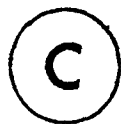


ALPHA-1-PROTEASE INHIBITOR,

AN ACUTE PHASE REACTANT

IN INFLAMMATION^e

By



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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

June, 1982

ALPHA-1-PROTEASE INHIBITOR

DOCTOR OF PHILOSOPHY (1982)
(Medical Sciences)

MCMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Alpha-1-Protease Inhibitor, an Acute Phase Reactant
in Inflammation

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NUMBER OF PAGES: xx, 278

ABSTRACT

Alpha-1-protease inhibitor (α 1Pi) is a plasma glycoprotein which plays a major role in limiting proteolysis during inflammation due to its broad spectrum of inhibitory activity.

The purification and partial characterization of α 1Pi have allowed for its investigation as an acute phase reactant during inflammation.

Mouse α 1Pi is a glycoprotein of molecular weight 53,500 with a half-life of approximately 15.5 hours. On two dimensional immunoelectrophoresis, two variants of α 1Pi were seen which exhibited immunological non-identity. The two variants showed trypsin binding activity, both were synthesized by hepatocytes, and both behaved as acute phase reactants.

The ubiquitous nature of α 1Pi was evident from its presence in a wide variety of body fluids. Using an immunohistochemical stain, the inhibitor was shown to be normally present in the cytoplasm of hepatocytes, islet cells of pancreas and in some villous epithelial cells of the small intestine. The hepatocyte was shown to be the major source of serum α 1Pi. Alveolar macrophages, shown to contain α 1Pi histochemically in particular inflammatory states of the lung, synthesized minimal quantities.

The acute phase response of α 1Pi was investigated in four models of inflammation consisting of either a subcutaneous injection of celite or infection with one of three parasites: *Nippostrongylus brasiliensis*; *Trichinella spiralis*; and *Trypanosoma congolense*. In addition, studies

have been initiated with other mouse acute phase reactants, namely, complement component C3, serum amyloid P (SAP) and serum amyloid A (SAA).

The induction of acute phase protein synthesis during inflammation was characterized by an increase in the number of hepatocytes staining for α 1Pi. During inflammation the level of hepatocyte staining was shown to correlate with the synthetic output of α 1Pi. The maximum staining activity for α 1Pi in hepatocytes preceded the peak increase in serum levels during inflammation. Moreover, there was a characteristic, progressive alteration in the distribution pattern of stained hepatocytes within the liver lobule. These results were consistent with an acute phase mediator perhaps originating from the site of inflammation, gaining access to the liver via the portal circulation causing an induction of acute phase protein synthesis.

The synthesis of an acute phase mediator (APM), probably interleukin 1 (IL1), by alveolar macrophages and the *in vitro* induction by APM of α 1Pi synthesis by hepatocytes has been demonstrated.

It was also shown that α 1Pi accumulated at the site of inflammation which may account for the apparent lack of increase in serum levels even though there was an increased hepatic output of α 1Pi at that time.

Collectively these results demonstrate that in the mouse the induction of synthesis of α 1Pi during an acute response to inflammation is mediated by an acute phase mediator originating from macrophages at the site of inflammation.

The increased synthesis of α 1Pi has been shown *in vivo* by a measure of serum levels and immunohistochemical examination of liver tissue as well as *in vitro* by cultures of isolated hepatocytes.

These studies constitute a basic framework upon which mechanisms for the induction and control of synthesis of acute phase proteins can be further explored.

A ma chère épouse, Danielle,
pour son amour, son support,
sa patience et ses sacrifices nombreux

et

A mes parents,
pour leur intérêt et encouragement

Je dédie-cette thèse.

ACKNOWLEDGEMENTS

To my supervisor, Dr. Jack Gauldie, my profound gratitude and deepest appreciation for his guidance, support and patience, and for providing every opportunity to express myself.

I wish to thank the members of my Supervisory Committee, Drs. Robert Hill, Shuen-Kuei Liao and Erwin Regoeczi for their continued interest and constructive criticism throughout this endeavour.

To Ms. Elizabeth Jenkins, my sincere thankfulness and appreciation for her invaluable technical assistance throughout this undertaking. I am especially grateful for her perseverance in times of research impasse.

I express special thanks to Drs. Dean Befus, John Bienenstock, David Clark, Peter Horsewood, Mark McDermott and Henry Solow for their encouragement and many fruitful discussions.

To my fellow graduate students, Tom Egwang, Shelagh Mirski, Carl Richards, Renata Slapsys, Andy Stadnyk and Doug Williams, I am very appreciative of their support and good humour. I wish them well.

I wish to thank Mrs. Joan Hickey for the meticulous preparation of this manuscript and her editorial assistance.

I would also like to thank Mrs. Barney McLaughlin, Ms. Betty Brown, Ms. Jane-Ann Smith and many others who have contributed in some way to this project.

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LIST OF ABBREVIATIONS

α 1Pi	alpha-1-protease inhibitor
APM	acute phase mediator
APR	acute phase reactant
BAPNA	N-benzoyl-DL-arginine p-nitroanilide
BSA	bovine serum albumin
C	complement
CLE	crude leukocyte extract
COLD	chronic obstructive lung disease
CRP	C-reactive protein
DAB	diaminobenzidine tetra-HCl
ELISA	enzyme linked immunosorbent assay
EP	endogenous pyrogen
Hepes	N-2-hydroxyethylpiperazine-N ¹ -2-ethanesulfonic acid
HF α	activated Hageman Factor
IgG	immunoglobulin G
IL1	interleukin 1
i.d.	internal diameter
i.v.	intravenously
LAF	lymphocyte activating factor
LEM	leukocyte endogenous mediator
LP	leukocyte pyrogen
LPS	lipopolysaccharide
MW	molecular weight
NaCl	sodium chloride

PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PHA	phytohemagglutinin
P/S	penicillin/streptomycin
SAA	serum amyloid A
SAASF	serum amyloid A stimulating factor
SAP	serum amyloid P
SDS	sodium dodecyl sulfate
spp.	members of the species
TCA	trichloroacetic acid
Tris	trishydroxymethylaminomethane

CHAPTER 1

REVIEW OF LITERATURE

1.1 The Acute Inflammatory Response

1.1.1 Historical background

Historically, the concept of Inflammation can be traced to approximately 1650 B.C. where the term "Shememet" was repeatedly used in Egyptian scrolls in association with wounds and injury (Ryan and Majno, 1977). "Shememet" was represented in hieroglyphs by a flaming brazier, with smoke rising and curving down and as such was believed to convey the sensations of "hotness" and "burning".

During Hippocrates' century (460 - 380 B.C.), the Greeks used the term "phlegmonē" in reference to inflammation which signified "burning thing". A synonym for inflammation is phlegmasia.

In 100 A.D., Cornelius Celsus, a Roman writer, provided one of the original definitions of inflammation:

"The major signs of inflammation are four: Rubor et Tumor cum Calore et Dolore."

that is, redness and swelling with heat and pain.

Up until the 17th century, inflammation was considered for the most part as a dreadful disease since in many instances, pus and local pain were a prominent feature of the lesion. This was likely accentuated by the lack of aseptic treatment of wounds. However, in 1793, John Hunter, a Scottish surgeon-scientist, strongly advocated that inflammation was not a disease but rather a beneficial and necessary requirement for wound repair and healing as well as for the destruction of certain pathogenic organisms.

Rudolph Virchow (1821 - 1902), the father of cellular pathology and one of his students, Julius Cohnheim (1839 - 1884), contributed a vast amount of knowledge towards the understanding of some of the underlying processes of the general inflammatory response. While studying the mesentery of the frog tongue, they were the first to observe dilatation of arterioles with concomitant increase of blood flow. Their greatest contribution was perhaps the description of the movement of white blood cells as they were lining the venule walls (margination) while the red blood cells flowed past them. Subsequent to this margination, there was migration of white cells across the venule wall into the extravascular spaces (diapedesis). It was also observed that in some venules, red blood cells were so tightly packed that the plasma had leaked out of the blood vessels (edema).

Although Virchow and Cohnheim had carefully documented the vascular changes and cell movement across blood vessels during tissue injury, the Russian biologist Elie Metchnikoff (1845 - 1916) is credited for explaining why these cells emigrated and their possible function(s) in the extravascular spaces.

In 1882, Metchnikoff, observing the mobile cells of a transparent starfish larva, postulated that similar cells might serve in the defense of the organism against invading foreign pathogens. To test this hypothesis, he introduced a wooden splinter into the body of a starfish larva and observed that after a period of time, the splinter became surrounded by a cellular infiltrate (Silverstein, 1979).

In 1884, Metchnikoff described the engulfment of anthrax bacillus by frog leukocytes and referred to this process as "phagocytosis". He

concluded that the purpose of inflammation was to bring phagocytes to the injured area to engulf bacteria or other organisms and as such constituted a first line of defense against invading pathogens.

Metchnikoff's theory was particularly difficult to integrate or assimilate with the then current concepts of host defense since cellular pathology was a relatively young science and Louis Pasteur's earlier discovery of a vaccine for chicken cholera was strong evidence for humoral defense mechanisms.

It is only in the past twenty years or so that the concepts of inflammation and humoral and cellular immunity have been integrated as part of one major defense mechanism against invading pathogens. It is now evident that inflammation and specific immunity are interdependent and the study of pathways by which these systems become activated, in response to an invading organism, provides for an interesting and highly dynamic field for investigation.

1.1.2 The cellular acute phase reaction

The acute phase reaction consists of two main components, the cellular response and the humoral events, and has been the subject of recent reviews (Ryan and Majno, 1977a; 1977b).

The acute inflammatory reaction is characterized by increases in blood flow and vascular permeability as well as by an infiltration of inflammatory cells. Increased vascular permeability can be induced by a variety of biochemical mediators which include bradykinin, vasoactive amines such as histamine and 5-hydroxytryptamine (5-HT) (serotonin); slow reacting substance of anaphylaxis (SRS-A), prostaglandins E_1 and

E₂, complement components C3a and C5a and neutrophil lysosomal cationic proteins.

Increased vascular permeability during acute inflammation is classified under three main categories. The first type is referred to as "immediate-transient leakage". In this case, the increased vascular permeability occurs within minutes of influx of mediator(s), is of brief duration (15 - 30 min) and is characterized by its high degree of specificity for venules up to about 100 microns in diameter. A typical example of immediate-transient leakage is the allergic wheal. The underlying mechanism for this transient leakage is not fully understood but the actual contraction of endothelial cells in venule walls has been suggested (Majno *et al.*, 1969).

A second type of increased vascular permeability is "immediate-prolonged leakage" and is induced by direct vascular injury. The mechanism is evident: the leakage occurs immediately following vessel disruption and proceeds until healing is complete.

The third type of increased vascular permeability is "delayed-prolonged leakage". This type of vascular leakage is often characterized by an initial short burst of increased permeability followed after two to four hours by a second and more persistent episode (Burke and Miles, 1958; Sevitt, 1958), and as such is considered biphasic.

Injurious agents which have been demonstrated to induce delayed-prolonged leakage include bacteria, bacterial toxins, carageenin and mild thermal injury (Burke and Miles, 1958; Vinegar *et al.*, 1969; Sevitt, 1958; Hurley *et al.*, 1967).

In this type of increased vascular permeability, capillaries are usually affected since they are closest to the surface of the skin and as such more vulnerable to the injurious stimuli. In some instances, venules may also be involved.

As for the mechanism for delayed-prolonged leakage, it is currently thought that it arises as a result of mild direct injury of the endothelium (Ryan and Majno, 1977b). Moreover, if the intensity of the injurious stimulus is increased such as in cases of severe burns, the delay period between the two bursts of vascular leakage becomes shorter and the reaction shows the characteristics of immediate-prolonged leakage.

One of the main cellular events in acute inflammation is leukocyte infiltration which is frequently accompanied by a subsequent release of lysosomal enzymes from leukocytes and phagocytosis of pathogens in the extravascular spaces. This cascade of events is initiated by leukocyte adherence (margination) against vessel walls in inflamed areas. The mechanism for leukocyte adherence is not fully understood but a role for Ca^{2+} ions has been implicated (Thompson *et al.*, 1967; Atherton and Born, 1972; Giddon and Lindhe, 1972) and it is likely that inflammation induces alterations in the endothelial cell surface, such as electrostatic changes, which may allow for a more intimate and stronger leukocyte to endothelial cell contact (Ryan and Majno, 1977b).

The emigration of leukocytes across vessel walls via inter-endothelial cell junctions (Marchesi and Florey, 1960; Marchesi, 1961) is referred to as diapedesis. Although the presence of increased

vascular permeability is not responsible for diapedesis, vascular leakage accentuates the degree of leukocyte infiltration into tissues during chemotactic stimulus-mediated inflammation (Issekutz, 1981). Chemotaxis is defined as the directed migration of cells against a chemotactic factor concentration gradient. △

During the course of an acute inflammatory reaction, usually both polymorphonuclear and monocytic leukocytes emigrate to the site of injury. The classic experimental models of leukocyte migration kinetics consisted of an injection of a mild irritant, such as serum, or a living microorganism, such as *Klebsiella pneumoniae*, into the pleural cavity of a rat (Hurley *et al.*, 1966). In these studies, neutrophils infiltrate the inflamed area within an hour, peak at approximately four hours and subsequently rapidly decline. Mononuclear cells; mainly macrophages derived from blood monocytes, appear at approximately four hours post-injurious stimulus and reach a sustained peak 18 to 24 h later.

Such studies, however, suffer from the fact that histological determinations of cellular influx and identification of cell types is relatively imprecise. Recently, the use of neutrophils and macrophages labelled with radioisotopes has greatly improved kinetic studies. It was shown that in the rabbit, neutrophils and monocytes migrate out of the circulation to the skin simultaneously in response to chemotactic agents or *Escherichia coli*, but the rate of accumulation of monocytes occurs at a lower rate than that of neutrophils. Neutrophils cease to migrate into lesions by six hours whereas monocytes continue for an extended period (7 to 24 h). The histologic predominance of neutrophils

in early acute inflammatory lesions likely arises as a result of the abundant neutrophil emigration compared with monocytes during that period of time. Furthermore, the half-life of neutrophils is relatively short (3.8 h) whereas monocytes differentiate into long-lived mononuclear phagocytes (39 h) and as such, the absolute number of neutrophils gradually decreases at the site of inflammation (Perper *et al.*, 1974; Jones *et al.*, 1977; Issekutz and Movat, 1980; Kopaniak *et al.*, 1980; Issekutz, 1981; Issekutz *et al.*, 1981). One can, therefore, conclude that the change from neutrophils to a predominantly macrophage/monocyte infiltrate in acute inflammation is a result of differences in emigration kinetics as well as in the lifespan of these two cell types.

These studies are amenable to models of inflammation in which the inflammatory lesion(s) is somewhat locally restricted to a particular tissue site. However, in situations in which the pathogenic organism gives rise to massive tissue destruction and is involved in a variety of tissue sites such as in natural parasite infections, the cell types involved may be quite different and kinetic studies are somewhat more complex. One has to rely heavily on histologic examination of tissues at successive time intervals. This task is rendered particularly difficult in tissue sections where a predominance of one cell type may mask the presence of other inflammatory cells.

1.1.3 The humoral acute phase reaction

The acute inflammatory response is characterized by a rapid rise in the serum concentration of a number of proteins with subsequent decline to normal levels as healing occurs. These proteins are classically known as acute phase reactants (APR) (Koj, 1970, 1974; Gordon, 1976; Kindmark, 1976).

Using this operational definition, a variety of proteins have been classified as acute phase reactants and these are listed along with some of their biological functions in Table 1.1.3. A more in depth description of the acute phase proteins α 1-protease inhibitor (α 1Pi), C-reactive protein (CRP), serum amyloid P (SAP) and serum amyloid A (SAA) is presented in section 1.2.

1.1.3.1 Acute phase reactants as humoral markers of inflammation

In the past, the main interest in acute phase reactants resided in their clinical potential as "Markers of Inflammation".

In a recent symposium in Lyon, France (Symposium sur les Marqueurs de l'Inflammation. Le profile protéique dans la malnutrition et les états inflammatoires. Lyon, France, April, 1981), the serum profiles of acute phase proteins in a wide variety of inflammatory conditions including bacterial infections, post-surgical trauma, cancer, burn victims, rheumatoid arthritis, gastrointestinal disease, myocardial disease and neonatal complications, were presented. The concluding remarks from the symposium were that although in many instances, there was a marked increase in serum concentrations of

TABLE 1.1.3

The Acute Phase Reactants

<u>Proteins</u>	<u>Biological Function</u>
Fibrinogen Prothrombin Factor VIII	Involved in the coagulation sequence
Plasminogen	Activator of complement, kinin and Factor XII
α 1-antitrypsin (α 1-protease inhibitor) α 1-antichymotrypsin	Protease inhibitors
C1s, C2, C3, C4, C5, C56, C9, Factor B, C1 INH, C3b INA, B1H	Involved in the complement sequence and generation of a variety of effector molecules
Ceruloplasmin	Eliminates excess free radicals
Hemopexin Haptoglobin	Function as transport proteins for iron from hemoglobin release
Cold insoluble globulin (fibronectin)	Cell to cell interactions
C-reactive protein (CRP) Serum amyloid A (SAA) Serum amyloid P (SAP)	Binds to a variety of ligands but biological function not known
Orosomucoid (α 1-acid glycoprotein) Gc globulin	Unknown <i>in vivo</i> biological function

some acute phase proteins during a particular inflammatory condition, there was no correlation with the intensity of the disease and there was also considerable variation between individual patients. It was also evident that the interpretation of serum profiles of acute phase reactants could be misleading in many instances and that the use of acute phase reactants as diagnostic tools required further development. It is clear from these studies that a greater knowledge of the biological function of these acute phase reactants, their site of synthesis, the mechanism(s) whereby the acute phase response is induced during an inflammatory process, and their role in inflammation is imperative.

Recent advances in methods of identification, isolation and quantitation of acute phase reactants should allow for the dissection at the molecular and cellular levels of their biological activities and hence their role in health and disease.

In conclusion, the acute inflammatory response is defined as an early host response to tissue injury and is characterized by the cell type(s) migrating to the site of injury as well as by a rapid rise in serum levels of a variety of acute phase reactants.

1.1.4 Humoral pathways of inflammation

The humoral pathways of inflammation consist of complex cascades of enzymatic reactions which involve proteins of the Coagulation, Kinin, Fibrinolytic and Complement systems. Each of these proteins either participate directly by mediating specific effector functions or act on other proteins in the cascade.

Although there are numerous mechanisms whereby each of these enzymatic cascades can be initiated, it is important to recognize that these pathways are all interrelated. The activation of a particular system gives rise to a number of active enzymes or products which can in turn activate the other pathways. This so called "tangled-web" is depicted in Figure 1.1.4.1 and has been the subject of recent reviews (Ryan and Majno, 1977a, 1977b; Leid and Williams, 1979; Wicher, 1981).

The activation of the humoral pathways of inflammation can be initiated by trauma or tissue injury. For example, tissue injury could result in the exposure of collagen and other negatively charged components of capillary basement membranes. This converts Hageman Factor (Factor XII) of the coagulation system into activated Hageman Factor (HFa). HFa initiates the clotting cascade by converting Factor XI to activated Factor XI (XIa). The coagulation sequence is terminated by the conversion of fibrinogen to fibrin by thrombin and stabilization of the fibrin clot by Factor XIII. Factor XIa amplifies the coagulation process by converting more Hageman Factor molecules to HFa.

HFa can recruit the fibrinolytic system by converting plasminogen proactivator to plasminogen activator. This is followed by the conversion of plasminogen to plasmin by plasminogen activator.

HFa fragments can activate the kinin system by generating kallikrein from prekallikrein which in turn interacts with kininogens to yield kinins. Kallikrein can also feed back on the coagulation sequences by activating Hageman Factor. In addition, activation of the kinin system produces a so-called Kf fragment which has been

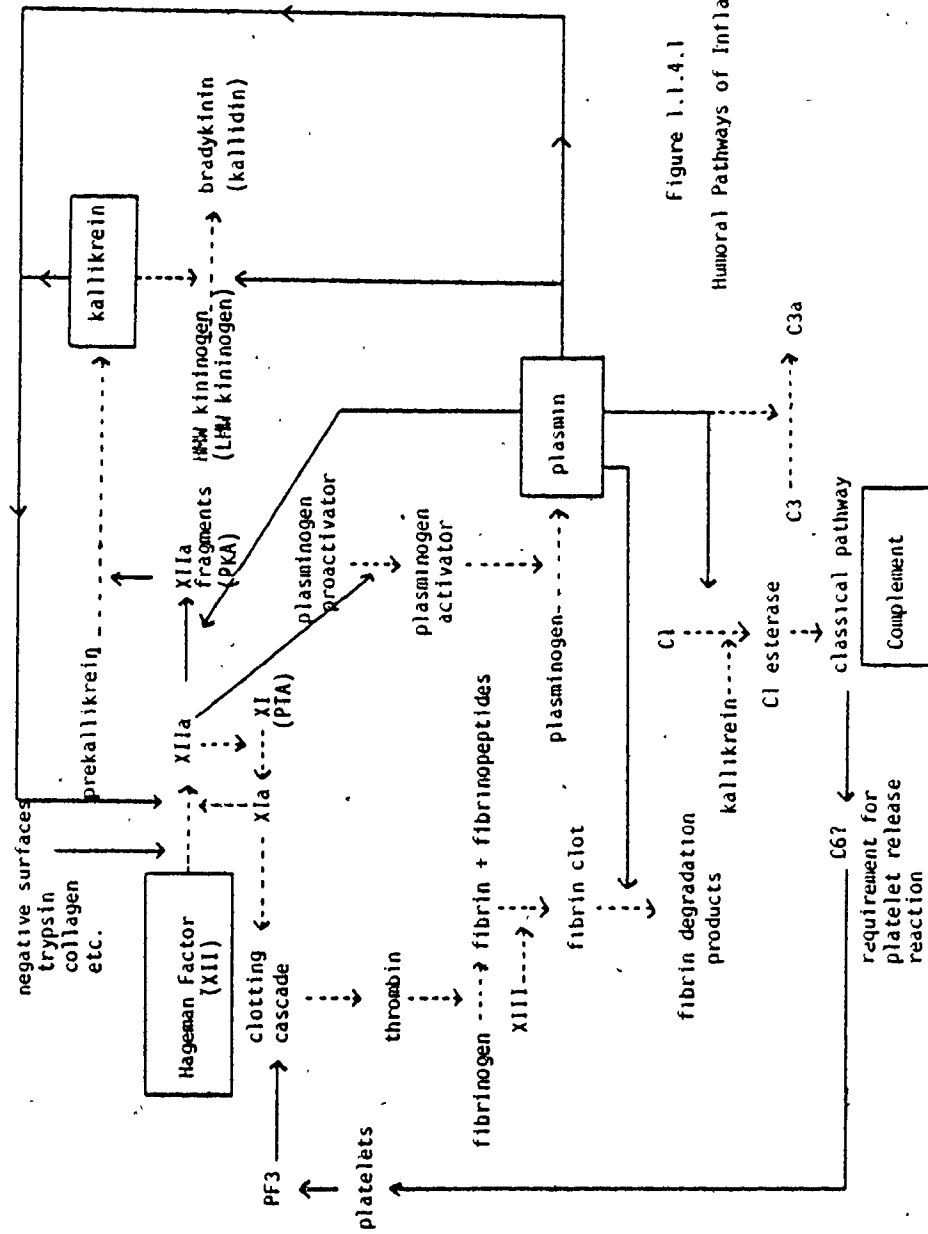


Figure 1.1.4.1 Humoral Pathways of Inflammation

implicated as a helper factor in the production of complement component C3 convertase by C1 esterase. Kallikrein can also directly activate C1 to $\overline{\text{C1}}$ s. Although a role for the complement system in coagulation is not evident, complement component C6 is required for normal blood coagulation to occur (Zimmerman and Muller-Eberhard, 1971; Siraganian, 1972).

The "tangled-web" becomes simpler to understand when the effects of plasmin are examined. Plasmin can activate Hageman Factor as well as convert HFa into HFa fragments. Plasmin has also been implicated in the conversion of kininogens to kinins and C1s of the first component of complement to activated $\overline{\text{C1}}$ s and C3 to C3a, a potent monocyte/neutrophil chemotactic factor. Plasmin is also responsible for the degradation of the fibrin clot at the terminal stages of the healing process.

Kinins cause constriction of smooth muscles and the release of vasoactive amines which lead to increased vascular permeability.

The activation of these humoral pathways of inflammation also initiates the recruitment of a variety of cell types at the site of inflammation. Kallikrein exhibits chemotactic activity for neutrophils, mononuclear phagocytes and basophils.

The complement system, once activated, is likely the most significant source of mediators for the recruitment of the cellular component of the inflammatory reaction. The anaphylatoxins C3a and

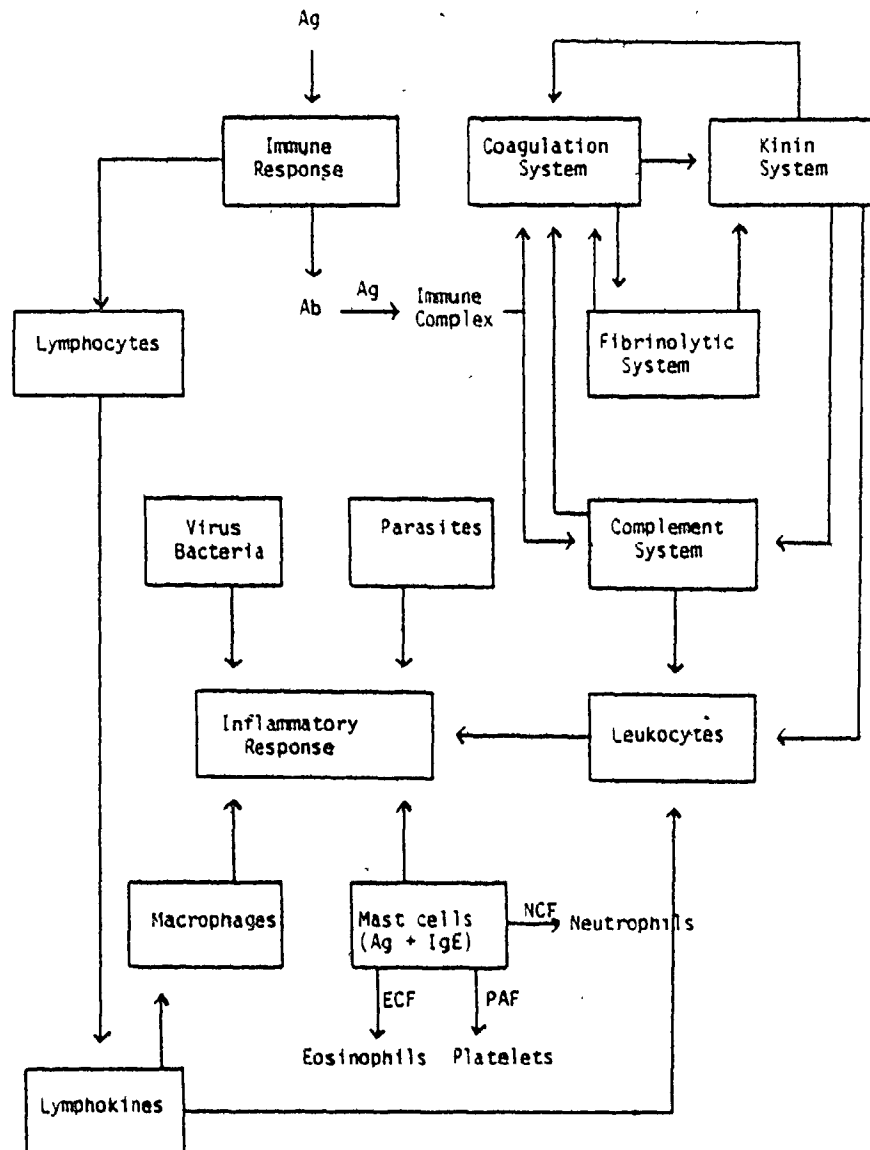
C5a cause histamine release from mast cells, resulting in arteriolar dilatation and increased vascular permeability. The chemotaxins C3a, C5a and $\overline{\text{C567}}$ cause migration of phagocytes into the area whereas the production of C3b on the surface of bacteria results in opsonization and enhancement of phagocytosis. During phagocytosis, the release of a variety of lysosomal enzymes can potentiate the acute inflammatory response by activating the fibrinolytic system and the complement pathway.

As depicted in Figure 1.1.4.2, direct tissue injury is not the sole mechanism whereby the acute inflammatory response is induced. One type of mechanism is immunologic injury which, as a result of immune complex formation and deposition, leads to the activation of complement and potentially other humoral pathways of inflammation.

A second mechanism for the induction of an inflammatory response is a delayed-type hypersensitivity reaction where the interaction of antigen with immunocompetent lymphocytes induces the production of a number of lymphocyte products termed lymphokines, which include macrophage migration inhibitory factor (MIF).

The induction of an acute inflammatory response may also occur by a third mechanism. During an immediate hypersensitivity reaction, the interaction of antigen and IgE-sensitized mast cells causes release of histamine and other vasoactive enzymes as well as an eosinophil chemotactic factor (ECF), a neutrophil chemotactic factor (NCF) and a platelet activating factor (PAF). These various cell types in turn release a variety of lymphocyte mitogenic products such that these systems are all amplified.

Figure 1.1.4.2 Pathways to Inflammation



A fourth mechanism whereby the inflammatory response is initiated is by direct invasion of the host by viruses, parasites and bacteria. These can lead to the direct activation of complement, via the alternative pathway, in the absence of an immune response. Certain bacterial products are also potent leukocyte chemotactic factors.

It is, therefore, quite clear that there are many distinct mechanisms whereby the acute inflammatory response can be initiated. Regardless of the initial injurious stimulus, the final result is the potential activation of all of the humoral pathways of inflammation with the concomitant release of cellular mediators of inflammation such as vasoactive amines, lymphokines, monokines and lysosomal enzymes.

One must, however, exercise caution in the extrapolation of these interactions to *in vivo* situations since most of the evidence for the various linking pathways has been derived from *in vitro* studies. It is not clear to what extent these pathways interact *in vivo* and how they are regulated.

1.1.4.1 Inhibitors

A number of potential inhibitors of various enzymes or proteins of the humoral pathways of inflammation have been described and include kininases, C1 esterase inactivator, β -lipoprotein, α 2-macroglobulin, antithrombin III and α 1-protease inhibitor (Ryan and Majno, 1977a, 1977b).

Of relevance to the present work is α 1-protease inhibitor (α 1Pi). α 1Pi is a major inhibitor of neutral proteases and exhibits

inhibitory activity towards kallikrein, Factor XIa, thrombin and possibly plasmin (Heimbürger *et al.*, 1971; Koj, 1974). The α 1Pi acute phase response during inflammation may play an important modulatory role during development of the acute inflammatory reaction as well as during the healing phase. It is worth noting that in patients with severe α 1Pi deficiency (section 1.2.1), there are no apparent defects in the activation and modulation of the humoral pathways of inflammation. It is, therefore, likely that compensatory inhibitory mechanisms are present. However, as discussed later (section 1.2.1), the impaired ability to neutralize certain proteolytic enzymes in the lung may play a major role in the development of pulmonary emphysema in young adults.

1.2 Acute Phase Reactants. Biochemical and Biological Functions

A major portion of the present study deals with the acute phase reactant α 1Pi and this molecule will therefore receive most of the attention. A brief description of the acute phase reactants SAA, SAP and CRP will also be presented. Although CRP was not investigated in the present studies, it will be incorporated into our understanding of the model (Chapter 6).

1.2.1 α 1-protease inhibitor (α 1Pi)

In 1897, Camus and Gley demonstrated that serum exhibited antitryptic activity. Jacobsson (1953) showed that 90% of the antitryptic activity was in the α 1-globulin fraction and, in 1955, reported on the increased serum concentration of α 1-globulin tryptic inhibitor in inflammatory-type diseases. Schultze *et al.* (1962) identified the

principal trypsin inhibitor as the $\alpha 1$ 3.5 S-glycoprotein they had isolated and characterized in 1955 and called this protein $\alpha 1$ -antitrypsin ($\alpha 1$ AT) which, because of its broad spectrum of inhibitory activities, is now designated as $\alpha 1$ -protease inhibitor ($\alpha 1$ Pi) (reviewed in Kueppers and Black, 1974; Fagerhol and Laurell, 1970).

1.2.1.1 Phenotypes and disease associations of human $\alpha 1$ Pi

Human $\alpha 1$ Pi is a single polypeptide glycoprotein of molecular weight 49,500 - 56,000 daltons and contains approximately 11 - 14% carbohydrate residues (Chan *et al.*, 1973; Crawford, 1973; Kress and Laskowski, 1973; Panell *et al.*, 1974; Morii *et al.*, 1978; Jeppsson *et al.*, 1978b).

Human $\alpha 1$ Pi exhibits marked polymorphism as well as considerable microheterogeneity within phenotypes. The polymorphism is under the control of 2-autosomal co-dominant alleles such that each allele in a pair codes for the synthesis of one molecular species of $\alpha 1$ Pi. The extensive polymorphism and microheterogeneity of $\alpha 1$ Pi is reflected by a series of protein bands on electrophoresis. The bands arising from the product of each allele exhibit a migration pattern independent of each other although some overlap may occur. Using acid starch gel electrophoresis in conjunction with two-dimensional-immunoelectrophoresis and more recently by isoelectric focusing, over 27 different phenotypes and corresponding alleles have been documented in the literature (reviewed in Fagerhol and Gedde-Dahl, 1969; Fagerhol and Laurell, 1970; Arnaud *et al.*, 1978).

The most widely studied $\alpha 1$ Pi phenotypes are the PiM, PiS and PiZ alleles. Homozygous MM individuals have normal $\alpha 1$ Pi serum levels

whereas SS homozygotes have intermediate α Pi deficiency with serum levels 40 - 60% of normal. ZZ individuals have severe α Pi deficiency with serum levels 5 - 10% of normal. There is, in addition, a recently described Pi-- allele which is associated with a totally absent α Pi level. Although there is some variation in gene frequencies for α Pi alleles in various populations, MM, SS and ZZ occur in approximately 90%, 0.14% and 0.07% of the population, respectively (Fagerhol, 1967; Janus *et al.*, 1975; Klasen *et al.*, 1977; Schwartz *et al.*, 1973; Fagerhol and Tenfjord, 1968; Cook, 1975; Frants and Eriksson, 1976; Szczeklik *et al.*, 1974; Harada and Omoto, 1970; Kellerman and Walter, 1970; Blundell *et al.*, 1975).

From a clinical standpoint, the interest in α Pi stems from the strong association of certain diseases with the deficiency state (reviewed in Kueppers and Black, 1974; Talamo, 1975).

There is little doubt that individuals with homozygous ZZ deficiency are at an increased risk of early onset of pulmonary emphysema (Eriksson, 1964) and liver disease, most frequently in the form of neonatal hepatitis and cholestasis followed by juvenile liver cirrhosis (Sharp, 1971; Sharp *et al.*, 1969; Berg and Eriksson, 1972; Kueppers *et al.*, 1976; Sveger, 1978; Larsson, 1978).

Efforts to find a correlation between intermediate α Pi deficiency (heterozygote individuals) and cirrhosis or emphysema have yielded highly conflicting and divergent results. Since a major part of the literature on α Pi deals with α Pi deficiency and disease associations and the present studies are not concerned with this aspect, the results are briefly summarized in Table 1.2.1.1.1.

TABLE 1.2.1.1.1

α 1Pi Intermediate Deficiency (PiMS, MZ and SS)
and Disease Association

<u>Disease</u>	<u>Positive Association</u>	<u>No Association</u>
Pulmonary emphysema	Lieberman, 1969; Kueppers <i>et al.</i> , 1969; Kueppers and Donhardt, 1974; Cox <i>et al.</i> , 1976; Eriksson <i>et al.</i> , 1975; Mittman <i>et al.</i> , 1974	Webb <i>et al.</i> , 1973; Morse <i>et al.</i> , 1975, 1977; Larsson <i>et al.</i> , 1977; Girard <i>et al.</i> , 1978; McDonagh <i>et al.</i> , 1979
Liver cirrhosis	Eriksson <i>et al.</i> , 1975; Blenkinsopp <i>et al.</i> , 1977; Hodges <i>et al.</i> , 1981	Morin <i>et al.</i> , 1975; Theodoropoulos <i>et al.</i> , 1976; Kelly <i>et al.</i> , 1979; Carlson <i>et al.</i> , 1981

From these controversial studies, it is inconclusive whether heterozygous α 1Pi deficient individuals are at an increased risk of early onset of pulmonary emphysema or childhood cirrhosis. The controversy likely arises as a result of a variety of factors in the different experimental designs such as age, screening methodology, populations under study, environmental factors (smoking, air pollution, work environment), medical history, pulmonary and liver function tests employed and criteria for determination of statistical significance.

The current hypothesis for α 1Pi deficiency (homozygous)-related lung disease proposes that lung tissue degeneration occurs as a result of lack of inhibition of proteases in the lung microenvironment (Gadek *et al.*, 1980; Fagerhol and Laurell, 1970; Lee *et al.*, 1981). In support for such a hypothesis is the experimental model of pulmonary emphysema of Weinbaum *et al.* (1974) and Janoff *et al.* (1977) which consists of the intra-alveolar administration of proteolytic enzymes.

The pathogenesis of α 1Pi deficiency-related liver disease remains unknown. In humans, the presence of α 1Pi in the cytoplasm of hepatocytes is restricted to homozygous PiZZ or heterozygous deficient individuals (see section 1.3). It is speculated that an excess of α 1Pi in the hepatocyte may interfere with important intracellular proteolytic enzymes which are involved in the detoxification of proteins and peptides arriving via the portal circulation and as such lead to hepatic destruction (Aagenaes *et al.*, 1972).

Other disease states have been investigated for their association with α 1Pi deficiency (Table 1.2.1.1.2). In all cases, results are as yet inconclusive with respect to an association between

TABLE 1.2.1.1.2

Homozygous and Heterozygous α 1Pi Deficiency in Other Disease States

Juvenile rheumatoid arthritis	Cox and Huber, 1976; Arnaud <i>et al.</i> , 1977; Sjoblom and Wollheim, 1977
Malignant hepatoma	Berg and Eriksson, 1972; Lieberman, 1974; Lieberman <i>et al.</i> , 1975
Gastrointestinal disease - intestinal mucosal atrophy peptic ulcers	Sharp, 1976 (review)
Panniculitis	Rubinstein <i>et al.</i> , 1977
Systemic sclerosis	Novis <i>et al.</i> , 1975
Kidney disease	Moroz <i>et al.</i> , 1978
Systemic lupus erythematosus	Karsh <i>et al.</i> , 1979

α 1Pi homozygous or heterozygous deficiency and increased prevalence of these various diseases.

1.2.1.2 Biochemical characterization of α 1Pi

Numerous studies have dealt with the isolation and characterization of some of the more common α 1Pi variants.

The extensive polymorphism and microheterogeneity of human α 1Pi is attributed in part to differences in the net charge of α 1Pi molecules as indicated by isoelectric focusing (Arnaud *et al.*, 1974). The microheterogeneity within the α 1Pi variants is likely due to a combination of slight differences in the content of charged amino acids (Jeppsson *et al.*, 1978b; Hercz *et al.*, 1978; Hercz and Barton, 1977) and/or differences in degree of sialylation (Yoshida *et al.*, 1976; Chan and Rees, 1975; Miller *et al.*, 1976; Jeppsson *et al.*, 1978b; Cox, 1975). However, Hercz and co-workers (1978) have demonstrated microheterogeneity in the ZZ variant isolated from the liver inclusion bodies suggesting that the heterogeneity occurs in the absence of sialic acid residues and becomes established prior to transportation from the RER.

The amino acid content of α 1Pi has been determined by numerous investigators and although there are slight differences in the number of residues of some amino acids, these differences are within the range of experimental error (Chan *et al.*, 1973; Crawford, 1973; Heimbürger *et al.*, 1964; Kress and Laskowski, 1974; Laurell and Jeppsson, 1975; Travis *et al.*, 1974; Jeppsson *et al.*, 1978b; Roll *et al.*, 1978).

The overall amino acid composition of the MM, SS and ZZ α 1Pi variants does not differ significantly. However, using tryptic peptide maps, the SS α 1Pi variant was shown to have a neutral valine residue substituted for a negatively-charged glutamic acid residue at position 22 whereas the ZZ α 1Pi variant has a positively-charged lysine residue substituted for a glutamic acid residue at position 100 (Jeppsson *et al.*, 1978b; Owen *et al.*, 1976; Yoshida *et al.*, 1976). Whether these, or other as yet unidentified amino acid substitutions, are in part responsible for the decreased serum levels of α 1Pi is not clear.

Ultraviolet and fluorescence spectra have indicated that MM and ZZ α 1Pi have different 3-dimensional structures (Glaser and Karic, 1978) with the ZZ variants having greater hydrophobicity. The increased hydrophobicity of the PiZZ molecule could lead to aggregation and precipitation of ZZ in the liver prior to complete glycosylation and secretion into the circulation (Jeppsson *et al.*, 1975).

The decreased serum levels of ZZ α 1Pi cannot be attributed to increased turnover rate since MM, SS and ZZ α 1Pi variants have similar catabolic rates (Miller *et al.*, 1976; Laurell *et al.*, 1977; Jeppsson *et al.*, 1978a; Moser *et al.*, 1978).

Although serum α 1Pi ZZ has perhaps slightly less carbohydrate residues than serum α 1Pi MM (Miller *et al.*, 1976; Chan and Rees, 1975; Yoshida *et al.*, 1976), there are major differences in carbohydrate residues between α 1Pi ZZ isolated from the cytoplasm of hepatocytes and serum α 1Pi MM (Jeppson *et al.*, 1975; Hercz *et al.*, 1978). Whether or not this difference in carbohydrate residues has

any relation to the decreased serum levels of α 1Pi in homozygous ZZ individuals, is unknown.

Interaction of human α 1Pi with proteases

α 1Pi inhibits proteases in a 1:1 molar ratio and the reactive site on the α 1Pi molecule involves a methionine residue (Kress and Laskowski, 1973; Cohen, 1973, 1979; Crawford, 1973; James and Cohen, 1978; Baumstark, 1978; Martodam and Liener, 1981; Travis and Johnson, 1978; Satoh *et al.*, 1979; Johnson and Travis, 1978, 1979; Janoff *et al.*, 1979). α 1Pi can be reversibly inactivated by the oxidation of the methionine residues to a sulfoxide by strong oxidizing agents such as chloramine-T, ozone and myeloperoxidase in the presence of hydrogen peroxide and halide ion (Matheson *et al.*, 1979, 1981; Johnson, 1980; Johnson and Travis, 1979; Janoff *et al.*, 1979; DelMar *et al.*, 1979; James *et al.*, 1980; Beatty *et al.*, 1980; Wong and Travis, 1980).

α 1Pi from other species

Rat α 1Pi

Rat α 1Pi has been isolated and partially characterized by several investigators (Rosenberg *et al.*, 1976; Ikehara *et al.*, 1981; Koj *et al.*, 1978a; Role and Glew, 1981; Takahara *et al.*, 1980; White *et al.*, 1981). Rat α 1Pi binds trypsin in a 1:1 molar ratio and shows microheterogeneity (5 bands) on isoelectric focusing. Ikehara and co-workers (1981) have suggested that post-translational

modification of amino acid residues causes a primary heterogeneity of rat α 1Pi, which is further modified by uneven sialylation during intracellular transport.

Rabbit α 1Pi

Rabbit α 1Pi was purified and characterized by Koj *et al.* (1978b, 1981) and Koj and Regoeczi (1978). Rabbit α 1Pi is present in serum as two distinct molecular variants designated S and F components for slow and fast electrophoretic variants and on isoelectric focusing, exhibits further microheterogeneity. These two variants are separately synthesized in the liver and independently metabolized in the circulation (Regoeczi *et al.*, 1980). The two α 1Pi variants are, however, functionally distinguishable by their binding kinetics as well as inhibitory capacities towards various proteases (Koj *et al.*, 1981).

Canine α 1Pi

Very little is known with respect to canine α 1Pi. The isolation of canine α 1Pi has revealed one band on SDS-PAGE (Schnitzlein *et al.*, 1980) and two bands on isoelectric focusing (Abrams *et al.*, 1978).

1.2.1.3 Biological function of α 1Pi

The main role of α 1Pi *in vivo* is the preservation of host tissue integrity during acute and chronic inflammation. This is achieved by the inhibition of proteolytic enzymes released by leukocytes in response to invading pathogens as well as by the inhibition of the humoral pathways of inflammation, as discussed in section 1.1.

In the recent past, a role for α 1Pi in the modulation of the immune response has been suggested. α 1Pi has been shown to be synthesized by lymphocytes (Ikuta *et al.*, 1982) and demonstrated on the surface of concanavalin-A stimulated lymphocytes (Lipsky *et al.*, 1979). The biological significance of the association of α 1Pi with lymphocytes is highly speculative. There is accumulating evidence that neutral proteases such as trypsin, elastase, chymotrypsin and thrombin are involved in the induction of lymphocyte activation (Chen *et al.*, 1976; Vischer, 1974; Hart and Streilein, 1976; Girard and Fernandes, 1976; Vischer *et al.*, 1976). Therefore, α 1Pi may function as a modulator of several cellular functions during the development of an immune response. This is supported by recent investigations in which α 1Pi was shown to prevent the *in vitro* and *in vivo* primary antibody responses to sheep erythrocytes in the mouse (Arora and Miller, 1978). Human α 1Pi was also shown to suppress DNA synthesis in stimulated human lymphocytes and to inhibit T- and B- lymphocyte cell surface protease activity (Bata *et al.*, 1981a, 1981b; Baranova *et al.*, 1978).

