To my parents

Tissue specific opsonins for phagocytic cells

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A B S T R A C T

The rapid and efficient uptake of intravenously injected foreign particles, such as drug carriers, by blood-sinus-lining mononuclear phagocytes provides an opportunity for passive or spontaneous targeting. However, the goal of passive targeting of drug carriers has a major drawback, that the targeting is not restricted to a diseased or a specific organ of mononuclear phagocytic system (MPS). Coating of particulate materials with plasma or serum components (opsonins and dysopsonins) is believed to be important in determining their recognition by the mononuclear phagocytic cells. Colloids of differing surface characteristics acquire different array of serum opsonins and dysopsonins on exposure to blood and these differences might account for the different patterns in their clearance. Identification of the serum components that enhance or inhibit the uptake of colloidal carrier by the blood-sinus-lining mononuclear phagocytes might thus offer new opportunities for manipulating the tissue distribution of drug carriers.

In this thesis the effect of serum opsonins on the interaction and subsequent uptake of liposomes (as an example of drug carrier) by rat bone-marrow, liver, spleen, and peritoneal phagocytes *in vitro* have extensively been studied. Evidence presented for the first time demonstrates that serum contains opsonins specific for hepatic, bone-marrow, splenic, and peritoneal phagocytic cells. Characterization and properties of these opsonins were studied and it was found that these opsonins have different affinities for liposomes of varied phospholipid composition and cholesterol content. Some properties of opsonins

specific for bone-marrow and splenic phagocytes are similar to each other, whereas liver-specific opsonin(s) have properties which are opposite to those of bone-marrow specific and spleen-specific opsonins. Some properties of peritoneal-specific opsonin(s) are similar to bone-marrow specific and spleen-specific opsonins, but it is suggested that the nature of peritoneal-specific opsonin(s) is different from that of bone-marrow and spleen specific opsonins.

Since the majority of intravenously injected colloids is sequestered by the largest organ containing blood-sinus-lining mononuclear phagocytes, i.e. the liver, an attempt was made to purify serum specific opsonin(s) for kupffer cells. A partial purification and characterization of this opsonin(s) from rat, rabbit, and calf serum was achieved. This opsonin(s) is a heat stable proteinaceous macromolecule which possesses an apparent molecular weight greater than that of bovine albumin.

A comparison between the opsonic activity of normal serum and serum from the tumour bearing rats was performed. The results demonstrated an increased opsonic activity of serum from tumour bearing rats for splenic and a diminished opsonic activity for hepatic uptake of liposomes. Further investigation on to the cause for the diminished opsonic activity towards hepatic phagocytosis was also conducted.

Finally, this thesis proposes that identification and purification of organ-specific opsonins may not only provide opportunities for targeting of drug carriers specifically to the desired organ containing blood-sinus-lining mononuclear phagocytes, but also enable us to evaluate their role in the altered opsonin states known to exist in certain diseases.

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Chapter I

INTRODUCTION

1.1. The mononuclear phagocytic system

Mononuclear phagocytes are widely distributed in the body, being present in blood, bone-marrow, connective tissue, liver, lungs, lymphoid tissue, nervous tissue, and the serous cavities. These cells constitute an important part of the defence mechanism of the body by clearing the blood, the lymph, and tissues of particulate pollutants, for instance, microorganisms and damaged body constituents.

The importance of phagocytic cells in antibacterial defence was first delineated in 1882 by Elie Metchnikoff. Starting with the observation of transparent starfish larvae, Metchnikoff examined phagocytosis in the various animal phyla. He found that vertebrates characteristically possessed two types of cells able to fight invading microorganisms. The cell types were "macrophages", i.e. 'big eaters', because they are capable of ingesting particles such as spermatozoa, protozoa, etc, as opposed to the polymorphonuclear leucocytes (PMN) which he termed "microphages" or 'small eaters', since these cells are able to take up only smaller particles (e.g. bacteria). Early in the twentieth century, other investigators showed that acid disazo dyes (trypan blue) injected into animals accumulated in certain cells in different tissues and organs. This collection of cells were named by Aschoff (1924) the "reticuloendothelial system" (RES), a term which unfortunately persists in the literature. Aschoff proposed that the RES was not only involved in defence but also had other functions such as haematopoiesis, blood destruction, and metabolism of iron, bilirubin, and fat. Several of the cells included in the RES (table 1.1) belong to the original macrophage population described by Metchnikoff. It is now known that the disazo

dyes are bound to plasma albumin and taken into cells by pinocytosis. Indeed, as the dyes administered *in vivo* are also captured by other cells such as kidney tubular epithelia, dye uptake alone cannot be used to define the macrophage system. Several cells included in RES, such as those lining the sinusoids of the spleen, or the fibroblastic reticular cells of the haematopoietic tissues, are only weakly phagocytic and differ from monocytes and macrophages in their recognition of particulate matter.

In recent years the RES has been re-examined and a new term, the mononuclear phagocyte system (MPS), has been proposed to replace RES (Langervoort *et al.*, 1970). As shown in table 1.1, the MPS excludes some of the cells of the RES and adds others. Minimal criteria for inclusion of cell types in the MPS are:

1) derivation from bone-marrow precursor cells,

2) characteristic cell structure, and

3) high level of phagocytic activity mediated by immunoglobulin and components of the serum complement system.

PMNs are arbitrarily excluded from the MPS classification, even though they share many properties with the cells of MPS.

Table 1.1. Comparison of RES and the MPS

RES	MPS
Endothelial cells	Promonocyte (bone-marrow)
Fibrocytes	⇓
Reticular cells (spleen & lymph nodes)	Monocytes (blood)
Reticuloendothelial cells (lymph and blood sinuses)	⇓
Histiocytes	Macrophages (tissues)
Splenocytes & monocytes	Connective tissue (histiocytes)
	Liver (kupffer cell)
	Lung (alveolar macrophage)
	Spleen (free & fixed macrophages)
	Lymph node (free & fixed)
	Serous cavity (peritoneal cells)
	Nervous system (microglia)
	Bone tissue (osteoclasts?)

1.2. Physiological roles of the MPS

The MPS is known to participate in a variety of biochemical and physiological events. In biochemical events the MPS is known to participate in secretion of numerous products that affect a wide range of host functions. These secretory products include complement components; coagulation factors; enzymes such as lysozyme, elastase, acid hydrolases, etc; enzyme inhibitors such as plasmin and phosholipase A2 inhibitors; factors regulating synthesis of substances by other cells such as serum amyloid A & P factors (e.g. hepatocytes); factors promoting replication of cells such as lymphocytes or fibroblasts; factors inhibiting replication of lymphocytes, tumour cells, etc; chemotactic factors and reactive metabolites of oxygen and other hormone-like factors (Reviews by Unanue, 1976; Adams & Hamilton, 1984; Nathan and Cohn, 1985).

Some major physiological activities performed by the fixed macrophages of MPS are phagocytic clearance from the blood of microorganism, effete autologous tissue debries, dead cells, erythrocytes, immune complexes, microaggregates of fibrin, inert and metabolizable foreign colloidal and particulate materials such as colloidal carbon, latex beads and particulate drug carriers (Reviews by Altura, 1980; Poste, 1983).

In addition to the above functional roles the MPS is also involved in a myriad of other functions, for example, determining the host response to haemorrhagic, traumatic, septic and endotoxin shock, burns, surgery, x-ray irradiation, prolonged tissue ischemia, response to drugs, bacteria

and viral infections, tumour growth, atherosclerosis and immunological depression (Reviews by Saba, 1970; Allen, 1988).

Phagocytosis is the most prominent overt physiological feature of the MPS. Since one of the most fascinating aspects of phagocytosis is the cell's ability to discriminate between normal self components on the one hand and damaged self or foreign particulates on the other (Stossel, 1974; Silverstein *et al.*, 1977), an understanding of the factors regulating this discrimination is critical in attempting to describe the basic physiopathology of the MPS.

1.3. The recognition process

The prerequisite for ingestion and subsequent digestion is the formation of the critical adhesive bond between the particle to be ingested and the phagocytic cell. Several biological entities are known to either promote (opsonins) or retard (dysopsonins) this event. The term "opsonins" is generally taken to refer to some physical entity which adsorbs onto a surface, thereby rendering that surface more palatable to phagocytes. Opsonins primarily affect the critical first step in phagocytosis, i.e., the attachment phase between the phagocyte receptor and the particle (Messner and Jelinck, 1970; Mantovani *et al.*, 1972; Reynolds *et al.*, 1975; Scriber & Fahney, 1976; Fearon 1980). Opsonins are not generally regarded as being those factors which influence subsequent events in the process, such as actual internalization of the particle and/or digestion. It should be emphasized, however, that even in the complete absence of any opsonins, the phagocytic ingestion of particles, including bacteria, can also occur (Van Oss, 1978). Such

ingestion is mediated by the non-specific recognition of the particle. The extent of such ingestion is determined by the relative surface energies of the interacting components, i.e., phagocyte, particle, and suspending liquid medium (Van Oss *et al.,* 1984). These aspects, i.e., the non-specific recognition and specific recognition (opsonins) are subject to brief review here.

1.3.1. Non-specific recognition

The hydrophobicity, charge, and chemical composition of the surface of a particle plays an important role in determining the nature of the particle-cell interaction. Mudd & Mudd (1924) first postulated that encapsulated bacteria are much less readily ingested by mammalian phagocytes than are unencapsulated variants of the same species because of the diminished hydrophobicity of the bacterial surface generated from the encapsulation. Further extension of this hypothesis determined the importance of particle charge from a variety of systems using both bacterial and fungal substrates (Stendahl & Edebo, 1972; Van Oss & Gillman, 1972; Stendahl *et al.,* 1973/ 1974/ 1977; Pesanti, 1979; Kozel *et al.,* 1980). Evidence also indicates that hydrophobic forces are perhaps involved in the phagocytosis of latex beads and erythrocytes (Walter *et al.,* 1968; Van Oss, 1978; Capo *et al.,* 1979), two of the most commonly phagocytic substrates used *in vitro* studies. Although non-specific recognition mediated by such forces is probably important in uptake of these particles, evidence is accumulating for participation of receptors for at least some of these particles, notably the participation of receptors recognizing sugar residues of glycoproteins on particle surface. One example is the involvement of the mannosyl receptor in

zymosan phagocytosis. This has been suggested by plating macrophages on a fixed layer of zymosan, which leads to loss of membrane mannosyl receptors and loss of ability to phagocytose zymosan (Berton & Gordon, 1983). Interestingly, macrophages can also internalize zymosan even in the presence of high concentrations of D-mannose which should block the receptor sites on the cell surface (Bodmer & Dean, 1983). This may demonstrate the involvement of other receptors for the phagocytosis of zymosan. Another example is participation of rat macrophage galactose-specific receptor on the removal of aged but not fresh erythrocytes, (see also page 36-39), (Schlepper-Schafer *et al.*, 1980).

The physical characteristics of the surface of the macrophages can also play a vital role in phagocytosis. Perhaps, the most important physical property of the phagocyte surface is its hydrophobicity. This parameter is reflected in measurements of the angle a droplet of saline placed on a cell monolayer makes with that monolayer (Van Oss and Gillman, 1972). Using this technique Van Oss & Gillman (1972) re-established a general physical model for phagocytosis. They stated that a phagocyte will engulf particles whose surface hydrophobicity is greater than its own, but not particles whose surfaces are more hydrophilic than its own. Thrasher *et al.* (1973) reported that a lymphokine preparation decreased the surface hydrophobicity of cultured macrophages, enhancing their ability to ingest opsonized erythrocytes. Subsequently, Griffin & Griffin (1979) and Vogel & Rosenstreich (1979) reported enhanced immune receptor function imparted to macrophages by lymphokines. Vogel & Rosenstreich (1979) found that macrophages derived from lipopolysaccharide-hyporesponsive C3H/HeJ mice

(macrophages known to exhibit a decreased ability to phagocytose immunoglobulin-opsonized sheep red blood cells) demonstrated normal phagocytic capability after exposure to a lymphokine preparation. Griffin & Griffin (1979) have also found that a lymphokine preparation converted the mouse peritoneal macrophage complement (C3b) receptor system from mediating only particle binding to promoting both the binding and internalization of complement-coated erythrocytes, but not affecting the phagocytosis via the Fc receptor system. Recently, Griffin & Mullinax (1981) suggested that this enhanced C3b receptor function may be due to increased mobility of the receptor within the plane of the plasmalemma.

1.3.2. Specific recognition (Opsonins)

Following the pioneering studies of Metchnikoff (1882) on the role of phagocytosis, it was soon established that optimum phagocytic ingestion of some particles required either the addition of serum to the system or the pretreatment of the particles with serum (Wright & Douglas, 1903). Indeed, as early as 1903 Wright & Douglas concluded from their studies that

> " We have conclusive proof that the blood fluids modify the bacteria in a manner which renders them a ready prey to the phagocyte. We may speak of this as an 'opsonic' effect (opsono-to cater for) and we may employ the term 'opsonins' to designate the elements in the blood fluid which produce this effect".

These early studies demonstrated the presence of two groups of opsonins, one heat labile and the other heat stable. The opsonic activity of molecules that belong to heat labile group can be destroyed by heating the serum at temperatures above 50° C for 30 minutes with complete

inactivation occurring in some experiments at 60-65° C for 10-15 minutes (Wright & Douglas, 1903; Hektoen and Ruedieger, 1905).

Neufeld and Rimpau (1904) documented the presence of opsonins in immune sera which were effective against the immunizing bacterial strain, even at high serum dilutions and which, in addition, were stable at 56° C . Such opsonins were initially referred to as " bacteriotropins " and later as heat stable opsonins.

It is now a generally accepted view that the specific opsonins which are heat stable are immunoglobulins and that specific heat labile opsonins are components of the complement system. These two systems act synergistically to prepare particles for ingestion. The heat stable immunoglobulins serve both to identify (through immune-complex formation) the invasive foreign particles and to activate the complement cascade. It should be emphasized, however, that several other serum factors can also play a role in opsonization. These non-specific opsonins are discussed in a separate section.

A. Specific opsonins

a) Immunoglobulins (Ig)

1. IgG - The opsonizing role of IgG has been well established in literature. This molecule can activate the ingestion by phagocytes of coated microorganisms (Nickerson *et al.*, 1970; Jones *et al.*, 1972), erythrocytes (Huber *et al.*, 1968), platelates (Handin and Stossel, 1974), and neutrophils (Boxer & Stossel, 1974) by monocytes, macrophages, and neutrophils. Ingestion of IgG-coated drug carriers by leucocytes (Finkelstein *et al.*, 1981), peritoneal macrophages (Hsu & Juliano, 1982) and kupffer cells (Derksen *et al.*, 1987) have also been documented.

Of human IgG molecules, IgG1 and IgG3 are mainly implicated in opsonization (Haeffner-Cavaillon *et al.,* 1979) and have been found to bind with the greatest efficiency to neutrophils. The remaining subclasses being relatively inactive (Huber *et al.,* 1968; Johnston *et al.,* 1973).

The opsonizing ability of IgG molecule is retained in the Fc moiety, of IgG subclasses IgG1 & IgG3, while the Fab and $F(ab')_2$ fractions totally lack the opsonizing ability (Van Oss *et al.,* 1973). Chemical treatment of Ig G with sodium metaperiodate, mercaptoethanol or pepsin resulted in the loss of IgGs opsonizing ability, since they all affect the Fc tail portion of this antibody molecule (Quie *et al.,* 1968). Papain or pepsin digested IgG, which lacks the Fc region, binds to particles but lacks the opsonic activity, i.e., it does not promote enhanced phagocytic ingestion (Handin & Stossel, 1974).

2. IgM - Some investigators were unable to demonstrate receptors for the IgM on neutrophils (Henson, 1969; Ward & Zvaifler, 1973). These results are in apparent contradiction with the earlier studies of Robbins *et al.* (1965), which suggested that particle-adsorbed IgM had a high degree of opsonic activity. However, opsonic activity of IgM has been shown to be dependent upon addition of complement. Dosseh *et al.* (1969) found that the addition of complement was required for the phagocytic ingestion of erythrocytes already sensitized with IgM which alone exhibited no opsonic activity.

Further, IgM has a profound hydrophilicity, and thus adsorption of this antibody, on to the surface of, for example, micro-organisms, will not result in the production of conditions which are favourable for non-

specific phagocytic ingestion (Van Oss *et al.*, 1981/ 1984).

3. IgA - This immunoglobulin as well as secretory IgA (sIgA) may be regarded as a true dysopsonin (substances known to inhibit phagocytic ingestion) for both neutrophils and macrophages (Reynolds *et al.*, 1978) despite the fact that subpopulation of both neutrophils and macrophages possess Fc receptors specific for IgA (Fanger *et al.*, 1980).

Dysopsonic activity of IgA may arise from its marked hydrophilicity, due to its large content of carbohydrates (Kobayashi, 1971), and this makes IgA uniquely suited for preventing viruses from penetrating a cell (Van Oss & Absolom, 1981). The dysopsonic functions of IgA and sIgA may be important in preventing infection (particularly viral infection) in the upper respiratory anatomy where exclusion and subsequent removal of microorganisms is feasible (e.g. by coughing) without assistance of phagocytosis (Heremans, 1975).

4. Other immunoglobulins - High density and affinity IgE receptors on a variety of phagocytic cell types including basophils and macrophages have been demonstrated (Ishizaka & Ishizaka, 1978). However, the opsonic role for this immunoglobulin and also IgD has not yet been clearly established.

b) Complements

A number of laboratories have shown that the sequential interaction of antibody and classic complement components C1, C4, C2 & C3 is sufficient to opsonize erythrocytes, various microorganisms, and possibly even urate crystals (Gigli and Nelson, 1968; Johnston *et al.*, 1969; Young, 1972; Naff & Byers, 1973). However, sera from various species deficient in antibody, C4, C2 or C1 were found to have heat-

labile opsonic activity equivalent to that of normal sera, suggesting that the complement system which can operate in the absence of antibody was activating C3 and thereby opsonizing as well (Johnson *et al.,* 1972; Root *et al.,* 1972). The dominant opsonin generated from complement is believed to be the high molecular weight C3b fragment of C3. Stossel *et al.* (1975) have suggested that opsonic activity per se does not reside in the C3b fragment as such but rather in a low molecular weight derivative.

It is well documented that C3b can bind to a wide variety of substance. The mechanism of this binding has not yet been elucidated (Absolom, 1986). Due to presence of C3b inactivators in serum (Lachmann and Muller-Eberhard, 1968), subsequent exposure of C3b bound particles to sera will result in the cleavage of C3b to C3c and C3d fragments (Muller-Eberhard, 1975). Thus, C3b attachment to C3b receptors on phagocytes must presumably occur rapidly if phagocytic ingestion is to be enhanced.

An opsonic role for C5 has been suggested on the basis that mice genetically deficient in C5 are more susceptible to experimentally induced bacterial and fungal pathogens than are normal mice (Shin *et al.,* 1969; Morelli and Rosenberg, 1971).

c) Activation of IgG & C3b receptors - Of macrophage receptors a great attention has been imposed on complement and Fc receptors. Activation of these receptors are of great importance for ingestion of opsonized particles by the phagocytes. The work of Griffien *et al.* (1975) showed that erythrocytes coated with IgG or C3b were not ingested when Fc or C3b receptors lying outside the zone of attachment were blocked with

antimacrophage IgG. Griffien *et al.* (1975) showed that the initial interaction of IgG or C3b ligands on the surface of a particle with receptors on the phagocyte is not sufficient to trigger the automatic ingestion of the particle and also that the phagocytic process requires the continuous apposition of receptors and ligands circumferentially until the particle is fully enclosed within a phagocytic vacuole. Shaw & Griffien (1981) further extended this hypothesis by demonstrating that continuous apposition of cell surface immune receptors and particle-immune ligand is necessary not only to guide pseudopod movement, but also for repeated generation of intracellular phagocytic signals required for particle ingestion. At the same time continuous apposition of receptor and ligand, though necessary, may not be sufficient to promote particle internalization. Michl *et al.* (1979) and Griffien & Mullinax (1981) strongly suggest that lateral mobility of the ligand-bound receptor within the plane of plasma membrane may be essential for generation of the phagocytic signal.

B. Non-specific opsonins

A number of reports have appeared in the literature describing the presence in serum of natural phagocytosis-enhancing factors which are distinct from opsonic immunoglobulins and activated complement components. Generally these are substances that can aspecifically adsorb or adhere onto particles thereby altering the physiochemical surface properties of the particles, i.e., charge and hydrophobicity.

1. C-reactive protein - This molecule belongs to group III acute phase proteins and has shown to enhance significantly the phagocytic ingestion of microorganisms by human neutrophils. C-reactive protein is

not an immunoglobulin and that substrate-C-reactive binding is not an antigen antibody type of reaction. The precise role of this protein is not clear. Included among the recognition functions is binding to phosphocholine moieties (such as those on leucocyte membranes) and polycations. It also has the capacity to recognize and bind to a number of substances such as exogenous materials derived from tissue breakdown. The mechanism by which complexed C-reactive protein enhances phagocytic ingestion is not completely understood. However, it is known that surface bound C-reactive protein activates the complement system (Gewurz *et al.*, 1982), and further that the complexed protein can bind with a high affinity to the membrane of various phagocytes (Morley & Kushner, 1982).

2. Fibronectin - This molecule is also known as cold insoluble globulin, antigelatin factor, microfibrillar protein, opsonic protein, opsonic surface binding alpha-2-glycoprotein, and also cell spreading factor (Yamada & Olden, 1978). This opsonin, first detected in rat serum, has a molecular weight of and electrophoretic mobility characteristics similar to those of human alpha-2-macroglobulin (Yamada and Olden 1978). This factor stimulates phagocytic uptake of gelatinized particles including gelatinized carbon (Saba *et al.*, 1966), gelatin coated beads (Molnar *et al.*, 1977; Doran *et al.*, 1981), erythrocytes (Weinberg *et al.*, 1985).

Binding of gelatin-coated particles to phagocytes was considerably stimulated by heparin (Saba 1970). For opsonic activity the entire fibronectin molecule was not required. Early plasmin derived fragments of molecular weight 190 000-200 000 containing a gelatin-binding and a

cell-binding site were still active although less than fibronectin itself (Molnar *et al.,* 1983). More processed fragments were inhibitory and acted as dysopsonins (Ehrlish *et al.,* 1981). Later serum was found to contain a co-factor in addition to fibronectin. This cofactor amplified binding of gelatinized particles by phagocytes (Blumenstock *et al.,* 1983).

There is still dispute about the role of the fibronectin as a direct, independent opsonin. For example, although fibronectin is capable of binding to various strains of *staphylococci,* this does not promote phagocytosis by human monocytes or alveolar macrophages (Verbrugh *et al.,* 1981). In another example, fibronectin enhanced internalization of erythrocytes coated by complement factor C3b in a dose-dependent manner (Pommier *et al.,* 1983). The same effect was observed when monocytes had been preincubated with fibronectin and washed before incubation with C3b-coated erythrocytes. If, however, the coated erythrocytes were pretreated with fibronectin prior to attachment to monocytes, they were not ingested. Evidently, fibronectin stimulated phagocytosis by interaction with the monocytes rather than by opsonization of the substrate.

3. Tuftsin - This opsonin was discovered as a result of an observation by Fidalgo & Najjar (1967) that a certain fraction (subsequently called leucokinin) of homologous IgG bound to autologous neutrophils. The active portion promoting such interaction was found to be a tetrapeptide, Thr-Lys-Pro-Arg, located in Fc portion of the leucophilic IgG (Nishioka *et al.,* 1972/1973). Two enzymes may be responsible for the production of tuftsin from leucokinin. The first is leucokinase

located on the outer surface of neutrophil membrane and which cleaves the terminal end of that threonine residue; the second is an enzyme produced in the spleen which cleaves at the terminal end of the L-arginine residue (Najjar and Nishioka, 1970; Nishioka *et al.*, 1973). Splenectomized animals have been shown not to produce tuftsin even though their leucokinin levels are normal (Najjar *et al.*, 1968).

Tuftsin enhances phagocytic ingestion 2-3 fold by both neutrophils and macrophages (Najjar & Constantopoulos, 1972). The mechanism by which tuftsin stimulates phagocytosis has not been completely unravelled. The tetrapeptide, remarkably binds to the phagocytic cell rather than to the ingested particle. Tuftsin is strongly basic peptide. Perhaps this may result in its attachment, via electrostatic bonds, to the negative charge groups already present in high density on phagocytes. It could be possible to speculate that negatively charged microorganisms (opsonized or non-opsonized) will then interact with the positively charged tuftsin, thereby promoting phagocyte-bacterial interaction and hence enhanced phagocytic ingestion (Review by Najjar *et al.*,1986).

4. Other non-specific opsonins - Several other serum components have been implicated as stimulators of phagocytosis and thus have been called opsonins. These include the heat-labile factors alpha1-globulin and ß-globulin reported by Tullis and Surgenor (1956).

Substances such as clotting factor VIII and heparin have also been implicated to show opsonic activity since they can reduce the surface hydrophobicity of the neutrophils and macrophages, thus increasing their phagocytic activity (Alexander *et al.*,1970).

1.4. Disorders of the MPS

Alterations in phagocytic activity of macrophages can result in increased or decreased clearance of materials from the circulation. As a consequence, a number of diseases can be caused by qualitative or quantitative disorders of phagocytosis. Phagocytic disorders may be classifed into extrinsic and intrinsic defects. Included in the extrinsic category are abnormalities in specific and deficiencies in non-specific serum opsonins. Intrinsic defects include disorders and abnormalities of ingestion due to hyperactive or impaired phagocytosis by macrophages.

1.4.1. Extrinsic disorders of macrophage system

1. Specific opsonins - A fall in concentration of IgG or C3 profoundly decreases the rate of clearance of phagocytic particles. Levels of IgG may be diminished because of decreased synthesis in inherited immunodeficiencies (Root and Ryan, 1985) and in acquired disorders such as lymphomas and monoclonal gammophaties (Millar, 1962). Levels of Ig G may also be decreased because of increased loss of protein, e.g. in burns (Bjornson, 1977) and protein losing enteropathies (Waldmann & Strober, 1969). Elevated levels of IgG may result in pathological conditions due to excessive clearance of materials from the circulation. Perhaps, the best example is the destruction of platelets in idiopathic thrombocytopenic purpura by macrophages of spleen and to a lesser extent, of the liver (Fehr *et al.*, 1982).

C3 levels may be decreased in a variety of inherited acquired disorders (Ross & Densen, 1984). Increased C3 catabolism may occur in states of increased circulating immune complexes or in disorders which activate the alternative pathway such as certain forms of nephritis (Parris,

1982).

2. Non-specific opsonins - Alteration in concentrations of circulating C-reactive protein and fibronectin during various clinical and pathological conditions have been well documented. Serum concentration of C-reactive protein may be increased to several fold following a large variety of inflammatory or injurious stimuli, including many infections, surgical or other traumas, fractures, and during chronic inflammatory states such as rheumatoid arthritis (Kuskner, 1982; Morley and Kushner, 1982).

Fibronectin is shown to be depleted after severe trauma including surgery (Scovill *et al.*, 1976), burns (Grossmann *et al.*, 1980) and in case of various infections and advanced tumour growth (Mosher & Williams, 1978).

1.4.2. Intrinsic disorders of macrophage system

A variety of human diseases is associated with decreased or increased numbers of monocytes and tissue macrophages. Macrophage dysfunction can also result from alteration in number or affinity of its receptor for opsonic ligands.

1. Alteration in the quantity of macrophages - A decrease in quantity of tissue containing macrophages will prolong the circulation of phagocytic particles. For instance, a striking example is observed in patients after splenectomy who have a markedly increased risk of overwhelming bacteremia with encapsulated bacteria (Hosea *et al.*, 1981). Individuals with hepatic cirrhosis have a decreased amount of functioning reticuloendothelial tissue (Jaffe *et al.*, 1978). The inability of leucopenic patients to contain or eliminate circulating pathogens is

also a consequence of the decreased number of neutrophils and mononuclear phagocytes (Stossel, 1974). However, in some conditions a reduction in the quantity of tissue containing phagocytic cells may be useful to prolong survival of vital components of the blood (Mc Millan, 1981). One example is the consumption of platelets in idiopathic thrombocytopenic purpura and is often reduced after splenectomy.

Cells of mononuclear phagocytes may proliferate in response to a stimulus, such as a variety of intracellular parasites. In such a circumstance, the response of mononuclear phagocytes is often termed "reactive hyperplasia" and this is clinically manifested by enlargement of reticuloendothelial organs such as liver and spleen. For example, in tuberculous lesions of man and animals, one sees macrophages at all stages of development, from newly arrived monocyte to the mature multinucleated giant cell (Dannenberg, 1968). Many of the phagocytes contain ingested organisms. However, one of the interesting features of this infection, as well as other infections with faculatative and obligate intracellular parasites, is that many organisms survive and even proliferate within the macrophage. Armstrong & D'Arcy-Hart (1971) have suggested that surviving organisms are sequestered in phagosomes that do not receive a complement of hydrolytic enzymes from the discharge of primary and secondary lysosomes.

The presence of large numbers of immature monocytes in the peripheral blood may also have important implications in the pathogenesis of human (HIV)-I infections, since these cells may also act as an important reservoir for viral persistence and dissemination (Roy and Wainberg, 1988). Storage of nononcogenic retroviruses, such as

those caused by lentiviruses, in mononuclear phagocytes has also been implicated (Narayan *et al.*, 1982; Geballe *et al.*, 1985). These agents cause persistent but debilitating infections, which lead to onset of arthritis, progressive pneumonia, and slow neurological diseases in ungulate mammals (Narayan *et al.*, 1982; Cheevers & McGuire, 1985).

Reactive hyperplasia may also be induced by response to chemicals, for example, chronic pulmonary disease can be caused by exposure to beryllium (Williams, 1958), or by response to haematopoietic cell destruction such as idiopathic thrombocytopenic purpura (as described before) and erythrophagocytosis. There is some evidence that erythrophagocytosis interferes with the ability of macrophages to phagocytose and destroy certain intracellular bacteria (Gill *et al.*, 1966). This may be important in the pathogenesis of the frequent infections of sickle cell disease.

