages can also be postulated. Before discussing the proposed mechanisms it is necessary to recall several points and to introduce new concepts.

It is well known that during cell proliferation and also probably during differentiation the need for iron is increased A consequence of this need is the expression of the Tf receptor on the cell membrane (Kaplan 1983). Hamilton et al (1984a) found that macrophages elicited by a number of agents including thioglycollate broth expresed significantly more TfR than either rM or activated macrophages (CpM included). The CpM expressed slighly more than rM but much less than tM. Blood monocytes also show greater expression of TfR than rM Weiel et al (1984) and Esparza and Brock (personal comunication) have found that monocytes are, like tM, poor at releasing iron. It is known that synthesis and expresion of TfR is increased if the iron in the labile pool is reduced by incubating cells with DFO (Mattia et al, 1980). Thus modulation of the expression of TfR probably represents the major control mechanism for iron uptake when the cell is in need of metabolically active iron. This is thought to be a long-term control due to involvement of synthesis of the TfR,

availability and uptake of Fe-Tf and the release of the iron from the Tf (Young and Bomford, 1985).

It has been suggested that the intracellular iron is in dynamic equilibrium with a low molecular weight iron pool, the so called labile iron pool (Jacobs, 1977a). This labile pool is apparently necessary for all metabolic processes involving iron (Crichton, 1984). Although the presence of this labile pool is highly controversial, a large number of molecules, mainly of small size such as free amino acids have been postulated as possible components (Romslo, 1980). Munro and Linder (1978) have also postulated that this labile pool consists of Fe^{2+}. Bakkeren et al (1985) suggested, as an explanation for the variety and relative lack of specificity of the molecules of the labile pool, that there are probably specific iron-binding and transporting factors with high turnover which lose their iron during isolation and fractionation procedures to the "next best" chelators, namely glycine and citrate.

The flux of iron through the labile pool is supposed to be coupled to several processes (Schneider and Erni, 1981), such as metabolic use of iron for haem synthesis and other enzymes (Wrigglesworth and Baum, 1980). If it is a common pool the flow will be dictated by the metabolic state of the cell The incoming Fe from Tf via TfR would contribute to the flux of iron through the labile pool (Crichton, 1984; Pollack and Campana, 1981). As well every intracellular event involving iron mobilization would modify the flow through the labile pool. These events

include the deposition and release from Ft (Harrison, 1980; Jacobs, 1977a, 1980; Crichton, 1984; Munro and Linder, 1978), synthesis of Ft (Munro and Linder, 1978; Zähringer et al, 1976; Rittling and Woodworth, 1984), extracellular release of iron (Lipschitz et al 1971; Haurani and O'Brien, 1972), and the reflux of iron from degradation of Ft, Hs, and iron containing enzymes, carried out in autophagosomes (Jacobs, 1980). In phagocytic cells an important flow of iron through the labile pool will come, in addition to those mentioned above, from the degradation of the phagocytosed particles including RBC. For the sake of clarity discussion of the controversial subject of transport of iron across membranes will be avoided, though it is believed that the translocation of iron across biological membranes is achieved by the transport of small iron complexes (May and Williams, 1980; Bekkeren et al, 1985), perhaps by the same type of molecules that play a role in the labile pool itself.

Although the exact nature of the labile pool is not clear, it is reasonable to postulate that under increased metabolic demands from iron the first source of iron to be used would be that present in this labile pool. This loss of iron for the labile pool could be replace from the iron core of Ft molecules, at a speed dependent upon the size of the cores and the metabolic demand for iron. From the work of Harrison et al (1980) it can be deduced that the iron flow from Ft would come principally from those molecules which are kinetically more able to release iron, leaving selectively those molecules from which the release is slower. Those Ft molecules left which are less able to

release, iron are also the less able to accept iron. The iron core of these molecules could be therefore considered as relatively "refractory". At the same time, when requirements for metabolically active iron are increasing, the synthesis de novo of Ft induced by iron should be reduced. Hence, a cell with increased need for metabolically active iron would pass through a stage when it is still needing iron and its existing ferritin contains an iron core less kinetically able to release or accept iron. This situation would be maintained and probably exacerbated if incoming iron via the Tf-TfR pathway did not keep pace with the metabolic need of the developing cell.