In conclusion, not only does α 1Pi play a major role in the modulation of the inflammatory response and in preserving tissue integrity but may also assume an important role in the regulation of the host immune response. This is particularly significant in inflammatory conditions caused by infections, whether bacterial, viral or parasitic, since the α 1Pi acute phase response may influence the subsequent development of immunity to the invading pathogens. It is only with a greater understanding of some of the mechanism(s) whereby α 1Pi synthesis is induced and regulated, that the *in vivo* functions and overall significance of the α 1Pi acute phase response can be put into proper perspective in the host response to pathogens.

1.2.2 C-reactive protein (CRP)

In 1930, it was discovered that human serum from patients with acute bacterial infections precipitated the C-polysaccharide substance (CPS) from pneumococcus. The precipitating protein was called C-reactive protein (CRP) and was shown to be an acute phase reactant (Tillet and Francis, 1930; Tillet *et al.*, 1930).

1.2.2.1 Biochemical characterization

Human CRP is a molecule with a molecular weight of 105,500 daltons and its structure consists of five identical, non-glycosylated, non-covalently associated subunits, in a disk-like arrangement with cyclic pentameric symmetry (Osmand *et al.*, 1977a; Pepys *et al.*, 1978a). Rat CRP has a slightly different configuration than human CRP due to

covalent association between subunits within the CRP molecule (De Beer *et al.*, 1982).

1.2.2.2 Biological functions of CRP

In vitro, human CRP binds to a number of ligands (Table 1.2.2.2). The binding of CRP to phosphoryl choline residues is particularly significant since materials containing such phosphoryl choline residues are ubiquitously distributed in diverse peptide-polysaccharides ("C-substances") of bacteria, fungus, plants and parasites including *Fasciola hepatica*, *Ascaris suum*, *Nippostrongylus brasiliensis* and *Haemonchus contortus* (Pepys and Longbottom, 1971; Tillet *et al.*, 1930; Baldo *et al.*, 1977). Such phosphoryl choline residues also occur in mammalian cell membranes.

Subsequent to binding of CRP to a particular ligand, CRP can mediate precipitation (Tillet and Francis, 1930), swelling of bacteria capsules (Lofstrom, 1944) and activation of complement (Kaplan and Volanakis, 1974; Siegel *et al.*, 1974).

Very little is known with respect to the *in vivo* biological function of CRP. The injection of complexed CRP into animals causes local vasculitis whereas subcutaneous deposition of CRP in man results in cutaneous vasculitis (Parish, 1976, 1977).

It has been suggested that an *in vivo* function for CRP is to enhance phagocytic activity and provide greater resistance to bacterial infection (Patterson and Higginbotham, 1965; Patterson *et al.*, 1968). However, according to Pepys (1981a), highly purified CRP has failed to reproduce these results.

TABLE 1.2.2.2

In vitro Biological Activities of Human CRP

<u>Biological Activity</u>	<u>References</u>
Calcium-dependent ligand binding to phosphorylcholine residues	Anderson <i>et al.</i> , 1978
Activates classical complement cascade upon complex formation with polyanions and polycations, soluble CPS, particulate CPS, CPS-coated erythrocytes, liposomes	Potempa <i>et al.</i> , 1981; Volanakis and Narkates, 1981; DiCamelli <i>et al.</i> , 1980; Kaplan and Volanakis, 1974; Siegel <i>et al.</i> , 1974; Mold and Gewurz, 1980; Mold <i>et al.</i> , 1981
Enhances phagocytosis of bacteria - <i>pneumococci</i> , <i>Staph. aureus</i> , <i>E. coli</i>	Kindmark, 1971
Binds to T-cells	Mortensen <i>et al.</i> , 1975; Croft <i>et al.</i> , 1976; Williams <i>et al.</i> , 1978
Inhibits spontaneous E-rosette formation, T-cell transformation, lymphokine production	Kinsella and Fritzler, 1980; Mortensen <i>et al.</i> , 1975, 1977
Suppressive effects on mixed lymphocyte cultures, generation of cytotoxic T-lymphocytes, antigen-induced proliferative response and antibody production	Mortensen and Gewurz, 1976; Mortensen <i>et al.</i> , 1977; Mortensen, 1979
Inhibits platelet function	Fiedel and Gewurz, 1976; Fiedel <i>et al.</i> , 1982

Based on all of these studies, Pepys (1981a) has proposed that the *in vivo* function of CRP is likely as an early broad-spectrum recognition mechanism for the products of pathogenic microorganisms as well as for products released from damaged cells such as poly-cations, polyanions and phospholipids. The main role for CRP is, therefore, to recognize, bind and mediate the clearance of potentially toxic materials, that is, detoxification during an inflammatory reaction. In addition, recent demonstrations of interaction of CRP with lymphocytes suggests a role for CRP in the modulation of the immune response.

In other species, mouse CRP is also an acute phase reactant whereas rat CRP serum levels do not rise to any significant extent during an acute inflammatory reaction. In addition, rat CRP does not precipitate upon complexing with CPS-coated particles and does not appear to activate complement (De Beer *et al.*, 1982). It is possible that the difference in functional properties of rat CRP may be related to its structural configuration.

1.2.3 Serum amyloid P-component (SAP)

1.2.3.1 Biochemical characterization

Serum amyloid P-component (SAP) was originally ~~described~~ by Cathcart *et al.* (1965, 1967) and is a polypeptide with a molecular weight of 235,000 daltons. SAP's structural configuration consists of ten identical glycosylated subunits, non-covalently associated in the form of two pentameric discs that interact face-to-face with a

tendency to stack one upon each other (Painter *et al.*, 1976; Pepys *et al.*, 1977a; Pinteric, 1976; Pepys, 1981b). SAP is closely related to CRP and there is 60% homology with CRP in the known amino acid sequences (Osmand *et al.*, 1977a; Skinner *et al.*, 1980), is also present in normal human vascular basement membranes (Dyck *et al.*, 1980a, 1980b) and on elastic fibre microfibrils in normal skin and blood vessels (Breathnach *et al.*, 1981).

SAP is, at the moment, the only acute phase reactant with potential diagnostic value for a specific type of diseases. Dyck *et al.* (1980c) have described distinctive abnormal immunohistochemical staining patterns for SAP in renal biopsy specimens from patients with diabetes and diverse nephritides. The recognition of such abnormal patterns of SAP staining may be of significant value as a diagnostic tool.

1.2.3.2 Biological functions

In vitro, SAP exhibits similar Ca^{2+} -dependence for ligand binding as CRP although it complexes with different molecules (Table 1.2.3.2).

Perhaps the most significant interactions are the binding of SAP with some microbial cell wall polysaccharides and to the fixed complement component, C3b. The *in vivo* functional significance of the binding to these ligands is unknown. SAP may facilitate agglutination and/or phagocytosis.

Other recently described ligands are fibronectin and C4 binding protein. Fibronectin is a major cell surface protein of many

TABLE 1.2.3.2

In vitro Biological Activities of SAP

<u>Biological Activity</u>	<u>References</u>
Binds to: isolated amyloid fibrils	Pepys <i>et al.</i> , 1979a; Pepys, 1981b
Galactans (agarose)	Pepys <i>et al.</i> , 1977b; 1977a
Microbial cell wall polysaccharides (zymosan)	Pepys <i>et al.</i> , 1979a
Fibronectin and C4 binding protein (C4bp) - (Ca ²⁺ dependent)	De Beer <i>et al.</i> , 1981
Complexes with fixed C3b (agglutination of complement- coated erythrocytes)	Hutchcraft <i>et al.</i> , 1981

different cell types and plays a role in cell-cell and cell-substratum interactions as well as assuming the function of a non-specific opsonin in the phagocytic function of the reticulo-endothelial system. SAP, upon complexing with fibronectin, may affect such functions.

C4 binding protein is a regulatory protein of the classical pathway of complement and binds to antibody-antigen complexes (Fujita *et al.*, 1978; Nagasawa *et al.*, 1980). It is possible that SAP may have an effect on this interaction with immune complexes. However, the exact *in vivo* significance of SAP binding to fibronectin and C4 binding protein is highly speculative.

Boxer *et al.* (1977) reported that SAP functions as a heparin antagonist, that is, a coagulation factor, although Pepys *et al.* (1980) have failed to demonstrate such activity.

Although SAP is an acute phase reactant in the mouse (Pepys *et al.*, 1979b; Pepys, 1979), it is not an acute phase protein in rats (De Beer *et al.*, 1982) nor in humans, although in some patients with chronic degenerative diseases, serum SAP is slightly elevated (Pepys, 1981; Pepys *et al.*, 1978b).

1.2.4 Serum amyloid A (SAA)

1.2.4.1 Biochemical characterization

The fibrils in secondary systemic amyloidosis are mainly composed of a fibrillar protein called amyloid A protein. Protein AA is thought to originate from SAA, a serum protein which is

antigenically and chemically related to AA (Rosenthal *et al.*, 1976; Linder *et al.*, 1976; Ignaczak *et al.*, 1977; Benson *et al.*, 1975). It has also been suggested that protein AA probably arises as a result of proteolytic cleavage of SAA (Lavie *et al.*, 1978).

SAA is a single polypeptide chain of molecular weight 11,000 - 14,000 (Anders *et al.*, 1975; Rosenthal *et al.*, 1976; Ignaczak *et al.*, 1977; Linke *et al.*, 1975) although its apparent molecular weight has been reported as 85,000 - 205,000 due to its association in plasma with high density lipoprotein (HDL) (Benditt and Eriksen, 1977). SAA may account for as much as 25% of the HDL apoproteins from acute phase serum (Bausserman *et al.*, 1980).

There are six polymorphic forms of human SAA all having a similar amino acid composition (Bausserman *et al.*, 1980). The heterogeneity does not apparently arise from varied neuraminic acid content. In another species, mouse SAA has also been shown to be polymorphic (Gorevic *et al.*, 1978).

1.2.4.2 Biological functions

SAA is an acute phase reactant in human and mouse (McAdam *et al.*, 1978; McAdam and Sipe, 1976; Rosenthal and Franklin, 1975; Gorevic *et al.*, 1976) but the *in vivo* function of SAA is not known.

SAA may play a role in the rapid clearance of HDL as a result of its strong association with the lipoprotein (Benditt *et al.*, 1980; Skogen *et al.*, 1979).

SAA has also been implicated as an immunoregulatory protein since it has been shown to inhibit specific induction of antibody to T-dependent antigens *in vitro* (Benson and Aldo-Benson, 1979).

In conclusion, the acute phase reactants, α 1Pi, CRP, SAP and SAA exert a wide range of biological and biochemical activities towards products or components of a variety of pathogenic organisms such as bacteria, parasites and possibly viruses. In the case of α 1Pi, CRP and SAA, an immunoregulatory function is also probable. However, we know very little with respect to the extent that these various functions occur *in vivo* as well as the individual and overall effects of these acute phase reactants on host-pathogen interactions.

It is, therefore, crucial to obtain a greater understanding of the mechanism(s) for the induction of the synthesis of these acute phase reactants, their sites of synthesis, their function(s) at the inflammatory site and their mode(s) of degradation.

1.3 Site of Synthesis and Tissue Localization of Acute Phase Reactants

The site of synthesis of acute phase reactants has been the subject of numerous investigations and although many cell types exhibit variable degrees of synthetic activity for some of the APR, it appears that the hepatocyte is the major source of these proteins.

1.3.1 α 1 protease inhibitor

Human α 1Pi

Using immunohistochemistry, human α 1Pi has been demonstrated in a variety of cell types (Table 1.3.1).

TABLE 1.3.1

Immunohistochemical Localization of Human α 1Pi

<u>Cell type</u>	<u>References</u>
Hepatocytes from homozygous and heterozygous α 1Pi deficiency	Ray and Desmet, 1975; Palmer <i>et al.</i> , 1974; Hadchouel and Gautier, 1976; Lieberman <i>et al.</i> , 1972; Gordon <i>et al.</i> , 1972; Sharp, 1971; Palmer and Wolfe, 1976; Palmer <i>et al.</i> , 1977
Hepatocytes from normal PiM allele	Feldmann <i>et al.</i> , 1976;
Bile duct epithelium of PiZ gene carriers	Hadchouel and Gautier, 1976
Proliferating epithelial cells of bile ductule	Tanaka <i>et al.</i> , 1980
Hepatoma cell lines	Reintoft and Hagerstrand, 1979; Gerber <i>et al.</i> , 1981 Turner and Turner, 1980
Peripheral blood macrophages, tissue macrophages of spleen, lymph nodes and small intestine, alveolar macrophages, Kupffer cells	Isaacson <i>et al.</i> , 1979, 1981; Gupta <i>et al.</i> , 1979; Cohen, 1973a; Geboes <i>et al.</i> , 1982; Olsen <i>et al.</i> , 1975
Concanavallin A stimulated lymphocytes (cell surface)	Lipsky <i>et al.</i> , 1979
Mast cells in the pancreas, small intestine, skin and connective tissue of the cervix and uterus	Geboes <i>et al.</i> , 1982; Ray <i>et al.</i> , 1977; Benitez-Bribiesca <i>et al.</i> , 1973
Platelets	Nachman and Harpel, 1976; Nalli <i>et al.</i> , 1977; Bagdassarian and Colman, 1975, 1978
Megakaryocytes	Nalli <i>et al.</i> , 1977

TABLE 1.3.1 (cont'd)

<u>Cell type</u>	<u>References</u>
Islet cells of the pancreas, parenchymal pancreatic cells	McElrath <i>et al.</i> , 1979; Ray <i>et al.</i> , 1977; Ray and Desmet, 1978
Polymorphonuclear leukocytes	Benitez-Bribiesca and Freyre-Horta, 1978.
Epithelial cells in villi and crypts of small intestine	Geboes <i>et al.</i> , 1982

It is still inconclusive whether the presence of α Pi in these various cell types represents *de novo* synthesis or uptake into the cytoplasm.

Monocytes have been shown to synthesize α Pi although some of the intracellular α Pi was attributable to endocytosis of protease-antiprotease complexes (Isaacson *et al.*, 1981; Wilson *et al.*, 1980). Lipski *et al.* (1979) have suggested that the expression of α Pi on the surface of concanavalin A stimulated lymphocytes was a result of active synthesis by the lymphocytes as opposed to passive uptake. More recently, it has been shown that α Pi synthesis by lymphocytes is greatly enhanced by a factor(s) released from concanavalin A stimulated macrophages (Ikuta *et al.*, 1982). However, the synthesis of α Pi by monocytes and lymphocytes is likely minimal in comparison to hepatocytes.

The hepatocyte has been implicated as the major site of synthesis of α Pi (Sharp, 1971; Hood *et al.*, 1980; Putnam *et al.*, 1977; Eriksson *et al.*, 1978). The best supportive evidence is the fact that in human liver transplantation, the serum α Pi phenotype of the recipient converts to that of the donor, following surgery (Hood *et al.*, 1980).

Rat α Pi

The major site of synthesis of rat α Pi is the liver (Koj *et al.*, 1978a) although rat alveolar macrophages have been shown to synthesize small amounts of α Pi (White *et al.*, 1981).

Rabbit α Pi

Rabbit α Pi is also synthesized primarily by the liver (Koj and Regoeczi, 1978).

1.3.2 C-reactive protein

The hepatocyte has been shown to be the major source of synthesis of rabbit CRP (Horowitz *et al.*, 1979; Hurlimann *et al.*, 1966; Kushner and Feldmann, 1978; Kushner and Sommerville-Volanakis, 1976) and it is likely that mouse CRP is also derived from the liver (Bodmer and Siboo, 1977).

1.3.3 Serum amyloid P

Serum amyloid P (SAP) has been shown to be present in human fibroblasts (Spark *et al.*, 1978) and in the cytoplasm of mouse hepatocytes as well as in perifollicular regions in the spleen in response to multiple injections of casein and hepatocytes have been implicated as the major site of synthesis of SAP (Baltz *et al.*, 1980).

1.3.4 Serum amyloid A

Serum amyloid A (SAA) has been localized in mouse hepatocytes and kidney when induced by endotoxin and also shown to be synthesized by hepatocytes (Benson and Kleiner, 1980; Selinger *et al.*, 1980).

SAA has also been reported to be synthesized by connective tissue fibroblasts (Linder *et al.*, 1976; Johnson *et al.*, 1977) and polymorphonuclear leukocytes (Rosenthal and Sullivan, 1978). The synthesis of SAA by lymphoid cells of the spleen is controversial (Watanabe *et al.*, 1977; Bauma *et al.*, 1970; Benson and Kleiner, 1980).

1.3.5 Other acute phase reactants

The immunohistochemical localization and sites of synthesis of various acute phase reactants is summarized in Table 1.3.5.

In conclusion, it is evident from these studies that acute phase reactants are widely distributed amongst the various cell types in the body. Although it is likely that the localization of acute phase reactants in the cytoplasm or the surface of these cell types is attributed to uptake and in some cases minimal synthesis, the major source of serum acute phase reactants, except perhaps C3, is hepatocyte synthesis.

The role for these acute phase reactants in the various cell types is unknown. However, one can speculate that a major role for α 1Pi synthesis other than hepatic may be to protect the cell from a highly proteolytic microenvironment during an inflammatory reaction and/or constitute an effective means of releasing α 1Pi directly into an inflammatory foci. Although synthesis *in vitro* may be minimal in comparison to hepatocytes, the effective concentration of α 1Pi in the microenvironment of the cell may be significant.

1.4 Endogenous Mediators of the Acute Phase Inflammatory Response

Koj (1974) originally proposed that the induction of acute phase protein synthesis by hepatocytes was mediated by a factor(s) originating from the site of tissue injury or trauma. Evidence in support of such a proposition was provided by Kushner and Feldmann

TABLE 1.3.5

Immunohistochemical Localization and Sites of Synthesis of Various Acute Phase Reactants

Acute Phase Reactant	Synthesis	Localization	Reference
α 2-macroglobulin		surface of B-lymphocytes	McCormick <i>et al.</i> , 1973
	hepatocytes		Gitlin and Biasucci, 1969; Courtoy <i>et al.</i> , 1981; Koj, 1980
	monocytes		Tunstall and James, 1974; Hovi <i>et al.</i> , 1977; Mosher <i>et al.</i> , 1977
	alveolar macrophages		White <i>et al.</i> , 1980
	hepatocyte		Courtoy <i>et al.</i> , 1981; Legrele <i>et al.</i> , 1980; Forman and Barnhart, 1964; Kwan and Fuller, 1977; Rupp and Fuller, 1979
α 1 acid glycoprotein	hepatocyte		Courtoy <i>et al.</i> , 1981
		surface of leukocytes	Gahmberg and Andersson, 1978

TABLE 1.3.5 (cont'd)

Acute Phase Reactant	Synthesis	Localization	Reference
haptoglobin	hepatocyte		Courtoy <i>et al.</i> , 1981; Hooper <i>et al.</i> , 1964, 1965; Mouray <i>et al.</i> , 1964; Peters and Alper, 1966
C3	organ cultures of mouse spleen, trachea, Peyer's Patches, liver, lung		McClelland and Van Furth, 1976
	organ cultures of small intestine		Whaley, 1980
	glass-adherent cells of bone marrow, blood, lung washings and subcutaneous tissues, peritoneal and alveolar macrophages		Whaley, 1980; Einstein <i>et al.</i> , 1977
	hepatocytes		Alper <i>et al.</i> , 1969

(1978) in their studies of the rabbit CRP acute phase response during acute inflammation. However, a subject of much debate is what cell type(s) is/are involved in the production of such factor(s) and whether all acute phase reactants are synthesized in synchrony. In addition, very little is known with respect to the triggering mechanism at the hepatocyte for APR synthesis.

The acute phase inflammatory reaction is generally characterized by the onset of fever, a decrease in plasma iron and zinc as well as an increase in neutrophil release into the peripheral circulation from the bone marrow and a rapid and transient elevation in serum levels of the acute phase reactants (Pekarek and Beisel, 1971; Pekarek *et al.*, 1969, 1970; Marsh *et al.*, 1967; Williams and Johnson, 1976; Rosensheim *et al.*, 1979).

Although bacterial endotoxins (LPS) have been shown to express pyrogenic activity (Gillman *et al.*, 1961) and induce hypoferremia (Kampschmidt and Upchurch, 1962; Kampschmidt *et al.*, 1965, 1969), zinc depression (Pekarek and Beisel, 1969), increased peripheral blood neutrophil count (Kampschmidt and Upchurch, 1980) as well as increased *in vivo* synthesis of SAA (Watson *et al.*, 1978; McAdam and Sipe, 1976; Sipe *et al.*, 1979) and fibrinogen (Weidner *et al.*, 1979), it is likely that these effects are mediated indirectly through the generation of endogenous acute phase mediator(s) (APM). That this is the situation is supported by the fact that these acute inflammatory serum changes can be initiated by many different stimuli including endotoxin, gram negative bacteria, gram positive bacteria, certain fungi, myxoviruses, DNA, poly I-poly C, colchicine, synthetic

muramyl dipeptides (adjuvants), phorbol myristic acetate and certain immunologic stimuli such as antigen-antibody immune complexes and antigens to which there is an established state of delayed-type hypersensitivity (Oken *et al.*, 1981; Bernheim *et al.*, 1979). Moreover, LPS (endotoxin) does not directly produce significant stimulation of haptoglobin synthesis relative to albumin in isolated hepatocyte monolayer cultures (Hooper *et al.*, 1981).

Beeson (1948) was the first to recognize that leukocytes were capable of producing a substance with temperature-elevating or fever inducing properties. Subsequent studies have revealed a variety of molecules with pyrogenic activities as well as numerous biological activities.

Endogenous pyrogen (EP), commonly referred to as leukocyte pyrogen (LP) or crude leukocyte extract (CLE) has been ascribed numerous biological activities (Table 1.4.1).

Pekarek *et al.* (1972a, 1972b, 1972c) have described a molecule with similar pyrogenic and biological activities and called it leukocyte endogenous mediator (LEM). The biological activities of LEM have been reviewed by Kampschmidt (1978).

Sipe and colleagues (1979) and Selinger *et al.* (1980) have recently described a serum amyloid A stimulating factor (SAASF) arising from LPS stimulated macrophages. This stimulating factor was capable of inducing the synthesis of SAA in isolated hepatocyte cultures as well as *in situ* upon injection to LPS non-responder mice.

Amongst the monokines elaborated by macrophages under appropriate stimuli is a small molecular weight peptide whose

TABLE 1.4.1

Biological Activities of EP (LP, CLE)

<u>Biological Activity</u>	<u>Reference</u>
Induce fever	Kozak <i>et al.</i> , 1968;
Decrease of plasma iron and zinc	Kampschmidt and Upchurch, 1970a, 1969a;
Increase release of neutrophils from bone marrow	Bornstein and Walsh, 1978; Gordon and Parker, 1980; Turchik and Bornstein, 1980; Merriman <i>et al.</i> , 1977; Kampschmidt and Upchurch, 1969; Kampschmidt and Pulliam, 1978
Increase serum concentration of: fibrinogen, α 2-macroglobulin, CRP, haptoglobin, SAA, ceruloplasmin	Merriman <i>et al.</i> , 1977; Bornstein and Walsh, 1978; Weidner <i>et al.</i> , 1979; Seligsohn and Klein, 1975; Turchik and Bornstein, 1980; Eddington <i>et al.</i> , 1972; McAdam and Dinarello, 1980; Sztein <i>et al.</i> , 1981
No significant effects on serum α 1Pi nor albumin synthesis	Bornstein and Walsh, 1978; Weidner <i>et al.</i> , 1979

biological activity consisted of inducing lymphocyte proliferation and hence referred to as lymphocyte activating factor (LAF). LAF was shown to exhibit a broad spectrum of *in vitro* immunological activities which are summarized in Table 1.4.2. According to accepted nomenclature, LAF is now designated as Interleukin 1 (IL1) (Mizel and Farrar, 1979).

Sztejn *et al.* (1981) have also shown that SAASF activity is indistinguishable from LAF (IL1) activity and as such these two factors likely represent the same molecular entity.

It has now become apparent that these putatively different endogenous mediators of the acute phase phenomenon all belong to the same class of molecules, if not identical molecules. Indeed, numerous studies in which these molecules have been purified to apparent homogeneity and their biological activities and biochemical properties compared, have been unsuccessful in separating or distinguishing them from each other.

Therefore, LEM, LP, EP, CLE, SAASF, LAF and IL1 probably represent one distinct molecular species. Although these molecules have been so far proven unseparable on the basis of biochemical and biological properties, it is only when the full amino acid sequence of these molecules is known and compared, that their biochemical relationship will be definitively established. Throughout the present work these molecules will be referred to as acute phase mediator (APM).

TABLE 1.4.2

Biological Activities of Lymphocyte Activating Factor (LAF)

<u>Biological Activity</u>	<u>Reference</u>
Enhancement of thymocyte proliferation in response to T-cell mitogens and antigens	Mizel and Farrar, 1979; Mizel, 1980; Mizel and Mizel, 1981
Initiation of <i>in vitro</i> antibody responses to particulate antigens	
Initiation of alloantigen specific cytotoxic T-lymphocytes	
B-cell activation	
Enhancement of Interleukin 2 (IL2) production	
Induces fever	
Increases serum concentration of SAA	Sztejn <i>et al.</i> , 1981

The mechanism(s) whereby APM induces all of these serum changes during acute inflammation is not fully understood.

With respect to the pyrogenic activity of APM, it is likely that fever is induced by direct interaction of EP with the anterior portion of the hypothalamus (Snell, 1971; Bornstein and Woods, 1969; Turchik and Bornstein, 1980).

With respect to the induction of acute phase reactant synthesis by the liver, LEM (IL1) has been shown to stimulate the incorporation of orotate into ribosomes, induce the intracellular accumulation of α -amino isobutyric acid, increase the influx of amino acids into the liver and increase the synthesis of hepatic RNA (Thompson *et al.*, 1976; Wannemacher *et al.*, 1972, 1975). Moreover, there is an accelerated movement of amino acids from muscle to liver within one hour after injection of LEM (Powanda *et al.*, 1973).

It is possible that the effect of LEM on hepatocytes is synergistic with another factor(s) as evidenced by only a moderate acute phase response when pure LEM is introduced into isolated hepatocyte culture systems (Kampschmidt and Upchurch, 1974; Rupp and Fuller, 1979a; Selinger *et al.*, 1980). To this extent, introduction of an "appropriate" amount of purified APM into the peritoneal cavity, the peripheral circulation or into the hypothalamus gives rise to increased serum levels of acute phase reactants comparable to that induced by conventional experimental inflammatory stimuli (Sipe *et al.*, 1979; Weidner *et al.*, 1979; McAdam and Dinarello, 1980; Kampschmidt and Upchurch, 1980a; Turchik and Bornstein, 1980; Bailey *et al.*, 1976; Szein *et al.*, 1981). In these *in situ* systems,

other putative factor(s) normally present in the circulation may act synergistically with *de novo* introduced purified APM to induce APR synthesis by hepatocytes. Hormones are likely involved since the simultaneous incubation of isolated hepatocytes with CLE and cortisol gives rise to increased fibrinogen output comparable in intensity to the increase observed *in vivo*, following turpentine stimulation (Rupp and Fuller, 1979, 1979a). Similar findings were reported by Hooper *et al.* (1981) although the extent of stimulation of haptoglobin was not as marked in comparison to the *in vivo* response. This may indicate that other factor(s) besides hormones may also be involved. Moreover, it has been suggested that the increased fibrinogen synthesis comes about by making more mRNA available, that is, a transcriptional stimulation.

A role for hormones in the mediation of the acute phase response is further indicated by the fact that the effects of IL1 on APR synthesis is blunted in hypophysectomized or adrenalectomized rats (Thompson *et al.*, 1976; Moretti *et al.*, 1966; Krauss, 1963).

A few investigators have also postulated that the central nervous system may play a role in the mediation of the acute phase response (Kampschmidt *et al.*, 1973; Bailey *et al.*, 1976; Turchik and Bornstein, 1980).

It was shown that far less LP was required to reproduce the serum changes in the acute phase reaction if introduced intracerebrally rather than intravenously. It is not known whether the pathway is neural, endocrine or neurohumoral or a combination of the above. The liver contains numerous adrenergic and parasympathetic fibers (Koo and

Liang, 1979; Koo *et al.*, 1977) and it is possible that the central nervous system plays a non-specific stimulatory function in that the hepatocytes may contain receptors for APM whereas the CNS controls the degree of stimulation (Turchik and Bornstein, 1980). Clearly, further studies are required in order to establish a role for the central nervous system in the induction of acute phase reactant synthesis.

1.5 Parasite Interactions with Host Humoral Pathways of Inflammation

In the present studies, parasitic infections are used as experimental models of inflammation. Their suitability as inflammation models is discussed in Chapter 2.

Metazoan helminth and protozoan parasites cause widespread disease primarily in tropical and sub-tropical developing countries. It is estimated that more than 1600 million people are afflicted by parasitic infections of some kind or other and in addition, millions of cattle die of parasitic diseases each year with a further 700 million diseased to the extent that they are not fit for human consumption nor can they be used for work on farms. Moreover, in Africa alone, 7 million square kilometres of grazable land capable of supporting 120 million head of cattle are essentially of no use for feeding (Bloom, 1979). The social and economic impact of parasitic disease is far-reaching even in North America and Europe where devastating parasitic infections such as schistosomiasis, leishmaniasis, filariasis and malaria are of rare occurrence.

The modes by which parasites infect humans and cattle, the vectors and intermediate host and the various migration routes of

these parasites within host tissues has been extensively described (Schmidt and Roberts, 1981). The biochemistry and physiology of parasites has also been documented (Brand, 1979) and have provided a firm basis for the understanding of how parasites adapt to a multitude of environmental conditions throughout their life cycle as well as to their requirements for survival.

Another area which has received considerable attention recently is the pathology associated with the parasites as it interacts with the host. Although the gross features of the pathology such as splenomegaly, elephantiasis, anemia, cutaneous lesions and many others have been extensively described, very little is known with respect to the pathological events occurring in the microenvironment of the host. The cell types involved in the various parasitic diseases have been the subject of many reviews (Henson *et al.*, 1979; Askenase, 1977; Tizard *et al.*, 1978; Askenase, 1980; Mitchell, 1979).

With the emergence of a more comprehensive account of the interrelationship of the humoral pathways of inflammation and the cellular components of the inflammatory response, a firm basis is provided for the study of the host-parasite interaction.

The interaction of parasites with host humoral pathways of inflammation has been the subject of recent reviews (Leid, 1981; Leid and Williams, 1979; Askenase, 1980; Santoro *et al.*, 1979) and is described in the following sections.

1.5.1 Coagulation system

The nematode *Ancylostoma caninum* produces an anticoagulant which interferes with activated Factor X (Thorson, 1956; Eiff, 1966; Spellman and Nossel, 1971). Similar effects were observed for *Strongylus* spp. (reviewed in Leid and Williams, 1979) and *Haemonchus contortus* (Schwartz, 1921). However, little effort has been made to characterize either the agents responsible for inhibition of clot formation or the site(s) at which the coagulation pathway is disrupted. It appears, however, that in the case of *A. caninum*, the terminal portion of the coagulation cascade is affected (Eiff, 1966) whereas in *Angiostrongylus vasorum*, both the extrinsic and intrinsic pathways are affected (Dodd, 1973).

A surface extract of the cestode *Taenia taeniaeformis* yields a macromolecular polysulfated polysaccharide (proteoglycan) which inhibits the coagulation sequence at the initial stages of the sequence (Hammerberg *et al.*, 1980). There is little or no interference with later phases of the coagulation cascade although Leid (1977) has shown that culture products of *T. taeniaeformis* prevent the stabilization of fibrin clots by interacting with Factor XIII. Extracts of *Echinococcus granulosus* when injected I.V. into dogs prevented blood coagulation (Rocha and Grana, 1945).

Perhaps one of the most significant interactions of parasite products with the coagulation cascade is the inhibition of activation of Factor XI by activated Hageman Factor by the interaction of adult trematode *Schistosoma mansoni* product with activated Hageman Factor

(Tsang *et al.*, 1977; Tsang and Damian, 1977). In this case, there is no thrombin formation around living parasites *in vivo* but death of the worms rapidly leads to the development of thromboembolic lesions around the dead parasite. Such an interaction has far more reaching implications since by direct interaction with activated Hageman Factor, the parasite can influence the activation of the other inflammatory mediator pathways due to the pivotal role of activated Hageman Factor. It would, therefore, appear that the interactions of parasite products with the coagulation sequence are beneficial to the parasite since such interference likely minimizes tendencies for blood clots to form around parasites thereby allowing for easier migration through tissues. Moreover, the prevention of Hageman Factor-dependent activation of biologically active molecules would exert a significant modulatory effect on the local tissue response by modulating the infiltration of host inflammatory cells.

1.5.2 Kinin system

There are very few examples of direct interaction of parasites with the enzymes of the kinin pathway. However, infection of rats with the nematode *Trichinella spiralis* results in significantly decreased plasma kininogen levels (Leid and Williams, 1979), suggestive of activation of the kinin system by some as yet undetermined mechanism.

Leid and Williams (1979) have described an experiment whereby the injection of bradykinin into rat gut allowed for greater number of cysts of *T. taeniaeformis* to form in the liver. It was postulated that increases in local vascular permeability allowed more activated

oncospheres to migrate from the intestinal epithelium to the liver.

Kinins have also been shown to be generated during trypanolysis in the protozoan infection with *Trypanosoma* spp. (Resch and Ferber, 1975; Veenendaal *et al.*, 1976).

The interaction of parasite products with enzymes of the kinin system may be both beneficial and detrimental to the parasite. In one instance, the activation of the kinin system leads to increased vascular permeability which may facilitate the migration of the parasite through the various tissues and may constitute a mechanism whereby nutrients may be concentrated in the immediate vicinity of the parasite. On the other hand, the activation of the kinin system gives rise to enzymes, which have chemotactic properties for inflammatory cells which have been implicated in the mediation of host-protective defense mechanisms against parasites (Askenase, 1977, 1980; Henson *et al.*, 1979; Mitchell, 1979).

1.5.3 Fibrinolytic system

There is one documented example of a potential interaction of parasites with the fibrinolytic system which describes an increase in serum plasmin concentration and fibrinogen degradation products by a protease released from *Trypanosoma brucei brucei* (Boreham and Facer, 1973). The significance of this interaction has not been further explored but since plasmin plays such a pivotal role in the activation of the other molecular pathways of inflammation, it is worth pursuing.

1.5.4 Complement system

The interaction of parasites and/or parasite products with complement components has been more extensively studied, partly because of its more obvious importance in host protective immunity to parasite infections either directly as a lytic system or by promoting cellular adherence to parasites with subsequent destruction.

Complement components have been found on the tegument of the nematode *Ascaris suum* (Ziprin and Jeska, 1975). Complement component C3b has been found on the surface of infective *Trichinella spiralis* larvae and adults as well as *Nippostrongylus brasiliensis* and appears to mediate eosinophil adherence and subsequent killing of the parasite *in vitro* (Mackenzie *et al.*, 1980).

Ascaris suum and the cestode *Echinococcus granulosus* release a phospholipid which can activate the alternate pathway of complement (Archer *et al.*, 1977) while *E. multilocularis* activates complement via the classical pathway (Kassis and Tanner, 1976, 1977). In either case, the activation of the complement cascade kills the parasite. C3 has also been found on the tegument of *Hymenolepis diminuta* and *H. microstoma* in the mouse (Befus, 1977; Threadgold and Befus, 1977).

Although it would appear that complement activation by parasites or parasite products is detrimental to the parasite due to direct killing of parasite as well as through the generation of inflammatory cell chemotactic factors and promotion of cell adherence, *T. taeniaeformis* and *T. crassiceps* have evolved a means of interfering

with C-mediated attack of their membranes by releasing a factor which "consumes" complement components around the parasite and prevents the assembly of attack sequences on the parasite surface (Hammerberg *et al.*, 1976, 1977; Hammerberg and Williams, 1978a, 1978b). This ability to "consume" complement components may allow for the successful evasion of the parasite from inflammatory cells by a decreased generation of chemotaxins.

The trematode *Schistosoma mansoni* has perhaps been the most widely studied with respect to its interaction with complement components (Santoro *et al.*, 1979). Incubation of *S. mansoni* cercariae and normal serum activates the alternate pathway of complement (Machado *et al.*, 1975). *S. mansoni* products have also been shown to generate anaphylatoxic activity in normal serum (Gazzinelli *et al.*, 1969).

In the case of protozoan parasites, *Trypanosoma cruzi* incubation with normal serum results in the activation of alternate complement pathway and the killing of all epimastigote forms (Nogueira *et al.*, 1975; Kierszenbaum and Weinman, 1977; Kierszenbaum *et al.*, 1976). Similar results were observed for *T. congolense* (Nielsen *et al.*, 1979) although the classical pathway of complement was also activated. Antigens from *T. brucei*, *T. lewisi* and *T. congolense* have also been shown to activate complement (Musoke and Barbet, 1977; Nielsen and Sheppard, 1977; Assoku *et al.*, 1977). The activation of complement by trypanosomes is usually detrimental to the parasite.

Plasmodium coatneyi, *P. berghei*, *P. vivax* and *P. falciparum* can also activate complement and schizont rupture usually occurs (Atkinson *et al.*, 1975; Neva *et al.*, 1974). However, *Babesia rodhaini* will also activate complement but unlike with *Plasmodium* spp. (Ward *et al.*, 1981), the ability of *B. rodhaini* to penetrate and infect red blood cells is dependent on factors of the alternate pathway of complement (properdin and Factor B) and C3 and C5 (Chapman and Ward, 1977; Jack and Ward, 1980, 1980a). Hence, with *Plasmodium* spp., the activation of complement is usually beneficial to the host whereas with *Babesia rodhaini*, activation of complement appears detrimental to the host.

Therefore, with respect to the direct interaction of parasites or parasite products with the complement cascade, that is, in the absence of antibody mediated activation of complement, it appears that there are both beneficial and detrimental effects. In other instances, activation of complement leads to parasite killing either by the direct assembly of lytic components on the surface of parasites or by mediating cell adherence with subsequent killing. On the other hand, the activation of complement leads to increased vascular permeability and affords ease of migration as well as nutrient provision. However, the generation of chemotactic factors for inflammatory cells may be detrimental to the parasite due to cytolytic activity.

In conclusion, most of the work described in relation to host-parasite interactions was generated *in vitro*. It is, therefore, difficult to extrapolate to *in vivo* situations. Clearly, a great deal of research is required in order to provide more evidence for the direct interaction of parasites with host humoral pathways of inflammation. It is evident, however, that some of the interactions described provide an important component of host protective mechanisms. The study of such interactions will provide a better understanding of host protective mechanisms against parasites as well as the role of the inflammatory response in specific and non-specific immune defense mechanisms.

CHAPTER 2

PURPOSE OF THE STUDY AND OUTLINE OF THE ANIMAL MODELS

2.1 Summary of the Literature and Conclusions

A large portion of the literature on acute phase reactants, in particular the acute phase proteins α Pi, SAP and SAA, focuses on their role in health and disease.

α Pi

Human α Pi is highly polymorphic and exhibits marked microheterogeneity. The most common phenotypes, PiM, PiS and PiZ, have been widely studied with respect to their association(s) with disease states. There is a strong association between the homozygous ZZ deficiency state and onset of pulmonary emphysema in young adults and childhood liver cirrhosis. In both instances, the current hypothesis for the pathogenesis of the diseases proposes that the reduced inhibitory capacity for proteolytic enzymes results in tissue destruction. However, studies in which individuals were heterozygous for α Pi deficiency (PiMS, MZ), that is, α Pi serum levels were in the intermediate range, yielded highly divergent results. It is still inconclusive whether such individuals are at an increased risk for development of pulmonary emphysema and/or liver cirrhosis.

SAP

Amyloidosis is characterized by the presence of amyloid fibrils in a variety of tissues but the role for SAP in both primary and secondary amyloidosis has not been delineated. Protein AP (P-component)

is a universal constituent of amyloid deposits and AP is identical to protein SAP, a normal serum protein. SAP has calcium-dependent binding affinity for a variety of ligands including isolated amyloid fibrils but how this is related to deposition of AP in amyloidosis is not known.

SAA

The fibrils in secondary systemic amyloidosis are mainly composed of a fibrillar protein called amyloid A protein. Protein AA is thought to originate from SAA, an acute phase reactant in normal serum which is antigenically and chemically related to AA. It has also been suggested that protein AA probably arises as a result of proteolytic cleavage of SAA.

In the past few years, there has been a considerable amount of literature describing the biochemical characteristics as well as the *in vitro* biological functions of α 1Pi, CRP, SAP and SAA. Unfortunately, we know very little with respect to the *in vivo* biological functions of these acute phase reactants. However, on the basis of the *in vitro* information, we can postulate on an *in vivo* role. It is important to realize that the *in vitro* data was generated under optimal conditions and as such, the extent to which the various interactions occur *in vivo* is subject to debate.

In summary, α 1Pi is an acute phase reactant in the rat, rabbit, mouse and likely in the human. It is a major inhibitor of neutral proteases, elastase, collagenase and trypsin. In addition, α 1Pi also

exhibits inhibitory activity against some effector molecules of the humoral pathways of inflammation such as thrombin, kallikrein and plasmin. The affinity constant for these effector molecules is considerably less than that of neutral proteases and elastase but by virtue of its relatively high concentration in serum, α 1Pi is a significant inhibitor. Therefore, the main function of α 1Pi is likely to preserve tissue integrity and limit tissue destruction due to proteolysis during inflammation. Because of its interaction with certain effector molecules of the humoral pathways of inflammation, α 1Pi may also function as a regulator of the acute inflammatory response.

SAP binds to a variety of ligands in a calcium-dependent fashion. Perhaps the most significant interactions are the binding of SAP with some microbial cell wall polysaccharides (zymosan) and to the fixed complement component, C3b. The functional significance of this ligand binding is highly speculative, SAP may facilitate agglutination and/or phagocytosis.

SAA, *in vivo*, is associated with high density lipoprotein (HDL) and likely mediates the rapid plasma clearance of the lipoprotein. Other biological functions of SAA are as of yet unknown.

CRP, in the presence of calcium, binds to numerous ligands and perhaps the most significant interaction is binding to phosphoryl choline residue. Phosphoryl choline is ubiquitously distributed amongst bacteria, fungi, parasites and mammalian cell membranes. The binding of CRP to various ligands can result in precipitation, agglutination and activation of the classical complement pathway.

Therefore, it has been proposed that CRP may function, *in vivo*, as an

early recognition system for pathogenic invasion and constitute a non-specific defense mechanism.

* A major portion of the literature on the biological functions of these APR has dealt with the interaction of these molecules with a variety of other proteins and enzymes. However, in the recent past, the interactions of these APR with a variety of cell types has received considerable attention. α 1Pi, CRP and to some extent, SAA, have been shown to interact with lymphocytes and exert marked effects on their *in vitro* response to a variety of stimuli.

The *in vitro* interactions of these acute phase reactants with cells directly involved in mediating both cellular and humoral immunity is potentially very significant. This would imply that not only do these acute phase reactants perform a vital function by either maintaining tissue integrity, or functioning as an early recognition and non-specific defense mechanism or serve as a "detoxification" system, but they may also modulate the subsequent development of specific immunity to the injurious stimuli. With the emergence of a better understanding of the interrelationships between the humoral pathways of inflammation as well as the relationships between acute inflammation and specific immunity, we now have a basis for investigating the role of acute phase reactants.

2.2 Specific Objectives of the Study

It is suggested that α 1Pi is an acute phase reactant in the mouse and that its synthesis in hepatocytes can be induced by various inflammatory reactions.

The specific objective of the study was to investigate, both *in vivo* and *in vitro*, the α 1Pi acute phase response in a variety of inflammatory conditions. In particular, using a mouse model, the partial biochemical characterization of α 1Pi, its distribution and localization throughout the body, its major site of synthesis, the induction mechanism(s) of acute phase protein synthesis and the possible sites of utilization during inflammation were investigated. Similar studies were also initiated with a variety of other acute phase reactants such as SAP, SAA and C3, in collaboration with other laboratories. The information obtained has been incorporated within the framework of a general model of inflammation (Chapter 6).

In order to achieve these objectives, answers to the following questions were sought:

- 1) Is α 1Pi an acute phase reactant in the mouse?
- 2) Using an immunohistochemical staining technique for the specific localization of α 1Pi in various cell types, are there alterations in staining intensity and tissue distribution associated with the onset of an acute inflammatory response?
- 3) If so, how do these changes correlate with the rise in serum levels of the acute phase reactant?
- 4) In order to gain some insight into the biological function(s) of α 1Pi, can we demonstrate sequestration of α 1Pi at the site(s) of inflammation and speculate on its role?
- 5) Can we demonstrate the release of an acute phase mediator (APM) originating from the site of inflammation?
- 6) If so, what is/are the cell type(s) involved?

- 7) Is there a correlation between the *in vivo* demonstration of an acute phase response and the *in vitro* synthetic output of acute phase reactants?
- 8) Is the α 1Pi acute phase serum profile identical during an inflammatory reaction at a particular tissue site, regardless of initial injurious stimuli?
- 9) How does the serum profile of other acute phase proteins compare with that of α 1Pi under the same inflammatory condition?
- 10) Is the serum profile of a particular acute phase reactant necessarily indicative of an ongoing acute inflammatory reaction?
- 11) Under the same inflammatory condition, is there strain variation in any given acute phase reactant serum profile?
- 12) Is there a correlation between the extent of susceptibility of various strains of mice to a particular infectious agent and the ability to generate an acute phase response?

2.3 Description of the Animal Models

Initially, in order to determine that α 1Pi is an acute phase reactant in the mouse and to establish the methodology for the study of α 1Pi during inflammation, celite was used as an injurious stimulus (Koj and Dubin, 1976). Celite is a particulate material and is broadly classified as a diatomaceous earth. Its composition is mainly siliceous in nature. The acute inflammatory response has a rapid onset of a few hours, is of brief duration (24 - 28 h) and the inflammatory focus is restricted locally to the injection site. For these reasons, the celite-induced inflammation model is advantageous for its relative simplicity.

and ease of generation.