Mononuclear phagocytes may also proliferate "inappropriately" to an extent that exceeds normal levels. These are described under neoplastic histiocytic proliferation disorders. Some examples are monocytic leukaemia, Hand-Schullar-Christian disease and the childrens' disease eosinophilic granuloma (Cline & Golde, 1973).

Another group of disorders in which one observes increased numbers and abnormalities of tissue macrophages is that in which substances accumulate intracellularly more rapidly than it can be disposed of by metabolic processes. These are known as storage diseases. Examples are Gaucher disease, due to deficiency of B-glucocerebrosidase, and Niemann-Pick disease, due to deficiency of sphingomyelinase, of liver, spleen, and bone-marrow macrophages.

2. Alteration in macrophage receptor number and affinity - Qualitative dysfunction of macrophages can also result from alteration in number or affinity of its cell surface receptors for opsonic ligands. For example, the number of unbound Fc receptors may be reduced by elevated levels of circulating immune complexes such as occurs in autoimmune disorders (Frank *et al.,* 1983). Monocytes and macrophages derived from AIDS patients have a diminished ability to remove ^{51}Cr-labelled opsonized red blood cells, implying a defect in Fc receptor-mediated clearance (Bender *et al.,*1985) but when tested *in vitro,* these cells retain their ability to phagocytose (Estevez *et al.,* 1986).

1.5. An approach for treating the diseases of MPS

Because of the diverse repertoire of host defence function performed by the MPS the treatment of phagocytic disorders must be directed at the underlying disease where possible. However, a common limitation to the usefulness of many therapeutic agents is their inability to reach selectively and in sufficient quantities to macrophages in order to influence their function. A promising approach involves the use of particulate drug carriers, such as oil emulsion (Davis, 1984), polymeric microspheres (Widder *et al.,* 1982), liposomes (Poste 1983), natural carriers such as lipoproteins (Vitols *et al.,* 1985) and erythrocytes (Ihler *et al.,* 1973), since the majority of these particles localize primarily in mononuclear phagocytes lining the vascular sinusoids of the liver, spleen and bone-marrow when administered by intravenous route. Among these carriers liposomes have been extensively studied.

1.5.1. Liposomes

Liposomes are closed microscopic vesicles that form spontaneously upon hydration of dry phospholipids above their transition temperature (Bangham *et al.*, 1965). These preparations are heterogenous with respect to size and are multilamellar with each lamella consisting of a single bilayer, separated from neighbouring bilayers by aqueous spaces (Stoeckenius, 1959; Luzzati, 1968). The multilamellar (MLV) structure can be reduced into smaller unilamellar vesicles (SUV) by ultrasonication (Papahadjopoulos and Miller 1967, Huang 1969). Although phospholipids are most often used, vesicles have also been prepared from single chain amphiphiles, niosomes, (Gebicki and Hicks, 1973; Tall & Small, 1977) and epidermal lipids, a non-phospholipid mixture, (Gray & White, 1979).

Liposomes have extensively been used as a model of lipid bilayer system and have also been implied for successful experimental manipulation of cellular functions, table1.2., (Reviews by Kimelberg and Meyhew, 1978; Pagano & Weinstein, 1978; Papahadjopoulos, 1978). Since liposomes are versatile, biodegradable and non-toxic, a wide application of these vesicles in the field of experimental and clinical medicine is abundant, table 1.2., (Reviews by Patel & Ryman, 1981; Poste, 1983). In the following section a brief review of the various applications of liposomes in treatment of a wide variety of macrophage diseases and disorders is presented.

Table 1.2. The use of liposomes in biology and medicine

Lipid bilayer	Experimental manipulation of cellular function	Clinical
Bilayer structure	Phospholipid exchange	Targeted drug delivery
Protein-lipid interaction	Fusion and endocytosis	Imaging
Ion-channels and pumps	Lipid metabolism	Vaccines
Membrane reconstitution	Cell recognition	Ophthalmic use
Membrane interaction	Antigen recognition	Dermatological use
Signal transduction	Cell sorting	Immunodiagnostics
		Skin care
		Biosensors

1.5.2. Applications of liposome formulations for the treatment of macrophage disorders

A) Treatment of infectious diseases of macrophage system

1) Bacterial infections - The delivery of antibacterial drugs to phagocytic cells by liposomes has been successfully demonstrated *in vitro* experiments. These studies have demonstrated enhanced intraphagocytic killing of *Staphylococcus aureus, Listeria monocytogenes, Brucella canis, brucella abortus, E.coli,* and *Salmonella typhimrium* by antibiotic encapsulated liposomes (Bonventre & Gregoriadis, 1978; Fountain *et al.,* 1981/ 1985; Desiderio & Campell, 1983; Stevenson *et al.,* 1983). In experimental infections caused by *Listeria monocytogenes* in mice a considerable enhancement (80 fold) in the therapeutic activity of ampicillin resulted from the intravenously injected liposomally entrapped ampicillin (Bakker-Woudenberg *et al.,* 1985) and a substantial amount of liposomal radioactive ampicillin was recovered from isolated kupffer cells, the target cell of *L. monocytogenes.*

Similarly, intravenous injection of streptomycin encapsulated in large unilamellar vesicles to mice infected with *Mycobacterium tuberculosis* prolonged survival time and produced a significant decrease in bacterial number in the spleen, but not in the lungs of these infected mice (Vladimirsky & Ladigina, 1982).

2) Parasitic infections - Treatment of leishmaniasis with pentavalent antimonial drugs is associated with marked hepatic, cardiac, renal, and gastrointestinal toxicities due to heavy metal contents of antimonial drugs (Alving, 1983). But, encapsulation of antimonial drugs in

liposomes have not only increased their efficiency, but also reduced the toxicity of these drugs as compared to unencapsulated agents in experimental models of intracellular *visceral leishmaniasis* (Alving *et al.*, 1978; New and Chance, 1980).

In one study, no enhanced activity of the liposomal entrapped primaquine could be demonstrated against malarial parasites that can possibly exist in kupffer cells. However, drug encapsulation resulted in a marked reduction of its toxicity (Pirson *et al.*, 1980).

3) Fungal infections - Cryptococcosis and histoplasmosis are fungal diseases that resemble leishmaniasis in that the organisms reside in macrophages. As in leishmaniasis, such fungal organisms present tremendous therapeutic problems because most commonly used drug amphotericin B (AMB) is exceptionally toxic. AMB, a polyene antibiotic, interacts with ergosterol in fungal cell membrane thus creating a transmembrane channel which permits the escape of vital ions and metabolites. The drug also interacts with cholesterol found in mammalian cell membranes causing similar permeability changes, which probably are the basis of AMB toxicity. Graybill *et al.* (1982) found that the maximal dose of commercial AMB that could be given in mice without acute cardiorespiratory arrest was 10 µg. When incorporated in liposomes containing ergosterol as much as 167 µg of liposome-entrapped AMB was well tolerated by the mice, and death was only observed after injection of 600 µg (Graybill *et al.*, 1982).

Systemic mycoses also represent a challenging therapeutic problem often in immunocompromised individuals such as cancer or renal transplant patients (Bodey, 1984). The incorporation of AMB in simple

phospholipid vesicles markedly reduced its toxicity with retention of excellent therapeutic activity during fungal infections of such patients (Lopez-Berestein *et al.*, 1985/1987; Sculier *et al.*, 1988).

4) Viral infections - In experimentally induced Rift valley fever, in mice, the viral replication occurs within the kupffer cells of the liver and later in the macrophages of other visceral organs (Kende *et al.*, 1985). Encapsulation of a low-dose regimen of ribavirin in liposomes had a therapeutic effect, similar to that achieved with higher but potentially more toxic doses of the free agent. The results suggest that liposome-entrapped antiviral agents might be of potential interest in the treatment of human viral pathogenes such as bunyavirus, flavi virus or even AIDS virus that possibly replicate within phagocytic cells.

B) Activation of macrophages with immunomodulators

The feasibility of exploiting the passive, natural homing, localization of intravenously administered liposomes within mononuclear phagocytes as a method for stimulating macrophage mediated host defence mechanisms has been demonstrated in several laboratories over the past decade. Systemic administration of liposome containing immunomodulators such as lymphokines (Poste *et al.*, 1979), muramyl dipeptide (Chedid *et al.*, 1978) and more recently gamma interferon (Saiki & Fidler, 1985) has been shown to activate macrophages *in situ* and to augment host destruction of spontaneous metastases. Successful treatment of metastases by the intravenous injection of liposomes containing immunomodulators have been reported for several mouse fibrosarcomas (Deodhar *et al.*, 1982; Lopez-Berestein *et al.*, 1984), melanomas (Phillips *et al.*, 1985) and colon carcinoma (Deodhar *et al.*,

1982).

Liposome encapsulated macrophage activators also appeared promising for the treatment of macrophage parasites such as leishmaniasis in mouse (Reed *et al.* 1987).

C) Destruction of macrophage system

Uptake of systemically administered liposomes by macrophages in liver, spleen and bone-marrow can also be used to achieve site-specific delivery of drugs to these cells in treatment of several disorders in which selective destruction of macrophages would be beneficial (Review by Ahn & Harrington, 1980). The elimination of macrophages in liver and spleen, but not in lungs, was achieved by intravenous injection of liposome-encapsulated dichloromethylene diphosphonate into mice (Claasen & Van Rooijen, 1984/85).

1.5.5. Metal poisoning and metal storage diseases

Metal chelators such as EDTA or EGTA are toxic due to their lack of specificity. On the other hand chelators such as desferrioxamine (DF), from a natural source, exhibits a very high specificity for iron and is non-toxic. But the hydroxamic acid functional groups of DF are rapidly degraded once DF is introduced into plasma. The liposome concept for chelator delivery in successful treatment of plutonium and experimental iron overload have been demonstrated by Rahman *et al.* (1973/1983) and Lau *et al.* (1981/1983).

The examples discussed above provide substantial evidence to support that passive targeting of drug carriers, such as liposomes, into macrophages of MPS offers realistic opportunities for site-specific drug delivery in the treatment of a number of macrophage diseases and

disorders. However, the goal of passive targeting of drug carriers has a major drawback that the targeting is not restricted to only a diseased or specific organ of the MPS. For example, in treatment of an infectious disease of spleen macrophages by means of liposome encapsulated agents; it is necessary to target liposomes only to spleen phagocytes and not to other blood-sinus-lining phagocytes such as kupffer cells and bone-marrow macrophages, as this may produce toxicity and could result in suppression of the essential defence system of the body.

The advantage of site-specific delivery can also be exploited in diagnostic imaging. For example, in selective spleen scanning by radionuclide colloids there is interference from the liver. This interference may either be anatomic or functional. The anatomic interference arises since the left lobe of the liver extends into the left upper quadrent, thus making it difficult or impossible to separate hepatic from splenic tissue, or even to be certain whether or not any splenic tissue is present (Chaudhuri & Bobbitt, 1976). Functional interference could result from the poor uptake of colloids by the spleen macrophages, at least in relation to liver phagocytes, making delineation of the organ suboptimal or impossible (Armas, 1985).

Hence, there is a need to target liposomes or any other drug carrier specifically to the required mononuclear phagocytic organ. But how can the aim of selective targeting of drug carriers be achieved?

1.6. Selective targeting within MPS

Earlier in this chapter the role of particle surface characteristics and serum opsonins on phagocytosis were described. The following section is

intended to provide natural evidences that surface characteristics of a particle and serum opsonins may allow for capture or "recognition" of a particle by one specific organ of MPS and avoidance or "non-recognition" in another. Such examples may provide opportunities for the concept of targeting of drug carriers to one specific organ of MPS.

1.6.1. The natural evidence

It is known that some microorganisms, by virtue of their surface capsules, are resistance to phagocytosis and thus escape destruction by MPS. For example, in gram negative bacilli, the somatic O-antigens may inhibit phagocytosis, as does the Vi surface antigen of *Salmonella typhi* (Freemann, 1979). Other striking examples are *Borrelia recurrentis* and *Borrelia duttoni* which cause relapsing fever. In the liver borreliae accumulate and multiply causing focal necrosis of the parenchymal cells which they invade. The fixed phagocytes do not respond to live borreliae but do ingest dead ones (Manson-Bahr & Bell, 1987).

In contrast to above examples, some microorganisms may be phagocytized primarily by macrophages of one specific mononuclear phagocytic organ. For example, uptake of pneumococci bacteria from the blood stream of immune animals occurs mainly in the liver; clearance occurs in the spleen to a much greater extent in the non-immune animals (Schulkind *et al.*, 1967). Subsequent investigations demonstrated that the complement depletion in experimental animals leads to increased clearance of pneumococci by the spleen, relative to the liver, which demonstrates the role of C3 in localizing pneumococcal uptake to the liver (Brown *et al.*, 1981a). Immunizing the experimental animals or precoating the pneumococci with antibody *in vitro* enhances uptake in

the liver, but immunization does not affect localization in complement-depleted animals; hence, serum antibody causes hepatic clearance solely via its ability to mediate complement deposition on the bacteria (Brown *et al.,* 1982). This makes complement deposition by antibody a major determinant of hepatic removal of bacteria. In the absence of sufficient opsonizing capacity in the host, e.g. in the absence of specific antibody or because of complement deficiency, the spleen assumes the major responsibility for clearance of bacteria. The ability of an animal effectively to opsonize pneumococci for hepatic clearance also depends on properties inherent in the bacteria. For example, an unencapsulated variant of very low virulence is almost totally cleared by the liver, while two different encapsulated organisms are cleared less by the liver and increasingly by the spleen in proportion to their virulence (Brown *et al.,* 1981b). These differences in clearance rate are not related to the amount of C3b bound to the pneumococci by the action of the alternative pathway, since equal amounts of C3b are deposited on the pneumococci under these conditions. Rather, the C3b, which is bound by the alternative pathway to the pneumococcal cell wall in the more virulents encapsulated strains, does not interact with complement receptors (Brown *et al.,* 1981b). Thus, the pneumococcal capsule prevents hepatic clearance by preventing kupffer cell complement receptors from interacting with C3b bound to the cell wall.

But what factors are responsible for the clearance of pneumococci by spleen macrophages? Amounts of IgG too small to activate complement may lead to phagocytosis through interaction with Fc receptors on splenic phagocytes, as occurs in the clearance of antibody-sensitized

autologous erythrocytes (Frank *et al.*, 1977). C3b fixed to the pneumoccocal cell wall by the alternative pathway may be inadequate to allow immune adherence of virulent encapsulated pneumoccocci in the liver, but may still be sufficient to mediate immune adherence in the spleen. The reason for the increased ability of the spleen compared to liver to clear lightly sensitized organisms is unknown. However, this may be related to fundamental differences in the macrophages of the two organs.

The above examples demonstrate that the recognition or non-recognition of a particle by macrophages of one specific organ of MPS may be either due to the presence of "specific" opsonins or dysopsonins in the blood or to specific characteristics of the particle surface. As a consequence the mechanism of the adhesion will be different depending on the nature of the opsonic component and the particular receptor-mediated process which may be organ specific. Hence, by manipulation of the surface characteristics of drug carriers it may be possible to gain control over normal physiological processes in order to direct particles to required sites within the vascular compartment as needs indicate. The following section describes such attempts with special reference to liposomes.

1.6.2. Manipulation of drug carrier surface characteristics

A. Manipulation of size, charge, and hydrophobicity

The fate of colloid administered into the vascular compartment will be determined by two main factors, particle size and particle surface. If the particles are large they will be removed from the blood rapidly and efficiently by the filtering propensity of the lung capillaries. This

physical trapping represents one of the easiest approach to target within the vascular compartment and has been exploited in radiodiagnostic imaging (Davis *et al.,* 1986). It is believed that the entrapment of drug loaded carriers in the lungs could allow higher concentration of drug to reach the required sites. Indeed, Hunt *et al.* (1979) observed that [^{3}H] Ara C in large MLV was preferentially taken up by the lung as compared with small MLV, while in other tissues was no significant influence of size on uptake.

The rate of clearance from the blood is also dependent upon the surface charge of colloids. Wilkins & Myers (1966) used isotopically labelled polystyrene latex modified with gum arabic or polylysyl-treated gelatin to give surface charges of negative and positive to colloids at pH 7.5 respectively as assessed by microelectrophoresis. The negatively charged colloid is largely taken up by the liver and this distribution is maintained, while the positively charged colloid shows an initial appreciable accumulation in the lungs and a later accumulation in the spleen after intravenous injection.

Similarly, the surface charge of liposomes has also played an important role on the rate of liposome clearance from circulation and, hence, their site of accumulation. In general, vesicles with positive or neutral charge are retained in the circulation for longer periods, whereas negatively charged liposomes are rapidly cleared (Gregoriadis & Neerunjun, 1974). But, it is interesting to note that regardless of initial charge of liposomes they all acquire the negative charge on contact with blood (Black & Gregoriadis, 1976). This raises the question as to how colloids with differing surface charge in saline interact with the MPS in

a variable manner, having all acquired the same charge in blood. A possible explanation is that upon entry into circulation a colloid may immediately acquire a layer of plasma components (opsonins & dysopsonins) which depend upon the initial surface charge and characteristics of the colloid. The surface properties of the colloid may then affect the steric arrangement of the adsorbed component, rendering the particle recognizable to a particular organ(s) containing blood-sinus-lining mononuclear phagocytic cells without necessarily giving rise to a difference in surface charge or acquiring the same overall charge. For example, large MLVs were more efficiently retained in the lungs when they contained the negative charged lipid phosphatidylserine than SUVs or MLVs composed exclusively of the neutral lipid phosphatidylcholine (PC), (Schroit & Fidler, 1982). Using two different molar ratios of dicetylphosphate, DCP, Tagesson *et al.* (1977) showed that MLV composed of PC and cholesterol in molar ratio of 4:1 with 10% DCP added are recovered predominantly in the liver. However, when 1% DCP is used, a larger proportion of liposomes accumulate in the spleen, suggesting that the density of negative charge on the surface may alter the distribution of liposomes, whereby the more negative the surface charge, the more liver uptake is favoured.

Further, by altering the lipid composition of liposomes some modification of their uptake by a particular organ of MPS has also been achieved. For example, Patel *et al.* (1983) demonstrated that the incorporation of 46.6 mol% cholesterol in liposomes suppresses their clearance by the liver as compared to cholesterol-poor (20 mol% cholesterol) and cholesterol-free liposomes. Interestingly, studies

conducted by Senior *et al.* (1985) demonstrated that 24 hours after intravenous injection, entrapped marker in cholesterol-rich liposomes was sequestered to a great extent in bones, perhaps by the resident macrophages of this system. In this study, sequestration of cholesterol-rich distearoylphosphatidylcholine, DSPC, was much higher than cholesterol-rich PC liposomes as determined by scanning. Incorporation of cardiolipin (Hnatowich *et al.,* 1979) in liposomal bilayer resulted in diminished liver uptake, whereas incorporation of sulfatides (Yagi & Naoi, 1986) increased the hepatic uptake of liposomes. Intravenous injection of liposome incorporated sphingomyelin resulted in a relatively strong preference to accumulate in lungs, whereas incorporation of gangliosides in liposome bilayer enhanced the splenic uptake of liposomes (Yagi & Naoi, 1986).

B. Active targeting

The study of liposome-cell interaction *in vitro* has led to the understanding that certain components of these mechanisms can facilitate targeting of liposomes to specific areas or cell types. The most common strategy for targeting liposomes to mononuclear cells has been to couple antibodies or carbohydrates to liposomes, which then serve as ligands for their binding to a specific cell surface determinent. Indeed, well characterized cell-surface components of the macrophage membrane, the Fc and sugar receptors such as mannose and galactose make this cell type amenable to a variety of targeting strategies.

1) Fc dependent targeting

In 1968 Huber & Fundenberg showed that the macrophage membrane possesses receptors for the Fc portion of immunoglobulin molecules.

This fundamental observation laid the foundation for understanding a variety of macrophage recognition mechanisms and more recently served the basic moiety to which liposomes can be directed. This involves binding of specific antibodies to antigen present in the liposome bilayer and subsequent interaction of the antibody-liposome complex with macrophage Fc receptors. Early attempts to target liposomes to phagocytic cells employed liposomes which were coated with heat-aggregated immunoglobulins (Finkelstein and Weissmann, 1978). Using more defined systems employing haptenated lipids and specific antibodies developed by Kinsky & Nicolotti (1977), other investigators were able to show enhanced uptake of liposomes by phagocytes. Thus, lipid haptens bearing either dinitrophenol (Leserman *et al.,* 1980; Hsu & Juliano, 1982) or nitroxide (Lewis *et al.,* 1980) groups were readily bound to Fc receptor-bearing cells upon interaction with specific hapten antibodies. Recently, covalent attachment of antibodies to the liposomal surface enhanced both their *in vitro* and *in vivo* endocytosis by kupffer cells (Derksen *et al.,* 1987; Derksen, 1987).

2) Carbohydrate dependent targeting

Lectins or lectin-like molecules are naturally occurring carbohydrate-binding cell surface receptors that have been implicated in a variety of cellular recognition processes (Reviews by Frazier & Glaser, 1979; Sharon, 1984). A number of investigators have tested the ability of glycolipid containing liposomes to interact with macrophages. For example, liposomes containing an aminomannose derivative of cholesterol were shown to have a dramatic influence on the ability of macrophages to bind and subsequently endocytose the vesicles (Wu *et al.,*

1981). In contrast, other cholesterol analogs, such as those containing glucose, galactose, xylose, fucose of mannose were shown not to enhance significantly liposome-cell binding (Ponpipom, 1980). These results have suggested that the presence of a sugar in combination with a positive charge is important in the recognition process. In addition to enhanced macrophage phagocytosis, the inclusion of aminomannose- cholesterol also resulted in increased lung localization following intravenous administration (Mauk *et al.,* 1980).

Sunamoto *et al.* (1984) have recently shown that the uptake of liposomes by monocytes and alveolar macrophages can be increased by coating vesicles with an amylopectin derivative. After intravenous injection the amylopectin modified liposomes were preferentially concentrated in the lungs. This observation was elegantly applied in the treatment of experimental legionnaire's disease in guinea pigs by intravenous injection of a mixture of free and entrapped sisomycin (Sunamoto *et al.,* 1984).

The idea of active or receptor targeting is attractive, but care should also be taken that through this approach the delivery of the therapeutic agents must be restricted only to the macrophages but not to the other cells within the concerned RE organ. One example is the presence of galactose-specific receptor on the surface of both hepatocytes and Kupffer cells in the liver. In recent years Kolb-Bachofen and her associates (1982) demonstrated the presence of a galactose receptor on the kupffer cells, in addition to the well-known one on hepatocytes. Inclusion of targeted ligand, lactosylceramide in SUV which gives an exposed galactose residue on the liposome surface resulted in enhanced

liver uptake (Szoka & Mayhew, 1983) mainly into parenchymal cells. In contrast, the uptake by non-parenchymal cells, due to only kupffer cells but not the endothelial cells, was only slightly increased (Spanjer & Scherpof, 1983). Thus, through this approach the majority of ligand-associated liposomes was targeted to hepatocytes rather than Kupffer cells. However this distribution can be altered by altering the bulk lipid composition of the liposomes. For example, Liposomes based on dimyristoylphosphatidylcholine, DMPC, as the major phospholipid constituent seemed to show a shift towards the hepatocytes upon lactosylceramide incorporation, whereas sphingomyelin-based liposomes were rather diverted to the kupffer cells as a result of this procedure (Scherpof *et al.,* 1986). It is conceivable that ligands, galactose in this case, anchored in a relatively rigid liposomal bilayer such as sphingomyelin allows a firmer "grip" by the receptor molecules in the plasma membrane of kupffer cells and hence, a preferable uptake as compared to DMPC-lactosylceramide liposomes by Kupffer cells can occur (Scherpof *et al.,* 1986).

In another study, intravenous injection of plasmid containing the rat proinsulin I gene entrapped in lactosylceramide incorporated reversed evaporated vesicles resulted in a substantial increase in the proportion of the exogenous gene in the hepatocytes and mainly in the endothelial cells, with a simultaneous decrease of this proportion in the kupffer cells (Soriano *et al.,* 1983). Firstly, it is somewhat confusing how such large liposomes have passed through the fenestration and reached the hepatocytes [In endothelial wall of liver the mean diameter of the fenestrations has been established at 100 nm with a maximum of 200-

300 nm (Wisse *et al.*, 1982). Although larger pores or gaps with a diameter of more than 250 nm in the endothelial cell lining have been reported (De Zanger & Wisse, 1982), their frequency is low and it is believed that, in general, particles with a diameter of more than 300 nm can not reach hepatocytes directly]. However, this may be due to mechanical interaction between the liposomes and the sinusoidal wall, resulting in processes like "forced sieving" and "endothelial massage" (Wisse *et al.*, 1982). Secondly, uptake by endothelial cells does not agree with the earlier findings of Spanjer & Scherpof (1983). Recently, Kolb-Bachofen *et al.* (1986) demonstrated the presence of galactose receptors on the surface of endothelial cells. These receptors are capable of forming the coated pit structures, but unlike kupffer cells these coated pits never really leave the plasma membrane and hence no true internalization of particles occur (Kolb-Bachofen *et al*, 1986).

1.7. The aim of the present study

The examples presented in the previous section indicate that by altering the surface characteristics of drug carriers for example, liposomes, some modification of their uptake, albeit not to a dramatic level, by a particular mononuclear phagocytic tissue may be achieved. The extent to which these modifications are related to serum opsonins remains unclear, since liposome properties can be markedly altered by adsorption of serum factors. Even liposomes that are initially prepared and coated with "targeting" groups, such as IgG, at their surfaces, it is possible that these groups could be masked in the blood environment (Finkelstein *et al.*, 1981). Therefore, the possible alterations of

liposome characteristics in blood should be an important consideration in the design of liposomes for therapeutic applications.

Hence, the present work is devoted to the investigation of the effect of serum opsonins on the association and subsequent uptake of liposomes (as an example of a drug carrier) of differing phospholipid composition and cholesterol content by bone-marrow, hepatic, splenic, and peritoneal phagocytes, in suspension, during both normal and pathological conditions.

Chapter II

Materials & General Methods

2.1. Materials

2.1.1. Chemicals

A - The following chemicals were obtained from Sigma Chemical Company, Poole, England: Bovine serum albumin; Cholesterol (Chol); Collagenase (type IV); Dicetylphosphate (DCP); L-α Dimyristoylphosphatidylcholine [(14:0), DMPC]; DL-α - Dipalmitoylphosphatidylcholine [(16:0), DPPC]; L-α - Distearoylphosphatidylcholine [(18:0), DSPC]; Iodoacetamide; Sphingomyelin, type I, (SM); Silver nitrate and Trypsin.

B - The following were obtained from Pharmacia Ltd., Bucks, Great Britain : Gelatin-Sephrose beads; Sephadex G-25 and G-200; Sephrose 4B; Phast gel gradient 10-15%; and Phast gel SDS buffer strips.

C - Egg phosphatidylcholine (PC) was from Lipid Products, Surrey, England.

D - Thioglycolate tablets were from Flow Laboratories, England.

E - Folin & Ciocalteau's reagent and Sodium azide were from BDH, Poole, England.

F - Ecoscint A was from Kimberly Research, England.

G - Heparin sodium was from Weddel Pharmaceutical Ltd., England.

2.1.2. Radiochemicals

The following radiochemicals were obtained from Amersham International, England : ^{3}H-Cholesterol; [^{14}C- Carboxylic acid]-inulin; and [^{125}I]-Poly(vinylpyrrolidone).

2.1.3. Animals

A - Male CFY rats of body weight 250 ± 10 g (from the breeding centre of Charing Cross and Westminster Medical School) were maintained at

15-17°C on a 12 hour-day/12 hour-night cycle (day started at 08.00 h). Animals were fed on a standard diet *ad libitum* on chow.

B - Animal transplantation technique for chondrosarcoma

Male CFY rats approximately six weeks of age were used in all experiments as tumour recipients. Chondrosarcoma was a gift from Prof. Mason. The chondrosarcoma is routinely maintained by serial transplant every 4-6 weeks (Breilkreutz *et al.,* 1979) and there is no spontaneous regression and no sign of metastases in the host. The transplantation of chondrosarcoma was accomplished according to the technique described by Breilkreutz *et al.* (1979). In this procedure, tumour donors were asphyxiated by carbon dioxide and the tumour was excised under sterile conditions. The viable periphery of the tumour was passed through a sieve and cells were collected in sterile Hanks' balanced salt solution, HBSS. Each recipient rat received subcutaneously 2.5 x 10^4 viable cells in an injection volume of 0.5 ml at each flank. Control rats were injected subcutaneously with the same volume of sterile saline at each flank. Full tumour growth occurs in 5-6 weeks following transplantation. Rats were killed 5-6 weeks following transplantation, and the opsonic activity of serum from tumour bearing and control rats was tested on liposome uptake by liver and spleen phagocytes prepared from both tumour bearing and control rats as described later in this chapter.

2.2. General Methods

2.2.1. Preparation of serum

A. Rat serum - Rats were anaesthetised with ether, and blood was collected by cardiac puncture and allowed to clot at room temperature for 30 min followed by centrifugation at 1500g for 20 min. The serum was kept in ice before use.

B. Rabbit serum - The blood was collected from the ear vein of rabbits and allowed to clot at room temperature followed by centrifugation as described above.

C. Foetal calf serum (Australian origin) - The serum was obtained in 100 ml batches from GIBCO BRL (Scotland).

2.2.2. Dialysis of serum

Fresh serum was dialyzed in 8/32" dialysis tubing (Medicell International Ltd., London) against a large volume of de-ionised water overnight at 4° C.

2.2.3. Freeze drying of serum dialysate

The serum dialysate was placed in a large round-bottomed flask and frozen in a container containing a mixture of methanol and dry ice with continuous rotation. After freezing the flask was placed in a freeze drying apparatus overnight. The freeze dried sample was resuspended in saline to the same volume of serum used for dialysis.

2.2.4. Depletion of fibronectin from serum (Engvall & Ruoslahti, 1977)

This was achieved by three times incubating serum for a 30 minute period with gelatin-sepharose beads (1.0 ml of serum per 0.25 ml beads) in an ice bath after which the beads were pelleted by centrifugation.

