STRUCTURAL AND FUNCTIONAL ASPECTS OF THE HEPATIC SINUSOIDS

by

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DECLARATION

With the exception of the *in vitro* biosynthesis experiments outlined in Chapter 4, all studies described in this thesis were initiated and designed by myself.

Sections used for the preparation of transmission electron micrographs in Chapter 1 were cut by Jane Hair. However, I was responsible for their interpretation and photography and for the preparation of all other material used in the illustrations in this introductory chapter. All of the experiments described in Chapter 2 were carried out by me. The scanning electron micrographs were prepared by Paul Schellinck but I was responsible for their interpretation and photography. I performed the light microscopic immunolocalization studies described in Chapter 3. The immuno-electron microscopic experiments were carried out with the help of Mrs. Maureen Griffiths under my direct supervision; I was responsible for the interpretation of all data. The *in vitro* biosynthesis studies using [^{3}H]-proline incorporation and SDS-PAGE analysis outlined in Chapter 4 were carried out in collaboration with, and under the supervision of, Dr. Bert Geerts. I performed the cell isolation procedures and assisted with the radiolabelling experiments.

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Quantitative analysis of nerve fibre density in studies discussed in Chapter 8 were performed with Dr. John Lee under my direct supervision on an image analyser in the CRC Oncology Department, University of Newcastle upon Tyne.

I was solely responsible for all other practical aspects of the work described in this thesis.

PUBLICATIONS

Material contained in this thesis has been included in the following scientific articles:

Burt, A.D. & MacSween, R.N.M. (1986) Immunolocalisation of desmin in fixed rat and human liver. In: *Cells of the Hepatic Sinusoid*, ed. Kirn, A., Knook, D.L. & Wisse, E. Vol.1. p. 234-5. Rijswijk, Holland: Kupffer Cell Foundation.

Burt, A.D., Robertson, J.L., Heir, J. & MacSween, R.N.M. (1986) Desmin-containing stellate cells in rat liver; distribution in normal animals and response to experimental acute liver injury. *Journal of Pathology*, **150**, 29-35.

Burt, A.D., Stewart, J.A., Aitchison, M. & MacSween, R.N.M. (1987) Expression of tissue polypeptide antigen (TPA) in fetal and adult human liver: changes in liver disease. *Journal of Clinical Pathology*, **40**, 719-724.

Burt, A.D., Tiniakos, D., MacSween, R.N.M., Gillon, M., Wisse, E. & Polak, J.M. (1989) Localization of adrenergic and neuropeptide tyrosine (NPY)-containing nerves in the mammalian liver. *Hepatology*, **9**, 839-845.

Geerts, A., Vrijsen, R., Rauterberg, J., **Burt, A.D.**, Schellinck, P. & Wisse, E. (1989) *In vitro* differentiation of fat storing cells parallels marked increase in collagen synthesis and secretion. *Journal of Hepatology*, **9**, 59-65.

Burt, A.D., Griffiths, M.R., Schuppan, D., Voss, B. & MacSween, R.N.M. (1990) Ultrastructural localization of extracellular matrix proteins in human liver biopsies using ultracryomicrotomy and immuno-gold labelling. *Histopathology*, **16**, 53-58.

Griffiths, M.R., Schuppan, D., Ferrier, R. & **Burt, A.D.** (1991) Type VI collagen: a component of the perisinusoidal extracellular matrix in human liver. In *Cells of the Hepatic Sinusoid*, ed. Wisse, E., Knook, D.L. & McCuskey, R. Vol.3. (in press) Rijswijk, Holland: Kupffer Cell Foundation.

Burt, A.D., Ahmed, Q., Hines, J. & Lee, J.A. (1991) Innervation of human hepatic sinusoids: alterations in liver disease. In *Cells of the Hepatic Sinusoid*, ed. Wisse, E., Knook, D.L. & McCuskey, R. Vol.3. (in press). Rijswijk, Holland: Kupffer Cell Foundation.

Lee, J.A., Ahmed, Q., Hines, J. & **Burt, A.D.** (1991) Disappearance of parenchymal nerve fibres in human liver cirrhosis. *Gut* (in press).

Ahmed, Q., Harrison, D., Johnson, S.J. & **Burt, A.D.** (1991) Expression of muscle-associated cytoskeletal proteins by human sinusoidal liver cells. In *Cells of the Hepatic Sinusoid*, ed. Wisse, E., Knook, D.L. & McCuskey, R. Vol.3. (in press). Rijswijk, Holland: Kupffer Cell Foundation.

Griffiths, M.R., Keir, S. & **Burt, A.D.** (1991) Basement membrane components in the space of Disse: a reappraisal. *Journal of Clinical Pathology* (in press).

Whaley, K. & **Burt, A.D.** (1991) Inflammation, healing and repair. In *Muir's Textbook of Pathology*, 13th edn., ed. MacSween, R.N.M. & Whaley, K. (in press) Edward Arnold.

Griffiths, M.R., Schuppan, D., Ferrier, R. & **Burt, A.D.** (1991) Ultrastructural localization of type VI collagen in human liver: evidence of zonal heterogeneity for this microfilamentous protein. Submitted to *Journal of Hepatology*.

Johnson, S.J., Hines, J. & **Burt, A.D.** (1991) Immunolocalization of proliferating perisinusoidal (Ito) cells in rat liver. *Histochemical Journal* (accepted for publication).

Johnson, S.J., Hines, J. & **Burt, A.D.** (1991) Macrophage and perisinusoidal cell kinetics following acute liver injury. Submitted to *Journal of Pathology*.

Data from experiments outlined in the thesis have also been presented at a large number of national and international scientific meetings. Published abstracts resulting from these presentations include:

Burt, A.D., Robertson, J.L. & MacSween, R.N.M. (1986) Identification of desmin-containing non-parenchymal cells in rat liver. *Journal of Pathology*, **148**, 65A.

Robertson, J.L., **Burt, A.D.** & MacSween, R.N.M. (1986) Morphometric study of desmin-containing non-parenchymal cells in experimental acute liver injury. *Journal of Pathology*, **148**, 66A.

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Burt, A.D., Tiniakos, D., MacSween, R.N.M., Wisse, E. & Polak, J.M. (1986) On the distribution of neuropeptide Y containing noradrenergic nerves in mammalian liver. *Proceedings of the 43rd Falk Symposium*, **85**.

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Burt, A.D., Gillon, M., Wisse, E., Polak, J.M. & MacSween, R.N.M. (1987) Distribution of calcitonin gene-related peptide (CGRP) and substance P containing nerves in liver: an immunohistochemical study. *Gut*, **28**, A1330.

Burt, A.D., Griffiths, M., Polak, J.M. & MacSween, R.N.M. (1988) Peptidergic innervation of the mammalian liver. *Hepatology*, **8**, 1295.

Burt, A.D., Griffiths, M.R., Schuppan, D., Voss, B. & MacSween, R.N.M. (1989) Localization of type IV collagen in human liver using immuno-electron microscopy. *Journal of Pathology*, **157**, 174A.

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Johnson, S.J., Hines, J. & **Burt, A.D.** (1991) Perisinusoidal (Ito) cell proliferation and phenotypic modulation in acute liver injury. *Gut*, **32**, A837.

PREFACE

This thesis describes experimental studies dealing with structural and functional aspects of the liver sinusoids which were carried out over a period of almost seven years. My interest in liver pathophysiology was initially stimulated as an intercalated BSc Honours student in Pathology when I spent one year with Professor R N M MacSween in the University Department of Pathology, Western Infirmary studying auto-immune reactions in alcoholic liver disease. I returned to the department in August 1982 and again became involved in hepatological research under the guidance of Professor MacSween. One of the first projects I completed as a postgraduate in the department was to study histological changes occuring within perivenular zones in progressive alcoholic liver disease (Burt & MacSween, 1986a). Around that time several reports appeared in the scientific literature which indicated that the perivenular fibrosis which is so characteristic of alcohol-induced disease,may result from the laying down of extracellular matrix proteins by one type of sinusoidal cell, the fat-storing cell. It became clear that many previous investigators of hepatopathology had rather neglected the sinusoidal cells, concentrating instead on parenchymal cells[1]. Stimultated by the report of Yokoi *et al* (1984) that fat-storing cells may express the intermediate filament protein desmin, I performed a series of experiments to establish whether immunolocalization of this protein could be used as a reliable marker for these cells in tissue sections. The method was applied to an animal model of acute liver injury (see Chapter 6); this was the first to demonstrate immunohistochemically that fat-storing cells accumulate in areas of injury. Several other groups subsequently used this approach to monitor the response of these cells to a variety of forms of liver damage.

In 1985, I serendipitously discovered intra-sinusoidal peptidergic nerves during experiments designed to identify neuroendocrine cells within liver sinusoids. As a result of fruitful collaboration with Professor J M Polak, Royal Postgraduate Medical School, I was able to

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[1]In scientific literature the principal epithelial cells of the liver are termed parenchymal cells or hepatocytes; the former has been adopted throughout this manuscript. In descriptions of topography, I have used the acinar concept of the hepatic micro-architecture although equivalent lobular zones are also given where appropriate.

expand this work to document the distribution of adrenergic and peptidergic nerves in rat, guinea-pig and human liver.

In October 1985, I had the opportunity of spending one year in the laboratory of Professor Eddie Wisse at the Free University of Brussels as a Peel Travelling Research Fellow. Professor Wisse is widely regarded as one of the doyens of sinusoidal cell research, having been responsible for the identification of the four distinct sinusoidal cell types in early morphological studies. During my stay in Belgium, I was introduced to the methods of sinusoidal cell isolation and culture. In the first instance these techniques were used to investigate the capacity of rat liver sinusoidal cells to synthesize and secrete complement proteins. Although these experiments demonstrated that Kupffer cells may play a role in complement biosynthesis (Chapter 2), they suggested that their contribution to total hepatic complement synthesis was small.

Of perhaps greater importance to a hepatopathologist, however is the role of sinusoidal cells in the production of extracellular matrix proteins, both in normal liver and during fibrogenesis. In addition therefore to my studies of complement proteins, I collaborated with Bert Geerts in Wisse's department in studies of *in vitro* collagen biosynthesis by fat-storing cells (Chapter 4). One of the powerful methods with which I gained experience in Brussels was ultracryomicrotomy and immuno-gold labelling for the demonstration of antigens at the ultrastructural level. On my return to Glasgow I was fortunate to be able to adapt this approach to study the composition of the extracellular matrix of the space of Disse in normal human liver and in a variety of liver diseases (Chapter 3).

In 1989 I moved to the University of Newcastle upon Tyne to take up my current position as Senior Lecturer in Pathology. Since then, I have resumed studies dealing with *in vivo* response of fat-storing cells to experimental liver injury, developing methods for studying their proliferation and activation in acute carbon tetrachloride-induced damage and in a model of biliary cirrhosis (Chapter 6). Methods for the identification of fat-storing cells in human liver have also been developed (Chapter 5). Finally, studies of human liver innervation have been extended to include an investigation of intra-sinusoidal nerves in various forms of liver disease (Chapter 8).

This thesis is not laid out in any historical order. Instead, the various studies referred to above have been grouped together. Thus, Chapter 2 deals with the synthesis of complement

proteins by sinusoidal cells. Chapters 3 to 6 deal with the extracellular matrix of the space of Disse and the role of fat-storing cells in its production and Chapters 7 and 8 deal with innervation of the sinusoids. Finally, interactions between different components of the sinusoids and perisinusoidal space are discussed in Chapter 9 with speculation about future directions in the study of sinusoidal cell pathobiology.

SUMMARY

The hepatic sinusoids were long regarded as simple, inert vascular channels lined by phagocytic and non-phagocytic cells derived from a common progenitor cell. Several ultra-structural studies reported in the late 1960's however, identified four distinct cell populations (Kupffer cells, sinusoidal endothelial cells, fat-storing cells and pit cells) within the walls of mammalian sinusoids and in the surrounding space of Disse; these cells types were subsequently shown to differ in topography, origin, population kinetics and function.

The morphological and functional characteristics of the individual cell types are reviewed in the first part of this thesis. Kupffer cells are the resident liver macrophages. The sinusoidal endothelial cells are a unique form of endothelium characterized by a fenestrated cytoplasm and the lack of any recognisable basement membrane. Fat-storing cells lie between the endothelial cells and parenchymal cells in the so-called space of Disse and play a pivotal role in the hepatic handling and storage of vitamin A metabolism. Pit cells are the least abundant of the sinusoidal cells; they are now known to represent a form of natural killer cell. In addition to these four cell types, electron microscopic studies identified extracellular matrix components (striated collagen fibrils) and nerve fibres within the space of Disse although these have hitherto been less well characterized than the cellular components of the sinusoids.

This thesis describes studies dealing with various structural and functional aspects of the mammalian sinusoids. The aims of the work included can be broadly summarized as follows:

(i) To investigate the role of sinusoidal cells in the synthesis of complement proteins.

(ii) To characterize the composition of the extracellular matrix of the space of Disse in normal liver and to examine alterations to this in liver disease.

(iii) To identify the cellular origin of such extracellular matrix proteins with particular attention to the role of sinusoidal cells.

(iv) Having identified fat-storing cells as a major source of extracellular matrix proteins in (iii), to study the response of these cells *in vivo* to acute and chronic liver injury.

(v) To identify and characterize intra-sinusoidal nerve fibres and to examine alterations to sinusoidal innervation in liver disease.

The recently developed methods of sinusoidal cell isolation and culture were used to investigate the capacity of Kupffer cells, sinusoidal endothelial cells and fat-storing cells to synthesize C3, the most abundant protein of the complement cascade pathway. C3 was detected in Kupffer cell supernatants using a sensitive ELISA. Release of C3 could be reversibly inhibited by addition of the protein synthesis inhibitor cycloheximide, indicating active synthesis of the protein by these cells. Secretion of C3 could be enhanced by the prior exposure of the cells to bacterial endotoxin. Smaller amounts of C3 were also detected in fat-storing cell cultures after 5 days in culture but were undetectable in sinusoidal endothelial cell cultures. This study was the first to demonstrate C3 synthesis and secretion by sinusoidal cells. Although it was calculated that Kupffer cells are likely to contribute less than 1% of total hepatic C3 production, the observation of enhanced secretion following exposure to endotoxin suggests that C3 release by these cells may play an important role in the local intrahepatic host response to bacterial toxins.

In order to characterize the extracellular matrix of the space of Disse in human liver, a panel of affinity-purified polyclonal antibodies to a wide range of matrix components was used for immunolocalization of the proteins by light microscopy and an ultracryomicrotomy/ immuno-gold labelling method was applied for their identification at the ultrastructural level in liver biopsy specimens. The normal space of Disse was found to contain several types of interstitial collagen (types I, III and V), a "minor" collagen (type VI), fibronectin, undulin and vitronectin. In spite of the paucity of a recognisable basement membrane at this site, the basement membrane components collagen type IV and laminin could also be identified. Increased immunolabelling for fibronectin was noted in the space of Disse in material from patients with acute liver disease. By contrast, in various forms of chronic (fibrotic) liver disease increased labelling was noted for interstitial collagens (types I and III) and the basement membrane proteins collagen type IV and laminin. Such changes in the extracellular matrix of the space of Disse may adversely affect parenchymal cell function by altering normal cell-matrix interactions and may perpetuate liver injury by interfering with the transport of nutrients between the sinusoidal blood flow and parenchymal cells.

Two methods were used to examine the role of sinusoidal cells in the synthesis of extracellular matrix proteins. First, immunohistochemistry was used to identify intracellular

proteins by light microscopy and at the ultrastructural level. Second, *in vitro* biosynthesis of matrix proteins by isolated rat liver sinusoidal cells was studied using [^{3}H]-proline incorporation and SDS-PAGE analysis. Evidence is presented which suggests that fat-storing cells are a major source of such proteins; these cells may therefore be important in hepatic fibrogenesis. In order to study the response of these cells *in vivo* to various forms of experimental liver injury, a method for their immunolocalization in rat liver using monoclonal antibodies to the intermediate filament protein desmin was developed. This method was used to monitor the response of fat-storing cells to two forms of experimental injury: acute carbon tetrachloride-induced damage and chronic cholestatic injury (common bile duct ligation). In both models, desmin-positive fat-storing cells accumulated in areas of parenchymal cell damage. A novel double-labelling method was used to demonstrate that expansion of the desmin-positive fat-storing cell population was, at least in part, due to local cell proliferation. Furthermore, in both models evidence of phenotypic modulation in these cells towards that of myofibroblasts was found by demonstration of expression of the α-(smooth muscle) isoform of actin. Thus, in response to experimental liver injury in rats, fat-storing cells proliferate and become activated towards myofibroblasts. Preliminary studies in human liver disease using PR2D3 as a marker for fat-storing cells are described.

A novel immunohistochemical approach was used for the identification and characterization of intra-sinusoidal nerves. Adrenergic nerves were identified in guinea-pig and rat liver using antibodies to tyrosine hydroxylase and dopamine β hydroxylase, enzymes essential for the biosynthesis of noradrenaline. In addition, fibres containing neuropeptide tyrosine (NPY) and its C-flanking peptide (C-PON) were immunolocalized; these regulatory peptides are frequently associated with adrenergic nerves. Finally, intrahepatic afferent sensory nerves were identified using antibodies to calcitonin gene-related peptide (CGRP) and substance P. These immuno-histochemical studies, in conjunction with chemical denervation experiments, indicated that abundant adrenergic and NPY-ergic fibres are present within portal tracts in both species. In guinea-pig liver but not rat liver however, abundant adrenergic/NPY-ergic fibres were also found within sinusoidal walls. It is proposed that such fibres may directly innervate parenchymal cells, fat-storing cells and sinusoidal endothelial cells; such nerve-cell interactions may play important roles in the control of intermediary metabolism and intra-sinusoidal blood flow.

CGRP- and substance P-containing nerves were also observed in portal tracts but in neither species was there any evidence of sensory innervation of the sinusoids.