Having established the methodology for the study of α 1Pi as an acute phase reactant in a simple inflammation model, these studies were extended to more complex model systems involving inflammatory reactions not only in muscle tissue but at mucosal sites such as lungs and small intestine. For that purpose, parasitic infections of mice were selected as models of inflammation.

These were selected for four main reasons: firstly, in most cases there are significant pathologic changes associated with the parasite as it migrates through the host; secondly, the kinetics of migration and the tissues involved have been well characterized in the literature; and thirdly, by selecting the appropriate parasite, one can generate an inflammatory reaction in a specific tissue site or at multiple sites, either simultaneously or sequentially. Finally, not only do these models provide information as to the mechanism(s) underlying the acute phase protein phenomenon but, in light of the recently growing interest in the host-parasite interactions, will perhaps give a better understanding of some of the initial events that occur in parasitic infections and how these in turn may influence the eventual outcome of the host-parasite interaction. It must be stressed, however, that the present studies deal exclusively with primary infections, that is, in the absence of an anamnestic specific immune response as would occur in a secondary infection.

The first parasite model of inflammation consisted of the infection of CBA/J mice with the nematode *Nippostrongylus brasiliensis*. *N. brasiliensis* is a parasitic metazoan helminth of the phylum nematoda.

The basic form of the parasite is elongate and tapered at both ends. The infective third stage larvae has a mean length of 300 μm . The life cycle of *N. brasiliensis* in CBA/J mice is depicted in Figure 4.6.1. Briefly, infective third stage larvae are introduced in the skin and by two days post-infection, these have migrated to the lung where an inflammatory response occurs. The parasites mature and subsequently migrate to the gastrointestinal tract, via the trachea and esophagus, where an inflammatory reaction is induced. This particular model, therefore, provides an inflammatory reaction at two different sites.

The second model of inflammation consisted of NIH mice infected with *Trichinella spiralis*.

T. spiralis is a metazoan nematode of the order Trichurata and in the Trichinellidae family and the infective larvae measure 14.0 - 16.0 μm long. The biology of this organism is unusual in that one animal serves as both definitive and intermediate host, with the juveniles and the adults located in different organs (Schmidt and Roberts, 1981).

The life cycle of *T. spiralis* in the mouse is depicted in Figure 4.15.1. Briefly, the parasites are intubated into NIH strain of mice where they proceed to the gut by day 1. The parasites reproduce, the male dies and the female burrows into the mucosa and submucosa. Newborn larvae emerge and may follow the lymphatic ducts to the mesenteric lymph nodes. The females die or remain in the intestine where they are spontaneously expelled by immune reactions at about day 12. Some newborns may escape into the intestinal lumen where they can remain infective to another intermediate host if transmitted in the feces. Most

newborn larvae are, however, carried by the hepatic portal system through the liver, then the heart, lungs and other parts of the body. When the young worms reach muscle tissue at approximately day 12 - 15 post-infection, they penetrate muscle fibers and growth is initiated. The larvae eventually become encysted. Some muscles are much more heavily invaded than others such as the muscles of the eye, tongue and diaphragm. The heavy muscles of the arms and legs are less susceptible. The larvae grow and become infective but remain in cyst-like habitat until the next host feeds on the infected animal (Schmidt and Roberts, 1981).

Therefore, this particular model gave rise initially to an inflammatory response in the small intestine and subsequently in muscle tissue.

The third parasite model of inflammation consisted of the infection of A/J and C3H/HeJ mice with *Trypanosoma congolense*.

T. congolense is a protozoan parasite of the order Kinetoplastida and is mainly a parasite of the bloodstream with minimal invasion of the lymphatics and certain tissues. The insect vectors are *Glossina morsitans*, *G. pallidipes* and *G. swynnerton*, common tse tse flies throughout much of Africa.

In the laboratory, the insect vector stage is not required as trypanosomes may be kept in culture. In this case, the morphology and metabolism of the trypomastigotes revert to that found in the fly midgut (Schmidt and Roberts, 1981).

The interest in this model stems partly from the fact that there is marked strain variation in susceptibility/resistance to *T. congolense*.

This strain variation has been fairly well documented but the underlying mechanism(s) are poorly understood. This model, therefore, provides an excellent opportunity to study acute phase protein synthesis in strains with variable susceptibility/resistance to infection.

In conclusion, the information obtained from these four models of inflammation has been incorporated into an overall model of the induction of acute phase protein synthesis (Chapter 6). The model also depicts certain events subsequent to the induction of acute phase reactant synthesis.

CHAPTER 3

MATERIALS AND METHODS

Materials

DEAE-Sephadex A-50, Sephadex G-25 fine, Sepharose-Concanavalin A, Sepharose CL-4B-Protein A and Percoll were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); Sepharose-heparin and Sepharose-Cibacron Blue were gifts from Dr. E. Regoeczi (McMaster University, Hamilton, Ontario, Canada); Ion Exchange Resin (GA-540, 100-200 mesh) was purchased from Baker Chemical Co. (Phillipsburg, NJ). Complete Freund's Adjuvant (CFA) and *E. coli* 0127:B8 LPS were obtained from Difco (Detroit, MI); rabbit anti-sheep IgG and horseradish peroxidase conjugated rabbit anti-sheep IgG and sheep anti-rabbit IgG were purchased from Cappel Laboratories (Cochranville, PA); N-benzoyl-DL-arginine p-nitroanilide (HCl) (BAPNA), porcine pancreas trypsin (T-0134), collagenase (Type I), Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and cycloheximide were obtained from Sigma (St. Louis, MO); Na¹²⁵I, ³⁵S-methionine, Bolton-Hunter reagent and ³H-thymidine were purchased from New England Nuclear (Boston, MA); ¹⁴C-isoleucine, ¹⁴C-lysine were obtained from Scharz/Mann (Division of Becton, Dickinson and Company, Orangeburg, NY); 5-aminosalicylic acid, p-nitrophenyl guanidinobenzoate and DAB (3,3'-diaminobenzidine tetra-HCl) were obtained from ICN Pharmaceuticals (Plainview, NY); 10% buffered formalin and activated charcoal (20-35 mesh) were purchased from BDH Chemicals (Toronto, Ontario, Canada); William's E medium, fetal calf serum, gamma G-free horse serum and Eagle's Minimum Essential Medium (MEM) were purchased from Gibco

(Grand Island, NY), whereas all other culture media were obtained from the Culture Media Centre Resources (Dr. W. Rawls, McMaster University, Hamilton, Ontario, Canada); polyethylene glycol-6000 (PEG) was obtained from Baker Chemical Co. (Phillipsburg, NJ); Tween-20 was purchased from Fisher Scientific Co. (Fairlawn, NJ); 1-O-methyl- α -D-glucopyranoside was obtained from General Biochemicals (Chagrin Falls, OH); agarose Seakem (Me, Le) was purchased from Marine Colloids (Rockland, ME); Chloramine-T was obtained from Eastman Organic Chemicals (Rochester, NY); heparin sodium (Hepalean) was purchased from Harriş Laboratories (Brantford, Ontario, Canada); DNase I was obtained from Boehringer Mannheim (Dorval, Quebec); aquacide II-A was purchased from Calbiochem-Behring Corp. (La Jolla, CA); plasminogen was purchased from KABI (Stockholm, Sweden); Permablend II from Packard Instrument Co. (Downers Grove, IL) and celite was a generous gift from Dr. P. Horsewood (McMaster University, Hamilton, Ontario, Canada).

All other reagents and solvents used were of analytical grade.

Methods

3.1 Animals

Mice of various inbred strains were obtained from The Jackson Laboratory, Bar Harbor, Maine. Animals were fed *ad libitum* and subjected to a 12 h diurnal light cycle and were age and sex matched within experiments.

For α PI ontogeny studies, breeding colonies were established in a controlled situation; the sighting of a vaginal plug was arbitrarily designated as day 1 of gestation.

3.2 Purification of α PI

Mouse α PI was purified with the aid of Dr. A. Koj (Institute of Molecular Biology, Jagiellonian University, Krakow, Poland), using an isolation procedure similar to that described for the purification of rabbit α PI (Koj *et al.*, 1978b) and human α PI (Pannell *et al.*, 1974).

The buffers used throughout the purification procedure consisted of:

Buffer A

Tris buffer (0.05 M Tris, pH 8.8, 0.05 M NaCl)

Buffer B

Tris buffer (0.05 M Tris, pH 8.8, 0.2 M NaCl)

Buffer C

Tris buffer (0.1 M Tris, pH 7.5, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$)

Buffer D

Tris buffer (0.05 M Tris, pH 8.0, 0.15 M NaCl)

Buffer E

Phosphate buffer (5 mM phosphate, pH 6.5, 0.05 M NaCl)

Buffer F

Phosphate buffer (5 mM phosphate, pH 6.5, 0.2 M NaCl)

Purification Protocol

1. Normal CBA mouse plasma was precipitated with saturated $(\text{NH}_4)_2\text{SO}_4$ solution to final saturation of 40%.
2. The supernatant was dialysed against 0.15 M NaCl and passed through a Sepharose-heparin column.
3. Saturated $(\text{NH}_4)_2\text{SO}_4$ was added to the effluent and the precipitate collected between 55 and 85% $(\text{NH}_4)_2\text{SO}_4$ saturation.
4. The dissolved precipitate was dialysed against several changes of buffer A.
5. The protein solution was chromatographed on a DEAE-Sephadex A-50 column (2 cm x 30 cm) equilibrated with buffer A.
6. The column was washed (15 ml/h) with buffer A (100 ml) and a linear gradient (200 ml buffer A and 200 ml buffer B) was applied.
7. Eluted fractions exhibiting antitryptic activity (section 3.5.1) were pooled and loaded onto a Sepharose-Concanavalin A column (2 cm x 20 cm) equilibrated with buffer C.
8. The column was washed with 5 column volumes of buffer C and glycoproteins eluted with 0.1 M 1-O-methyl- α -D-glucopyranoside in buffer A.

9. The eluate was dialysed against buffer D and passed through a Cibacron Blue-Sepharose column (5 cm x 50 cm) equilibrated with buffer D.
10. The effluent was concentrated and dialysed against buffer E and loaded onto a DEAE-Sephadex A-50 column (1 cm x 15 cm) equilibrated with buffer E.
11. After washing with 50 ml of this buffer, a linear gradient (100 ml of buffer E and 100 ml of buffer F) was applied.
12. Fractions exhibiting maximum antitryptic activity were pooled and dialysed against Tris buffer (5 mM, pH 8.0).
13. Preparative acrylamide gel electrophoresis was carried out in an apparatus as described by Pajdak (1973). The acrylamide gel (7.5%) was cast in a column (2.8 cm x 2.8 cm) with Tris buffer (5 mM, pH 8.2, 39 mM glycine) in the anode and cathode chambers. 1 ml of α 1Pi (10 mg/ml) was added and electrophoresis carried out at a constant current of 20 mA (approx. 300 V) for 2 h at 4°C until the Bromophenol Blue tracking dye had reached the anode chamber. Fractions were collected at 3 min intervals and examined for protein and antitryptic content. Those fractions exhibiting high specific activities were pooled, examined for homogeneity by analytical acrylamide gel electrophoresis, dialysed against Tris buffer (5 mM, pH 8.0) and stored at -20°C.
14. Analytical electrophoresis in polyacrylamide tube gels was carried out at pH 8.2 (Clarke, 1964). Gels were stained with 0.05% Amido black in 7% acetic acid (v/v) and destained in 7% acetic acid.

3.3 Immunization Protocols and Antisera Production

Anti-mouse α lPi was obtained by immunizing a sheep with 100 μ g of pure α lPi emulsified in 1 ml of saline and 1 ml of complete Freund's adjuvant (CFA). The immunogen was injected intramuscularly in approximately equal dosage in each of the four legs. Four weeks following the primary challenge, the sheep was boosted with 100 μ g of pure α lPi in 1 ml of normal saline, injected intramuscularly into the back legs. The animal was bled at weekly intervals (approximately 250 ml) and the specificity and reactivity monitored by double diffusion analysis and nephelometry (section 3.5.3). The sheep was subsequently boosted at approximately 6 wk intervals with the same antigen dose and continued to be bled weekly. Individual antiserum bleeds were aliquoted and stored at -70°C .

Rabbits were immunized intramuscularly with a 25 μ g dose of purified α lPi in complete Freund's adjuvant and boosted with 25 μ g doses of α lPi in saline. Antiserum bleeds were processed as described above.

3.4 Characterization of α lPi

3.4.1 Molecular weight

The molecular weight of α lPi was determined by SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970).

3.4.2 Two-dimensional immunoelectrophoresis

Barbital buffer

Barbital Sodium 3.0 mM

Barbital 6.1 mM

Tris 0.19 M

Glycine 0.36 M

pH 8.82

This buffer system was used for all electrophoresis work and is characterized by a high buffering capacity and low ionic strength ($\mu = 0.08$) resulting in minimal pH changes during electrophoresis (Axelsen *et al.*, 1973). Throughout the electrophoresis procedures, the above buffer was diluted 1/1.6 with distilled water to an ionic strength of $\mu = 0.05$.

Protocol

α IPi variants were identified by two-dimensional immunoelectrophoresis (Laurell, 1965) against sheep anti-mouse α IPi. Agarose gel (1% in barbital buffer) of uniform thickness was obtained by pouring the gel between two glass plates (8.3 cm x 10.2 cm) (Kodak, Rochester, NY) spaced 1 mm apart and a well of uniform size (10 μ l volume) was punched out. The same agarose preparation was used as "wicks" for conducting the electrical current. The sample to be analysed was appropriately diluted with the barbital buffer and electrophoresed in the first dimension at 50 mA, 12 volts/cm at 4°C for 4 h. Subsequent to this separation, the sample was electrophoresed, in a direction

perpendicular to the first dimension, into a 1% agarose gel containing 3.2% sheep anti-mouse α lPi at 25 mA, 7 volts/cm for 18 h at 4°C. The gels were washed extensively with 0.9% saline, pressed, dried and stained with Coomassie blue (Axelsen *et al.*, 1973).

3.4.3 Trypsin binding studies

3.4.3.1 Radioiodination of α lPi

Purified α lPi was iodinated with iodide-125 by using the strong oxidizing agent chloramine-T or under mild conditions using the Bolton-Hunter reagent.

A) Chloramine-T method

Purified α lPi was iodinated by the chloramine-T method of Hunter and Greenwood (1962). To 100 μ g/50 μ l (PBS) of purified protein, 20 μ g of chloramine-T (4 mg/ml in H₂O) (Eastman Kodak, Lot A8C, Rochester, NY) were added. 1 m Ci of ¹²⁵I (sodium iodide, New England Nuclear, Boston, MA) in 20 μ l (PBS) was subsequently introduced in the reaction mixture. The iodination was allowed to proceed at room temperature for 90 sec with constant mixing. The iodination was terminated by quenching with sodium meta bisulfite (25 μ l of 10 mg/ml in PBS) followed by potassium iodide (25 μ l of 13 mg/ml in PBS).

Radioiodinated protein was separated from free iodide by ion exchange chromatography. A 1 cm x 5 cm column of Ion Exchange Resin CGA-540 was saturated with 1% BSA in 0.85% NaCl. The column was subsequently washed with 0.85% NaCl. The iodinated material was applied to the column and eluted with 0.85% NaCl. Protein-associated

radioactivity was determined by precipitation with a 10% trichloroacetic acid solution.

B) Bolton-Hunter Method

α lPi was iodinated under non-oxidizing conditions by the Bolton-Hunter technique (Bolton and Hunter, 1973). Briefly, protein (50 μ g/50 μ l in PBS) was added to 25 μ l of 0.1 M borate buffer, pH 8.5 and reacted with the iodinated ester Bolton-Hunter reagent (New England Nuclear, Boston, MA). The reaction mixture was agitated for 15 min at 0°C and excess reagent hydrolyzed by the addition of 0.2 M glycine.

Iodinated material was separated from unreacted products by chromatography on Sephadex-G25 fine (0.1 M borate, pH 8.5). The column was initially washed with 0.5% gelatin in borate buffer followed by a borate buffer wash. The iodinated material was subsequently applied to the column and eluted with borate buffer. Protein-associated material was determined by precipitation with a 10% trichloroacetic acid solution.

For the trypsin binding studies, labelled α lPi was incubated in a 1:1 molar ratio with porcine pancreas trypsin in Tris buffer (0.05 M, pH 8.0) at 25°C for 1 h (Koj *et al.*, 1981). The sample was subsequently subjected to two-dimensional immunoelectrophoresis (section 3.4.2) and autoradiography on x-ray film (X-Omat-R, Kodak, Rochester, NY).

3.4.3.2 Radioiodination of trypsin

In a second series of experiments, binding of iodine-125 labelled trypsin to α 1Pi was investigated.

Porcine pancreas trypsin was iodinated using chloramine-T as described earlier (section 3.4.3.1). Separation of iodinated material from free iodine was achieved by chromatography on Sephadex-G25-fine. Binding of ^{125}I -trypsin to α 1Pi was investigated by two-dimensional electrophoresis using two different approaches:

- i) Normal CBA mouse serum was electrophoresed in the first dimension and the gel was overlayed with ^{125}I -trypsin (3×10^6 dpm) for 15 min at room temperature. The reaction mixture was then electrophoresed in a perpendicular direction into the antibody-containing gel.
- ii) In the second approach, the serum sample was electrophoresed in both dimensions prior to overlaying with ^{125}I -trypsin.

In both methods, the gels were exhaustively washed to remove unbound ^{125}I -trypsin and electrophoresis plates exposed to x-ray films.

3.4.4 Plasma clearance and placental transfer of α 1Pi

3.4.4.1 Plasma clearance of α 1Pi

CBA/J female mice (12 wks old) were used. Each animal was injected intravenously via the tail vein with 200 μ l of iodine-125 labelled α 1Pi in saline (Chloramine-T method; section 3.4.3.1) and adjusted to approximately $1-2 \times 10^6$ dpm. After an initial period of 30 min, a serum sample was collected from the retroorbital plexus

and designated time $T(0)$. The same animals were then bled out by cardiac puncture, at selected times, $T(n)$, over a period of 60 h, to determine the *in vivo* α PI plasma half-life. The half-life was estimated from the terminal portion of the plasma clearance curve.

3.4.4.2 Placental transfer of α PI

Purified mouse α PI (100 μ g) was iodinated by the chloramine-T method (section 3.4.3.1). CBA/J pregnant mice (CBA x CBA) were injected with 100 μ l (1 μ g) of radioiodinated α PI (approximate specific activity 3×10^6 dpm/ μ g) intravenously via the tail vein. Mice were injected both at 17 and 19 days gestation and in each group, serum was obtained from the female mice by intracardiac puncture and from fetuses by decapitation. Serum from fetuses of one pregnancy were pooled but kept separate from other pregnant mice. These serum samples were collected at 1, 2.5 and 4 h post-injection of radioiodinated material. A small aliquot of serum was counted in a Beckman 8000 gamma counter and protein-associated radioactivity assessed by trichloroacetic acid precipitation. Controls included the injection of Na^{125}I .

3.4.5 α PI distribution in body fluids

The presence of α PI in various body fluids was investigated by collecting fluids from various tissues in the mouse and quantitating by nephelometry and ELISA assays (sections 3.5.3; 3.5.4).

For the detection of α 1Pi in the lumen of the gut, a section of small intestine (20 cm in length), consisting of the jejunum and ileum, was excised, filled with 2 ml of saline and clamped at both ends. After gentle massage, the lumen washing was collected, centrifuged to remove fecal material and 0.1% sodium azide was added.

Breast milk was obtained from nursing mice by manual manipulation of the nipple after stimulation by a single injection of oxytocin (1 I.U.). The milk was centrifuged and the supernatant stored with 0.1% sodium azide.

Bile from the gall bladder was collected by aspiration with a syringe and pooled prior to concentration and dialysis against PBS.

Bronchiolavage fluid was obtained by infusion of 10 x 1 ml aliquots of PBS (37°C) via cannulation of the trachea (Godleski and Brain, 1972) as fully described in section 3.9.2.

Fetal blood was obtained by clamping the umbilical cord and removing the fetuses from pregnant CBA/J mice at various times of gestation. Fetuses were rinsed in PBS, blotted on filter paper and blood samples collected by decapitation.

Amniotic fluid was obtained by aspiration from the amniotic sac.

3.5 Quantitation of α 1Pi

3.5.1 Antitryptic activity assay

Reagents

Porcine pancreas trypsin (2.1×10^{-7} M in 0.0025 M HCl)

N-benzoyl-dl-arginine para nitroanilide (BAPNA) (2×10^{-4} M in distilled water)

Tris buffer (0.5 M, pH 7.8, 0.02 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)

Protocol

Serum antitryptic activity was determined by a modified chromogenic substrate assay (Hoffmann *et al.*, 1976; Eriksson, 1965; Cohen *et al.*, 1978; Takada *et al.*, 1979).

The specific protocol consisted of a preincubation of a mixture of 45 μl of heat inactivated (56°C , 30 min) diluted mouse serum samples (1/50 - 1/300 in Tris buffer), 50 μl of Tris buffer and 50 μl of trypsin stock solution for 5 min at 30°C . The remaining uninhibited tryptic activity was determined by the addition of 300 μl of the BAPNA substrate solution and the release of p-nitroanilide monitored by spectrophotometric recording of the absorbance at 405 nm in a 1 cm^2 cell for a period of 1 to 5 min using a Beckman Spectrophotometer Acta III. The percent inhibition was expressed as initial rate of substrate cleavage for the sample over initial rate of substrate cleavage for a control sample with no αPi .

3.5.2 Rocket immunoelectrophoresis

αPi serum levels were determined by Laurell rocket immunoelectrophoresis (Laurell, 1966).

Protocol

The barbital buffer used for the electrophoresis was as

described in section 3.4.2. A 1% agarose gel of uniform thickness was obtained by pouring the gel between two glass plates spaced 1 mm apart. The gel contained 3.2% sheep anti-mouse α IPi and wells of uniform size (10 μ l volume) were punched out. Purified mouse α IPi was used to standardize a pool of normal mouse serum which was subsequently used as the standard for α IPi measurements. Electrophoresis was carried out at 15 V/cm (25 mA) for 18 h at 4°C. Gels were washed, dried and stained as described earlier (section 3.4.2). Quantitation of α IPi was obtained by measuring peak heights and plotting against antigen concentration in the standard curve.

3.5.3 Nephelometry

Serum α IPi was measured by nephelometry on a Technicon Auto-analyzer II Fluoronephelometer (Walker and Gauldie, 1978).

Reagents

Saline Solution (0.9%, 0.05% Tween-20)

Polyethylene glycol solution (PEG-6000) (4% in saline solution)

Protocol

Sheep anti-mouse α IPi was diluted 1/30 with polyethylene glycol solution and filtered through 0.45 μ m pore size filters and serum samples were appropriately diluted (1/400 - 1/2000) in saline solution.

For the determination of α IPi serum level, the antiserum stock solution and antigen preparation were mixed at a rate of 0.80 ml/min and 0.32 ml/min, respectively. The sampling rate was 100/h with a 2:1

sample to saline solution wash ratio.

α lPi was quantitated by measuring peak height and the concentration obtained from the standard curve.

3.5.4 Enzyme-linked immunosorbent assay

In order to achieve levels of sensitivity for the quantitation of α lPi in the range 10 - 500 ng/ml, an ELISA (Voller *et al.*, 1978) was developed using 96 well microtitre plates (NUNC, Gibco Laboratories, Grand Island, NY).

Reagents

i) Rabbit anti-mouse α lPi

Rabbit IgG was isolated from rabbit anti-mouse α lPi antiserum by affinity chromatography on a column of protein A-Sepharose CL-4B. The column (1 cm x 15 cm) was washed with PBS (pH 7.6) and 1 ml of antiserum was applied. The column was subsequently washed with 10 ml of PBS and the eluate discarded. The absorbed IgG was eluted with 3 ml of 0.1 M glycine (pH 2.8). The acidic eluate fraction was neutralized with 1 N sodium hydroxide and dialysed against PBS. The dialysed material was essentially pure IgG with specificity for mouse α lPi as determined by double diffusion immunoassay.

ii) Substrate

The substrate for the enzyme horseradish peroxidase was prepared by dissolving 20 mg of 5-aminosalicylic acid in 25 ml of warm distilled water. The pH was adjusted to 6 with 1N NaOH.

iii) Catalyst

The reaction between horseradish peroxidase and substrate was catalyzed by the addition of 10% of 0.16% hydrogen peroxide solution in distilled water.

iv) Bicarbonate buffer (0.05 M, pH 9.6, 0.02% azide)

v) Saline solution (0.9% NaCl, 0.025% tween-20)

Protocol

Each of the following steps is interspersed with three saline solution washes and steps 2 - 6 were performed at room temperature.

1. Rabbit IgG anti-mouse α PI was diluted 1/400 with bicarbonate buffer and 100 μ l (approximately 1 μ g IgG) were added to each well and incubated at room temperature for 2 h with subsequent overnight incubation at 4°C.
2. 150 μ l of 2% BSA in PBS (pH 7.2) was added for 30 min as a blocking step.
3. 100 μ l of test material was added for 2.5 h.
4. To the wells, 100 μ l of sheep anti-mouse α PI antiserum, diluted 1/1000 with PBS, was added and incubated for 2 h.
5. 100 μ l of rabbit anti-sheep IgG-conjugated with horseradish peroxidase diluted 1/500 with PBS was added to the wells and incubated for 1½ h.
6. 100 μ l of substrate containing 10% of hydrogen peroxide catalyst solution was added and the colorimetric reaction was terminated by the addition of 50 μ l of 1N NaOH. The absorbance was recorded on a Microelisa Minireader MR590 (Dynatech).

3.6 Immunohistochemistry

α lPi was demonstrated in tissues and in the cytoplasm of various cell types by immunohistochemical means.

Protocol

1. Tissues were fixed in 10% buffered formalin.
2. Fixed tissues were processed in fresh absolute ethanol for three cycles of 2 h each; xylol for three cycles of 2 h each; 54°C paraffin for two cycles of 2 h each, except for lung tissue which was processed in paraffin under vacuum. Tissues were subsequently embedded in paraffin.
3. Tissue sections (5 μ m) were dewaxed in absolute ethanol and background peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 7 min.

Each of the subsequent steps are interspersed with three PBS washes of 5 min each.

4. Tissue sections were overlayed with sheep anti-mouse α lPi diluted 1/150 with PBS (0.01 M, 0.1% Triton X-100, 2.5% BSA, 0.25% sodium azide, pH 7.3) for 30 min.
5. Tissue sections were overlayed with rabbit anti-sheep IgG diluted 1/50 with PBS for 30 min.
6. The tissue sections were incubated (30 min) with sheep anti-rabbit IgG-conjugated with horseradish peroxidase diluted 1/100 with PBS.
7. Slides were washed in Tris - saline (0.05 M NaCl, pH 7.6) and the substrate DAB (6 mg/10 ml in Tris - saline solution, 0.016% hydrogen peroxide) added for 10 - 15 min.

8. Slides were washed in tap water, counterstained with methyl green (0.1% in distilled water), dehydrated and cover slipped.

3.7 Ontogeny Studies

Fetal serum was collected as described earlier (section 3.4.5). Neonatal (birth - day 20) serum samples were also collected by decapitation whereas adult serum samples were collected by cardiac puncture. Except for fetal blood, where several samples were pooled, the serum α 1Pi levels of neonatal and adult mice were expressed as an average of seven animals. Serum α 1Pi concentration was determined by nephelometry and rocket immunoelectrophoresis.

3.8 Inflammation Models

3.8.1 Celite induced inflammation

Celite (gift from Dr. Peter Horsewood, McMaster University, Hamilton, Ontario, Canada), a diatomaceous earth, was routinely used to induce an inflammatory response in CBA/J mice. 150 μ l of sterile celite suspension (30 mg/ml) was injected subcutaneously into both scapular regions (Koj and Dubin, 1976).

At selected time intervals, mice were bled by cardiac puncture and serum collected for quantitation of α 1Pi. A variety of tissues were also taken and fixed (section 3.6) for immunohistochemical and histological studies.

3.8.2 Parasite infections

3.8.2.1 *Nippostrongylus brasiliensis*

N. brasiliensis was obtained from Dr. N. Croll at the Institute of Parasitology, Montreal, Quebec in 1976. The nematode was maintained by serial passage (5000 L3 larvae injected subcutaneously at the back of the neck) in Sprague-Dawley rats, 150 - 250 g (Biobreeding, Ottawa, Ontario, Canada) (Befus *et al.*, 1979).

Infective third stage larvae (L3) were cultured *in vitro* by routine techniques (Jennings *et al.*, 1963). Briefly, rat feces were collected at days 6 to 9 post-infection and mixed with an equal weight of activated charcoal (20 - 35 mesh) (BDH Chemicals, Toronto, Ontario, Canada). The fecal-charcoal mixture was soaked with water and a suspension obtained by grinding the feces with a mortar and pestle. A small amount of this mixture was placed on filter paper supported by a piece of foam, to retain moisture, in a glass petri dish. Eggs were allowed to hatch (10 - 18 days) by incubating the plates at 30°C while maintaining a moist environment. Upon hatching, the first stage larvae migrate out towards the edge of the filter paper as they mature to the second and third stage (infective) larvae (L3).

L3 larvae were collected by cutting small pieces of the infected filter paper, and setting them on cotton gauze placed on top of a funnel partially filled with water (room temperature). The larvae migrate off the filter paper into the water and settle by gravity to the bottom of the funnel.

For the inflammation model, mice were infected with 500 L3 larvae via the subcutaneous route. Serum samples were obtained at various time intervals by cardiac puncture and tissues removed and stored at 4°C in buffered formalin until processing (section 3.6).

3.8.2.2 *Trypanosoma congolense*

Mice (A/J and C3H/HeJ) were infected by Dr. Terry Pearson (Department of Biochemistry and Microbiology, University of Victoria, Victoria; British Columbia, Canada) by intraperitoneal injection of 10^3 *T. congolense* strain 5E-12 organisms (Pearson *et al.*, 1979; Morrison *et al.*, 1978). Mice were sacrificed at selected time intervals and serum samples (frozen) and buffered formalin-fixed tissues sent to our laboratory for α Pi quantitation as well as immunohistochemical and histological studies.

3.8.2.3 *Trichinella spiralis*

Mice (NIH strain, Anglia Laboratory ~~Animals~~) were infected by Dr. Derek Wakelin (Wellcome Laboratories for Experimental Parasitology, University of Glasgow, Scotland) with 400 *T. spiralis* infective muscle larvae, by intubation. Lyophilized serum samples and buffered formalin-fixed tissues were subsequently sent to our laboratory for α Pi quantitation as well as immunohistochemical and histological studies.

3.9 In vitro Culture Systems

3.9.1 Isolated hepatocyte cultures

i) Perfusion protocol

Hepatocytes were isolated from mice by a two-step perfusion procedure similar to that described by Seglen (1973) and Deschênes *et al.* (1980) with a few modifications. Briefly, 20 - 30 g mice were anesthetized with a 500 µl intraperitoneal injection of 10% sodium pentobarbital containing 10 U/ml heparin sodium (Harris Laboratories, Brantford, Ontario, Canada). The abdominal cavity was exposed and a 25-gauge butterfly cannula was inserted into the portal vein and clamped in place.

The perfusate consisted of a Ca^{2+} free, sterile Hepes buffer (100 mM Hepes, 67 mM KCl, 1.4 M NaCl, pH 7.3) and maintained at a temperature of 37°C using a circulating water bath. Immediately after perfusion was initiated, the superior vena cava was severed to allow perfusate to flow out and thus prevent excessive build up of pressure in the liver and as such minimize rupture of blood vessels.

In the first part of the perfusion procedure, the liver was perfused at a rate of 6.6 ml/min with a 1/10 dilution of the above stock Hepes buffer. Uniform blanching of the liver, which occurred immediately upon perfusion, indicated an extensive removal of blood. The perfusion was allowed to proceed for 6 - 7 min in order to irreversibly remove Ca^{2+} from the desmosomes, thereby causing cleavage of intercellular junctions. In order to optimize Ca^{2+} removal, the superior vena cava was periodically clamped with forceps in order to

allow pressure build up in the liver, as judged by swelling of the organ.

The second step consisted of perfusing the liver, at the same rate and volume, with a freshly prepared stock collagenase solution (Sigma, Type I, St. Louis, MO; 0.375% collagenase, 54.4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.12 M NaCl, 10 mM Hepes, pH 7.3 with 0.1 N NaOH) diluted 1/10 with Hepes buffer from the first perfusate solution. It was crucial that Ca^{2+} ions be present in the perfusate buffer since the activity of the collagenase enzyme is Ca^{2+} -dependent. In each of the two perfusion steps, a single-pass, non-recirculating, system was used.

Upon completion of perfusion, the liver was excised from the animal and held in the portal region (with forceps). The liver capsule was disrupted with forceps and with continuous gentle mixing, liver cells were collected in Ca^{2+} -free Hepes buffer at room temperature. Large aggregates and clumps were removed by decantation and the resulting suspension consisted of a mixture of intact and damaged parenchymal and non-parenchymal cells. Viable hepatocytes were obtained by sedimenting twice through 50 ml of Ca^{2+} -free Hepes buffer in a 50 ml conical tube for 15 min under unit gravity and room temperature. Viability was assessed by staining with ethidium bromide/fluorescein diacetate (Takasugi, 1971).

ii) Preparation of collagen coated plates

Collagen solution was prepared as described by Sirica *et al.* (1979). Briefly, collagen fibers (2 - 3 cm) were mechanically extracted from rat tails and stored in PBS at -20°C . To prepare the collagen

solution, 1 g of fibers were suspended in 300 ml of a 0.1% glacial acetic acid solution and stirred at 4°C for 24 - 48 h. The undissolved fiber remnants were removed by filtration, first through coarse filter paper followed by a 0.45 µm filter and the acid soluble collagen stored at -20°C.

Collagen-coated plates were prepared by introducing 3 ml of acid soluble collagen solution into a 25 cm² tissue culture flask (Falcon^R, Division of Becton, Dickinson and Co., Oxnard, CA). The plates were incubated at room temperature for 24 h in a sterile hood, under ultraviolet light. Following this incubation period, the acetic acid and any excess collagen were removed by rinsing the culture flasks twice with 5 ml volumes of Ca²⁺-free HEPES buffer and once with pH indicator containing culture medium (3 ml) to monitor for neutrality. This procedure allows for a thin film of collagen to attach to the bottom of the culture flasks.

iii) Hepatocyte culture conditions

Hepatocyte cultures were initiated by adding 5 ml of liver cell preparation, containing 3×10^6 viable hepatocytes, in William's E medium supplemented with 1% L-Glutamine, 1% penicillin/streptomycin, 1% HEPES, 10% gamma G-free horse serum or fetal calf serum and 10^{-6} M dexamethasone sodium phosphate (Hexadrol[®], Organon Canada Ltd., Toronto, Ontario, Canada) to each plate. The hepatocytes were allowed to adhere for 1.5 h at 37°C in a closed-cap system. Non-adherent dead/damaged hepatocytes and non-parenchymal cells were washed off with 5 ml of William's E medium and 10 ml of fresh medium added. Hepatocytes

were maintained in culture for 24 h with no significant loss of viability.

3.9.2. Alveolar macrophage cultures

i) Isolation of lung alveolar cells

Murine (CBA/J female mice) alveolar macrophages were isolated as essentially described by Godleski and Brain (1972). Briefly, mice were anesthetized with a 500 μ l intraperitoneal injection of 10% sodium pentobarbital containing 10 U/ml heparin sodium. In order to maximize alveolar macrophage yields, 14-18 wk old mice were selected. Under aseptic conditions, the peritoneal cavity was exposed and the abdominal aorta was transected to exsanguinate the mouse. The lungs were collapsed by lightly puncturing the diaphragm. The trachea was exposed by removing the thyroid gland and excising the oesophagus. A small incision was made and a polyethylene cannula (I.D. 0.76 mm, O.D. 1.33 mm) was inserted into the trachea and immobilized, above the main bronchi, by surgical thread. Alveolar macrophages were collected by ten *in situ* preliminary lavages of 0.8 - 1.0 ml each of PBS maintained at 32 - 37°C. The infusion and recovery of bronchiolavage fluid was performed relatively slowly to prevent bursting of lungs and collapse of trachea. Slow motion of the syringe plunger back and forth during the lavages as well as gentle massage of the rib cage also maximized cell yields. Cells were collected on ice in 12 ml polyethylene tubes and centrifuged at 200 g on an MSE centrifuge (Measuring of Scientific Equipment, Ltd., England) for 12 min at 4°C. Cells were pooled in PBS, centrifuged once more and resuspended in RPMI medium.

Viability was assessed by ethidium bromide/fluorescein diacetate staining and cell differentials by May-Giemsa-Grunwald staining.

ii). Culture conditions

Alveolar macrophages ($5 \times 10^5/2$ ml) were allowed to adhere to plastic petri dishes (35 x 10 mm) (Falcon^R, Division of Becton, Dickinson and Co., Oxnard, CA) for 1.5 h at 37°C, 6% CO₂, in RPMI-1640 medium containing 10% fetal calf serum or γ G-free horse serum, 1% bicarbonate, 1% P/S, 1% Hepes and 1% L-glutamine. Non-adherent cells were removed by rinsing the petri dishes with 1 ml volumes of medium. Adhered alveolar macrophages were cultured in 2 ml of medium at 37°C, 6% CO₂.

3.9.3 Peritoneal macrophage cultures

Peritoneal macrophages were obtained as described by Conrăd (1981). CBA/J female mice, 10 - 14 wk, were anesthetized with ether and sacrificed by cervical dislocation. The abdominal skin was washed with alcohol and retracted taking care not to tear the peritoneal wall. A 20-gauge needle was attached to a syringe filled with 8 ml of PBS (4°C) containing 10 U/ml of heparin and inserted into the peritoneal cavity. The buffer was injected and the needle pulled out of the cavity, the point of entry was sealed by pinching the peritoneal wall with forceps. The abdominal cavity was gently massaged and the peritoneal lavage fluid was subsequently aspirated with the syringe.

The peritoneal exudate cells were collected on ice in 50 ml polyethylene tubes and spun at 250 g (MSE centrifuge, England) for

12 min at 4°C. The cells were resuspended in RPMI-1640 medium (1% L-glutamine, 1% P/S, 15 mM Hepes, 1% bicarbonate, pH 7.1) and enriched for peritoneal macrophages by adherence on plastic for 2.5 h at 37°C, 6% CO₂. After removal of the non-adherent cell population, peritoneal macrophages were cultured at a density of $1.1 \times 10^6/\text{ml}$ (75 cm² culture flask) (Falcon^R) in 30 ml of RPMI medium.

3.9.4 Isolated pancreatic islets of Langerhans cultures

i) Isolation of islets of Langerhans

Pancreatic islets of Langerhans were isolated by the collagenase digestion procedure described by Buitrago *et al.* (1977). Briefly, three CBA/J female mice (14 wk) were sacrificed by cervical dislocation and the pancreas excised, transferred to ice cold RPMI-1640 medium and cut into small pieces. The pieces were pooled and added to 1.5 ml of RPMI-1640 medium containing 2.5 mg of collagenase (Sigma, St. Louis, MO, Type I). Enzymatic digestion of the pancreas was allowed to proceed for 20 min at 37°C, with constant vigorous agitation on a Fisher Roto-Rack. The pancreas digest was subsequently diluted to a volume of 10 ml with ice cold RPMI-1640 medium and allowed to sediment at unit gravity for 5 min. The supernatant was subsequently discarded and 25 μl of DNase I solution (10 mg/ml) added.

Pancreatic islets were separated by density centrifugation on Percoll. A stock solution of Percoll was prepared by diluting 2 ml of 10x concentrated RPMI-1640 medium (Gibco, Grand Island, NY) with 18 ml of Percoll. The density gradient was prepared by diluting 14 ml of the above Percoll stock solution with 26 ml of RPMI-1640 medium and

layered over 2 ml of stock Percoll solution in a 2.5 cm x 10 cm round bottom polypropylene tube. The pancreas digest was layered on top of the Percoll separating medium and allowed to sediment for 15 min under unit gravity. The pancreas digest was subsequently aspirated and pancreatic islets of Langerhans collected at the bottom interface.

ii) Culture conditions

Islets of Langerhans were cultured for 24 h in RPMI-1640 supplemented with 15% gamma G-free horse serum, 1% P/S, 1% bicarbonate and 1% L-glutamine.

3.9.5 Whole organ cultures

Supernatants from whole organ cultures of rat (Sprague Dawley, Biobreeding, Ottawa, Ontario, Canada) spleen, lung, trachea, Peyer's Patches, jejunum, ileum and mesenteric, mediastinal, peripheral, popliteal and inguinal lymph nodes were kindly provided by Ms. Gudrun Goetsche (McMaster University, Hamilton, Ontario, Canada). These various tissues were cultured for 24 h in Minimum Essential Medium (Eagle, Gibco, Grand Island, NY) in the presence of ^{14}C -isoleucine and ^{14}C -lysine (Scharz/Mann - Division of Becton, Dickinson and Co., Orangeburg, NY).

3.10 In vitro Amino Acid Incorporation Studies

Murine hepatocytes were isolated and cultured as described earlier (section 3.9.1). For carbon-14 lysine incorporation, hepatocytes were cultured in Eagle's lysine free MEM medium (Gibco, Grand Island, NY)

supplemented with 10% dialysed gamma G-free horse serum, 2.2 g 1% bicarbonate, 1% P/S, 1% L-glutamine, pH 7.4. Hepatocytes (3×10^6) were cultured in 10 ml of Eagle's lysine-free medium, containing 1.05 $\mu\text{Ci}/\text{ml}$ of ^{14}C -lysine for 24 h.

Similar labelled amino acid incorporation studies were performed using ^{35}S -methionine. 1×10^6 hepatocytes/2ml of methionine free medium were pulsed for 2.5 h with 20 μCi ^{35}S -methionine/ml. After the pulse, 100 μl of "unlabelled" methionine (0.3 mg/ml) (Sigma, St. Louis, MO) was added and the hepatocytes cultured for 24 h at 37°C , 6% CO_2 . As controls, 3.6 $\mu\text{g}/\text{ml}$ of the protein synthesis inhibitor, cycloheximide, was added to the hepatocytes for 30 min. The hepatocytes were then washed with methionine-free medium and pulsed with ^{35}S -methionine as described above.

Culture supernatants (^{35}S -methionine labelled) from the murine hepatoma cell lines Hepa-1, 125, 11ab, 19/2 and HH were kindly provided by Dr. G. Darlington (Cornell University, NY).

Murine alveolar macrophages were isolated and cultured as described (section 3.9.2) and ^{14}C -lysine incorporation was performed using the hepatocyte protocol.

Alveolar macrophages were also pulsed with ^{35}S -methionine using the incorporation protocol described for hepatocytes.

Islets of Langerhans were isolated and cultured as described earlier (section 3.9.4) and pulsed with ^{14}C -lysine.

3.11 Autoradiography

The ability of these various cell types to synthesize α PI was determined by autoradiography. Culture supernatants and cell lysates (successive rapid freezing-thawing method) were exhaustively dialysed against PBS to remove free labelled amino acids and concentrated using aquacide II-A (CalBiochem - Behring Corp., La Jolla, CA). An appropriate amount of these supernatants was diluted with normal mouse serum and two-dimensional crossed immunoelectrophoresis carried out. Electrophoregrams were processed in Enhance^R (New England Nuclear, Boston, MA) and autoradiography performed on x-ray film.

3.12 Alveolar Macrophage Functional Assays

3.12.1 Morphology

The morphology of the alveolar macrophage from normal and *N. brasiliensis* infected animals was determined by conventional microscopy using May-Grunwald-Giemsa (MGG) staining as well as by electron microscopy (kindly carried out by Ms. B. Brown, Department of Pediatrics, McMaster University, Hamilton, Ontario, Canada).

3.12.2 Plasminogen activator release assay

The extent of activation of the alveolar macrophage was determined by an assay for the release of plasminogen activator (Vassalli and Reich, 1977).

Reagents

i) Kreb's Ringer Buffer

100 parts 0.15 M NaCl

4 parts 1.15% KCl

1 part 3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

20 parts 0.1 M phosphate buffer, pH 7.4

glucose 100 mg %

ii) Tris/HCl Buffer

0.024 M Tris

0.1 M NaCl

0.05 M KCl

0.0037 M Na_2HPO_4

pH 7.45

Protocol

1. Preparation of iodine 125-fibrinogen coated plates

Fibrinogen was labelled with iodine-125 by the Department of Nuclear Medicine (McMaster University, Hamilton, Ontario, Canada).

The labelled fibrinogen was adjusted to approximately 3×10^6 dpm/ml with Kreb's Ringer Buffer and 60 $\mu\text{g/ml}$ of cold fibrinogen added.

For plating, the stock of ^{125}I -fibrinogen solution was diluted 1/10 with Kreb's Ringer Buffer and 300 μl added to each well (Linbro Dispo Trays; Linbro Chemical Co., New Haven, CT). The plates were subsequently dried at 45°C over a period of 5 days in a sterile incubator.

2. Conversion of ^{125}I -fibrinogen to ^{125}I -fibrin

Two-3 h prior to addition of macrophages, 500 μl of 10% heat inactivated fetal calf serum in Dulbeccos (1% P/S, 1% bicarbonate) was added to each well and incubated at 37°C , 6% CO_2 for 2 h. The plates were subsequently washed twice with Hank's balanced salt solution and washes kept for counting.

3. Fibrinolysis by macrophages

Alveolar macrophages ($1 \times 10^6/0.4 \text{ ml}$) were plated onto ^{125}I -fibrin-coated wells in Dulbecco's medium containing 100 $\mu\text{g/ml}$ soybean trypsin inhibitor. After an incubation period of 1.5 h (37°C , 6% CO_2), the plates were washed with Tris/HCl buffer and washes kept for counting. To the wells, 8 $\mu\text{g/ml}$ of plasminogen in Dulbecco's medium was added and plates incubated for $2\frac{1}{2}$ h at 37°C , 6% CO_2 . A 100 μl aliquot of the supernatant medium was taken from each well and kept for counting. Plates were incubated for a further $2\frac{1}{2}$ h and the remaining supernatant medium collected. Maximum fibrinolysis in control wells was obtained by adding urokinase (10 I.U.) to the well.