To ensure the complete removal of fibronectin, the pelleted beads were incubated at 4°C with 1.0 ml of 4.0M urea/0.02M NaH_2Po_4/0.15M NaCl/0.05M Tris-HCl, pH 7.4, for 30 minutes. After incubation the beads were pelleted by centrifugation and the supernatent was monitored for protein absorbance at 280 nm (the absorbance at 280nm from the fourth supernatent onward was basically zero). The materials eluted by 4.0M-urea were also subjected to Phast System SDS-PAGE using a 10-15% gradient gel (see 2.2.9). The material on the stained gel contained primarily fibronectin, molecular weight 220 000.

2.2.5. Preparation of liposomes

Liposomes were prepared as described by Souhami *et al.* (1981) from the combination of the following lipids: egg PC, Chol and DCP in molar ratio of 7:7:1 for Chol-rich, 7:2:1 for Chol-poor and 7:0:1 for Chol-free liposomes respectively. In some preparations DMPC, DPPC, DSPC, and SPHG replaced egg PC. The rotary evaporated lipid films (60 mg) were shaken off the sides of a round-bottomed flask with either 5.0 mM phosphate buffered saline (PBS), pH 7.4, or 0.9 (%w/v) saline containing 12-15 µCi (^{125}I)poly(vinylpyrrolidone). In some preparations the lipid membrane of liposomes were labelled by incorporation of 5-10 µCi ^{3}H-cholesterol into lipid mixture and the aqueous phase contained 20-25 µCi of ^{14}C-inulin. The lipid suspension was allowed to stand on bench for 1 h with occasional shaking. This preparation of liposome is referred to here as large multi-lamellar liposomes (MLV). Non-entrapped radioisotopes from such preparations were removed by centrifuging in an MSE Superspeed 65 centrifuge at 200000 X g for 30 min. The resulting liposomal pellet was suspended in 5.0 mM PBS, pH 7.4, and centrifuged

once again as described above. Finally, the pellet was suspended in PBS and just before use was diluted with the buffer to give a suitable concentration of liposomes for cell incubations. Liposomes containing DPPC and DSPC were prepared at their transition temperature, 41°C and 55°C respectively.

2.2.6. Liposome leakage studies

Fresh rat serum, 0.25 ml, was mixed with ^{125}I-PVP entrapped liposomes (0.3 mg lipid) in a total volume of 1.0 ml in 5.0 mM PBS, pH 7.4, and incubated for 30 minutes at 37°C. In control experiments serum was replaced with the same volume of PBS. After incubation 0.5 ml of the mixture was passed through a sephrose-4B column (0.7x19 cm) equilibrated with PBS, to separate the free and liposome associated ^{125}I-PVP.

2.2.7. Binding of serum proteins to liposomes (Opsonization)

Liposomes, 15 mg, in 0.5 ml of 0.9% saline were incubated with 50% serum at 37° C for 15 min. The suspension was then diluted to 10 ml with 0.9% saline and centrifuged in an MSE Superspeed 65 centrifuge at 200 000 X g for 30 min. The pellet was resuspended in saline to give a suitable concentration of opsonized liposomes for cell incubations. In some cases the resulting pellet was washed in saline and centrifuged once again as described above.

2.2.8. Protein assay (Lowry *et al.*, 1951)

Reagents:

A. 1% $CuSO_4.5H_2O$

B. 2% sodium potassium tartrate

C. A solution containing 20 mg sodium carbonate (anhydrous) and 4 mg sodium hydroxide per litre.

D. Folin and Ciocalteau reagent, diluted with an equal volume of distilled water.

E. Standard protein solution, 200 µg/ml, was prepared using bovine serum albumin as the standard protein.

A calibration curve was prepared as follows. A series of suitable aliquots (0.1 to 1.0 ml) of standard protein solution within the range 20-200 µg/ml were taken in duplicate and diluted to 1.0 ml with water. A volume of 5.0 ml of freshly prepared "reagent mixture", prepared by mixing A (1.0 ml) with B (1.0 ml), and made up to 100 ml with C, was added to each tube. After 10 minutes, 0.5 ml of the diluted Folin and Ciocalteau reagent was added to each tube and mixed immediately. The colour was allowed to develop for 30 minutes and was read at 750 nm on a SP8-400 Pye Unicam spectrophotometer. Blanks were included which contained 1.0 ml of water in place of the protein solution and with the addition of all reagents.

2.2.9. Pharmacia PhastSystem SDS-PAGE (Pharmacia manual file, 1987)

Materials:

A. PhastGel gradient 10-15% - The gel is approximately 0.45 mm thick and has a13 mm stacking gel zone and a continuous 10-15% gradient gel zone with 2% crosslinking. The buffer system in the gel is of 0.112 M acetate (leading ion) and 0.112 M Tris, pH 6.4.

B. PhastGel SDS Buffer Strips - The buffer strips are made of 2% Agrose IEF. The buffer system in the strips is of 0.20 M tricine (trailing ion), 0.20 M Tris and 0.55% SDS (analytical grade), pH 7.5.

Sample preparation:

Sample buffer: 10 mM Tris/HCl; 1mM EDTA, pH 8.0, containing 0.01% bromophenol blue.

Treatment: To the samples (10-40 ng/µl for silver staining), SDS and ß-mercaptoethanol were added at a final concentration of 2.5 and 5% respectively. Samples were then boiled for 5.0 minutes. Any insoluble materials were removed by centrifugation to prevent streaking patterns in the developed gel.

Staining and development:

Silver staining method optimized for SDS-PAGE with PhastGel gradient was programmed into the development method file according to the Pharmacia manual. The program is described in table 2.1.

Table 2.1. Procedures of silver staining and development for Phast gel

Step No.	Solution	Time	Temp
1	50% ethanol, 10% acetic acid	2 min	50°C
2	10% ethanol, 5% acetic acid	2 min	50°C
3	10% ethanol, 5% acetic acid	4 min	50°C
4	8.3% glutarialdehyde	6 min	50°C
5	10% ethanol, 5% acetic acid	3 min	50°C
6	10% ethanol, 5% acetic acid	5 min	50°C
7	de-ionised water	2 min	50°C
8	de-ionised water	2 min	50°C
9	0.25% silver nitrate	13 min	40°C
10	de-ionised water	0.5 min	30°C
11	de-ionised water	0.5 min	30°C
12	developer *	0.5 min	30°C
13	developer	4 min	30°C
14	5% acetic acid	2 min	50°C
15	10% acetic acid, 5% glycerol	3 min	50°C

** Developer: 12.5% sodium carbonate (anhydrous) diluted X5 with de-ionised water followed by addition of 0.04% formaldehyde.*

Note: All solutions were prepared fresh for each run and none was recycled. For each step 80 ml solution was required.

2.2.10. Cell isolation

A. Spleen cells - (Kleiman *et al.*, 1984)

Buffers:

1. Oxygenated phosphate buffered saline, pH 7.4 :

Sodium chloride 137 mM, potassium chloride 2.6 mM, disodium hydrogen phosphate 8.1 mM, potassium dihydrogen phosphate 1.5 mM, and D-glucose 5.5 mM.

2. Tris-Ammonium chloride, pH 7.2 at 4°C:

A) Tris-(hydroxymethyl)-methylamine 170 mM and

B) Ammonium chloride 0.83%.

For use 10 ml of A and 90 ml of B were mixed and the pH was adjusted to 7.2 at 4°C with 5 M HCl.

Cell isolation:

Rats were killed by cervical dislocation and the spleens were immediately removed and placed in ice cold petri dishes containing a few ml of oxygenated PBS. Cells were gently released from the cut end of the capsule by stroking the spleen with curved forceps. The residual tissue and the cell suspension were filtered through a wire gauze sieve and were gently pressed against the sieve with the aid of a flat rubber end of the plunger from a disposable syringe. The spleen cell suspension was contaminated with red blood cells and these were lysed with a large volume of ice-cold Tris-NH_4Cl buffer. The suspension was kept in ice, and the whole procedure of lysis occured within 5 minutes. The cell suspension was then centrifuged at 300 g at 4°C for 5.0 minutes to obtain the white spleen cell population. The supernatent was discarded and the pellet was resuspended in ice-cold oxygenated PBS. The

suspension was allowed to stand for a few minutes in order to sediment the clumps and cell aggregates by gravity. After removal of the clumps, the cell suspension was centrifuged again as described above. The final pellet was resuspended in PBS to give the desired cell concentration.

B. Bone-marrow cells

Rats were killed by cervical dislocation and the femoral bones were removed and rinsed with PBS, pH 7.4. The bone ends were cut and the bone-marrow lumen was perfused with ice-cold oxygenated PBS, pH 7.4. The cell suspension was filtered through a nylon filter cloth (pore size 75 µm) followed by centrifugation at 300 g for 5.0 minutes. Contaminated red blood cells were hemolysed by Tris/ammonium chloride and the suspension was centrifuged at 300g for 5.0 minutes. The sediment was resuspended in PBS and the centrifugation step was repeated as above. Finally the sediment was resuspended in PBS to give the required cell concentration.

C. Liver non-parenchymal cells (Doolittle & Richter, 1981)

Perfusion Buffers:

1 - Hank's balanced salt solution, HBSS, (Calcium and Magnesium free) pH 7.4 at 37°C and

2 - Hank's balanced salt solution, pH 7.2 at 37°C supplemented with 0.025% collagenase type IV (Sigma).

Procedure:

The rat was anaesthetised with ether and secured to a surgical board. The abdomen was opened through a mid-line incision, and mid-transverse incisions to right and left of the the mid-line were made. The intestines were then placed to the animal's left, so that the liver, portal vein, right

kidney and inferior vena cava became exposed. A ligature of silk (Ethicon Ltd) was placed around the vena cava above the right renal vein. A loose ligature was passed around the portal vein at an interval of 3-4 mm below the points where the vein is divided to enter the separate lobes of the liver, and a second ligature was tied around the vein at a point distal to the liver. The portal vein was then cannulated with a No.16 Frankis-Evans needle,and the loose ligature was tied. Buffer A (100 ml) was perfused through the liver at 8 ml/min.

The thorax was quickly opened, the chest wall was flapped back and then cut off. A loose ligature was placed around the inferior vena cava close to the heart. A cannula (which is a portex tubing heated in a gas flame, drawn out to an outside diameter of 2 mm and cut in angle to penetrate the right artium) was pushed down the vein towards the diaphragm and tied in position, to collect the draining perfusate. Buffer B (50 ml) was then perfused at a rate of 8ml/min.

Following perfusion the liver was excised, placed into a large petri dish containing approximately 80 ml of calcium and magnesium free HBSS, pH 7.4, at 4°C. The liver capsule was peeled off, and the majority of liver cells were librated by gentle shaking. Any remaining cells were mechanically dispersed from the liver with curved forceps. The connective tissue framework which remained was lifted out, and the suspension was cleared of large clumps of tissue debris by filteration through a wired gauze sieve and then forced through a 250 µm nylon mesh. From this total cell suspension, separate populations of intact hepatocytes and non-parenchymal cells were prepared by differential centrifugation.

The total cell suspension was centrifuged at 50g for 2 minutes. The supernatent (S1) was saved and the pellet was resuspended in 50 ml of cold HBSS (Ca^{2+} & Mg^{2+} free) and centrifugation was repeated again. The supernatent (S2) from this step was collected and combined with S1 for purification of non-paranchymal cells. The pulled supernatents S1 & S2 were centrifuged twice at 450g for 3 minutes to separate non-parenchymal cells from cell debris. Each time the non-parenchymal cell pellet was resuspended in 20 ml of HBSS (Ca^{2+} & Mg^{2+} free). Any remaining hepatocytes were sedimented by twice centrifugation at 50g and discarded. A final non-parenchymal cell pellet containing endothelial and kupffer cells were collected after centrifugation at 450g. All centrifugation steps were performed at 4°C.

D. Resident peritoneal cells (Mc Carron *et al.*, 1984)

Rats were killed by cervical dislocation and secured to a surgical board. The abdomen was swabbed with 70% ethanol. The skin of the anterior abdomen region was retracted with forceps and a longitudinal incision along the midline was made. The fur above and below incision was genetly pulled apart until the abdominal wall was exposed from the neck to the pelvic gridle. The exposed abdomen was washed with 70% ethanol to remove any loose fur. Cold HBSS (Ca^{2+} & Mg^{2+} free), pH 7.4, (25.0 ml) was injected into the side of abdominal wall, avoiding puncture of viscera. Once the peritoneal cavity became distended, the abdomen was massaged to ensure maximum suspension of cells. Finally a small incision was made on the abdomen and the fluid was collected with the aid of a Pasture pipette.

The collected cells were washed 2-3X in HBSS (Ca^{2+} & Mg^{2+} free), pH 7.4, by centrifugation (225g for 10 minutes) and resuspended in HBSS (Ca^{2+} & Mg^{2+} free) by gentle agitation. Contaminated red blood cells were removed with tris-ammonium chloride buffer.

E. Elicitated peritoneal cells (Mc Carron *et al.*, 1984)

Elicitating reagent : Brewer's thioglycolate medium, prepared according to manufacturer's instruction, sterilized by autoclave, and stored in dark at room temperature for one week prior to use.

Procedures : Three days before harvesting the peritoneal cells, rats were given intraperitoneal injection of 5 ml thioglycolate medium. Cell recovery and preparation was performed as described for resident peritoneal cells.

2.2.11. Determination of cell viability

Trypan blue solution : 0.5 mg trypan blue was dissolved in 100 ml of PBS, pH 7.4, filtered and stored at room temperature.

To 80 µl of cells (approximately 1-2 x 10^6), trypan blue solution (20µl) was added. An aliquot was placed in haemocytometer and after 4-5 minutes the fraction of darkly stained cells were counted. Cell viability for both spleen and liver non-parenchymal cells were in order of 70-75%, and for bone-marrow and peritoneal cells were greater than 95%.

2.2.12. In vitro experiments

A. Spleen cell incubations:

Spleen cell suspension, (7-10)X10^7 cells/ml, was incubated together with liposomes, 0.6 mg lipid, in polythene scintillation vials in the presence or absence of 25% fresh serum. Total volume of each incubation

was adjusted to 2.0 ml with PBS, pH 7.4, containing 5.0 mM glucose and 1% bovine serum albumin, BSA. After addition of cell suspension each vial was gassed with carbogen, capped tightly and then placed in a shaking water bath at 37°C for the required time of incubation (usually 1 h). After incubation, a large volume (5.0 ml) of ice-cold PBS was added to each vial and the contents were transferred to conical base centrifuge tubes followed by rapid centrifugation at 300X g on a bench centrifuge for 3 minutes. The pellet was washed twice with PBS followed by counting for radioactivity.

B. Bone-marrow cell incubations

All procedures were the same as described for the spleen cells except that 10^7 cells were placed in each vial and BSA was omitted from the incubation buffer.

C. Liver non-parenchymal and peritoneal cell incubations

All procedures were the same as described for the spleen cells except that 10^7 cells were placed in each vial and the incubation buffer was changed to calcium/magnesium free HBSS, pH 7.4. BSA was omited from incubations.

2.2.13. Measurement of cell associated radioactivity

Cell associated ^{125}I-label was measured in a Packard ∂-countre. For measurement of ^{3}H & ^{14}C label, cell pellet was digested with 0.1 ml of 33% KOH for 1 hour at 70°C followed by neutralization with 1.0 ml of 1.33 M HCl. To the neutralized sample 10 ml of scintillation fluid , Ecoscint A, was added and the radioactivity was measured in a searle ß-counter.

2.2.14. Discrimination between liposome adsorption to cell surface and liposome uptake

Liposome adsorption to the cell surface was measured by one or a combination of the following methods:

1 - Incubating the cell suspension at 4°C (Dijkstra *et al.*, 1984).

2 - Treatment of cells with trypsin (Pagano & Takeichi, 1977). Thus , after incubations, the cells were pelleted by centrifugation and resuspended in 1.0 ml of warm (37°C) incubation buffer containing 0.05% trypsin. This suspension was incubated for 5 minutes at 37°C. Finally, the cells were separated by centrifugation, washed twice with PBS and subjected to radioactivity measurement.

3 - The use of metabolic inhibitors (Stendahl & Tagesson, 1977). The cell suspension was preincubated for 20 minutes with 5.0 mM iodoacetic acid or iodoacetamide and 10 mM sodium azide at 37°C to block glycolysis and mitochondrial electron transport. Liposomes and serum (if necessary) were then added and the incubations were continued for an extra period of 60 minutes. Measurement of the cell associated radioactivity was performed as described before.

2.2.15. Procedures for the purification of liver specific opsonin(s)

A - Ammonium sulphate fractionation of serum

Serum from rabbit, rat, and calf were subjected to ammonium sulphate fractionation in an attempt to isolate a protein fraction containing the liver specific opsonic activity. Serum (10.0 ml) was brought to 35% saturation by slow addition, while gently stirring, with solid ammonium sulphate at 4°C. The pH was adjusted to 7.0-7.2 throughout the addition of ammonium sulphate. After the ammonium sulphate had been added, the

suspension was stirred gently for an additional 45 min at 4°C. The 35% saturated precipitate was removed by centrifugation at 12000 g for 15 min at 4°C.

To 6.0 ml of 35% saturated supernatent (from the above centrifugation step), ammonium sulphate was added slowly over 20 min to bring the final saturation to 50% at 4°C. This mixture was allowed to stir gently for an additional hour and the precipitate was removed by centrifugation as described in previous step. Finally, 3.0 ml of the 50% saturated supernatent from the previous step was brought to 65% saturation by addition of solid ammonium sulphate at 4°C. This suspension was allowed to stir gently for 150 min and the precipitate was removed by centrifugation.

All precipitates were dissolved in a small volume of cold (4°C) 10mM tris-0.15M NaCl (pH 7.0). The dissolved precipitates and remaining supernatents from the fractionation steps were dialyzed extensively against tris-NaCl at 4°C and tested for opsonic activity on freshly isolated liver non-parenchymal cells (chapter II).

B - Gel-filteration studies

After dialysis, 15.0 mg of 35-50% ammonium sulphate saturated precipitate was passed through a sephadex G-200 column (0.7 X 50 cm) equilibrated with 10 mM tris-0.15 M NaCl, pH 7.0, at 4°C. The collected fractions were monitored for protein by measuring the absorbance at 280 nm on a SP8-400 Pye Unicam spectrophotometer. The protein containing fractions were tested for opsonic activity on liposome uptake by freshly isolated liver non-parenchymal cells.

C - Pharmacia Phast System SDS-PAGE

Protein containing fractions from gel-filteration step were subjected to Pharmacia Phast-System SDS-PAGE. The gels were developed by silver staining as described earlier (see section 2.2.9. in this chapter).

D - Expression of protein purification results

Results are expressed either as % of initial liposomal radioactivity associated with cells or as specific activity of the protein fractions.

Specific activity (SP) is defined as follows:

SP = [(A - B) / (mg serum protein fraction)] X 100,

where A = % of initial liposomal radioactivity associated with cells in the presence of serum protein fractions, and

B = % of initial liposomal radioactivity associated with cells in the absence of serum proteins.

Chapter III

Tissue specific opsonins for phagocytic cells and their different affinity for cholesterol-containing liposomes

3.1. Introduction

The rate of clearance of intravenously injected liposomes depends on their structural stability in the circulation and the rate of phagocytosis by blood-sinus-lining mononuclear phagocytes. For example, inclusion of cholesterol in liposomes is known to increase their half-life in the bloodstream (Kirby & Gregoriadis, 1980/1981). This is achieved by cholesterol playing a dual role (Patel *et al.*, 1983); firstly, it stabilizes the liposomal membrane and makes it less susceptible to destruction by various serum components (Scherphof *et al.*, 1978; Kirby *et al.*, 1980). Secondly, it makes liposomes unfavourable for phagocytosis by hepatic Kupffer cells (Patel *et al.*, 1983; Claassen & Van Rooijen, 1984; Dave & Patel, 1986). The reason why Kupffer cells have a poor affinity for cholesterol-rich liposomes is not yet known. Cholesterol influences the fluidity of liposomal membranes and binding of serum opsonin(s) on liposomes (Papahadjopoulos *et al.*, 1973; Van der Bosch *et al.*, 1973; Tall & Lange, 1978) and may perhaps affect the uptake of liposomes by these cells.

Since the liver has poor affinity to sequester chol-rich liposomes, in a further study, Dave & Patel (1986) suggested that an effective way to saturate the hepatic phagocytic activity would be to predose animals with chol-free empty liposomes rather than chol-rich liposomes. In this study liver and spleen responded differently to chol-free and chol-rich liposomes when injected to produce "hepatic blockade". Surprisingly, the predosed chol-free liposomes which caused the maximum "spillover" of the test liposomes from the liver did not achieve maximum enhancement in the splenic uptake. Instead, the maximum enhancement was recorded

with the predosed chol-rich liposomes. The mechanism by which these organs handle the two types of liposomes differently is not yet known. Hence, to investigate the possible mechanism responsible for the suppression of cholesterol-rich liposome uptake in liver and factors which stimulate a differential response in hepatic and splenic phagocytic activity, we have studied the uptake of liposomes in the absence and presence of rat serum by using the isolated hepatic and splenic cells in suspension.

3.2. Results

A. The role of serum opsonins on the uptake of cholesterol containing liposomes by hepatic and splenic phagocytes

The results in fig 3.1.a demonstrate that both hepatic and splenic cells take up chol-free liposomes much more than cholesterol containing vesicles in the absence of serum. Inclusion of serum in the incubations suppressed the uptake of chol-free liposomes by both hepatic and splenic cells, fig 3.1.b. This may be attributed to the fact that chol-free liposomes are degraded in serum (Scherphof *et al.*, 1978; Kirby & Gregoriadis, 1980/1981), as a consequence a smaller number of intact vesicles may be available to cells which may apparently result in the poor uptake (Patel *et al.*, 1983). Among cholesterol containing liposomes, chol-poor are taken up much more by hepatic and splenic cells in the absence of serum than chol-rich liposomes. Serum enhances the uptake of chol-poor vesicles by hepatic cells but suppresses that of chol-rich liposomes, in comparison to the results obtained in the absence of serum. In contrast, serum stimulates uptake of cholesterol-

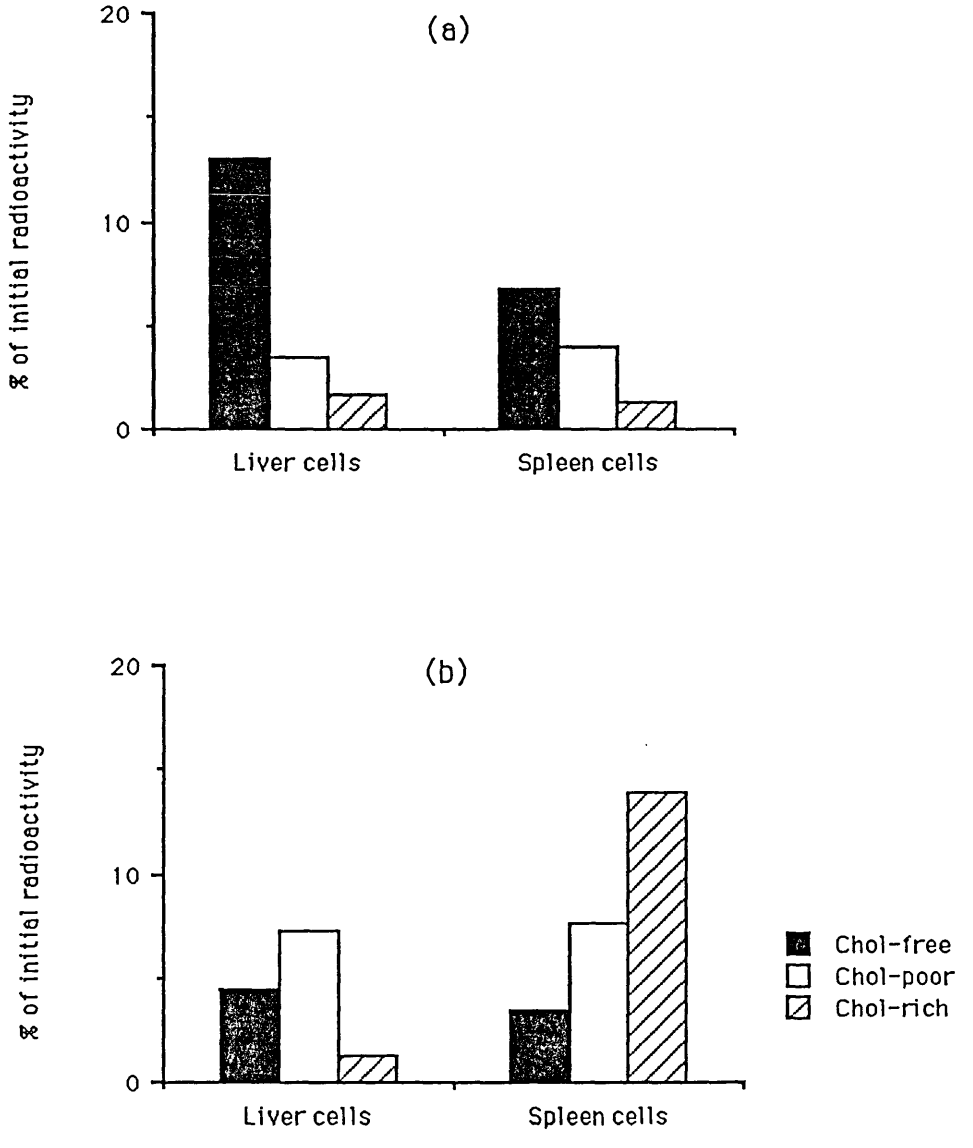


Fig 3.1. Uptake of liposomally entrapped ^{125}I-PVP by hepatic and splenic phagocytic cells in the absence (a) and in the presence (b) of rat serum.

containing liposomes by splenic cells (fig 3.1.a & b) but its effect is greater on the uptake of chol-rich than chol-poor liposomes.

B. Assessment for liposome adsorption and uptake

At 4°C, liposomes are predominantly adsorbed on to the cell surface and not phagocytozed (Dijkstra *et al.*, 1984). Thus, the effect of cholesterol and serum on the adsorption of liposomes on to the cell surface at 4°C was examined. It was found (table 3.1.) that the presence of cholesterol in liposomes reduces their adsorption and serum further hinders this process for all liposomes in hepatic and splenic cells except the adsorption of chol-rich liposomes on splenic cells which, in contrast, is enhanced. The amount of liposomes adsorbed onto the cells did not constitute more than a few percent of total liposomes taken up by the cells at 37°C, except in the case of chol-free liposomes in hepatic cells where the results of adsorption at 4°C and association at 37°C in the presence of serum are similar. This makes it difficult to assess the role of serum on the uptake of chol-free liposomes by these cells and hence in subsequent studies chol-free liposomes were omitted.

Phagocytosis is an energy dependent process and the presence of metabolic inhibitors during incubations can inhibit the uptake of liposomes by phagocytes (Dijkstra *et al.*, 1984). When liver cells were incubated at 37°C in the presence of the metabolic inhibitors (table 3.2.) the association of the radioactive label was reduced to 50% of control values both in the absence and presence of serum, indicating a combination of both fusion and endocytosis as the major mechanism of interaction. Similarly, association of the radioactive label with spleen cells was reduced to 15 and 65 percent of the control values in the

Table 3.1.

Adsorption of liposomes on to liver and spleen cells at 4°C

Liposomes	Adsorption (% of initial [^{125}I]-PVP radioactivity)*					
	Chol-Free		Chol-Poor		Chol-Rich	
Serum	−	+	−	+	−	+
Liver cells	4.50	3.80	0.90	0.27	0.16	0.19
Spleen cells	1.60	0.67	0.17	0.13	0.23	0.99

* *Mean of duplicate incubations.*

N.B. These experiments were repeated twice and the results of a typical experiment are presented.

Table 3.2.

Effect of metabolic inhibitors on liposome uptake by liver and spleen phagocytes in the absence and presence of serum

Treatment	% of initial [^{125}I]-PVP	
	37°C	37°C + Metabolic inhibitors
Liver cells:		
Buffer	0.9 ± 0.1	0.5 ± 0.1
Serum	6.9 ± 0.4	3.7 ± 0.1
Spleen cells:		
Buffer	3.5 ± 0.1	2.8 ± 0.2
Serum	6.4 ± 0.2	2.4 ± 0.2

Metabolic inhibitors : Iodoacetic acid (5.0 mM) + Sodium azide (10.0 mM).

Cells were preincubated for 20 min with inhibitors followed by addition of liposomes and serum.

Cholesterol-poor PC liposomes were used for incubations.

absence and presence of serum respectively (table 3.2.), suggesting fusion and endocytosis as the major mechanisms of interaction in the absence and presence of serum respectively.

C. Effect of liposome opsonization on their uptake by hepatic and splenic phagocytes

The effect of serum on interaction of liposomes is further confirmed when opsonized chol-poor and chol-rich liposomes were incubated with hepatic and splenic cells at 37°C. The results in table 3.3. show that opsonization enhances the uptake of chol-poor but suppresses that of chol-rich liposomes by liver cells. When opsonized chol-poor vesicles were washed once with saline prior to the incubation with liver cells, the opsonic activity was lost. On the other hand, although serum enhances the uptake of both chol-poor and chol-rich liposomes by splenic cells, opsonization of chol-poor liposomes has no effect on their uptake by these cells. Interestingly, opsonized chol-rich liposomes are taken up 2 fold more than unopsonized control liposomes, but this represents only 12% of the total liposomes taken up by splenic cells in the presence of serum. This small opsonic activity of the opsonized chol-rich liposomes is not lost when liposomes were washed prior to the incubation with cells.

Electrophoretic studies of the opsonized chol-poor and chol-rich liposomes show that more serum proteins are associated with chol-poor than chol-rich liposomes (fig 3.2.). This may be attributed to the increased rigidity of the membrane due to high concentration of cholesterol (46.6 mol%) in chol-rich liposomes (Van der Bosch *et al.*, 1973). Washing opsonized liposomes with saline removes most of the

Table 3.3.