6.1 Mechanism in tM

The fact that tM retained relatively more of a pulse of iron introduced as Tf-antiTf immune complexes, but had a lower content and depressed synthesis of Ft compared with the rM, indicates that these cells may divert iron to an apparently non-reactive intracellular pool. This putative non-reactive pool may be composed of several entities. Firstly, there is iron entering unidentified proteins (the intermediate M.W. fraction), as this portion of ingested iron is increased in tM. Part of this iron in the intermediate M.W. fraction should be excluded from the putative non-reactive pool, as it probably consist of iron being incorporated into enzymes. The other entities of the putative non-reactive pool could be iron deposited in relatively overloaded Ft molecules and deposition of iron to an insoluble fraction.

Considering that the iron to be released has to pass through the labile pool, the flow of iron coming from Ft would be reduced in these macrophages due to the 'refractory' core condition discussed above. The extracellular release of iron will, therefore, be reduced too. This condition of 'refractory' Ft cores is supported by the findings that tM have a low content of Ft with a relatively high Fe:Ft ratio, and depressed Ft synthesis, which indicates that the iron deposited into Ft in these cells would be more difficult to release. It is known that iron recently acquired by Ft is more readily released than iron which has been bound for a longer period (the so called "last in, first out principle"), and also that iron can be more readily mobilised from Ft molecules containing about 1500 iron atoms (i.e. about 1/3 loaded) than from molecules of greater loading, probably because of the greater surface area of the iron core in the former (section 1.5.2; Harrison et al, 1974).

The other part of the putative non-reactive pool, the buffer-insoluble fraction found in cell debris, could represent a really inert fraction i.e. Hs or a Hs-like compound. Although the size of this fraction is small and no evidence of the rate of formation are available, it could be the starting point for deposition of more iron into an inert form.

6.2 Mechanism in CpM

The CpM released more ^{59}Fe and ^{125}I than rM, but the release ratio (^{59}Fe:^{125}I) is very similar in both types of macrophage. This could indicate that the intracellular

mechanism determining iron release by both cells is similar, but not exactly the same.

The CpM contain less Ft than the rM, but the ratio Fe:Ft is very similar. Even though they showed enhanced synthesis of Ft, the CpM gave a larger extracellular release of iron. Considering that CpM have a fast turnover of Ft, the Ft present in these cells will be on average less loaded, making it easier to release and deposit iron from and to these Ft molecules. Furthermore the high turnover of Ft could indicate that the flow of iron into the labile pool will be enhanced, and therefore the extracellular release would be increased. Rittling and Woodworth (1984) have shown in rat L-6 cells that the rate of accumulation of Ft was constant up to 92h after incubation with FeNTA. Even though the rate of Ft accumulation was constant its synthesis and degradation were both increased up to two-fold at the beginning of the induction (0-4 h) compared with later stages. Thus, during induction of Ft by iron, in which a priori the flow of iron through the labile pool should be increased, there is no excessive accumulation of Ft because there is also an accompanying enhanced degradation. If these findings applied to the CpM which showed similar features to L-6 cells, i.e. increased Ft synthesis with accelerated degradation, it could be inferred that the flow of iron through the labile compartment is increased.

Although increased synthesis of Ft has been thought to be the underlying factor for iron retention during inflammation (Konijn and Hersko, 1977; Konijn et al, 1981),

this suggestion has to be qualified. This proposal was based on results from whole liver, without identifying which cells were responsible. The results presented here suggest that increased Ft per se does not lead to abnormal intracellular iron retention. Probably enhanced Ft synthesis is part of the monocyte differentiation process in response to an abnormal process (inflammation). Therefore there would be an increase of Ft during inflammation reflecting the infiltration and differentiation of young macrophages in order to reach the levels of Ft found in the normal macrophages.