The higher the amount of plasminogen activator released, the more activated the alveolar macrophage (Unkeless *et al.*, 1974).

3.12.3 Adherence/phagocytosis

The assay was carried out by a modified Leighton tube method using human D-positive red blood cells (RBC) coated with anti-D antibodies. Briefly, the macrophages were incubated for 90 min at

37°C for attachment to Leighton tubes and subsequently for 1 h at 37°C with sensitized red blood cells, airdried and stained with MGG to assess attachment and phagocytosis of RBC.

3.13 Generation of LAF (IL1)

Interleukin I (LAF) production was induced in murine peritoneal and alveolar macrophages as described by Mize [1981] and Simon and Willoughby (1981).

Murine (CBA/J) peritoneal macrophages were isolated and enriched as described previously (section 3.9.3). $85 \times 10^6/75 \text{ cm}^2$ adhered peritoneal macrophages were cultured for 24 h (5% CO₂) in 30 ml of RPMI-1640 medium (1% L-glutamine, 1% P/S, 15 mM HEPES, 1% bicarbonate, pH 7.2) containing 200 µg of LPS. The culture supernatants were removed and centrifuged to remove cellular debris and dialysed exhaustively (dialysis tubing, 6000-8000 Mw cut-off) against 0.03 M PBS. The supernatants were stored frozen (-20°C).

The generation of interleukin 1 (LAF) by alveolar macrophages was investigated in alveolar macrophages from normal and *N. brasiliensis* infected lungs either with or without LPS (40 µg) stimulation. In all cases, 3×10^6 alveolar macrophages were cultured in 5 ml of medium in a 25 cm² culture flask at 37°C, 6% CO₂ for 24 h.

3.14 Thymocyte Assay for Interleukin 1 (IL1 \equiv LAF)

Production of interleukin 1 (LAF) by peritoneal and alveolar macrophages was determined using a thymocyte proliferation assay

(Mizel, 1981). Lipopolysaccharide (LPS) - unresponsive C3H/HeJ female mice (Jackson Laboratories, Bar Harbor, ME) are generally interleukin 1 (LAF) responsive mice and as such were used as a source of thymocytes. The thymus from 8 - 10 wk old mice was excised and thymocytes obtained by gently pressing through 60-gauge stainless steel wire sieves into RPMI-1640 medium supplemented with 1% HEPES, 1% L-glutamine, 1% bicarbonate, and 1% P/S. The thymocyte suspension was subsequently layered over gamma G-free horse serum and allowed to sediment at unit gravity for 30 min at 4°C. Large aggregates rapidly settled in the horse serum and thymocytes in the medium were carefully aspirated and layered over gamma G-free horse serum. The cell suspension was centrifuged at 250 g for 15 min at 4°C. The thymocytes were suspended in RPMI-1640 medium at a concentration of $15 \times 10^6/\text{ml}$.

The interleukin 1 assay consisted of adding 1.5×10^6 thymocytes (100 μl) into each well of 96-well microtiter plates, 10 μl of phytohemagglutinin (PHA, 200 $\mu\text{g}/\text{ml}$) and 90 μl of macrophage culture supernatant. The presence of interleukin 1 and PHA have a synergistic effect on thymocyte proliferation and at high concentrations of IL1 inhibition of proliferation occurs. Therefore, serial dilutions of culture supernatants were added to the wells in order to titrate the IL1 activity. The thymocytes were cultured for 72 h at 37°C and 6% CO₂ and pulsed with ³H-thymidine (0.5 $\mu\text{Ci}/\text{well}$) for 4 h.

Incorporated ³H-thymidine was measured by harvesting the cells on cellulose filter paper using a cell harvester (Skatron, Norway), drying the filter papers, placing each of them into 10 ml of scintillation

fluid (20 gm of Permablend II, Packard Instrument Co., Downers Grove, IL; 4 l of scintillation grade toluene) and counting in a Beckman LS-233 beta counter.

3.15 Alveolar Macrophage Derived Acute Phase Mediator (APM)

Twenty-four h culture supernatants from normal alveolar macrophages and from day 2 post-*N. brasiliensis* infection were obtained as described in section 3.9.2.

Hepatocytes from normal mice were isolated and cultured as described in section 3.9.1 but the culture medium was supplemented with 6 - 10% alveolar macrophage supernatants. Hepatocytes were cultured for 24 h under closed-cap system at 37°C and supernatants assayed for α 1Pi by ELISA (section 3.5.4).

3.16 Statistical Analysis

Tests for significant differences between groups were conducted by use of the two-sample Student's t test. The two-tailed hypotheses, $H_0: \mu_1 - \mu_2 = 0$ and $H_A: \mu_1 - \mu_2 \neq 0$ were tested in each case. All data is presented as mean \pm standard error of the mean (SEM).

CHAPTER 4

EXPERIMENTAL RESULTS

4.1 Isolation of α 1-antitrypsin (α 1Pi)

The trypsin inhibitory capacity of pooled CBA/J mouse plasma was 1.42 mg trypsin inhibited/ml plasma which contained 51 mg of protein. The specific activity of the final α 1Pi preparation was 336 μ g trypsin inhibited/mg α 1Pi, representing a purification factor of 12 (Table 4.1).

The purified α 1Pi from the final DEAE-Sephadex column exhibited a single homogeneous band on analytical polyacrylamide gel electrophoresis (Fig. 4.1.1). This purified α 1Pi represented 27.4% of the total plasma antitryptic activity. The molecular weight of α 1Pi, as determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) was $53,500 \pm 2,000$ daltons.

The plasma clearance of ^{125}I -radiolabelled α 1Pi over a period of 50 hours suggested a half-life of approximately 15.5 hours (Fig. 4.1.2). The initial time points of the plasma clearance curve do not show the expected compartmentalization of α 1Pi into extravascular sites following injection of radiolabelled material. This may be due to the actual experimental protocol where $T(0)$ was taken at approximately 30 min post-injection of ^{125}I - α 1Pi. In the mouse, this initial 30 min period may be sufficient for compartmentalization to have occurred. However, this should not have any effect on the remainder of the plasma clearance curve which was used for estimating $T_{1/2}$.

TABLE 4.1

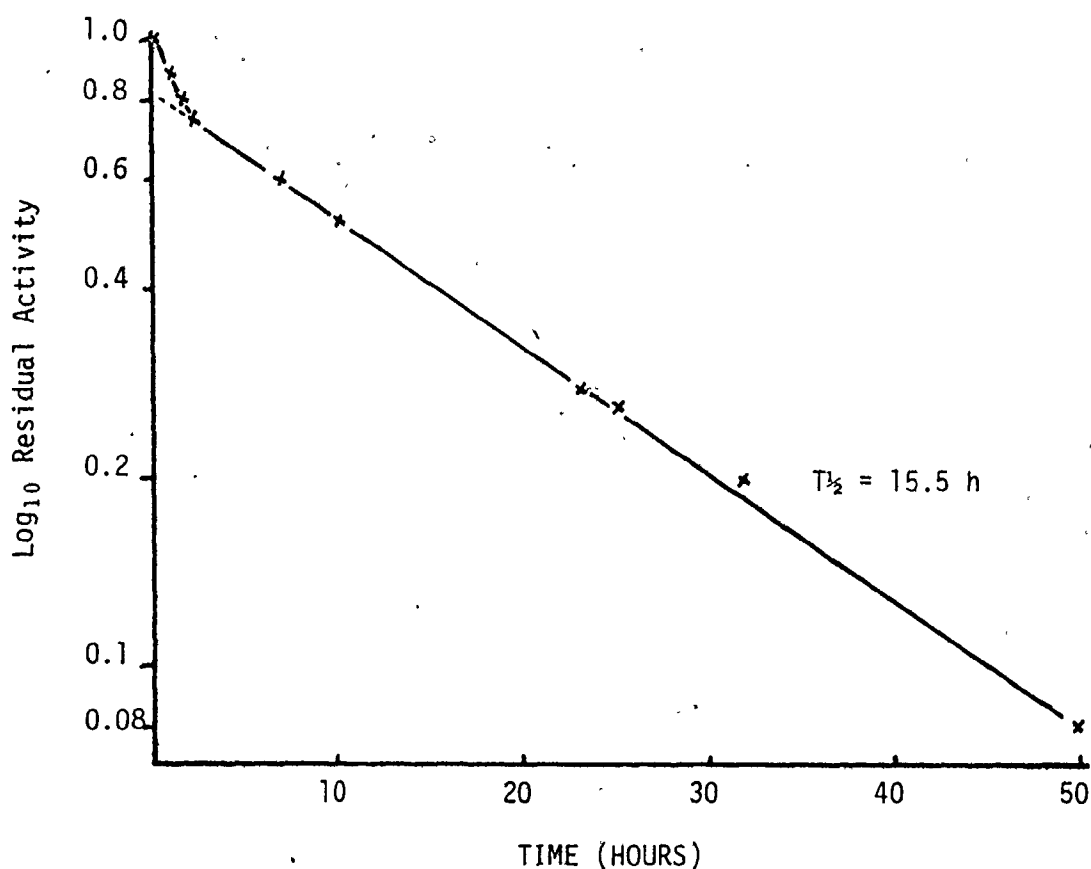
Purification of Mouse α_1 Protease Inhibitor

Purification step	Volume (ml)	Protein content (mg/ml)	Antitryptic activity (units/ml)	Specific activity (units/mg protein)	Purification (-fold)	Recovery of inhibition activity (%)
1 Plasma	20	51.0	1420	27.8	1.00	100
2 Sepharose-Heparin $(\text{NH}_4)_2\text{SO}_4$ 55-85%	25.0	23.0	920	40.0	1.44	83
3 DEAE 8.8	73.0	3.9	225	57.7	2.07	59
4 Con A-Sepharose	10.0	4.5	1270	282.2	10.15	52
5 C.B.-Sepharose	17.0	2.5	758	303.2	10.90	46
6 DEAE 6.5	3.8	6.1	2050	336.0	12.08	28



Figure 4.1.1 Polyacrylamide gel electrophoresis of purified mouse $\alpha 1\text{Pi}$

- a) DEAE, pH 6.5 active fraction
- b) final purified $\alpha 1\text{Pi}$
- c) contaminant after preparative polyacrylamide gel electrophoresis

Figure 4.1.2 Plasma Clearance of ^{125}I - αIPi 

4.2 Immunochemical Characterization of αIPi

Sheep anti-mouse αIPi antiserum reacted with a single precipitin line in double-diffusion against normal mouse serum showing identity with the purified αIPi fraction. The same antiserum exhibited marked cross-reactivity with normal rat serum (Fig. 4.2.1) whereas sheep, goat, rabbit and guinea pig sera did not react.

On two-dimensional immunoelectrophoresis against normal mouse serum, two crossing precipitin arcs are observed (Fig. 4.2.2), suggesting that αIPi in the mouse occurs as two antigenically distinct entities or

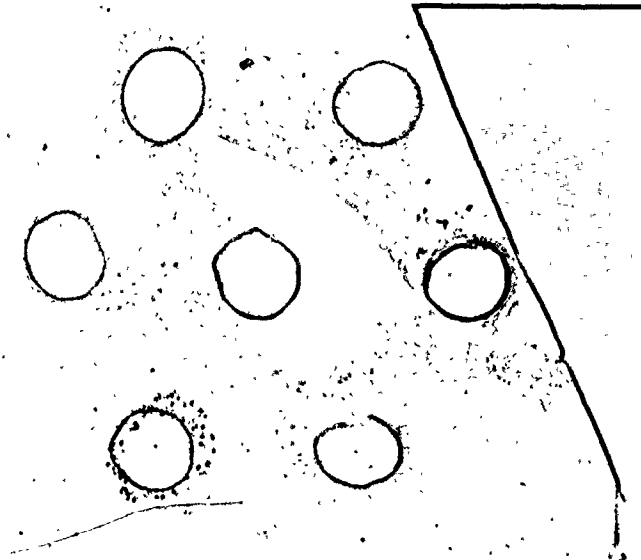
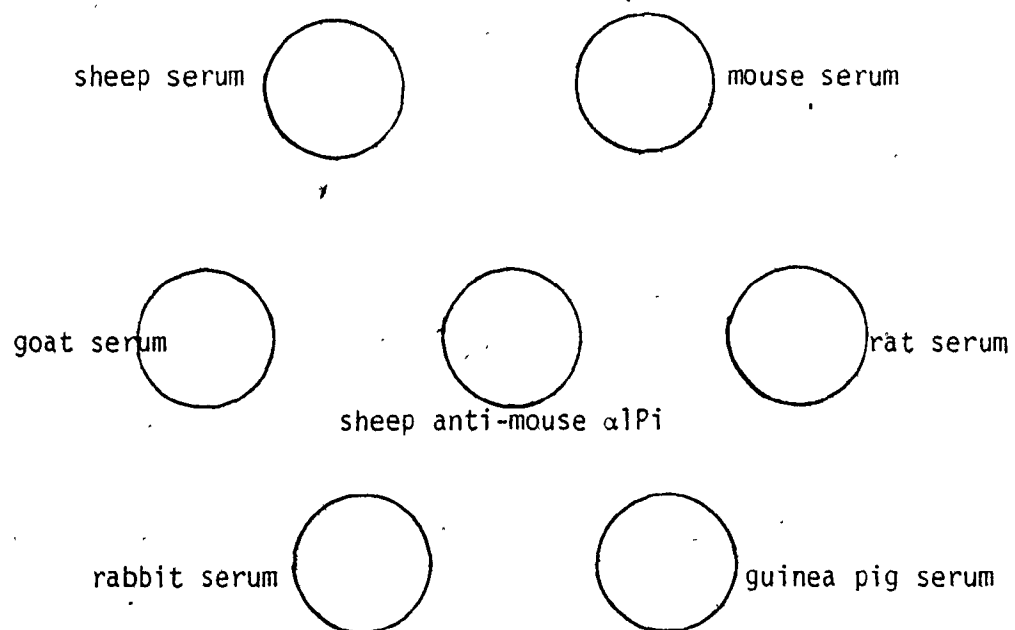


Figure 4.2.1 Specificity of $\alpha 1\text{Pi}$ antiserum



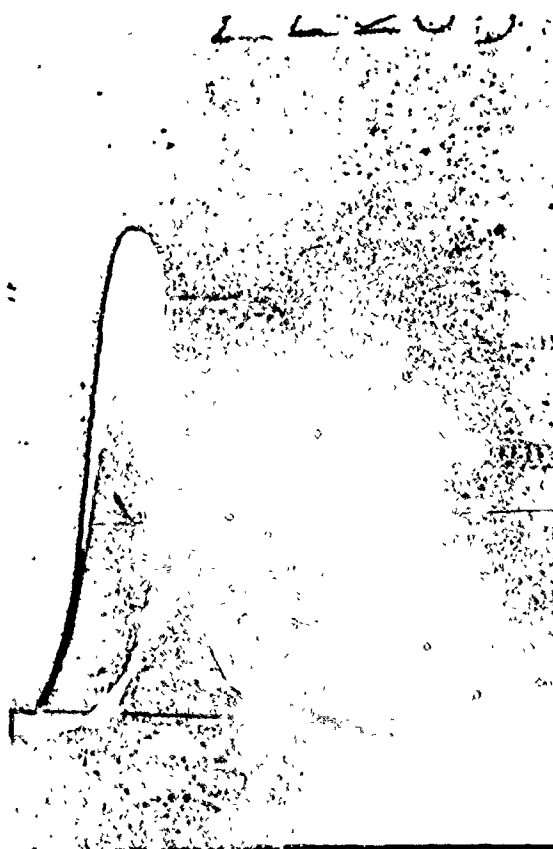


Figure 4.2.2 $\alpha 1\text{Pi}$ variants

Mouse $\alpha 1\text{Pi}$ was electrophoresed in the first dimension (cathode at left) in 1% agarose and electrophoresed in the second dimension (cathode at bottom) against 3.2% sheep anti-mouse $\alpha 1\text{Pi}$

variants. Such results are consistent with those of Myerowitz and co-workers (1972a). The ratio of slow to fast variant indicates that both molecules are present in substantial amounts in normal mouse serum (Table 4.2.1) although there is considerable variation in peak ratios amongst different mouse strains. Within an individual strain, however, the two α 1Pi variants are present in the same relative concentrations in fetal circulation, gut washings, breast milk, as well as in supernatants of cultured hepatocytes. Moreover, during the onset of an acute inflammatory reaction, both α 1Pi variants rise proportionally such that the relative ratio of the two peaks remains constant (data not shown).

Both α 1Pi variants bind to porcine pancreas trypsin although there were some differences depending on the iodination procedures used for α 1Pi. Chloramine-T, which is a strong oxidizing agent, interferes considerably with the trypsin binding activity of the fast variant of α 1Pi as evidenced by the nearly complete absence of fast variant in the slower migrating α 1Pi-trypsin complex (Fig. 4.2.3). Iodination of α 1Pi by the mild Bolton-Hunter reagent has no apparent effect on the two variants with respect to their trypsin binding activities (Fig. 4.2.4). These results are consistent with the work of Carp and Janoff (1978) who showed that under oxidizing conditions, the methionine reactive site of α 1Pi was inhibited. ^{125}I -labelled porcine pancreas trypsin bound to both α 1Pi variants.

Normal adult α 1Pi serum levels showed variation between strains of mice (Table 4.2.2).

TABLE 4.2.1

Ratios of Electrophoretically Heterogeneous α 1Pi Variants in Mouse Strains

Strain	Ratio slow : fast variants
CBA/J	2.7
RF/C57B1	2.6
Ba1b/C	2.3
DBA	2.3
RJ/J	2.1
nu/nu	2.1
C3H	2.1
A/J	1.9
C57B1	1.6

TABLE 4.2.2

 α 1Pi Concentration in Normal Mouse Serum

	mg/ml
CBA/J	3.0 \pm 0.7
C3H	3.5 \pm 0.6
C3D2	4.6 \pm 1.0
n = 10	

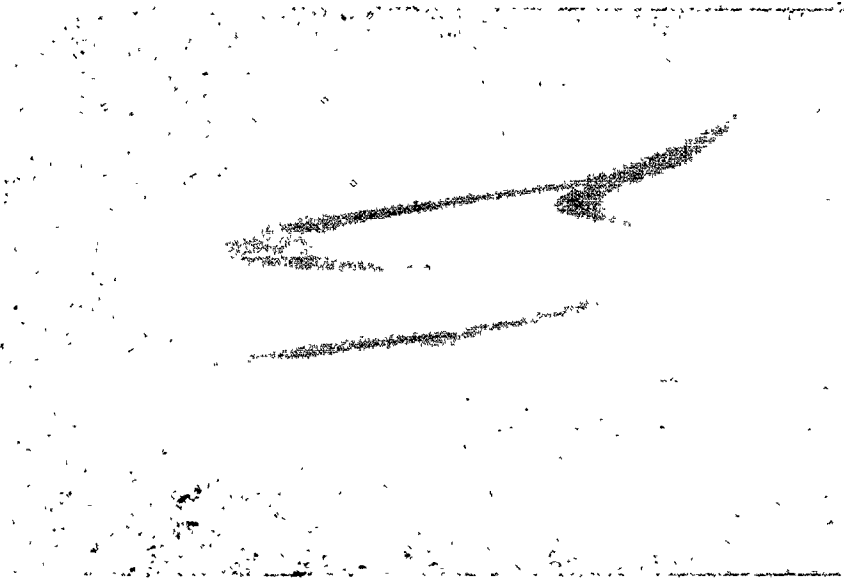


Figure 4.2.4 Trypsin binding activity of α PI
initiated by Bolton-Hunter reagent.
Slower migrating α PI-trypsin
complex at left.

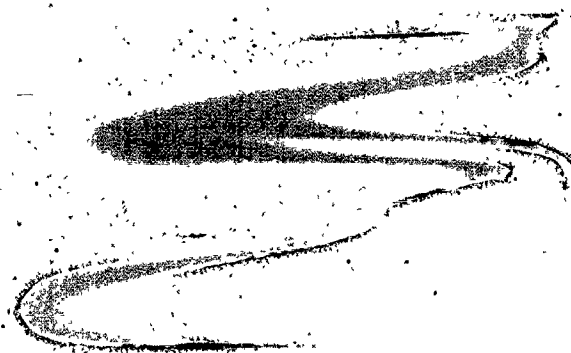


Figure 4.2.3 Trypsin binding activity of α PI
iodinated by chloramine-T. Slower
migrating α PI-trypsin complex at
left.

The ubiquitous nature of α Pi was indicated by its presence in various body fluids (Table 4.2.3) as well as its localization in a variety of tissues such as liver hepatocytes (Fig. 4.2.5; control, Fig. 4.2.6), pancreatic islets of Langerhans (Fig. 4.2.7), some epithelial cells in the small intestine villi as well as interstitially in the lamina propria (Fig. 4.2.8). α Pi was thus found in all body fluids examined and although the amounts varied, these never exceeded plasma levels.

Using *in vitro* incorporation of ^{35}S -methionine/ ^{14}C -lysine in isolated cell culture systems, it was shown that the major site of synthesis of α Pi was the hepatocyte (Fig. 4.2.9) whereas alveolar macrophages synthesized minimal quantities of α Pi in comparison to hepatocytes (Fig. 4.2.10).

Islets of Langerhans did not synthesize detectable amounts of α Pi under these conditions but there was release of cytoplasmic α Pi as evidenced by iodination of culture supernatant proteins and subsequent specific immunoprecipitation of ^{125}I -labelled α Pi on two-dimensional electrophoresis.

Both α Pi variants were also synthesized by the murine hepatoma cell lines Hepa-1, 12S, 11ab, 19/2 and HH.

There was no detectable synthesis of α Pi by whole organ cultures of rat spleen, lung, trachea, Peyer's patches and mesenteric, mediastinal, peripheral and inguinal lymph nodes. However, a labelled protease(s) was synthesized and secreted by jejunum and ileum tissues and was bound to exogenous cold α Pi as evidenced by altered electrophoretic mobility of α Pi.

TABLE 4.2.3

Presence of α 1Pi in Various Body Fluids (CBA/J mice)

Fluid	α 1-protease inhibitor concentration	
Adult serum	3 mg/ml	N
Fetal serum	0.4 mg/ml	N
Amniotic fluid	28 μ g/ml	E
Bile	19 μ g/ml	E
Breast milk	2 mg/ml	N
Gastrointestinal washings	7.2 μ g/20 cm gut	E
Bronchiolavage fluid	65 μ g/lung	E

N, assayed by nephelometry

E, assayed by ELISA

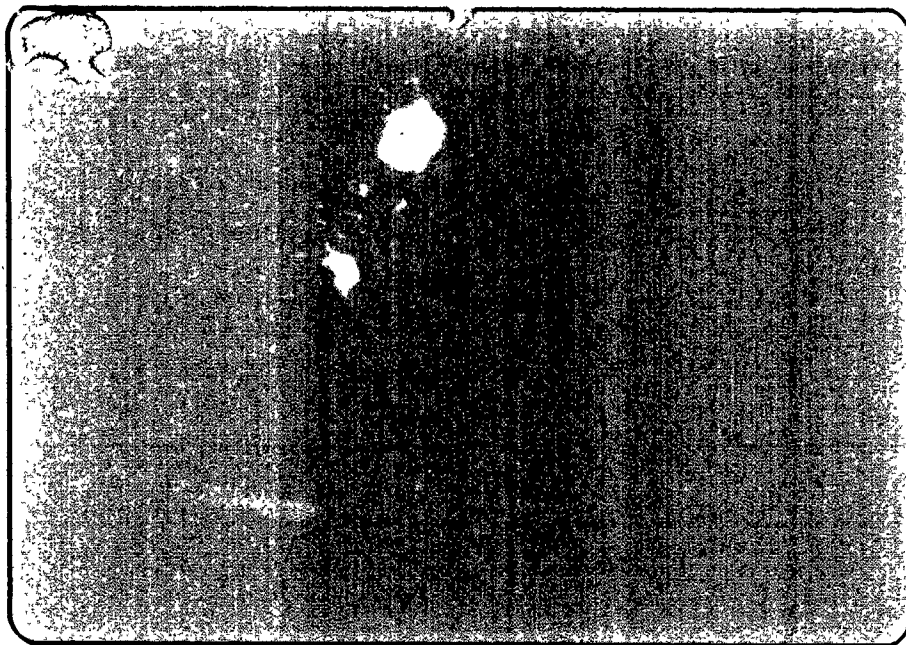


Figure 4.2.5 Adult liver stained for $\alpha 1\text{Pi}$ with horseradish peroxidase conjugated immunoreagents (x100)

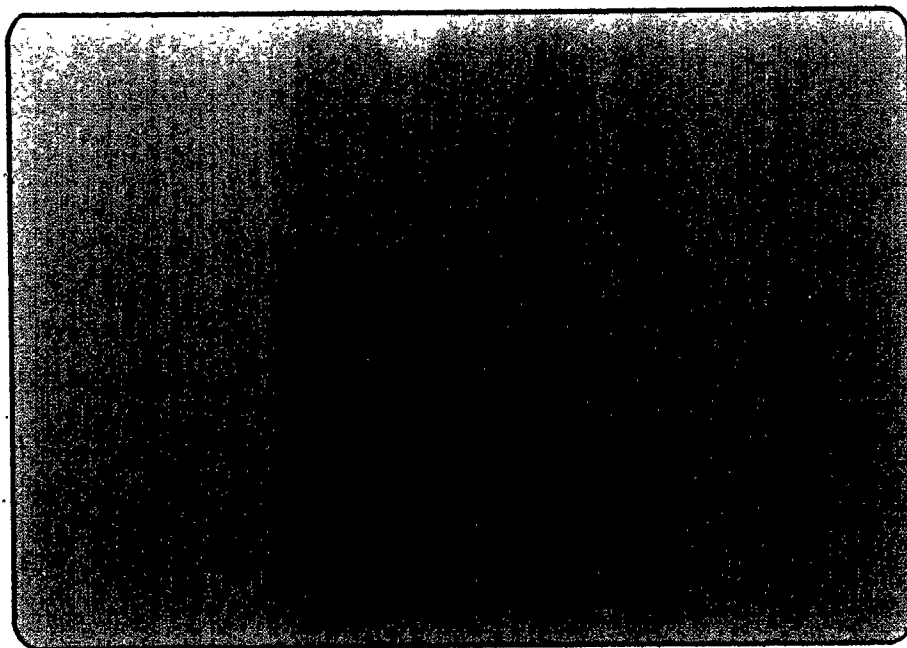


Figure 4.2.6 Negative control for $\alpha 1\text{Pi}$ staining in adult liver (Counterstain methyl green)

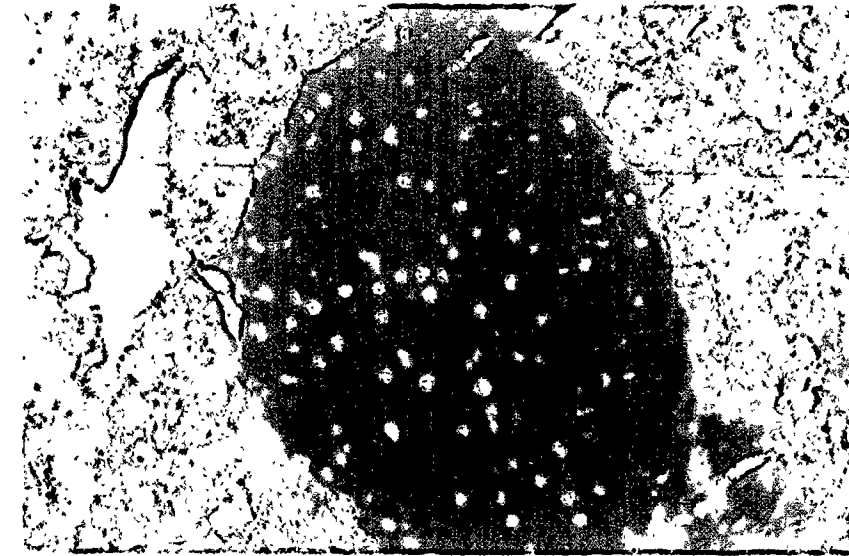


Figure 4.2.7. Immunohistochemical localization of $\alpha 1\text{Pi}$ in islets of Langerhans. Horseradish peroxidase conjugated immunoreagent stain (x400)

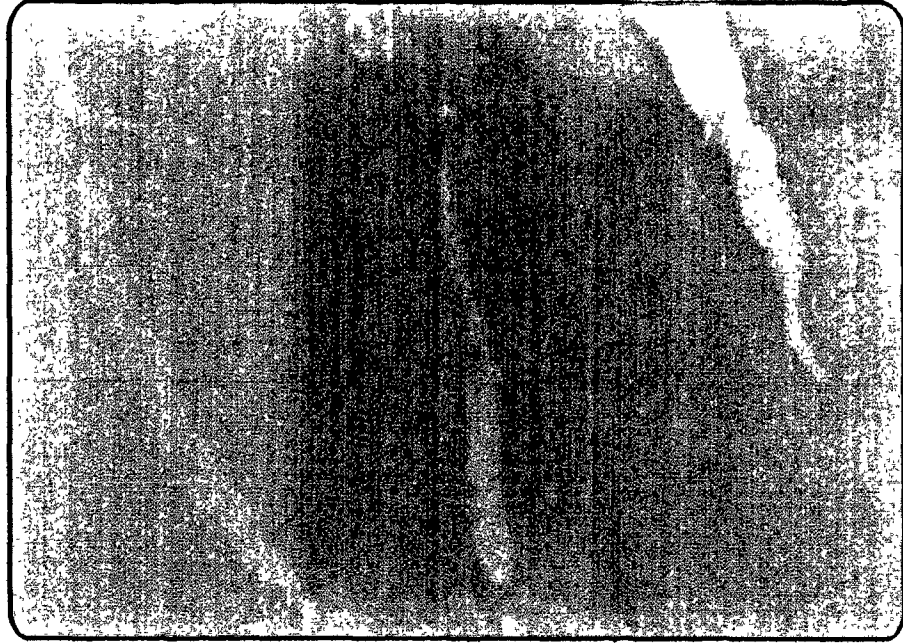


Figure 4.2.8. Immunohistochemical localization of $\alpha 1\text{Pi}$ in mouse small intestine using horseradish peroxidase conjugated immunoreagent (x250)

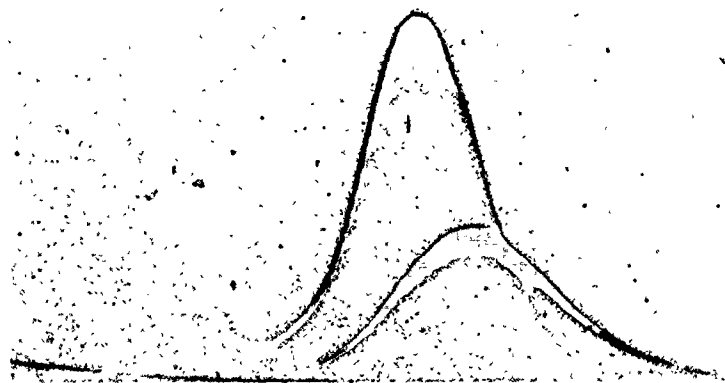


Figure 4.2.9. *In vitro* synthesis of $\alpha_1\text{Pi}$ by isolated hepatocytes in a 24 h culture period. ^{35}S -methionine incorporation revealed by autoradiography. The fast variant is at right.

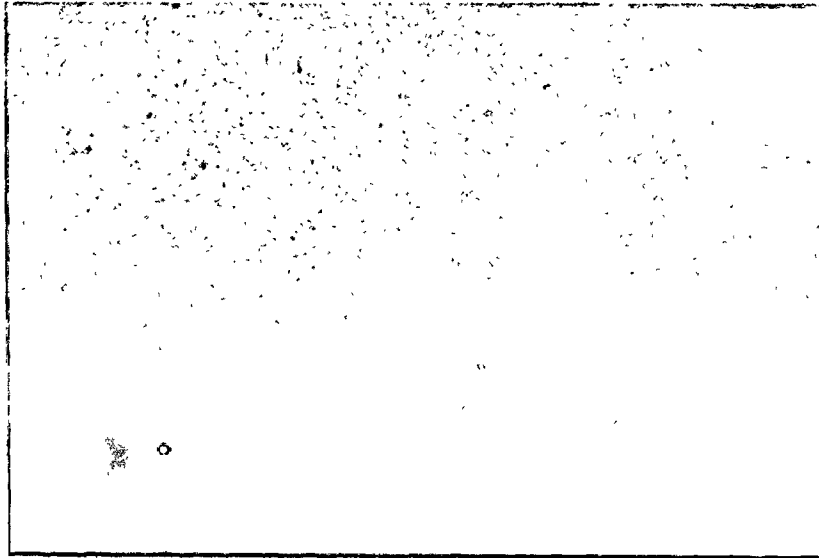


Figure 4.2.10 (a) *In vitro* synthesis of $\alpha 1\text{Pi}$ by alveolar macrophages from normal lung in a 24 h culture. ^{35}S -methionine incorporation revealed by autoradiography

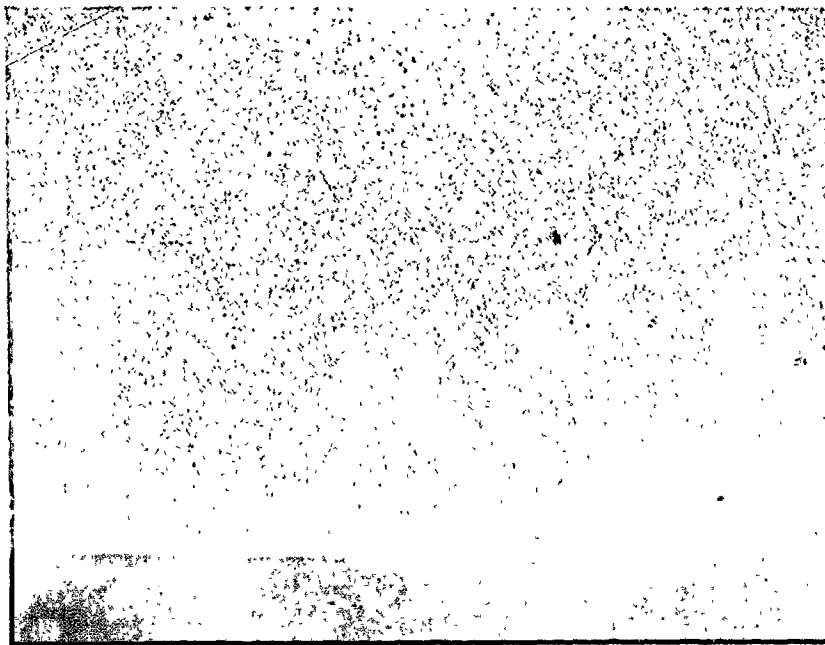


Figure 4.2.10 (b) *In vitro* synthesis of $\alpha 1\text{Pi}$ by *N. brasiliensis* infected lungs in a 24 h culture. ^{35}S -methionine incorporation revealed by autoradiography

4.3 Ontogeny of α 1Pi

The serum levels of α 1Pi in the fetus represent approximately 15% of the normal adult level (Fig. 4.3.1). There is a progressive rise in serum levels to the adult range approximately 25 days post-parturition and the antitryptic activity of serum correlates with the immunoquantitation profile (Fig. 4.3.1). Throughout the pregnancy, the maternal serum level is within the normal adult range.

In order to determine whether the low levels present in the fetal circulation represent synthesis of α 1Pi by the fetus or trans-placental transfer of maternally derived α 1Pi, ^{125}I - α 1Pi was injected intravenously into pregnant mice at 17-19 days gestation. Maternal and fetal sera were obtained at selected time intervals and total serum radioactivity as well as protein-associated iodine-125 determined. As shown in Figure 4.3.2, there is a progressive linear increase in the ratio of total radioactivity of fetal to maternal serum with time. However, the ratio of protein-associated iodine-125 in fetal and maternal serum is constant with time and is less than 1%. Such data would indicate that fetal α 1Pi is not maternally derived to any significant extent, if at all and hence, the low fetal levels likely represent synthesis by the fetus. The small amount of protein-associated radioactivity could potentially arise from non-specific binding of free iodine-125 to serum proteins, such as IgG.

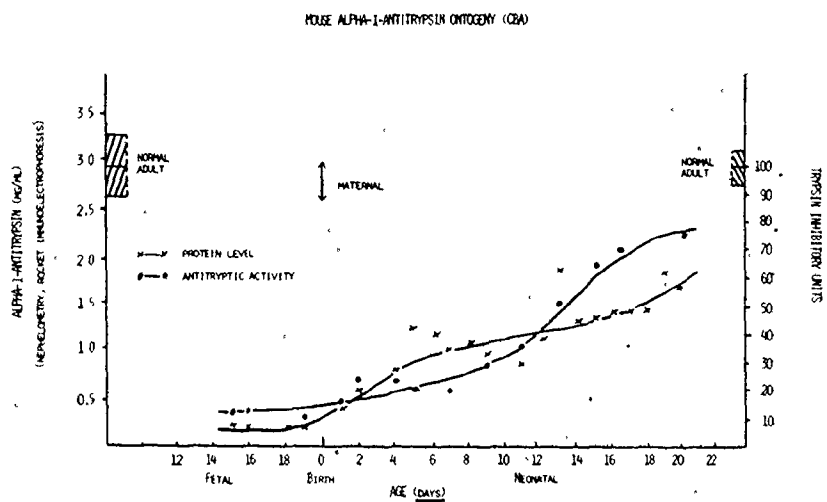
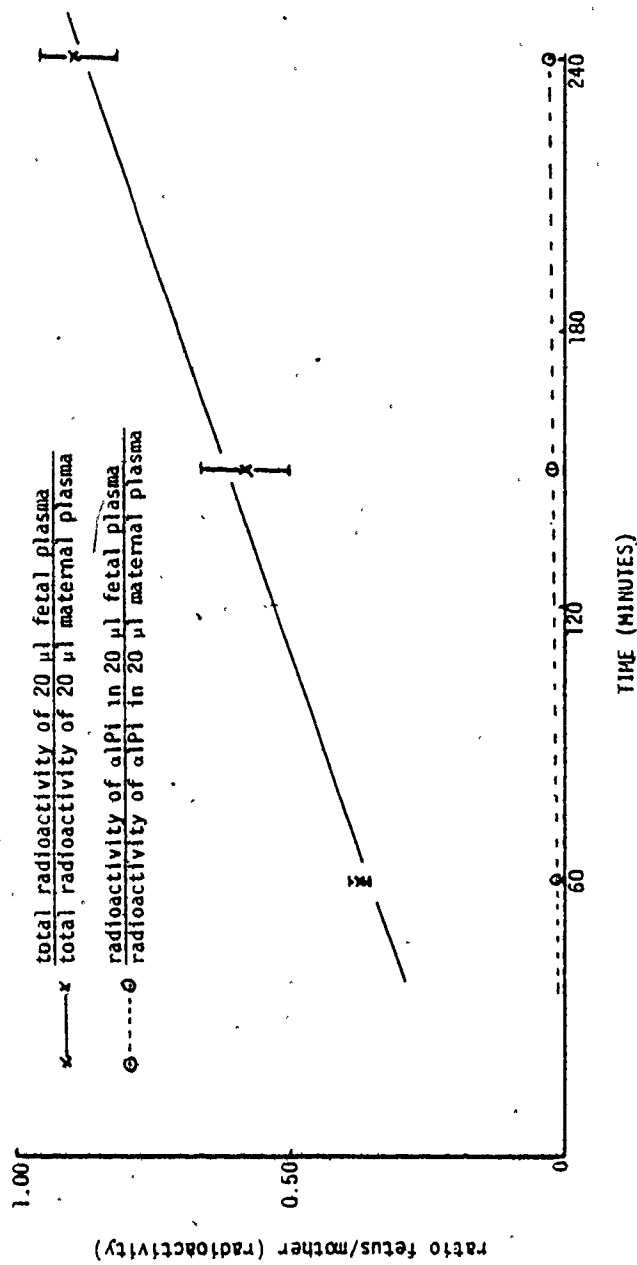


Figure 4.3.1

Ontogeny of $\alpha 1\text{Pi}$ in CBA/J mice. Each data point represents a pool of sera from seven mice. $\alpha 1\text{Pi}$ serum levels (x—x) were determined using sheep anti-mouse $\alpha 1\text{Pi}$ and antitryptic activity (o-----o) using BAPNA as substrate

Figure 4.3.2 Transplacental transfer of ^{125}I - αPI 

4.4 Celite Induced Acute Inflammation

Following a single subscapular injection of a sterile suspension of celite, α lPi levels in serum showed a steady increase approximately five hours post-injection and reached a maximum of 125% of normal adult levels by 24 h (Fig. 4.4.1). Subsequently, α lPi serum levels decreased to within a normal range by approximately 45 h. The initial decrease in serum α lPi levels (1 - 4 h) is likely due to initial rapid sequestration of α lPi at the site of the inflammatory stimulus although experiments to demonstrate this phenomenon have not been carried out.

Using immunohistochemistry, there was a marked alteration in the number of hepatocytes which stained for α lPi and also in the overall distribution of such hepatocytes throughout the liver lobule during celite induced inflammation. In normal mouse liver, most hepatocytes exhibit low-level diffuse cytoplasmic staining for α lPi although the intensity of staining varies within the liver lobule. There are a limited number of hepatocytes (1 - 3%) which stain intensely for α lPi (Fig. 4.4.2.1) whereas others exhibit weaker staining intensity. The intense α lPi cytoplasmic staining does not appear to be restricted to a particular size of hepatocytes and is found in both uni- and multi-nucleated hepatocytes. These hepatocytes are distributed mainly in areas around the portal triads as well as being randomly distributed throughout the liver lobule. Under high power magnification, α lPi was located in diffuse granular-like deposits throughout the cytoplasm (Fig. 4.4.3).