Interaction of opsonized liposomes with liver and spleen cells

Treatment	(% of initial [^{125}I]-PVP radioactivity)* Liver cells	Spleen cells
Chol-Poor liposomes:		
Liposomes (control)	7.6	5.3
Liposomes + Serum	22.7	9.1
Opsonized liposomes	15.4	5.3
Washed opsonized liposomes	7.2	N.D
Chol-Rich liposomes:		
Liposomes (control)	5.0	1.6
Liposomes + Serum	2.1	31.7
Opsonized liposomes	2.8	3.8
Washed opsonized liposomes	N.D	3.1

** Mean of duplicate incubations.*

N.D : Not done.

N.B. The whole experiment was repeated three times and only the result of a typical experiment is presented here.

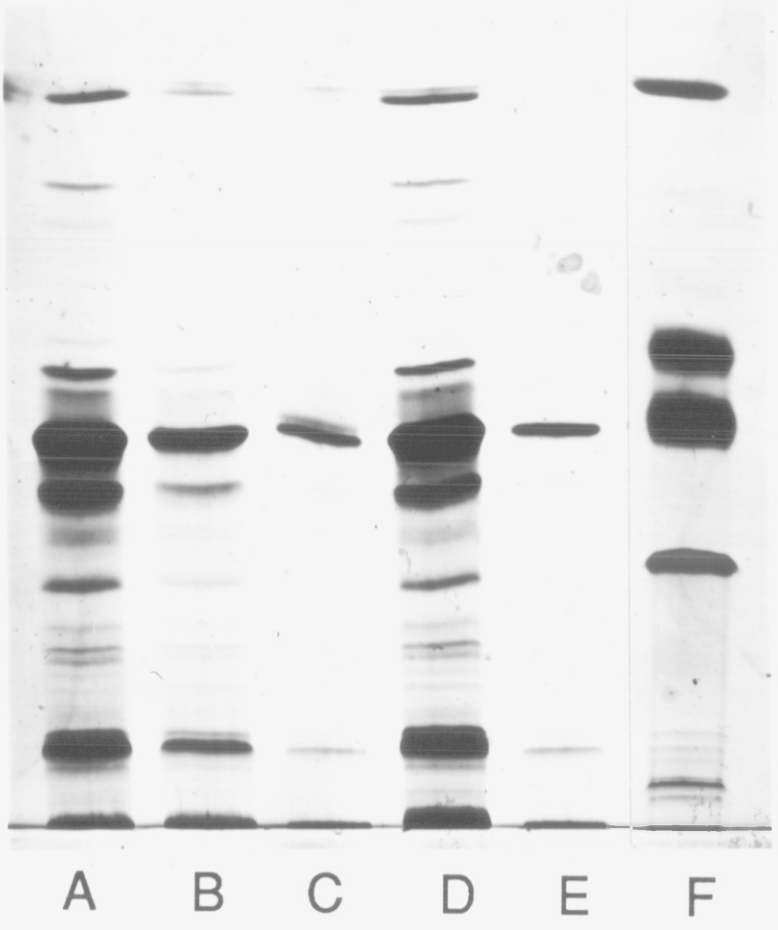


Fig 3.2. SDS-PAGE electrophoresis of opsonized cholesterol containing liposomes by Pharmacia Phast-Electrophoresis system on a 10-15% gradient gel. Direction of migration is from bottom to top. A, serum; B, opsonized chol-rich liposomes; C, washed opsonized chol-rich liposomes; D, opsonized chol-poor liposomes; E, washed opsonized chol-poor liposomes; F, standards (from bottom to top: phosphorylase a, bovine albumin, ovalbumin, and cytochrome *c*).

serum proteins associated on both chol-poor and chol-rich opsonized liposomes and the proteins retained on the gels gave very similar bands in both preparations.

3.3. Discussion

The results presented here confirm the earlier findings of *in vivo* studies of Patel *et al.* (1983) and Dave & Patel (1986), and show that Kupffer cells take up avidly egg chol-poor but not chol-rich PC liposomes, whereas splenic cells take up preferentially chol-rich more than chol-poor liposomes in the presence of serum (fig 3.1.). The experiments with opsonized liposomes (table 3.3.) suggest that there are opsonins present in serum which are specific for liver and spleen phagocytes. Opsonin(s) specific for liver cells has no affinity for chol-rich liposomes since no enhancement in uptake of these liposomes is observed either in the presence of serum (fig 3.1.) or when liposomes are pre-opsonized with serum (table 3.3.). This opsonin exerts its action probably on liver phagocytic cells by being adsorbed loosely on the surface of egg PC chol-poor liposomes, since, when the opsonized liposomes are washed prior to incubation with these cells, its opsonic effect is lost (table 3.3.). Cholesterol (46.6 mol%) incorporated in cholesterol-rich liposomes decreases the fluidity of their membrane (Demel & De Kruijff, 1976) and this could interfere with the interaction of liposomes with Kupffer cells, since it is known that the fluidity of membrane determines the ability of liposomes to interact with cells (Papahadjopoulos *et al.*, 1973; Margolis, 1984). The results also indicate that certain serum components hinder the adsorption and eventually

uptake of egg PC chol-rich liposomes by liver cells. However, the most likely explanation for the poor uptake of chol-rich vesicles by Kupffer cells is that cholesterol reduces the interaction of liposomes with serum proteins (Tall & Lange, 1978), (fig 3.2.) and serum components (Scherphof *et al.*, 1978; Kirby *et al.*, 1980). It is therefore not surprising that incorporation of 46.6 mol% cholesterol in these experiments renders them less attractive for the interaction with liver specific opsonin and hence no stimulant for Kupffer cells.

On the other hand, the opsonin(s) specific for splenic cells stimulates phagocytosis of both preparations of cholesterol-containing liposomes but its effect is greater on the uptake of chol-rich liposomes (fig 3.1.). However, unlike the opsonin specific for liver cells, this factor(s) does not exert its effect on splenic phagocytes by being adsorbed onto the surface of liposomes, since no enhancement in uptake of the opsonized liposomes is observed (table 3.3.). This probably suggests that, unlike in the case of liver, this opsonin may exert its effect by binding to the site other than the liposome-binding site on the spleen cell surface. However, since a small increase in uptake of the opsonized chol-rich liposomes is found, it may indicate that more than one opsonin specific for spleen cells may be present in serum. Among these opsonins, one probably binds tightly to chol-rich liposomes and enhances their uptake to a smaller extent than that observed in the presence of serum. It is also possible that this opsonin may act on other cells like lymphocytes in spleen cell preparation which may be stimulated to take up liposomes (Dave & Patel, 1986).

Finally, the results discussed here probably explain why early

attempts to show the opsonic effect of serum on phagocytosis of liposomes by kupffer cells *in vivo* (Kao & Juliano, 1981; Ellens *et al.*, 1982) and *in vitro* (Dijkstra *et al.*, 1984) were not successful. These investigators used chol-rich liposomes (approx. 50 mol% cholesterol content) and sometimes they employed phospholipids other than egg PC. The experimental data (see chapter IV) suggest that like cholesterol, certain phospholipids have a poor affinity for opsonin specific for liver and hence their uptake in liver and consequently distribution in the whole body is altered.

Chapter IV

Serum opsonins and phagocytosis of saturated and unsaturated phospholipid liposomes

4.1. Introduction

Liposome containing cholesterol prepared from either sphingomyelin (SM) or saturated phospholipids such as dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC), when injected intravenously, have an extremely long half-life in the circulation (Senior & Gregoriadis, 1980; Ellens *et al.,* 1981; Senior & Gregoriadis, 1982). These liposomes are less susceptible to destruction by serum components (Senior & Gregoriadis, 1982; Gregoriadis *et al.,* 1983) and hence it is suggested that their stability in the circulation contributes to their long half-life. However, the clearance of liposomes from the blood by blood-sinus-lining mononuclear phagocytes may be the major factor in determining their half-life (Patel et al 1983; Dave & Patel, 1986) and hence it is important to investigate the reasons for the slow uptake of these liposomes by phagocytic cells.

The clearance of liposomes from the circulation by blood-sinus-lining phagocytes depends on several factors such as the physical and chemical properties of liposomes and serum components associated onto the surface of liposomes in the circulation. Various investigators (Tyrrell *et al.,* 1977; Juliano, 1982; Ellens *et al.,* 1982; Dijkstra *et al.,* 1984) have examined the possible involvement of serum opsonins in phagocytosis of liposomes by macrophages from the same animal species - both *in vivo* and *in vitro.* Apart from the studies of Tyrrell *et al.* (1977) others have failed to demonstrate the opsonic effect of serum on uptake of liposomes by macrophages and yet these authors (Juliano, 1982; Ellens *et al.,* 1982; Dijkstra *et al.,* 1984) speculated the possible role of

opsonins in enhancing the uptake of liposomes by macrophages. Recently, we have for the first time demonstrated (Moghimi & Patel, 1988; Chapter III) the presence of tissue-specific opsonins in serum which exhibit a different affinity for cholesterol-poor and cholesterol-rich liposomes. This suggests that the failure of the earlier attempts (Juliano, 1982; Ellens *et al.*, 1982; Dijkstra *et al.*, 1984) to demonstrate the opsonic effect of serum on phagocytosis of liposomes by macrophages may possibly be attributed to the different affinity of these opsonins for liposomes prepared from various phospholipids used in these studies. Hence, in this chapter the role of serum opsonins on the uptake of liposomes prepared from saturated and unsaturated phospholipids by rat hepatic and splenic phagocytes is examined.

4.2. Results

A. The role of serum on the interaction of liposomes composed of saturated and unsaturated phospholipids with liver phagocytes

The results in table 4.1. show the interaction of negatively charged chol-free liposomes prepared from various saturated and unsaturated phospholipids with liver non-parenchymal cells at 37°C. In the absence of serum, liposomes prepared from SM interact poorly with liver cells, while substitution of SM by egg PC considerably increases their interaction with these cells. In the case of liposomes prepared from saturated phospholipids the amount of the vesicles associated with the cells in the absence of serum increases with increase in the chain length of the phospholipid used. For instance, DSPC (chain length:18) liposomes are associated with cells much more than DPPC (chain length:16) liposomes, and DPPC liposomes are in turn more associated with cells

Table 4.1.

Interaction of negatively charged saturated and unsaturated phospholipid liposomes with liver non-parenchymal cells in the absence and presence of serum

Liposome composition (mole ratio)	% of initial liposomal [^{125}I]-PVP	
	Control	Serum
A) Chol-free liposomes		
PC : DCP (7 : 1)	13.4 ± 0.7	10.3 ± 0.1
SM : DCP (7 : 1)	0.6 ± 0.1	0.3 ± 0.1
DMPC : DCP (7 : 1)	1.2 ± 0.2	0.2 ± 0.1
DPPC : DCP (7 : 1)	2.7 ± 0.3	0.1 ± 0.1
DSPC : DCP (7 : 1)	13.0 ± 0.2	0.2 ± 0.1
B) Chol-poor liposomes		
PC : CHOL : DCP (7 : 2 : 1)	1.4 ± 0.3	4.1 ± 1.0
SPHY : CHOL : DCP (7 : 2 : 1)	0.6 ± 0.1	< 0.1
DMPC : CHOL : DCP (7 : 2 :1)	1.1 ± 0.3	< 0.1
C) Chol-poor & Chol-rich liposomes		
DPPC : CHOL : DCP (7 : 2 : 1)	7.5 ± 0.2	0.2 ± 0.05
DPPC : CHOL : DCP (7 : 7 : 1)	1.2 ± 0.1	0.2 ± 0.05

(A), (B), and (C) are independent experiments.

For experimental details see Materials & Methods (Chapter II).

than DMPC (chain length:14) vesicles.

The amount of liposomes interacting with cells at 37°C is reduced when serum is included in the incubation medium. This effect of serum is much more pronounced on the saturated phospholipid liposomes than unsaturated PC vesicles. When adsorption of these liposomes onto the surface of the cells were examined, it was found in the absence of serum less than 1% of all but PC liposomes is adsorbed onto the surface of these cells (results not shown).

Incorporation of cholesterol in SM and DMPC liposomes increases the rigidity and in DPPC liposomes increases the fluidity of liposomal membrane. When 20 mol% cholesterol is included in the preparation of SM, DMPC, and PC liposomes, the uptake of SM and DMPC in the presence of serum is inhibited to a great extent, but the uptake of PC liposomes is increased by 3-fold in the presence of serum as compared to that in the absence of serum (table 4.1.). Thus, in the presence of serum the uptake of cholesterol-poor SM and DMPC liposomes is reduced.

Incorporation of either 20 or 46.6 mol% cholesterol in DPPC liposomes failed to show any opsonic effect of serum (table 4.1.).

B. Assessment for the stability of liposomes in serum

The degradation of liposomes by serum components (Kirby & Gregoriadis,1981; Patel *et al.*, 1983) may reduce the number of liposomes available for phagocytosis by the cells and this could have attributed to the suppression in uptake of liposomes in the presence of serum. Hence, to evaluate the true role of serum on the interaction of liposomes with hepatic cells, the degree of destruction of liposomes by serum was determined by measuring the release of the entrapped marker.

The results in table 4.2. show that over 50% of the entrapped marker leaks out of the chol-free SM and DMPC liposomes and this is reduced to 30% if PC or DPPC is substituted instead of the former lipids. Incorporation of 20 mol% cholesterol into these liposomes, particularly in SM and DMPC, significantly reduces the leakage of the marker. These results are in agreement with the earlier reports of Senior & Gregoriadis (1982).

C. The effect of serum on the uptake of liposomes by spleen cells

Splenic phagocytes respond differently to hepatic cells in handling SM and DPPC liposomes in the presence of serum (table 4.3.). For example, the uptake of entrapped marker from SM and DPPC liposomes is very similar in the presence and absence of serum. Incorporation of cholesterol into these liposomes reduces their uptake in the absence of serum. However, incorporation of cholesterol into these liposomes enhances their uptake in the presence of serum and this effect of cholesterol is dependent upon the cholesterol concentration of liposomes. For example, inclusion of 20 mol% cholesterol in SM and DMPC liposomes enhances their uptake by nearly 2- to 3-fold, but 46.6 mol% cholesterol enhances their uptake by 6- to 7-fold as compared to the respective control incubations in the absence of serum (table 4.3.).

D. Mode of liposome-cell interaction in the presence of serum

The possible mechanism of liposome interaction with cells was examined by using the double-labelled liposomes with ^{3}H-Chol as membrane marker and ^{14}C-Inulin as an aqueous marker. The ratio of two markers associated with the liver and spleen cells in the presence and absence of serum is different as shown in table 4.4. The amount of the

Table 4.2.

Leakage of liposome entrapped ^{125}I-PVP in the absence and presence of serum

Liposome composition (mole ratio)	% of initial liposomal [^{125}I]-PVP	
	Buffer	Serum
PC : DCP (7 : 1)	10	30
SM : DCP (7 : 1)	10	51
DMPC : DCP (7 : 1)	11	67
DPPC : DCP (7 : 1)	8	30
PC : CHOL : DCP (7 : 2 : 1)	6.5	22
SM : CHOL : DCP (7 : 2 : 1)	1	4
DMPC : CHOL : DCP (7 : 2 :1)	8	8

Negatively charged cholesterol-free and -poor liposomes containing [^{125}I]-PVP were incubated in the absence and presence of serum at 37°C for 30 minutes. The amount of [^{125}I]-PVP released from the liposomes was measured by separating free from entrapped [^{125}I]-PVP by gel-chromatography. Results are expressed as percentage of radioactivity leaked. For experimental details see Materials & Methods, chapter II.

Table 4.3.

Interaction of negatively charged saturated and unsaturated phospholipid liposomes with splenic phagocytes in the absence and presence of serum

Liposome composition (mole ratio)	% of initial liposomal [^{125}I]-PVP	
	Control	Serum
A) Chol-free liposomes		
PC : DCP (7 : 1)	6.5 ± 0.2	3.4 ± 0.1
SM : DCP (7 : 1)	1.7 ± 0.6	1.4 ± 0.3
DPPC : DCP (7 : 1)	0.9 ± 0.1	1.0 ± 0.1
B) Chol-poor liposomes		
PC : CHOL : DCP (7 : 2 : 1)	2.0 ± 0.2	4.4 ± 0.8
SM : CHOL : DCP (7 : 2 : 1)	0.2 ± 0.1	0.6 ± 0.1
DPPC : CHOL : DCP (7 : 2 : 1)	0.4 ± 0.1	0.9 ± 0.1
C) Chol-rich liposomes		
PC : CHOL : DCP (7 : 7 : 1)	1.5 ± 0.2	13.1 ± 0.1
SM : CHOL : DCP (7 : 7 : 1)	0.3 ± 0.1	2.2 ± 0.2
DPPC : CHOL : DCP (7 : 7 : 1)	0.6 ± 0.1	3.9 ± 0.1

(A), (B), and (C) are independent experiments.

For experimental details see Materials & Methods (Chapter II).

Table 4.4.

Interaction of double-labelled saturated and unsaturated phospholipid liposomes with liver and spleen phagocytes in the absence and presence of serum

Liposome composition (mole ratio)	% of initial liposomal radioactivity			
	Control		Serum	
	[^{3}H]-Chol	[^{14}C]-Inulin	[^{3}H]-Chol	[^{14}C]-Inulin
A) Liver cells				
PC : CHOL : DCP (7 : 2 : 1)	14.2 ± 0.7	4.2 ± 0.2	8.6 ± 0.8	7.3 ± 1.0
SM : CHOL : DCP (7 : 2 : 1)	0.7 ± 0.4	0.2 ± 0.02	0.3 ± 0.1	< 0.1
DMPC : CHOL : DCP (7 : 2 : 1)	2.7 ± 0.1	1.1 ± 0.2	1.3 ± 0.1	1.3 ± 0.1
B) Spleen cells				
PC : CHOL : DCP (7 : 7 : 1)	8.5 ± 0.5	2.0 ± 0.2	13.6 ± 0.41	11.7 ± 0.3
DMPC : CHOL : DCP (7 : 7 : 1)	5.5 ± 0.1	0.5 ± 0.1	6.9 ± 0.1	7.2 ± 0.2

For experimental details see Materials & Methods (Chapter II).

exchangeable ^{3}H-Chol associated with the cells in the absence of serum is greater than of an aqueous marker ^{14}C-Inulin in DMPC and PC liposomes. This suggests that a few intact liposomes are associated with the cells and a large amount of ^{3}H-Chol is exchanged with plasma membrane. Whereas in the presence of serum the mechanism of interaction is different since the ratio of the two markers is close to 1, indicating the association of intact liposomes with liver and spleen cells.

4.3. Discussion

Liposomes prepared from various phospholipid composition can interact with cells at different rates and by different mechanisms (Pagano & Weinstein, 1978). In the present study, in the absence of serum, liposomes may interact with hepatic and splenic phagocytic cells by fusion, endocytosis or a combination of these two mechanisms. Both of these mechanisms involve direct interaction of liposomal membrane with the plasma membrane of the cells, and hence, their interaction is regulated by properties such as charge, fluidity, and hydrophobicity of the liposome membrane (Pagano & Weinstein, 1978; Pagano *et al.*, 1981). These factors can explain the variation in the amount of liposomes prepared from different cholesterol content and lipid composition in experiments with liver and spleen cells in the absence of serum here. For example, fusion may perhaps be the major mechanism for the association of fluid chol-free egg PC liposomes with both hepatic and splenic phagocytes at 37°C (see also chapter III). In experiments with liposomes composed of saturated phospholipids (table 4.1.) the amount of the

associated aqueous marker with hepatic cells increases by increasing the chain length of these lipids. Since DMPC, DPPC, and DSPC are more rigid as compared to egg PC, fusion may perhaps not be responsible as the major mechanism of saturated phospholipid liposome uptake by kupffer cells. The most probable mechanism(s) for the association of these liposomes with liver cells is a combination of both strong adsorption and endocytosis; as 30% of the aqueous marker was released after trypsinization following the liver cell incubation with, for example, DSPC liposomes (data not shown). Studies with double labelled liposomes (table 4.4.) indicate an increase association of the cholesterol label than the aqueous phase marker, inulin with both liver and spleen cells in the absence of serum. The difference in cell association of the two labels during the incubation period is possibly explained by a favourable exchange of cholesterol between the cells and liposomes. However, a partial release of liposomal aqueous content upon contact with the cell surface, as described earlier for hepatocytes and hepatoma cells (Van Renswoude & Hoekstra, 1981), African green monkey cells (Fraley *et al.*, 1981), and cultured kupffer cells (Dijkstra *et al.*, 1984), may also be responsible for diminished uptake of the aqueous marker by the cells in here.

The main interest of the present work has been to study the effect of serum on the uptake of liposomes by liver and spleen cells. In the presence of serum the mode of interaction of liposomes with the cells is different since their interaction will be mediated via serum components associated on the surface of liposomes (see table 4.4.). The serum components may be opsonins which may stimulate or dysopsonins which

will suppress phagocytosis of liposomes by liver and spleen phagocytes. Binding of the opsonins onto the liposome surface may depend on the chemical and physical properties of liposomes and these properties can be manipulated by selecting the lipid composition and phospholipids of the vesicles (Szoka & Papahadjopoulos, 1981). For example, the opsonin(s) specific for liver phagocytic cells has a poor affinity for cholesterol-rich liposomes, probably, due to the rigidity of these liposomal membrane (Moghimi & Patel, 1988; Chapter III). Sphingomyelin liposomes are less fluid than PC liposomes at 37°C (Szoka & Papahadjopoulos, 1981) and this may explain the fact that no enhancement in the uptake of SM liposomes is observed in the presence of serum, since the liver-specific opsonin(s) may not associate with less fluid SM liposomes. Perhaps other serum components that can act as dysopsonins may adsorb onto surface of such liposomes and inhibit their uptake by liver phagocytes (see also illustrations in fig 4.1.). Similarly, the liver-specific opsonin(s) has no affinity for 'solid' saturated phospholipid vesicles, DMPC, DPPC, and DSPC, since no enhancement in their uptake by liver cells is observed in the presence of serum. However, the rigidity of DPPC and DSPC liposomes can be reduced by incorporation of cholesterol into these preparations (Demel *et al.*, 1977), but there is no evidence of serum stimulating the uptake of DPPC vesicles containing even 46.6 mol% cholesterol by liver cells (table 4.2.). This probably suggests that the fluidity of the membrane may not be the only factor regulating the binding of the liver-specific opsonin onto liposomal surface. Other factors such as cholesterol content, hydrophobicity, and the physical state of phospholipids could play an

important role in attracting the right serum components.

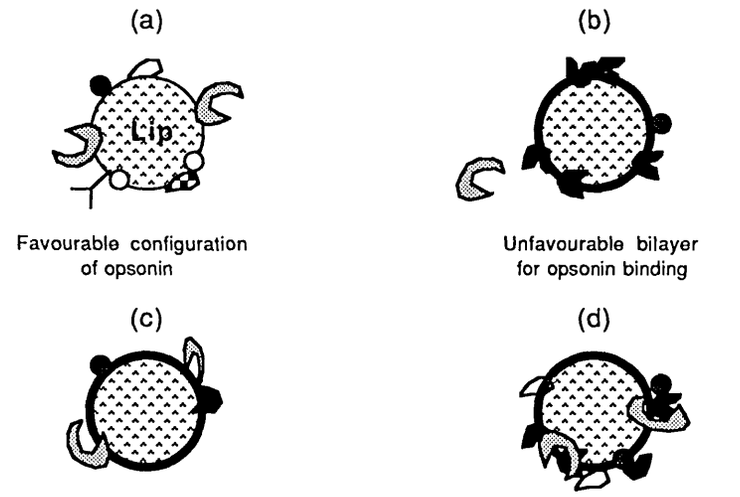
The opsonin(s) for splenic phagocytic cells is different from that for Kupffer cells (Chapter III). The results in here show that spleen-specific opsonin(s) like those of liver has no affinity for adsorption onto the surface of SM or saturated phospholipid vesicles, perhaps due to different physical properties of these phospholipids as compared to those of unsaturated egg PC liposomes. However, in contrast to liver, serum does not inhibit the uptake of these vesicles by the spleen cells. This suggests that serum components which may act as dysopsonins and inhibit the uptake of SM and saturated phospholipid vesicles by liver cells have no effect on the uptake of these liposomes by splenic cells. However, serum enhances the uptake of cholesterol-containing liposomes, particularly cholesterol-rich (46.6 mol% cholesterol) liposomes, despite their phospholipid composition. This suggests that by increasing the hydrophobicity of the liposomal membrane by inclusion of cholesterol perhaps spleen-specific opsonin(s) can be attracted to these liposomes, resulting in enhancement in their uptake by spleen cells.

Finally, the results discussed here probably explain why early attempts to show the opsonic effect of serum on SM and saturated phospholipid liposomes were not successful (Juliano, 1982; Ellens *et al.*, 1982; Dijkstra *et al.*, 1984). The opsonins, particularly liver-specific, do not bind to these liposomes and as a result these liposomes are not readily phagocytosed by liver phagocytes. This probably explains why these liposomes have a long half-life in the circulation. For investigation of the reasons for the variation in the degree of interaction of these opsonins with liposomes prepared from various

phospholipids it is necessary to characterize and purify liver and spleen specific opsonins.

Fig 4.1.

Interaction of serum components with liposomes



Key : Lip = liposomes, (a) composed of unsaturated phospholipid (b, c, and d) composed of saturated phospholipids.
Opsonin; Dysopsonin

C h a p t e r V

Differential properties of liver and spleen specific opsonins

5.1. Introduction

The understanding of the interaction of liposomes with serum components is important, since such interactions may alter the *in vivo* properties and tissue distribution of intravenously injected liposomes. Various studies demonstrated that a variety of serum components such as albumin, blood coagulation factors, globulins, fibronectin, and lipoproteins can interact with liposomes depending on the surface properties and lipid composition of liposomes (Reviews by Morisett *et al.*, 1977; Scherphof *et al.*, 1981; Juliano, 1983; Bonte & Juliano, 1986). The role of some serum components on the uptake of liposomes by hepatocytes (Hoekstra & Scherphof, 1979), lymphocytes (Blumenthal *et al.*, 1977), fibroblasts (Tyrrell *et al.*, 1977), polymorphonuclear leucocytes (Finkelstein *et al.*, 1981), and peritoneal macrophages (Hsu & Juliano, 1982) have been the subject of study for the last ten years. In spite of this, there is lack of information concerning the effect of serum components on the uptake of liposomes by blood-sinus-lining mononuclear phagocytes, particularly those of liver and spleen. Apart from the early studies of Tyrrell *et al.* (1977) on perfused rat liver, and more recently Dijkstra *et al.* (1984) on kupffer cells no information concerning the effect of serum or its components on the uptake of liposomes by liver and spleen phagocytes is cited.

The stimulatory effect of serum on the uptake of liposomes by both hepatic and splenic phagocytes was demonstrated in chapters III &IV. The evidence presented in chapters III & IV illustrates the presence of opsonins specific for liver and spleen phagocytes. The adsorption of these opsonins onto the surface of liposomes depends on the surface

property and lipid composition of liposomes. In the present chapter, experiments are designed to determine some properties of liver and spleen specific opsonins.

5.2. Results

A. Effect of temperature on the opsonic activity of rat serum

To investigate the temperature sensitivity of opsonins specific for liver and spleen serum was heated at different temperatures and its opsonic activity was studied. The stimulatory effect of fresh rat serum on interaction of liposomes by both hepatic and splenic phagocytes is altered by heating the serum at 55°C and at different time intervals prior to incubation, fig 5.1. In case of liver cells, the opsonic activity of rat serum is increased when heated for only 10 minutes in comparison to control incubations containing fresh unheated rat serum. However, this increased opsonic activity gradually starts to decline, if serum is heated for longer periods; and yet after 1 hour of heating the opsonic activity of serum is still above that of fresh unheated serum (fig 5.1.). In contrast, for splenic phagocytes, the opsonic activity of serum is gradually reduced and eventually lost after 30 minutes of heating prior to incubation, fig 5.1. with these cells.

The stimulatory effect of fresh rat serum on interaction of liposomes with liver cells is reduced on heating the serum at higher temperatures than 55°C prior to incubation. For instance, the opsonic activity of serum heated at 60°C for 10 minutes was reduced by 35% and, indeed, is completely abolished when the temperature was raised by a further 5 °C, in comparison with fresh unheated serum (table 5.1. Exp A).

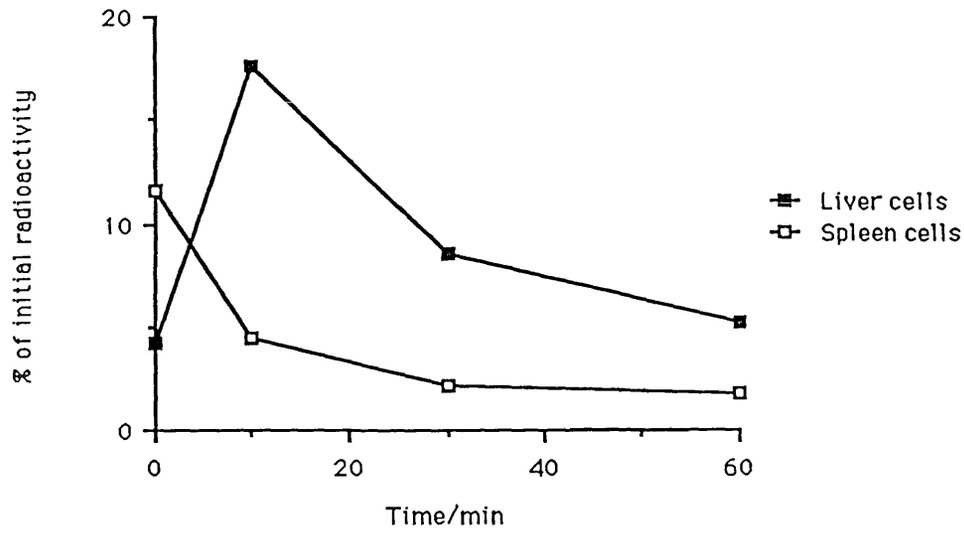


Fig 5.1. Effect of heated serum at 55°C on its opsonic activity specific for liver and spleen phagocytes.