Intra-sinusoidal nerve fibres were also identified in normal human liver using antibodies to a constitutive cytosolic neuronal protein (PGP 9.5) and a cytoplasmic protein of Schwann cells (S-100 protein). PGP 9.5-immunoreactive fibres were found throughout acinar zones I to III. These antibodies were also used to study ontogenesis of intrahepatic innervation and to investigate changes occurring to intrahepatic nerves in liver disease. In acute liver injury, intrasinusoidal fibres were identified with a normal distribution and density. However, in established cirrhosis intra-sinusoidal fibres were completely absent. This sinusoidal denervation may contribute to alterations of metabolic function and intrahepatic blood flow in advanced liver disease.

In the final chapter, possible interactions between different components of the sinusoids are discussed with particular reference to cell-cell interactions in the biosynthesis of complement and other plasma proteins and in the stimulation of proliferation and activation of fat-storing cells in response to injury. The importance of cell-matrix interactions and nerve-cell interactions in liver pathophysiology are discussed and the concept of a perisinusoidal functional unit is presented.

In conclusion, the studies presented in this thesis indicate that the hepatic sinusoids are structurally complex. Far from being inert, the components of the sinusoidal walls and the space of Disse are metabolically active and may contribute enormously to normal liver function. It is likely that they play an important role in the pathogenesis of many forms of liver disease.

CHAPTER 1. THE HEPATIC SINUSOIDS: AN INTRODUCTION

1.1 INTRODUCTION

Clinical and experimental hepatologists have traditionally regarded the liver as a homogeneous organ composed of a single cell type, the parenchymal cell. Although these cells account for over 70% of total liver volume (Blouin, 1977; Blouin *et al*, 1977) and are responsible for many of the biosynthetic and excretory capabilities of this metabolically active tissue, such an obsession with the parenchymal cell has resulted in comparative scientific neglect of other intrahepatic structures such as the liver capillaries. These vascular channels, termed sinusoids because of their tortuous course lie adjacent to, and run parallel with, the plates or cords of parenchymal cells. Blood flows through the sinusoids from portal vein and hepatic artery radicles, towards hepatic vein branches. Between the cells which line the sinusoidal wall and the adjacent parenchymal cells is the so-called space of Disse (Figure 1.1).

The sinusoids are easily overlooked in immersion-fixed tissue at the light microscope level; they are more readily visualized in tissue which has been perfusion-fixed at a physiological pressure (Wisse *et al*, 1984). Even in such optimally processed material, the cells of the sinusoid can be shown by morphometry to contibute less than 7% of total liver volume (Blouin *et al*, 1977). It is hardly surprising therefore that the sinusoids have been largely ignored by investigators of liver pathophysiology. Nevertheless, in recent years there has been a growing awareness that far from being inert blood channels, the sinusoids are highly complex and metabolically active, contributing significantly to normal hepatic function.

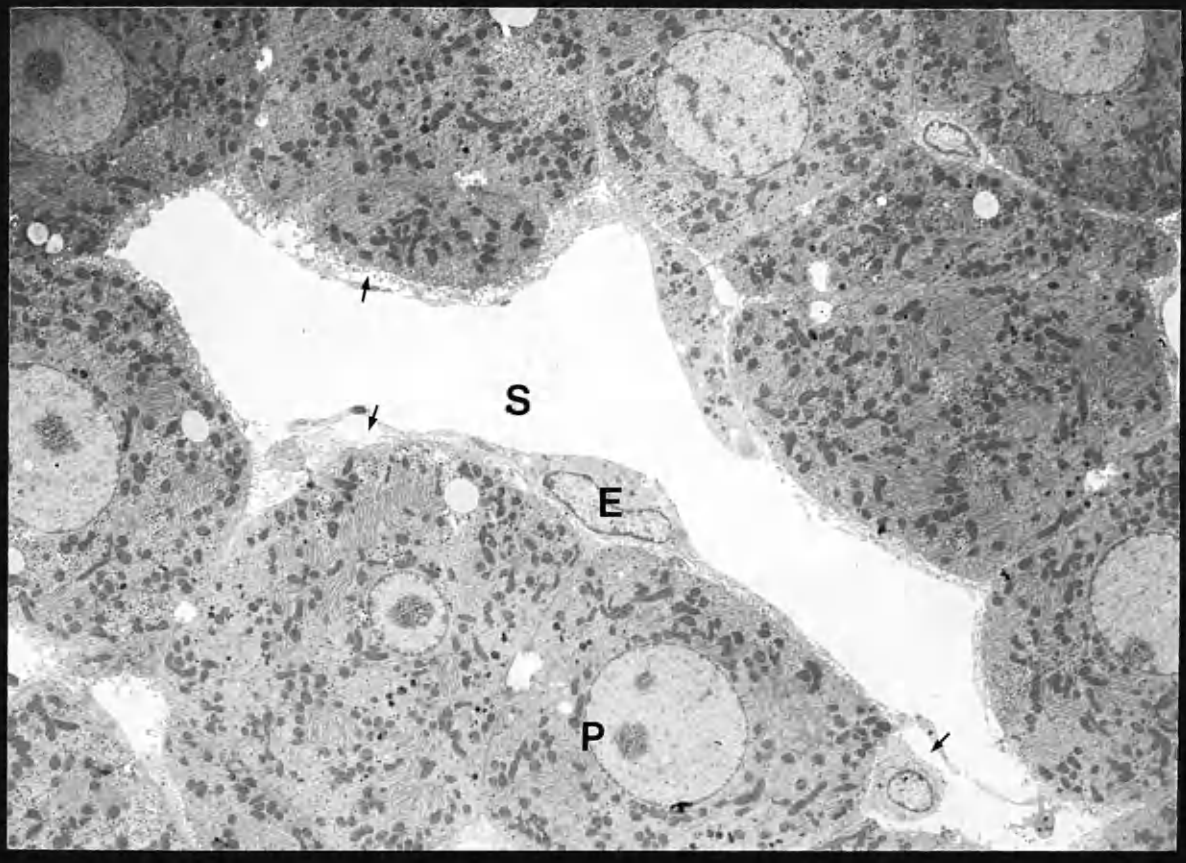
1.2 HISTORICAL PERSPECTIVES

Probably the first reference to the existence of sinusoidal lining cells was Wedl's description in 1854 of pigment-containing "connective tissue" cells in the liver from a patient with malaria (Aterman, 1977; 1986). It is almost certain that these were sinusoidal macrophages which had phagocytosed malarial pigment. Several years later, pioneering experimental biologists such as Hoffmann & Von Recklinghausen (1867) and Ponfick (1869) recognised that a population of non-parenchymal cells had the capacity to phagocytose intravenously administered exogenous agents including dyes such as cinnabar. Hoffmann & Von Recklinghausen (1867)

Figure 1.1 Transmission electron micrograph of perfusion-fixed normal rat liver.

The liver of a 250g male Wistar rat was perfused through a cannula in the portal vein with a solution containing 1.8% glutaraldehyde, 0.067 M cacodylate (pH 7.4) and 1% sucrose (310 m Osmol) at a flow rate of approximately 10ml/min and with a perfusion pressure of 10cm H_2O.

The sinusoids (S) can be seen to be lined by fenestrated endothelial cells (E). Between these cells and the cords of parenchymal cells (P) is the so-called space of Disse (arrows). (Uranyl acetate/lead citrate; x 3,900)


S
E
P

further demonstrated that cinnabar-containing cells often simultaneously contained red blood cells, suggesting that they may be involved in phagocytosis of endogenous as well as exogenous particles. Several morphologists concurrently provided further descriptive accounts of "connective tissue" cells of the liver (Wagner, 1860; Kölliker, 1867). Boll (1869) noted the existence of a "network of anastomosing areolar connective tissue cells" containing osmiophilic material which he interpreted as being either "pigment or of fatty nature". Seven years later, Kupffer detected a population of stellate-shaped sinusoidal cells ("Sternzellen") (Kupffer, 1876) using a gold chloride histochemical method. These cells were in contact with the hepatic "blood capillaries" and were closely applied to parenchymal cells. Although he initially regarded these as being perivascular connective tissue cells, he subsequently suggested that they were identical to those capable of phagocytosing India ink (Kupffer 1898; 1899). It is now clear that this was a misinterpretation as the gold chloride method detects a non-phagocytic cell population referred to as fat-storing cells (vide infra) (Wake, 1971, 1980). In spite of this misunderstanding, the principal phagocytic cells or macrophages of the liver sinusoids are now widely referred to as Kupffer cells.[1]

Development of vital staining methods with the introduction of synthetic dyes such as phyroll blue and trypan blue, permitted the identification of further sites at which phagocytic cells were present including spleen, lymph nodes and bone marrow. On the basis of these observations, Aschoff (1924) formulated the concept of a "retikulo-endotheliales system". This was considered to be composed of (i) reticular cells of lymphoid organs, (ii) all sinusoidal lining cells of the liver, spleen and bone marrow, (iii) histiocytes of the connective tissue, and (iv) circulating monocytes. This paradigm was widely accepted; all cells lining the hepatic sinusoids were seen as belonging to a common lineage.

At that time however, Zimmermann (1923, 1928) in a series of careful morphological studies demonstrated three distinct cell types in and around the hepatic sinusoids: (i) endothelial cells, which were elongated flattened cells, (ii) "endocytes", which projected into the sinusoidal lumen, and (iii) pericytes, which surrounded the sinusoids. Zimmermann believed the three cell

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[1]In a series of erudite reviews, Aterman (1977, 1986) has argued that for historical accuracy they should be referred to as Wedl-Wagner cells!

types to be distinct cell populations, a view which was disputed by proponents of Aschoff's reticulo-endothelial concept (Aterman, 1977).

In the early 1950's Ito and co-workers (Ito & Nemoto, 1952) reported the existence of a "new" sinusoidal cell which was apparently perivascular and which contained cytoplasmic fat droplets; these were considered to be distinct from Kupffer cells. In retrospect, it is clear that rather than describing a novel cell type, Ito was merely rediscovering cells identified as early as 1869 by Boll. It seems likely that Ito's cells also corresponded to Zimmmermann's pericytes.

In spite of these observations, the notion that the sinusoids were lined by a single cell type (referred to as the Kupffer cell or reticuloendothelial cell) persisted in standard textbooks of histology until the late 1960's (Aterman, 1963; Bloom & Fawcett, 1968; Ham, 1969). Based on observations made in fetal liver, it was also believed that the sinusoidal lining cells were derived from a primitive stem cell which was capable of differentiating towards haemopoietic cells. To quote Ham (1969): "the primitive cell of the sinusoidal lining can differentiate along two main lines of differentiation that are thought to occur in developing haemopoietic tissues ..... (1) free cells (the precursors of blood cells) and (2) phagocytic reticuloendothelial cells".

A more detailed characterization of the cellular components of the hepatic sinusoids was made possible only with the advent of electron microscopy. When this was combined with the use of enzyme histochemistry and intravenous particle injections, it was firmly established that rather than being composed of one cell type, the sinusoids contained four distinct cell populations each with their own characteristic morphology, topography, and population dynamics: (i) Kupffer cells, (ii) sinusoidal endothelial cells, (iii) fat-storing cells and (iv) pit cells. Electron microscopy also provided information on the possible function of the different cell types. However, our understanding of the functional diversity of sinusoidal cells was greatly enhanced by the development of methods for the isolation and culture of the sinusoidal cells. Before considering the morphological features and principal functions of the cellular components of the sinusoids, methods previously used for their study will be reviewed.

1.3 METHODS USED IN THE STUDY OF SINUSOIDAL CELLS

1.3.1 Electron microscopy

The enhanced optical resolution afforded by transmission electron microscopy allowed investigators to obtain more detailed images of the sinusoidal cells than could be achieved using conventional light microscopy. Although early studies dealt principally with the ultrastructure of the parenchymal cells and biliary epithelium (Fawcett, 1955; Rouiller, 1956; Novikoff & Essner, 1960), they provided preliminary observations on the fine structure of the sinusoids. Hampton (1958) was probably the first to apply electron microscopy to study the phagocytic properties of intrahepatic cells in an investigation of the uptake of colloidal mercuric sulphide and thorotrast. In the 1960's there was a profusion of literature (reviewed in Wisse, 1972) dealing with the electron microscopic appearances of hepatic sinusoids in a variety of species including rat (Burkel & Low, 1966), calf (Wood, 1963), goat (Kuhn & Olivier, 1965) and human (Schaffner *et al*, 1963). Many of the early studies reinforced the belief that the endothelial cells and the Kupffer cells simply represented different forms of a single cell type; endothelial cells were commonly considered to represent the quiescent precursor stage of the Kupffer cell. This was based on the apparent observation of transitional stages between the two cell types. Schaffner *et al* (1963) were unable to identify any such transitions in human liver and considered that they may represent different cell populations. However, they added a further complication by suggesting that there may be transitional forms between the Kupffer cells and "plasmacytoid cells". It can be concluded that these early studies added little to our understanding of the nature of and relationship between the sinusoidal cells.

The clear distinction between different sinusoidal cell types was due largely to the work of Wisse (1970, 1972, 1974a, 1974b) and Fahimi (1970). The experimental approach adopted by these investigators had two advantages over those used in the earlier studies: (i) careful attention was paid to the preparation of the tissue (in particular with respect to fixation), and (ii) other methods such as enzyme histochemistry were combined with transmission electron microscopy. Fahimi (1967) showed that fixation of liver tissue by perfusion rather than the traditional method of immersion, resulted in substantial improvement in the preservation of

ultrastructural detail within the sinusoids. Wisse and co-workers subsequently defined the optimal conditions for fixation of rat liver for transmission and scanning electron microscopy of the sinusoids. Stress-free animals are anaesthetized (with rapid induction) and the portal vein is cannulated. The superior vena cava is transected and the liver is perfused with isotonic buffer followed by 1.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4; osmolality: 310 mOsmol) at room temperature. A perfusion pressure of 10 cm H_2O is used to maintain flow at a physiological pressure during the fixation procedure. This method not only results in better preservation of intracellular structures, but maintains the spatial relationship between different sinusoidal cells. Wisse (1970, 1972, 1974a, 1974b) used this technique in a series of meticulous studies of rat liver sinusoids. On the basis of morphological features, he established that the sinusoids were principally lined by two distinct cell types: (i) flattened cells which had fenestrated cytoplasm (endothelial cells) and (ii) larger cells with surface microvilli and abundant lysosomes (Kupffer cells); he could not identify any transitional cell types nor could he detect any specialized junctions between these cells. Application of the perfusion-fixation method also permitted a more detailed investigation of Ito's lipid-containing cells within the space of Disse (fat-storing cells). Finally, this approach enabled Wisse to later identify a fourth sinusoidal cell, the pit cell (Wisse *et al*, 1976).

That Kupffer cells and endothelial cells were morphologically and functionally distinct was further confirmed when perfusion-fixed tissue was used to study endogenous peroxidase activity. The larger, lysosome-rich Kupffer cells were found to exhibit peroxidase activity within their endoplasmic reticulum, nuclear envelope and annulate lamellae, whereas the flattened and fenestrated endothelial cells were entirely negative for this enzyme. Furthermore, when material was studied from animals which had been injected intravenously with 0.8 μm latex beads one hour prior to death, it was found that the peroxidase-positive cells were actively phagocytic for these particles while the peroxidase-negative cells did not exhibit this property (Wisse, 1974 a and b).[1]

[1]Subsequent studies have indicated that under certain circumstances the peroxidase-negative cells can take up these particles (Steffan *et al*, 1986) and that endothelial cells in fact possess a strong endocytotic capacity (see 1.5.3).

The low-pressure perfusion method developed by Wisse's group also allowed investigation of the surfaces of sinusoidal cells by scanning electron microscopy using freeze-fracture, critical point drying and gold sputtering (Motta, 1975). While providing useful information on the nature of the endothelial fenestrae (Wisse *et al*, 1985), this approach has probably contributed less to our understanding of the nature of the different cell types than transmission electron microscopy.

The observations of Wisse and Fahimi were subsequently confirmed by numerous other investigators. Thus, by the late 1970's it was clearly established, and widely accepted, that the liver sinusoids contained four cell types.

1.3.2 Isolation and culture of sinusoidal cells

The earliest attempts at isolation of sinusoidal cells involved loading the phagocytic (Kupffer) cells *in vivo* with iron particles, washing the cells out of the liver and isolating them from the resultant cell suspension using a magnet (Rous & Beard, 1934). Although the method was subsequently refined (Jacob & Bhargava, 1962), it provided poor yields of viable cells. Howard *et al* (1967) introduced the use of collagenase for the isolation of parenchymal cells from rat liver. This served to degrade the extracellular matrix of the liver, producing a cell suspension of parenchymal and sinusoidal cells. Berry and Friend (1969) later modified this method using the enzymes as a perfusate; parenchymal cells were purified from the resultant cell suspension in high yield and viability by low speed centrifugation. Mills & Zucker-Franklin (1969) used pronase in place of collagenase and discovered that this enzyme selectively destroyed parenchymal cells; perfusion of the liver with pronase therefore produced a crude suspension of non-parenchymal cells. Kupffer cells could be purified from this by virtue of their capacity to bind avidly to the plastic of tissue culture dishes (Munthe-Kaas *et al*, 1975; Emeis & Planqué, 1976). However, although this method provided relatively high yields of Kupffer cells, it was not possible to isolate sinusoidal endothelial cells. Furthermore, during the stage of dish adherence Kupffer cells were found to take up considerable amounts of cell debris; this may affect their functional activities. Such problems were overcome by the method described by Knook & Sleyster (1976). Following perfusion with pronase and collagenase, the resultant cell

suspension was centrifuged through a density gradient (Metrizimide). This removed contaminating parenchymal cell debris, fat-storing cells and pit cells and resulted in a fraction containing Kupffer cells and endothelial cells which were in turn separated by virtue of volume and density by the method of centrifugal elutriation. Although there have been subsequent minor modifications to the original protocol to enhance the yield and viability of isolated cells (Irving *et al*, 1984; Knook *et al*, 1986), this method is still widely used for the isolation and purification of Kupffer cells and endothelial cells. While the technique has principally been applied to studies of rat liver sinusoidal cells, several groups have demonstrated that it can be used to isolate the cells from a variety of species including mouse (Kirn *et al*, 1982), guinea-pig (Shaw *et al*, 1984) and human (Steffan *et al*, 1981; Brouwer *et al*, 1988a). For cell surface receptor studies, a collagenase-only method has been used (Nagelkerke *et al*, 1982) limiting the enzyme degradation of cell membrane proteins on isolated cells. For endocytosis experiments, a cold pronase method (perfused at 8 - 10°C) has also been described (Praaning-Van Dalen & Knook, 1982).