In marked contrast, six hours post-inflammatory stimulus (celite), at a time when there were no significant changes in serum

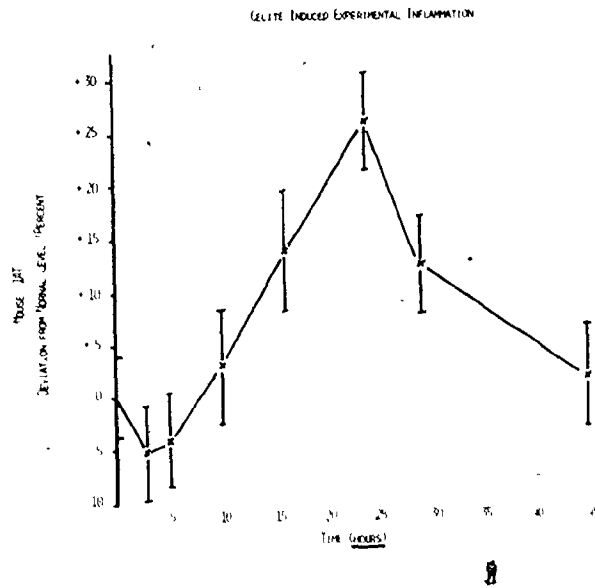


Figure 4.4.1 α 1Pi serum levels in celite induced inflammation. At 24 h, serum levels are significantly different from normal levels ($0.025 < P < 0.05$)

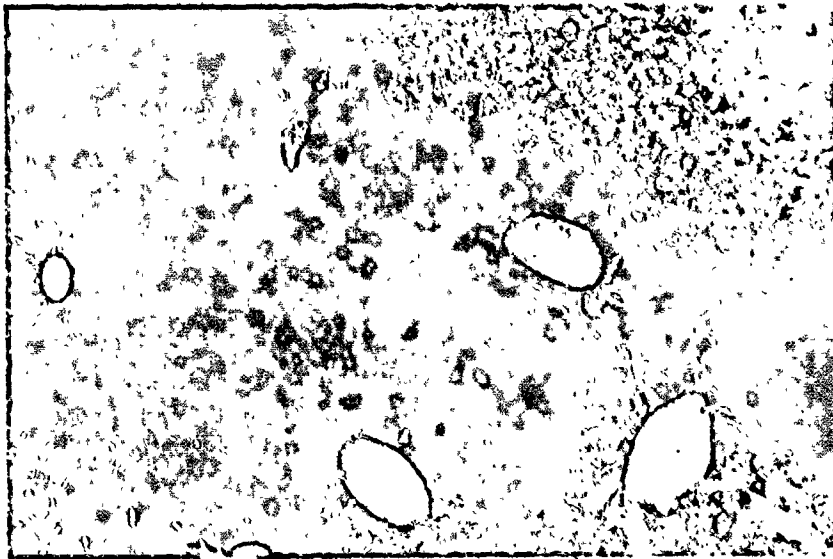


Figure 4.4.2.1 Normal adult liver stained for α Pi with horseradish peroxidase conjugated immunoreagents (x100)

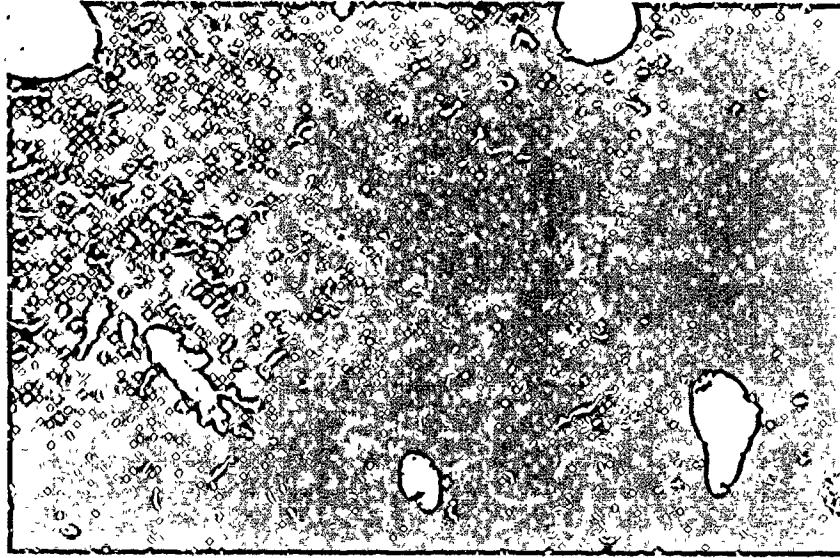


Figure 4.4.2.2 Adult liver stained for α Pi 9 h post-celrite injection (x100)

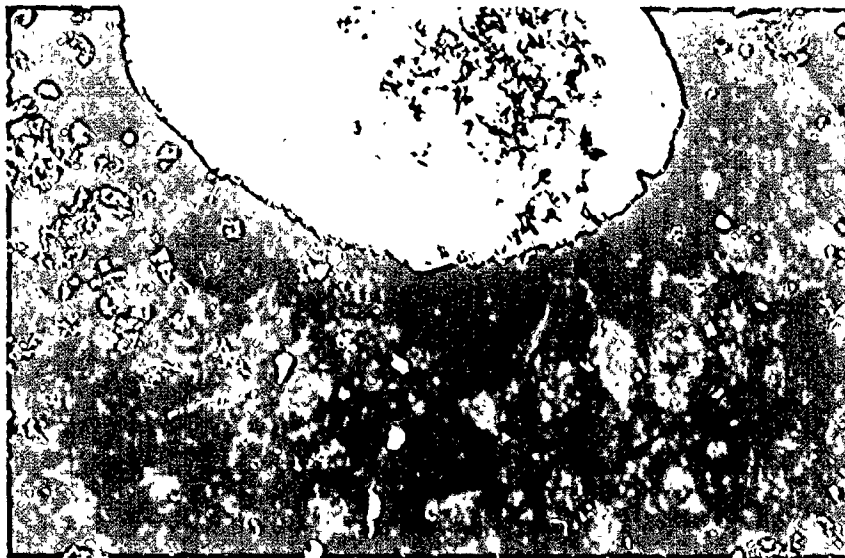


Figure 4.4.3 Normal adult liver stained for α 1Pi with horseradish peroxidase conjugated immunoreagents (x400)

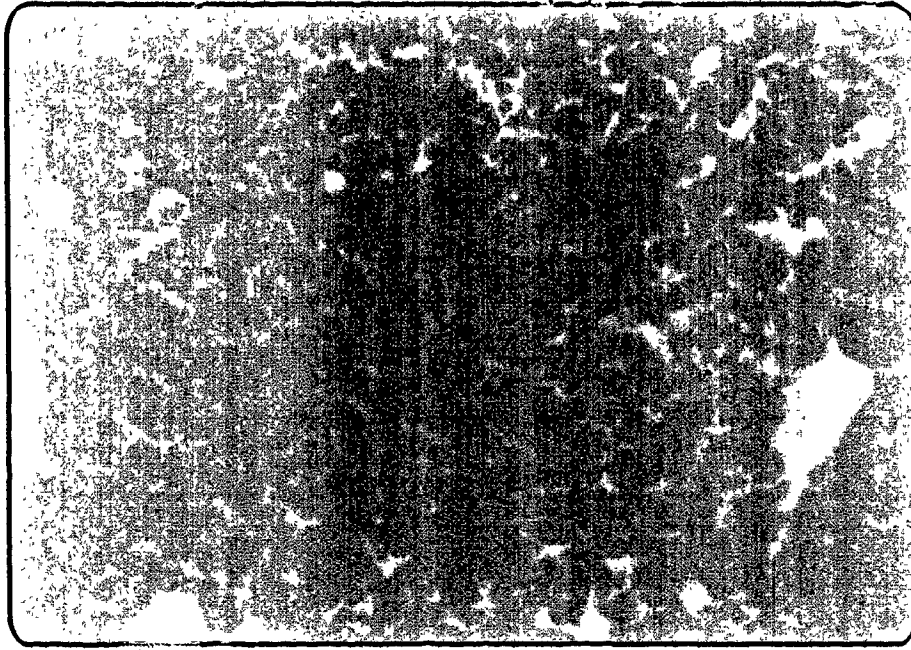


Figure 4.4.4 Day 15 fetal liver stained for α 1Pi with horseradish peroxidase conjugated immunoreagents (x250)

α lPi level, a greater number of hepatocytes which stained intensely for cytoplasmic α lPi were observed. Furthermore, there was a significant increase in the intensity of the low level "background" staining of hepatocytes for α lPi. This increase in the number of hepatocytes which stained intensely for cytoplasmic α lPi was more accentuated at nine hours (Fig. 4.4.2.2) and maximum staining activity was observed at approximately 9 - 12 h. Concurrent with this increase in α lPi cytoplasmic staining activity there was a marked alteration in the distribution pattern of such hepatocytes. These now tended to be located in the vicinity of the central vein as well as being distributed throughout the liver lobule. At 24 h, corresponding to maximum increase in α lPi serum level, the number and distribution of intensely staining hepatocytes was more characteristic of a normal liver.

Fetal liver hepatocytes also stained for α lPi (Fig. 4.4.4) as did neonatal liver. In both instances, as was the case with adult liver, there were a few hepatocytes which stained intensely for cytoplasmic α lPi. Kupffer cells and epithelial cells lining the bile ducts were negative for α lPi.

4.5 *In vitro* Hepatocyte Output of Acute Phase Reactants in Celite Induced Inflammation

As described in section 4.4, there were marked alterations in α lPi staining intensity and distribution of such hepatocytes in the liver during the course of a celite induced inflammatory response. These immunohistochemical findings suggested an increased hepatic output of α lPi with maximum activity at approximately 9 - 12 h post-

stimulus. In order to substantiate this, the *in vitro* output of α 1Pi by isolated hepatocytes was studied.

Hepatocytes from mice injected with celite were isolated (section 3.9.1) at 9 - 12 h post-stimulus and cultured for a 24 h period. At selected times, a small aliquot of culture supernatant was collected and assayed for α 1Pi by ELISA (section 3.5.4). This output was compared to that obtained from hepatocytes from non-treated (normal) mice.

Over 24 h, there was a 1.5 to 3-fold increase in α 1Pi hepatic output in celite treated animals as compared to normal mice (Fig. 4.5.1). The increased hepatocyte output of α 1Pi *in vitro*, in celite induced inflammation, correlates with the increased cytoplasmic α 1Pi activity as determined by immunohistochemistry.

The *in vitro* hepatocyte output of SAA in celite induced inflammation also demonstrated an acute phase reaction although the immunohistochemical localization of SAA in this model was not studied. There was approximately a 25-fold increase in SAA from hepatocytes from celite stimulated animals as compared to hepatocytes from non-treated mice (Fig. 4.5.2).

4.6 *Nippostrongylus brasiliensis* Induced Inflammation

The life cycle of *N. brasiliensis* in mice is shown in Figure 4.6.1. Previous workers have described the migration kinetics and associated pathology of this parasite. In the skin, there is evidence of dissolution of

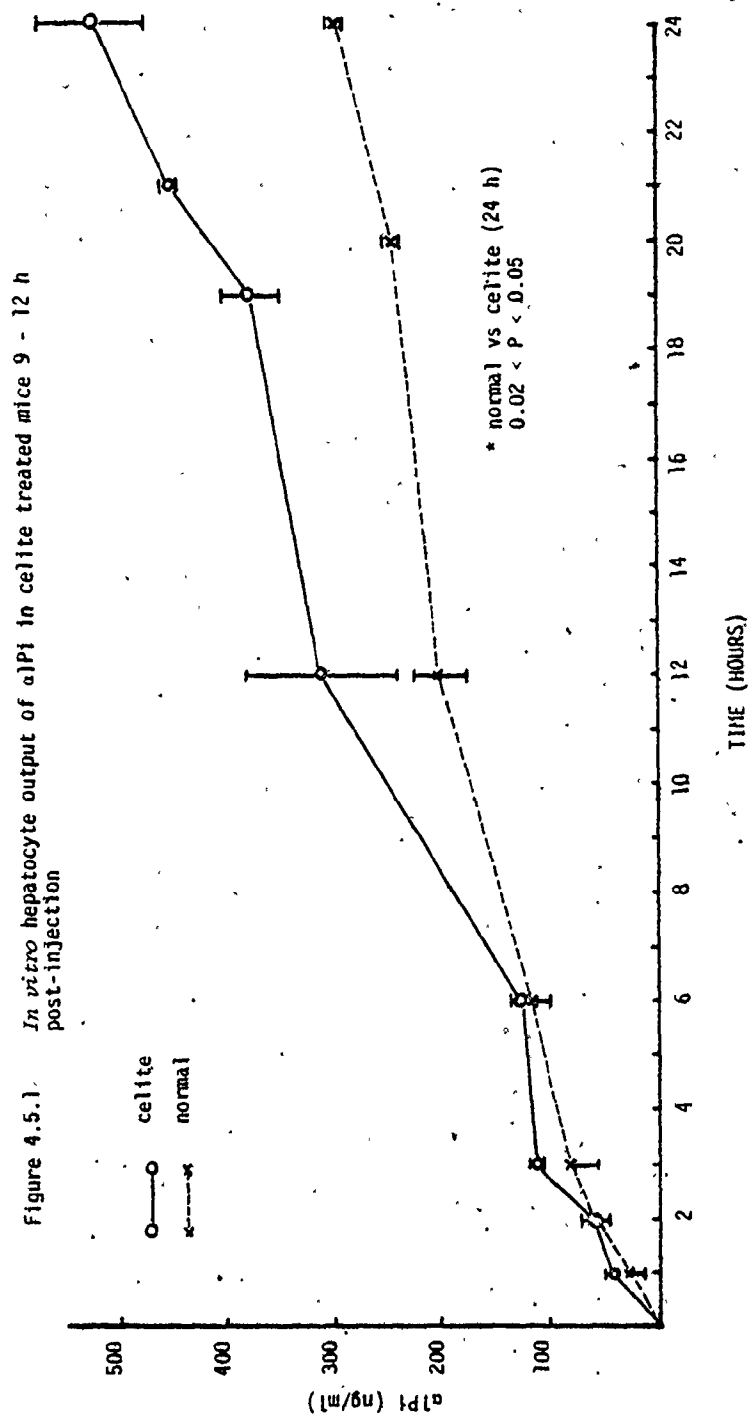
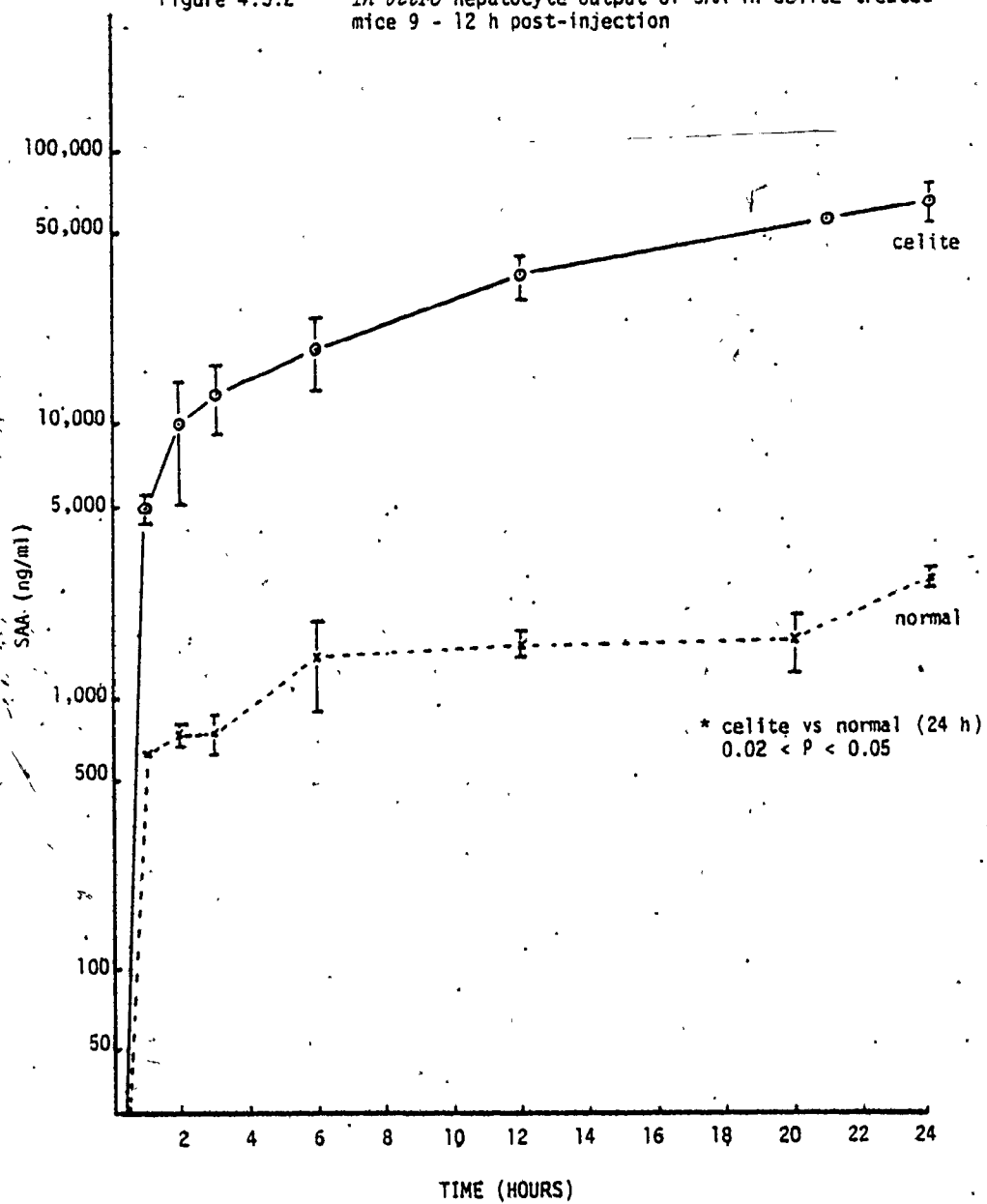


Figure 4.5.2 *In vitro* hepatocyte output of SAA in celite treated mice 9 - 12 h post-injection



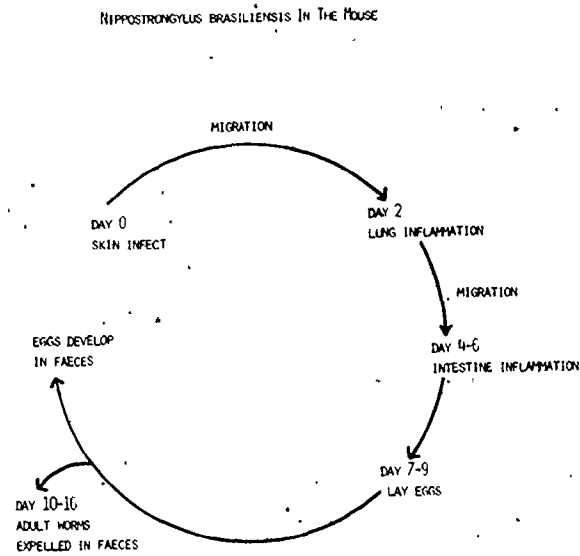


Figure 4.6.1 Life cycle of *N. brasiliensis* in the mouse

collagen around the nematode but this need not necessarily be an enzymatic degradation of the collagen as an alteration in pH of the microenvironment could bring about dissociation of the collagen fibres (Lee, 1972). Within two days, L3's migrate, via the central stream of the arteries, to capillary beds and are passively trapped in the lungs (Croll and Ma, 1978). At this stage, the parasites initiate a local acute inflammatory response with marked edema, hemorrhage and destruction of alveolar spaces (Fig. 4.6.2; 4.6.3). Morphologically, the external lung surface is characterized by large hemorrhagic foci giving it a "patchy" appearance. Such a distinguishing feature of a parasitized lung has been used as a rapid qualitative assessment of the "success" of an infection protocol and the intensity of the worm burden. The parasites then develop into fourth stage larvae (L4) and migrate to the gastrointestinal tract via the trachea and esophagus. The parasites are transported in the mucus stream which lines the whole trachea as well as by the flagellated motion of the cilia (Croll and Ma, 1978). An acute inflammatory reaction develops in the small intestine by day 5 causing edema and villous atrophy with crypt hyperplasia (Symons and Fairbairn, 1962) (Fig. 4.6.4; 4.6.5). Upon maturation to the adult stage, the nematodes reproduce and maximum number of eggs are layed between 7 - 9 days. The eggs and adult stage parasites are subsequently expelled in the feces by days 14 - 19 (Wescott and Todd, 1964).

4.7 Serum and Tissue Acute Phase Response in *N. brasiliensis* Infection

During the initial five days following the infection of mice with *N. brasiliensis*, the serum levels of α 1Pi fluctuated within the



Figure 4.6.2 Normal mouse lung.
Haematoxylin and eosin stain
(H & E) (x100)

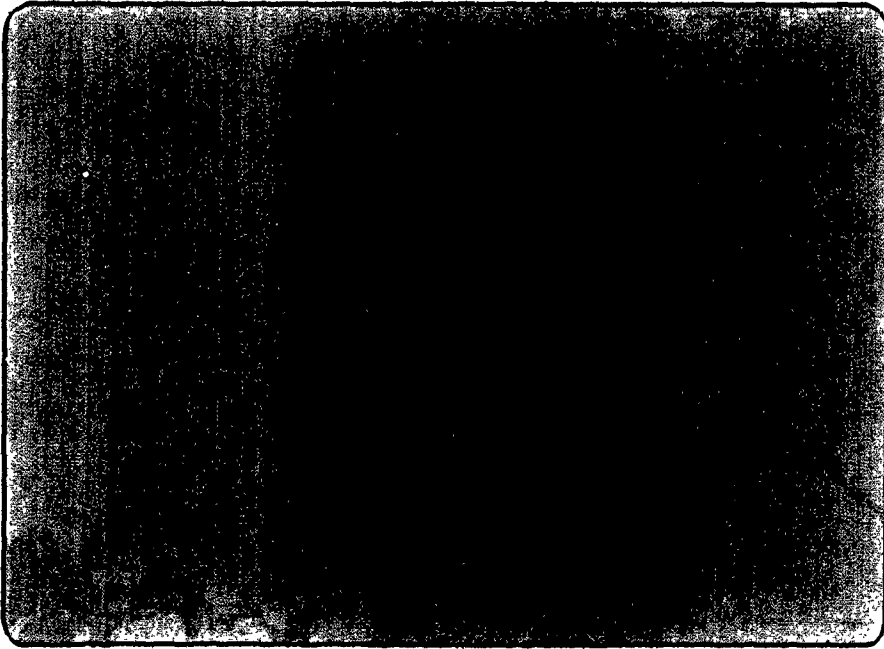


Figure 4.6.3 Mouse lung 2 days post-
infection with *N. brucei*
(H & E) (x100)



Figure 4.6.4 Normal mouse intestine (H & E) (x100)



Figure 4.6.5 Mouse intestine 6 days post-infection with *N. brasiliensis* (H & E) (x100)

normal range with an indication of a slight but insignificant increase in serum levels at day 2 (Fig. 4.7.1), a time corresponding to massive lung inflammation. Subsequently, there was a gradual increase in α Pi serum levels to a maximum of 120% of normal adult by day 10 post-infection, a time corresponding to gastrointestinal inflammation. The α Pi levels subsided to normal by approximately 20 days post-infection.

As indicated in Figure 4.7.1, at the time of massive lung inflammation (day 2), there was no significant increase in α Pi serum concentration. However, the immunohistochemical localization of α Pi in the liver revealed an intense acute phase response at this particular time in the course of the parasite infection (Fig. 4.7.2). As was the case for celite induced inflammation, there was a significant increase in the number of hepatocytes which stained intensely for cytoplasmic α Pi. The lobular distribution of such hepatocytes was consistent with previous findings in that the intensely staining hepatocytes were situated around the central vein as well as throughout the liver lobule whereas the areas around the portal triads were relatively devoid of such intense staining hepatocytes. With this inflammation model, the liver acute phase response was of such intensity that individual liver lobules were clearly demarcated. This was of considerable significance since, unlike the human liver, individual lobules in the mouse liver were not clearly defined under common H & E staining.

During the intestinal inflammatory stage, the liver was still undergoing an acute phase response as evidenced by a large number of intensely staining hepatocytes for cytoplasmic α Pi, but there were areas within the lobule in which α Pi hepatic content was markedly

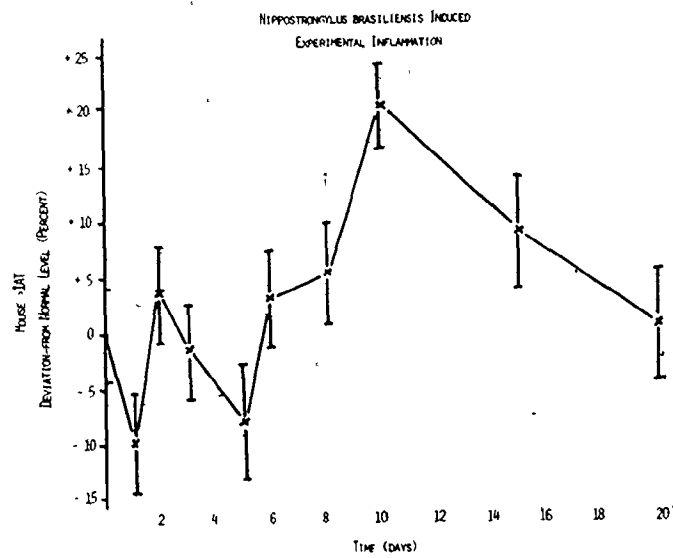


Figure 4.7.1 Serum levels of $\alpha 1\text{Pi}$ in CBA/J mice after subcutaneous infection with 500 *N. brasiliensis* infective larvae. Serum levels at 10 days are significantly different from normal levels ($0.025 < P < 0.05$)

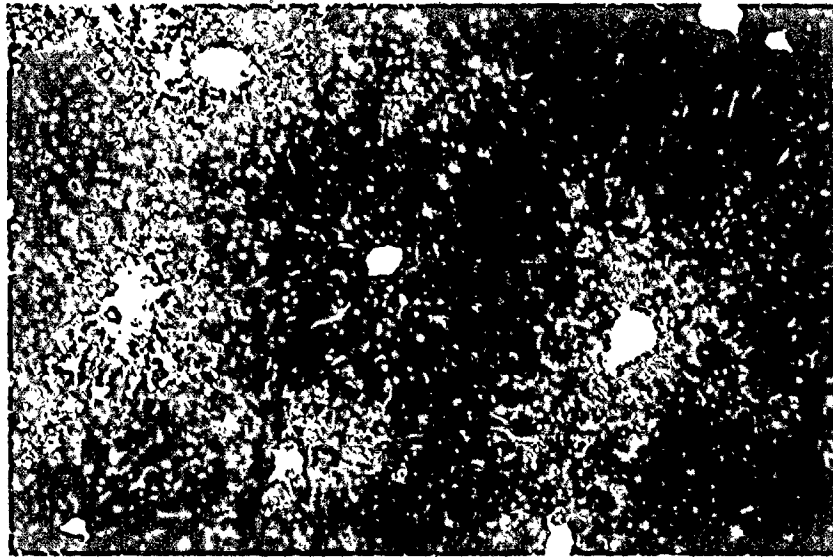


Figure 4.7.2 Mouse liver stained for $\alpha 1\text{Pi}$ 2 days post-*N. brasiliensis* infection (x100)

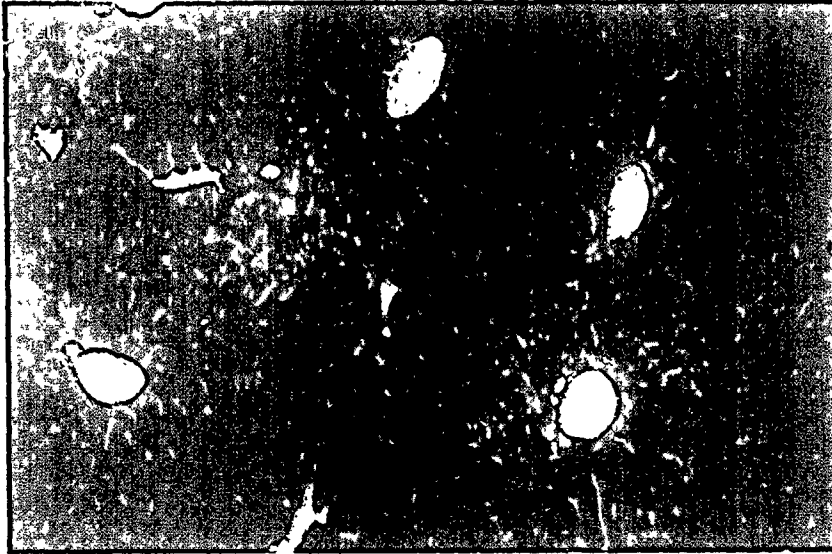


Figure 4.7.3 Mouse liver stained for $\alpha 1\text{Pi}$ 6 days post-*N. brasiliensis* infection (x100)

reduced (Fig. 4.7.3). The distribution and number of intense staining hepatocytes for cytoplasmic α Pi returned to normal following expulsion of worms from the host.

By immunohistochemistry, there were no apparent alterations in α Pi staining in intestinal tissue during the gut stage of *N. brasiliensis*. Interestingly, however, on some occasions, the α Pi positivity in the islets of Langerhans in the pancreas progressively diminished to undetectable levels during the gut stage of *N. brasiliensis* (Fig. 4.7.4 versus Fig. 4.2.7) and α Pi positivity was restored following expulsion of the worms. The exact nature of this α Pi pancreatic activity was not further investigated.

In an attempt to account for the apparent lack of serum α Pi acute phase reaction, the immunohistochemical localization of α Pi at the site of inflammation (lung) was investigated. At day 2 post-infection, at a time corresponding to maximal parasite burden in the lung, there was a marked increase in interstitial staining for α Pi (Fig. 4.7.5). Perhaps of greater significance was the intense α Pi cytoplasmic staining of alveolar macrophages, particularly those surrounding the parasites (Fig. 4.7.5). The identity of these alveolar macrophages was confirmed by fluoride ion inhibitable non-specific esterase activity. Alveolar macrophages from day 1 and 3 post-infection did not exhibit α Pi cytoplasmic staining nor did uninfected normal lung. Moreover, in these latter instances, background interstitial staining for α Pi was minimal.

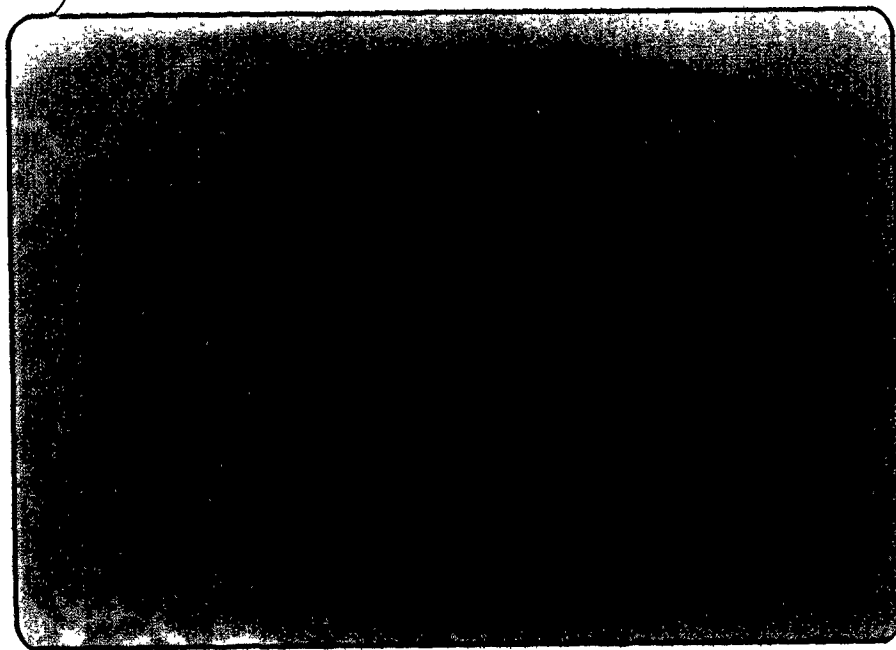
Increased localization of α 1Pi in infected lungs, as compared to normal, was shown using ^{125}I - α 1Pi (Table 4.7.1). There was a significant increase in ^{125}I - α 1Pi in bronchiolavage fluid and washed lung tissue of infected animals. This data is consistent with the increased interstitial staining for α 1Pi in infected lung tissue.



Figure 4.7.4 Absence of α 1Pi in islets of Langerhans 8 days post-*N. brasiliensis* infection as determined by horseradish peroxidase conjugated immunoreagents (x400)



Figure 4.7.5. Mouse lung stained for $\alpha 1\text{Pi}$ with horseradish peroxidase conjugated immunoreagents 2 days post-*N. brasiliensis* infection



Negative control

TABLE 4.7.1

Lung Distribution of ^{125}I - αPI 24 h After Intravenous Administration

	dpm sample/dpm serum ¹	
	<u>Non-infected</u>	<u>Infected</u>
Bronchiolavage Fluid (1 ml volume)	0.037 \pm 0.009	0.246 \pm 0.036*
Washed Lung Tissue (1 gm wet weight)	0.039 \pm 0.005	0.130 \pm 0.028**

* Significant at $0.002 < P < 0.005$

** Significant at $0.02 < P < 0.05$

¹ dpm in serum were similar in both groups of mice

4.8 In vitro Hepatocyte Output of Acute Phase Reactants in *N. brasiliensis* Induced Inflammation

In order to correlate the increased α 1Pi cytoplasmic activity in hepatocytes day 2 post-*N. brasiliensis* infection (Fig. 4.7.2) with increased hepatocyte synthesis, α 1Pi was measured by ELISA in culture supernatants from hepatocytes isolated two days post-infection.

Over a 24 h period, in culture, there was a 5- to 10-fold increase in α 1Pi output by hepatocytes from day 2 infected mice as compared to normal mice (Fig. 4.8.1). As in celite induced inflammation, there is good correlation between the increased α 1Pi cytoplasmic presence in hepatocytes, as determined by immunohistochemistry and *in vitro* hepatocyte synthesis.

The *in vitro* hepatocyte synthesis of SAA was also indicative of an acute phase response as evidenced by a three-fold increase in SAA synthesis in day 2 *N. brasiliensis* infected mice (Fig. 4.8.2).

4.9 Other Acute Phase Reactants in *N. brasiliensis* Induced Inflammation

i) C3 and SAP

Serum levels of the acute phase reactants C3, the third component of complement, and serum amyloid P protein (SAP), were measured by Drs. M. Bältz and M. Pepys (Royal Postgraduate College, London, England).

Following infection of mice with *N. brasiliensis*, C3 serum levels fluctuated within the normal range up to approximately day 8 post-infection (Fig. 4.9.1). As was the case for α 1Pi, there was no apparent C3 serum acute phase response during the lung stage inflammation.

Figure 4.8.1 In vitro hepatocyte output of a1P1 in *N. brasiliensis* infected mice 2 days post-infection

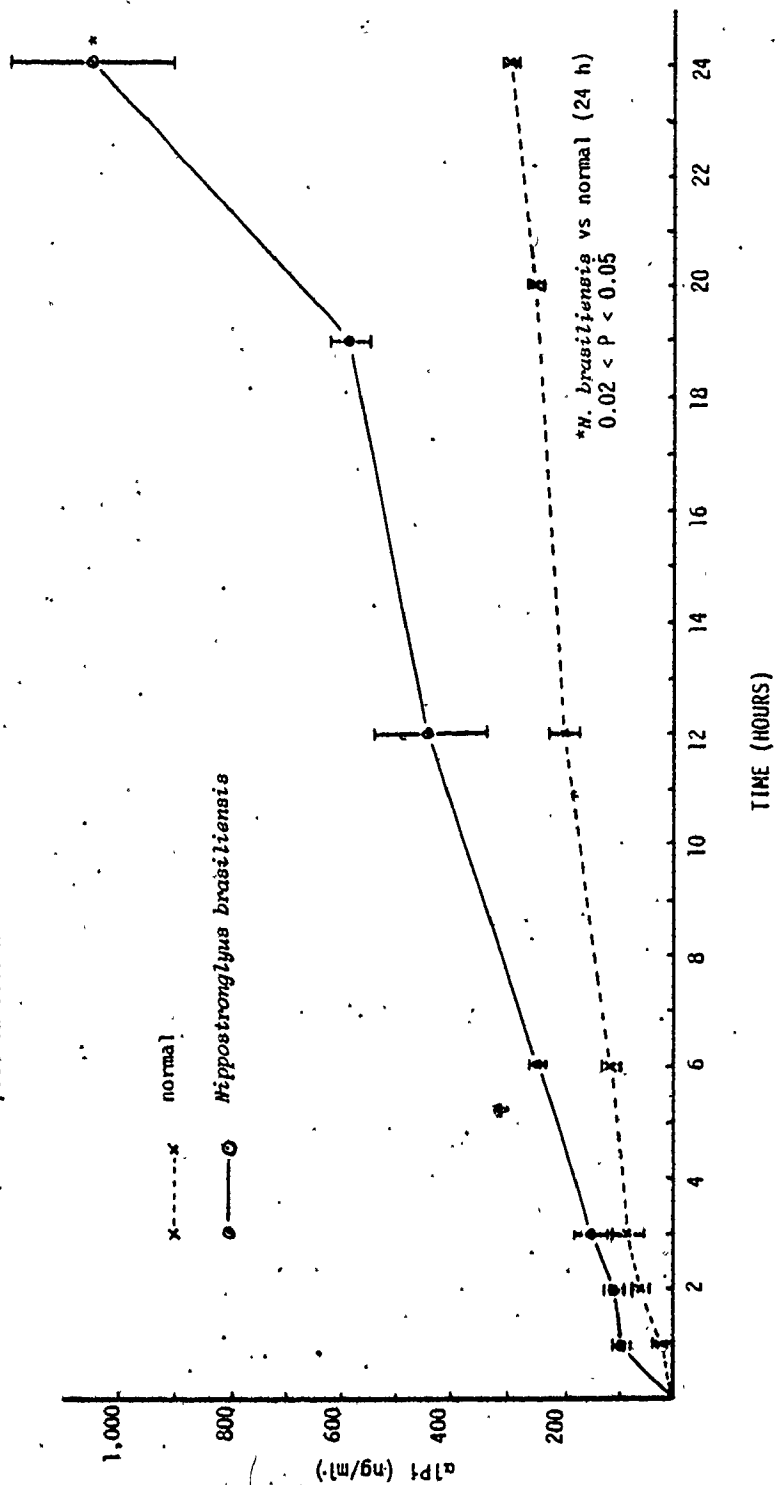


Figure 4.8.2 *In vitro* hepatocyte output of SAA in *N. brasiliensis* infected mice
2 days post-infection

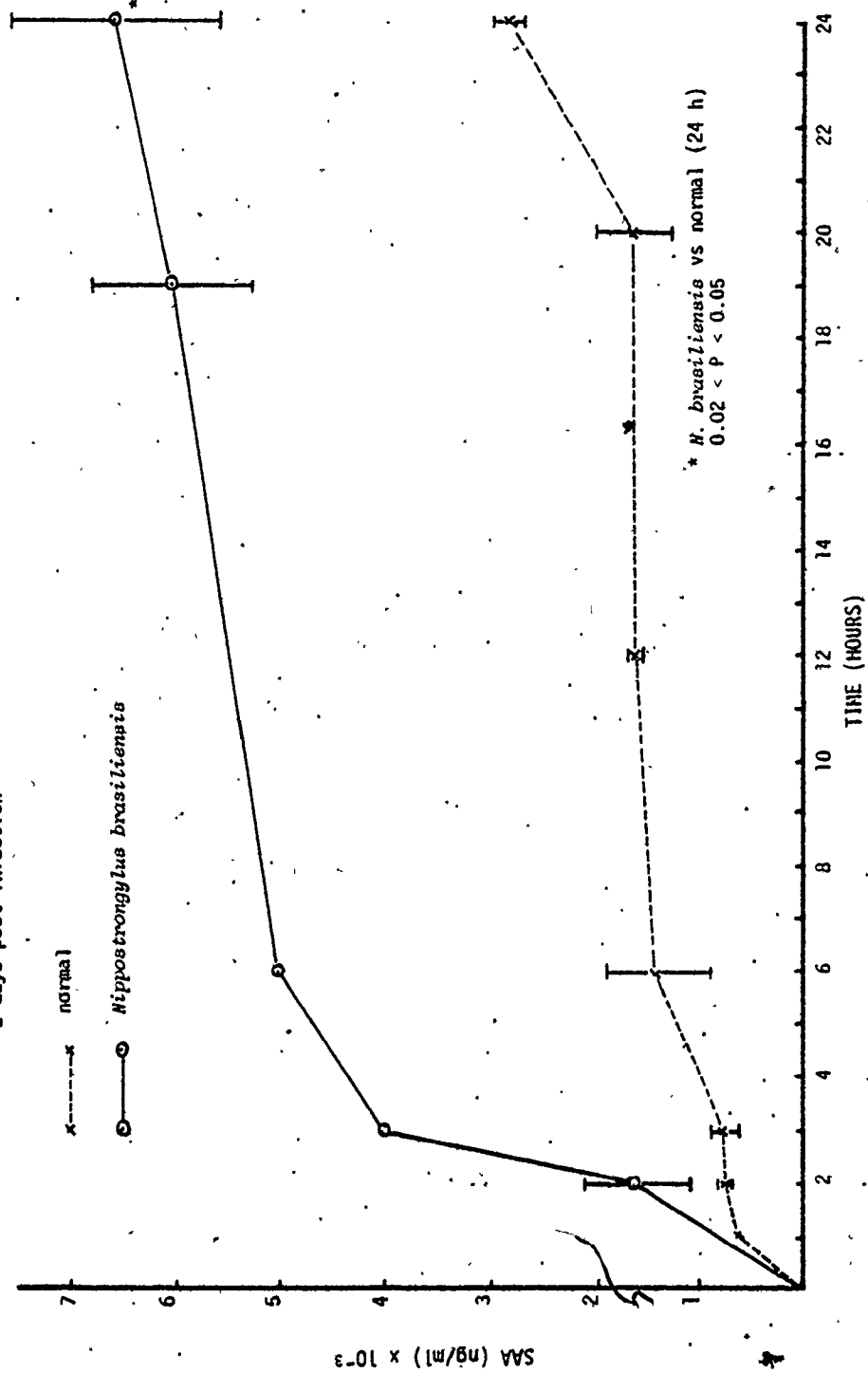
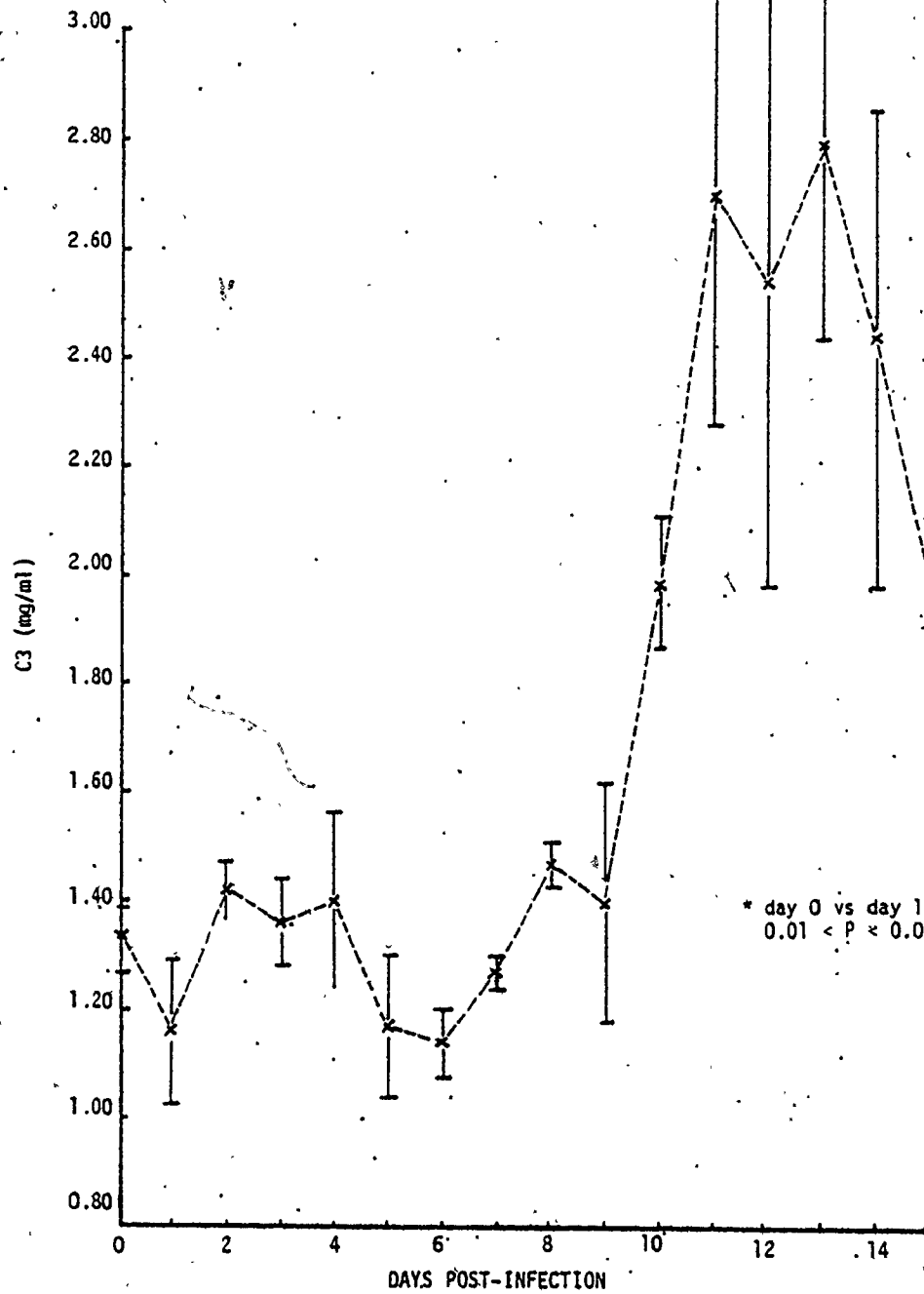


Figure 4.9.1 Serum levels of C3 during *N. brasiliensis* infection



However, during the gut inflammatory stage, there was a marked C3 serum acute phase response (Fig. 4.9.1) with approximately a two-fold increase over normal.

Serum amyloid P component (SAP) showed a significant rise in serum levels (two-fold) at approximately 2.5 days post-infection, corresponding to the lung inflammation stage (Fig. 4.9.2). There was a subsequent decline to the normal range at days 5 - 7. During the gut inflammation stage, there was a marked increase in SAP serum levels (three-fold) peaking at days 10 - 12 post-infection.

ii) SAA

A fourth acute phase reactant, serum amyloid A (SAA), was measured by Dr. K. McAdam (Tufts University, Boston, MA) and the serum profile is depicted in Figure 4.9.3. There was a marked increase in SAA serum levels (13-fold) in the early stages of the infection with the maximum occurring at 1 - 1.5 days post-infection. Subsequently, there was a rapid decline to normal levels by day 3 and at day 8 - 9, there was a gradual increase in serum levels with an eight-fold increase at approximately day 12.