Negatively charged cholesterol-poor egg PC liposomes containing [^{125}I]-PVP were used for the above experiments. For experimental details see Chapter II.

Table 5.1.

Effect of heat treatment and dialysis of serum on its opsonic activity specific for liver phagocytes

Treatment	% of initial liposomal [^{125}I]-PVP
Exp A	
Control	2.2 ± 0.1
Serum	12.4 ± 0.1
Heated serum (55°C/30 min)	14.1 ± 0.8
(55°C/10 min)	17.5 ± 1.3
(60°C/10 min)	9.2 ± 0.1
(65°C/10 min)	< 0.1
Exp B	
Control	2.3 ± 0.6
Serum	13.4 ± 0.6
Dialyzed serum (DS)	28.6 ± 2.0
Serum dialyzate (DZ)	2.1 ± 0.2
DS + DZ	13.6 ± 0.1
DS + Boiled DZ	10.1 ± 0.1

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

B. Effect of cold-storage on the opsonic activity of serum

In a series of studies rat serum was stored at -20°C for a period of 6 months. When tested for its opsonic activity, the frozen serum retained its stimulatory effect on liposome interaction with liver cells and, indeed, its opsonic activity was increased similar to elevated opsonic activity of heated serum (table 5.2.).

The opsonic effect of cold-stored serum on splenic phagocytes was partially lost as compared to fresh serum (table 5.2.).

C. Effect of dialysis on the opsonic activity of serum

The opsonic activity of serum specific for liver was enhanced by 2-fold when serum was dialyzed prior to incubation (table 5.1. B). Addition of serum dialyzate to liver cell incubations did not affect liposome-cell interaction, whereas addition of either unheated or boiled dialyzate to dialyzed serum abolished the increased opsonic activity of the dialyzed serum (table 5.1. Exp B.)

In contrast to liver cells, the stimulatory effect of serum on interaction of Chol-poor liposomes with splenic cells was partially lost when serum was dialyzed prior to incubation, table 5.3. This decrease in opsonic activity was even more pronounced and, indeed, completely abolished when tested for the interaction of Chol-rich liposomes with splenic cells. Addition of serum dialyzate to dialyzed serum failed to restore the opsonic activity of dialyzed serum (table 5.3.)

Table 5.2.

Effect of cold-storage of serum on its opsonic activity specific for hepatic and splenic phagocytes

Treatment	Opsonic activity (% of fresh serum)	
	Liver	Spleen
Fresh serum	100	100
Fresh serum heated at 55°C/10 min	142	not done
Cold-stored serum (-20°C/6 months)	124	81
Cold-stored serum heated at 55°C/10 min	131	not done

Negatively charged cholesterol-poor egg PC liposomes containind [^{125}I]-PVP were used for the above experiments.

Table 5.3.

Effect of different treatments of serum on its opsonic activity specific for splenic phagocytes

Serum	% of initial liposomal [^{125}I]-PVP	
	Chol-poor liposome (A)	Chol-rich liposome (B)
None	2.8 ± 0.2	0.5 ± 0.1
Fresh	7.9 ± 0.2	8.5 ± 0.4
Heated (55°C/30min)	1.9 ± 0.1	0.8 ± 0.1
Fibronectin depleted	7.5 ± 0.2	not done
Dialyzed	4.4 ± 0.5	0.6 ± 0.1
Serum dialyzate (DZ)	3.0 ± 0.1	not done
Dialyzed + DZ	3.1 ± 0.4	not done

Negatively charged liposomes were used for the above experiments.

A & B are independent experiments.

D. Mode of liposome-liver cell interaction in the presence of dialyzed serum

The interaction of liposomes with liver cells in the presence of dialyzed serum was greatly reduced by incubating the mixture at 4°C, while addition of metabolic inhibitors had no effect on blocking the liposome uptake at 37°C during the incubation period (data not shown). The possible mechanism of liposome-liver cell interaction in the presence of dialyzed serum was further examined by using the double-labelled liposomes with [^{3}H]-cholesterol as membrane marker and [^{14}C]-inulin as an aqueous marker. The ratio of the two markers associated with the liver cells in the presence of dialyzed serum is close to one, indicating the association of intact vesicles with the cells (table 5.4.). However, upon trypsinization the great majority of vesicles were removed, demonstrating the presence of a bridge formation between the liposomes and liver cells mediated by serum components present in dialyzed serum.

E. Effect of fibronectin depletion of serum on its opsonic activity

Fibronectin is known to stimulate the phagocytic uptake of liposomes (Hsu & Juliano, 1982). Depletion of fibronectin from serum did not influence the opsonic activity of the rat serum for both hepatic and splenic phagocytes (tables 5.3. & 5.5.). The opsonic activity of fibronectin is known to be increased by addition of heparin (Chang *et al.*, 1985). Addition of heparin to liver cell incubations in dosages of 5 or 20 units did not influence the opsonic activity of serum (table 5.5.). Addition of higher dosages was toxic and caused cell aggregation.

Table 5.4.

Effect of trypsinization on the association of double-labelled liposomes with liver non-parenchymal cells in the absence and presence of dialyzed serum

Treatment	% of initial radioactivity			
	[^{3}H]-Chol		[^{14}C]-Inulin	
	Before trypsin	After trypsin	Before trypsin	After trypsin
Buffer	2.8 ± 0.1	1.6 ± 0.3	1.8 ± 0.1	0.8 ± 0.1
Dialyzed serum	20.7 ± 0.5	3.4 ± 0.3	18.6 ± 0.3	2.3 ± 0.3

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

Liposomes were labelled with [^{3}H]-cholesterol as membrane marker and [^{14}C]-inulin as an aqueous marker.

For experimental details see Materials & Methods (Chapter II).

Table 5.5.

Effect of fibronectin depletion of serum and the effect of heparin addition to serum on its opsonic activity specific for liver phagocytes

Treatment	% of initial liposomal [^{125}I]-PVP
Control	1.5 ± 0.1
Serum	12.3 ± 0.6
Serum (fibronectin depleted)	11.8 ± 0.5
Heparin (5U)	1.3 ± 0.1
Heparin (5U) + Serum	13.5 ± 3.2
Heparin (20 U)	0.7 ± 0.1
Heparin (20U) + Serum	10.2 ± 1.3

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

Table 5.6.

Opsonic effect of serum from various species on liver non-parenchymal and spleen cells in suspension

Species	% of initial liposomal [^{125}I]-PVP	
	Serum	Dialyzed serum
A) Liver cells:		
Rat (CFY)	7.9 ± 0.7	23.9 ± 3.6
Foetal calf (Australian)	8.0 ± 0.6	28.9 ± 2.0
Rabbit (English)	11.4 ± 0.1	20.6 ± 1.7
B) Spleen cells *:		
Rat (CFY)	8.5 / 7.6	5.6 /4.9
Rabbit (English)	4.0 / 4.2	12.6 / 10.9
Foetal calf	9.1 / 8.4	6.1 / 6.4
Human (male)	3.0 / 3.6	3.7 / 3.4

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

** Duplicate incubations, individual values are given.*

F. Opsonic effect of serum from various species on liver and spleen cells

Both foetal calf and rabbit serum stimulated the uptake of liposomes by liver cells (table 5.6. a). Upon dialysis, their stimulatory effect was increased by 2-3 fold and the bridge formation between liposome and the cells was enhanced (table 5.6. a.)

Foetal calf serum possessed the same degree of stimulatory effect as rat serum on liposome uptake by spleen cells, and prior dialysis causes a partial loss of its opsonic activity (table 5.6. b.) In contrast, the stimulatory effect of rabbit serum on liposome-spleen cell interaction is only achieved by prior dialysis of serum (table 5.6. b.). Both fresh and dialyzed human serum had no stimulatory effect on liposome uptake by spleen cells (table 5.6. b).

5.3. Discussion

The results presented in this chapter for the first time demonstrate the differential effect of organ-specific serum opsonins on the interaction of liposomes with hepatic and splenic phagocytes. Heating (55°C/30 min), freezing and thawing, and dialysis of serum prior to incubation increase the interaction of liposomes with liver phagocytes while the same treatments reduce or completely abolishe liposome-spleen cell interaction.

The liver specific opsonin(s) is heat stable as its opsonic activity is preserved by prolonged heating at 55°C. This supports the hypothesis that the liver specific opsonin(s) may not be a component of complement pathway (Smith & Wood, 1969). The increase in opsonic activity of serum upon heating at 55°C/10 min may suggest a change in the conformation

of this opsonin and as a result a favourable interaction between opsonized liposomes and the cell-surface receptors occurs. Although, inactivation or denaturation of a possible heat sensitive inhibitor for liver specific opsonin can also explain the apparent increase in the stimulatory effect of the heated serum (see also discussion of chapter VII). Similarly, the probable conformational change of the liver specific opsonin(s) or the inactivation/denaturation of a heat sensitive serum dysopsonin may have also occurred during the cold-storage of serum (-20°C), since the opsonic activity of cold-stored serum was comparable to that of fresh heated serum. In contrast to liver, the opsonic activity of serum specific for spleen phagocytes is lost on heating (55°C/30 min). This may suggest the involvement of heat labile proteinaceous serum factors, such as components of complement pathway (Smith & Wood, 1969) as spleen specific opsonin(s).

The opsonic activity of serum specific for both liver and spleen phagocytes also varies on dialysis of serum. The opsonic activity of liver specific opsonin(s) is retained and elevated on dialysis of serum. This suggests that the liver specific opsonin(s) is a macromolecule and its activity is partially inhibited by a dialyzable serum component(s). This dialyzable inhibitor of liver specific opsonin(s) is heat stable even at boiling temperature. Since serum dialyzate had no effect on the interaction of liposomes with liver cells may suggest that the dialyzable factor has no inhibitory effect on the interaction of liposomes by acting on liver cells. Hence, its effect must be mediated by its interaction with the liver specific opsonin(s) and thus, interfering with either the process of liposome opsonization or with the interaction

of opsonized liposomes with cell-surface receptors. This argument is further supported by the observation in loss of the elevated opsonic activity of the dialyzed serum on addition of serum dialyzate (table 5.1.). However, a full investigation as to the nature and the mechanism of action of this inhibitor has been carried out in the forthcoming chapter. The results of experiments with dialyzed serum (table 5.4.) demonstrate that dialysis of serum stimulates the binding of liposomes to the cells by formation of a bridge between the liposomes and liver cells as judged from the metabolic inhibitor and trypsinization experiments. The binding effect of dialyzed serum is not observed at low temperature of incubation (4°C) suggesting a temperature dependent alteration in the functional state of kupffer cells. This may perhaps reflect an alteration in the fluidity of the cell-membrane lipids and hence a reduction in accessibility of appropriate receptor sites in the cell surface (Stendahl & Tagesson, 1977).

In contrast to liver, the opsonic activity of serum specific for spleen phagocytes is reduced on dialysis. This may suggest the involvement of small molecular weight serum components as either spleen specific opsonin(s) or as co-factor(s) for spleen specific opsonin(s). However, the small amount of the spleen specific opsonic activity retained in the dialyzed serum totally disappeared when chol-poor liposomes were replaced with chol-rich vesicles (table 5.3.). This observation was unexpected since chol-rich vesicles are taken-up much more than chol-poor liposomes by spleen cells in the presence of serum (chapters III & IV) and hence one would expect that dialysis of the serum should bring about proportionally the same amount of the reduction in the uptake of

chol-rich as well as that of chol-poor vesicles. Probably, this discrepancy suggests and supports the earlier hypothesis (chapter III) that there are more than one spleen specific opsonins in serum. Hence, the spleen specific opsonins are macromolecules which have a differential degree of dependency on a "dialyzable co-factor" for their opsonic activity. Therefore, the dialysis of serum may bring about irreversible conformational changes in the structure of these opsonins which cause partial or total loss of their activity.

Fibronectin, a serum opsonin, is known to interact with phospholipid vesicles of various composition (Rossi & Wallace, 1983) and enhance their phagocytosis by peritoneal phagocytes (Hsu & Juliano, 1982). The opsonic activity of fibronectin is dramatically reduced on either heating or cold-storage of serum (Boughthon *et al.,* 1984). Since, these properties do not match with those of liver specific opsonin(s) fibronectin can not be the liver specific opsonin in here (see also discussion of chapter VII). This conclusion is further supported by the evidence that fibronectin depletion from serum does not alter its liver specific opsonin activity. Also, heparin, which is known to enhance fibronectin function (Chang *et al.,* 1985), has no significant effect on the opsonic activity when added to the serum. On the other hand, some properties of fibronectin are similar to those of spleen specific opsonin(s). Both of these opsonins are heat-labile but unlike fibronectin, the spleen specific opsonin(s), on cold-storage, loses only part of its activity. Moreover, fibronectin-depleted serum does not show significant reduction in the uptake of liposomes. However, this discrepancy apparently rules out the possibility of fibronectin as spleen specific

opsonin in here, but at the same time the discrepancy can be explained by following the earlier suggestion (chapter III) that there are more than one spleen specific opsonins so that the depletion or inactivation of one of the serum opsonins, which may be fibronectin, will produce only partial loss in the opsonic function of the serum. Whereas, the total loss of the serum activity on heating at 55 °C suggests that the other spleen specific opsonin may also be heat labile. It should also be noted that an independent and direct role of fibronectin as an opsonic molecule is still not established (see page16), but its complementary role in the enhancement of complement C3b-coated particles is well known (Pommier *et al.*, 1983). Complement C3b and fibronectin are both heat-labile and hence it is tempting to suggest that the spleen specific opsonins may include both fibronectin and complement components for optimum opsonic activity.

Serum from various species, except human sera, possessed the same stimulatory effect as rat serum when tested for hepatic and splenic phagocytes (table 5.6. a. & b.). But surprisingly the rabbit serum possessed its stimulatory effect on liposome uptake by spleen cells only by prior dialysis before incubation. Perhaps, this suggests that there is one specific opsonin which stimulates the liposome uptake by both liver and splenic phagocytes in this species. However, this point requires further investigation by using isolated liver and spleen phagocytes from rabbit rather than rat. Further, differences exist in mononuclear phagocytic activity between the mouse, rat, rabbit, dog, human, etc. In this regard, experimental observations have shown that MPS is most active in mouse, and moves in decreasing activity in the rat, guinea pig,

rabbit and human, respectively (Benacerraf *et al.,* 1957). The relative liver and spleen size has been emphasized as an explanation for the species mononuclear phagocytic variability, such that the enhanced mononuclear phagocytic activity by smaller animals appears to be due to relatively larger liver and spleen mass. However, from the results obtained in here it can be speculated that the decreased activity of the mononuclear phagocytes in larger animals may perhaps be due to the lack of opsonic effect of their serum on phagocytosis. For example, the stimulatory effect of rabbit serum for splenic phagocytes appears only by prior dialysis, whereas human sera lack the stimulatory activity even after dialysis.

Thus, the evidence presented in this chapter supports the earlier findings of chapter III & IV that serum contains specific opsonins which may selectively enhance phagocytosis by the phagocytes of liver and spleen. The knowledge of differential properties of liver and spleen specific opsonins may allow strategies for their purification and hence evaluation of their role in normal and pathological conditions.

Chapter VI

Regulatory role of calcium on liver specific opsonin activity

6.1. Introduction

Previously (chapter V) it was shown that the activity of liver specific opsonin(s) is elevated on dialysis of serum, whereas that of spleen specific opsonin activity is reduced. The enhancement on the opsonic activity of serum specific for liver appeared to be due to removal of small molecular weight heat stable (even at boiling temperature) serum components on dialysis. The most easily removed heat stable (at boiling temperature) small molecular weight serum components that can possibly affect the process of either opsonization or phagocytosis are diavalent cations such as calcium and magnesium. For example, low concentrations (1-2 mM) of Ca^{2+} and Mg^{2+} are a necessary prerequisite for the process of phagocytosis (Allison *et al.*, 1963; Greendyke *et al.*, 1963; Wilkins & Bangham, 1964; Stossel, 1973), while higher concentrations tend to inhibit the process (Stossel, 1973). The role of cations in phagocytosis is complex and they may affect various stages in attachment or ingestion of the particles. For example, during phagocytosis of albumin-coated paraffin-oil particles or glutaraldehyde-treated erythrocytes by alveolar or peritoneal macrophages, the internalization phase was stimulated by calcium and magnesium (Rabinovitch, 1967; Stossel, 1973). However, in receptor-mediated endocytosis of various proteins, the binding step was Ca^{2+} dependent (Kaplan & Nielsen, 1979; Weigel, 1980; Dickson *et al.*, 1981). Recently, it was suggested that Ca^{2+} may also control the number of receptors which are oriented at the exterior of the cell (Blumenthal *et al.*, 1980).

Hence, in the present investigation the effect of various diavalent

cations on the activity of liver and spleen specific opsonin(s) are examined.

6.2. Results

A - Effect of EGTA on the opsonic activity of serum

The effect of EGTA on the interaction of negatively charged liposomes with liver cells is shown in table 6.1. In the absence of serum, EGTA reduced the interaction of liposomes with liver cells in comparison to control incubation. In contrast, the stimulatory effect of serum on liposome-cell interaction is greatly enhanced in the presence of EGTA; a situation similar to the enhanced stimulatory effect of dialyzed serum (table 6.1.). Similarly, the opsonic activity of dialyzed serum can further be increased by addition of EGTA to the incubation media (table 6.1.).

EGTA has the ability to chelate diavalent cations, particularly Ca^{2+}, hence, the increase in the opsonic activity of serum after dialysis or by addition of EGTA can perhaps be attributed to the removal of Ca^{2+} or other diavalent cations from serum. The effect of increasing concentrations of Ca^{2+} on the interaction of liposomes with liver non-parenchymal cells in the absence of serum and in the presence of either serum or dialyzed serum is evaluated in fig 6.1. The results demonstrate that low concentrations of extracellular Ca^{2+} has no significant effect on liposome-cell interaction. However, increasing concentrations of Ca^{2+} above 1.5 mM resulted in slight inhibition of liposome-cell interaction.

In contrast, addition of 0.25 mM Ca^{2+} was sufficient to decrease the opsonic activity of serum and, indeed, addition of higher concentrations

Table 6.1.

The effect of EGTA on the activity of liver specific opsonin(s)

Treatment	% of initial liposomal [^{125}I]-PVP
Buffer	1.1 ± 0.2
Buffer + EGTA (1.25 mM)	0.3 ± 0.1
Serum	6.1 ± 0.7
Serum + EGTA	25.7 ± 0.3
Dialyzed serum	25.6 ± 0.4
Dialyzed serum + EGTA	30.3 ± 1.2

Cholesterol-poor egg PC liposomes were used for the above experiments. For experimental details see Materials & Methods (Chapter II).

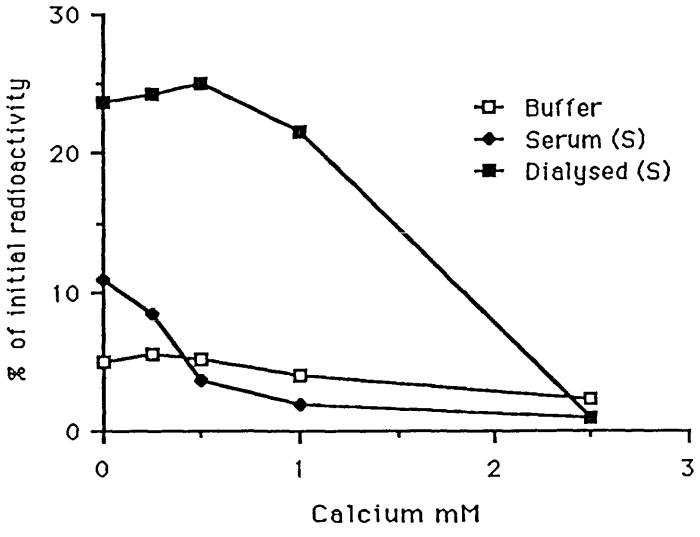


Fig 6.1. The effect of $CaCl_2$ on the interaction of negatively charged cholesterol-poor egg PC liposomes with liver non-parenchymal cells in the presence and absence of serum and dialyzed serum.

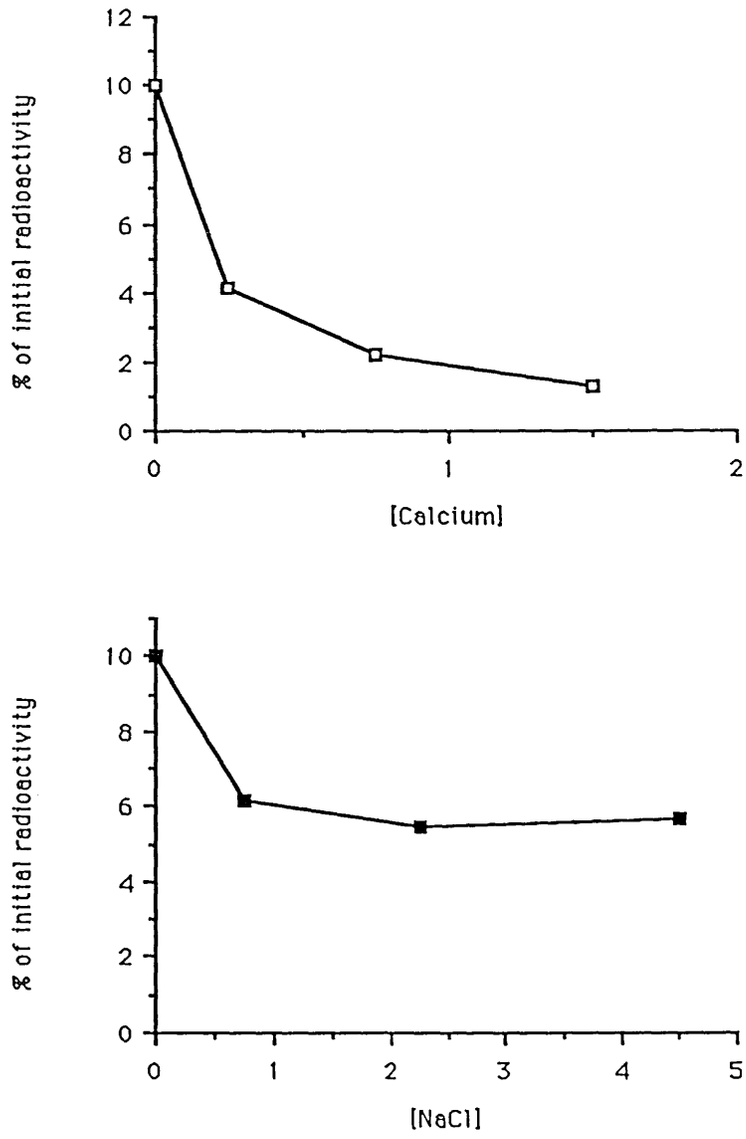


Fig 6.2. The effect of ionic strength of incubation media on the interaction of negatively charged cholesterol-poor egg PC liposomes with liver non-parenchymal cells in the presence of serum.

of Ca^{2+} completely abolished the stimulatory effect of serum (fig 6.1.). In order to determine whether the decrease in the opsonic activity of serum was due to increase in the ionic strength of the medium, similar incubations were performed but NaCl at a concentration three times higher than Ca^{2+} (equivalent to same ionic strength) replaced the Ca^{2+} (fig 6.2.). The inhibitory effect of Ca^{2+} on liposome-cell interaction was 2- to 3-fold higher than incubations of similar ionic strength made from NaCl (fig 6.2).

The stimulatory effect of serum on liposome-cell interaction can be increased by prior dialysis of serum to incubation (table 6.1.). Total concentration of Ca^{2+} in serum and dialyzed serum was measured by atomic absorption (data not shown) and the results demonstrated that on dialysis 1-1.5 mM Ca^{2+} is removed from serum. The increased opsonic activity of dialyzed serum was reduced by addition of Ca^{2+} in concentrations greater than 1.0 mM as indicated in fig 6.1.

B - Effect of other diavalent cations on the opsonic activity of serum

Since EGTA can influence the opsonic activity of serum, the effects of other diavalent cations at a concentration of 1.5 mM were tested and the results are shown in table 6.2. Diavalent cations Mg^{2+}, Co^{2+}, and Mn^{2+} stimulated the interaction of liposomes with liver cells in the absence of serum; and this effect was more pronounced with Co^{2+}. However, in the presence of dialyzed serum, Mn^{2+} completely abolished the stimulatory effect of the dialyzed serum, whereas Mg^{2+} and Co^{2+} reduced 60% of the opsonic activity of the dialyzed serum.

Table 6.2.

The effect of various diavalent cations on the opsonic activity of liver specific opsonin(s)

Salt [1.5 mM]	% of initial liposomal [^{125}I]-PVP	
	Buffer	Dialyzed serum
None	1.4 ± 0.1	35.4 ± 2.9
Ca^{2+}	2.4 ± 0.1	1.6 ± 0.2
Mg^{2+}	2.0 ± 0.4	9.8 ± 0.9
Co^{2+}	5.5 ± 1.0	11.3 ± 0.1
Mn^{2+}	3.0 ± 0.1	1.0 ± 0.3

C - An investigation on to the mechanism(s) of action of diavalent cations

Previously it was shown that the prior incubation of liposomes in serum causes weak adsorption of liver specific opsonin(s) onto the surface of liposomes and this stimulates their interaction with liver phagocytes (Chapter III, table 3.3.). The results in table 6.3. demonstrate that there is no significant difference on the interaction of liposomes that have been pre-opsonized with either serum or dialyzed serum with liver phagocytes.

The interaction of serum pre-opsonized liposomes with liver phagocytes in the presence of extracellular Ca^{2+} was also studied, fig 6.3. The results show that the presence of 1.5 mM extracellular calcium can prevent the interaction of serum pre-opsonized liposomes with liver cells in comparison to the respective control incubations in the absence of extracellular calcium. Washing of the serum pre-opsonized liposomes prior to incubation removes the liver-specific opsonin(s) (Chapter III, table 3.3.). The interaction of washed-opsonized liposomes with liver cells both in the presence and absence of extracellular calcium was similar to the respective incubations that have been performed in the presence of unopsonized liposomes, fig 6.4.

Results of electrophoretic studies of the serum pre-opsonized negatively charged chol-poor egg PC liposomes are presented in fig 6.4. These results show that there is no difference in the pattern of serum proteins associated with liposomes that have been opsonized with serum in the absence of additional calcium and those liposomes that have been opsonized with serum but in the presence of different Ca^{2+} concentration

Table 6.3.

Interaction of serum pre-opsonized liposomes with liver cells

Treatment	% of initial liposomal [^{125}I]-PVP*
Liposomes pre-opsonized in serum	9.9
Liposomes pre-opsonized in dialyzed serum	7.9

** Mean of duplicate incubations. The results from each individual incubation was within 5% of the mean value.*

For experimental details see Materials & Methods (Chapter II).

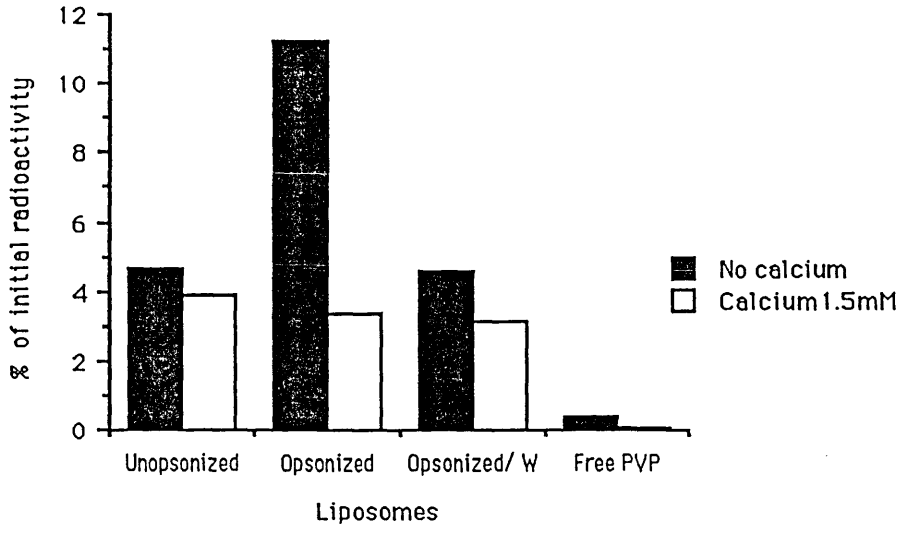


Fig 6.3. Effect of calcium on interaction of serum pre-opsonized negatively charged cholesterol-poor egg PC liposomes with liver non-parenchymal cells. Serum pre-opsonized or unopsonized liposomes were incubated with liver cells in the presence of 1.5 mM $CaCl_2$. Similarly, the effect of Ca^{2+} on the interaction of pre-opsonized liposomes washed once with saline was studied. Opsonized/W : washed opsonized liposomes.

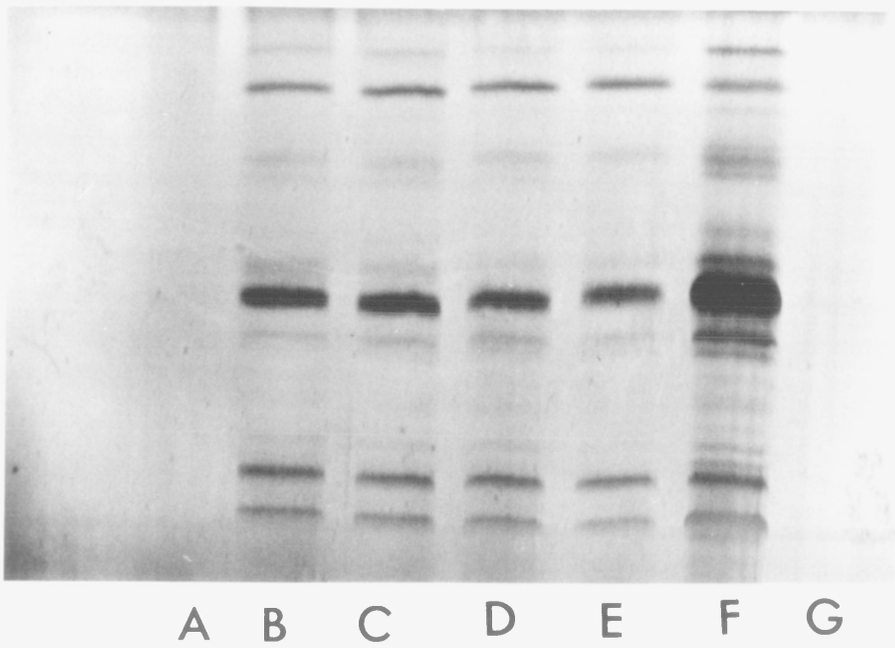


Fig 6.4. SDS-PAGE electrophoresis of serum opsonized negatively charged cholesterol-poor egg PC liposomes by Pharmacia Phast-Electrophoresis system on a 10-15% gradient gel. A & G, are void; B, liposomes opsonized in serum; C, D, and E, are liposome opsonized in serum and in the presence of additional 2.0, 5.0, and 10.0 mM calcium respectively; and F, serum.