Knook *et al* (1982) showed that a similar approach could be used to obtain isolates of fat-storing cells from rat liver. This group subsequently described a shorter purification procedure by which fat-storing cells could be obtained using density gradient centrifugation alone using either Metrizimide or Nycodenz. Although the cell yield was only half that of the original method, this approach obviated the need for centrifugal elutriation, providing a cell fraction which was approximately 80% pure. An alternative method, using Stractan in place of Nycodenz (Friedmann & Roll, 1987) was reported to provide better cell yields. Similar methods have also been used to isolate fat-storing cells from mouse (Chen *et al*, 1989a and b) and human liver (Rockey *et al*, in press).

A method for the isolation of pit cells from rat liver was described by Bouwens *et al* (1987). This involves sinusoidal lavage where the liver is perfused at supra-physiological pressure (50cm H_2O). The pit cells, which are less adherent than the other sinusoidal cell types, are then purified from the perfusate on a Percoll (45%/47.5%) density gradient providing a fraction with a purity of 90%.

With the exception of pit cells, all isolated sinusoidal cells can be maintained in primary culture. Fat-storing cells proliferate *in vitro* and can be serially passaged. Optimal conditions

for sinusoidal cell culture were reviewed by Van Bossuyt *et al* (1988a) and are discussed further in Chapters 2 and 4.

1.3.3 *In vivo* microscopy

Another experimental approach which has yielded important data is *in vivo* microscopy. This method permits the observation of living cells in the intact organ of an anaethetized animal with an undisturbed microcirculation, at a magnification and resolution comparable to normal light microscopy of sectioned material (McCuskey, 1981) The livers of anaethetized animals[1] are exteriorised at laparotomy and transilluminated on a special microscope stage using a high pressure Xenon lamp. The use of a water immersion objective lens provides clear images which can be transmitted to a TV monitor or recorded on video tape. Modifications to the apparatus can provide epifluorescence to follow dynamic processes such as endocytosis of fluorescent particles injected into the circulation of the animal.

The principal application of *in vivo* microscopy has been in the study of the response of the sinusoidal blood flow to vasoactive substances such as adrenergic (Reilly *et al*, 1981) and cholinergic (Reilly *et al*, 1982) compounds. In addition, it has been used to study micro-circulatory changes occuring within the sinusoids following exposure of animals to various toxic substances such as alcohol (Rappaport *et al*, 1970), murine hepatitis virus (Bloch *et al*, 1975) and bacterial endotoxin (McCuskey *et al*, 1982), following experimental procedures such as portacaval anastomosis (McCuskey *et al*, 1983) or induction of circulatory shock (Koo & Liang, 1977).

Data provided by this technique on the contractility of sinusoidal walls and the responses to vasoactive compounds could not have been generated using conventional cell biological techniques. Its use however, has been limited by the restriction that only sinusoids in the thin, outermost regions of the liver lobes can be visualized; the local microanatomy may differ at this site from that elsewhere in the liver.

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[1]Most published studies have used rats, although the livers of several other species including frogs, cat, guinea pig and dog have also been used.

1.4 KUPFFER CELLS

1.4.1 Morphology

Although Kupffer cells may have a variable shape and position within the sinusoid, they are generally stellate in outline and lie on, or are juxtaposed between, the sinusoidal endothelial cells; their cell bodies protrude into the sinusoidal lumen (Figures 1.2 and 1.3). They have an irregular surface due to the presence of numerous microvilli. By transmission electron microscopy, short cytoplasmic processes can often be seen extending along the sinusoidal wall; these extensions may penetrate the fenestrae of underlying endothelial cells (vide infra). Although Kupffer cells and endothelial cells are thus in close apposition, no specialized junctions between the cells have ever been described. Rarely, Kupffer cells may be found within the space of Disse; contact with fat-storing cells and pit cells has also been described (Gendrault *et al*, 1988).

A substantial part of the Kupffer cell surface is exposed to the sinusoidal lumen allowing direct interaction with cellular, particulate and soluble components of the circulating blood. Several authors have described a so-called fuzzy coat over the cell surface (Wisse 1974a; 1974b; 1977); this coat, which is about 700 Å in width, can only be observed using certain methods of tissue preparation (eg. osmium tetroxide primary fixation). Although the precise nature of this structure is uncertain, it is thought to be involved in pinocytosis; it has not been observed on the surface of other sinusoidal cells. Another organelle peculiar to the Kupffer cell, the "worm-like structure" (micropinocytosis vermicularis) may represent invagination of the fuzzy coat together with cell membrane. The cytoplasm contains several other organelles believed to participate in pinocytosis and phagocytosis; these include different forms of lysosomes, pinocytotic vesicles and large vacuoles. The rough endoplasmic reticulum is generally well developed as is the Golgi apparatus, although a recognisable smooth endoplasmic reticulum is absent. Wisse (1977) has drawn attention to a structure closely associated with the rough endoplasmic reticulum, the so-called annulate lamellae. Although this organelle has been described in cells from a wide range of extrahepatic tissues, within the sinusoids it is restricted to Kupffer cells. It is composed of parallel arrays of intracytoplasmic membranes in close apposition to the

Figure 1.2 Enzyme histochemistry/transmission electron microscopy : perfusion-fixed normal rat liver.

This electron micrograph illustrates the typical position of Kupffer cells (K), lying on top of the thin, fenestrated cytoplasm of sinusoidal endothelial cells (E); microvilli protrude into the sinusoidal lumen. A fat-storing cell (F) can be seen lying within the space of Disse and in a recess between parenchymal cells. In this preparation, endogenous peroxidase activity has been demonstrated using the method outlined in Appendix 1. Enzyme activity is identified within the Kupffer cell but not in the endothelial cell or fat-storing cell. (Modified Graham and Karnovsky's method; uranyl acetate/lead citrate; x 5200)

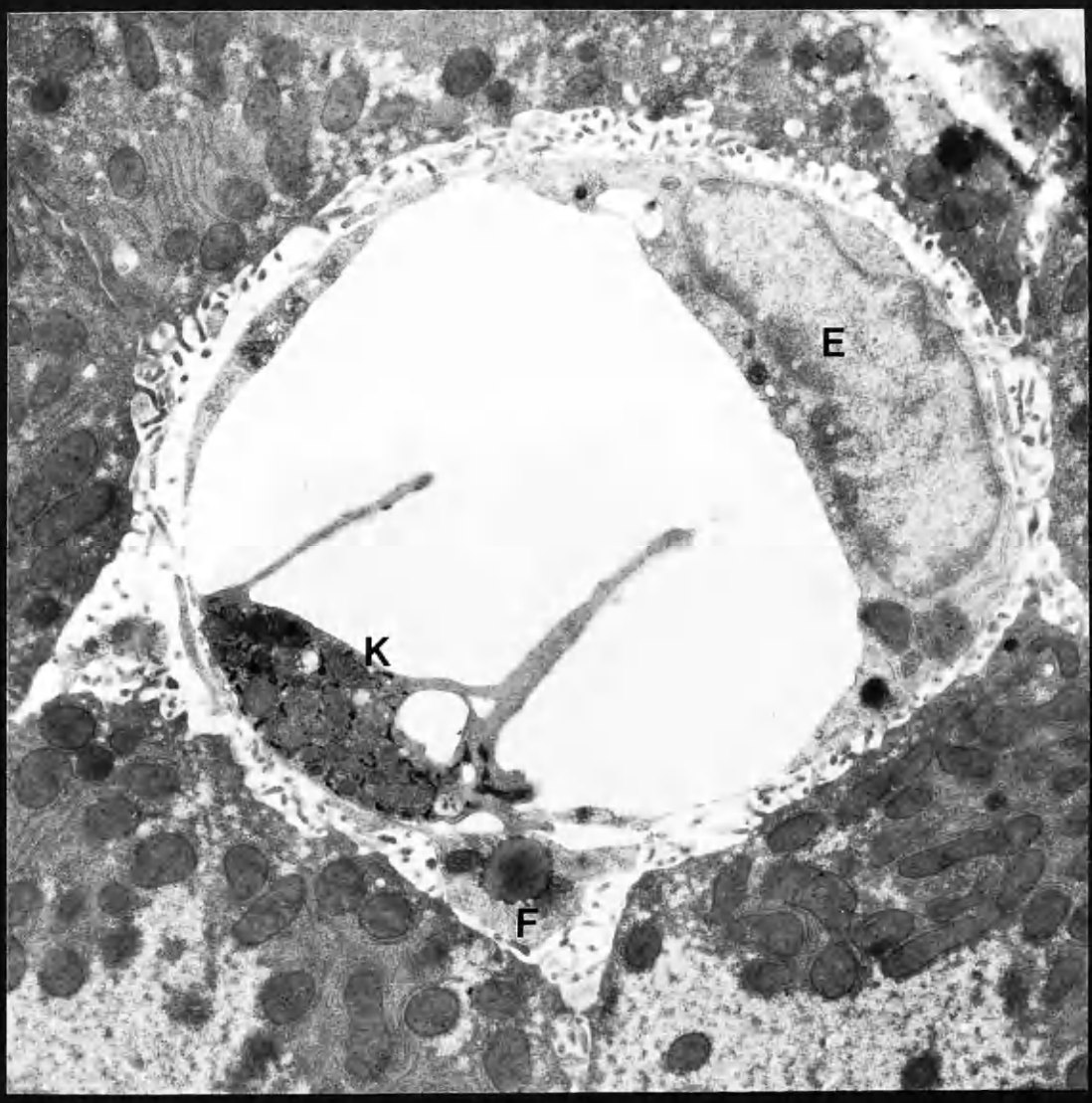
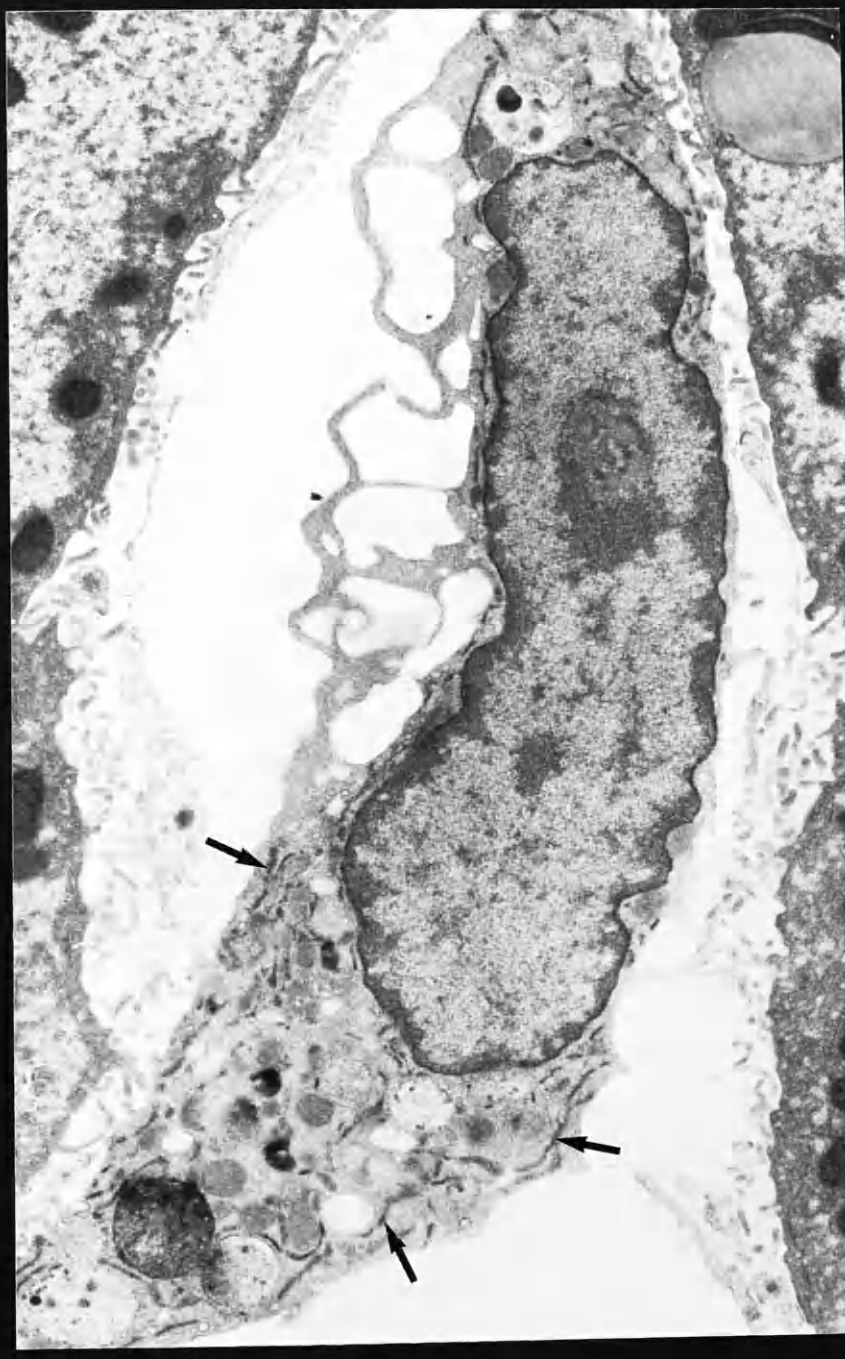

E
K
F

Figure 1.3 Enzyme histochemistry/transmission electron micrograph of Kupffer cell in normal rat liver.

As in Figure 1.2, this preparation demonstrates endogenous peroxidase activity. It is identified in the endoplasmic reticulum (arrows) and in the nuclear envelope. This distribution of enzyme activity differs from that seen in monocytes where it is limited to lysosomes. (Modified Graham and Karnovsky's method; uranyl acetate/lead citrate; x 6,100)



nuclear envelope. Although its precise function remains unclear, it is thought to play a role in protein synthesis.

Kupffer cells are not uniformly distributed throughout the hepatic acini. Sleyster & Knook (1982) found a ratio of 4:3:2 between numbers of Kupffer cells in acinar zone 1 (periportal), acinar zone 2 (midzone) and acinar zone 3 (perivenular) of normal rat liver. Although slightly different ratios have been obtained by other authors (Bouwens *et al.*, 1986a) there is general agreement that they are most abundant in periportal zones. There is also evidence for functional heterogeneity of Kupffer cells. Sleyster & Knook (1982) showed that periportal Kupffer cells were larger than those in zones 2 and 3, exhibited higher lysosomal enzyme activities and were more actively phagocytic for 0.81 μm latex particles.

The most reliable method for the identification of Kupffer cells in rat liver is the histochemical demonstration of endogenous peroxidase activity. As discussed above, enzyme activity can be localized in Kupffer cells to the rough endoplasmic reticulum and nuclear envelope; other sinusoidal cells do not exhibit peroxidase activity (Figure 1.2). Unfortunately, this method cannot be applied to other species. In mouse liver for example, 60% of sinusoidal endothelial cells are also peroxidase-positive (Stöhr *et al.*, 1978). Zafrani *et al* (1982) found peroxidase activity in all Kupffer and sinusoidal endothelial cells in normal human liver.[1]

Other methods that have been used for the demonstration of Kupffer cells in tissue sections include identification of specific lectin binding and localization of cytoplasmic enzymes or membrane determinants using immunohistochemistry with monoclonal or polyclonal antibodies; some of these are applicable to human liver. McMillan *et al* (1984) demonstrated that by virtue of exposed α-D-galactose residues on cell membranes, Kupffer cells bind the lectin Bandieraea simplicifolia whereas other sinusoidal cells do not. Kupffer cells also contain the enzyme lysozyme (muramidase) (Mason & Taylor, 1975) (Figure 1.4a); immunolocalization of this protein has been used in quantitative studies of Kupffer cells in human liver diseases (Manifold *et al.*, 1982). However, Kelly *et al* (1989) have shown that some Kupffer cells may be negative for lysozyme and that this protein may be down-regulated in liver disease. In

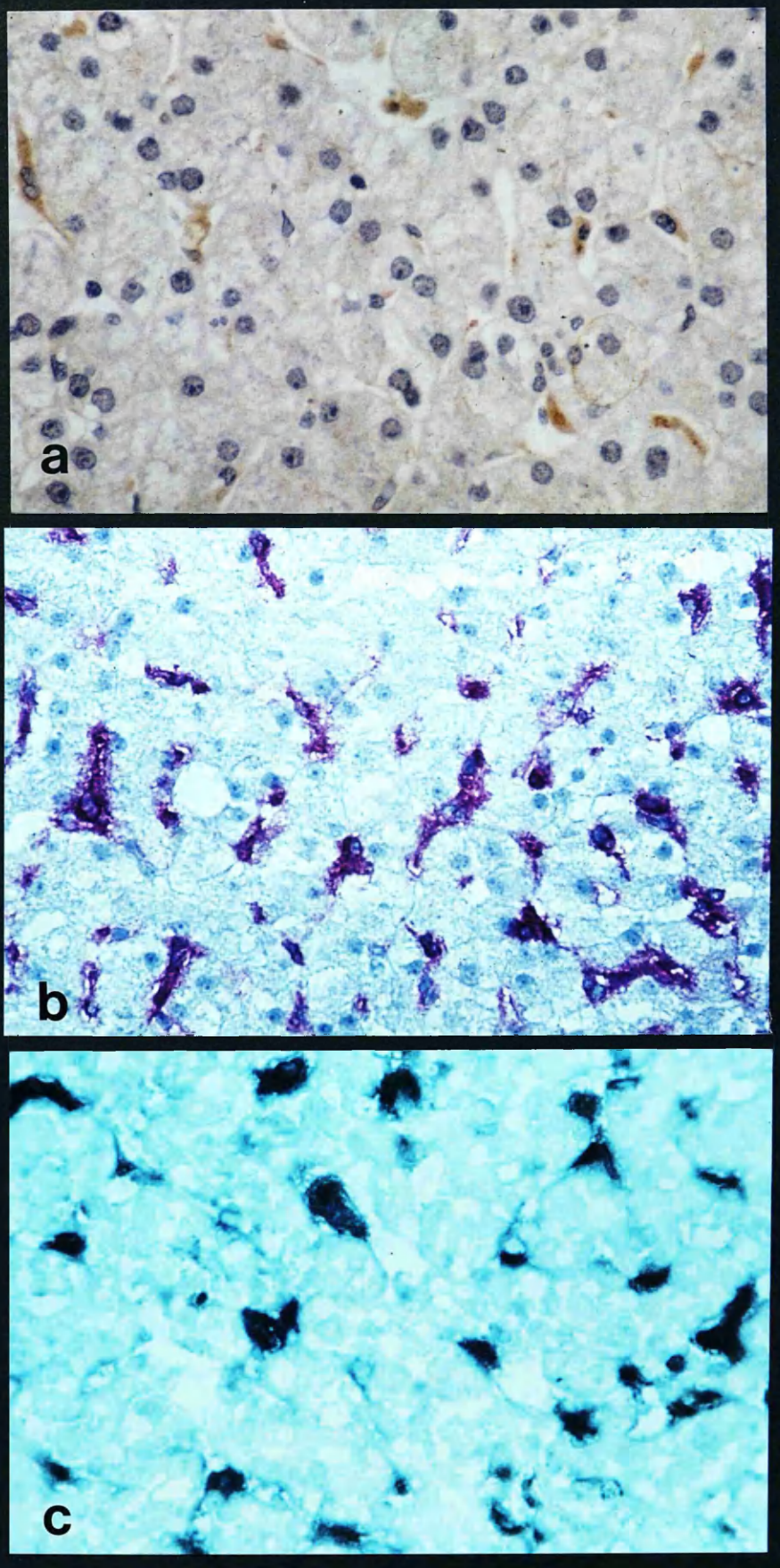
[1]By contrast, I have been unable to demonstrate any endogenous peroxidase activity in human liver sinusoidal cells using identical methods to those of Zafrani et al.