As shown in composite Figure 4.9.4, the overall serum C3 and α 1Pi profiles were very similar to each other whereas SAP and SAA demonstrate remarkable similarity. Of the four acute phase reactants, only SAP and SAA indicated a serum acute phase response during lung inflammation whereas all four behaved as "classical" acute phase reactants during gut inflammation. Although the maximum serum response

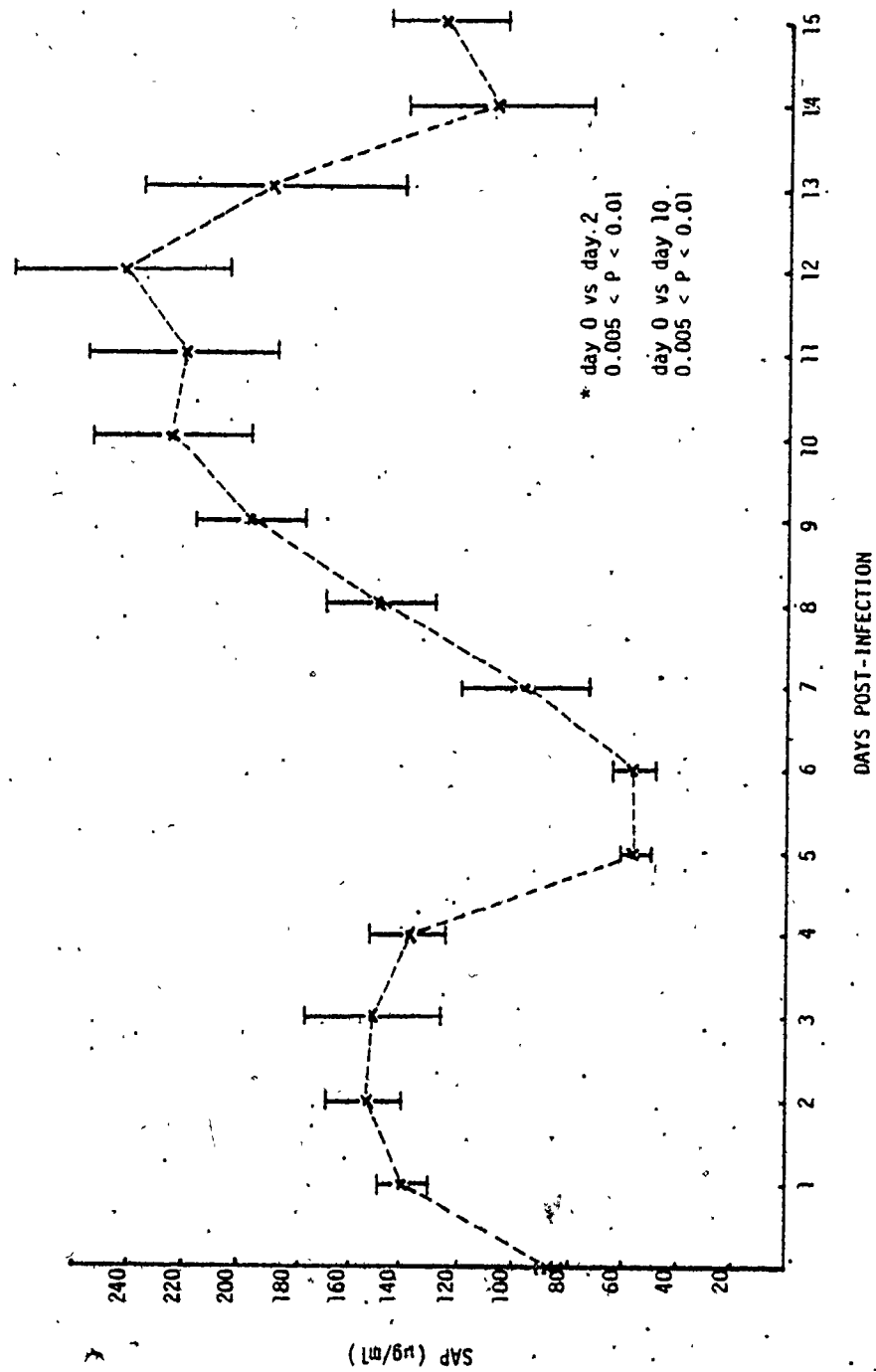
Figure 4.9.2 Serum levels of SAP during *N. brasiliensis* infection

Figure 4.9.3 Serum levels of SAA during *N. brasiliensis* infection

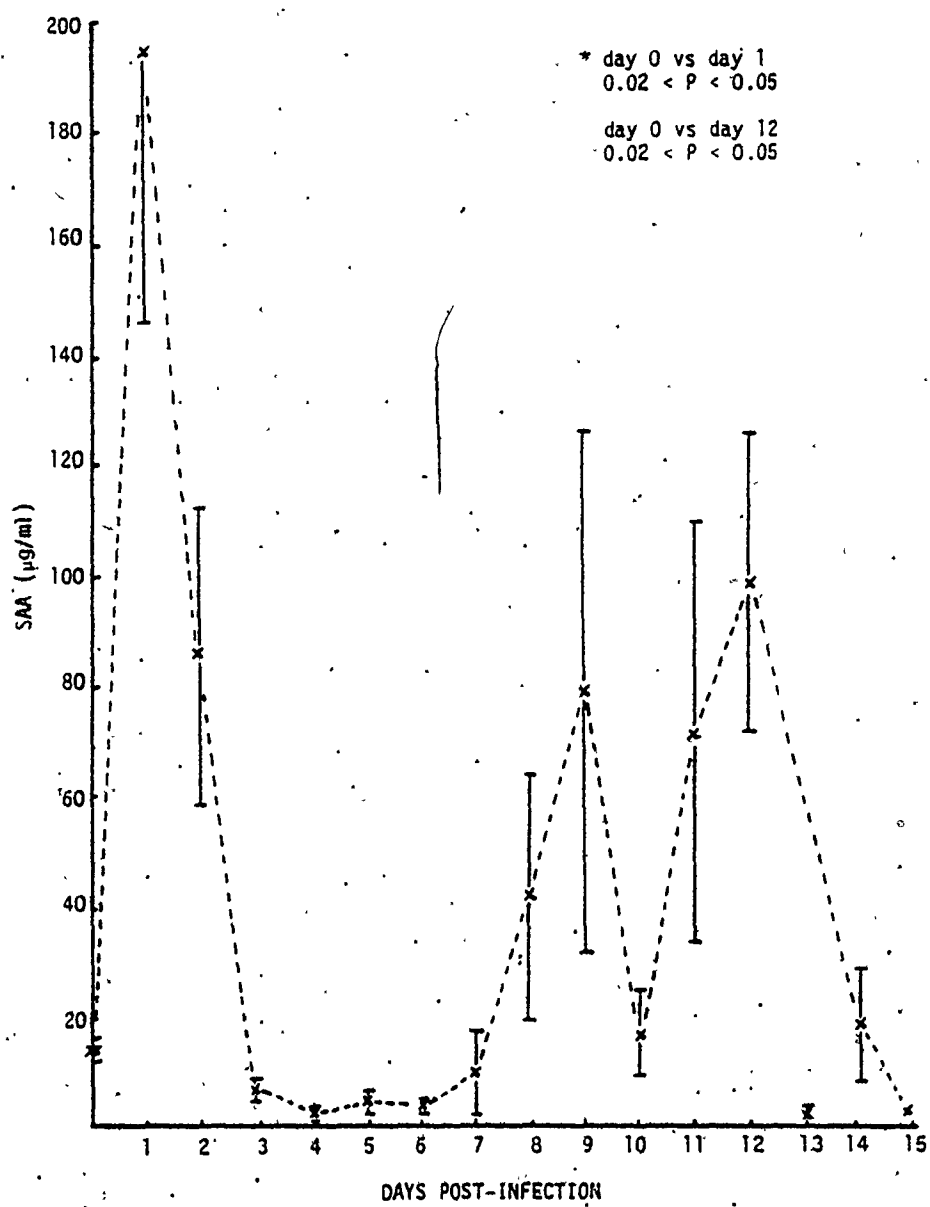
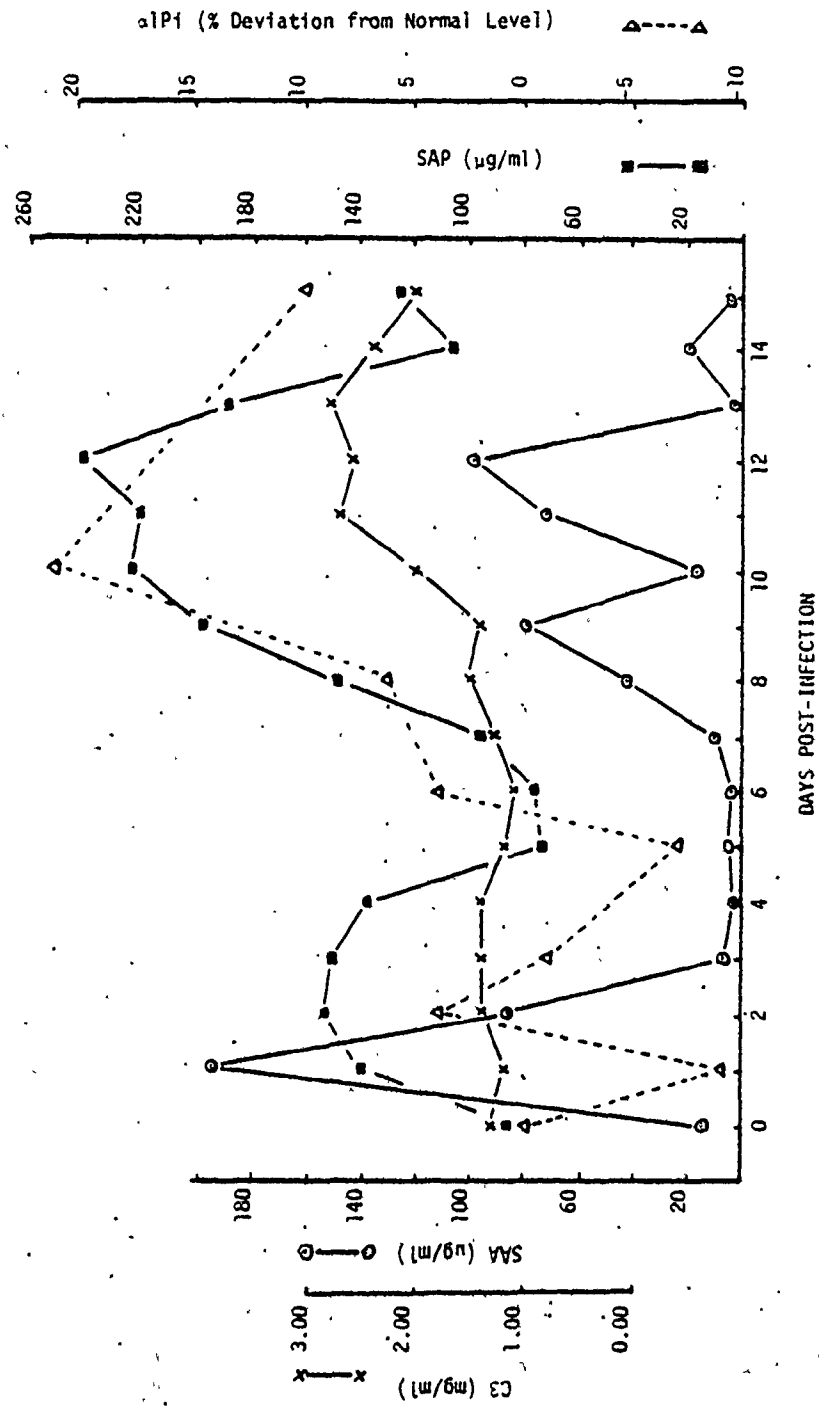


Figure 4.9.4: (composite diagram)
Serum levels of α Pi, C3, SAP and SAA during *N. brasiliensis* infection



for each acute phase reactant may not peak on exactly the same day(s) following infection, there was nevertheless significant overlap.

4.10 State of Activation of Alveolar Macrophages in *N. brasiliensis* Parasitized Lung

Alveolar macrophages were obtained by bronchiolar lavage and their state of activation assessed by morphology, the adherence and phagocytosis of opsonized red blood cells (RBC) and, the release of plasminogen activator.

i) Morphology

Morphologically, the alveolar macrophages from the day 2 infected lung as compared to the normal lung were larger, had a higher cytoplasm to nuclear ratio, a greater number of mitochondria and more endoplasmic reticulum, and were usually more extensively vacuolized (Fig. 4.10.1; 4.10.2; 4.10.3; 4.10.4).

ii.) Adherence and Phagocytosis

Alveolar macrophages from normal uninfected lungs had a weak capacity for adherence and phagocytosis while most alveolar macrophages from parasitized lungs had 5 - 10 RBC bound to their surface and on average ingested 2 - 3 RBC (Fig. 4.10.2) although as much as 8 RBC have been observed.

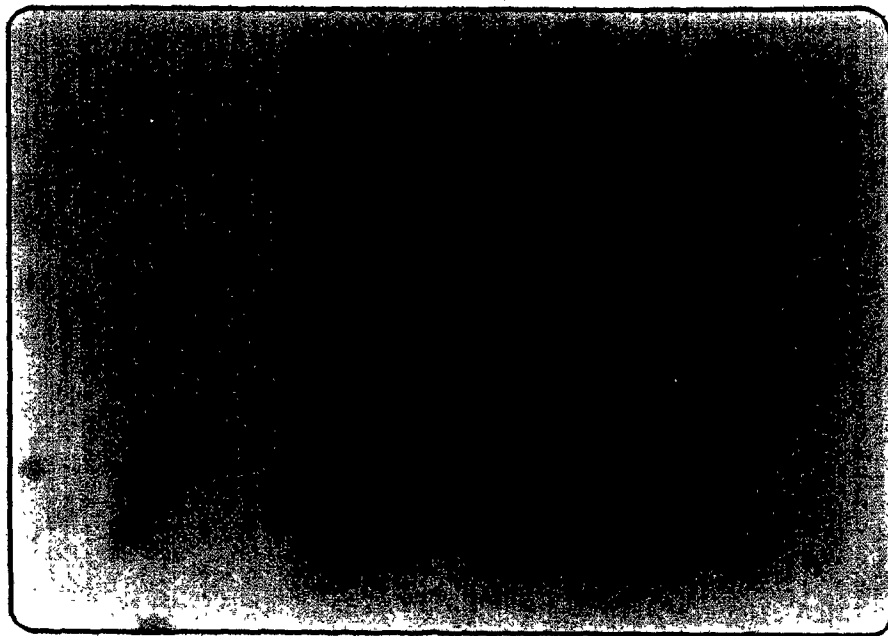


Figure 4.10.1 Mouse alveolar macrophages from uninfected lungs.
May-Grunwald-Giemsa stain (x400)

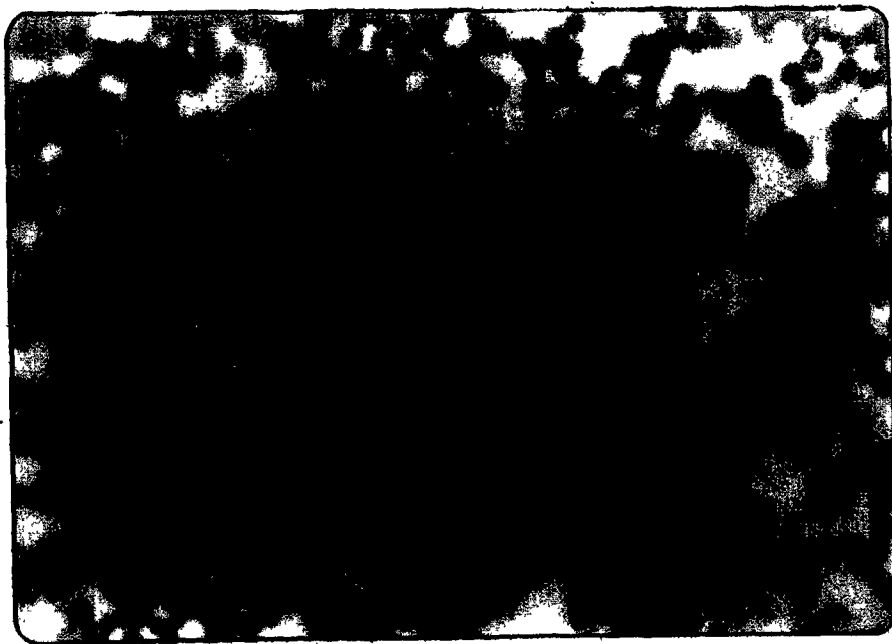
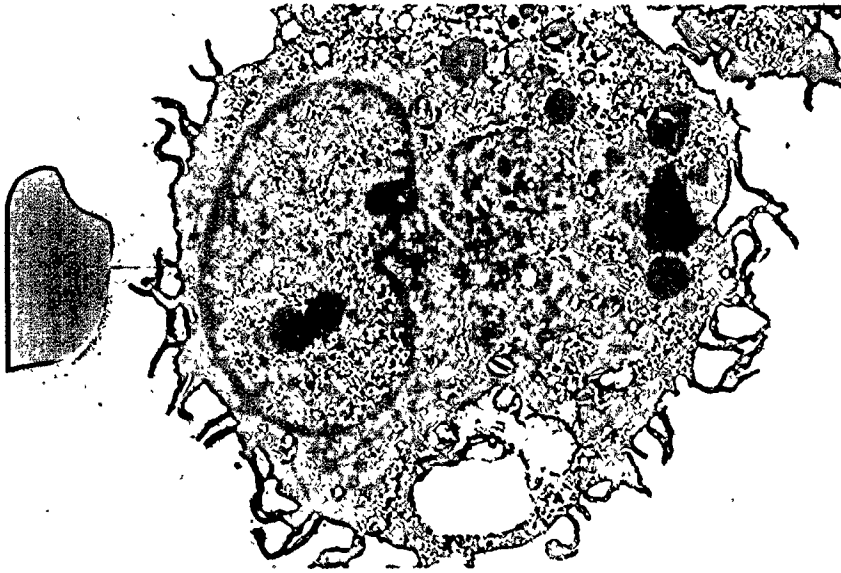
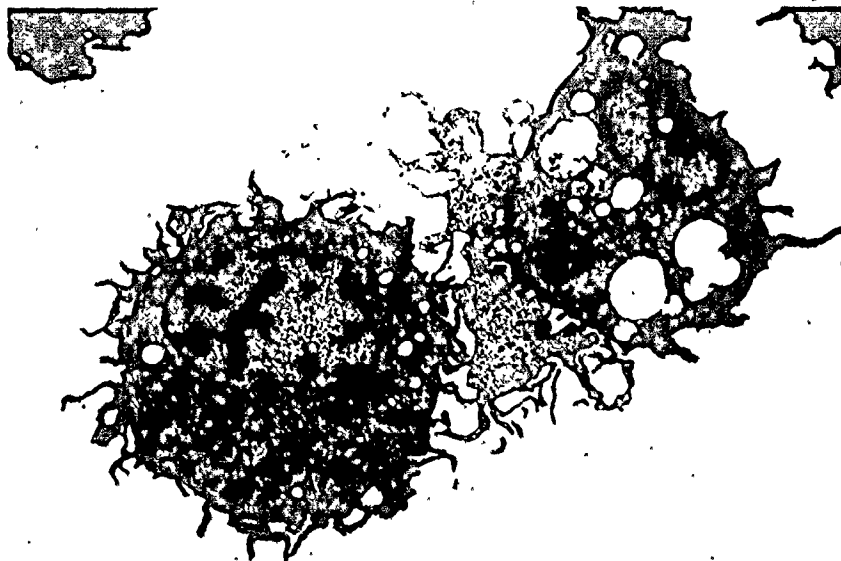


Figure 4.10.2 Mouse alveolar macrophages from *N. brasiliensis*
infected lungs (x400)



Electron micrograph of mouse alveolar macrophages from *N. brasiliensis* infected lungs (x3800)



Electron micrograph of mouse alveolar macrophages from uninfected lungs (x3800)

Figure 4.10.3

Figure 4.10.4

iii) Plasminogen Activator Release

Plasminogen activator release by alveolar macrophage was indirectly quantitated by measuring the amount of ^{125}I -fibrin degradation products released from ^{125}I -fibrin coated plates.

Alveolar macrophages from uninfected lungs induced the release of 18% of plated ^{125}I -fibrin whereas there was 42% release induced from alveolar macrophages from parasitized lungs. The maximum inducible release, using urokinase, was 80%.

4.11 Trypanosoma congolense Induced Inflammation

Preliminary studies of the acute phase response in *T. congolense* infected mice were initiated with the collaboration of Dr. T. Pearson (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada).

Mice of different strains with variable resistance to *T. congolense* were infected with 10^3 *T. congolense* strain 5E-12 organisms into the peritoneum and the pathology of the infection and serum profiles of αPi and SAA are described in the following sections.

4.12 Pathology of Experimentally Induced *T. congolense* Infection in Mice

T. congolense infection is characterized by the appearance of successive waves of parasitemia, each containing one or more populations of organisms with distinct surface antigenicity. In these preliminary

studies, levels of parasitemia were not evaluated in the different strains of mice but the strain of *T. congolense* and the protocol for infection were identical to that of Morrison *et al.* (1978) and as such their data for relative strain susceptibilities and levels of parasitemia will be quoted.

A/J mice

The A/J mouse is the most susceptible strain and the mean prepatent period, that is, the time from inoculation until first detectable parasitemia is 5.8 days (Morrison *et al.*, 1978). This period of time is significantly shorter than that for any of the other more resistant strains. The mean parasitemia level was approximately $2 \times 10^5/\mu\text{l}$ serum. This level of parasitemia was much higher than that with the other strains and was attained by day 10 - 12 post-inoculation. Moreover, this level of parasitemia was maintained until death of the animals which had a mean survival time of 15.8 days.

In our studies, at four days post-infection, the liver histology was normal.

In the lung, there was evidence of edema but no mononuclear cell infiltration.

At the onset of the parasitemia (day 5), the lung alveolar structures appeared slightly distorted and there was evidence of a slight mononuclear cell infiltration.

At day 8 post-infection, there was a slight increase in the number of mononuclear cells which were especially prominent in the

sinusoidal spaces of the liver. In addition, there were small foci of mononuclear cell infiltrates and these were randomly distributed throughout the liver lobule:

The lung tissue was edematous as well as hemorrhagic as indicated by the presence of red blood cells in the alveolar and interstitial spaces.

At 12 days post-infection, the liver morphology was intact and there were numerous trypanosome organisms with some mononuclear cell infiltration in the sinusoidal spaces.

The lung was generally swollen and there was a significant mononuclear cell infiltration extending into the lining of the bronchials (Fig. 4.12.1).

The histological findings indicated that in A/J mice, the early (day 1 - 4) inflammatory reaction to the parasite infection was minimal and it was only at a later time that there was any indication of a significant inflammatory response and mononuclear cell infiltration. By such a time, the parasitemia had reached a lethal level.

C3H/HeJ mice

C3H/HeJ mice had intermediate levels of resistance to *T. congolense* infection with a mean survival time of 59 days (Morrison *et al.*, 1978). Although the prepatent period was the same as the susceptible A/J strain of mice (5.8 days), the first wave of parasitemia was considerably lower (6×10^3 organisms/ μ l blood) at days 9 - 10. Moreover, there was a marked drop in parasitemia to barely detectable levels on days 11 - 13, before rising again with a second wave

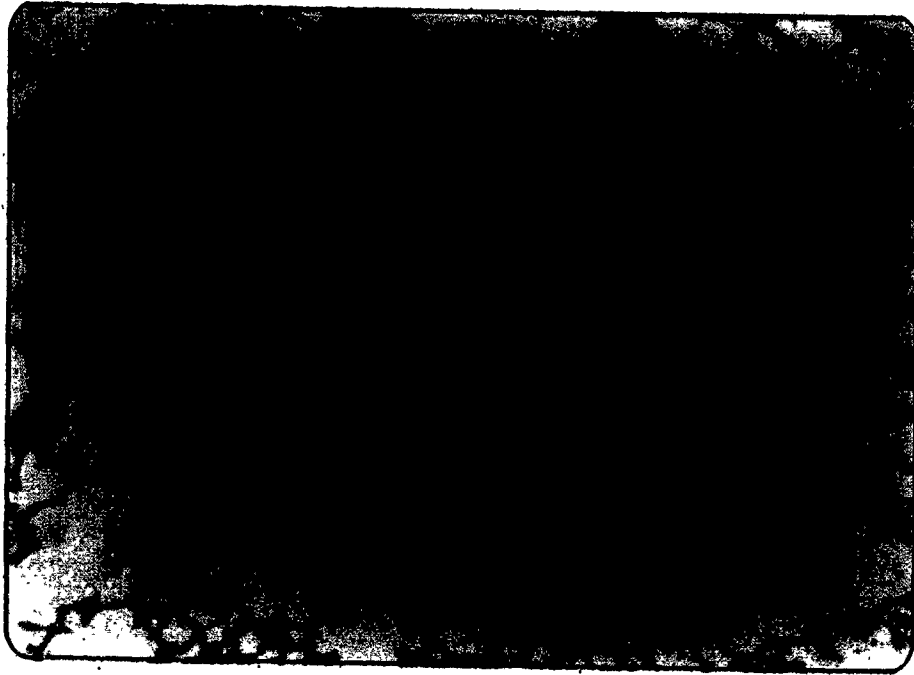


Figure 4.12.1 Lung 12 days post-infection of A/J mice with *T. congolense* (H & E) (x100)

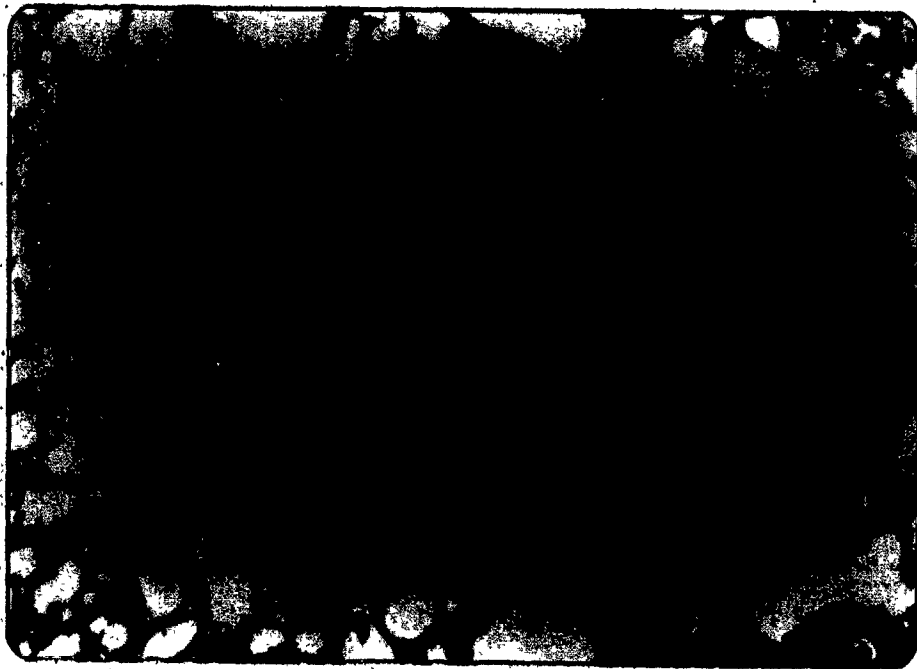


Figure 4.12.2 Lung 12 days post-infection of C3H/HeJ mice with *T. congolense* (H & E) (x100)

at approximately day 17 post-inoculation. This cycle was repeated and a third peak occurred on day 25. Of greater significance, perhaps, is the fact that with each successive wave of parasitemia, the maximum number of organisms peaked at 6×10^3 , 5×10^4 and $2 \times 10^4/\mu\text{l}$ for the first, second and third waves, respectively. This progressive change in parasitemia coincided with onset of death (13% at day 40) (Morrison *et al.*, 1978).

In our study, six days following the infection, the lung already showed evidence of mononuclear cell infiltration and at day 8, both the liver (Fig. 4.12.3) and the lungs exhibited prominent mononuclear cell infiltration. By day 12, liver architecture was altered with prominent areas of tissue necrosis. There was an increase in the number of mononuclear cells, presumably Kupffer cells. The lung tissue was also characterized by heavy mononuclear cell infiltration (Fig. 4.12.2).

The histological findings indicated that in contrast to A/J mice, there was a prompt and heavy mononuclear cell infiltration in both liver and lung tissue during *T. congolense* infection.

4.13 Immunohistochemical Localization of αPi in *T. congolense* Infection

There were marked differences in αPi staining activity in liver tissue in the resistant C3H/HeJ strain in comparison to the more susceptible A/J mice. In the susceptible strain, there was a slight but significant increase in background αPi cytoplasmic staining intensity as well as a slight increase in the number of hepatocytes which stained intensely for cytoplasmic αPi on days 6 - 8 post-infection.

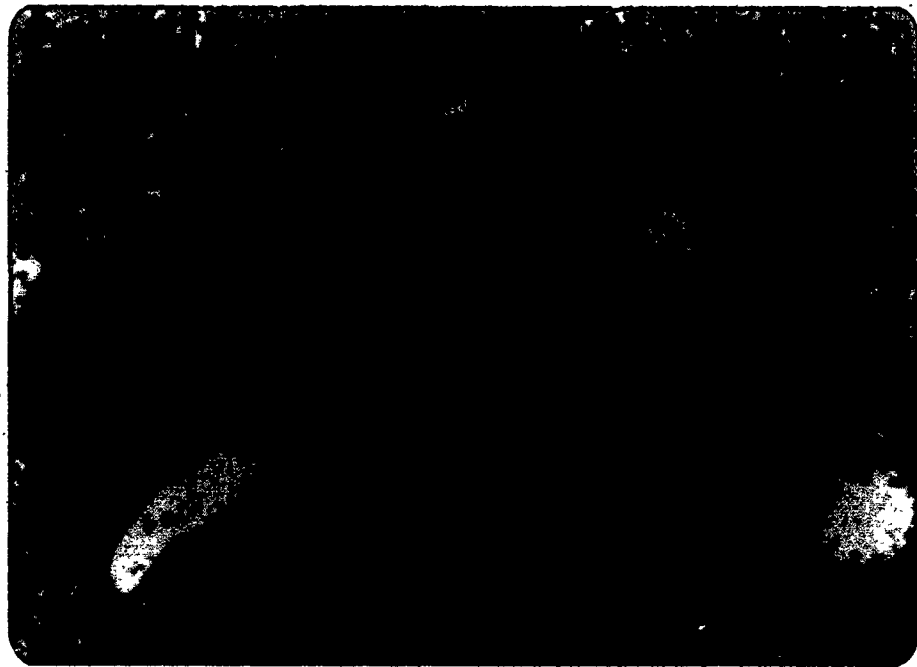


Figure 4.12.3 Liver 12 days post-infection of C3H/HeJ mice with *T. congolense* (H & E) (x100)

In contrast, the α 1Pi acute phase response in the liver occurred much more rapidly in the more resistant C3H/HeJ mice. Background α 1Pi cytoplasmic staining was significantly increased four days post-infection and an increase in the number of hepatocytes with intense α 1Pi cytoplasmic staining was observed on day 6 post-infection. The distribution of such intense staining hepatocytes was as described previously. There was a progressive increase in the number of these hepatocytes as well as background α 1Pi cytoplasmic activity from days 6 - 12.

4.14 α 1Pi and SAA Serum Profiles During *T. congolense* Infection

As shown in Figure 4.14.1, there was no apparent serum acute phase response for α 1Pi nor SAA in the susceptible A/J strain. In both cases there was a marked decrease below normal in serum level. In contrast, there was a SAA and α 1Pi serum acute phase response in the more resistant C3H/HeJ mice (Fig. 4.14.2) and the maximum response coincided with the onset of the mononuclear cell infiltration in liver and lung tissues. During the peak parasitemia, however, there was a decrease to normal levels of both acute phase reactants. Thus, the acute phase reactants α 1Pi and SAA had similar serum profiles in *T. congolense* infection but there were marked differences in the serum acute phase response in the two strains of mice which can be correlated with their relative susceptibilities to *T. congolense* infection.

Figure 4.14.1 Serum levels of α PI and SAA in *T. congolense* infected A/J mice
(n = 1)

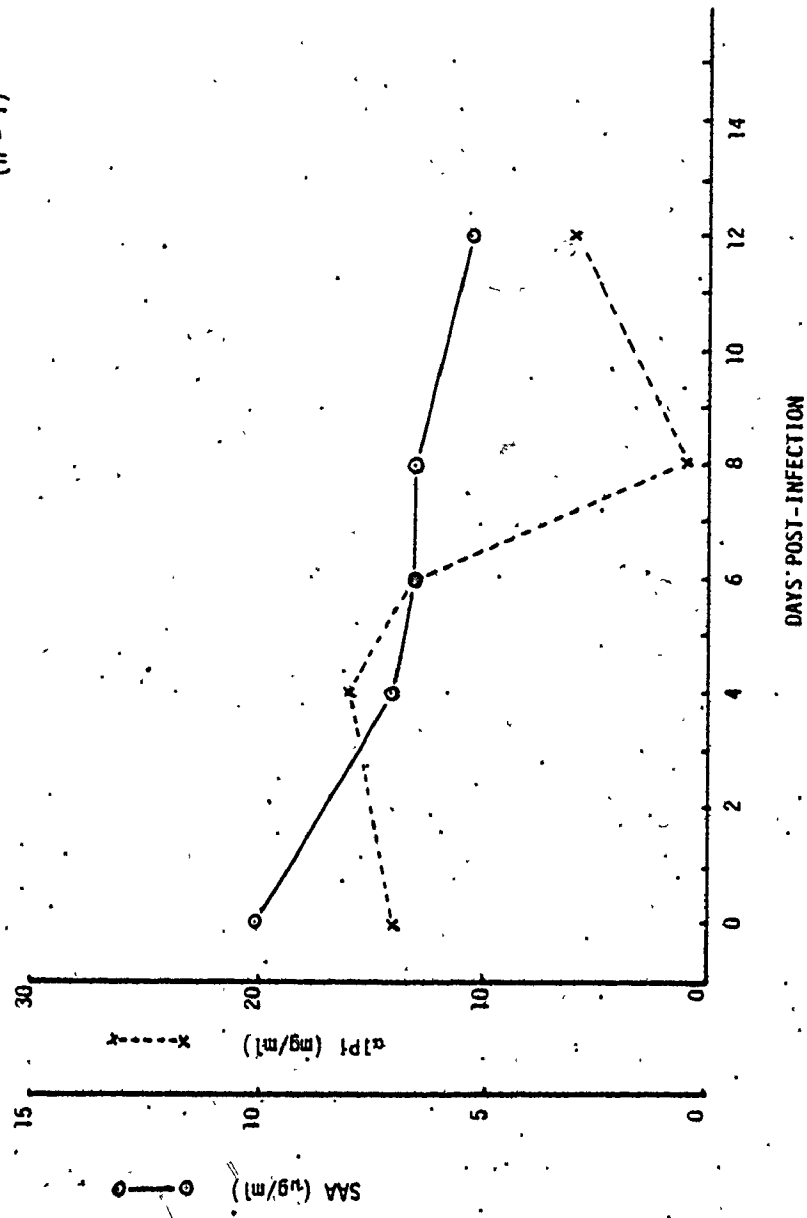
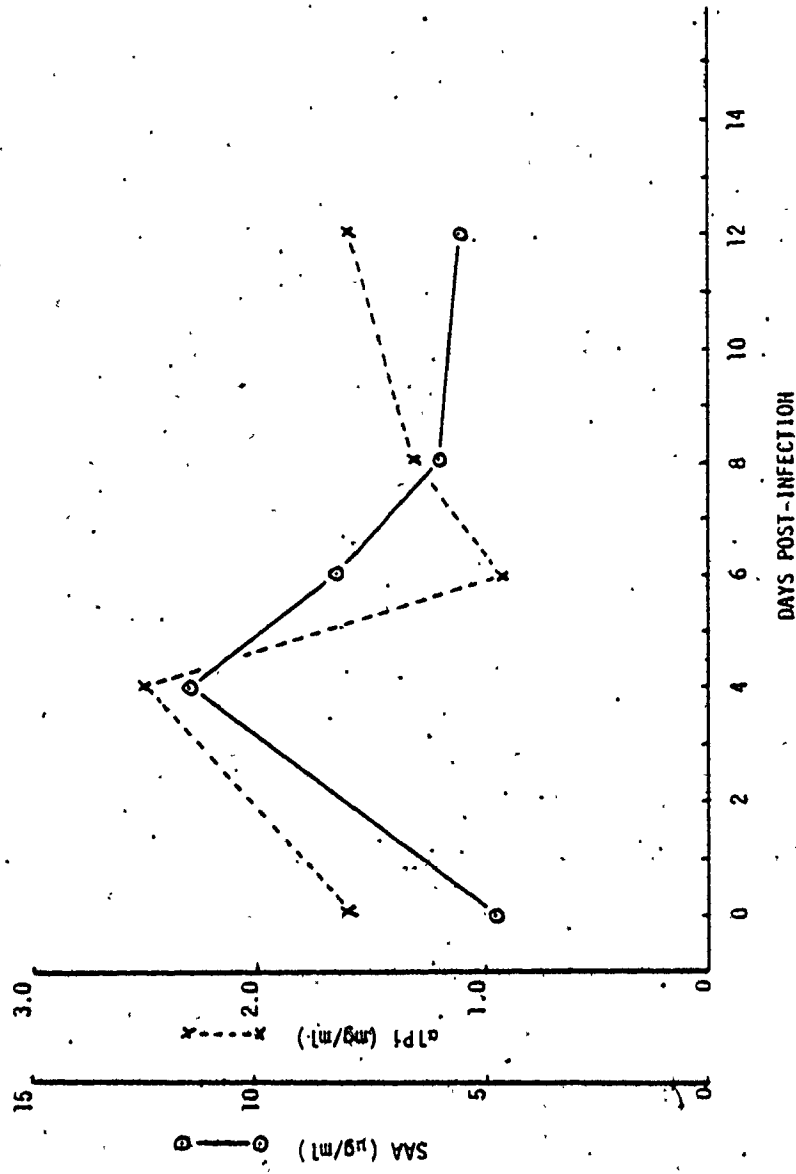


Figure 4.14.2 Serum levels of α PI and SAA in *T. oostrogolense* infected C3H/HeJ mice ($n = 1$)

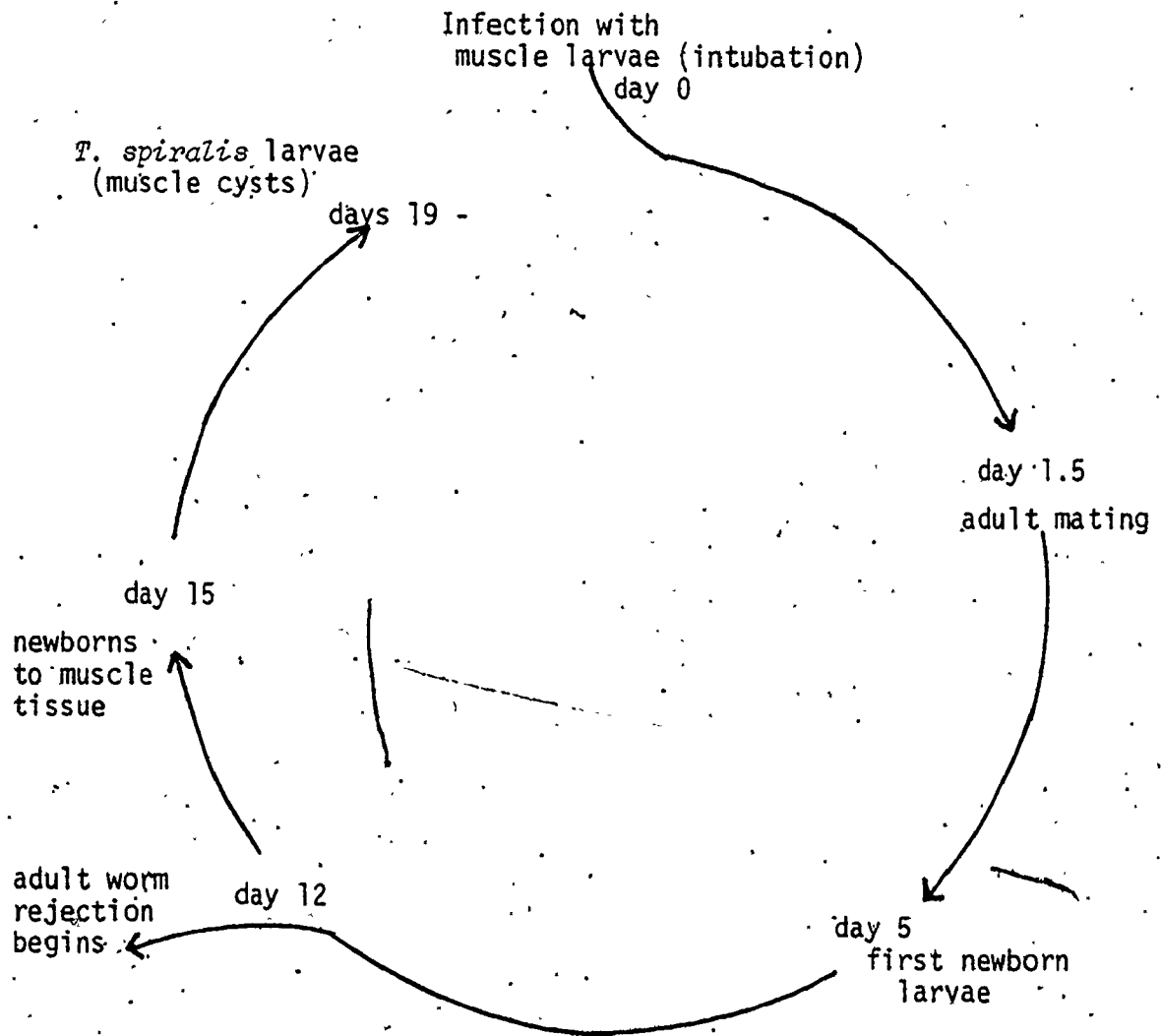


4.15 Trichinella spiralis Induced Inflammation

The life cycle of *T. spiralis* in the mouse is depicted in Figure 4.15.1 and was derived from Bell *et al.* (1979).

4.16 Pathology of T. spiralis Induced Inflammation

In the initial 24 h following intubation of *T. spiralis* muscle larvae, the gut mucosa remained intact. However, in a number of areas of the gut, there was a pronounced mononuclear cell infiltrate in the lamina propria, the basal lamina propria as well as in the epithelial-like cell layer lining the villi. Day 3 post-infection, subsequent to mating, the muscularis mucosa was infiltrated with mononuclear cells as well as the lamina propria. The villi were swollen and had a "ruffled" appearance. In general, the gut architecture is distorted. *T. spiralis* parasites were occasionally observed, not in the lumen of the gut, but lodged usually at the basal part of the villus near the crypt. At day 6 when the first newborn larvae had emerged and had begun to penetrate the gut mucosa, villus atrophy was prominent and, goblet cell and paneth cell hyperplasia was discernible (Fig. 4.16.1; 4.16.2). *T. spiralis* adults and newborn were present in the basal part of the villi at the surface of the gut wall. Over the next few days (day 8 - 10) there was marked alterations in gut villi morphology and spatial orientation. There was a mononuclear cell infiltration and goblet cell hyperplasia and marked villus atrophy and villus tip destruction. There were large areas with marked destruction of villi structure and there were a few areas where the basal lamina propria was partially

Figure 4.15.1 Life cycle of *Trichinella spiralis*

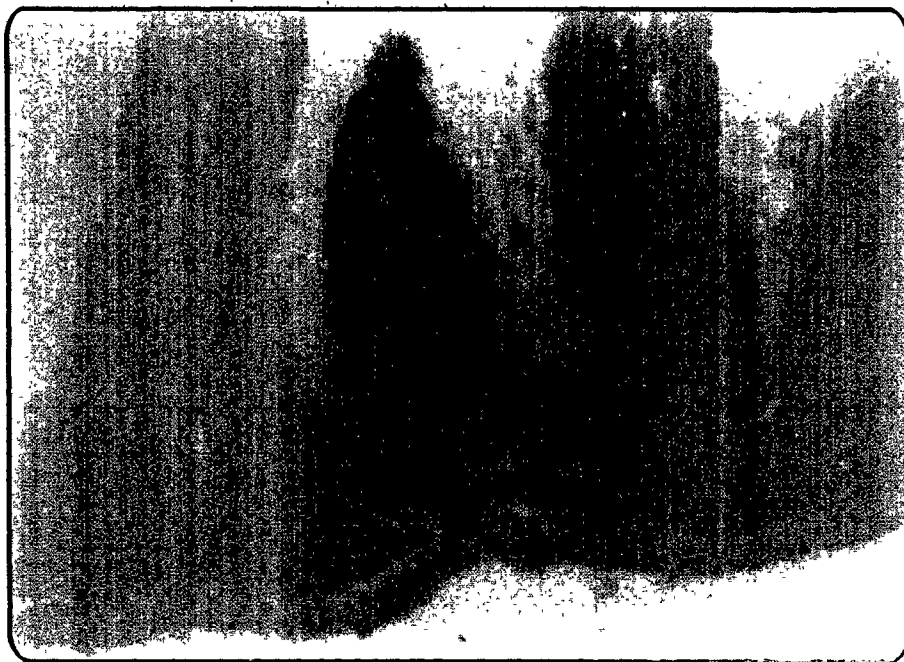


Figure 4.16.1 Normal small intestine (NIH mice)
H & E stain (x250)



Figure 4.16.2 Small intestine from day 8 post-infection
with *T. spiralis* (H & E) (x250)

detached from the muscularis mucosa. At day 14, when the majority of adult worms had been expelled from the gut and newborn larvae had migrated to muscle tissue, the gut inflammatory condition was less severe. There were still indications of villus atrophy but the goblet cell hyperplasia was not as prominent. During the muscle stage of *T. spiralis* (days 19 - 28) (Fig. 4.16.3), the gut regained its normal appearance but the liver began to show signs of mononuclear cell infiltration. At days 19 - 28 post-infection, the gross morphology of the liver was intact but there were numerous foci of mononuclear cell infiltration, especially around the central and portal veins and throughout the liver lobule. There were also abundant mononuclear cells in the sinusoidal spaces. The mononuclear cell infiltration became more intense 35 days post-infection but subsided to a relatively normal appearance by days 60 through 80. The larval cysts were also surrounded by prominent mononuclear cell infiltration (Fig. 4.16.3).

4.17 Immunohistochemical Localization of α Pi in *T. spiralis* Infection

The introduction of *T. spiralis* larvae in the small intestine gave rise to an acute-phase response as evidenced by the significant increase in the number of hepatocytes staining intensely for cytoplasmic α Pi. These hepatocytes appeared singly as well as in large clusters (Fig. 4.17.1). The general appearance therefore consisted of large areas with intense α Pi cytoplasmic activity interspersed with large areas of minimal α Pi specific staining.