In a further study, the cells were preincubated with double-labelled liposomes in the presence of dialyzed serum for 30 min. After this preincubation 1.5 mM Ca^{2+} was added to the mixture and the incubation was continued for another 30 min. The results in table 6.4. show that the addition of calcium reduces the association of already cell-associated liposomes to 50% of the respective control incubations (table 6.4.).

E - Effect of diavalent cations on the opsonic activity of dialyzed rat serum on liposome uptake by splenic phagocytes

The opsonic activity of rat serum on liposome interaction with spleen phagocytes is abolished by prior dialysis of serum, and serum dialyzate had no stimulatory effect either on its own or by its addition to dialyzed serum (Chapter V, table 5.3.). The results in table 6.5. demonstrate that the opsonic activity of dialyzed serum can not be restored by addition of 1.0 mM Ca^{2+} or Mg^{2+} in incubations of spleen cells and liposomes (table 6.5.). Similarly, none of these cations had any effect on the interaction of liposomes with spleen phagocytes at an extracellular concentration of 1.0 mM (table 6.5.). Interaction of liposomes with spleen cells were also unaffected in the presence of EGTA.

Table 6.4.

Effect of calcium on interaction of negatively charged double-labelled cholesterol-poor egg PC liposomes with liver non-parenchymal cells *in vitro*

Treatment	% of initial liposomal radioactivity	
	[^{3}H]-Chol	[^{14}C]-Inulin
Dialyzed serum (incubated 60 min)	10.2 ± 0.5	7.2 ± 0.2
Dialyzed serum + Calcium (1.5 mM) (incubated 60 min)	2.5 ± 0.1	0.6 ± 0.1
Dialyzed serum (incubated 30 min)	8.8 ± 0.3	5.8 ± 0.3
Dialyzed serum (incubated 30 min) followed by addition of 1.5mM calcium and incubated for another 30 min	5.7 ± 0.2	3.3 ± 0.2

Liposomes were labelled with [^{3}H]-cholesterol as membrane and [^{14}C]-inulin as an aqueous marker.

Liposomes were initially present in each incubation.

Table 6.5.

The effect of diavalent cations on the uptake of negatively charged cholesterol-poor egg PC liposomes by spleen cells in the absence and presence of dialyzed serum

Serum	% of initial liposomal radioactivity	
	[^{3}H]-Chol	[^{14}C]-Inulin
Exp (a)		
None	10.6 ± 0.2	2.9 ± 0.2
None + Ca^{2+}	10.3 ± 0.3	3.3 ± 0.2
None + Mg^{2+}	9.3 ± 0.3	2.6 ± 0.1
None + Ca^{2+} + Mg^{2+}	8.9 ± 0.7	2.5 ± 0.3
Serum	19.2 ± 0.9	20.3 ± 0.8
Dialyzed	8.0 ± 0.7	6.4 ± 1.0
Dialyzed + Ca^{2+}	8.3 ± 0.6	6.2 ± 0.8
Dialyzed + Mg^{2+}	9.3 ± 0.2	8.0 ± 0.5

Liposomes were labelled with [^{3}H]-cholesterol as membrane and [^{14}C]-inulin as an aqueous marker.

The final concentration of each diavalent cation is 1.0 mM.

6.3. Discussion

The results in fig 6.1. and table 6.2. suggest that the interaction of liposomes with liver phagocytes in suspension appears to be regulated by calcium. Liposome-cell interaction is enhanced at low calcium concentration, whereas liposome-cell interaction is reduced at higher concentrations (2-2.5 mM) of calcium. These observation supports the earlier reports of Dijkstra *et al.* (1984) that low concentration of extracellular calcium enhances liposome-cell interaction. Similar to calcium, various diavalent cations (table 6.2.) affected liposome-liver cell interaction. The results in table 6.2. demonstrate that the order of effectiveness of cations in promoting the interaction of liposomes with liver cells is as follows: $Co^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. This is somehow similar to the earlier studies of Stossel (1973). In his studies, Stossel (1973) reported that uptake of unopsonized albumin-coated paraffin-oil droplets was stimulated by various diavalent cations and the order of increasing effectiveness in promoting ingestion the cations were ranked as follows: $Mn^{2+} > Co^{2+} > Mg^{2+} > Ca^{2+}$. The greater stimulatory action of cobalt and manganese in liposome-liver cell interaction, as compared to calcium, may be attributed to the respective size of these cations or different affinity of the involved cell surface receptors for such cations. Among these cations, the ionic radii increase from Co^{2+} to Mg^{2+}. Perhaps, the smaller size of Co^{2+} & Mn^{2+} may allow better interaction between the liposomes and the specific receptor sites on the cell surface, in comparison to Ca^{2+} and Mg^{2+}. However, it should be emphasized that the concentration of cations used in the present study was not physiological and this concentration was only selected in order to compare the results

with those of the physiological concentrations of calcium and magnesium.

In contrast to liver phagocytes, the interaction of liposomes with spleen cells is independent of diavalent cations (table 6.5.). This is a process similar to an earlier study of Stendahl & Tagesson (1977) on peritoneal phagocytes. Fraley *et al.* (1981) suggested that negatively charged liposomes might be recognized by the so-called scavenger receptors, which bind molecules carrying multiple negative charges (Goldestein *et al.*, 1979). Further, scavenger receptor-mediated uptake of, for example, malondialdehyde low-density lipoprotein by monocytes was reported to be Ca^{2+} independent (Schlechter *et al.*, 1981). Such receptors may probably be involved on the interaction of negatively charged liposomes with spleen cells in the absence of serum.

In chapter V (table 5.1.), the presence of a heat-stable small molecular weight factor in serum which prevents the interaction of liposomes with liver phagocytes was discussed. The present investigation may suggest that the identity of this factor is similar to diavalent cations, particularly Ca^{2+}, since the stimulatory effect of dialyzed serum is similar to EGTA treated serum and further the opsonic activity of both serum and dialyzed serum declines by addition of calcium. There are several explanations for the possible mechanism(s) of action of Ca^{2+} on the interaction of liposomes with liver phagocytes in the presence of serum as illustrated in figs 6.5 & 6.6. One possible explanation is that the Ca^{2+} in serum may prevent, at least to a certain extent, the binding or the adsorption of liver specific opsonin(s) onto the surface of liposomes (fig 6.5.). This possibility can be excluded since

pre-opsonization of liposomes with either serum or dialyzed serum (table 6.3.) produces basically the same effect on their interaction with liver phagocytes. Further, if liver specific opsonin(s) is of proteinaceous nature (Chapter V), there is no difference in the pattern of proteins bound to liposomes that have been incubated in the presence of serum and high concentrations of Ca^{2+}, as demonstrated by the electrophoretic studies (fig 6.4.). The second possibility arises if calcium can bind to the liver specific opsonin(s) (see fig 6.5.). This may cause a probable conformational change in the structure of liver specific opsonin(s) and as a result it can no longer interact with the concerned receptor on the cell surface. Since the opsonic molecule is loosely adsorbed onto the surface of liposomes (chapter III) a probable conformational change induced by Ca^{2+} may possibly release the loosely bound opsonin(s) from the surface of liposomes. The inability of serum pre-opsonized liposomes to interact with liver phagocytes in the presence of extracellular Ca^{2+} supports the above hypothesis (see fig 6.3.). However, this observation may also suggest a third possible mechanism of Ca^{2+} action. This third possibility arises if there is a competition between calcium and the opsonic molecule(s) for the same receptor on the cell surface (see fig 6.6.). In order to discriminate between these two possibilities liver cells were pre-incubated with liposomes in the presence of dialyzed serum to allow the formation of bridges between liposomes and liver cells (chapter V) in order to block the concerned receptors. Addition of Ca^{2+} after the pre-incubation period resulted in approximately the release of 50% of the bridges between liposomes and liver cells. Unfortunately this observation did not discriminate between

these possibilities as the release of the bridges may occur either by binding of Ca^{2+} to opsonin even when it is bound to the cell surface receptor or due to higher affinity of the concerned receptor for Ca^{2+} than opsonin and as a result displacement and release of the opsonin(s) occurs. To further discriminate between these two possibilities a series of experiments were conducted by pre-incubating the liver phagocytes with Ca^{2+} in order to block the specific receptor sites on the cell surface. After this preincubation the cells were pelleted, resuspended in calcium free buffer and incubated in the presence of liposomes and dialyzed serum. Unfortunately, no comparison on the uptake of liposomes by these cells and the respective controls was possible as these treatments depressed the uptake of liposomes by both the test and control incubation for an unknown reason (data not shown).

In case of spleen cells, diavalent cations failed to restore the lost opsonic activity of spleen-specific opsonin(s) thus confirming our earlier observation (chapter V) of a possible irreversible conformational change in the structure of this opsonin(s). However, the possibility of involvement of diavalent cations for the activity of spleen-specific opsonin(s) can not be excluded and hence further investigation is required.

In conclusion, the results discussed here explain why other investigators (Dijkstra *et al.*, 1984) have failed to demonstrate the opsonic activity of serum on liposome uptake by kupffer cells in culture, as these investigators have not only used chol-rich liposomes (Chapters III & IV) but also selected buffers that have been supplemented with 1-2 mM Ca^{2+} and Mg^{2+}. Meanwhile, the study of detailed mechanism(s) of the

regulatory role of calcium on liver specific opsonin activity requires purification of liver specific opsonin(s).

Fig 6.5.

Possible roles of calcium on interaction of liver specific opsonin with liposomes

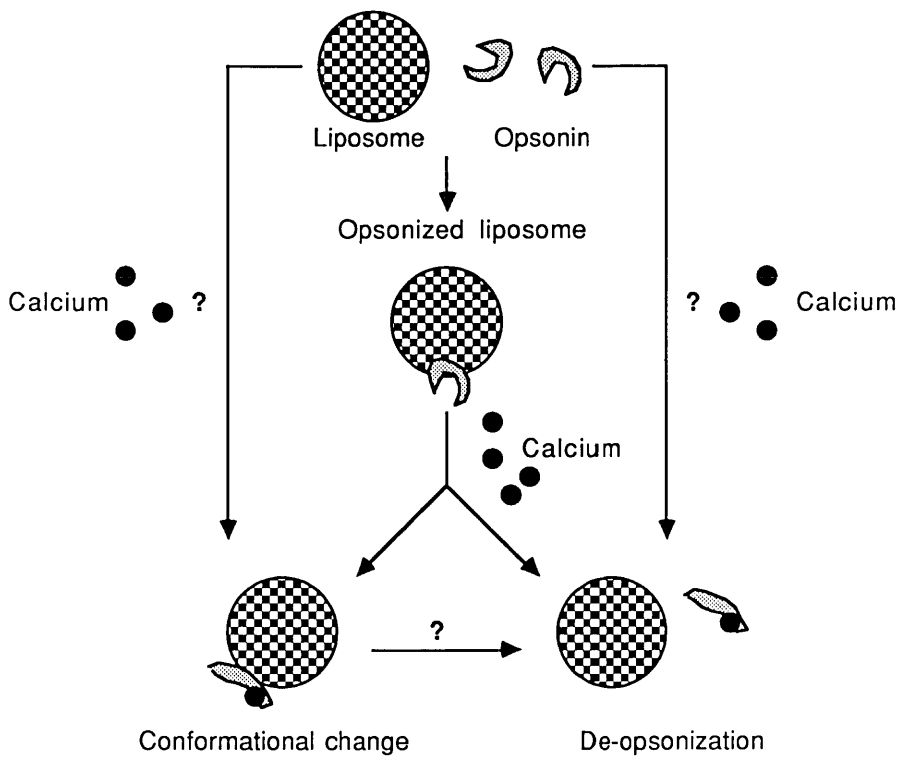
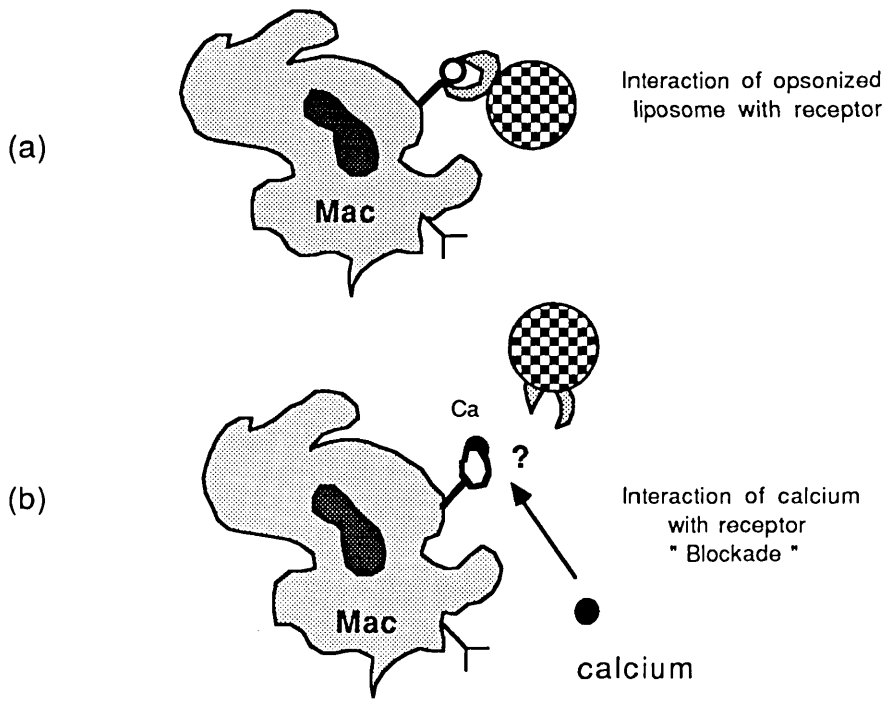


Fig 6.6.

Possible roles of calcium on interaction of opsonized liposomes with liver phagocytes



Chapter VII

Partial purification and characterization of liver specific opsonin(s)

7.1. Introduction

Clearance of intravenously injected particulate materials such as drug carriers occurs mainly in liver by Kupffer cells. This provides a major problem for targeting of drug carriers such as liposomes to other organs containing blood-sinus-lining mononuclear phagocytes. The presence of tissue specific opsonins in serum of various species which stimulate the interaction of liposomes with liver and spleen phagocytes have been previously demonstrated (Chapters III & IV) and their properties were characterized (Chapter V). Identification and purification of these opsonins, particularly the liver specific opsonin(s), may provide strategies for preventing liposome uptake by liver phagocytes.

Since the majority of both specific and non-specific serum opsonins are of proteinaceous character (Absolom, 1986) the present study describes an attempt for the purification of the liver specific opsonin(s) by conventional protein purification methods.

7.2. Results

A - Ammonium sulphate fractionation and gel-filteration of serum

An initial attempt was made to purify the liver specific opsonin from rabbit serum. Fractionation of rabbit serum by stepwise ammonium sulphate precipitation produced a fraction that contained high opsonic activity. The results of these studies are presented in table 7.1. The majority of the opsonic activity of serum is retained in 35-50% ammonium sulphate precipitated proteins, whereas the precipitate from 0-35% did not possess any opsonic activity and, in contrast, inhibited liposome interaction with liver cells (table 7.1.). The liver specific opsonin was further purified by passing the 35-50% ammonium sulphate

Table 7.1.

Opsonic activity of rabbit serum ammonium sulphate precipitated fractions on the interaction of liposomes with rat liver phagocytes

Treatment	% of liposomal radioactivity	Serum protein (mg)	Specific activity	Fold purification
Control	1.5	—	—	—
Serum	9.0	30.0	0.2	1
$(NH_4)_2SO_4$ fractions :				
0-35% Sup	21.4	11.6	1.7	7
0-35% Pellet	0.7	6.2	Nil	—
35-50% Sup	11.3	9.2	1.1	4
35-50% Pellet	22.6	3.6	5.9	23
50-65% Sup	0.4	5.7	Nil	—
50-65% Pellet	4.5	5.3	0.6	2

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

For experimental details see Materials & Methods (Chapter II).

precipitated fraction through a sephadex G-200 column and the elution profile is represented in fig 7.1. When tested for opsonic activity, fractions 17-19 possessed substantial stimulatory effect on liposome-cell interaction (table 7.2.). Calibration of the column with molecular weight markers demonstrated that the liver specific opsonin elutes just after the void volume and its molecular weight is in excess of albumin.

Studies on rat serum basically produced the same results as rabbit serum fractionation studies; the majority of the opsonic activity was accumulated in 35-50% ammonium sulphate precipitated proteins (data not shown). The elution profile of 35-50% ammonium sulphate precipitated fraction from rat serum (fig 7.2.) was slightly differed from rabbit fraction and the opsonin was accumulated in fractions 20-22 (table 7.3.). In contrast to late fractions, early fractions (14-17) from gel-filteration step inhibited liposome-cell interaction (table 7.3.). SDS-PAGE of rat serum fractions 14-26 from gel-filteration step are shown in fig 7.3.a & b. The results shows the presence of a number of protein bands in the active fraction.

Fractionation of calf serum by stepwise ammonium sulphate precipitation produced two fractions that contained opsonic activity (table 7.4.). Both 35-50% and 50-65% ammonium sulphate precipitates possessed stimulatory effect on liposome-cell interaction. Subsequent precipitation experiments revealed that 40-58% ammonium sulphate precipitated proteins contained the majority of opsonic activity (table 7.5.). The gel-filteration elution profile of this fraction was similar to that of 35-50% ammonium sulphate precipitate of rat serum elutions profile (fig 7.4.). The maximum opsonic activity was recovered in

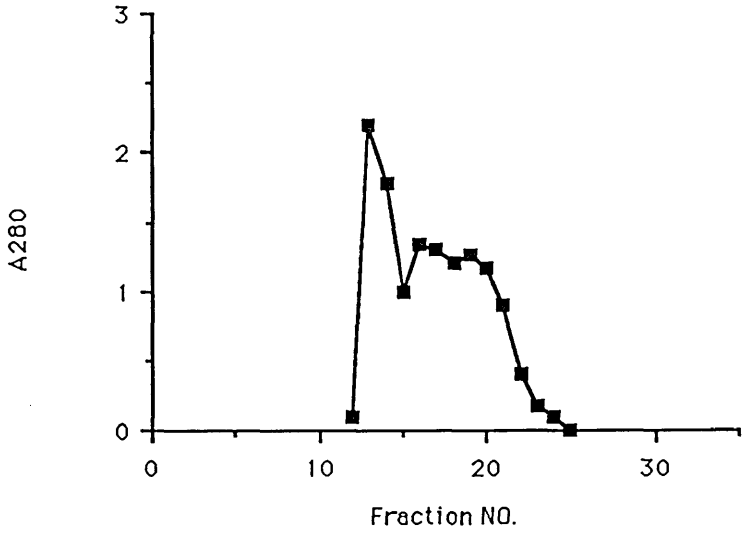


Fig 7.1. Elution profile of rabbit serum 35-50% ammonium sulphate precipitated fraction on sephadex G-200. For experimental details see Materials & Methods (Chapter II).

Table 7.2.

Opsonic activity of 35-50% ammonium sulphate precipitated fraction of rabbit serum separated on sephadex G-200

Tested fraction	% of liposomal radioactivity	Serum protein (mg)	Specific activity	Fold purification
Control	2.0	—	—	—
Serum	8.3	25.0	0.2	1
*35-50% pellet	19.8	7.2	2.5	10
Gel-filteration fractions :				
F 16	2.3	0.7	0.4	2
F 17	8.2	0.6	10.3	41
F 18	11.8	0.5	17.8	71
F 19	12.2	0.5	13.6	54

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

** Represent the $(NH_4)_2SO_4$ precipitated fraction.*

N.B. No activity was detected below fraction No.16.

For experimental details see Materials & Methods (Chapter II).

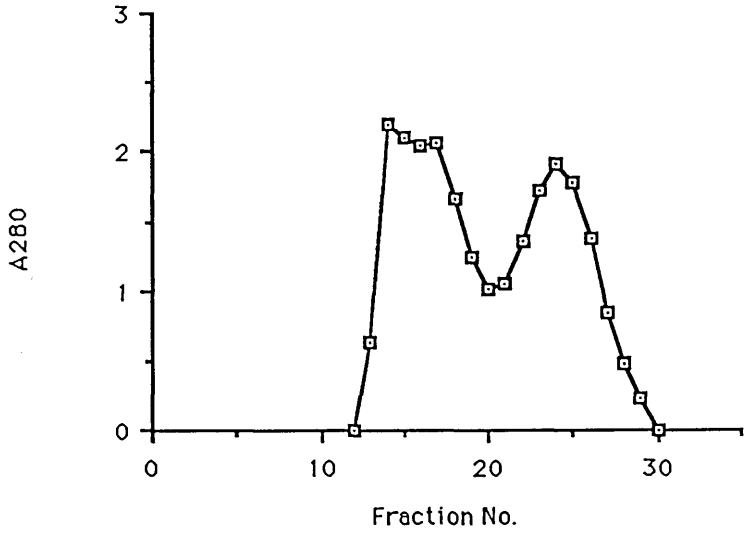


Fig 7.2. Elution profile of rat serum 35-50% ammonium sulphate precipitated fraction on sephadex G-200. For experimental details see Materials & Methods (Chapter II).

Table 7.3.

Opsonic activity of 35-50% ammonium sulphate precipitated fraction of rat serum separated on sephadex G-200

Tested fraction	% of liposomal radioactivity	Serum protein (mg)	Specific activity	Fold purification
Control	1.8	—	—	—
Serum	9.0	36.0	0.2	1
*35-50 % Pellet	31.7	10.0	3.0	15
Gel-filteration fractions :				
F 14-17	0.7	1.3	—	—
F 18&19	6.7	1.0	5.1	25
F 20	15.5	0.5	27.4	137
F 21	25.3	0.5	44.3	221
F 22	29.2	0.7	39.1	200
F 23	14.6	0.9	14.5	72
F 24	20.1	1.1	16.6	83
F 25&26	9.5	1.1	7.0	35

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

** Represent the pellet from (NH4)2SO4 precipitated fractionation.*

For experimental details see Materials & Methods (Chapter II)

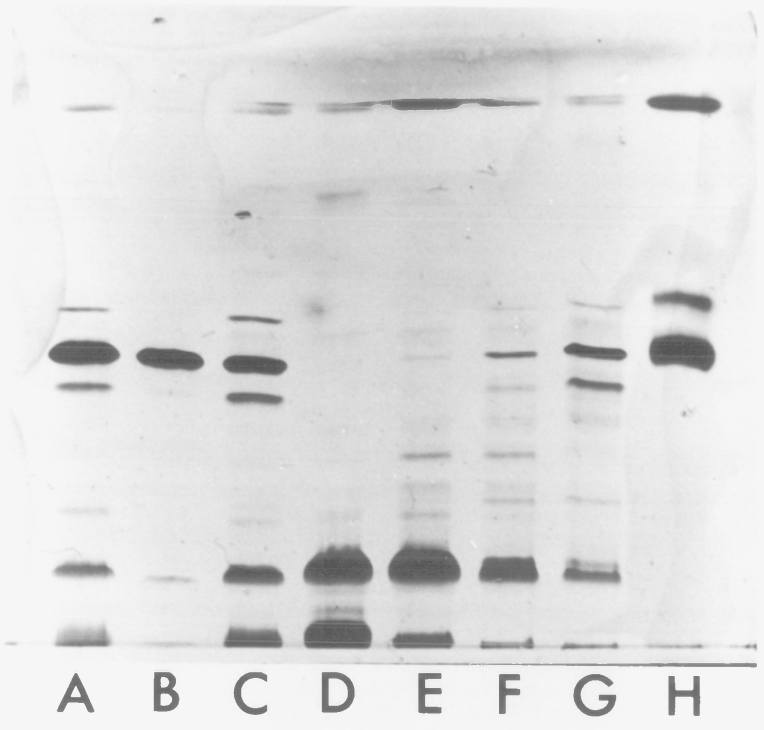


Fig 7.3.a. SDS-PAGE electrophoresis of rat serum fractions by Pharmacia Phast-Electrophoresis system on a 10-15% gradient gel. A, serum; B, supernatent of rat serum 35-50% ammonium sulphate precipitated materials; C ; rat serum 35-50% ammonium sulphate precipitated materials; D,fractions 14-17; E, fractions18 &19; F, fraction 20 of gel-filteration step (fig 7.2. and table 7.3.) and H are standards (from top to bottom: cytochrome C, ovalbumin and bovine albumin), G Fraction 21.

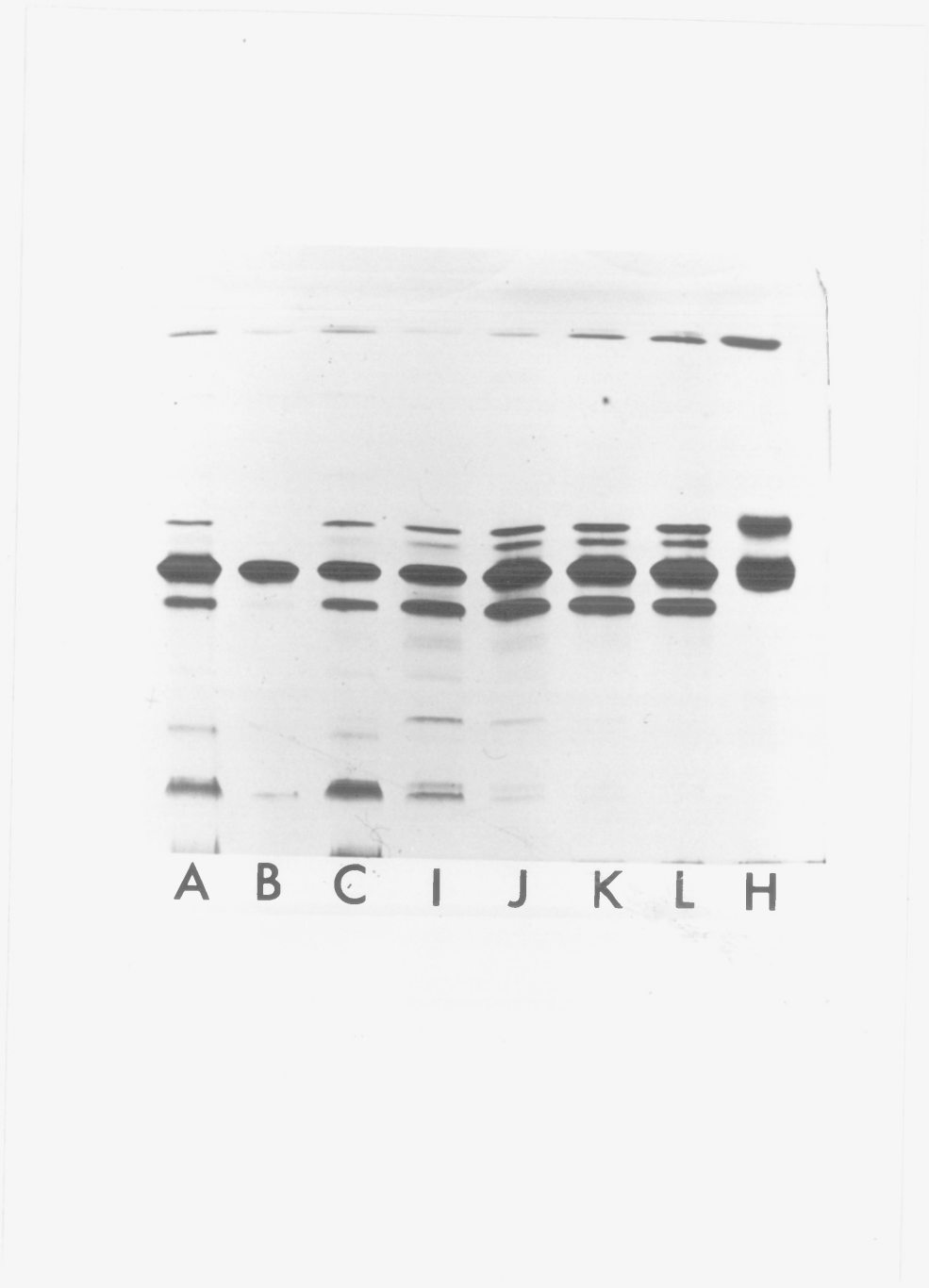


Fig 7.3.b. SDS-PAGE electrophoresis of rat serum 35-50% ammonium sulphate precipitated fractions separated on sephadex G-200. See also legends of fig 7.3.a. I, fraction 22; J, fraction 23; k, fraction 24; and L, fraction 25 from the gel-filteration step (see fig 7.2. and table 7.3.).

Table 7.4.

Opsonic activity of calf serum $(NH_4)_2SO_4$ precipitated fractions on the interaction of liposomes with rat liver phagocytes *in vitro*

Treatment	% of liposomal radioactivity	Serum protein (mg)	Specific activity	Fold purification
Control	0.5	—	—	—
Serum	7.9	25.8	0.3	1
$(NH_4)_2SO_4$ fractions :				
35-50% pellet	22.9	6.2	3.6	13
35-50% sup	24.8	13.2	1.8	7
50-65% pellet	10.3	2.1	4.5	16
50-65% sup	1.2	7.5	0.1	—

N.B. Subsequent analysis has shown that most of the opsonic activity can be concentrated at 40-58% ammonium sulphate pelleted fraction (see table 6.5.).

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

For experimental details see Materials & Methods (Chapter II).

Table7.5.