Figure 1.4 Expression of macrophage-associated proteins by human Kupffer cells.

a. Immunolocalization of lysozyme (muramidase) in a 3 μm dewaxed formalin-fixed section of normal human liver. Immunoreactivity (brown cytoplasmic staining) can be seen within some sinusoidal cells. (Polyclonal anti-lysozyme (Dako; 1:400); peroxidase-antiperoxidase method; haematoxylin counterstain; x 450)

b. Immunohistochemical staining using the monoclonal antibody Leu M5 on a 5 μm cryostat section of normal human liver. This antibody recognizes an epitope expressed by cells of the mononuclear phagocyte system. (Leu M5 (Becton Dickinson, 1:10); immuno-alkaline phosphatase method (Appendix 3); haematoxylin counterstain; x 450)

c. Immunoreactivity for the cluster differentiation antigen CD68. A greater number of sinusoidal cells can be seen to be immunoreactive for this than with anti-lysozyme. (Monoclonal antibody EBM/11 (Dako; 1:100); indirect immunoperoxidase method (Appendix 4) with Nickel/cobalt enhancement (Appendix 5) on 4μm cryostat section; fast green counterstain; x 550).


a
b
c

common with macrophages at other sites, Kupffer cells also synthesize the proteinase inhibitors α-1-antitrypsin (Theaker & Fleming, 1986) and α-1-antichymotrypsin; immunolocalization of these proteins can also be used for the identification of Kupffer cells as they are not found within other sinusoidal cell types although the former is synthesized by parenchymal cells. Monoclonal antibodies which detect cell surface determinants on macrophages can also be used. Although many of these antibodies (eg. Leu M3, Leu M5) require to be used on unfixed cryostat sections (Figure 1.4b), in recent studies I have demonstrated that the monoclonal antibody EBM11, which recognises the macrophage-associated cluster differentiation antigen CD68 (Figure 1.4c), can be used with paraffin-embedded, paraformaldehyde-fixed human liver tissue (Ahmed *et al*, 1991)); this is in keeping with the experience of Greywoode *et al* (1990). Monoclonal antibodies reacting with rat macrophage-specific membrane proteins have also been generated (Dijkstra *et al.*, 1985; Sugihara *et al.*, 1990); some of these such as ED1, ED2, and ED3 have now been used in quantitiative studies dealing with the response of Kupffer cells to experimental liver injury (Jonker *et al*, 1990). The monoclonal antibody KCA-1 appears to detect an epitope on an antigen expressed by Kupffer cells but not other tissue macrophages (Sugihara *et al.*, 1990).

1.4.2 Origin, ontogeny and population kinetics

The origin of Kupffer cells has been the subject of long (and sometimes acrimonious!) debate (Wisse, 1980; Van Furth, 1980). Proponents of the concept of a mononuclear phagocyte system (Van Furth *et al.*, 1975) believe that Kupffer cells are non-dividing end cells derived from circulating monocytes (Crofton *et al.*, 1978). Others believe Kupffer cells to be capable of local proliferation, representing a self-renewing macrophage population (Volkman, 1976).

Evidence in favour of a monocyte derivation has been provided by observations in (i) bone marrow transplants in sublethally irradiated animals (Shand & Bell, 1972; Freundenberg *et al.*, 1986) and in human subjects (Gale *et al.*, 1978), and (ii) human (Porter, 1969) and rat orthotopic liver transplantation (Kaneda *et al.*, 1989). In animal models it has been demonstrated that several weeks after bone marrow transplantation, Kupffer cells in the recipient

liver are of donor origin as defined by sex chromatin pattern, chromosomal markers or Northern blot analysis (Paradis *et al.*, 1989).

Those who support a self-replicating model of Kupffer cell steady state kinetics have drawn attention to the (albeit infrequent) identification of mitotic figures in cells with morphological and histochemical features of Kupffer cells (Wisse, 1974b). Bouwens *et al* (1986) have shown a slow turnover in normal liver with a mitotic index of 0.06% in stathmokinetic experiments. Probably the strongest argument against a monocytic derivation is the lack of identifiable transitional forms between those of monocytes and Kupffer cells. Although both cells exhibit intracellular endogenous peroxidase activity, the distribution of this in histochemical preparations is distinct. Unlike Kupffer cells, monocytes show peroxidase activity only in lysosomes and not in endoplasmic reticulum or nuclear envelope. In spite of extensive ultrastructural investigations of Kupffer cell-monocyte relationships (Wisse, 1974a; Bouwens *et al.*, 1984; Bouwens & Wisse, 1985) there are only three reports claiming the existence of cells with histochemical features intermediate between Kupffer cells and monocytes (Deimann & Fahimi, 1979; Kaneda *et al.*, 1989; Ukai *et al.*, 1990). Several authors have also demonstrated that in rat liver a population of sinusoidal macrophages with histochemical features of Kupffer cells is established by day 11 of gestation (Naito & Wisse, 1977; Pino & Bankston, 1979; Bankston & Pino, 1980); this ante dates the formation of bone marrow or the appearance of circulating monocytes by several days.

Further support for the local proliferation concept was provided by the carefully executed cell kinetic experiments of Bouwens and co-workers in normal rat liver (Bouwens *et al*, 1986a) and in the livers of animals following (i) stimulation of hepatic macrophages by zymosan and, (ii) partial hepatectomy with or without partial body irradiation (Bouwens *et al.*, 1984; Bouwens & Wisse, 1985; Bouwens *et al.*, 1986b & c). These studies established beyond doubt that Kupffer cells were at least capable of local replication. Bouwens *et al* (1984) calculated that 75% of the Kupffer cell population expansion seen following zymosan administration could be accounted for by local proliferation. This clearly means however, that there is an additional component derived from extrahepatic sources. These data have led Wake *et al* (1990) to conclude that Kupffer cells may have a dual origin, although the predominant mechanism in the steady state and in response to most forms of injury is one of local proliferation. It remains

to be established whether cells derived from local proliferation represent a functionally distinct subset of Kupffer cells from those derived from extrahepatic recruitment.

Finally, it should be noted that Kupffer cells are not static within the sinusoids but are capable of migration. Zajicek *et al* (1988), who proposed the concept of a "streaming liver", used [^{3}H]-thymidine labelling to demonstrate progressive movement of Kupffer cells from periportal zones towards hepatic vein radicles; they estimated that this process occurred at a rate of 2μm per day in normal rat liver. Hardonk *et al* (1986) have also demonstrated migratory capability for Kupffer cells using carbon and colloidal gold labelling. In contrast to Zajicek *et al* (1988) they suggested that the flow is in the opposite direction (ie. towards portal tracts). They also demonstrated that Kupffer cells labelled by low dose carbon administered via the portal vein could subsequently be identified within hilar lymph nodes.

1.4.3 Functions

Endocytosis

The ability of Kupffer cells to take up particulate matter was established in the early intravenous dye administration studies referred to above (Ponfick, 1869). The capacity of these cells for clearance of a wide range of exogenous substances was further demonstrated in numerous *in vivo* studies including some in which the phagocytic function of Kupffer cells was inhibited (so-called reticuloendothelial blockade) (Dinsdale *et al.*, 1981). Kupffer cells are involved in the endocytosis of many exogenous and endogenous substances including effete red blood cells, denatured proteins, hormones, immune complexes, glycoproteins and infectious agents (Table 1.1). These cells can also clear several types of pathogen including viruses (Kirn *et al*, 1982b), fungi such as Candida albicans (Schwocho & Moon, 1981) and protozoa such as malaria (Meis *et al*, 1982).

Kupffer cells provide the first line of defence against gut-derived foreign material in the portal venous system. In this respect one of their most important functions is the uptake and detoxification of endotoxin or bacterial lipopolysaccharide, a product of gram negative bacteria (Ruiter *et al*, 1981; Van Bossuyt & Wisse, 1988; Van Bossuyt *et al*, 1988b, 1989).

Senescent erythrocytes	Khansari & Fudenberg (1983)
Altered platelets	Kaplan & Saba (1978)
Endotoxin	Van Bossuyt *et al* (1988a & b, 1989)
Immune complexes	Rifai & Mannik (1984)
Cell membranes and organelles	Rieder & Decker (1984)
Hormones (Parathormone, growth hormone)	Segre *et al* (1981) Kover & Moore (1984)
Denatured albumin	Brouwer *et al* (1980)
Fibrin-fibrinogen	Sherman *et al* (1975)
Enzymes released from damaged cells	De Jong *et al* (1982) Bijsterbosch *et al* (1983)
Thrombin	Oka *et al* (1983)
Glycoproteins with exposed mannose or galactose residue	Summerfield *et al* (1982)
Carcinoembryonic antigen	Toth *et al* (1985)
Viruses	Kirn *et al* (1982b)
Experimental test substances (latex particles, Thorotrast, colloidal gold)	Hardonk *et al* (1986)

Table 1.1 Examples of materials which may be endocytosed by Kupffer cells.

Although it is recognised that Kupffer cells have the capacity for fluid phase pinocytosis (Minniksona *et al*, 1980), a process which does not require contact with cell membranes for initiation, most macromolecules are taken up by absorptive endocytosis. This process consists of three basic steps: (i) binding of molecules or particulate matter to the Kupffer cell membrane, (ii) internalization by either micropinocytosis or phagocytosis and (iii) intracellular transport of endocytosed material. Endocytosed substances are transported mainly to the lysosomal compartment where the endocytic vesicles fuse with primary lysosomes in which degradation of the material occurs. Non-degradable substances can remain in the lysosome leading to the formation of the so-called residual body.

The initial stage of binding to the cell surface involves several receptors of variable specificity. Some receptors show a high degree of specificity for certain ligands; others are much less specific and can be regarded as general binding sites. Several Kupffer cell membrane receptors have been characterized (Praaning-Van Dalen *et al.*, 1982): (i) N-acetyl-D-galactosamine receptors, (ii) N-acetylglucosamine (mannose) receptors, (iii) Fc γ receptors, (iv) C3 receptors (CR1, CR3, CR4 (Hinglais *et al.*, 1989)), (v) receptors for insulin and glucagon, (vi) apolipoprotein B receptors, and (vii) receptors for infectious agents such as FV3 virus. In addition, it is recognised that particulate matter may bind to so-called foreign body receptors through non-specific anionic/cationic interactions.

Release of reactive oxygen metabolites and eicosanoids

One of the early events observed after phagocytosis is the so-called respiratory burst. Various agents have been used to induce this *in vitro* in Kupffer cells; these include immunomodulatory agents such as calcium ionophores and phagocytosable material such as Corynebacterium parvum, zymosan and glucan (reviewed by Jones & Summerfield, 1988). Oxygen is reduced by NADPH with the formation of the superoxide anion radical $O_2^{\cdot -}$. The large amount of NADPH required during phagocytosis is provided by a flux of glucose through the pentose phosphate pathway. In the presence of the enzyme superoxide dismutase, the superoxide anions are converted to hydrogen peroxide as follows:

$$2\ O_2^{\cdot -} + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

Hydroxyl radicals can in turn be formed via the Haber-Weiss reaction or the Fenton reaction. Although the reactive species generated through these pathways are important physiological mechanisms by which Kupffer cells destroy bacteria, there is evidence that their release following endocytosis may lead to concomitant parenchymal cell injury by peroxidation of cell membrane lipids (Arthur, 1988).

Several other biochemical events occur following phagocytosis. These include: (i) a rapid influx of calcium ions from the extracellular compartment, (ii) activation of phospholipase A_2 and (iii) synthesis and secretion of various arachidonic acid derivatives; Kupffer cells are the most potent producers of eicosanoids amongst the different liver cell types. The principal arachidonic acid metabolite released after activation (eg. following phagocytosis) is prostaglandin D_2 (PGD_2), although smaller quantities of PGE_2, $PGF_2\alpha$, thromboxane and prostacyclin (PGI_2) are also released. The effects of these mediators are complex but include stimulation of glycogenolysis by parenchymal cells and alterations in sinusoidal blood flow; some (eg. PGE_2) may be cytoprotective (Decker, 1989, 1990). Several peptide mediators (eg. tumour necrosis factor α and interleukin 1 and 6) are also released by activated Kupffer cells; these are discussed in greater detail in Chapter 9.

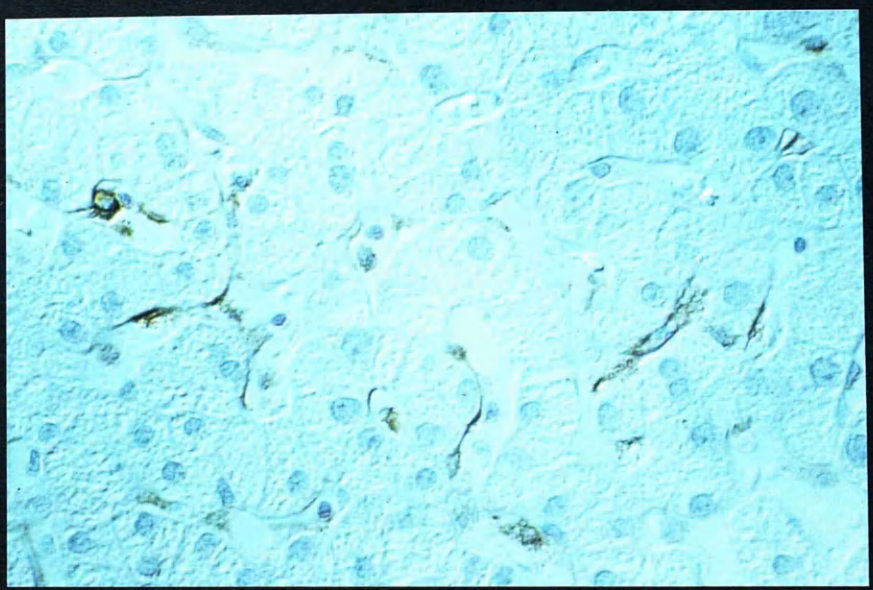
Antigen processing and presentation

Macrophages at other sites are known to play a crucial role in induction and regulation of immune responses (Nelson, 1981) through interactions with T lymphocyte subpopulations. The ability of Kupffer cells to function as antigen-presenting cells has been studied (Heil & Garvey, 1982). They have been shown to express Class II histocompatibility antigens (Ramadori *et al*, 1986; Barbatis *et al*, 1987), an essential property for antigen presentation (Figure 1.5). *In vitro* studies have demonstrated that they can function as antigen-presenting cells in immunological responses to antigens such as keyhole limpet haemocyanin and to allogeneic cells (Richman *et al*, 1979; Nadler *et al*, 1980) but they appear to be considerably less efficient than macrophages derived from other tissues (Rogoff & Lipsky, 1980, 1981). Their principal roles in the immune response would therefore appear to be (i) antigen sequestration by phagocytosis and (ii) clearance of immune complexes (Hopf *et al*., 1981; Rifai & Mannik, 1984).

Figure 1.5 Immunolocalization of Class II histocompatibility antigens (HLA DR) on sinusoidal cells in normal human liver.

In this preparation immunoreactive cells can be seen along sinusoidal walls. Many HLA DR-positive cells were found to be large and to protrude into the sinusoidal lumen; these are likely to be Kupffer cells. However, in keeping with the findings of Barbatis *et al* (1987) some cells with morphological features of sinusoidal endothelial cells also appear to express this molecule.

(Monoclonal antibody TAL 1B5 (Epenetos *et al*, 1985) (1:20); indirect immunoperoxidase method on 3μm dewaxed formalin-fixed section; haematoxylin counterstain; photographed under Nomarski filters; x 380).