6.3 Mechanism of iron release by normal macrophages

The character of the following propositions are a reflection of the shortage of experimental evidence related to release of iron by cells, despite its importance in maintaining the homeostasis of iron metabolism of the body. The main area of controversy is still the processes of transport of iron across bio-membranes and for the release of iron there is at least one and perhaps many membranes to be crossed. Several elements that are relevant to release of iron by macrophages are discussed.

Although the ingestion of infectious microorganisms is one of the most dramatic roles of the MPS, it is almost certainly of secondary importance in quantitative terms (Silverstein et al, 1977). Quantitatively, senescent and moribund cells, cell fragments and a variety of small particles and soluble macromolecules probably represent the bulk of materials endocytosed daily by the MPS. This is most easily illustrated by the normal turnover of

erythrocytes in man. A 60-70 Kg adult has about 2.5 x 10^{13} RBC, calculated from the total volume of blood which is 6-8% of body weight (Wintrobe, 1942) and the normal concentration of RBC in human bood (man) which is 5.4 x 10^{12}RBC/l (Coulter Counter, Coulter Electronics Ltd.,). The normal life span of erythrocytes in man is around 120 days (Bezkorovainy, 1980). Thus each day 1/120th of the total RBC, i.e. ~2 x 10^{11} erythrocytes have to be removed from circulation. This degradation of senescent erythrocytes requires the handling of 20-23 mg of iron per day, and in addition the macrophages of the bone marrow handle 7.0-7.5 mg of iron from ineffective erythropoiesis. (Cook et al, 1970; Cavill et al, 1977). The ingestion of just one human erythrocyte represents 70-120 fg of iron to be handled by the phagocyte (section 5.3.2), which could be considered as a sudden large quantity of iron. Despite this large quantity of iron in the erythrocytes the macrophages are well equipped to handle this challenge. The macrophage strategy to handle this quantity of iron, could be summarised as compartmentalization of the RBC degradation within a phagolysosome, extracellular release of iron and efficient buffering of the iron which enters the cytoplasm (section 1.7). After phagocytosis of the erythrocytes, in order to return the iron in Hb to circulation, the macrophage has to degrade the RBC, and this occurs after the fusion of a lysosome to form a phagolysosome or secondary lysosome (Alberts et al, 1983).

The release of iron from the MPS (or RES) is well known to occur in two continuous stages, these being an early fast and late slow phases (Fillet et al, 1974; Hershko et al, 1974; Haurani and Ballas, 1985). In dogs, the biphasic release of iron after the infusion of heat damaged ^{59}Fe-RBC has a $t_{1/2}$ of 34 minutes and 7 days, respectively for the two phases (Fillet et al, 1974). The release of iron by resident peritoneal macrophages following the ingestion of ^{59}Fe-Tf-αTf immune complexes also follows a biphasic patern (Esparza and Brock, 1981a; Brock et al, 1984). It is well known that during the initial phase of release there is a proportion of iron in the incubation medium due to free Hb, in those experimental models in which iron release has been studied following ingestion of RBC by macrophages (Custer et al, 1982; Haurani and O'Brien, 1972, Haurani and Meyer, 1976, Haurani and Quinn, 1985). In the present work, as well as in that of Esparza and Brock (1981a), it was observed that a proportion of the iron being released following the ingestion of Tf-αTf was in the form of undegraded immune complexes, though this form of iron found in the incubation media was a small proportion of the total, most of which was bound to Tf in the culture medium. Thus it is reasonable to postulate that a part of the iron release during the early phase could be iron that never enters the cytosol of the macrophages. This iron could be released by regurgitation from the phagocytic vesicle and/or exocytosed from the phagolysosome. Regarding the in vivo situation, although this partial regurgitation of debris of ingested erythrocytes could appear to be a fault in the process of ingestion-degradation, it might also be