Opsonic activity of 40-58% ammonium sulphate precipitated fraction of calf serum separated on Sephadex G-200

Tested fraction	% of liposomal radioactivity	Serum protein (mg)	Specific activity	Fold purification
Control	0.4	—	—	—
Serum	9.4	25.40	0.35	1
*40-58% pellet	28.7	3.30	8.6	24.5
*40-58% sup	24.0	11.60	2.0	6
Gel-filteration fractions :				
F 19	3.2	0.32	8.7	25
F 20	5.1	0.38	12.4	35
F 21	7.0	0.43	15.3	44
F 22	7.9	0.43	17.6	50
F 23&24	3.8	0.41	8.3	24

** Represent the fraction precipitated by $(NH_4)_2SO_4$*

N.B. No activity below fraction 19 was detected.

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

For experimental details see Materials & Methods (Chapter II).

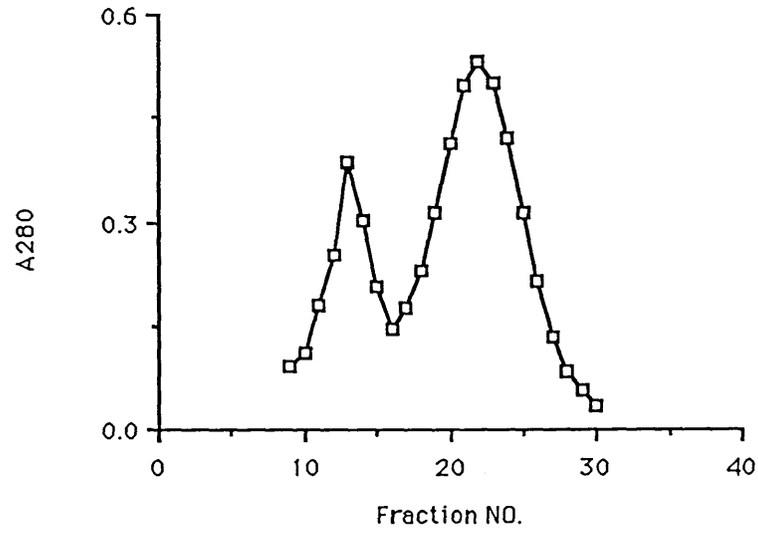


Fig 7.4. Elution profile of calf serum 40-58% ammonium sulphate precipitated fraction on sephadex G-200. For experimental details see Materials & Methods (Chapter II).

fractions 21 & 22, a situation similar to the recovery of the opsonic activity from the gel-filteration of 35-50% ammonium sulphate precipitated proteins of rat serum.

B - Mode of liposome-cell interaction in the presence of the active fractions from the gel-filteration step

Active fractions from rat serum collected from the gel-filteration step were pulled together (fractions 20-22) and tested for opsonic activity on liposome uptake by liver cells that have been preincubated with metabolic inhibitors as described in Chapter II. The results demonstrate a substantial decrease in association of the liposomal aqueous marker with liver cells in the presence of metabolic inhibitors (table 7.6.). Further, the association of liposomes with liver cells in the presence of the purified factor was temperature dependent and inhibited at low temperature of incubation, table 7.7. The results suggest that the interaction of liposomes with liver cells in the presence of purified factor at 37°C is an energy dependent process and appears to be phagocytosis (Poste & Papahadjopoulos, 1976).

C - Partial characterization of liver specific opsonin

The active fractions from the gel-filteration step were examined for their sensitivity to calcium and temperature in exhibiting their opsonin activity on the uptake of liposomes by liver cells.

As in the case of normal serum and dialyzed serum, the opsonic activity of fractions from the gel-filteration steps were Ca^{2+} sensitive (fig 7.5.). The presence of low concentrations of Ca^{2+} (0.5-1.0 mM) during incubation decreased the opsonic activity of the partially purified factor(s) obtained from the gel-filteration step. Higher concentrations

Table 7.6.

Effect of metabolic inhibitors on the uptake of liposomes by liver phagocytes in the presence of partially purified rat serum opsonin

Treatment	% of liposomal [^{125}I]-PVP*
Control	0.3
Gel-filteration active fraction (AF)**	7.6
AF + [IAA (5mM) and NaN_3 (10mM)]	1.7
AF + Cytochalasin B (50μM)	4.4

** Mean of duplicate incubations.*

*** 0.6 mg in each incubation.*

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

Table 7.7.

Effect of temperature on liposome-cell interaction in the presence of partially purified calf opsonic factor

Treatment	% of liposomal [^{125}I]-PVP	
	37°C	4°C
Control	1.5 ± 0.1	0.3 ± 0.1
Gel-filteration active fraction*	15.0 ± 4.9	0.4 ± 0.2

** 1.02 mg in each incubation.*

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

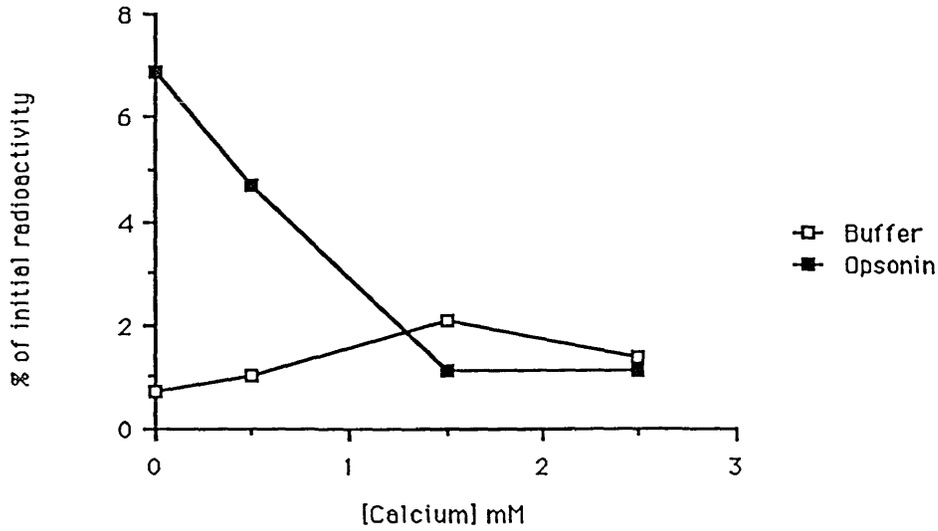


Fig 7.5. Effect of extracellular calcium on the uptake of negatively charged cholesterol-poor egg PC liposomes by liver phagocytes in the presence and absence of partially purified rat serum 35-50% ammonium sulphate fraction separated on sephadex G-200. Incubations contained 0.6 mg of partially purified factor.

Table 7.8.

Heat treatment of partially purified calf serum factor

Treatment	% of initial liposomal [^{125}I]-PVP	
	Exp I	Exp II
Control	0.7 ± 0.1	1.1 ± 0.2
Serum	3.6 ± 0.4	3.8 ± 0.3
Gel-filteration active fraction	5.8 ± 0.9	4.3 ± 0.5
Gel-filteration active fraction heated at :		
55°C/30 min	3.9 ± 0.5	4.7 ± 1.2
55°C/60 min	3.8 ± 1.4	*3.3 / 6.7
60°C/10 min	5.0 ± 1.0	4.0 ± 0.7
65°C/10 min	*4.4 / 4.2	4.7 ± 1.6

** Represents duplicate incubations (individual values are given). All other incubations are in triplicate.*

Negatively charged cholesterol-poor egg PC liposomes were used for the above experiments.

Experiments I & II describe two different batches of commercially obtained calf serum.

For experimental details see Materials & Methods (Chapter II).

than 1.0 mM completely abolished the stimulatory effect of the purified factor. Similar results were also obtained from the purified fractions of calf serum (data not shown).

The effect of temperature on the opsonic activity of the partially purified calf factor is presented in table 7.8. In this study the active fractions from the gel-filteration column, i.e. fractions 20-22, were mixed and heated at various temperatures prior to incubation with liposomes and cells. The results in table 7.8. demonstrate that partially purified factor is heat stable and its opsonic activity is preserved by heating over a wide range of temperatures prior to incubation with liver cells.

7.3. Discussion

Preliminary attempts at the purification of the protein-like factor responsible for the stimulation of liposome interaction with liver phagocytes, by classical salt fractionation techniques using ammonium sulphate, demonstrate that significant opsonic activity could be concentrated in 35-50% saturated ammonium sulphate precipitate of rabbit and rat serum and 40-58% saturated ammonium sulphate precipitate of calf serum, as demonstrated by the fact that the specific activity of these fractions was greater than that of the starting materials. Further, gel-filteration of these fractions resulted in 70, 220, and 50 fold purification of the opsonin from rabbit, rat and calf serum respectively (tables 7.2., 7.3. & 7.5.). These data suggest that in normal rabbit, rat, and calf serum there is a large excess of a proteinaccous molecule which enhances the interaction of chol-poor egg

PC liposomes with liver phagocytes.

The materials in 0-35% saturated ammonium sulphate precipitate from rabbit and rat serum inhibited liposome-cell interaction as compared to control incubations (tables 7.2. & 7.3). This can be attributed to the presence of dysopsonins in serum which is precipitated at this fraction and prevents the liposome-cell interaction. The materials in 0-35% ammonium sulphate precipitated fraction contain mainly macromolecules such as δ-globulins (Cohn *et al.,* 1940), α1 and α2 macroglobulin (Allen *et al.,* 1973) and fibronectin, one of the largest known polypeptides, (Blumenstock *et al.,* 1976; Molnar *et al.,* 1977), hence rejecting the involvement of such serum components as liver specific opsonin(s). This further confirms the earlier suggestion of lack of involvement of fibronectin as liver specific opsonin (chapter V). The materials above 50% saturated ammonium sulphate precipitate of rabbit and rat serum usually contains crystalline albumin and various small molecular weight enzymes (Cohn *et al.,* 1940). Hence, involvement of albumin and small molecular weight serum components as liver specific opsonin(s) may also be rejected. Also, 99% commercially purified albumin had no opsonic activity on liposome uptake by liver phagocytes (data not shown). Therefore, these observations together with the patterns of gel-filteration fractions on SDS-PAGE gels (figs 7.3.a & b) suggest that the opsonic activity of serum for hepatic phagocytosis of liposomes is probably retained in α or β globulin fractions of serum. This is in agreement with the earlier study of Tyrrell *et al.* (1977), that commercially purified bovine α and β globulins stimulated the uptake of chol-poor egg PC liposomes by perfused rat liver. Mucoglobulins are

usually precipitated in the range of 35-50% ammonium sulphate saturation, depending on the nature of serum (Cohn *et al.*, 1940). Hence, involvement of such molecules as liver specific opsonin(s) can not be rejected and this requires full investigation.

The opsonic activity of partially purified opsonin(s) from the gel-filteration step was retained on heating in the range of 55-65°C prior to incubation with cells and liposomes. This may represent that the earlier apparent increase in the opsonic activity of serum by its prior heating (chapter V) may be due to denaturation or inactivation of some dysopsonins in serum rather than a conformational change of the opsonin. However, heat sensitivity of purified serum dysopsonins was not tested here and this requires further investigation in order to justify the above statement.

The results in fig 7.5. demonstrate that the opsonic activity of the partially purified opsonin is calcium dependent. This supports the earlier observations of Chapter VI. The molecular mechanism(s) underlying the above observation is not clear and requires homogenous purification of liver specific opsonin(s).

In conclusion, the present investigation is the first to demonstrate the partial purification of an opsonin from serum which stimulates the interaction of liposomes with liver phagocytes. Purification of this factor to homogeneity may allow a powerful access of either targeting the drug carriers to liver or preventing their clearance by liver phagocytes.

Chapter VIII

Opsonins in cancer

8.1. Introduction

Macrophage defence mechanisms are involved in the host's response to counteract the growth and spread of cancer (Omori, 1964; Hibbs, 1974; Saba & Antikatzides, 1975; Fidler, 1985). Macrophage stimulation either before or during the early stages of tumour growth will inhibit tumour growth and spread; and depression of the macrophage system will accentuate tumour growth. For example, syngeneic mouse macrophages activated *in vitro* and then injected intravenously reduced the formation of B16 melanoma metastases (Fidler, 1974) and the intravenous injection of non-specifically activated macrophages prevented the formation of spontaneous fibrosarcoma metastases (Liotta *et al.*, 1977). In contrast, treatment of mice with macrophage toxins shortened the latent period of skin cancer induction (Norbury & Kripke, 1979).

The above observations probably suggest a regulatory influence of MPS over the course and pattern of tumour development and growth. The findings by Stern (1960) on MPS activity in inbred mice which manifest clear differences in spontaneous incidence of malignant tumours further emphasize the potential role of the MPS in neoplasia. Thus, phagocytosis by hepatic kupffer cells and splenic phagocytes was greatest in animals manifesting the lowest incidence of spontaneous tumours, while animals exhibiting the greatest incidence of spontaneous tumours exhibited lower basal level of mononuclear phagocytic activity. Stern (1960) postulated that macrophage failure may be a critical factor in tumour growth.

However, a variety of pathophysiological conditions associated with a distinct functional alteration of phagocytosis and host resistance can be

related to a significant change in serum opsonin activity. For example, a consistent observation made with respect to the macrophage system and neoplasia is the striking functional change that develops by the host MPS following tumour cell challenge. This response is typically an early activation of the macrophage system, followed by a decline in its capacity at least with respect to phagocytosis (Saba & Antikatzides, 1975) and this sequential phasic alterations of MPS phagocytic capacity was closely correlated with the circulating plasma levels of opsonins (Saba & Antikatzides, 1975).

Previously, the presence of opsonins specific for liver and spleen phagocytes in serum were demonstrated (chapter III & V). Opsonins specific for liver and spleen facilitate the interaction of liposomes with liver and spleen phagocytes respectively (chapter V). Thus, activity of such opsonins during neoplastic diseases can play an important role for targeting liposomes containing immunomodulators either specifically to liver or spleen. Therefore, in the present investigation the activity of serum opsonins specific for liver and spleen phagocytes during the terminating growth phase of an experimentally induced tumour (chondrosarcoma) model is examined.

8.2. Results

A - Comparison of liver and spleen weights from tumour bearing and control rats

The gross and net weight of animals at 5-6 weeks following transplantation (tumour growth occurs fully at this time and its weight remains constant) are given in table 8.1. There is no significant change in the weight of liver between tumour bearing, at 5-6 weeks following

transplantation, and control rats. In contrast, the spleen weights from tumour bearing rats at 5-6 weeks post transplantation were significantly higher than the control rats ($p < 0.02$).

B - Comparison of serum liver specific opsonin activity of tumour bearing and normal rats

The opsonic activity of serum from normal and tumour bearing rats on liposome interaction with liver phagocytes are shown in figs 8.1. & 8.2. The hepatic phagocytic cells obtained from normal rats (fig 8.1.) as well as from rats at 5-6 weeks after transplantation (fig 8.2.) manifest similar phagocytic activity when incubated in normal serum. In contrast, both cell populations exhibit suppression on interaction with liposomes in the presence of serum derived from tumour bearing rats at 5-6 weeks post transplantation. However, prior dialysis or addition of EGTA to serum derived from tumour bearing rats enhanced the interaction of liposomes by several fold as compared to undialyzed or untreated serum from tumour bearing rats. Since in the previous studies presented in chapters VI & VII it was suggested that calcium may play a regulatory role on the liver specific opsonin(s), the concentration of calcium in the serum of normal and tumour bearing rats was measured. Serum calcium concentration was significantly higher ($p < 0.001$) in tumour bearing than normal rats (table 8.2.).

C - Comparison of the opsonic activity of the partially purified liver opsonin of normal and tumour bearing animals

Earlier, it was demonstrated that the liver specific opsonin can be accumulated in 35-50% ammonium sulphate saturated precipitate of serum. The specific activity of this fraction from normal and tumour

Table 8.1.

Comparison of the organs weight from normal and tumour bearing rats

Rats	Gross Weight (g)	Net weight (g)	Liver [a] (g)	Spleen [b] (g)
Normal	250 ± 25	250 ± 25	9.53 ±1.20	0.56 ± 0.01
Tumour	*350 ± 25	260 ± 25	10.05 ±1.02	0.71 ± 0.01

a $p < 0.65$

b $p < 0.02$

Gross weight = body weight + tumour weight

Net weight = body weight - tumour weight

** Tumour weight : 90 ± 24 g*

The results are mean of 5 animals ± SD.

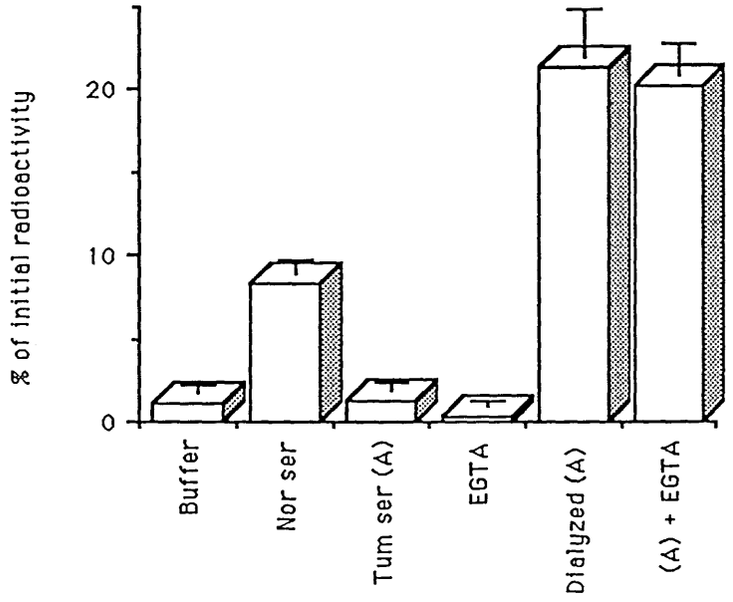


Fig 8.1. Comparison of liver specific opsonin activity of serum from normal and tumour bearing rats on interaction of cholesterol-poor egg PC liposomes with liver phagocytes of normal rat. Nor ser, normal serum; Tum ser, tumour serum. For experimental details see Materials & Methods (chapter II).

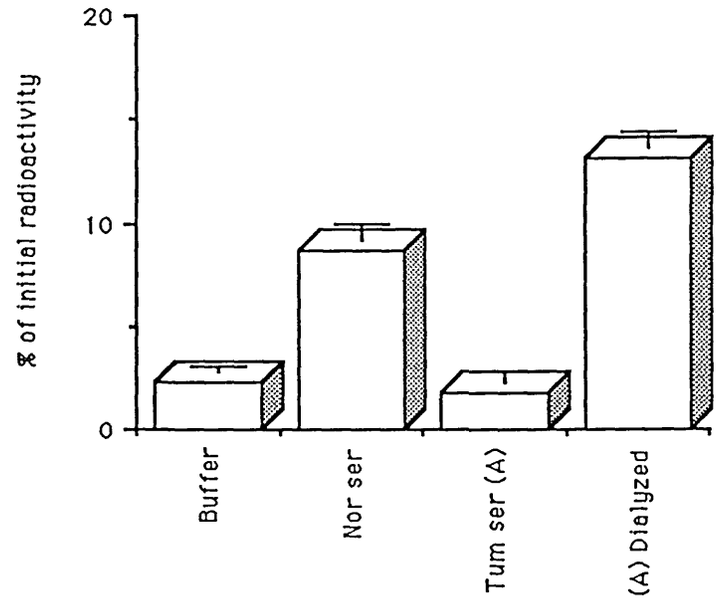


Fig 8.2. Comparison of liver specific opsonin activity of serum from normal and tumour bearing rats on interaction of cholesterol-poor egg PC liposomes with liver phagocytes of tumour bearing rats.

Table 8.2.

Serum calcium concentrations in control and tumour bearing rats

Rats	*Serum calcium concentration (mM)	
	Control	Tumour bearing
1	2.77	2.90
2	2.80	2.96
3	2.77	2.90
4	2.74	3.12
5	2.82	3.08
6	2.82	2.97
7	2.84	3.01
Average	2.80 ± 0.03	2.99 ± 0.08

** Determined by atomic absorption.*
$p < 0.001$

bearing sera on liposome interaction with normal phagocytes is presented in table 8.3. The specific activity of this fraction from normal serum is considerably higher than the corresponding values obtained from the serum of 5-6 weeks post transplanted rats.

In the next step of purification, 15 mg of 35-50% ammonium sulphate precipitated materials from normal and tumour rats were passed through a sephadex G-200 column , as described previously in chapter VI, and the elution profiles are presented in fig 8.3. The elution profile of of the tumour serum shows a broader second peak with higher A280 values as compared to corresponding normal serum. When these fractions were tested for opsonic activity, the majority of opsonin was accumulated in fractions 20 & 21 for normal serum, whereas in tumour serum is distributed between fractions 19-24 (table 8.4.). However, the specific activity of the active fractions from tumour serum was considerably lower than that of the normal serum.

D - Comparison of serum spleen specific opsonic activity of tumour bearing and normal rats

Serum from both normal and 5-6 week transplanted animals displayed stimulatory effect on liposome interaction with splenic phagocytes. However, normal splenic phagocytes as well as those obtained from transplanted rats exhibit enhanced interaction in the presence of serum from tumour bearing rats, as compared to incubations with normal serum (table 8.5.).

Table 8.3.

Comparison of the specific activity of 35-50% ammonium sulphate precipitated serum proteins from control and tumour bearing rats

Treatment	Specific activity
*Tumour sup	0.70 ± 0.02
Tumour pellet	2.51 ± 0.50
**Normal sup	0.69 ± 0.06
Normal pellet	3.87 ± 0.13

** Represents the supernatent from the ammonium sulphate fractionation of tumour serum.*
*** Represents the supernatent from the ammonium sulphate fractionation of normal serum.*
Results are average of triplicate incubations ± SEM.

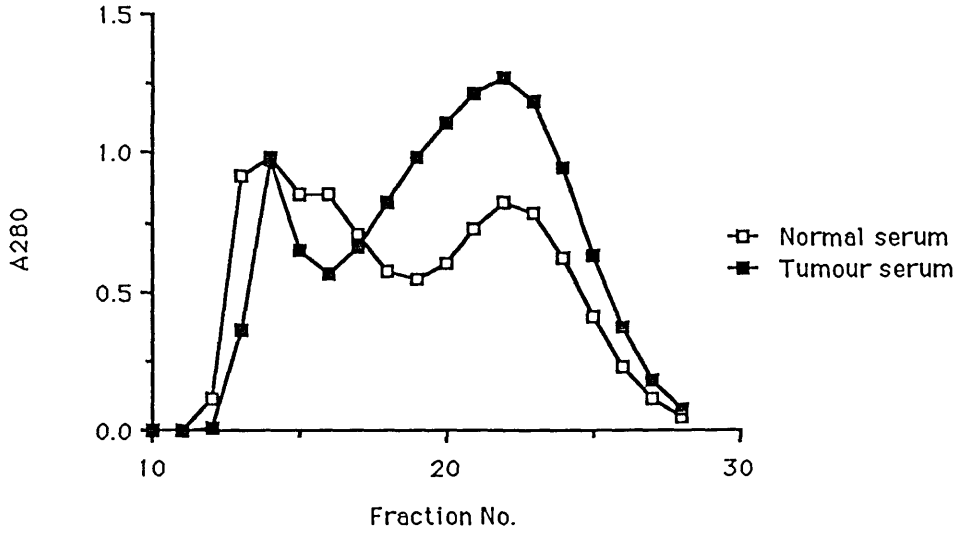


Fig 8.3. Comparison of elution profiles of 35-50% ammonium sulphate precipitated materials from normal and tumour bearing rats on sephadex G-200. For experimental details see Materials & Methods (chapter II).

Table 8.4.

Comparison of the specific activity of purified liver specific opsonin(s) from normal and tumour serum from gel-filteration on sephadex G-200

Treatment	Normal serum			Tumour serum		
	% of initial radioactivity	Serum protein(mg)	Specific activity	% of initial radioactivity	Serum protein (mg)	Specific activity
Fraction A	0.96	0.54	0.12	0.75	0.34	—
B	3.20	0.33	7.12	1.56	0.39	1.82
C	7.99	0.31	23.03	4.73	0.54	7.18
D	7.22	0.34	18.73	3.73	0.63	4.57
E	6.54	0.49	11.61	4.00	0.60	5.25
F	4.75	0.46	8.48	2.07	0.31	3.94

Results are mean of duplicate incubations.
A= Fraction No. 17& 18 in normal and16 in tumour,
B= Fraction No. 19 in normal, 17&18 in tumour,
C= Fraction No. 20 in normal,19&20 in tumour,
D= Fraction No. 21 in normal, 21&22 in tumour,
E= Fraction No. 22&23 in normal and 23&24 in tumour,
F= Fraction No. 24&25 in normal and 25&26 in tumour serum G-200 profile.

Table 8.5.

Comparison of spleen specific opsonin activity of serum from normal and tumour bearing rats on interaction of negatively charged cholesterol-poor and cholesterol-rich PC liposomes with spleen phagocytes of normal and tumour bearing rats

	% of initial liposomal [^{125}I]-PVP			
	Chol-poor		Chol-rich	
Source of cells:	Normal rat	Tumour rat	Normal rat	Tumour rat
Treatment:				
Buffer	3.8 ± 0.3	6.0 ± 0.5	0.8 ± 0.1	1.6 ± 0.1
Normal serum	4.8 ± 0.2	7.8 ± 0.2	12.2 ± 0.1	15.8 ± 0.4
Tumour serum	8.8 ± 0.8	14.7 ± 0.7	17.4 ± 0.1	22.4 ± 1.0

7.3. Discussion

The present investigation has demonstrated that the diminished phagocytosis of kupffer cells during terminating phase of tumour growth could be closely correlated with the activity of serum opsonin(s). This suggestion is based on the following observations. Firstly, liver cells derived from tumour bearing rats manifest similar phagocytic activity as to normal cells in the presence of normal serum. This indicates no impairment of cellular phagocytic capacity of liver phagocytes from tumour bearing rats. Secondly, liver phagocytes derived from both control and tumour bearing rats manifest suppressed phagocytic activity in the presence of serum from tumour bearing rats. Thirdly, comparison of the activity of partially purified liver-specific opsonin(s) from the serum of normal and tumour bearing animals by classical ammonium sulphate fractionation and gel-filteration techniques demonstrates a considerable lower specific activity of opsonin(s) from tumour serum in comparison to normal serum. Similar observations were made earlier by Saba (1974) in which both hepatic phagocytes from normal and Walker 256 tumour bearing rats manifested hypophagocytosis when incubated in plasma from tumour bearing rats at 30 days post transplantation. However, Saba & Antikatzides (1975) demonstrated that the diminished opsonic activity of serum was due to decreased concentration of an opsonic protein which presently is known as fibronectin (Yamada & Olden, 1978) but the results discussed earlier in chapters V & VII eliminated the possibility of fibronectin as liver specific opsonin(s) in our study and since the identity of our opsonin(s) is not known, we can

not yet consider a decrease in its circulatory concentration during terminating phase of tumour growth.

However, previous analysis of serum ionic composition from experimental (Hilgard *et al.*, 1970/1973) and clinically encountered (Buckle *et al.*, 1970; Ferenczy *et al.*, 1971; Haskell *et al.*, 1971; Elte *et al.*, 1987; Morton *et al.*, 1988) neoplasms, with or without bone metastases, have reported a frequent pronounced hypercalciemia during tumour growth. Upon medically induced regression (Morton *et al.*, 1988) or surgical resection of these tumours (Buckle *et al.*, 1970) the calcium levels have been observed to return to normal levels, while tumour recurrence is accompanied by a reappearance of hypercalciemia. Interestingly, the opsonic activity of our liver specific opsonin(s) is regulated by calcium (chapter VI & VII). Hence, a significant increase in serum calcium concentration, as in the case of the present study, may affect the activity of the liver specific opsonin(s), see table 8.2. and figs 8.1. & 8.2. Ryder *et al.* (1975) also demonstrated that the elevation of serum calcium levels above normal inhibited phagocytosis of gelatin coated lipid emulsions by liver slices. Hence, it is tempting to speculate that the tumour could be facilitating its own growth process and escape from the immune surveillance via a tumour induced hypercalciemia leading to depression of kupffer cell clearance. The partial restoration of the opsonic activity of tumour derived serum by dialysis, the significant increase ($p < 0.001$) in serum calcium concentration of tumour bearing rats, and the inhibitory action of increased serum calcium concentration on opsonic activity of serum (chapter VI & VII), all support the above hypothesis.

In contrast to liver specific opsonin, the activity of spleen specific opsonin(s) appears to be increased during the terminating phase of tumour growth since its stimulatory effect on interaction of liposomes with spleen cells of either normal or tumour bearing rats is greater than normal serum. The cause for this observation is not clear but this may probably explain why the spleen size of tumour bearing animals is usually increased .

Recently, increased lung uptake of technetium tin colloid (TTC) during liver imaging has been noticed in numerous malignant and benign diseases (Lantto *et al.,* 1986). In contrast, bone-marrow uptake of TTC was noted in patients with gastrointestinal malignancies, while patients with lymphoreticular malignancies seemed to cause abnormally increased spleen uptake of TTC (Lantto *et al.,* 1986). The cause for these discrepancies has been hypothesized to be related to regional stimulation of the MPS by the tumour (Lantto *et al.,* 1986). The variation of opsonic activity of tumour serum on liver and spleen phagocytes in the present investigation may suggest an alteration in circulatory level of a particular tissue specific opsonin, during different malignancies, may account for the above observations.

Chapter IX

Opsonin(s) specific for bone-marrow phagocytes

9.1. Introduction

In previous chapters it was shown that serum contains opsonins specific for liver and spleen phagocytes. Since bone-marrow is one of the major organs of MPS and plays a small but significant role in the clearance of foreign particulates such as drug carriers, e.g. liposomes, it is important to investigate whether or not serum contains opsonin(s) specific for bone-marrow mononuclear phagocytes. Hence, in this chapter the possible presence of specific opsonin(s) in serum which may enhance the uptake of liposomes by bone-marrow phagocytes is investigated.