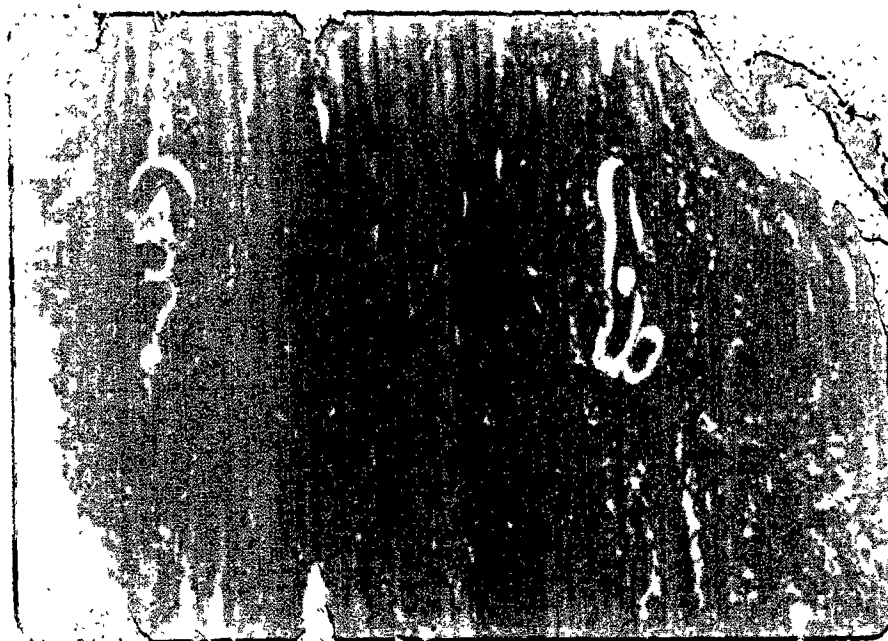


Figure 4.16.3 *T. spiralis* muscle larvae
(H & E) (x100)

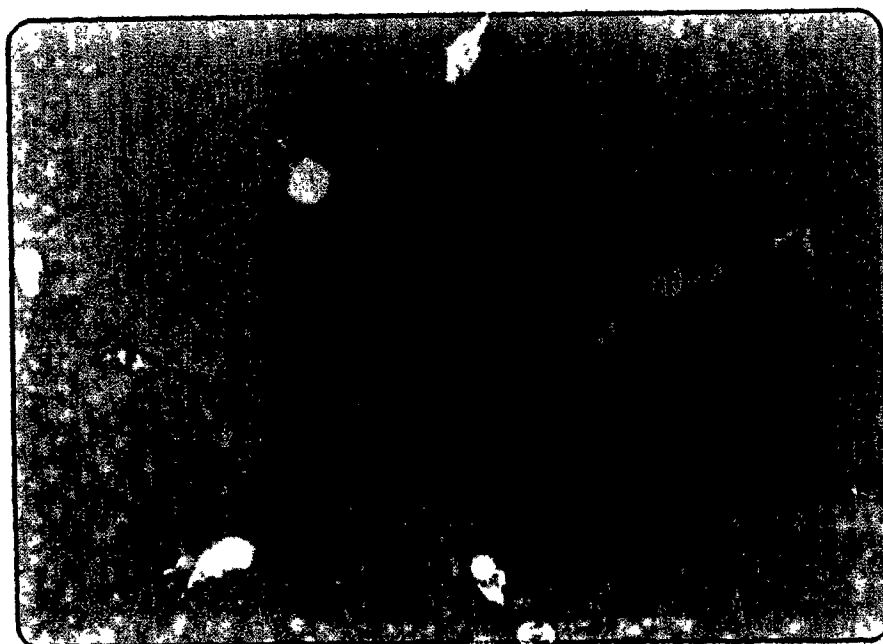


Figure 4.17.1 Immunohistochemical localization of α Pi in
liver day 8 post-infection with *T. spiralis*
(x100)

A liver α IPi acute phase response also occurred during the formation of *T. spiralis* cyst-like structures in muscle tissue. During the muscle stage of the parasite there was also evidence of α IPi in the interstitial spaces in muscle tissue and some of the mononuclear cells surrounding the larvae were also positive for α IPi.

4.18 α IPi and SAA Serum Profiles During *T. spiralis* Infection

There was a progressive decline in α IPi serum levels to 50% below normal levels at day 8 post-infection (Fig. 4.18.1). Subsequently, there was a gradual rise in serum levels, during the migration of the juvenile larvae from the gut mucosa to the muscle tissue, to 25% below normal levels. This was followed by a decrease in serum levels to 45% below normal.

In contrast, there was a serum SAA acute phase response during the gut stage of the parasite (Fig. 4.18.1) followed by a rapid decline to normal levels at days 12 - 20. During the muscle stage of the larvae, there was a minimal serum SAA acute phase response.

4.19 Synthesis of LAF (IL1) by Alveolar Macrophages

The synthesis of lymphocyte activating factor (LAF) by peritoneal macrophages and alveolar macrophages is shown in Table 4.19.1.

Culture supernatants from LPS stimulated peritoneal macrophages produced maximal ^3H -thymidine incorporation in thymocytes at a 1/8 dilution whereas alveolar macrophages from uninfected animals were optimal at a $\frac{1}{2}$ dilution. Culture supernatants from alveolar macrophages

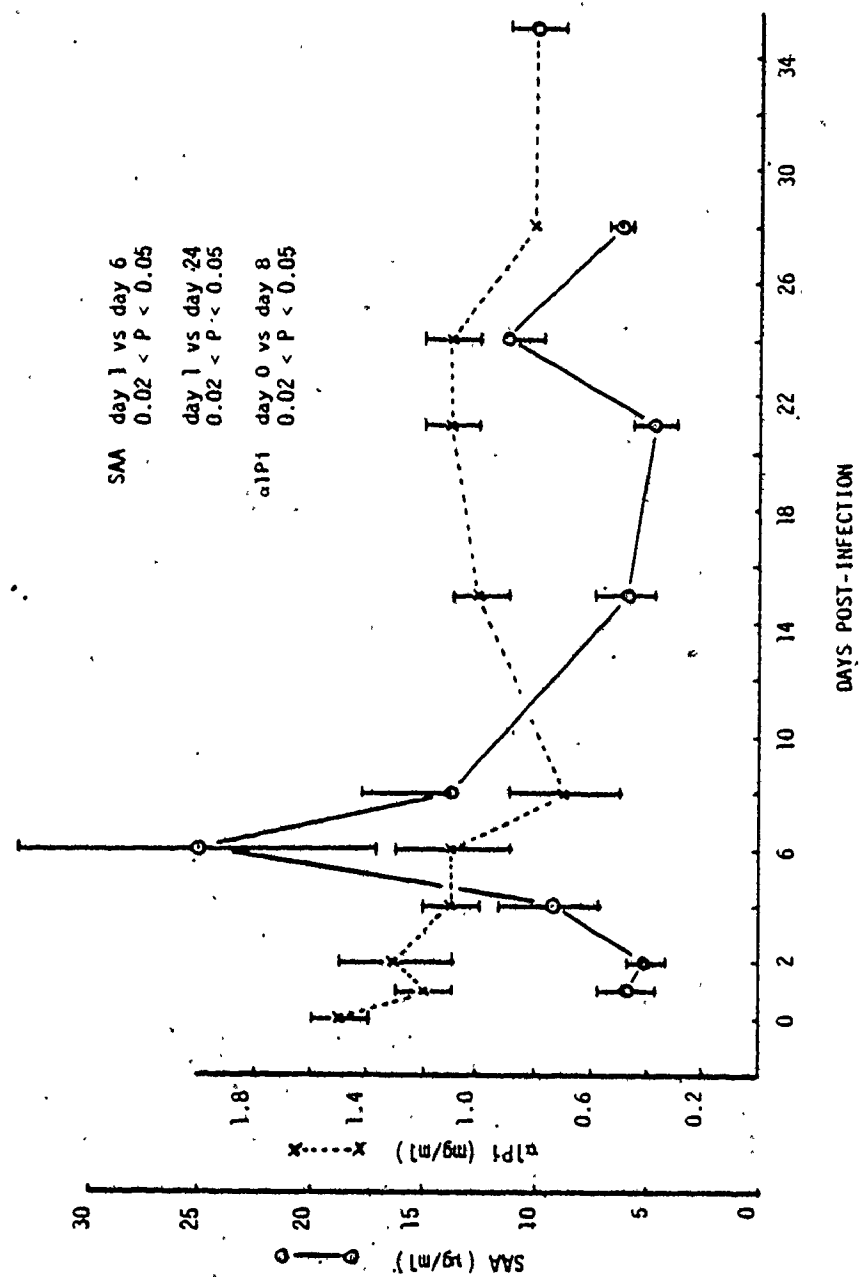
Figure 4.18.1 α 1Pi and SAA serum levels during *T. spiralis* Infection

TABLE 4.19.1

Synthesis of Lymphocyte Activating Factor (LAF) by Alveolar and
Peritoneal Macrophages

Cell population	Dilution	Counts Incorporated (³ H-thymidine) cpm
LPS alone	-	200
Peritoneal exudate cells + LPS	1/2	820
	1/8	1080
	1/32	202
Alveolar macrophage from uninfected animals + LPS	1/2	1823
	1/8	460
	1/32	197
Alveolar macrophage from infected animals	1/2	203
	1/8	440
	1/32	1010

from *N. brasiliensis* infected lungs, in the absence of LPS stimulation, showed maximal stimulation at a 1/32 dilution.

This preliminary data would indicate that alveolar macrophages from both normal and infected animals are capable of producing LAF and that the latter synthesize greater amounts of LAF in comparison to LPS stimulated alveolar macrophages from normal animals. Clearly, further experiments are required to further compare LAF producing capabilities of these various cell types.

4.20. In vitro Induction of Hepatocyte Synthesis of α PI by Alveolar Macrophage Culture Supernatants

As shown in Table 4.20.1, hepatocyte cultures supplemented with: i) 6.6% alveolar macrophage (from normal lung) culture supernatants synthesized 1170 ± 60 ng/ml of α PI; ii) 10% alveolar macrophage (from *N. brasiliensis* infected lungs) culture supernatants synthesized 1140 ± 90 ng/ml of α PI; iii) 15% alveolar macrophage (LPS stimulated) culture supernatants synthesized 1125 ± 15 ng/ml α PI and; iv) 15% LPS control medium synthesized 880 ± 56 ng/ml of α PI. Hepatocyte cultures not supplemented with alveolar macrophage culture supernatants synthesized 260 ± 40 ng/ml of α PI.

TABLE 4.20.1

In vitro Induction of α 1Pi Synthesis, in Hepatocyte Cultures, by Alveolar Macrophage Culture Supernatants

Additions to hepatocyte cultures	Final concentration	α 1Pi (ng/ml) (24 h culture period)
Medium (control) (RPM 1-1640)	10%	260 \pm 40
Alveolar macrophage culture supernatant from normal lungs	6.6%	1170 \pm 60*
Alveolar macrophage culture supernatant from <i>N. brasiliensis</i> infected lungs	10%	1140 \pm 90*
Culture supernatant from normal alveolar macrophages pre-stimulated with LPS	15%	1125 \pm 15*
LPS (control)	15%	880 \pm 56

* Statistically significant from medium (control) at $0.01 < P < 0.02$
 Statistically significant from LPS (control) at $0.02 < P < 0.05$

CHAPTER 5

DISCUSSION

5.1 Purification and Characterization of α 1Pi

The isolation of immunologically pure mouse α 1-protease inhibitor has been achieved by a combination of ion exchange and affinity column chromatography. The affinity columns included Sepharose-heparin to specifically remove antithrombin III; Sepharose-concanavallin-A for the generation of a glycoprotein-rich fraction; and, Sepharose-cibacron blue for the selective removal of albumin. The final preparative polyacrylamide gel purification step yielded a homogeneous protein band on analytical polyacrylamide gel electrophoresis (Fig. 4.1.1) with MW $53,500 \pm 2,000$ and $t_{1/2}$ of 15.5 hours.

The specific activity of the purified α 1Pi was 336 μ g of trypsin inhibited per mg of α 1Pi (Table 4.1). Theoretically, the optimum specific activity is approximately 440 μ g of trypsin inhibited per mg of α 1Pi, assuming a 1:1 stoichiometry for the inhibition reaction and molecular weights of 23,500 and 53,500 for trypsin and α 1Pi, respectively. Based on the specific activity of whole mouse plasma (Table 4.1), the α 1Pi preparation represented a purification factor of 12 with a 28% recovery. However, since whole plasma contains a variety of other inhibitor proteins with some degree of activity against trypsin, such as antithrombin III, α 2-macroglobulin and antichymotrypsin, the purification factor and overall yield were probably slightly higher. Myerowitz *et al.* (1972b) have isolated mouse α 1Pi from serum with a purification factor of approximately seven and a recovery rate of 13%. Recently, Takahara and Sinohara (1982) have reported the isolation of

mouse α PI with a purification factor of 6.7 and overall yield of 14%. The present isolation of mouse α PI represents a significant advance in purification as well as in overall yield.

This purified α PI was used as antigen in the immunization protocols described in section 3.3. The specificity of sheep and rabbit anti-mouse α PI antisera was determined by double immunodiffusion and two-dimensional electrophoresis. In addition to the reactivity against α PI, the antisera exhibited minor reactivity against albumin. The antisera were made monospecific for α PI by liquid phase absorption with pure mouse albumin. For the immunohistochemical localization of α PI in tissues, albumin absorbed sheep anti-mouse α PI was used in the staining protocol. This pre-absorption assured that cytoplasmic staining was specific for α PI. For the quantitation of α PI by nephelometry, unabsorbed antiserum was used since the addition of pure albumin to the samples did not have any measurable effect. This lack of interference with the quantitation of α PI is probably due to the low anti-albumin antibody titer in the original antiserum. Moreover, the dilution at which the antiserum was used in addition to the relatively large concentration of albumin in the serum samples to be analysed, did not constitute optimal conditions for quantitation by nephelometry. In the ELISA assay, specificity for α PI was obtained by using monospecific rabbit IgG anti-mouse α PI as the first layer.

Using these antisera, it was shown by double immunodiffusion, that mouse α PI cross-reacts strongly with rat α PI (Fig. 3.2.1), indicating some degree of homology in these two rodent α PI although Roll and Glew (1981) have not found any such cross-reactivity but did

not show experimental proof of these results. Cross-reactivity of mouse and rat α 1Pi is not totally unexpected since their respective amino acid compositions are similar (Takahara and Sinohara, 1982).

The sheep and rabbit anti-mouse α 1Pi antisera were also used in rocket immunoelectrophoresis (section 3.5.2), nephelometry (section 3.5.3) and ELISA (section 3.5.4) for the quantitation of α 1Pi in the various models of inflammation (section 5.3). In addition, the sheep antiserum was also used in the immunoperoxidase staining technique for the specific localization of α 1Pi in tissue sections and within the cytoplasm of a variety of cell types (section 3.6). As such, these anti- α 1Pi antisera were invaluable to the study of α 1Pi as an acute phase reactant.

On two dimensional immunoelectrophoresis in agarose gel, the purified mouse α 1Pi exhibits electrophoretic heterogeneity (Fig. 4.2.2) and the two precipitin peaks can be referred to as the fast (f) and slow (s) electrophoretic variants. These two variants are not artifacts of the purification procedure since they are present in the same relative proportion in purified α 1Pi fractions, normal mouse serum and hepatocyte cell lysates and culture supernatants. The slow and fast electrophoretic components bind trypsin (Fig. 4.2.3, 4.2.4; section 4.2) and are therefore both likely to be α 1Pi molecules.

In addition, the two α 1Pi variants are immunologically non-identical as indicated by the crossing precipitin lines of fast and slow molecules. Myerowitz *et al.* (1972a), using polyacrylamide gel electrophoresis in the first dimension and agarose in the second

dimension, have also demonstrated two distinct non-fusing precipitin peaks. Lee and Janoff (personal communication) have observed similar electrophoretic heterogeneity with purified rat α Pi. Electrophoretic heterogeneity of α Pi is also observed in rabbit (Koj *et al.*, 1978, 1981), rat (Takahara *et al.*, 1980; Ikehara *et al.*, 1981; Roll and Glew, 1981), human (Pannell *et al.*, 1974), dog (Abrams *et al.*, 1978; Schnizlein *et al.*, 1980) and rhesus-monkey (Berninger and Mathis, 1976). However, unlike mouse and rat α Pi, rabbit, human and rhesus-monkey α Pi variants are immunologically identical as revealed by two-dimensional immunoelectrophoresis (Koj *et al.*, 1981; Berninger and Mathis, 1976).

There are two hypotheses which could account for the two immunologically distinct α Pi variants in the mouse. They could arise as a result of post-translational modification of a single α Pi gene product either before or after its release from the hepatocyte. Or alternatively, as suggested for rabbit α Pi, the mouse α Pi variants could be coded for by two autosomal genes or two sets of autosomal allelic genes (Regoeczi *et al.*, 1980).

My preliminary studies, in collaboration with Dr. R.K. Murray (University of Toronto, Toronto, Canada), have shown that the antiserum to mouse α Pi precipitates an apparent single precursor polypeptide from mRNA extracts of hepatocytes in a reticulocyte cell free system. Although the detection of a single band was by single dimension SDS-acrylamide gel, and may not resolve two closely electrophoresing polypeptides, such results are consistent with post-translational modification of a single gene product. This modification would

likely occur prior to secretion since hepatocyte cell lysates express both forms in relative proportions similar to that observed in serum (data not shown). Ikehara *et al.* (1981) have proposed a similar mechanism for the heterogeneity of rat α Pi. It was suggested that post-translational modifications of amino acids causes a primary heterogeneity of rat α Pi and uneven sialylation during intracellular transport causes further modifications.

As mentioned earlier, both α Pi variants exhibit antitryptic activity but the binding kinetics, the stoichiometry of the reaction and the relative inhibitory activities of both α Pi variants have not been determined in these studies. However, the oxidation of the reactive site of α Pi (methionine residue) by chloramine-T, resulted in greater impairment of the antitryptic activity of the fast variant (Fig. 4.2.3, 4.2.4). These results suggest that the reactive site of the fast α Pi variant is more susceptible to attack by strong oxidants and as such, it is tempting to speculate that the tertiary structure of slow and fast variants, in the vicinity of the reactive site, is different. Such configurational differences could result in differential inhibitory activities towards proteases. This is consistent with results obtained for rabbit α Pi variants (Koj *et al.*, 1981). In their studies, it was shown that the fast component was more reactive than the slow variant when tested against plasmin, trypsin, chymotrypsin, elastase and leucocyte proteinases. It is interesting to note that in the rabbit, the slow α Pi variant has a higher catabolic rate than the fast component (Regoecki *et al.*, 1980), with the synthetic rate of the slow variant being greater than that of the fast α Pi variant. Therefore,

the lower reactivity of the slow variant is likely compensated for by the ability to be replenished at a faster rate.

In summary, we have purified mouse α 1Pi and have raised monospecific antisera in sheep and rabbits. The partial characterization of α 1Pi has revealed two immunologically non-identical electrophoretic variants (fast and slow components) and both exhibit trypsin binding activity. On the basis of preliminary studies, it is suggested that α 1Pi is coded by a single gene and the two variants arise as a result of post-translational modifications. However, as the two molecules react differently to oxidation and exhibit different substrate specificities, it is possible that these are separately coded molecules.

The assays for the quantitation of α 1Pi and an immunohistochemical staining technique for its localization in tissues and cells constitute the basic investigative tools for the study of α 1Pi as an acute phase reactant.

5.2 Distribution of α 1Pi in Body Fluids and Tissues

Prior to the study of α 1Pi as an acute phase reactant in a variety of inflammation models, we have investigated the distribution of the inhibitor in body fluids and tissues and documented its development through ontogeny.

5.2.1 Ontogeny

The serum levels of α 1Pi seen during ontogeny in the mouse (Fig. 4.3.1) differ markedly from those seen in the human (Gitlin and Gitlin, 1975) and pig (Westrom *et al.*, 1982). Early in gestation, mouse

(day 8) and human (wk 6) fetal serum levels are similar at approximately 6 - 15% of normal adult levels. However, midway through gestation, fetal serum levels increase to 100% and 1500% of normal adult in the human and pig respectively, while α Pi fetal serum levels in the mouse remain unchanged at that time. At parturition, mouse levels have not changed whereas human fetal α Pi rise to 175% of normal adult and pig fetal α Pi decrease to 400%. During neonatal development, mouse α Pi serum levels rapidly increase to adult levels while human and pig decrease to normal.

Maternal serum levels of α Pi remain unchanged in the mouse (normal adult levels) throughout gestation as well as at birth whereas in the human, maternal serum concentration at term averages 200% of that in the normal adult. The increased maternal serum levels suggest that the high fetal serum levels in the human could be due to placental transfer from the mother or perhaps the same mediator(s) which induces increased synthesis in the mother crosses the placenta and induces increased synthesis in the fetus.

The reasons for the dissimilarities in the α Pi serum levels during ontogeny in the mouse as compared to the human and the pig, are not apparent. The concentration of any particular protein in fetal serum is a function of fetal synthesis and degradation rates as well as maternal-fetal and fetal-maternal exchange rates.

Maternal proteins can be transferred to the fetus by two main mechanisms: 1) amniotic fluid protein exchange and; 2) placental transfer. Gitlin and Gitlin (1975) have shown that there is little absorption of intact protein from amniotic fluid by the human fetus,

either through the lungs, skin, umbilical cord or gastrointestinal tract. As such, amniotic fluid protein exchange as a mechanism for maternal-fetal transfer of proteins constitutes a minor contribution.

Most of the protein that is transferred from the mother to the fetus proceeds via the placenta. The transfer of plasma proteins across the maternofetal barrier is independent of the molecular weight of the protein and as such, the fact that IgG (MW 165,000) crosses the murine placenta does not necessarily imply that α 1Pi (56,000) will. In fact, albumin with a molecular weight of approximately 65,000 does not cross the placenta as readily as IgG (Gitlin and Koch, 1968).

In the human, fetal α 1Pi is not likely maternally derived since the α 1Pi phenotype present in the fetus is not necessarily that of the mother (Adinolfi, 1971).

In the mouse, it is likely that the low serum levels of α 1Pi in the fetus represent fetal synthesis with minimal contribution from maternal-fetal transfer. This is derived from the fact that fetal hepatocytes stain for α 1Pi (Fig. 4.4.4) as early as day 10 gestation and more prominently at day 15. As discussed in greater detail in later sections, the specific localization of α 1Pi within the cytoplasm of hepatocytes correlates with synthetic activity and does not represent uptake of α 1Pi into the hepatocytes. There is an increasing number of these intense staining hepatocytes during fetal development and the cytoplasmic staining progressively changes from a granular- to a diffuse-like appearance. This alteration in cytoplasmic distribution during ontogeny likely reflects a functional change during hepatocyte development. Fetal hepatocytes staining positive for mouse

(Kuhlmann, 1975), and human α -fetoprotein (Peyrol *et al.*, 1977) and rat fibrinogen (Legrele *et al.*, 1980) have also been described.

Therefore, the immunohistochemical localization of α 1Pi within fetal hepatocytes is supportive of fetal synthesis but does not rule out maternally-derived α 1Pi as an additional source.

In order to assess the maternal contribution, the extent of placental transfer of radiolabelled purified α 1Pi was determined. As shown in Figure 4.3.2, although there was a linear increase in the fetal/maternal ratio of total plasma radioactivity over a four hour period, the corresponding fetal/maternal ratio of α 1Pi-associated radioactivity remained very low over this time period. Greater than 95% of fetal plasma radioactivity was free ^{125}I as revealed by TCA precipitation.

Collectively, these results suggest that the low α 1Pi serum levels during gestation represent fetal synthesis and placental transfer of α 1Pi is a minor component. Similar results have been obtained for rat fetal fibrinogen synthesis and lack of placental transfer (Legrele *et al.*, 1980).

5.2.2 Normal adult mouse

The presence of α 1Pi in the various body fluids (Table 4.2.3) reflects the broad distribution of this protease inhibitor.

Normal adult serum contains the highest concentration of α 1Pi. In CBA/J mice, the concentration of α 1Pi was 3.0 ± 0.7 mg/ml whereas C3H and C3D2 mice contained 3.5 ± 0.6 mg/ml and 4.6 ± 1.0 mg/ml of α 1Pi, respectively. Pepys *et al.* (1979b) have reported similar

interstrain variation in normal serum levels of SAP, with as much as a 50-fold difference between some strains. It remains to be established if this interstrain variation results from a direct effect on the synthetic and/or catabolic rates of the protein.

In comparing sex differences, Takahara and Sinohara (1982) have found a significant difference in α 1Pi levels in male and female mice of the ICR strain although we have not observed such a sex difference in the above strains.

The fact that α 1Pi is present in amniotic fluid is not surprising since most serum proteins have been found in amniotic fluid and the majority (95%) are maternally derived, except for α -fetoprotein which is totally of fetal origin. About 85 to 90% of amniotic fluid protein turnover is attributable to fetal degradation after swallowing of amniotic fluid. Virtually all of the protein swallowed is hydrolyzed in the fetal gastrointestinal tract and less than 0.1% is absorbed by the fetus through the gastrointestinal tract, skin, umbilical cord and lungs (Gitlin and Gitlin, 1975).

The role for α 1Pi in amniotic fluid is highly speculative. Another protease inhibitor, α 1-antichymotrypsin, has been shown to be present in the form of a protease-antiprotease complex in human amniotic fluid (Burnett and Bradwell, 1980). By analogy, α 1Pi perhaps plays an important function in the control of degradative enzymes in the amniotic compartment, thereby protecting surrounding tissues from proteolytic attack.

The presence of α 1Pi in considerable amounts in bile and gastrointestinal luminal washings and in large quantities in breast milk

suggests a possible new function for α Pi. In an earlier report, Tomasi and Hauptman (1974) found a significant quantity of α Pi in human colostrum and gastrointestinal fluids but more interestingly, they were able to demonstrate binding of α Pi to a human IgA myeloma. The exact nature of the binding mechanism is not known although a variety of other proteins such as albumin and haptoglobin also bind to IgA (Tomasi and Hauptman, 1974). α Pi may play a protective role for IgA by shielding the immunoglobulin from proteolysis, especially in the intestinal environment. It is not known, however, if α Pi bound to IgA still retains its inhibitory capacity towards proteolytic enzymes. Another possible role for IgA- α Pi complex formation is that IgA may act as a transport molecule for α Pi in secretions. This is substantiated by the fact that incubation of IgA- α Pi complexes with colostrum or intestinal contents liberates intact and biologically active α Pi. Whether this is operative in the mouse is not clear since a preliminary experiment has failed to demonstrate binding of mouse α Pi with the mouse myeloma IgA (MOPC 315) (Underdown, B.J., Gauldie, J., Lamontagne, L. and Socken, D., unpublished observations). Clearly, further investigations in this area are warranted.

An area of research which has recently received a great deal of attention is the role of protease inhibitors in chronic obstructive lung diseases (COLD).

The presence of α Pi in bronchiolar fluid is particularly significant in light of the current "protease-antiprotease theory" for the pathogenesis of chronic destructive lung diseases, such as pulmonary

emphysema. These disorders are characterized by the destruction of alveolar walls and distortion of alveolar spaces. The "protease-antiprotease theory" proposes that the integrity of alveolar structures is maintained by the inhibition of proteolytic enzymes by antiprotease complex formation (Gadek *et al.*, 1980). Lower respiratory tract imbalances in the inhibition of proteases result in excess proteolytic activity in the lung with subsequent degradation of lung tissue. This theory is supported by the findings that: 1) there is increased prevalence of early onset of pulmonary emphysema in young adults with homozygous $\alpha_1\text{Pi}$ deficiency (Eriksson, 1964; Laurell and Eriksson, 1963) and; 2) instillation of proteases, in particular, neutrophil-derived elastase, into the lower respiratory tract of animals produces lung disease similar to human pulmonary emphysema (Janoff *et al.*, 1977; Weinbaum *et al.*, 1974; Senior *et al.*, 1977).

The antielastases of human alveolar tissues include α_2 -macroglobulin (Gadek *et al.*, 1980), the newly recognized bronchial mucus inhibitor (Ohlsson and Tegner, 1976) and $\alpha_1\text{Pi}$ (Tuttle and Jones, 1975; Olsen *et al.*, 1975), but in the lower respiratory tract, only $\alpha_1\text{Pi}$ plays a significant inhibitory role since α_2 -macroglobulin represents less than 1% of the total antielastase activity at the alveolar level (Gadek *et al.*, 1980). The bronchial mucus inhibitor is an 11,000 dalton antielastase produced locally by the cells of the upper respiratory tract and is the major elastase inhibitor of the central airways. It is not present in the lower respiratory tract of man (Ohlsson and Tegner, 1976; Hochstrasser, 1976).

The role for lung α 1Pi in maintaining lung integrity is not only apparent in the hereditary form of emphysema (α 1Pi deficiency) but also in the cigarette smoking induced form. Compared to nonsmokers, the lower respiratory tract epithelial fluid from heavy smokers is up to 50% less efficient in antielastase activity (Gadek *et al.*, 1979). Exposing animals to cigarette smoke will also yield similar results (Janoff *et al.*, 1979). The mechanism for this decreased antielastase activity is due to the oxidative inactivation of α 1Pi by an oxidant in cigarette combustion products, causing a reversible decrease in the affinity of α 1Pi for elastase (Carp and Janoff, 1978; Janoff and Carp, 1977; Johnson and Travis, 1979). In addition, products released from activated neutrophils and possibly alveolar macrophages, which are present in increased numbers in smokers, can oxidize the reactive methionine site of α 1Pi and decrease its inhibitory capacity (Johnson *et al.*, 1978; Hunninghake *et al.*, 1980).

Therefore, the presence of α 1Pi in human alveolar fluid is very significant from a clinical standpoint. The finding of α 1Pi in mouse alveolar fluid and the existence of a rodent model for pulmonary emphysema (Janoff *et al.*, 1977) makes this system very amenable to gain further insight into the delicate balance between proteases and antiproteases in the lung microenvironment.

Although α 1Pi is found in a number of body fluids, it is not conclusively known whether the inhibitor is synthesized locally in these tissues or if it is derived from a common source.

5.2.3 Site of synthesis of α 1Pi

The liver, which is the major site of synthesis of α 1Pi (Fig. 4.2.9) and other acute phase reactants (reviewed in section 1.3), contains numerous hepatocytes which stain intensely for α 1Pi (Fig. 4.2.5 and 4.2a) and the remaining hepatocytes exhibit low-level background staining intensity. The intense staining hepatocytes are located in areas surrounding the portal triads as well as randomly distributed throughout the liver lobule. The specific localization of α 1Pi in hepatocytes is particularly significant in view of the fact that there is good correlation between synthetic activity and staining intensity. The evidence for this correlation will be discussed in greater detail in section 5.3. The distribution pattern of α 1Pi positive hepatocytes in the normal adult liver is very similar to that of fibrinogen in the dog (Forman and Barnhart, 1964), haptoglobin in the human (Peters and Alper, 1966), and CRP in the rabbit (Kushner and Feldmann, 1978).

As discussed elsewhere (section 5.3.1), during an acute inflammatory response, there is a marked alteration in the number of intense staining hepatocytes as well as their distribution in the liver lobule (Fig. 4.4.2, 4.7.2). The recognition of this progressional change in distribution patterns for intense staining hepatocytes during inflammation allows us to detect an acute phase response at the actual site of synthesis of the acute phase reactant.

The specific tissue localization of α 1Pi in the small intestine (Fig. 4.2.8), more specifically, within the cytoplasm of some

epithelial cells lining the villi and in significant amounts in the interstitial spaces may implicate this organ as a local source of α 1Pi in the lumen. However, whole organ cultures (section 4.2) of small intestine have failed to demonstrate detectable α 1Pi synthesis. It is possible that the staining activity within epithelial cells represents uptake, perhaps from the lumen, since a phenomenon of "leakage" of proteins into cells of columnar epithelium has been described for transferrin (Mason and Piris, 1980).

The exact biological significance of α 1Pi in the small intestine is open to speculation. It is not known how relevant these findings are to the human α 1Pi-IgA complex formation.

Recently, Geboes *et al.* (1982) have confirmed the specific localization of human α 1Pi in the small intestine. Although there were alterations in staining pattern in certain inflammatory bowel diseases, the exact relationship between these staining patterns and diseases is unknown.

In the lung, the source of proteins, including α 1Pi, in broncho-lavage fluid is probably as a transudate from serum. The alveolar macrophage has been shown to synthesize and secrete α 1Pi (Fig. 4.2.10) but the amounts produced are so small in comparison to hepatocytes (Fig. 4.2.9) that the overall contribution is difficult to assess. However, in the alveolar macrophage microenvironment, the amounts of α 1Pi synthesized may be quite significant.

In breast milk, α 1Pi may be present as a result of transport with IgA although no direct evidence for this mechanism exists in our model.

The demonstration of α Pi in islets of Langerhans in the human pancreas (McElrath *et al.*, 1979; Ray *et al.*, 1977; Ray and Desmet, 1978) confirms the present findings in the mouse (Fig. 4.2.7).

Although the intracellular α Pi is released during culture *in vitro*, the cells do not appear capable of synthesizing the protease inhibitor (section 4.2). The exact biological function of α Pi in the pancreas is unknown, however it may be to protect the islet cells against the various exocrine enzymes in the parenchymal cells of the pancreas (Bendayan and Ito, 1979).

In the mouse, the lack of α Pi staining in the bile ducts contrasts with the finding of human α Pi in a number of patients with cholangiocarcinomas (Reintoft and Hagerstrand, 1979). This may, however, reflect species differences or perhaps the fact that the human study was performed on diseased patients.

Finally, we were unable to confirm in the mouse the finding of α Pi in human mast cells (Ray and Desmet, 1978; Benitez-Bribiesca *et al.*, 1973), polymorphonuclear leukocytes (Benitez-Bribiesca and Freyre-Horta, 1978) and on the surface of concanavallin-A stimulated lymphocytes (Lipsky *et al.*, 1979). Although human platelets and megakaryocytes were positive for α Pi (Nachman and Harpel, 1976; Nalli *et al.*, 1977), we have not examined these cell types in the mouse.

In summary, using monospecific anti-mouse α Pi antisera, we have been able to quantitate α Pi in a variety of mouse body fluids and demonstrate its presence in a variety of tissues and cells.

Although the levels of α Pi in the body fluids are lower than those in serum, they are nevertheless significant

considering the wide range of inhibitory activities of α 1Pi. Regardless of the location of α 1Pi in the body fluids, its major biological function appears to be common, that is, the protection of tissues from excess proteolytic degradation.

The major source of α 1Pi in the serum is undoubtedly the hepatocyte and its presence in milk, bile, amniotic fluid, bronchiolavage fluid and lumen of the gut may also be indirectly of hepatic origin. However, in bronchiolavage fluid, the alveolar macrophage is capable of synthesizing α 1Pi, albeit in very low amounts.

Of all the cell types in which α 1Pi has been demonstrated within their cytoplasm, the hepatocyte is the only cell in which α 1Pi staining activity indicates active synthesis. In all other cases, it appears that uptake is likely the source of cytoplasmic α 1Pi.

5.3 Acute Phase Reactants in Inflammation

The establishment of the methodology for looking at α 1Pi in the intravascular, extracellular and intracellular compartments as well as the description of the presence of mouse α 1Pi in the normal adult allows us to study the acute phase reactant, α 1Pi, during inflammation. In addition, the isolated hepatocyte monolayer culture system, described in section 3.9.1, provides us with a means of investigating the acute phase protein response *in vitro*.

Prior to discussing the experimental results in celite-induced inflammation, certain considerations concerning the validity of isolated hepatocyte culture systems as an investigative tool have to be clarified.

The isolation of hepatocytes by the Seglen technique (Seglen, 1973) allows for the study of a single type of liver cell, although minimal contamination of cultures with kupffer cells and other non-parenchymal cells may not be entirely ruled out.

The maintenance of monolayer cultures of hepatocytes on a collagen substratum (Michalopoulos *et al.*, 1978, 1979), in addition to supplementation of the culture medium with the hormone dexamethasone (Laishes and Williams, 1976), significantly increases the longevity of hepatocytes.

This is not to say, however, that the morphological and functional integrity of hepatocytes remains entirely intact *in vitro*. The use of isolated hepatocyte monolayer cultures is amenable to a restricted number of studies and all results have to be interpreted with respect to endogenous controls within the same experiment. Thus, in short term cultures (0 - 24 h), isolated hepatocytes maintain their ability to incorporate amino acids and synthesize proteins (Williams *et al.*, 1978; Haars and Pitot, 1979; Gurr and Potter, 1980; Hertzberg *et al.*, 1981) and have been used for the study of the pathways of protein degradation by hepatocytes (Grinde and Seglen, 1980). Isolated hepatocytes have also proven to be valuable in the study of growth and functional activities of hepatocytes (Marceau *et al.*, 1982).

Nevertheless, in long term cultures (24 - 96 h), and to some extent as early as 12 h, metabolic adaptations to the culture conditions are often apparent. The isolated hepatocyte culture system is not immune to the inevitable inadequacies of the *in vitro* environment. Thus, in our system, although hepatocyte viability was approximately 85% after

24 h in culture, cell morphology was altered. Hepatocytes were flattened on the collagen substratum and in many instances had established contact with neighbouring cells. In other studies (Tarentino and Galivan, 1980), hepatocyte membranes were shown to become unstable under similar circumstances.

In the present studies, short term (24 h) cultures were used and cell viability was maintained over that period of time.

5.3.1 Celite induced inflammation

The most widely used model of acute inflammation for the study of acute phase reactants consists of a single subcutaneous injection of sterile turpentine oil into rats or rabbits (Koj *et al.*, 1978a; Koj and Regoeczi, 1978; Urban *et al.*, 1979; Courtoy *et al.*, 1981; Baglia *et al.*, 1981; Kwan *et al.*, 1977; Kushner *et al.*, 1980; Rupp and Fuller, 1979; Koj, 1980; Kwan and Fuller, 1977; Kushner and Feldmann, 1978; Legrele *et al.*, 1980).

α 1Pi has previously been shown to be an acute phase reactant in the rat and rabbit and in both instances, the liver has been established as the major site of synthesis of α 1Pi, although additional extrahepatic sites of synthesis have not been excluded.

In the rat, turpentine-induced local inflammation moderately stimulated α 1Pi synthesis, in comparison to fibrinogen. Twenty-four hours post-irritation, plasma α 1Pi concentration reached a maximum of 25 - 30% above normal levels and subsequently subsided to normal by 72 h. Perfusion of livers at 24 h indicated that there was approximately a 3-fold increase in output of α 1Pi in the perfusate

at the end of a three hour perfusion (Koj *et al.*, 1978a).

In the rabbit, α 1Pi serum levels rose progressively over a 72 h period to 75% above normal whereas, in comparison, fibrinogen levels rose rapidly to a maximum of 237% above normal by 48 h and subsided rapidly thereafter (Koj and Regoeczi, 1978).

One of the effects of turpentine-induced inflammation in rabbits is to accelerate the disappearance of α 1Pi and albumin although fibrinogen appears unaffected. During the period of accelerated disappearance, the half-life ($T_{1/2}$) of α 1Pi was shortened to 70 - 74% of the respective pretreatment values. The increased clearance of α 1Pi from the circulation could be accounted for by transient alteration in capillary permeability although some of the α 1Pi may have also interacted with proteases at the site of inflammation (Koj and Regoeczi, 1978).

The specific localization of α 1Pi at the site of inflammation along with a slight decrease in $T_{1/2}$ has been demonstrated in a granuloma model of inflammation by Ishibashi *et al.* (1978). In the present studies (the parasite model of inflammation; section 5.3.2) we have demonstrated the specific localization of α 1Pi to an inflammatory site (Fig. 4.7.5) with a concomitant significant increase in the sequestration of α 1Pi in bronchiolar fluid and lungs (Table 4.7.1).

In the mouse, turpentine is not a suitable model for acute inflammation due to its toxicity and for that reason celite was used as injurious stimulus in the present studies. Celite is a diatomaceous earth and is mainly a siliceous material. Koj and Dubin (1976) have previously used this model of inflammation and have demonstrated an acute phase response for fibrinogen and orosomucoid.

The induction of an acute inflammatory response in CBA/J mice by the administration of a single subcutaneous injection of sterile celite suspension in both scapular regions, resulted in a rapid increase in serum α 1Pi levels to a maximum of 25% above normal at 24 h (Fig. 4.4.1). The magnitude of the serum α 1Pi response was comparable to that observed in the rat (Koj *et al.*, 1978a).

It has been shown that the increased serum concentrations of α 1Pi in the rat and rabbit were due to accelerated synthesis by the liver during acute inflammation (Koj *et al.*, 1978a; Koj and Regoeczi, 1978). Furthermore, it was proposed that the induction of increased synthesis of acute phase reactants by the liver was mediated by a factor(s) originating from the site of inflammation (Koj, 1970, 1974).

Although the hepatocyte has been implicated as the major site of synthesis of α 1Pi, it was not known whether the increased synthesis of α 1Pi during an acute inflammatory state results from an increased output by a fixed number of hepatocytes or from an increase in the number of α 1Pi synthesizing cells. An increase in the number of hepatocytes synthesizing α 1Pi could be achieved by cell replication and/or activation of otherwise low-level α 1Pi synthesis in the "non-inflammatory" state.

Answers to these questions were sought in this study and the approach used was to examine the specific localization of α 1Pi in hepatocytes during an inflammatory reaction. The assumption being made is that the specific localization of α 1Pi in the cytoplasm of hepatocytes represents synthesis as opposed to uptake of denatured or desialylated α 1Pi as part of the normal catabolic pathway. The

validity of this assumption is supported by the fact that in normal rabbits, CRP is not detectable in hepatocytes by immunohistochemistry nor is there detectable CRP in circulation (Kushner and Feldmann, 1978). However, during turpentine-induced inflammation, CRP-positive hepatocytes are observed coincident with the detection of CRP in plasma. Ultrastructurally, CRP is located on the rough endoplasmic reticulum and less markedly, in the lumen of rough endoplasmic reticulum, smooth endoplasmic reticulum and Golgi apparatus; that is, CRP is associated with protein assembly, transport and secretion organelles. Similar ultrastructural findings are observed for albumin in human liver (Feldmann *et al.*, 1972). Moreover, treatment of rabbits with colchicine, an inhibitor of secretion of a variety of plasma proteins (Redman *et al.*, 1975; Banerjee *et al.*, 1976), results in generally more intense staining reactions with greater numbers of CRP-containing hepatocytes than are detected without colchicine treatment.

In a similar study, the appearance of SAA positive hepatocytes, during casein induced inflammatory response in mice coincides with the appearance of SAA in serum (Benson and Kleiner, 1980). Moreover, the intensity and number of hepatocytes positive for SAA increases with colchicine treatment. Similar results are seen in mice treated with casein to induce the synthesis of the acute phase protein SAP (Baltz *et al.*, 1980).

Our findings that hepatocytes isolated from celite treated mice at a time corresponding to maximum hepatocyte staining activity showed an *in vitro* increased output of α 1Pi (1.5- to 3-fold) over normal

hepatocytes (Fig. 4.5.1), confirms the assumption that the staining intensity of α 1Pi in hepatocytes correlates with synthetic activity. This is even more apparent during the parasite model of inflammation (*N. brasiliensis*) where most hepatocytes stain intensely for α 1Pi (Fig. 4.7.2) and hepatocyte output *in vitro* is increased 5- to 10-fold (Fig. 4.8.1).

The current demonstration of a progressive increase in the number of hepatocytes staining intensely for α 1Pi during the course of the celite induced inflammatory response suggest that the effect of inflammation is to induce or activate synthesis of α 1Pi in hepatocytes which are normally secreting low quantities of α 1Pi.

The increased output of α 1Pi is likely not due to increased synthesis by a fixed number of hepatocytes since there was an increase in the number of hepatocytes staining intensely for α 1Pi during the inflammatory reaction (compare Fig. 4.4.2.1 and 4.4.2.2). Alternatively, replication of hepatocytes which are actively synthesizing α 1Pi is not likely since one would have expected to see clusters of intense staining hepatocytes in the liver lobules.

There are two findings which have emerged from this model which are consistent with the hypothesis that the induction of increased synthesis of acute phase reactants by the liver is mediated by a factor(s) originating from the site of inflammation.

In the first instance, during celite induced inflammation, there is a marked alteration in the distribution of hepatocytes staining intensely for α 1Pi. As described in section 4.4, in normal animals, hepatocytes staining intensely for α 1Pi were found mainly in areas

surrounding the portal triad as well as scattered randomly throughout the liver lobule. In marked contrast, during inflammation, intense staining hepatocytes tended to be located mainly around the central veins as well as throughout the liver lobule. The striking feature was that portal triad areas did not contain intense staining hepatocytes. This progression from portal areas to central vein areas is particularly evident in the acute phase response to infection with the parasite *N. brasiliensis* (Fig. 4.7.2). This is consistent with an acute phase mediator gaining access to the liver via the portal vein, entering sinusoidal spaces and inducing increased acute phase reactant synthesis in adjacent hepatocytes with subsequent release of newly synthesized proteins into the central veins.

This marked alteration in the distribution of intense staining hepatocytes during inflammation is not unique to α 1Pi. Other acute phase reactants have been demonstrated to respond in a similar fashion. Kushner and Feldmann (1978) have demonstrated CRP initially in hepatocytes in periportal areas with subsequent positivity in the centrilobular areas during turpentine-induced inflammation. Benson and Kleiner (1980) have observed similar hepatocyte distribution patterns with mouse SAA in casein-induced experimental amyloidosis. SAP has also been detected in mouse hepatocytes after repeated injections of casein but the localization of intense staining hepatocytes was restricted to the areas surrounding the portal triad whereas centrilobular hepatocytes remained negative (Baltz *et al.*, 1980). However, these results require special consideration. SAP, similar to SAA, is usually not detectable in the cytoplasm of hepatocytes in normal animals.

Unlike SAA, however, the ability to detect the localization of SAP within the cytoplasm of hepatocytes requires up to 15 - 30 injections of casein on a daily basis and positivity is first detected at approximately day 19 with maximal intensity at day 32. Serum SAP levels are increased 5-fold by day 11. The proposed explanation for these differences is that perhaps SAP is being secreted so rapidly that the quantity present within the hepatocytes never achieves minimal detectable levels using an immunohistochemical staining technique. Hence, only after repeated casein injections, does the synthetic rate for SAP exceed cellular secretory capacity resulting in a net accumulation of cytoplasmic SAP. This is supported by the fact that a single dose of Freund's complete adjuvant gives rise to increased serum levels comparable to multi-casein treated animals but SAP remains undetectable in the cytoplasm of hepatocytes (Baltz *et al.*, 1980). It would, therefore, appear that, at least for SAP, the detection of intracellular SAP varies depending upon the type and mode of administration of the acute phase stimulus.

The alteration in distribution pattern of intense staining hepatocytes for these acute phase reactants, during an inflammatory response, may reflect a functional difference in certain hepatocytes. There is considerable ultrastructural and size heterogeneity in hepatocytes from the periphery and from the centre of the liver lobule (Drochmans *et al.*, 1975; Deschenes *et al.*, 1976). Rat liver consists of "light" hepatocytes which sediment in Ficoll solution of density 1.10 whereas "heavy" hepatocytes sediment in Ficoll solution of density 1.14 (Drochmans *et al.*, 1975).