that nature has evolved a mechanism to correct this probable leakeage of Hb from macrophages and to remove Hb from intravascular erythrocyte lysis by the presence of haptoglobin that binds free Hb in circulation (Hershko et al, 1981). And if this regurgitation occurs normally along with phagolysosome exocytosis, there will also be release of iron associated with low M.W. molecules present within the phagolysosome. The phagolysosome membrane shrinks in size as the particle inside is digested, but the membrane remains at all times closely applied to the content (Silverstein et al, 1977; Steinman and Cohn, 1972). Although the precise compounds and mechanism by which Fe is translocated across membranes is highly controversial it is a fact that the iron is transferred. Thus it is possible to postulate that during the degradation of an erythrocyte in the phagolysosome, which is gradually shrinking, there is a gradient of iron across the membrane towards the cytosol, and probabably some of the iron will also be released extracellularly depending on the proximity of the lysosome membrane to the cytoplasm menbrane. It is known that the internalization of macrophage membrane (in cell culture) is extremely fast and during phagocytosis is even faster (Steinman, et al, 1981). In normal conditions this membrane influx accounts for approximately twice their cell-surface area each hour. This internalization of cell membrane is principally due to "constitutive" pinocytosis, probably secondary to ligand binding and/or secretory activity. Sometimes these pinocytic vesicles can be considered as a transmembrane shuttle due to the fact that some of these

pinocytotic vesicles in macrophages do not fuse with other vesicles in the cytoplasm but instead fuse with the plasma membrane (Silverstein et al, 1977). Could it be possible that all vesicles, or some of them, function as a vehicle to carry iron outside the cell boundaries to be chelated by Tf? Probably the answer is that only a limited proportion of them could perform this as a passive mechanism, perhaps only those generated from a secondary lysosome. Brock et al (1984) have shown that the release of iron by peritoneal macrophages is not regulated by the availability of free binding sites of extracellular Tf. They also demonstrated that when the macrophages were incubated with 100% iron-saturated Tf, the iron released by the cells was found in a compound of high molecular weight. It was suggested that this iron was released in the ferrous form and that it precipitated as Fe^{3+} when leaving the cell. This is in keeping with the idea that the release of iron is mediated via vesicles, principally by acid vesicle derived from lysosomes, since the presence of ferrous iron is more feasible within the interior of a vesicle, especially in an acid eviroment.

Alternately there is the suggestion that the early phase of release of iron by the RES represents iron which enters the labile pool and is then released by the cell (Hershko et al, 1974; Haurani and Meyer, 1976; Haurani and Ballas 1985; Brock et al, 1984). The proposal that the release of iron by macrophages could be via vesicles is not incompatible with the idea of involving the labile pool. The concept of a labile iron pool, used in the context of iron release by cells does not specify compartmentalization, and it could

be that in macrophages a large part of the so called-labile pool is in vesicles. Also this iron in vesicles probably represents the pre-release pool postulated by Lipschitz, et al (1971). Pippard et al (1985) found that blocking lysosomal degradation with chloroquine prevented chelation of ^{59}Fe by desferrioxamine from ^{59}Fe-Ft-laden hepatocytes. This finding clearly shows that the availability of chelatable iron is conected with lysosomal free iron. Thus it is reasonable to think that in macrophages a large part of the labile pool is in phagolysosomes.

The late phase of release of iron is known to occur at a slower speed than the early phase, and is thought to be due to iron which is first taken up by Ft and then slowly exchanged with the labile pool, a proportion of which is released (Brock et al 1984; Esparza and Brock, 1981a). Expanding this suggestion with the results presented in this work (sections 5.2.2.4, 5.2.4, 5.2.5), it could be postulated that the high content of Ft found in rM would serve as a buffering system, to prevent adverse effects of the incoming iron from the phagolysosome, by deposition in preformed Ft molecules, and, if the influx of iron increases, by de novo synthesis of Ft. This iron deposited in Ft could in turn exchange with the labile pool. But as the normal macrophage is not in metabolic need of iron, as can be inferred from the lack of Tf receptors in rM (Hamilton et al, 1984) and Kupffer cells (Soda and Tavassoli, 1984), the iron in the labile pool would be diverted preferentially to extracellular release. The majority of the iron from Ft would be released

extracellularly, by coupling the release of iron during Ft degradation with the formation of iron-containing vesicles from autophagolysosomes and/or exocytosis of the content of those autophagosomes. But this Ft degradation in turn will introduce more iron into the cytoplasm. Thus the slow release of iron characteristic of the late phase would be achieved by the reflux of iron from degradation of Ft, due to partitioning between iron being reincorporated into Ft and the iron released extracellularly by the possible vesicular mechanism mentioned above.