Iron metabolism

Clearance of senescent erythrocytes is another important function of Kupffer cells. Intracellular degradation of erythrocytes occurs within the lysosomal compartment and leads to release of iron from haemoglobin (Young & Aisen, 1988). At least some of the ferrous ions become bound to transferrin for export from Kupffer cells and transport within the circulation. However *in vitro* studies have also demonstrated that iron-rich ferritin may be released by Kupffer cells and taken up by parenchymal cells via a specific membrane receptor. Ferritin may therefore function as an intrahepatic carrier involved in the redistribution of iron between Kupffer cells and parenchymal cells (Sibille *et al*, 1988). Kupffer cells do not appear to express transferrin receptors (Soda & Tavassoli, 1984; De Vos *et al*, 1988). In conditions of secondary iron overload such as transfusional siderosis, iron is less efficiently mobilized from Kupffer cells and accumulates as haemosiderin.

1.5 SINUSOIDAL ENDOTHELIAL CELLS

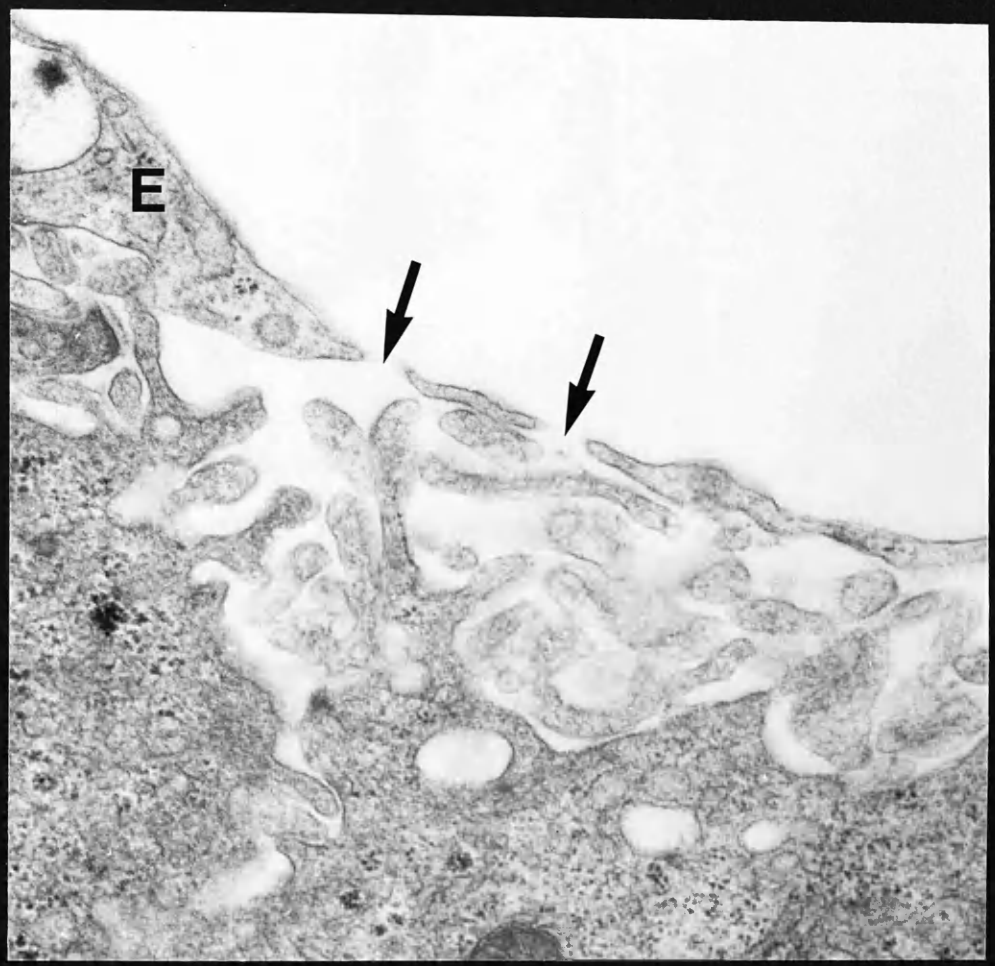
1.5.1 Morphology

These cells, which account for over 50% of sinusoidal cells (Blouin, 1977) form a unique type of endothelial lining. They are long flattened cells whose cytoplasm is perforated by numerous small fenestrae of approximately 0.1μm in diameter (Wisse, 1970; 1972). The fenestrae lack diaphragms and are arranged in groups (so-called sieve plates). By transmission electron microscopy, no basement membrane can be seen beneath the sinusoidal endothelial cells. This property, combined with the presence of cytoplasmic fenestrae leads to an open communication between the sinusoidal lumen and the underlying space of Disse, thereby providing access for fluid and solutes to the microvilli at the sinusoidal domain of parenchymal cells (Figure 1.6).

Fenestrae have been observed in ultrathin plastic-embedded preparations and in freeze-etch preparations by transmission electron microscopy (Wisse, 1970; Montesano & Nicolescu, 1978) and have been demonstrated in several species by scanning electron microscopy (Motta & Porter, 1974; Wisse *et al*, 1985). Larger pores (so-called gaps) have also been identified but

Figure 1.6 Transmission electron micrograph of sinusoidal endothelial cell in perfusion-fixed normal rat liver.

In this preparation, fenestrae (arrows) can be identified adjacent to the cell body of a sinusoidal endothelial cell (E). Note the absence of any underlying basement membrane. This structural property permits the free access of materials between the sinusoidal lumen and the space of Disse (and hence the microvilli of parenchymal cells). (Uranyl acetate/lead citrate; x 28,000).


E

were considered by Wisse and co-workers to represent artifacts (Wisse *et al*, 1985) formed during perfusion, due either to hypoxia or over-zealous perfusion at pressures exceeding physiological levels.

The nucleus is generally situated along the plasma membrane adjacent to the space of Disse (Figure 1.7). The overlying perinuclear cytoplasm may protrude into the sinusoidal lumen although this is much less marked than in Kupffer cells, and microvilli are absent. Apart from the presence of invaginations of micropinocytotic vesicles, the cells have a smooth surface. Within the cytoplasm there are numerous vesicular structures including bristle-coated micropinocytotic vesicles and macropinocytotic vesicles; lysosomes are also present. The rough endoplasmic reticulum is rather poorly developed although the cells generally possess more than one Golgi apparatus.

In addition to the ultrastructural appearances, sinusoidal endothelial cells show other phenotypic differences compared to vascular endothelium (Jaffe, 1987). They do not express Factor VIII-related antigen (von Willebrand factor) in normal human liver (Fukuda *et al*, 1986) rat liver or guinea-pig liver (Lenzi *et al*, 1990) and do not appear to bind Ulex europaeus (Petrovic *et al*, 1989).[1] Immunohistochemical studies have also demonstrated that, in common with Kupffer cells, human sinusoidal endothelial cells express the T lymphocyte-associated cluster differentiation antigen CD4, albeit at low levels (Figure 1.9). The functional significance of this in normal liver is uncertain but it is of relevance to the pathogenesis of HIV infection since CD4 is the receptor for HIV. The sinusoidal cells may act as a reservoir for the virus (Scoazec & Feldmann, 1990).

Unlike Kupffer cells, at present there are no antibodies available which can be used to specifically immunolocalize sinusoidal endothelial cells. Localization of fluorescence-labelled acetylated LDL has been used as a marker in rats but this requires prior loading of animals (Irving *et al*, 1984) and is not entirely specific since Kupffer cells also (weakly) express receptors for acetyl-LDL.

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[1]In my own experience however, the lectin binding properties are fixation dependent. Thus in Bouins-fixed normal human liver some sinusoidal endothelial cells do show positive staining (Figure 1.8).

Figure 1.7 Transmission electron micrograph of sinusoidal endothelial cell in normal rat liver : micropinocytotic vesicles.

In this sinusoidal endothelial cell, organelles are seen principally along the sinusoidal domain. Numerous micropinocytotic vesicles can be identified within the cytoplasm, close to the cell surface; these are derived from coated pits present on the cell membrane (inset: arrow). (Uranyl acetate/lead citrate; x 10,200).

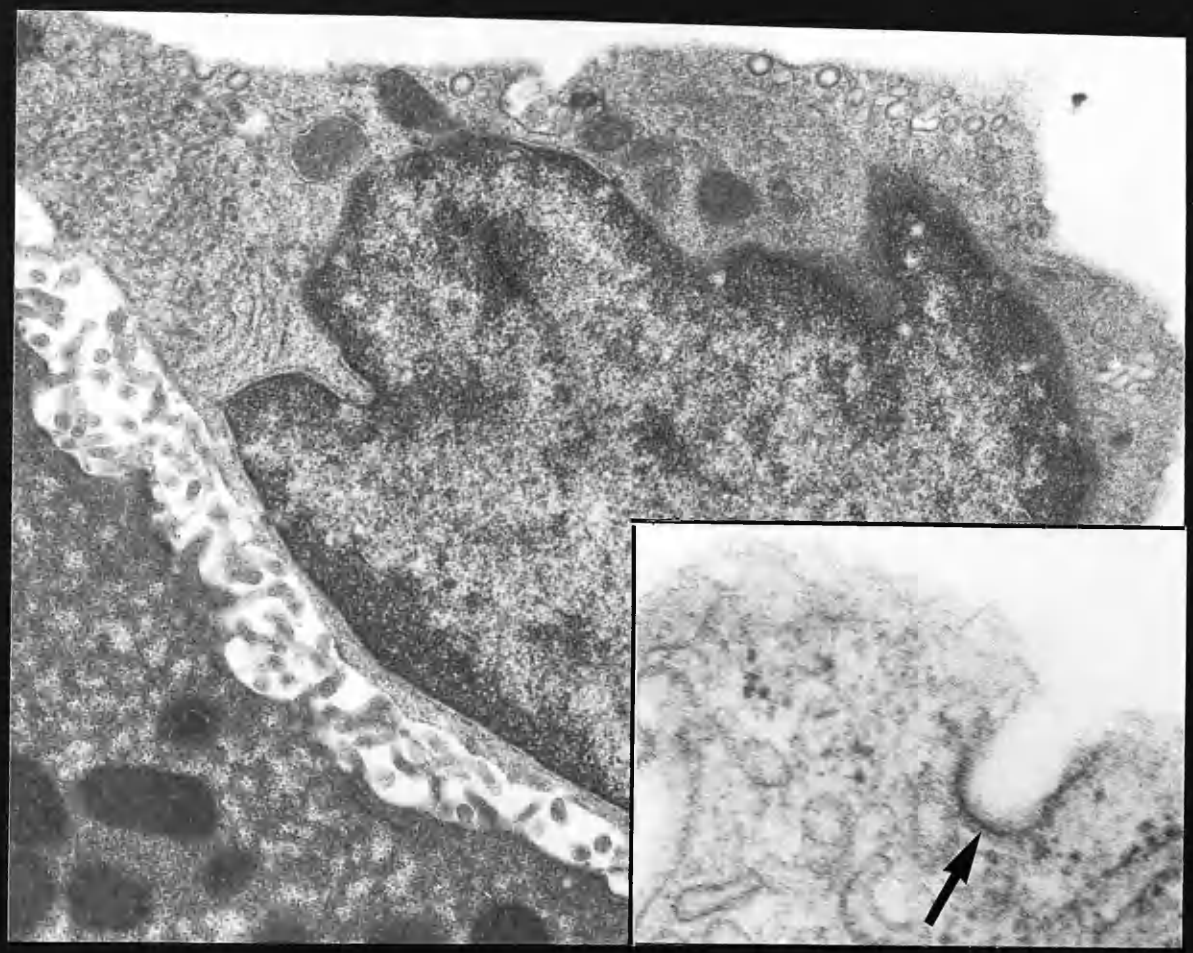
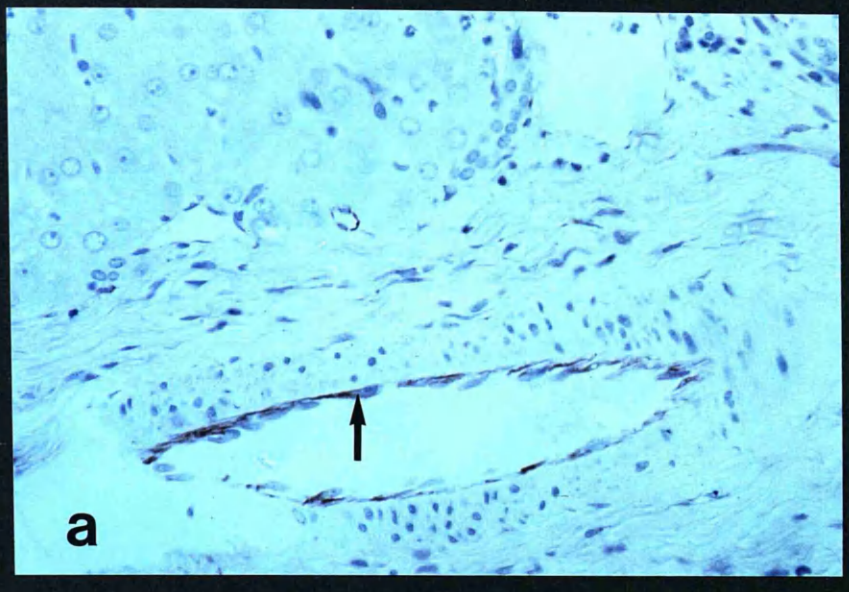


Figure 1.8 Immunolocalization of endothelial cell-associated markers in normal human liver.

a. Immunoreactivity for Factor VIII-related antigen (Von Willebrand Factor). This is restricted to vascular endothelial cells of hepatic artery (arrow) and hepatic vein and portal vein branches; sinusoidal endothelial cells are negative. (Polyclonal anti-Factor VIII-RAg (Dako, 1:250); indirect immunoperoxidase method on 3 μm dewaxed formalin-fixed section; haematoxylin counterstain; x 250).

b. Lectin histochemistry for demonstration of binding of Ulex europaeus. Strong staining can be seen on vascular endothelial cells in hepatic artery branches (A). In most preparations, sinusoidal endothelial cells were negative. This, however, is fixation-dependant; in Bouins-fixed material occasional sinusoidal endothelial cells can be seen to be positive (arrowhead).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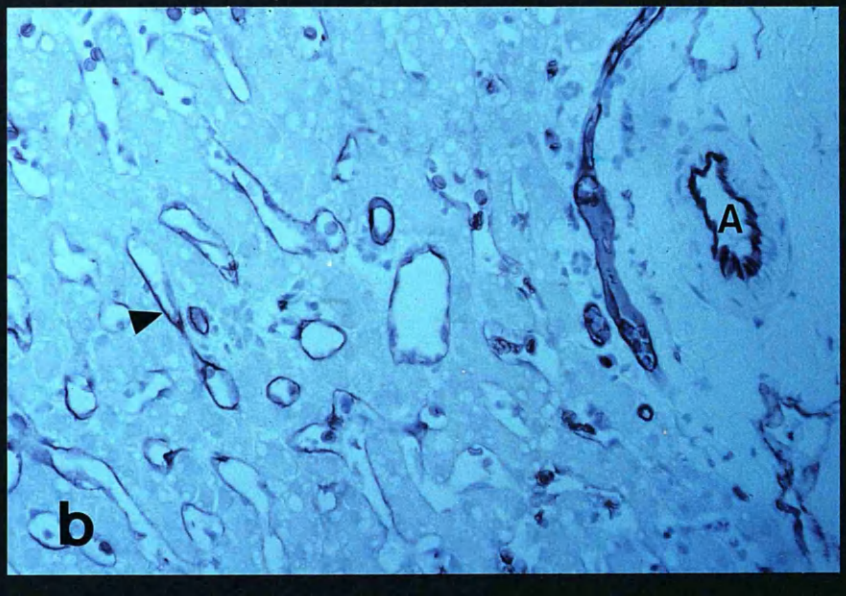
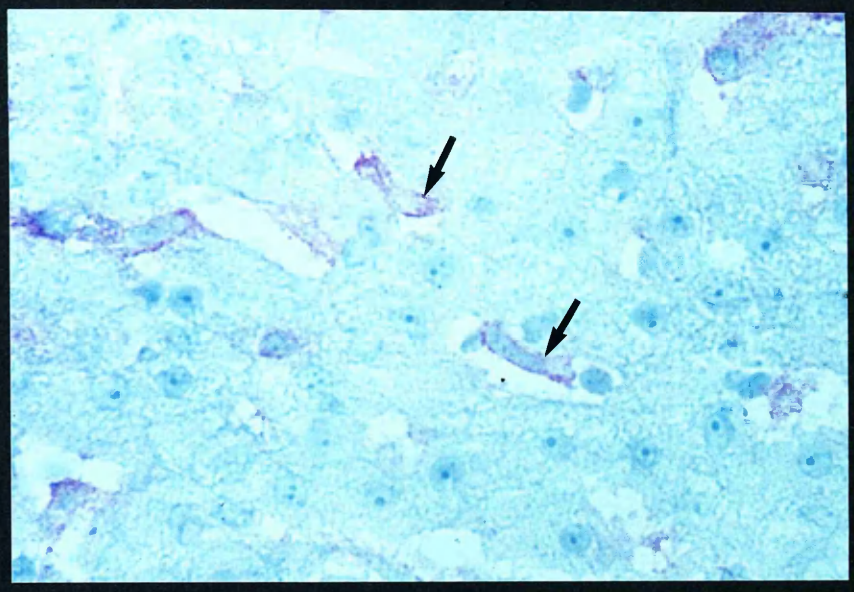

A
b

Figure 1.9 Immunoreactivity for the cluster differentiation antigen CD4 on sinusoidal cells in normal human liver.

Weak immunoreactivity can be seen on sinusoidal cell membranes (arrows). Although some CD4-positive cells have the morphological features of Kupffer cells, others have elongated nuclei and are likely to be sinusoidal endothelial cells. (Monoclonal antibody OKT4 (Orthoclone; 1:10); immuno-alkaline phosphatase method on 4 μm cryostat section; haematoxylin counterstain; x 580).