The "light" hepatocytes have a particular abundance of smooth endoplasmic reticulum with few glycogen particles and are more often found in the centrilobular regions. The "heavy" hepatocytes differ in that they contain less developed smooth endoplasmic reticulum and exhibit large accumulations of glycogen. These are more frequently located at the periphery of the liver lobule (Drochmans *et al.*, 1975; Wanson *et al.*, 1974).

Therefore, this heterogeneity in cell size, cytoplasmic organelles and preferential localization may reflect differences in the ability of peripheral and centrilobular hepatocytes to respond to the acute phase mediator (APM) as well as overall synthetic capacity for APR.

The second finding which is consistent with the concept of an acute phase mediator lies in the kinetics of maximum hepatocyte synthetic activity and maximum rise in serum levels of α 1Pi.

The maximum liver response during celite-induced inflammation precedes the maximum rise in α 1Pi serum levels. At 9 - 12 h post-injurious stimulus, there is a maximum number of hepatocytes staining intensely for α 1Pi and the overall staining background of the liver is more intense. In contrast, the peak rise in α 1Pi serum levels occurs at 24 h, by which time, liver staining intensity is significantly lower. Peters and Alper (1966) have reported similar findings for haptoglobin in the dog during turpentine-induced inflammation. Although Kushner and Feldmann (1978) have not commented on this aspect, the same basic phenomenon was present in their study. CRP serum levels attained a maximum at 38 h following turpentine injection whereas maximum CRP

hepatocyte activity was observed at 24 h. In another report, SAA serum levels, during casein-induced inflammation, peaked at 18 - 24 h which coincided with maximum hepatocyte activity (Benson and Kleiner, 1980). However, the immunohistochemical localization of SAA at earlier time points (10 - 15 h) was not investigated.

Using a slightly different approach, the same phenomenon has been described for fibrinogen. Kwan *et al.* (1977) and Kwan and Fuller (1977), in a turpentine-induced inflammatory reaction in rats, have measured the fibrinogen concentration in the post-microsomal fraction of hepatocytes at various time intervals. There was a 3- to 5-fold increase in cytoplasmic fibrinogen at 16 h post-stimulus whereas plasma fibrinogen was elevated 3-fold at 24 h (Bouma *et al.*, 1975). Thus an increase in fibrinogen synthesis was detected in the hepatocyte a few hours prior to the actual increase in serum concentration. In addition, on the basis of anti-fibrinogen antibody binding to polysomes which carried nascent polypeptide chains of fibrinogen, these investigators have concluded that the rate of translation of fibrinogen messenger RNA was unaffected during the acute phase response and the increase in fibrinogen synthesis is due to increased fibrinogen transcription. In a similar study, Baglia *et al.* (1981) have demonstrated a 10-fold increase in intracellular haptoglobin at the peak of turpentine-induced injury in rats. Their results also suggest an increase in functional haptoglobin mRNA during the acute phase response.

Courtoy *et al.* (1981) have simultaneously investigated the acute phase reactants fibrinogen, α_1 acid glycoprotein, α_2 -macroglobulin

and haptoglobin in a turpentine inflammation model in the rat. In all four cases, the intracellular concentration reached a maximum at 24 h whereas the peak plasma levels occurred at 40 h.

In conclusion, the findings in the celite-induced inflammatory reaction, in addition to that described in the literature for other acute phase reactants, are consistent with there being an acute phase mediator(s), either directly originating from the inflammatory site or indirectly from another source. Such an acute phase mediator has been demonstrated in the *N. brasiliensis* model of inflammation (section 5.3.2) and as shown in Table 4.20, induces increased synthesis of α 1Pi in isolated hepatocyte monolayer cultures. These results will be described in greater detail in section 5.3.5.

The mechanism for the induction of acute phase protein synthesis by an acute phase mediator(s) is not fully resolved. An important question that arises is whether the various acute phase reactants require individual "trigger" signals or if their synthesis occurs in response to a single acute phase mediator, that is, in a synchronous fashion.

Previous studies in man, using surgical trauma as an acute inflammatory model have yielded conflicting results. Werner and Odenthal (1967) have concluded that the synthesis of acute phase reactants is synchronous whereas Fisher *et al.* (1976), Crockson *et al.* (1966) and Minchin Clarke *et al.* (1971) have reported results which would tend to support an asynchronous mechanism. The inherent flaw in these studies is that the determination of whether a synchronous or asynchronous mechanism is operative, is based entirely on the assessment

of serum levels of various acute phase reactants. As will be shown with our studies in parasite infections, the lack of a serum acute phase protein response is not necessarily indicative of an absence of an ongoing acute inflammatory reaction (sections 5.3.2, 5.3.3).

In the present studies, the 25-fold increase in SAA synthesis (Fig. 4.5.2) and the 1.5- to 3-fold increase in α 1Pi output by the same hepatocytes, is consistent with the concept of synchronous synthesis of acute phase reactants by the liver. These same culture supernatants are currently being analysed for the acute phase reactants SAP and C3.

Evidence in support of a synchronous mechanism for the synthesis of acute phase reactants has been provided by other investigators. Courtoy *et al.* (1981), have demonstrated the simultaneous synthesis of four acute phase reactants in serial tissue sections, both at the light and electron microscope levels. The acute phase reactants were also localized in sinusoidal and Disse's spaces, intercellular spaces up to the tight junctions and in some instances were also observed in large coated pits and coated vesicles of kupffer cells. This study, therefore, provides strong evidence for the synchronism of the acute phase reaction in the liver and also suggests a single acute phase mediator.

In summary, we have demonstrated that α 1Pi is an acute phase reactant in the mouse as defined by a rapid rise in α 1Pi serum concentration with an equally rapid decline to normal levels thereafter.

The specific localization of α 1Pi within hepatocytes provides a means for studying the underlying mechanism(s) for the induction of

acute phase protein synthesis (α 1Pi) at the site of synthesis. The progressive alteration in lobular distribution of intense staining hepatocytes during acute inflammation is consistent with there being an acute phase mediator (APII), perhaps originating from the site of inflammation, which induces acute phase protein synthesis.

The demonstration of an α 1Pi and SAA acute phase response *in vitro* using primary hepatocyte monolayer cultures provides a model system for the delineation of mechanisms involved in the induction of acute phase reactant synthesis.

In the celite-induced inflammatory response, the extent of tissue injury is limited to the site of injection and constitutes a simple model of inflammation with restricted tissue involvement. The findings in this model form the basis for further investigations in more complex inflammatory models where the nature of the inflammatory reaction is constantly changing as well as being of greater intensity.

5.3.2 *Nippostrongylus brasiliensis* infection

Parasitic infections are ideal models for the study of acute phase reactants in infectious diseases. The route and kinetics of migration of parasites through the various host tissues have been well described in the literature and furthermore, there is considerable knowledge with regards to various products (enzymes, proteins, etc.), released by parasites as they migrate as well as some of their interactions with host components. In most cases, there is considerable pathology caused in the various tissues through which the parasites have migrated and the number of infective organisms used for the

inoculation and the mode of infection can be controlled. In addition, by appropriately selecting the parasite and the host, an animal model can be generated with site specific inflammatory changes such as an inflammatory reaction predominantly in the lungs, the gut or muscle tissue or a combination of the above. Moreover, such models potentially allow for the study of specific inflammatory cell types depending on the parasite used and the infected tissue involved.

5.3.2.1 α 1 Protease inhibitor

The life cycle of *N. brasiliensis* in CBA/J mice is depicted in Figure 4.6.1. Histopathologic findings clearly demonstrate that during the lung stage of the parasite (2 days post-infection) there was a massive inflammatory response, as evidenced by distortion of alveolar spaces, thickening of alveoli walls, edema and hemorrhage (compare Fig. 4.6.2 and 4.6.3).

The second major site of inflammation during *N. brasiliensis* infection is the small intestine. Larvae migrate out of the lungs and infect the small intestine (approximately 4 - 6 days post-infection) by migration via the trachea and esophagus causing inflammatory changes in the small intestine (compare Fig. 4.6.4 and 4.6.5).

As shown in Figure 4.7.1, there was no significant change in the serum levels of α 1Pi during the lung stage of the parasite infection where a massive inflammatory response had been documented. It was only during gut inflammation that the serum levels of α 1Pi were truly indicative of an acute phase reaction.

Using immunohistochemistry, it was shown that during the lung stage, virtually 95% of hepatocytes stained intensely for cytoplasmic α PI (Fig. 4.7.2). As discussed earlier in the celite model of inflammation, the intensity of cytoplasmic staining for α PI in hepatocytes correlated with relative synthetic activity. These findings strongly suggest that the inflammatory reaction in the lung does give rise to an α PI acute phase response. This was confirmed when we examined the synthetic output of α PI by monolayer cultures of hepatocytes isolated from mice infected with the parasite two days previously. In these *in vitro* cultures, synthesis of α PI was increased 5- to 10-fold (Fig. 4.8.1) in comparison to hepatocytes isolated from normal animals.

During the gut stage of the parasite, there was an increase in the number of hepatocytes staining intensely for α PI over normal (Fig. 4.7.3) although the overall impression was that the intensity of the α PI acute phase response was reduced in comparison to the corresponding lung inflammation (compare Fig. 4.7.2 and 4.7.3). This could be due in part to the involvement of different inflammatory cells such as macrophages in the lamina propria and Peyer's patches. Alternatively, the seemingly lower liver acute phase response may have been a direct reflection of the extent of tissue injury.

We have, therefore, an anomaly, in that the inflammatory reactions in the lungs and the small intestine both gave rise to an α PI acute phase response, as assessed by immunohistochemistry and *in vitro* synthesis

of α Pi by hepatocytes. However, it was only during gut inflammation that a concomitant increase in serum levels occurred.

The apparent lack of serum acute phase response of α Pi during the lung inflammatory reaction while there was increased hepatic synthesis suggests that α Pi was being cleared just as rapidly from the circulation as it was being supplied. Sequestration of α Pi at the site of inflammation could account for the rapid disappearance of α Pi from the circulation.

The specific localization of α Pi in parasitized lung tissue was seen in significant quantities in the interstitial spaces of infected lungs (Fig. 4.7.5) where in comparison to normal lung tissue, the intensity of staining was much greater. These findings suggest an increased accumulation of α Pi to the site of inflammation. Supportive evidence is provided by the increased accumulation of ^{125}I α Pi in both bronchiolar lavage fluid and lung tissue of *N. brasiliensis* infected animals (Table 4.7.1).

The presence of α Pi in lung tissue is not unexpected. Tuttle and Jones (1975), using immunofluorescence, have demonstrated human α Pi lining the terminal airways and alveoli and Mathis *et al.* (1973) have identified α Pi in hyaline membranes of children with respiratory distress syndrome.

More interesting and perhaps of greater significance was our finding of α Pi in the cytoplasm of alveolar macrophages in the tissues adjacent to the worm (Fig. 4.7.5). It is important to stress that the presence of α Pi within alveolar macrophages was specific for day 2 post-infection and was not observed in these cells from non-infected

animals nor was it present in comparable amounts in days 1 or 3 post-infection.

The presence of α 1Pi in the cytoplasm of alveolar macrophages where lung pathology is prominent has also been observed by Olsen *et al.* (1975), who demonstrated an increased concentration of α 1Pi in cell lysates of alveolar macrophages from heavy smokers. Using immunofluorescence, Cohen (1973a) has demonstrated that α 1Pi is normally present in the cytoplasm of human alveolar macrophages. α 1Pi was also demonstrated in human peripheral blood monocytes as well as in reactive macrophages within and around tumors (Isaacson *et al.*, 1979, 1981).

Although the α 1Pi synthetic capacity of human alveolar macrophages was not directly examined by Cohen (1973a), it was suggested that the presence of α 1Pi in the cytoplasm of alveolar macrophages likely represented uptake as opposed to synthesis. However, peripheral blood monocytes have been shown to synthesize α 1Pi (Isaacson *et al.*, 1981).

Collectively, the findings in our studies suggest that during the lung stage of the parasite, α 1Pi is both synthesized and sequestered from the circulation at an increased rate with the sequestration being predominantly to the lung. It is not known whether the localization of α 1Pi in the infected lung is specific for this protease inhibitor or whether other proteins are also found as a result of non-specific "leakage". The sequestration of other acute phase reactants at an inflammation site has been documented for rat α 1Pi (Ishibashi *et al.*, 1978) and α 1 acid glycoprotein (Shibata *et al.*, 1978), in other systems.

In both cases, albumin also accumulated at the site of inflammation but perhaps to a lesser extent than the acute phase proteins. Regardless of the non-specific nature of the sequestration, α 1Pi and α 1 acid glycoprotein have unique chemical and biological properties and as such assume distinct functions at the site of inflammation.

As discussed earlier, during the gut stage of the parasite, there was a significant progressive increase in serum levels of α 1Pi. Unlike the lung, there did not appear to be an increased localization of α 1Pi in the infected gut mucosa as compared to normal intestine and the luminal content of α 1Pi also remained within the normal concentration. This apparent lack of sequestration of α 1Pi in the small intestine may in part account for the increased serum concentration and indicates perhaps a different involvement of α 1Pi at a gastrointestinal site of inflammation as compared to the lung.

At this stage, one can only postulate on the biological function of α 1Pi in the parasitized lung. It is not inconceivable that α 1Pi may interact with host- and/or parasite-proteases released during the infection. In the present study, although the release of specific proteases and other parasite products has not been fully documented, it is reasonable to speculate that proteolytic enzymes inhibitable by α 1Pi may be released from the parasite since the release of amino-peptides, esterase and acetylcholinesterase by *N. brasiliensis* has previously been reported (Lee, 1970). Other parasites have been shown to release collagenase- and tryptic-like enzymes (Munoz *et al.*, 1982; Brand, 1979). Such an interaction between α 1Pi and host- and/or

parasite-proteases may be both beneficial and detrimental to the parasite depending on the biological function and source of the inhibited proteases.

α lPi may also play a role in the modulation of alveolar macrophage function. It has been shown that purified canine α lPi, at a concentration of 200 μ g/ml, significantly inhibits alveolar macrophage migration *in vitro* (Schnitzlein *et al.*, 1980). David and Remold (1976) have also reported that human α lPi enhances the macrophage response to macrophage inhibitory factor (MIF).

5.3.2.2 Alveolar macrophage synthesis of α lPi

In the present studies, the immunohistochemical localization of α lPi in alveolar macrophages of parasitized lungs raised several questions. Does the intense cytoplasmic staining represent *de novo* synthesis or uptake from the extracellular milieu? Is the presence of α lPi in the alveolar macrophage cytoplasm associated with an "activated" state of the cell and what is the nature and biological significance of α lPi in these cells?

In order to answer these questions, alveolar macrophages from normal and infected lungs were isolated, partially characterized as to their state of activation and their α lPi synthetic capacity was determined by *in vitro* incorporation of 35 S-methionine. However, prior to a discussion on these findings, certain technical considerations need further clarification:

The total macrophage population of the lung, at any one time, is usually referred to as pulmonary macrophages. However, pulmonary

macrophages consist of two main populations of cells; the major portion is located at or in the alveolar spaces whereas the remainder is in the interstitial lung tissue. The former is defined as alveolar macrophages while the latter is referred to as pulmonary tissue macrophages (van Oud Alblas *et al.*, 1981; van Oud Alblas and van Furth, 1979).

Macrophages isolated from the lung by bronchiolavage are by definition alveolar macrophages. However, in the infected lungs, the extensive tissue destruction and hemorrhage into the alveolar spaces may introduce some blood monocyte contamination into the alveolar macrophage preparations.

In tissue sections (Fig. 4.7.5), the cells that contain large amounts of cytoplasmic α Pi are macrophages since they are positive for non-specific esterase which is inhibited by fluoride ions. The location of these macrophages in the lung tissue, that is, associated with the alveolar walls, is consistent with these cells being alveolar macrophages.

Alveolar macrophages isolated from *N. brasiliensis* infected lungs were in a highly activated state in comparison to alveolar macrophages from normal lungs.

Morphologically, the alveolar macrophage from the infected lung was larger (Fig. 4.10.1, 4.10.2, 4.10.3, 4.10.4) and there was an increase in cytoplasm to nuclear ratio. There was also an increase in the number of mitochondria and in many instances the cell was vacuolized to a greater extent. These morphological criteria and others are routinely used to assess the "activated" state of macrophage populations

and have been extensively reviewed by David and Remold (1976).

Functionally, the alveolar macrophage from the infected lung had a greater propensity for the adherence and phagocytosis of opsonized red blood cells. Although increased phagocytic activity of macrophages is usually indicative of an activated state, there are instances in which decreased phagocytosis is observed (David and Remold, 1976).

Biochemically, there was a significant increase in the release of plasminogen activator by alveolar macrophages from parasite infected lungs. The release of plasminogen activator has been widely used as a relatively sensitive index of macrophage activation (Gordon *et al.*, 1981; Neumann and Sorg, 1981; Unkeless *et al.*, 1974; Vassalli and Reich, 1977; Davies and Allison, 1976).

In summary, based on morphological, functional and biochemical criteria, the alveolar macrophage isolated from a *N. brasiliensis* infected lung is in an "activated" state in comparison to alveolar macrophages from normal lungs.

The mode of activation of alveolar macrophages in the parasitized lung is not clear but the direct interaction of parasites and/or parasite products with alveolar macrophages, in a primary infection, is likely the main mechanism.

As mentioned earlier, the localization of α 1Pi in the cytoplasm of alveolar macrophages could represent *de novo* synthesis and/or uptake. The intensity of staining of α 1Pi in these cells was comparable, if not greater, to the intense staining hepatocytes described earlier (compare Fig. 4.2.5 and 4.7.5). Since the intensity of staining in hepatocytes correlated with α 1Pi synthetic activity, it was possible that alveolar

macrophages synthesized α 1Pi.

The incorporation of ^{35}S -methionine into secreted products of both normal and activated alveolar macrophages revealed synthesis of α 1Pi by both cell populations (Fig. 4.2.10). Although in both cases, there was only a single faint precipitin line on the photograph, corresponding to the fast variant of α 1Pi, the slow variant could be discerned on the original autoradiograph. (The intensity of the line was such that it was lost during photographic reproduction.) The incorporation of ^{35}S -methionine was inhibited by cycloheximide and there was no alteration in electrophoretic mobility of α 1Pi suggesting that the incorporated radio-label material was in α 1Pi and not a protease released by alveolar macrophages which subsequently bound to exogenous unlabelled α 1Pi added during electrophoresis.

On a semi-quantitative basis, there was no apparent difference in synthetic output of α 1Pi by normal and activated alveolar macrophages. Moreover, in comparison to hepatocytes, cultured under exactly the same experimental conditions, the amounts of α 1Pi synthesized by alveolar macrophages were minimal (compare Fig. 4.2.9 and 4.2.10).

Since both groups of alveolar macrophages synthesized similar and very low amounts of α 1Pi but only the activated alveolar macrophage stained intensely for cytoplasmic α 1Pi, then it is likely that the presence of α 1Pi within the cytoplasm of these cells in infected lungs represents uptake with a very minor contribution from *de novo* synthesis. The possibility that α 1Pi in activated alveolar macrophages was synthesized at an increased rate but not secreted is not likely since the activated cells released significantly greater amounts of other

proteins in these same culture supernatants, as assessed by immunoprecipitation with anti-whole mouse antiserum. The fact that numerous other proteins were secreted indicates that under our experimental conditions, the protein synthesis and secretion "apparatus" was intact in both normal and activated cells.

Concurrent with these studies, White *et al.* (1981) have demonstrated the synthesis of rat α 1Pi by isolated alveolar macrophage cultures using the same labelling procedures. They did not, however, compare normal and activated cells nor did they investigate the intracellular presence of α 1Pi by immunohistochemistry.

The mechanism(s) for the uptake of α 1Pi into the cytoplasm of the activated alveolar macrophages is not known. Dolovich *et al.* (1975) have previously shown that rabbit alveolar macrophages do not endocytose α 1Pi-trypsin or α 1Pi-subtilopeptidase A complexes. However, α 2-macroglobulin complexes were readily endocytosed under these conditions. From these findings, it was concluded that the alveolar macrophage expresses a receptor for α 2-macroglobulin-protease complexes but lacks an equivalent receptor for α 1Pi complexes.

Recently, it has been shown that neutrophil-derived elastase was bound and internalized by human alveolar macrophages without the requirement for prior complex formation with α 2-macroglobulin, as is necessary for pancreatic and bacterial proteases (Campbell *et al.*, 1979; Campbell and Senior, 1982). Not only is this pathway for uptake of neutrophil-elastase important in sites where low concentrations of α 2-macroglobulin prevail, such as the lower respiratory tract, but it may also be a means for the uptake of α 1Pi since neutrophil-elastase is a prime substrate

for α Pi. Thus α Pi could be endocytosed in a bystander mechanism without the requirement of a specific α Pi receptor.

In the present studies, it is postulated that α Pi is endocytosed by alveolar macrophages in infected lungs as either a host- and/or parasite-protease complex. The interaction of α Pi with products released from *N. brasiliensis* and host inflammatory cells is currently being investigated.

Recently it has been reported that rabbit alveolar macrophage elastase is not inhibited by α Pi but rather binds and inactivates the inhibitor (Banda and Werb, 1982). Since, in the infected lung, there is a predominance of alveolar macrophages which are in an activated state and as such are likely secreting a variety of proteases, it is possible that the α Pi in the alveolar macrophage cytoplasm is degraded but still immunologically reactive.

The actual biological significance of α Pi synthesis by alveolar macrophages is still speculative but the role of α Pi is presumably one of neutralization of proteases either intracellularly or after release of the inhibitor to the extracellular environment. Although the alveolar macrophage synthesized minimal amounts of α Pi in comparison to hepatocytes, this output of α Pi may be very significant in lung tissue. Hence in the immediate microenvironment of the alveolar macrophage, the effective concentration of α Pi may be of considerable magnitude. In addition, the fact that the alveolar macrophage is capable of synthesizing α 2-macroglobulin (White *et al.*, 1980), another important protease inhibitor, implicates this cell in the local regulation of protease-antiprotease balance in the lung. As such, the alveolar

macrophage may play a vital role not only in the destruction of pathogenic organisms but also in limiting the extent of tissue damage.

5.3.2.3. Other acute phase reactants - C3, SAA and SAP

The C3 serum levels, of mice infected with *N. brasiliensis*, had a pattern similar to that of α 1Pi (Fig. 4.9.1). There was no apparent serum acute phase response during lung inflammation although there was a significant increase in serum levels during the gut stage of the parasite.

Unlike α 1Pi and C3, the serum concentrations of SAA (Fig. 4.9.3) and SAP (Fig. 4.9.2) were indicative of an acute phase response during lung inflammation. The increased SAA serum level was also reflected in the three-fold increase over normal in SAA synthesis by monolayer cultures of hepatocytes isolated from day 2 infected mice (Fig. 4.8.2).

During inflammation in the small intestine, there was a significant increase in serum levels of SAA and SAP, similar to that observed for α 1Pi and C3. Although the kinetics of the maximum serum response for each of the four acute phase reactants did not coincide exactly, there was significant overlap of peak responses.

The activation of C3 in a variety of inflammatory processes has been well documented in the literature. Since there were no apparent changes in serum levels during lung inflammation, one would speculate that, similar to α 1Pi, C3 is synthesized in greater amounts by hepatocytes but it is rapidly sequestered and utilized in the infected lung.

As for the acute phase reactants SAA and SAP, there was a rapid decrease to very low serum levels (approximately days 3 - 8) following

the early stages of the parasitic infection. Since during this time period histopathologic findings revealed inflammatory changes in the lung, this decrease in serum levels may represent sequestration of SAA and SAP in extravascular sites where these may perform their respective biological functions. The roles and/or fate of SAP and SAA, synthesized in response to inflammation, are ill-defined and as such, the *N. brasiliensis* model of inflammation provides an excellent opportunity for their study.

5.3.3 Trichinella spiralis

This particular parasite-induced model of inflammation was selected to further investigate the acute phase response induced by gut inflammation. The *T. spiralis* model was perhaps more suitable than *N. brasiliensis* since in this model the small intestine is the initial site of tissue injury and as such the intestinal inflammatory response occurs in the absence of any other ongoing inflammatory reaction at a distant site.

The life cycle of *T. spiralis* in the mouse is depicted in Figure 4.15.1. In addition to an early inflammatory response in the small intestine, muscle tissue was also affected (Fig. 4.16.3) as the newborn larvae migrate out of the gut into muscles at a later time.

The histopathologic findings in gut, induced by *T. spiralis*, revealed marked villous architecture distortion with remarkable goblet and Paneth cell hyperplasia (compare Fig. 4.16.1 and 4.16.2). In fact there was actual penetration of intestinal mucosa by *T. spiralis* at the base of villi as well as in the crypts.

Using immunohistochemistry, there was a marked liver α Pi acute phase response (Fig. 4.17.1) during the gut inflammation which, on a semi-quantitative basis, was of similar magnitude to that induced by *N. brasiliensis* infected gut. However, in marked contrast to *N. brasiliensis*, α Pi serum levels failed to indicate an acute phase response at this time. In fact, there was a marked decrease below normal in α Pi serum levels. On the other hand, SAA serum levels were indicative of an acute phase response with a 5-fold increase at day 6 post-infection (Fig. 4.18.1). As was the case for α Pi during the lung stage of *N. brasiliensis*, the reduced levels of α Pi during *T. spiralis*-induced gut inflammation could represent utilization of α Pi at the site of inflammation. There was, however, no apparent difference in interstitial and intracellular staining for α Pi in the gut tissue at any time during the infection. This does not, however, rule out the possibility that α Pi was being consumed in the lumen of the gut.

During the muscle stage of *T. spiralis*, there was marked mononuclear cell infiltration in the muscle tissue, especially in the vicinity of larvae. Immunohistochemistry showed that there was an α Pi acute phase response in the liver at this time (Days 10 - 30) and serum levels did increase towards normal (Fig. 4.18.1) but subsequently decreased thereafter. There was a similar increase in SAA serum levels which occurred over a narrower time span (days 20 - 28).

Similar to the *N. brasiliensis* infected lung, there was evidence of increased interstitial α Pi staining in muscle tissue. This is suggestive of utilization of α Pi at this particular site of inflammation.

In conclusion, this model of inflammation illustrates very well the point that an acute inflammatory reaction induced in the same tissue-type but by two different injurious stimuli (i.e. gut inflammation induced by *N. brasiliensis* and *T. spiralis*) does not necessarily give rise to the same alterations in serum levels of a given acute phase reactant. It is likely that the different inflammatory cell types involved in the two parasite-induced gut inflammation, the relative intensity of the inflammatory reactions and the various products released during the host-parasite interaction, play a role in determining the net serum concentration of α PI.

5.3.4 *Trypanosoma congolense*

T. congolense is mainly a parasite of the bloodstream with minimal invasion of the lymphatics and certain tissues.

In this particular parasite-induced model of inflammation, one of the main interesting findings is the variation in strain susceptibility to the infection. Susceptibility is defined as the relative inability to limit parasitemia which results in early death (10 - 15 days). Alternatively, resistant animals do not achieve these high levels of parasitemia and the infection becomes lethal at a later time (60 - 80 days).

In recent years, considerable efforts have focused on determining the reasons why certain strains of mice were particularly more resistant or susceptible than other strains to a given infectious organism. To that effect, numerous studies have concentrated on the ability of the animal to mount an immune response to the infectious agent, that is,

whether a particular strain is a "low responder" or a "high responder". If strain susceptibility/resistance is related to the ability to generate immunity, then it is likely under the influence of immune response genes (Ir genes), located in the major histocompatibility complex (H-2). However, it is also possible that events prior to the generation of specific immunity, such as the ability to mount an acute phase response in addition to mononuclear cell infiltration, in response to an infectious agent, may have an effect on strain susceptibility/resistance.

Resistance to infection has been investigated in a variety of murine model systems which include *Listeria monocytogenes* (Stevenson *et al.*, 1981), *Plasmodium chabaudi* and *Babesia microtti* (Eugui and Allison, 1980), *Taenia taeniaeformis*, *Leishmania tropica*, *Giardia muris*, *T. spiralis* and *N. brasiliensis* (reviewed in Mitchell, 1979a) and *Trypanosoma congolense* (Morrison *et al.*, 1978; Murray and Morrison, 1979). In all cases, the C57Bl/6J was invariably the most resistant strain whereas the A/J mouse was the most susceptible. C3H/HeJ and Balb/C were usually of intermediate level of resistance.

The striking feature of the *T. congolense* induced inflammation was the apparent lack of an early acute phase-protein serum response for α 1Pi and SAA in the susceptible strain of mice (A/J) as compared to the more resistant C3H/HeJ mice (Fig. 4.14.1; 4.14.2). In fact, on a semi-quantitative basis, the immunohistochemical findings for α 1Pi in the hepatocyte revealed only a moderate induction of α 1Pi synthesis in the A/J mice whereas the more resistant strain showed a more pronounced α 1Pi acute phase response in the liver at that particular time. In addition, the mononuclear cell infiltration in both liver and lungs appears at a

later time in the susceptible strain (10 - 12 days post-infection as compared to 6 - 8 days for C3H/HeJ mice) and the infiltrate was not as prominent. By that time, the parasitemia had reached lethal levels.

On the basis of these findings, one can speculate that the degree of resistance to *T. congolense* in mice is related not only to an early infiltration of mononuclear cells in infected tissues, but also to the intensity of this cellular infiltrate. The intensity of the mononuclear infiltration is important since the *in vivo* activation of the mononuclear phagocyte system by a previous bacterial infection with *Bordetella pertussis*, *Corynebacterium parvum* or *Bacillus Calmette-Guérin* results in a significantly enhanced resistance of both the susceptible and resistant strains to the parasite infection (Murray and Morrison, 1979). Hence, a prompt and heavy mononuclear cell response may limit the parasitemia and allow the animal to survive the parasitemia.

It is also interesting to note that the level of resistance of inbred strains to *T. congolense* is also reflected in the ability of the mouse to mount an acute phase response as indicated by the staining of hepatocytes for α 1Pi and the serum levels of α 1Pi and SAA during the infection.

Stevenson *et al.* (1981), using mice infected with *Listeria monocytogenes*, have similarly shown that strain susceptibility resistance is related to an early infiltration of mononuclear cells as well as to the relative intensity of the infiltrate.

In our model, the exact nature of the relationship between the significantly reduced α 1Pi acute phase response at the hepatocyte level and susceptibility to infection, is not known. Since mononuclear

phagocytes in susceptible strains have been shown to exhibit markedly reduced chemotactic function *in vitro* (Stevenson *et al.*, 1981), it is possible that these cells may also be impaired in their ability to respond to inflammatory stimuli and release an acute phase mediator. Although this particular aspect has not been studied here, the *T. congolense* model of inflammation is potentially useful for such investigations.

5.3.5 Release of acute phase mediator (APM) from the site of inflammation

As discussed in detail in section 1.4, there is strong evidence that the macrophage is a potential source of acute phase mediator (APM), which is now known to be similar to interleukin 1 (IL1), if not identical.

It was of considerable interest to determine whether murine alveolar macrophages, from *N. brasiliensis* infected lungs, were capable of synthesizing/secreting an acute phase mediator. Murphy *et al.* (1980) have demonstrated the synthesis of EP (LAF) by rabbit alveolar macrophages stimulated *in vivo* with heat-killed *Bacillus Calmette-Guérin*.

Preliminary results (Table 4.20.1) indicated a slight but significant increase in the *in vitro* hepatocyte output of α Pi when the cells were incubated with crude culture supernatant from both normal and *N. brasiliensis* activated alveolar macrophages. LPS alone also exhibited a stimulatory effect on α Pi synthesis although it is not known whether this is mediated by the direct action of LPS on hepatocytes or on contaminating Kupffer cells. The Kupffer cell will respond to LPS by secreting an LAF-like molecule (McAdam, K.P.W.J., private communication).

-In these preliminary studies, there was, however, no apparent difference in the degree of induction of α 1Pi synthesis by normal or activated alveolar macrophages. This might be related to a dose-dependent response of hepatocytes to APM as was shown by Ritchie and Fuller (1981) for fibrinogen secretion by isolated hepatocytes in response to LEM (APM).

The identity of the factor(s), which originates from alveolar macrophages and exhibits α 1Pi synthesis induction in hepatocytes, has not been conclusively established. However, recent reports in the literature indicate that the α 1Pi stimulating factor is IL1 (Rupp and Fuller, 1979a; Hooper *et al.*, 1981; Selinger *et al.*, 1980; Ritchie and Fuller, 1981). The alveolar macrophage culture supernatants which stimulated α 1Pi synthesis by hepatocytes also possessed IL1 (LAF) activity, as assessed by the thymocyte proliferation assay (Table 4.19.1).

The mechanism(s) for the induction of APR synthesis in hepatocytes by APM is not fully understood. Koj and Dubin (1974) have suggested that the acute phase mediator(s) originating from the inflammatory site is a primary factor in the stimulation of the hepatocyte to synthesize APR and that hormones, such as cortisol, insulin and growth hormone, exert permissive or anabolic action and therefore are secondary effector molecules of the acute phase reaction. Hooper *et al.* (1981), Rupp and Fuller (1979) and Ritchie and Fuller (1981) have reported similar hormonal effects on acute phase protein synthesis.

A question which is still unresolved is whether the continued presence of APM is required for stimulation of acute phase protein synthesis or if it is a one hit mechanism. Kushner *et al.* (1980) found that isolated perfused rabbit liver synthesized CRP at constant rates. Moreover, the increase in rate of CRP synthesis that occurred in intact animals, undergoing a turpentine-induced inflammatory response, was not observed in isolated livers, suggesting that continuing exposure to factors of extrahepatic origin is required for the continuing increase in rate of CRP synthesis.

In conclusion, significant findings with respect to the study of acute phase reactants have emerged from this particular model of parasite-induced inflammation. The finding of a normal serum level of a single acute phase reactant is not necessarily indicative of the absence of an ongoing inflammatory response. However, the concurrent determination of serum levels of other acute phase reactants would provide a better index of acute inflammation. This is particularly applicable to complex models of inflammation such as parasitic infections. This is reasonable since the serum level of a particular protein during inflammation is determined by its synthesis and secretion rate, its catabolic rate and relative distribution to extravascular sites.

The demonstration of increased *in vitro* hepatic output and greater staining intensity of α 1Pi in hepatocytes during inflammation appears to be a much more reliable index of acute phase response since it is independent of the various parameters that affect the concentration of serum proteins. Such studies also allow for the investigation of

possible sites of utilization of newly synthesized acute phase reactants and provide insight into their interaction with host inflammatory systems, mechanisms of induction of synthesis and modes of degradation.

The establishment of isolated hepatocyte monolayer cultures provides additional information with regards to the acute phase phenomenon. In particular, this *in vitro* model provides a system for isolating and characterizing molecules which induce increased hepatocyte synthesis of acute phase reactants. 7

CHAPTER 6

MODEL OF ACUTE PHASE REACTANTS IN INFLAMMATION

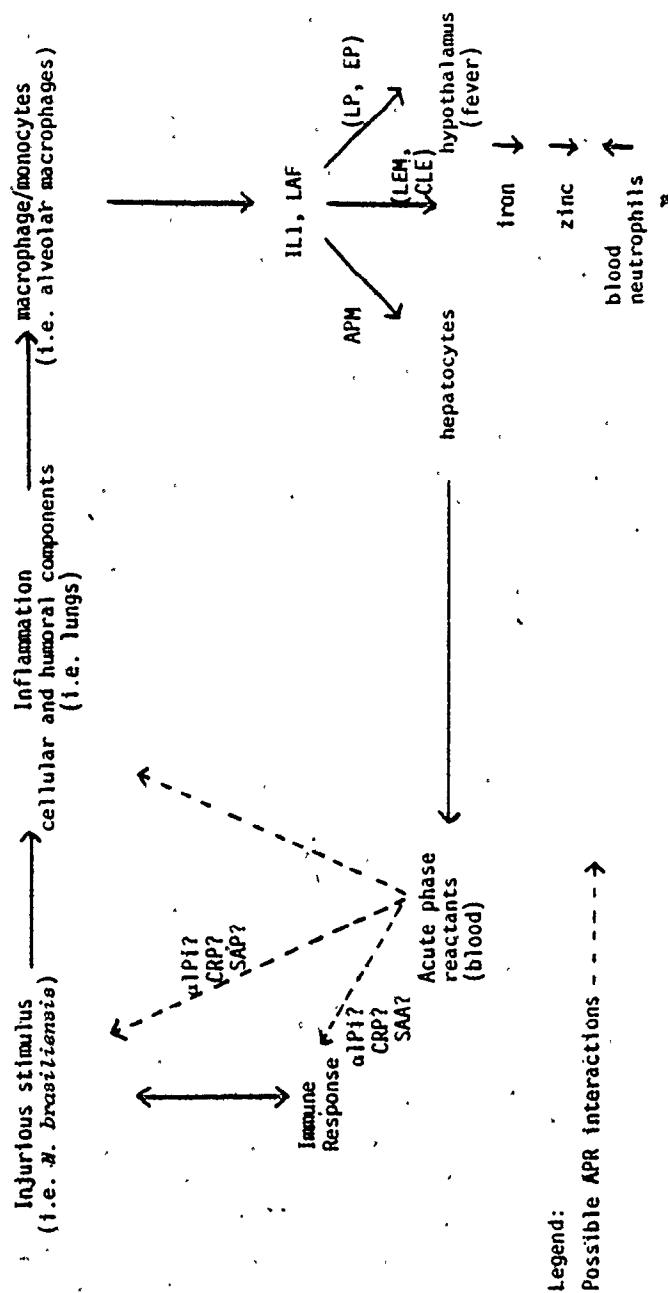
6.1 Model of Acute Phase Reactants in Inflammation

An overview of the acute phase phenomenon is depicted in Figure 6.1. This particular model was constructed on the basis of data generated in the present studies as well as from information available in the literature. The following is a description of the model and some of its implications. In this general discussion, specific examples from the four experimental models of inflammation will be alluded to.

An injurious stimulus at a particular tissue site initiates both a cellular and a humoral inflammatory reaction. The numerous pathways that are activated and the interrelationship between the humoral and cellular components of inflammation have been described in greater details in Chapter 1 and are depicted in Figures 1.1.4.1 and 1.1.4.2. In our experimental models, *N. brasiliensis* gives rise to an inflammatory reaction in the lungs and subsequently in the small intestine. *T. spiralis* induces an inflammatory response in the small intestine and muscle tissue whereas *T. congolense* infection is accompanied by prominent mononuclear cell infiltration in a variety of tissues.

At the site of inflammation, an acute phase mediator (APM) with numerous biological activities (section 1.4) is released, presumably from macrophages. Some of the biological functions of this APM include the induction of fever (EP, LP), a decrease in plasma iron and zinc in addition to an increase in the release of bone marrow derived neutrophils into the circulation (LEM, CLE), the induction of acute phase reactant synthesis by hepatocyte (APM, SAASF) and finally induction of thymocyte

Figure 6.1 Acute Phase Reactants in Inflammation



proliferation (LAF, IL1). So far, the molecules APM, SAASF, EP, LP, LEM, CLE, LAF and IL1 are inseparable on the basis of their biological activities and as such likely represent one molecular species.

During the lung stage of *N. brasiliensis*, alveolar macrophages are in an activated state and their culture supernatants can be shown to contain a factor(s) with LAF and APM activity *in vitro*.

There is increased α PI staining intensity in hepatocytes as well as a progressive alteration in their distribution pattern throughout the liver lobule. During celite induced inflammation, these changes are consistent with an acute phase mediator gaining access to the liver via the portal circulation with subsequent induction of APR synthesis and release into the central veins.

There are a number of proteins which have been classified as acute phase reactants (Table 1.1.3) and their respective biological functions during inflammation has been described earlier. In the present studies, α PI has been shown to be sequestered at the site of inflammation, more specifically, in the interstitial spaces of parasitized lungs as well as within the alveolar macrophages. In the *T. spiralis* model, α PI was also shown in muscle tissue during encystment of larvae.

Although the mechanism of uptake and the nature of α PI in the alveolar macrophage is unknown, it is likely that α PI is taken up either as a host- or parasite-protease complex.

In the lung, α PI and other acute phase reactants, in particular CRP, may also interact directly with the parasite or secreted parasite products.

The recent findings that α 1Pi, SAA and CRP may serve as modulators of the immune response may be particularly significant in this model since the ability to mount an acute phase response may have an effect on the subsequent development of immunity to the parasite.

In conclusion, this model constitutes a basic framework upon which mechanisms for the induction and control of APR synthesis can be studied. In addition, the further characterization of their biological functions will enable us to assess their overall role in inflammation and immunity. The model also stresses the importance of developing methods for investigating these APR at various tissue sites in addition to the monitoring of serum levels.

CHAPTER 7

SUMMARY AND CONCLUSIONS

7.1 Summary

α 1Pi (MW 53,500) has been purified to apparent homogeneity from normal mouse plasma with a purification factor of 12. On two-dimensional immunoelectrophoresis, two immunologically non-identical α 1Pi variants are present and both exhibit antitryptic activity.

During gestation, α 1Pi fetal mouse serum levels are approximately 15% of normal mouse adult levels and at birth, serum levels rise progressively to normal adult concentration within three weeks. Studies using labelled α 1Pi indicate that very little α 1Pi, if any, in the fetus is maternally derived and hence low levels during gestation likely represent fetal synthesis. This is confirmed by the immunohistochemical localization of α 1Pi in fetal hepatocytes at a relatively early gestational age.

α 1Pi is an acute phase reactant in the mouse as evidenced by increased serum levels and/or increase in the number and intensity of α 1Pi staining hepatocytes during acute inflammation. This was demonstrated in the classical model of celite-induced experimental inflammation and, in addition, the studies were extended to three different models of inflammation, consisting of a primary infection with the parasites *Nippostrongylus brasiliensis*, *Trichinella spiralis* and *Trypanosoma congolense*.

There are several salient features in these four models of inflammation:

- i) In all cases, an inflammatory reaction at any particular tissue site gives rise to an α 1Pi acute phase response as demonstrated by an increase

in the number as well as the intensity of α 1Pi staining in hepatocytes.

ii) There is a marked alteration in the distribution of intense staining hepatocytes during the onset of an acute inflammatory response. This was particularly evident in the classical celite-induced experimental model of inflammation. The fact that the maximum α 1Pi staining intensity as well as the number of positive hepatocytes preceded the maximum rise in α 1Pi serum levels, is consistent with the hypothesis that a soluble factor, now referred to as acute phase mediator (APM), originates at the site of inflammation and selectively induces the increased synthesis of acute phase reactants, by hepatocytes.

iii) In the *N. brasiliensis* model of inflammation, preliminary studies indicate that alveolar macrophages from a parasitized lung, that is, at the inflammatory site, are capable of secreting an α 1Pi stimulating factor (presumably IL1) *in vitro*, in the absence of an exogenous stimulus, which induces an increase in the synthetic output of α 1Pi by isolated hepatocyte monolayer cultures. No attempts were made to further purify and characterize this putative acute phase mediator.

iv) The lack of a serum acute phase response with respect to increased levels of APR, is not necessarily indicative of the absence of an underlying inflammatory response since many different parameters are involved in determining the final concentration of a particular protein in the intravascular pool. Such parameters include the rates of breakdown and patterns of distribution of the APR, as well as their respective synthetic rates.

v) In a given inflammatory condition, the serum profiles of many APR are not necessarily similar although the induction of their synthesis

was likely synchronously initiated by a single APM. Again, this reflects on the broad spectrum of biological functions of the various APR which will have an effect on their utilization kinetics. Moreover, an acute inflammatory reaction induced by two different injurious stimuli does not necessarily give rise to the same serum level profile for a given APR. This was particularly evident for α IPi during the gut stages of the two parasites, *N. brasiliensis* and *T. spiralis*. The reasons for the dissimilarity in serum level profiles are not clear. It is likely that the different inflammatory cell types involved in the two parasite-induced gut inflammation, the relative intensity of the inflammatory reactions and products released during the host-parasite interaction, account for these dissimilarities.

vi) Preliminary studies with *T. congolense* induced experimental inflammation have indicated that there is good correlation between the relative degree of resistance to *T. congolense* infection in different strains of mice and the intensity of the acute phase response as assessed by the immunohistochemical localization of α IPi in hepatocytes. Strain susceptibility/resistance also appears to be related to the ability of the particular strain of mice to mount a prompt and pronounced mononuclear cell response in a variety of infected tissues.

7.2 Conclusions

From a basic scientific standpoint, the characterization of the α IPi acute phase response in a classical model of inflammation, both at the hepatocyte level and in the serum, and the extension of such studies to the more complicated parasite-induced inflammatory reactions, have

provided further insight into the underlying mechanism for the induction of increased synthesis of APR.

The isolated hepatocyte monolayer culture system has provided a means for understanding the various cellular and humoral events occurring both at the site(s) of inflammation as well as at the site of synthesis of APR.

With appropriate immunological reagents, such studies can likely be extended to other acute phase reactants as well as other model systems such as bacterial and viral infections and other inflammatory conditions.

Such studies also form the basis for the investigation of the acute phase phenomenon in secondary infections where a concurrent immune response may provide additional parameters.

From a clinical point of view, the use of immunohistochemical staining for specific APR has its obvious limitations and is likely not very amenable to human studies. In the human, the interest in APR has been mainly as "Markers of Inflammation". Numerous studies have been carried out in an attempt to find one or more APR with potential as a diagnostic and prognostic tool in a variety of inflammatory conditions, but yielded limited success.

Our findings in the animal models provide an explanation for the difficulty in correlating APR serum profiles with the presence as well as intensity of disease. It is critical that, for a given inflammatory condition, the simultaneous determination of the serum profile of a variety of APR be characterized. It is also important to note that variation within individuals having a similar inflammatory disease will render the task more difficult.

Finally, the preliminary findings that there is perhaps a relationship between the ability to initiate an acute phase response and susceptibility to infection may have some implications in the human where an individual may be particularly prone to primary and/or secondary infections. The exact causal relationship is not known and clearly requires a great deal of further investigations. It would be interesting and important to establish whether there is a genetic component to the acute phase phenomenon, that is, whether there are "high-responders" and "low-responders" for the induction of acute phase reactant synthesis.

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