Chapter 7

Conclusions

7 Conclusions

The relationship between the results reported here and retention of iron by macrophages *in vivo* during inflammation cannot, at this stage, be clearly defined. But several suggestions can be made.

If the results of this work were applied to the *in vivo* situation, it would suggest that macrophages elicited non-specifically, for example, by cell debris from damaged tissue, might exhibit increased iron retention, whereas macrophages activated as a result of an ongoing immune response might show no such property. Clearly, further *in vivo* studies would be required before such conclusions could be firmly established. However, it might be relevant that Hs-like deposits are seen in macrophages at the site of chronic local lesions in diseases such as rheumatoid arthritis (Muirden and Senator, 1968; Blake *et al*, 1984) or allergic encephalomyelitis (Bowern *et al*, 1984) and these are thought to result from ingestion of extravasated red cells and other debris. Failure to release the iron so acquired in the normal manner could exacerbate iron accumulation in the lesion and potentiate possible toxic effects of free iron (Gutteridge *et al*, 1981) arising when these iron-laden cells break down.

Deposits of Hs-like compounds (iron demonstrable by positive Perls' Prussian blue) and the presence of Ft was not observed in all macrophages seen in rheumatoid synovitis (Blake *et al*, 1984). These authors suggested from their histochemical observations that Ft production fails in a proportion of macrophages. Thus the iron derived from

RBC would probably finish up in Hs-like deposits. The results presented in this work regarding tM have shown a possible mechanism for the start of iron accumulation into Hs-like compounds. Thus there may be a certain population within synovial macrophages which cannot release and handle intracellular iron in a normal manner. Interestingly the amount of Perls' positive iron bore no relation to the activity of the disease at the time of the analysis, but was associated with a poor prognosis, and the presence of Ft, which was associated with the activity of the disease, had no relation with the outcome.

Thus a probable mechanism that perpetuates the iron accumulation seen in the synovia could be as follows. At the beginning of the infiltration the release of reactive oxygen intermediates (ROI) triggered by phagocytosis (in neutrophils or macrophages) could damage their plasma membrane and may also damage surrounding tissues (Blake et al, 1984). The death of phagocytes leads to the liberation of proteases from lysosomes, free iron, and also Lf (from neutrophils, if it was not released during phagocytosis). It is known that Lf, if not fully iron-loaded, inhibits hydroxyl radical generation (Gutteridge, et al, 1981). Therefore, due to degranulation of neutrophils, cell-self destruction, tissue damage, and incoming RBC, there will be a vast quantity of cell debris, Lf and erythrocytes which will be ingested by the infiltrating macrophages. This massive ingestion could be responsible for abnormal handling of iron from the phagocytosed erythrocytes and inhibition of ROI formation (common features of elicited

macrophages; Nathan , 1981; Thompson, 1985). As this process proceeds chemotactic factors would continue to attract more phagocytes. Activated macrophages would arrive, with the functional peculiarities of inceased Ft synthesis, normal release of iron and enhanced production of ROI. Therefore more tissue destruction would occur. The presence of these activated macrophages could explain the observation of Blake et al (1984), regarding the activity of synovitis and the accumulation of Ft in macrophages in the early development of the disease. Also as the inflammatory process continues, the appearance of younger cells (similar to tM), with depressed Ft synthesis would continue to increase. Thus the accumulation of large deposits of Hs-like compounds would be established. Furthermore the death in situ of these macrophages (either activated or elicited) would leave free iron, free Ft and free Hs all of which can catalyze ROI generation via the Fenton reaction (Gutteridge et al, 1981; Blake et al, 1984; 1985a) to amplify the inflammatory process.

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