9.2. Results

A - Characteristics of liposome-cell interaction

If the association of liposomes with bone-marrow cells were due to phagocytosis, the process would be inhibited at low temperature of incubation (4°C) and in the presence of metabolic inhibitors at 37°C (Poste & Papahadjopoulos, 1976). At low temperature of incubation, the total amount of liposomes adsorbed on the cells did not constitute more than 15% of total liposomes associated with cells at 37°C in the absence of serum (table 9.1.). Further, the association of vesicles in the absence of serum and in the presence of metabolic inhibitors is reduced by 25% as compared to the control incubation at 37°C (table 9.1.). In contrast, in the presence of serum and metabolic inhibitors the inhibition is more pronounced and, indeed, 75% reduction in liposome uptake, in comparison to control incubation, has occured (table 9.1.). The adsorption of liposomes to cell surface (at 4°C) is also hindered in the presence of serum. Hence, phagocytosis appears to be the major mechanism of

Table 9.1.

Effects of temperature and metabolic inhibitors on the uptake of negatively charged cholesterol-rich egg PC liposomes by bone-marrow phagocytes *in vitro*

Treatment	% of initial liposomal [^{125}I]-PVP		
	4°C	37°C	37°C + Metabolic inhibitors
Control	0.2 ± 0.1	1.6 ± 0.2	1.2 ± 0.2
Serum	0.1 ± 0.1	3.3 ± 0.1	0.9 ± 0.1

Metabolic inhibitors : Iodoacetic acid (5.0 mM) + Sodium azide (10.0 mM).

liposome uptake by bone-marrow cells in the presence of serum.

B- Effect of lipid composition of liposomes on their interaction with bone-marrow phagocytes

The data presented in table 9.2. demonstrate the interaction of various liposomes with bone-marrow phagocytes in the absence and presence of rat serum. Bone-marrow phagocytes interact favourably with fluid chol-poor egg PC liposomes rather than less fluid chol-rich egg PC or solid chol-rich SM and DMPC liposomes at 37°C in the absence of serum. In contrast, serum stimulates the interaction of chol-rich rather than chol-poor vesicles with bone-marrow cells despite their phospholipid composition. However, this effect is more pronounced with egg PC vesicles than DMPC and SM liposomes (table 9.2.).

C - Effects of heated and dialyzed serum on bone-marrow phagocytes

The results in table 9.3. show the effect of heated and dialyzed serum on the uptake of liposomes by bone-marrow phagocytes. The stimulatory effect of serum on liposome uptake by bone-marrow phagocytes is abolished on heating of serum at 55°C/30 min prior to incubation (table 9.3.). Similarly, the opsonic activity of serum is lost on dialysis, in comparison to the fresh undialyzed serum (table 9.3.). Experiments on serum dialyzate demonstrated that freeze dried serum dialyzate has no stimulatory effect on liposome interaction with bone-marrow cells either on its own or on its addition to dialyzed serum (table 9.3.).

D - Effect of diavalent cations

Diavalent cations are involved in the process of phagocytosis (Stossel, 1973; Chapter VI) and they are usually removed on dialysis of serum.

Table 9.2.

Interaction of negatively charged cholesterol-containing liposomes with bone-marrow phagocytes in the absence and presence of serum

Liposome composition (molar ratio)	% of initial liposomal [^{125}I]-PVP	
	Control	Serum
PC : CHOL : DCP (7 : 2 : 1)	0.8 ± 0.1	1.0 ± 0.1
PC : CHOL : DCP (7 : 7 : 1)	0.4 ± 0.1	2.1 ± 0.1
SPHY : CHOL : DCP (7 : 7 : 1)	0.2 ± 0.1	0.6 ± 0.1
DMPC : CHOL : DCP (7 : 7 : 1)	0.3 ± 0.1	0.6 ± 0.1

Table 9.3.

Effects of heating and dialysis of serum on its opsonic activity specific for bone-marrow phagocytes

Treatment	% of initial liposomal [^{125}I]-PVP
Control	0.8 ± 0.2
Serum	2.1 ± 0.1
Heated serum (55°C/30 min)	0.5 ± 0.2
Dialyzed serum	0.8 ± 0.1
Serum dialyzate	0.5 ± 0.1
Serum dialyzate + Dialyzed serum	0.5 ± 0.1

Negatively charged cholesterol-rich egg PC liposomes containing [^{125}I]-PVP were used for the above experiments.

Table 9.4.

Effect of diavalent cations on the uptake of negatively charged cholesterol-rich egg PC liposomes by bone-marrow phagocytes in the absence and presence of dialyzed serum

Treatment	% of initial liposomal radioactivity	
	[^{3}H]-Chol	[^{14}C]-Inulin
Exp I		
Buffer	3.8 ± 0.4	0.9 ± 0.1
Buffer + Ca^{2+}	4.7 ± 0.2	1.0 ± 0.2
Buffer + Mg^{2+}	5.1 ± 0.1	0.9 ± 0.1
Buffer + Ca^{2+} + Mg^{2+}	5.2 ± 0.1	1.0 ± 0.1
Exp II		
Buffer	4.9 ± 0.1	0.4 ± 0.1
Buffer + EGTA	5.3 ± 0.2	0.5 ± 0.1
Serum	10.8 ± 1.4	11.3 ± 1.5
Dialyzed serum	2.1 ± 0.2	1.0 ± 0.1
+ Ca^{2+}	2.2 ± 0.1	0.9 ± 0.1
+ Mg^{2+}	2.6 ± 0.1	1.4 ± 0.2
+ Ca^{2+} + Mg^{2+}	2.7 ± 0.1	1.3 ± 0.1

Liposomes were labelled with [^{3}H]-cholesterol as membrane and [^{14}C]-inulin as an aqueous marker.
Final concentration of each diavalent cation was 1.0 mM in each incubation.
Final concentration of EGTA was 1.25 mM during the incubation.

Since the opsonic activity of serum specific for bone-marrow phagocytes was lost on dialysis of serum (table 9.3.), the role of diavalent cations on the opsonic activity of bone-marrow specific opsonin(s) was investigated, and the results are presented in table 9.4. Addition of diavalent cations to dialyzed serum had no effect on restoring its lost opsonic activity on liposome uptake by bone-marrow phagocytes (table 9.4.).

Also, interaction of liposomes with bone-marrow phagocytes is not affected in the presence of either extracellular or tightly bound cell-membrane diavalent cations (table 9.4.).

9.3. Discussion

The role of serum opsonins and dysopsonins on interaction of liposomes with liver and spleen phagocytes was described earlier in this thesis. The results of the present investigation demonstrate that, as well as liver-specific and spleen-specific opsonins, serum also contains opsonin(s) specific for bone-marrow phagocytes.

Bone-marrow specific opsonin(s) only stimulates the uptake of chol-rich vesicles (despite their phospholipid composition) by bone-marrow cells and hence, the results confirm the earlier reports of *in vivo* studies (Kimelberg, 1976; Senior *et al.*, 1985) that liposomes with high cholesterol content are preferentially taken up by bone-marrow cells. The greater stimulatory effect of serum on the uptake of chol-rich PC vesicles than either chol-rich SM or DMPC liposomes in here suggests that factors such as hydrophobicity and physical state of liposomal lipids may also play an important role on the extent of serum

opsonization of liposomes (see also chapter IV). The enhanced uptake of chol-rich liposomes in the presence of serum may suggest that the opsonin(s) probably recognizes the cholesterol moiety of the liposomes or some interior parts of the phospholipid molecules that have probably been exposed because of distortion in liposomal bilayer in the presence of high cholesterol concentration. Vesicles composed of SM or DMPC may also attract some serum dysopsonins and these probably hinder the binding of bone-marrow specific opsonin(s) to these vesicles.

Bone-marrow specific opsonin(s) is heat labile since its opsonic activity is abolished on heating of serum at 55°C and this may support its proteinaceous character.

The opsonic activity of bone-marrow specific opsonin(s) is also abolished on dialysis of serum. Further, the serum dialyzate did not possess any opsonin activity either on its own or on its addition to dialyzed serum. These observations may suggest that the bone-marrow specific opsonin(s) is either a dialyzable small molecular weight serum component(s) which is inactivated/denatured by the process of dialysis and freeze drying or it is an undialyzable serum macromolecule which requires a dialyzable co-factor for its activity and, hence, the removal of this co-factor may bring irreversible conformational changes (which are not restored even by addition of diavalent cations, see table 9.4.) on the opsonin molecule.

In conclusion, the results in this chapter demonstrate that as well as liver and spleen specific opsonins serum also contains opsonin(s) specific for bone-marrow phagocytes. The characteristics and properties of the bone-marrow specific opsonin(s) resemble those of spleen-

specific opsonin(s) which mediates the interaction of cholesterol-rich liposomes with spleen phagocytes (see chapters IV & V). Hence, the involvement of the same opsonin(s) molecule for spleen and bone-marrow phagocytes can not be excluded and this awaits further investigation.

C h a p t e r X

Opsonic effect of serum on peritoneal phagocytes

10.1. Introduction

Since peritoneal macrophages have the advantage of being readily available and susceptible to study in a tissue culture or suspension format, a number of investigators have studied the uptake of liposomes by these cells in the presence of serum. However, the results reported by various investigators are inconsistent (Torchilin *et al.,* 1980; Juliano, 1982; Stirk & Baldeschwieler, 1986). For example, Juliano (1982) observed the inhibition of uptake of liposome by mouse peritoneal macrophages in the presence of serum, whereas Stirk & Baldeschwieler (1986) reported that the uptake of vesicles by mouse peritoneal macrophages is promoted by inclusion of serum during incubation. Such inconsistency may probably have emerged from the difference in peritoneal macrophages that have been obtained either from different or similar species, lipid composition of liposomes, composition of media, and the source of serum used in the experiments.

However, the peritoneal macrophages have many properties which are different from the resident macrophages of liver, spleen, and bone-marrow,which are predominantly involved in the clearance of liposomes from blood *in vivo*. For example, the expression of some antigens and plasma membrane receptors of peritoneal macrophages differ from those of liver, spleen, and bone-marrow phagocytes. Resident macrophages isolated by collagenase digestion from bone-marrow, liver, and spleen possess a receptor which binds unopsonized sheep erythrocytes, whereas both circulating monocytes and peritoneal macrophages do not display this receptor (Gordon *et al.,* 1986). Another example is type 3 complement receptor (CR3) mac-1-antigen. CR3 is thought to participate

with lectin-like receptors such as the mannosyl, fucosyl receptors in macrophage adhesion to various cellular targets (Ezekowitz *et al.*, 1984). Both freshly isolated liver and bone-marrow macrophages express very low levels of or lack this antigen, unlike monocytes and peritoneal phagocytes (Gordon *et al.*, 1986). However, after isolation, these resident macrophages express mac-1-antigen on further cultivation. It is not known whether mac-1-antigen is initially absent, blocked by ligand or down regulated. Thus, the study of liposome uptake by peritoneal macrophages in the presence of serum may not correctly reflect the role of clearance of liposomes by the resident blood-sinus-lining macrophages of the MPS.

Hence, in this chapter, the effect of serum on the uptake of liposomes by peritoneal phagocytes has been studied, and the results are compared with the earlier studies of liposome uptake by macrophages of liver, spleen and bone-marrow (see chapters V & IX).

10.2. Results

A - Evidence of uptake of liposomes by peritoneal phagocytes

The results in table 10.1 demonstrate the effect of incubation temperature and metabolic inhibitors on liposome uptake by peritoneal phagocytes. In the absence of serum, either at 4°C or at 37°C, but in the presence of metabolic inhibitors the amount of cell associated liposomal aqueous marker is reduced by about 70 % as compared with control incubations at 37°C. These observations indicate that the association process at 37°C is energy dependent, suggesting phagocytosis as a major mechanism of interaction (Poste & Papahadjopoulos, 1976; Silverstein

et al., 1977), thus, supporting the earlier observations of Raz *et al.* (1981) and Hsu & Juliano (1982). In the absence of serum, the amount of radioactive label that become cell associated in the presence of metabolic inhibitors parallels that at 4°C and this probably represents those vesicles that bind to the cell surface without being internalized.

In contrast, serum stimulates liposome-cell interaction by several folds as compared to control incubation at 37°C. This enhancement effect of serum is greatly reduced either in the presence of metabolic inhibitors at 37°C or by lowering the temperature of incubation to 4°C. These observations confirm phagocytosis as the major mechanism of liposome uptake by peritoneal phagocytes in the presence of serum.

B - Role of serum opsonins and cholesterol moiety of liposomes

The results in table 10.2. demonstrate that peritoneal phagocytes take up chol-poor more than chol-rich liposomes in the absence of serum at 37°C. In contrast, serum enhances the uptake of both chol-poor and chol-rich liposomes, almost to the same extent, by peritoneal phagocytes at 37°C.

C - Effect of heated and dialyzed serum on the uptake of liposomes

The effect of heating and dialysis of serum on its opsonic activity is demonstrated in table 10.3. Both heating (55°C/30 min) and dialysis abolished the opsonic activity of serum as compared to fresh serum (table 10.3.).

Table 10.1

Effect of temperature and metabolic inhibitors on the uptake of negatively charged cholesterol-poor egg PC liposomes by peritoneal phagocytes

Treatment	% Uptake of initial liposomal [^{125}I]-PVP		
	4°C	37°C	37°C + Metabolic inhibitors
Buffer	0.25 ± 0.05	0.8 ± 0.1	0.2 ± 0.1
Serum	0.1 ± 0.1	10.6 ± 0.3	0.6 ± 0.1

Metabolic inhibitors : Iodoacetic acid 5.0 mM + Sodium azide 10 mM.

For experimental detail see Materials and Methods (chapter II).

Table 10.2.

Effect of cholesterol content of negatively charged liposome on the uptake of vesicles by peritoneal cells in the absence and presence of serum

Liposomes	% of initial liposomal radioactivity	
PC:CHOL:DCP	Control	Serum
7 : 2 : 1	1.6 ± 0.1*	8.2 ± 0.1**
7 : 7 : 1	0.6 ± 0.2*	10.8 ± 0.5**

* $p < 0.04$

** $p < 0.04$

Table 10.3.

The effect of various treatments of serum on the phagocytosis of negatively charged cholesterol-poor egg PC liposomes by peritoneal macrophages

Serum	% Uptake of initial liposomal [^{125}I]-PVP
None	1.0 ± 0.1
Serum	6.9 ± 0.1
Heated serum (55°C/30 min)	0.9 ± 0.2
Dialyzed serum	1.4 ± 0.1
None + EGTA (1.25 mM*)	0.7 ± 0.1
Serum + EGTA	3.0 ± 0.1

** Final concentration in incubation.*

D - Effect of diavalent cations on the uptake of negatively charged liposomes by peritoneal cells

Since dialysis can remove diavalent ions, the role of diavalent cations on liposome uptake by peritoneal phagocytes was firstly examined by the addition of EGTA to the serum (table 10.3.). The results in table 10.3. demonstrate that EGTA can partially reduce the stimulatory effect of serum on liposome-peritoneal cell interaction. Secondly, the diminished opsonic activity of dialyzed serum was partially restored by the addition of either Ca^{2+} or Mg^{2+}, while the simultaneous presence of both cations resulted in full recovery of the opsonic activity of dialyzed serum, table 10.4.

However, in the absence of serum but in the presence of EGTA a 30% inhibition on liposome uptake was observed (table 10.4.). This may indicate the involvement of the possible presence of diavalent cations, particularly calcium, on the cell surface for the phagocytosis of liposomes by peritoneal cells. Alternatively, the removal of some adsorbed vesicles from the cell surface by EGTA may attribute to the observed inhibition.

The effects of Ca^{2+} and Mg^{2+} on the uptake of negatively charged liposomes by peritoneal phagocytes in the absence and presence of dialyzed serum are shown in table 10.4. In the absence of dialyzed serum, Ca^{2+} stimulated the liposome-cell association by 30% as compared to the control incubation. In contrast, replacement of Ca^{2+} by Mg^{2+} resulted in 70% inhibition on liposome uptake by peritoneal phagocytes (table 10.4.). Further, Mg^{2+} competitively inhibited the stimulatory effect of Ca^{2+} when 1.0 mM of each diavalent cation was

Table 10.4.

Effect of diavalent cations on the uptake of negatively charged cholesterol-poor egg PC liposomes by peritoneal cells in the presence and absence of dialyzed serum

Serum	% of initial liposomal radioactivity
None	0.9 ± 0.1
Serum	4.4 ± 0.8
Dialyzed	1.9 ± 0.2
None + Ca^{2+}(1.0 mM)	1.2 ± 0.1
None + Mg^{2+}(1.0 mM)	0.3 ± 0.1
None + Ca^{2+} & Mg^{2+} (1.0 mM of each cation)	0.6 ± 0.2
Dialyzed + Ca^{2+}(1.0 mM)	3.6 ± 0.2
Dialyzed + Mg^{2+}(1.0 mM)	3.9 ± 0.2
Dialyzed + Ca^{2+} & Mg^{2+}(1.0 mM of each ion)	4.6 ± 0.2

added to the incubations.

E - Comparison of the opsonic effect of serum on resident and elicited peritoneal phagocytes

Table 10.5. shows the opsonic effect of normal rat serum in comparison with serum derived from thioglycolate treated rats on liposome uptake by both resident and thioglycolate stimulated peritoneal phagocytes. Thioglycolate elicited peritoneal cells take up liposomes to a similar extent as compared to resident cells in the absence of serum. In contrast, the opsonic activity of both normal serum and serum derived from thioglycolate treated rats is greater on thioglycolate elicited peritoneal phagocytes than normal resident cells.

Table 10.5.

Opsonic effect of serum on phagocytosis of negatively charged cholesterol-poor egg PC liposomes by resident and thioglycolate elicited peritoneal phagocytes

Treatment	% of initial radioactivity	
	Resident cells	Thioglycolate elicited cells
Buffer	1.5 ± 0.1	1.6 ± 0.2
Control serum	7.5 ± 0.2*	10.3 ± 0.2*
Thioglycolate serum	7.7 ± 0.1**	9.1 ± 0.3**

** $p < 0.011$*

*** $p < 0.048$*

Control rats were injected I.P with 4.5 ml of sterile saline.

Thioglycolate rats were injected I.P with 4.5 ml of thioglycolate medium.

Cell harvesting was performed 3 days after the injection of medium.

9.3. Discussion

Earlier the role of serum opsonins specific for liver, spleen (chapter III,IV, V) and bone-marrow phagocytes (chapter IX) were described. The present investigation demonstrates that serum contains opsonin(s) specific for peritoneal phagocytes with distinct characteristics and properties to those of liver, spleen, and bone-marrow specific opsonins. The peritoneal-specific opsonin(s) promotes the uptake of both cholesterol-poor and cholesterol-rich vesicles by peritoneal phagocytes. This observation is similar to the behaviour of spleen-specific opsonin(s) (chapter III & IV), but not to that of liver-specific opsonin(s) which promotes the interaction of only cholesterol-poor liposomes (chapter III) and bone-marrow specific opsonin(s) which promotes the interaction of only cholesterol-rich vesicles with the liver and bone-marrow phagocytes respectively.

The peritoneal specific opsonin(s), like spleen-specific (chapter V) and bone-marrow specific opsonins (chapter IX), is heat labile since its opsonic activity is lost on heating of serum at 55°C. This observation supports the hypothesis that the peritoneal-specific opsonin(s) has a proteinaceous character and may, perhaps, be a component of complement pathway (Smith & Wood, 1969)

Further, like spleen-specific (chapter V) and bone-marrow specific opsonins (chapter IX), the opsonic activity of peritoneal-specific opsonin(s) is also lost on dialysis of serum. But unlike the opsonins specific for spleen and bone-marrow phagocytes, its opsonic activity is partially recovered by addition of either calcium or magnesium to the dialyzed serum while simultaneous addition of both cations is necessary

for restoring its maximum activity (table 10.4.). These observations suggest that the peritoneal-specific opsonin(s) is an undialyzable serum macromolecule and its opsonin activity is dependent on diavalent cations. Activation of the complement system via either the classical or alternative pathways require the presence of diavalent cations; Ca^{2+} being required for the classical pathway and Mg^{2+} for the alternative pathway (Fine *et al.,* 1972). The physical properties of serum observed in the present investigation, (table 10.3. & 10.4.) may suggest the involvement of both classical and alternative complement components as opsonins specific for peritoneal macrophages; since the opsonic activity of serum is abolished by heating and further addition of either Ca^{2+} or Mg^{2+} partially restored the opsonic effect of the dialyzed serum, whereas the simultaneous addition of both cations restored the full opsonic activity of the dialyzed serum. The mechanism by which calcium and magnesium facilitate enhanced ingestion in the present work is not known. It has been suggested that diavalent cations may play a role in facilitating the attachment of particle-associated opsonins to the corresponding receptor sites on the phagocytic cell membrane (Lay & Nussenweing, 1969; Banks & Mc Guire, 1970). For example, C3b-coated particles require the presence of magnesium to facilitate attachment and thus subsequent ingestion (Roos *et al.,* 1977), whereas the necessity of cations to facilitate the attachment of IgG-opsonized particles has not been demonstrated (Roos *et al.,* 1977). However, it is also possible to speculate that the heat labile opsonin, in here, facilitates the action of diavalent cations at site(s) which activate the engulfment.

Various agents, including thioglycolate can elicit altered macrophage

populations, which differ functionally from each other and from resident cells. For example, Bianco *et al.* (1975) have reported that thioglycolate cells, but not normal, can ingest particles bound solely to the macrophage receptor for a fragment of third component of complement. The results (table 10.5.) in here demonstrate that there is no significant difference on liposome uptake by resident and elicitated peritoneal phagocytes. However, sera from either animals stimulate the uptake of liposomes by both resident and elicited cells, but their opsonic activity is significantly higher for thioglycolate elicited cells, thus, suggesting an increase in the number of functional receptor sites on already resident cells for the opsonin or an increase in the number of cells possessing the functional receptor sites for the opsonin.

In conclusion, the present investigation suggests that serum as well as liver, spleen, and bone-marrow specific opsonins contain opsonin(s) specific for peritoneal phagocytes and the characteristics of this opsonin(s) differ from those specific for blood-sinus-lining macrophages. Hence, the study of liposome-peritoneal macrophage interaction in the presence of serum does not solely resemble liposome-blood-sinus-lining macrophage interaction since the nature of the opsonins in either cases is different .

Chapter XI

General discussion

A number of investigators (Tyrrell *et al*, 1977; Kao & Juliano, 1981; Ellens *et al*, 1982; Dijkstra *et al*, 1984) have examined the possible involvement of serum opsonins in phagocytosis of liposomes by blood-sinus-lining mononuclear phagocytes at *in vivo* and *in vitro* . Apart from the studies of Tyrrell *et al.* (1977) various investigators (Kao & Juliano, 1981; Ellens *et al*, 1982; Dijkstra *et al*, 1984) have failed to demonstrate the opsonic effect of serum on interaction of liposomes with blood-sinus-lining macrophages, and yet these authors (Kao & Juliano, 1981; Ellens *et al*, 1982; Dijkstra *et al*, 1984) have speculated the possible role of serum opsonins in enhancing the uptake of liposomes by these macrophages. The work described in this thesis for the first time provides evidence for the presence of organ-specific opsonins in serum which enhances the formation of the critical adhesive bond between the liposomes and the correspondent specific blood-sinus-lining macrophages. Characterization of these opsonins was achieved, and the results presented describe their properties which differentiate between liver-specific, spleen-specific, and bone-marrow-specific opsonins (chapters III, IV, V, IX). Some properties of opsonins specific for spleen and bone-marrow phagocytes are very similar to each other and thus the involvement of the same opsonin molecule for these two organs of MPS can not be excluded (chapters V, IX). Also, our preliminary investigation suggests the presence of opsonin(s) specific for lung cells and the properties of this opsonin(s) is very similar to that of liver-specific opsonin(s) (data not presented in this thesis). The properties of organ-specific opsonins may probably explain failure of the earlier attempts (Kao & Juliano, 1981; Ellens *et al*, 1982; Dijkstra *et al*, 1984)

to demonstrate the opsonic effect of serum on phagocytosis of liposomes by blood-sinus-lining macrophages.

Firstly, these investigators (Kao & Juliano, 1981; Ellens *et al*, 1982; Dijkstra *et al*, 1984) have used cholesterol-rich liposomes and vesicles that were composed of either sphingomyelin or solid-state phospholipids. The liver-specific opsonin(s) can not enhance the interaction between cholesterol-rich or solid-state phospholipid liposomes and liver phagocytes for the reasons discussed in chapters III & IV. These lipids may attract dysopsonins and hence the association of serum dysopsonins may retard the interaction of such vesicles with liver phagocytes (chapters III, IV; for a brief summary see fig 4.1.). However, our results do confirm the earlier observations of Tyrrell *et al.* (1977), since serum opsonin(s) stimulates the interaction of cholesterol-poor unsaturated phospholipid liposomes with liver phagocytes. Similarly, serum also stimulated the uptake of cholesterol-poor unsaturated phospholipid liposomes by human blood leukocytes (Finkelstein *et al*, 1981). Interestingly, the *in vitro* results of the present investigation suggest that spleen and bone-marrow specific opsonins do have the ability to enhance the interaction of cholesterol-rich liposomes, despite their phospholipid composition, (chapters III, IV, IX) with the correspondent phagocytes. However, their opsonic activity is far more greater for vesicles containing unsaturated rather than saturated phospholipids. These observations may probably explain why cholesterol-rich saturated phospholipid liposomes have a prolonged circulatory half-life and why they were eventually removed by spleen and bone-marrow at a slow rate (Patel *et al*, 1983; Senior *et al*, 1985).

It should also be emphasized that other factors such as liposome charge or the exposed configuration of the charged groups of liposomal lipids may also control the extent of liposome opsonization and dysopsonization. For example, in our *in vitro* studies serum retarded the interaction of positively charged liposomes (stearylamine was incorporated instead of dicetylphosphate) with liver phagocytes (data not presented in this thesis) and this is in agreement with the *in vivo* results of Gregoriadis & Neerunjun (1974). Similarly, by substituting phosphatidylinositol, PI, instead of dicetylphosphate in cholesterol-poor egg PC liposome their interaction with both liver and spleen phagocytes was not enhanced or retarded in the presence of serum (data not presented). These observations may perhaps explain the pronounced increase in half-life with a parallel decrease in uptake by liver and spleen for PI liposomes *in vivo* (Gabizon & Papahadjopoulos, 1988).

Secondly, the use of buffers supplemented with calcium and magnesium may have accounted for the inability of serum to possess its opsonic activity specific for liver phagocytes (Dijkstra *et al* , 1984) as this opsonin is sensitive to diavalent cations, in particular to calcium, and hence the presence of extracellular calcium may regulate the opsonic activity of liver specific opsonin(s), (chapter VI; see also figs 6.5 & 6.6). It is also interesting to note that some states of altered mononuclear phagocytic activity, such as in neoplastic diseases (Saba & Antikatzides, 1975), have been related to a change in serum or plasma opsonin activity and, indeed, under such circumstances, for example as in the case of neoplastic diseases with or without bone metastases, there is an increase in serum calcium concentration (Morton *et al*, 1988).

Hence, a significant increase in serum calcium concentration may affect the opsonic activity of liver-specific opsonin and as a result depression of phagocytosis by liver phagocytes (chapter VIII). In support of our view, elevation of serum calcium levels above normal inhibited phagocytosis of gelatin coated lipid emulsions by liver slices (Ryder *et al,* 1975). Since the characteristics of the opsonin (fibronectin) which promotes the phagocytosis of gelatin coated oil droplets is different from our liver-specific opsonin, it is tempting to speculate in here that the tumour could be facilitating its own growth process and escape from the immune surveillance via a tumour induced hypercalciemia leading to depression of liver phagocyte clearance. Similar to neoplastic diseases, alteration in serum opsonic activity and serum calcium concentration is known to occur during different stages of pregnancy (Graham & Saba, 1973; Pitkin, 1977). Therefore, the degree of regulation of calcium on opsonic activity of serum may play an important part in the Kupffer cell clearance ability of the mother. However, although opsonin involvement may be important in terms of mononuclear phagocytic regulation, it is possible that variations in body weight, age, organ size, blood flow, temperature, cellular metabolism, mononuclear cellular population dynamics, etc, can profoundly influence intravascular phagocytic activity.

Thirdly, the inability of serum to stimulate the interaction of liposomes with liver cells *in vitro* (Dijkstra *et al,* 1984) may possibly be accounted for at cellular level rather than serum opsonin activity. In the present investigation liver non-parenchymal cells were prepared by mild collagenase perfusion rather than pronase treatment (Dijkstra *et al,*

1984), as pronase induces marked surface damage on liver phagocytes (Steer & Clarenburg, 1979) and perhaps, even after a few days in culture the recovery of the those receptors which recognize the serum opsonized-liposomes may not occur and hence the opsonic effect of serum is not apparent.

Finally, a number of investigators (Juliano, 1982; Stirk & Baldeshwieler, 1986) studied the role of serum on interaction of liposomes with peritoneal phagocytes. Unfortunately, the results of such studies have led to believe a similar role of serum opsonins on interaction of liposomes with resident phagocytes of liver, spleen, and bone-marrow which are predominantly involved in the clearance of liposomes from the blood. Since peritoneal phagocytes have many properties, such as expression of some antigens and plasma membrane receptors, which are different from those of resident blood-sinus-lining phagocytes of liver, spleen, and bone-marrow (see introduction in chapter X), the study of liposome-peritoneal cell interaction in the presence of serum may not necessarily demonstrate the true role of serum on the uptake of liposomes by liver, spleen, and bone-marrow phagocytes. Indeed, the results in chapter X indicate that serum contains opsonin(s) specific for peritoneal phagocytes and the characteristics of this opsonin(s) are different from those specific for liver but have some similarities to those of spleen and bone-marrow specific opsonins.

In conclusion, this thesis has provided for the first time evidence for the presence of opsonins with distinct properties which are specific for liver, spleen, bone-marrow, and peritoneal phagocytes. Purification and identification of these organ-specific opsonins may provide an

opportunity to target liposomes containing therapeutics selectively to the particular organ of the MPS for an efficient treatment of infectious diseases, scanning purpose, etc. Their identification may also help to evaluate their potential role during both normal and pathophysiological conditions, such as infections or tumours.

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