1.5.2 Ontogeny and population kinetics

Endothelial cells are present along sinusoidal walls from early stages of hepatic development. Bankston & Pino (1980) showed that in day 10 gestation fetal rat liver, endothelial cells had relatively few fenestrae and those that were present were spanned by a diaphragm. By day 17 of gestation, diaphragms had disappeared and fenestrae increased in number, the endothelial cells then adopting the ultrastructural appearances of adult rat liver. Mitotic figures have been observed within sinusoidal endothelial cells in neonatal and fetal liver (Naito & Wisse, 1977), partial hepatectomy (Wisse, 1972) and in response to exogenous oestrogen treatment in rats (Widmann & Fahimi, 1976) indicating that these cells may also form a self-replicating population.

1.5.3 Functions

Filtration

By virtue of their structural properties, the sinusoidal endothelial cells provide a selective barrier between the sinusoidal blood and the space of Disse. Soluble compounds can pass freely between these two compartments permitting easy exchange of substances between the hepatic microcirculation and parenchymal cells. However, the diameter of the endothelial cell fenestrae are such that some substances are excluded. Newly generated chylomicrons transporting alimentary lipid are thought to be too large to pass through the fenestrae and require to be metabolized within the circulation before they can gain access to the space of Disse. Naito & Wisse (1978) showed that chylomicron remnants in the space of Disse of weaning neonatal rats were never larger than the fenestrae. A similar filtration effect was also demonstrated in adult animals given varying intragastric doses of unsaturated corn oil (De Zanger & Wisse, 1982).

Wisse *et al* (1985) have suggested that the transport of materials through the fenestrae may be facilitated by a pressure effect of blood cells within the sinusoidal lumina. In morphometric studies they identified that many cellular constituents of the sinusoidal microcirculatory flow are larger than the actual diameter of the sinusoids; *in vivo* microscopy studies confirmed that red and white cells may compress the endothelial lining during their

transit through the sinusoids. Wisse *et al* (1985) have postulated that the blood cells may promote the transport of fluid or solid phase particles through the fenestrae by a direct pressure effect ("forced sieving"). Furthermore, they believe that white blood cells, as a result of their size and relative rigidity, may influence trans-endothelial flow by a second mechanism which they have termed endothelial massage. One of the effects of compression of the space of Disse by the white cells may be displacement of fluids downstream. The fluids may then flow out of the space of Disse into the sinusoidal blood flow. Wisse *et al* (1985) suggested that upstream of the white blood cell there may therefore be a negative pressure effect promoting the influx of plasma to the space of Disse. Although attractive, these hypotheses have not yet been adequately tested.

Endocytosis

The presence of abundant micropinocytotic vesicles and coated pits in sinusoidal endothelial cells indicates that they are actively involved in endocytosis; such high endocytotic activity is unusual in endothelial cells. The process appears to be directed towards uptake and degradation of compounds within lysosomes rather than providing an alternative route for the transport of materials from the sinusoidal lumen to the space of Disse. *In vivo* experiments have demonstrated that these cells are capable of endocytosing a large variety of exogenous and endogenous particles and molecules (Table 1.2). The spectrum of substances is as great as for Kupffer cells. However, it should be noted that they are generally less efficient than Kupffer cells in the uptake of large particulate particles such as latex and colloidal carbon. Several of the cell surface receptors involved are specific for endothelial cells although some, such as the receptor for mannose-residues on glycoproteins, are also found on Kupffer cells (Praaning-van Daaling *et al*, 1982). There is some evidence that they may possess Fc receptors and contribute to hepatic uptake of immune complexes (Gudmundson *et al*, 1986).

Denatured albumin	Eskild & Berg (1984)
Transferrin	Soda & Tavassoli (1984)
Immune complexes	Gudmundsen *et al* (1986)
α-galacto- and α-hexosamino orosmucoid	Hubbard *et al* (1979)
Hyaluronic acid	Smedsrød *et al* (1984)
Collagen type I	Smedsrød *et al* (1985)
Very low density lipoproteins	Gustafson *et al* (1985)
Modified low density lipoproteins	Nagelkerke *et al* (1984)

Table 1.2 Examples of compounds which may be endocytosed by sinusoidal endothelial cells.

1.6 FAT-STORING CELLS

1.6.1 Morphology

These cells, referred to in the scientific literature under a wide range of terms (Wake, 1980) are located within the space of Disse. They have long cell processes which at least partially surround the sinusoidal endothelial cells. The perikaryon of the cells is frequently present within recesses between parenchymal cells (Figure 1.2). No junctional complexes are found between adjacent fat-storing cells or between fat-storing cells and surrounding endothelial cells or parenchymal cells. Although some investigators have indicated that the fat-storing cells may be unevenly distributed throughout the acini (Giampieri *et al*, 1981), recent morphometric studies suggest that there is no zonal heterogeneity (Sztark *et al*, 1986).

The most characteristic morphological feature is the presence of lipid droplets within the cytoplasm; these frequently indent the nucleus (Wisse 1977). They autofluoresce to produce evanescent green fluorescence when excitation light of wavelength 328 nm is used with fluorescence microscopy (Wake, 1971). This property is due to the presence of retinol and retinyl esters (vitamin A) (Popper, 1941). A sensitive technique for the demonstration of vitamin A using cathodoluminescence electron microscopy has been described by Yamamoto *et al* (1986).

By transmission electron microscopy, the lipid droplets are of variable size. As a result of sampling errors not all fat-storing cells will be seen to contain lipid droplets in ultra-thin sections (Sztark *et al*, 1986). Species differences with respect to droplet size have been recorded (Ito, 1973); their number and size are also influenced by the age and vitamin A status of the animal (Brouwer *et al*, 1988b). Wake (1974) has described two types of lipid droplet. Type I droplets are electron-dense and membrane-bound. These are smaller and more numerous than type II droplets which have a diameter of around 2 μm and are not membrane-bound. Wake (1974) has suggested that type II droplets are formed by the fusion of several type I droplets. This was disputed by Yamamoto & Ogawa (1983) who believe the converse to be true, type I droplets being formed by (lysosomal) degradation of type II droplets.

Another conspicuous ultrastructural feature is the presence of a well developed rough endoplasmic reticulum and Golgi apparatus (Wood, 1963; Wake, 1971; Blouin *et al*, 1977; Wisse, 1977). The cisternae of the rough endoplasmic reticulum are generally expanded and often contain filamentous material; these appearances suggest active protein synthesis. By contrast, the smooth endoplasmic reticulum is poorly developed. Mitochondria are not abundant and there is little cytoplasmic glycogen (Tanuma *et al*, 1982). Centrioles may be present, sometimes in the form of a basal body of a cilium projecting into the space of Disse (Ito, 1973; Tobe *et al*, 1985). Although some authors have denied the existence of lysosomes within these cells (Bronfenmajer *et al*, 1966), others have identified small acid phosphatase-positive lysosomes (Wisse, 1974; Yamamoto & Ogawa, 1983). Furthermore, multivesicular bodies, often seen in relation to type I lipid droplets (Wake, 1974; Wisse, 1977) are considered to represent a form of lysosome. However, there is no evidence to indicate that fat-storing cells are actively phagocytic. Lysosomes and multivesicular bodies in these cells are not thought therefore to form phagolysosomes but probably contribute to intracellular vitamin A handling (vide infra) (Wake, 1980).

Cytoskeletal structures are found within the long cytoplasmic extensions (Sztark *et al*, 1986). Microtubules may be present but these are less abundant than microfilaments and intermediate filaments. These are clearly visible in tangential sections by transmission electron microscopy, running parallel to the plasma membrane and showing focal condensations beneath the membrane similar to those seen in smooth muscle cells and myofibroblasts. Micropinocytotic vesicles may also be present close to the surface of the cell processes.

1.6.2 Nomenclature

These cells have been referred to by a number of terms (Table 1.3); their nomenclature remains a controversial topic among those interested in sinusoidal cell biology. An international workshop in 1985 failed to reach a consensus as to their most appropriate name. Indeed, the situation became even more confused by the introduction of a new term, parasinusoidal cell (Aterman, 1986)! Although Ito cell is a widely used name, several investigators prefer to avoid the use of eponymous terms. Aterman (1986) has argued that if eponyms are to be attached

"Sternzellen" or stellate cells	Kupffer (1876); Wake (1971)
Granular cells	Berkley (1893)
Pericytes	Zimmermann (1928)
Fat-storing cells	Ito & Nemeto (195)
Metalophil cells	Marshall (1956)
Perisinusoidal cells	Wood (1963)
Fat-storage cells	Ladman (1964)
Lipocytes	Bronfenmajer *et al*, (1966)
Adventitious connective tissue cells	Schnack (1967)
Sinusoidal mesenchymal cells	Rubin *et al* (1970)
Ito cells	Hruban *et al* (1974)
Parasinusoidal cells	Aterman (1986)

Table 1.3 Terms previously applied to fat-storing cells

Other terms used in Japanese literature such as lipophagic, Disse space cells, transmittal cells, vitamin A-uptake and vitamin A-storage cells are cited by Wake (1980).

it should be that of Boll! Wake (1980) has suggested that the term fat-storing cell is inappropriate as the lipid is vitamin A rather than triglyceride; use of this term may lead to confusion with lipid storage in parenchymal cells in fatty liver. McGee & Patrick (1972) also maintain that it is unjustifiable to regard these cells as specialised exclusively for the storage of "fat" as it is apparent that they may have other important functions (vide infra).

In this manuscript I have used the term fat-storing cell. While recognising the constraints of this term, it is at present the most widely used by European authors (lipocyte is favoured by many North American investigators).

1.6.3 Ontogeny

Fat-storing cells are thought to be derived from primitive mesenchymal cells originating in the septum transversum. In fetal rat liver, they can be detected by day 15 of gestation (Naito & Wisse, 1977); only small cytoplasmic droplets are present during this period of development. In fetal human liver, fat-storing cells can be identified by 6 weeks gestation (Enzan *et al*, 1973). Localization of cellular retinol-binding protein in these cells during fetal development suggests that they may be involved in vitamin A metabolism although they contain little stored retinol (Kato *et al*, 1985). In addition to the paucity of lipid droplets, fetal fat-storing cells differ from those of adult liver by the following features: (i) they are proportionately more abundant, (ii) they form a complete "sleeve" around the endothelial cells and (iii) occasional desmosomal junctions may be observed between them and adjacent parenchymal cells (Bioulac-Sage & Balabaud, 1985). The presence of mitotic figures in fetal fat-storing cells has been taken as evidence that may also be a self-proliferating cell population (Naito & Wisse, 1977).

1.6.4 Functions

Retinoid metabolism

The liver plays a central role in the metabolism of retinol (vitamin A); over 95% of total body retinoids are found in this organ (Knook *et al*, 1989). Dietary retinoid, absorbed as chylomicron retinyl esters in the small intestine, are taken up in the liver and hydrolyzed to

retinol. Although a small proportion is then converted into retinoic acid or retinyl glucuronide, the majority is re-esterified and stored as retinyl ester in the liver or exported into the blood for distribution to other tissues.

There is now substantial evidence that fat-storing cells play a pivotal role in the hepatic handling and storage of retinol (Hendriks *et al*, 1987). Studies with isolated cell preparations have indicated that up to 90% of hepatic retinoids are present within these cells in the form of retinyl esters (Hendriks *et al*, 1985); these esters are contained within the cytoplasmic lipid droplets of the fat-storing cells. However, recent work has indicated that although retinoids are principally stored in the fat-storing cells, parenchymal cells are also involved in vitamin A metabolism. It has been proposed that dietary retinoids are initially taken up by parenchymal cells via chylomicron remnant receptors but are in turn transferred to the fat-storing cells for storage (Blomhoff *et al*, 1985; Blaner *et al*, 1987). Although the precise mechanisms remain to be elucidated, it is thought likely that retinoid transport between the cells involves retinol rather than retinyl esters. Fat-storing cells have been shown to contain the binding proteins and enzymes necessary to participate in this complex intercellular exchange (Blaner *et al*, 1987).

Contractile properties: regulation of intrasinusoidal blood flow

The microscopic observation that fat-storing cell processes ensheath the sinusoids in a similar manner to that of pericytes in the microvasculature at other sites has raised the possibility that these cells may also function in the regulation of intrasinusoidal blood flow (Wisse *et al*, 1989). As discussed in Chapter 5, fat-storing cells can be shown to express several muscle-associated proteins which would be in keeping with this hypothesis.

Synthesis and secretion of extracellular matrix proteins

This is dealt with in detail in Chapters 4, 5 and 6.

1.7 PIT CELLS

1.7.1 Morphology

During the course of his early ultrastructural studies, Wisse identified a fourth cellular component of the rat liver sinusoids (Emeis & Wisse, 1970); he later described these in detail, referring to them as pit cells because of their characteristic cytoplasmic granules. These cells are normally found in close contact with the luminal surfaces of sinusoidal endothelial and Kupffer cells although no junctional complexes with these cells have ever been identified. Rat pit cells have a diameter of around 7 μm. They have a low nuclear:cytoplasmic ratio and are often polarized with an eccentrically placed nucleus and aggregation of cytoplasmic organelles towards one side of the cell. The cytoplasmic granules, which have a diameter of around 0.3 μm, are osmiophilic and are surrounded by a single membrane (Figure 1.10). These are often closely applied to lysosomes and multivesicular bodies. Kaneda *et al* (1982) described a second type of vesicle in pit cells, the "rod-cored vesicle". These appear to be formed from the Golgi apparatus, which is well developed in pit cells. They are also membrane-bound structures but are smaller than the granules and contain a central rod shaped inclusion of approximately 40 nm width. Other organelles are sparse; both rough and smooth endoplasmic reticulae are poorly developed. Pseudopodia are often seen at the cell surface; these may extend through fenestrae in the endothelial cells allowing contact with fat-storing cells and the microvilli of parenchymal cells. However, pit cells are only rarely seen within the space of Disse.

Cells with identical morphological appearances have also been found within portal tracts and in other organs including spleen and peripheral blood (Wisse *et al*, 1976; Kaneda *et al*, 1982). Subsequent studies have shown that pit cells are not restricted to rat liver but can also be found in human livers (Bioulac-Sage *et al*, 1986) (Figure 1.11).

In human liver, pit cells appear to be few in number and to have smaller and less abundant granules than rat liver (Bouwens *et al.*, 1989). Indeed, the paper of Bioulac-Sage *et al.* (1986) is based on a study of just 25 pit cells from 13 liver biopsies! However, they contain an organelle absent from rat pit cells, "parallel tubular arrays" (Bouwens *et al.*, 1989). These are highly ordered arrangements of tubular structures each with a diameter of 15 to 18 nm. Some

Figure 1.10 Transmission electron micrograph of pit cell in perfusion-fixed normal rat liver.

a. A pit cell can be seen in close apposition to cell processes of an endothelial cell and a Kupffer cell. This cell shows the characteristic polarity of organelles towards the luminal surface.

b. At a higher magnification the characteristic electron-dense granules can be identified (arrows), as can ocassional rod-cored vesicles (arrowhead). (Uranyl acetate/lead citrate; a: x 4,800, b: x 14,300)

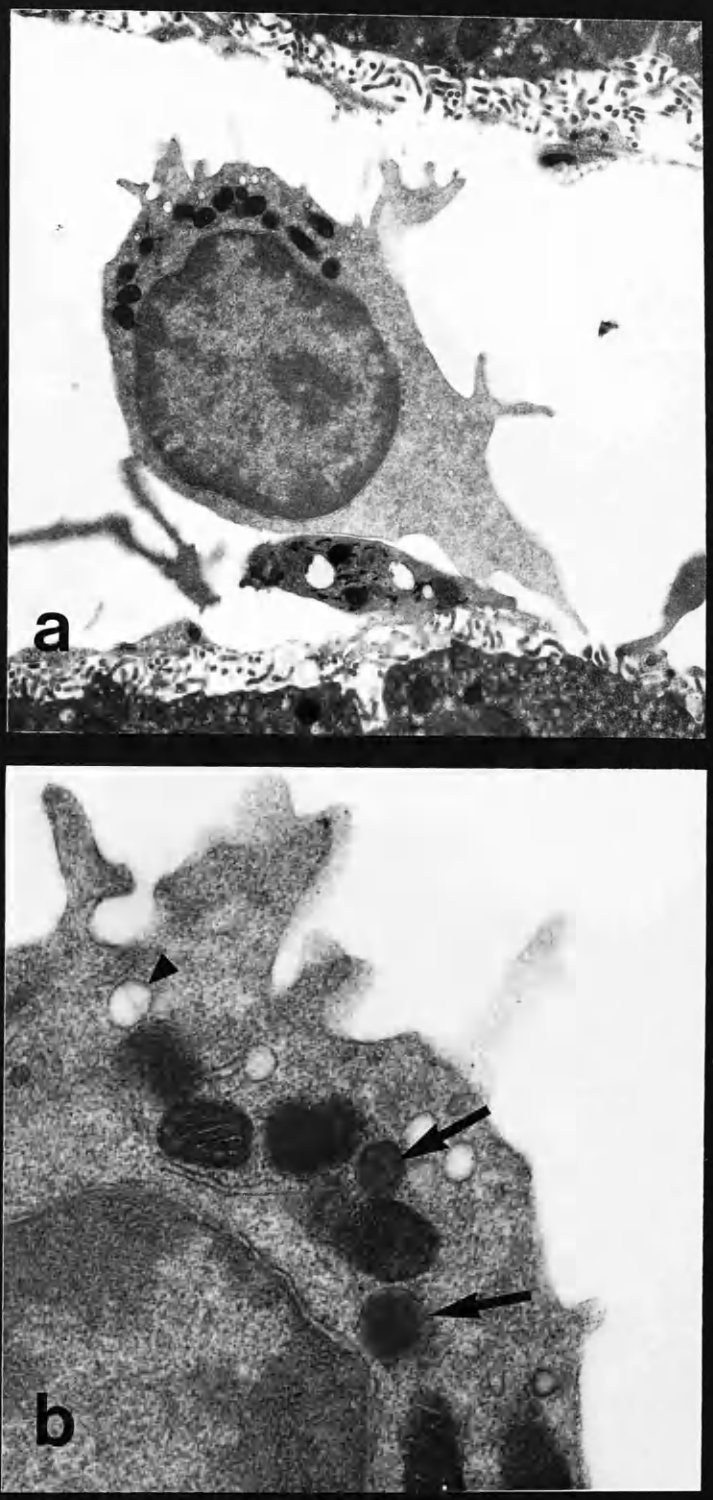
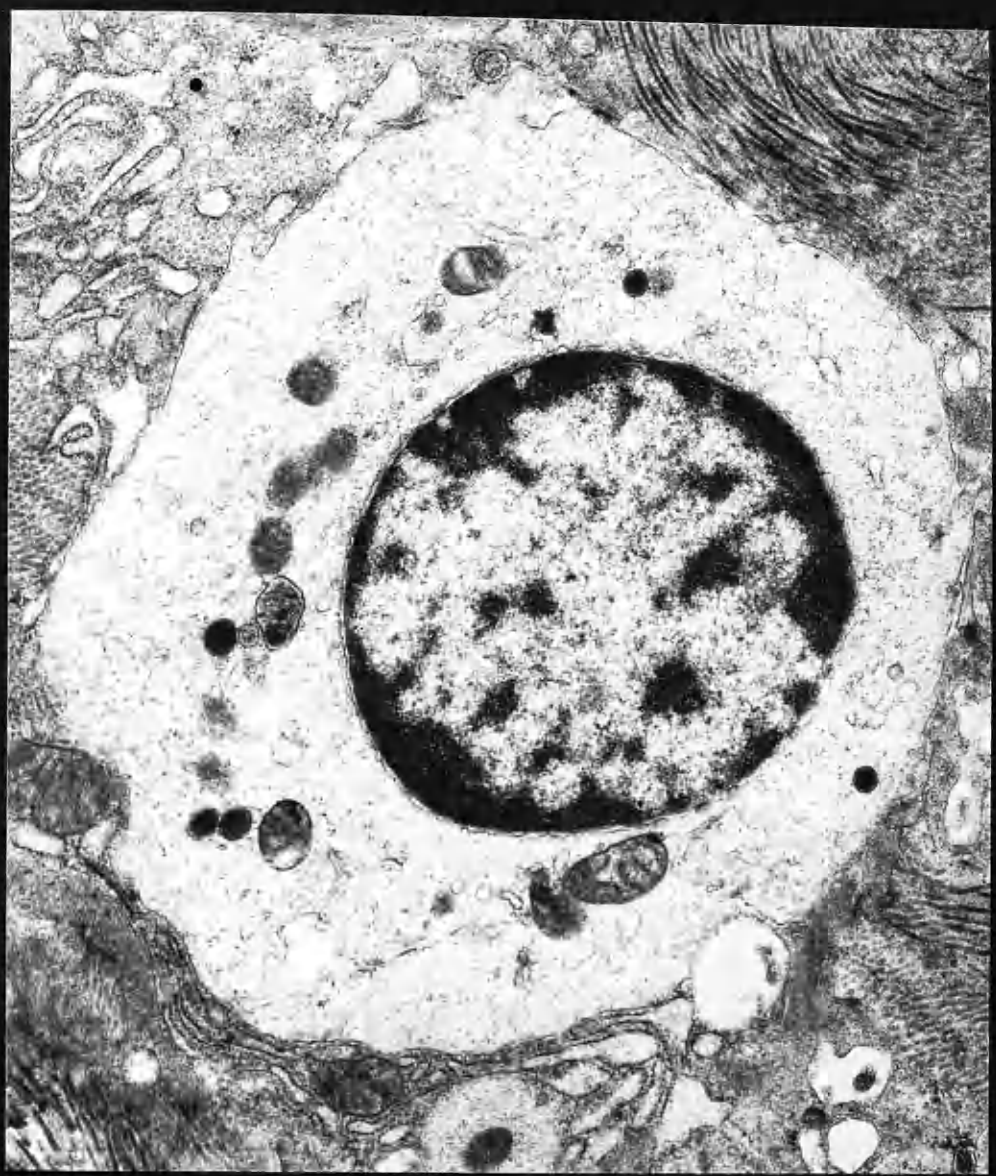

a
b

Figure 1.11 Transmission electron micrograph of pit cell in human liver.

Pit cells are less readily identified in human liver than in rat liver. In this preparation from a patient with auto-immune chronic active hepatitis, only occasional electron-dense granules can be identified in a pit cell which is present in an area of fibrosis. Note surrounding striated collagen fibrils. (Uranyl acetate/lead citrate; x 12,200).



of these can be observed in close apposition to granules leading Bouwens *et al.* (1989) to suggest that parallel tubular arrays may originate from the dense granules by polymerization of the protein content of the granule (vide infra).

1.7.2 Ontogeny and population kinetics

Naito & Wisse (1977) were unable to identify these cells in fetal rat liver but found them in neonatal livers. Bouwens & Wisse (1988) showed that the total numbers of pit cells in adult rat liver increased 4 to 6-fold when animals were treated with the following biological response modifiers: zymosan, Proiobacterium acnes and OK-432. Using similar stathmokinetic experiments to those used in their studies of Kupffer cell kinetics, they demonstrated that this increase was largely due to local proliferation within the liver although their results indicated that with P. acnes and OK-432 it was contributed to by recruitment from cells in the peripheral blood. They also showed that pit cells were absent from the livers of animals 9 days following total body x-irradiation, indicating that this cell population has a rapid turnover.

1.7.3 Function

Natural killer cell activity

Wisse *et al* (1976) initially suggested that pit cells may be neuroendocrine because of the presence of the membrane-bound granules. More recently however, Wisse's group have demonstrated that rat pit cells express cell surface determinants of natural killer lymphocytes (Bouwens & Wisse, 1987). They showed that these cells can be immunolabelled using antibodies against asialo GM1 and the monoclonal antibody OX8 but not with OX19; this phenotype is identical to that of circulating natural killer cells. Kaneda & Wake (1983) drew attention to the morphological similarities between the so-called large granular lymphocytes of the peripheral blood (now considered to represent the natural killer cell population) and hepatic pit cells. They were the first to suggest that pit cells were likely to function as natural killer cells in the host response to tumour cells (Kaneda *et al.*, 1983). Conclusive evidence for their natural killer capacity was recently presented by Bouwens *et al* (1987). Isolated pit cells from

normal rat liver were shown to be spontaneously cytotoxic against YAC1 lymphoma cells, an established killer cell-sensitive tumour cell line; this effect could be completely abolished by pre-treatment of the cell isolates with anti-asialo GM1 antiserum and complement. Natural killer and pit cell-induced cell lysis involves the release of molecules such as perforin that may be contained within the cytoplasmic granules (Ortaldo, 1986).

Bouwens & Wisse (1989) have subsequently shown a similar cytotoxic effect against solid tumour-derived cell lines. It has been postulated therefore, that pit cells may play a role in the response to intrahepatic metastatic tumour (and possibly primary hepatic malignancies) (Bouwens & Wisse, 1989). They may also play a role in the pathogenesis of viral hepatitis (McIntyre & Welsh, 1986) and autoimmune chronic active hepatitis (Kaneda *et al*, 1984).

Occasional sinusoidal cells can be identified in human liver which are immunoreactive with the monoclonal antibody Leu 7 (Si & Whiteside, 1983) (Figure 1.12). As Leu 7 immunoreactivity is not entirely restricted to NK cells immuno-electron microscopy would be required to confirm that these are indeed pit cells.

1.8 OTHER POSSIBLE CELLULAR CONSTITUENTS OF THE HEPATIC SINUSOIDS

Several other cell types have been proposed as possible cellular constituents of the hepatic sinusoids. Jézéquel *et al* (1986) suggested that mast cells could frequently be observed within the space of Disse in normal human liver. However, Baradadin & Scheuer (1986) could not demonstrate these cells in the sinusoids of livers from patients with histologically normal liver. The presence of neuroendocrine cells within the sinusoids was suggested by the studies of Martinez *et al* (1974) in which chromaffin cells were identified in mouse, rat and rabbit liver. As discussed above, there was earlier speculation that Wisse's pit cells may be neuroendocrine. Prior to their identification as natural killer cells, I performed a series of immunohistochemical studies (unpublished observations) of normal rat, human, rabbit and guinea pig liver using antibodies to proteins expressed by neuroendocrine cells including neuron-specific enolase, chromogranin A and several regulatory peptides. Although occasional chromogranin-positive and somatostatin-positive cells could be identified within portal tracts in close proximity with bile duct radicles, no sinusoidal neuroendocrine cells could be identified (Figure 1.13). This is in

Figure 1.12 Immunoreactivity for Leu 7 on sinusoidal cells in normal human liver.

Occasional sinusoidal cells in normal liver can be seen to express natural killer cell-associated proteins such as those detected using the monoclonal antibody Leu 7. (Leu 7 (Dako; 1:20); immuno-alkaline phosphatase method on 4 μm cryostat section; haematoxylin counterstain; x 300).

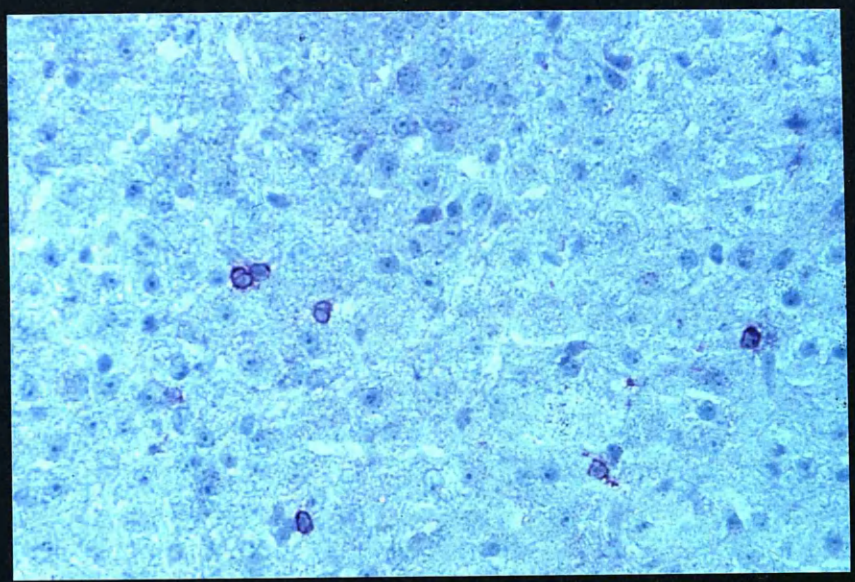
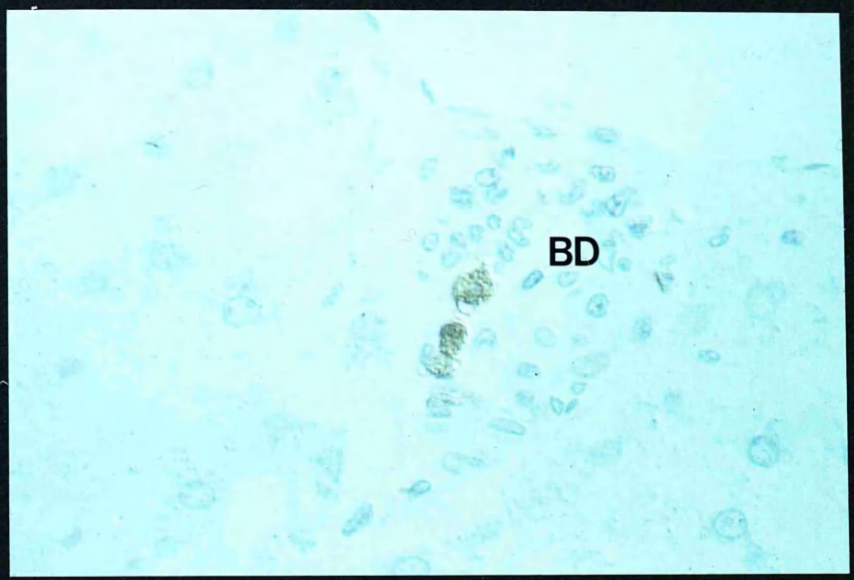


Figure 1.13 Immunolocalization of neuroendocrine cells in rabbit liver.

In this section of normal rabbit liver, cells containing chromogranin A are seen within a portal tract, lying adjacent to a bile duct radicle. In an unpublished study, I have identified similar cells in rat and human liver; occasional cells were shown to contain somatostatin although no other regulatory peptides were found. It should be noted, however, that no sinusoidal cells were found to contain neuroendocrine cell proteins in any species. (Monoclonal anti-chromogranin A (Clonatec; 1:10); indirect immunoperoxidase method (Appendix 4); haematoxylin counterstain; photographed under Nomarski filters; x 450).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keeping with recent data from Kurumaya *et al* (1989) and Roskams *et al* (1990). Finally, the possible existence of dendritic reticulum cells in the sinusoids was raised by the ultrastructural observations of Baradadin & Desmet (1984) in liver biopsies from patients with chronic hepatitis, and in the immunohistochemical study of Van den Oord *et al* (1988) in a variety of inflammatory liver disorders. To my knowledge, however, such cells have not been described within normal sinusoids.

It can be concluded that insufficient evidence exists to suggest that mast cells, neuroendocrine cells or dendritic reticulum cells should be considered as true sinusoidal cells. Most would subscribe to the view that the hepatic sinusoids contain the four distinct cell populations described earlier (Bradfield, 1984), each subserving a wide range of functions (Figure 1.14). The principal role of Kupffer cells is phagocytosis of endogenous proteins and foreign material, in particular gut-derived antigen such as bacterial endotoxins. The sinusoidal endothelial cells are a structurally and functionally unique form of endothelium being fenestrated and lacking any underlying basement membrane; their principal functions are filtration of material in the sinusoidal blood and endocytosis of a range of molecules. The fat-storing cells are involved in retinoid metabolism and, as discussed below, in production of the extracellular matrix. Finally, the pit cells are natural killer cells which may play an important role in host defence against tumours; indeed, in some species the liver may represent the principal site of NK cell activity.

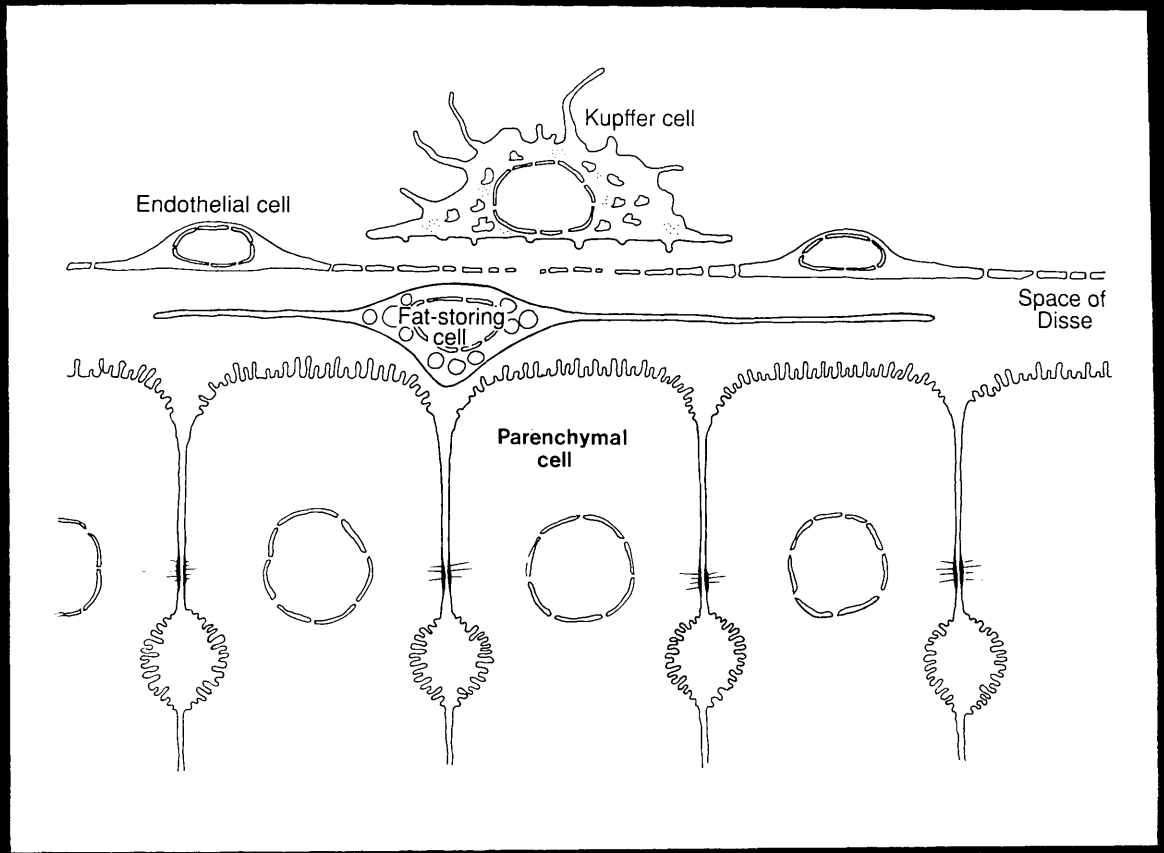
1.9 OUTLINE OF WORK CONTAINED IN THESIS

Chapter 2 of this manuscript describes studies designed to investigate the role of sinusoidal cells in the synthesis and secretion of components of the complement system. Previous studies had indicated that the liver was the principal site for synthesis of most of this important family of plasma proteins but had not considered the contribution of sinusoidal cells.

A major part of this thesis however, deals with two other components of the sinusoids which have hitherto received much less attention than the four types of sinusoidal cell : (i) the extracellular matrix of the space of Disse and (ii) intra-sinusoidal nerve fibres. In recent years it has been established that the extracellular matrix is a complex structure which comprises over

Figure 1.14 Cells of the hepatic sinusoids : relationship to parenchymal cells and the space of Disse.

This diagram outlines the positions of the sinusoidal cells. The sinusoids are lined by the endothelial cells. Kupffer cells lie on these cells and protrude into the sinusoidal lumen. Between the endothelial cells and parenchymal cells is the space of Disse; fat-storing cells are found in this position. For simplicity, pit cells have not been included in the diagram. Like Kupffer cells, these generally rest on the luminal aspect of the sinusoidal endothelial cells.

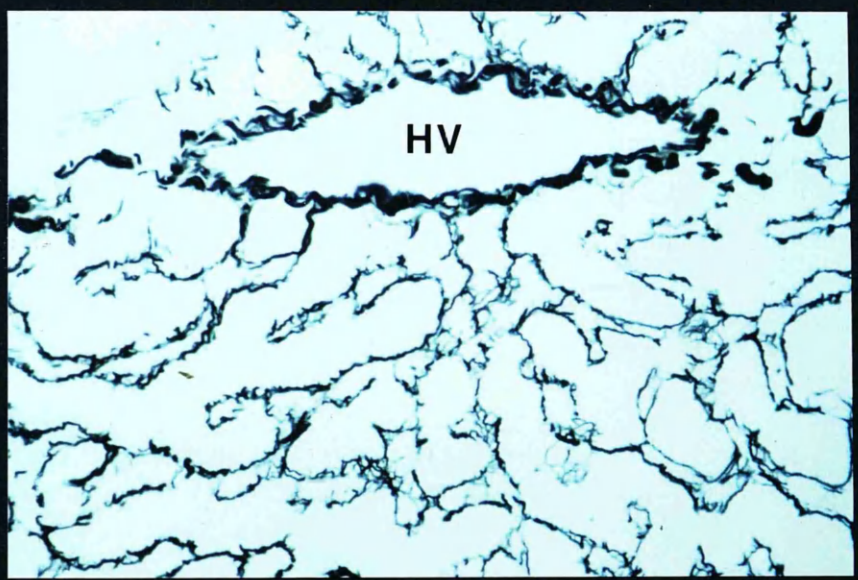

Kupffer cell
Endothelial cell
Space of
Disse
Fat-storing
cell
Parenchymal
cell

13 different types of collagen and a large number of non-collagenous glycoproteins and that the composition of the matrix varies between tissues and at different sites within an individual tissue. An understanding of the nature of the extracellular matrix of the space of Disse (Figure 1.15) is of great importance for two reasons. First, it is now apparent that matrix components not only provide structural support for the integrity and spatial arrangement of tissues but may, through complex cell-matrix interactions, modulate function in the constituent cells of a tissue. Second, it is also known that as part of the response to many forms of acute and chronic liver injury, there is accumulation of extracellular matrix proteins in which the relative amounts of individual components may vary; in chronic liver disease this leads to replacement of functional parenchyma by fibrous tissue. A more detailed knowledge of the distribution of these proteins in normal and diseased liver should lead to a clearer understanding of the mechanisms of the process of hepatic fibrogenesis. Chapter 3 describes studies designed to map the distribution of extracellular matrix components within the liver with particular reference to the space of Disse. In order to define their ultrastructural distribution in normal human liver and in various forms of liver disease, a novel method using ultracryomicrotomy and immuno-gold labelling was applied.

The liver contains few true fibroblasts; these are limited to portal tract stroma and Glisson's capsule. The origin of the extracellular matrix proteins of the space of Disse has therefore been uncertain. In Chapter 4, I describe studies which investigated the contribution of sinusoidal cells to their synthesis. Evidence from immunolabelling and *in vitro* biosynthesis experiments is presented which indicates that fat-storing cells are important producers of matrix proteins. An understanding of the response of fat-storing cells to liver injury is therefore essential for the elucuidation of mechanisms of hepatic fibrogenesis. As discussed in Chapter 5, until recently kinetic studies of fat-storing cell responses have been limited by the lack of a reliable method for their identification by light microscopy. This chapter describes the development of immunohistochemical methods for their detection in tissue sections. I established that in rat liver fat-storing cells express the muscle-associated intermediate filament protein desmin and that this can be used as an immunohistochemical marker for these cells. Chapter 6 describes experiments in which this has been used to investigate their response to two forms

Figure 1.15 Connective tissue proteins within the space of Disse : histochemical demonstration of "reticulin" fibres.

The presence of extracellular matrix proteins along sinusoidal walls can be demonstrated using conventional histochemical methods such as Gordon and Sweets' reticulin stain. In this preparation staining can be seen adjacent to parenchymal cell cords radiating from a terminal hepatic vein (HV). Reticulin stains are now considered to bind to several different extracellular matrix proteins including collagens and fibronectin. (Gordon and Sweets' reticulin; 4 μm dewaxed section of normal human liver; x 420).


HV

of experimental liver injury. In each model, expansion of the fat-storing cell population was demonstrated; the factors which contribute towards this are discussed.

The presence of nerve fibres within the space of Disse had been documented in earlier transmission electron microscopic studies and in histochemical experiments; these investigations however have generated conflicting data. I applied a novel approach using immunohistochemical methods with antibodies to regulatory peptides to define the presence and nature of intra-sinuoidal nerve fibres in rat and guinea-pig liver (Chapter 7). Antibodies to constitutive proteins of peripheral nerves were also used to map intra-sinusoidal nerves in normal adult human liver (Chapter 8) and to investigate (i) ontogenesis of intrahepatic nerves and (ii) abnormalities of innervation in a variety of liver diseases.

In the concluding chapter, the importance of cell-cell, cell-matrix and nerve-cell interactions is discussed and the concept of the sinusoids as a multi-functional "unit" is considered.

CHAPTER 2. SYNTHESIS OF THE COMPLEMENT COMPONENT C3 BY SINUSOIDAL CELLS

2.1 INTRODUCTION

2.1.1 The complement system

The complement system comprises a group of over 25 proteins that play important roles in host defence (Frank, 1988). Activation of the system results in the generation of a series of biologically active peptides which mediate (i) the lysis of cells, bacteria and viruses (Podack & Tschopp, 1984), (ii) opsonization of foreign material to enhance phagocytosis (Fearon & Wong, 1983), (iii) features of the acute inflammatory response (Jose, 1987) and (iv) regulation of the immune response (Hugli & Morgan, 1984).

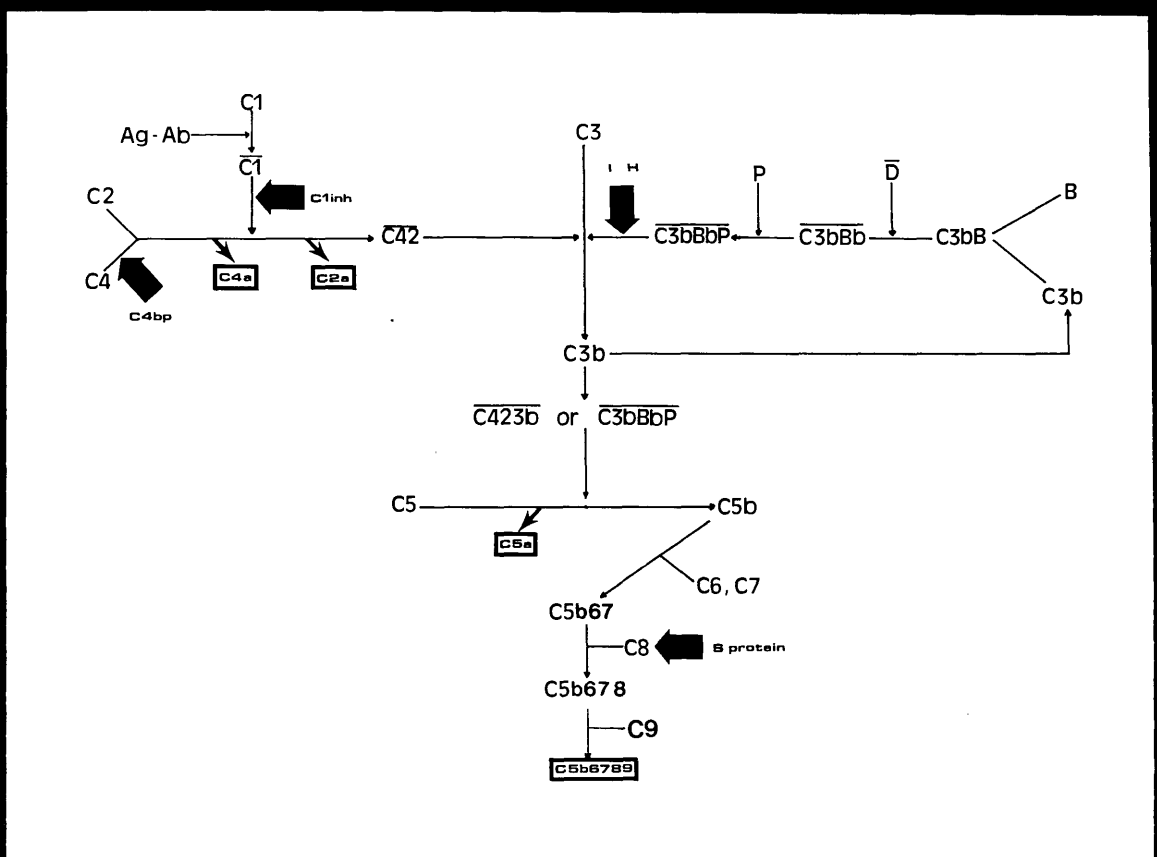
It is activated principally via two pathways, the classical pathway (Cooper, 1985) and the alternative pathway (Pangburn & Müller-Eberhard, 1984). Both of these involve the sequential activation and assembly of a series of proteins resulting in the formation of enzymes (C3 convertases) which can mediate the proteolytic cleavage of the third component of complement (C3) to the smaller fragments, C3a and C3b (Figure 2.1). This in turn leads to the generation of C5 convertases which initiate the terminal "attack" sequence. The end result of this process is the formation of a hydrophobic multimolecular complex which can insert into the lipid bilayer of cells and bacteria, leading to lysis. The classical pathway is generally initiated by the binding of antigen to specific antibody of IgM, IgG1, IgG2 or IgG3 class although it is also recognised that certain materials such as urate crystals and the surface of some viruses and bacteria can activate C1 in the absence of antibody (Cooper, 1983; 1985). The alternative pathway is activated by endotoxin of gram negative bacteria; specific antibody is not a requirement.

The chemistry of the individual complement components and of their complex interactions in the activation sequence has been extensively studied and is now well established (Reid & Porter, 1981). Furthermore, the factors which regulate the cascade system have been elucidated (Lublin & Atkinson, 1990; Vik *et al*, 1990). In recent years, attention has focused on the biosynthesis of complement components (McPhaden *et al*, 1982; Cole & Colten, 1988; Johnson & Hetland, 1988). These studies have dealt with (i) the site of biosynthesis, (ii) intracellular transcriptional and translational events and (iii) factors which may modulate the synthesis and secretion of individual components.

Figure 2.1 Outline of the complement system

The complement cascade pathway is activated through either the classical or alternative pathways, both of which lead to the generation of C3 convertases. All activated components in the system are designated by a bar above the symbol (eg $\overline{C1}$). As a result of activation, several biologically active peptides are generated (denoted here by solid box surround) which may act as acute inflammatory mediators or bacterial lysins. Some components act as control proteins (denoted in diagram by bold arrows). The activity of the by-products is also controlled by enzymes such as carboxypeptidase N (not shown).

The alternative pathway is initiated by the binding of bacterial endotoxin to the control protein factor H. There is thought to be constant low-level turnover of this system; loss of factor H-control leads to amplification with increased C3 convertase function.


C1
Ag-Ab
C1
C1inh
C2
C4
C4bp
C4a
C2a
C42
C3
I H
P
D
B
C3bBbP
C3bBb
C3bB
C3b
C3b
C423b or C3bBbP
C5
C5b
C5a
C6, C7
C5b67
C8
S protein
C5b678
C9
C5b6789

2.1.2 The liver as the principal site of complement protein synthesis

Several observations led to the suggestion that the liver is the principal source of complement proteins : (i) the liver is the major site of synthesis of other plasma proteins, (ii) studies in orthotopic liver transplant patients showed that following transplantation there was conversion of the serum C3, C6, C8 and factor B allotypes to those of the donor (Alper *et al*, 1969, 1980; Hobart *et al*, 1977) and (iii) *in vitro* cultures derived from fetal human liver fragments and whole guinea-pig liver were found to synthesize C2, C4, C3 and C1 inhibitor (Colten, 1972; Colten & Frank, 1972).

Transformed cells derived from rat and human hepatocellular carcinomas were also shown to synthesize several complement components (Strunk *et al*, 1975; Morris *et al*, 1982). Furthermore, *in vitro* synthesis of complement proteins by primary cultures of guinea-pig and rat liver parenchymal cells was demonstrated (Ramadori *et al*, 1984; Anthony *et al*, 1985). These observations indicated that parenchymal cells may be important in the production of complement components. However, the possibility that sinusoidal cells may also contribute to the hepatic synthesis of these proteins received little attention.

At the time the work contained in this chapter was initiated, the ability of various cell types from extrahepatic sites to synthesize and secrete complement components had already been established. Several *in vitro* studies had demonstrated that peritoneal and alveolar macrophages and monocytes may produce a range of components including C4, C3, C2, C5, factor B, D, P, H, I and C1 inhibitor and had identified pharmacological agents which could modulate synthesis and/or secretion of complement proteins by these cells (Whaley *et al*, 1981; Brade & Kreuzpainter, 1982; Newell *et al*, 1982; Lappin & Whaley, 1983). It seemed likely therefore, that Kupffer cells may also exhibit this property. The aim of the work described in this chapter was to investigate the role of these and other sinusoidal cells in the synthesis of the most abundant complement protein, C3 using two experimental approaches: (i) study of C3 biosynthesis in isolated sinusoidal cells and (ii) immunolocalization of intracellular protein in sinusoidal cells.

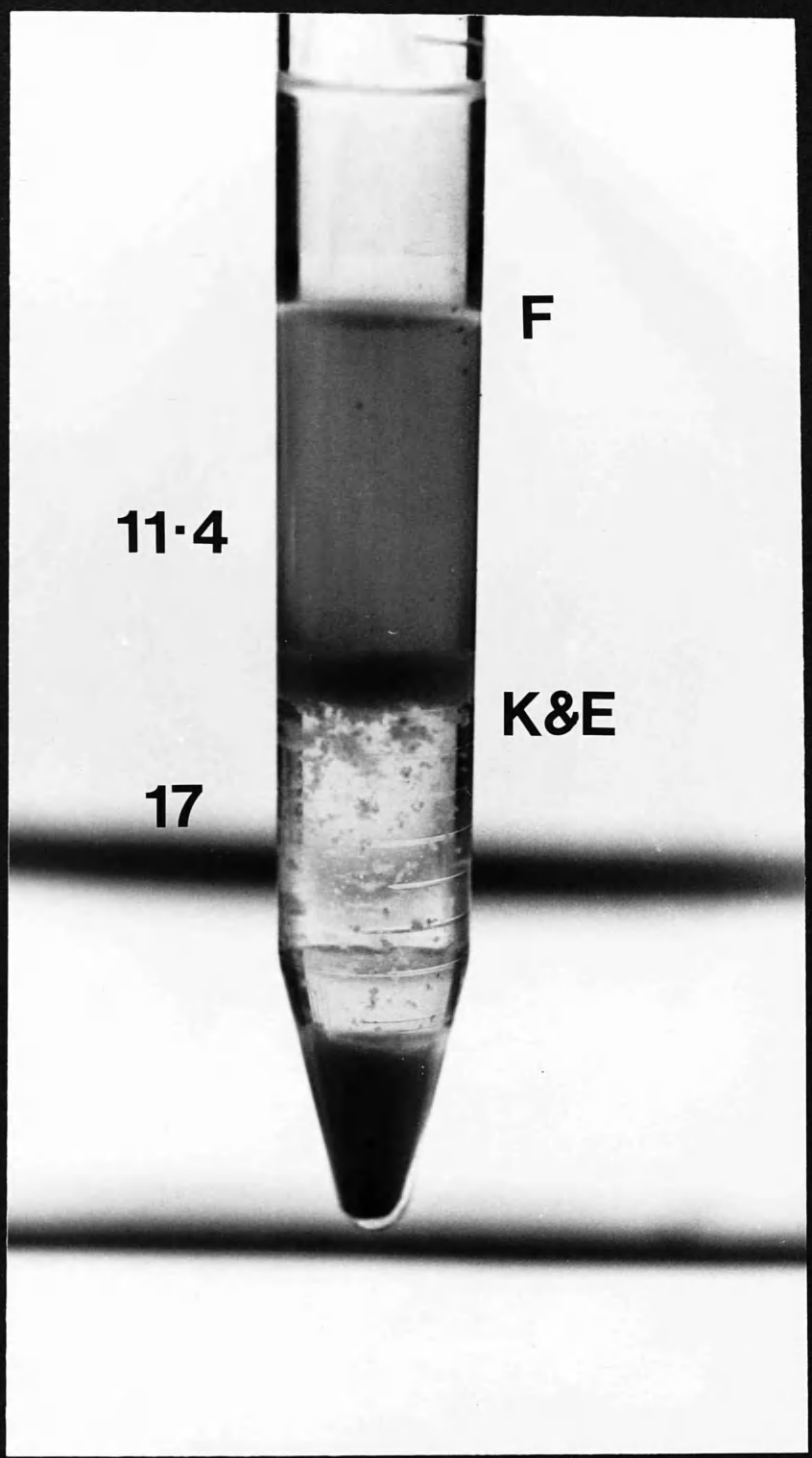
2.2 MATERIALS AND METHODS

2.2.1 Isolation of rat liver sinusoidal cells

Male Wistar rats (200-250g) were used in all experiments. Under ether anaesthesia, the abdomen was opened and the portal vein cannulated. The liver was perfused with 50 ml calcium-free Gey's balanced salt solution (GBSS) and subsequently with 120 ml of a solution containing 0.08% pronase (Merck, Germany; 95,000 U/g) and 0.05% collagenase (Cooper Biomedical, England; 131U/g) in GBSS/1.6M calcium chloride, at a pressure of 10 cm H_2O and a flow rate of 10 ml/minute. The liver was dissected free, the capsule disrupted and the resultant suspension incubated in 0.025% collagenase in GBSS for 15 minutes at 37°C. This was followed by filtration through nylon filter (mesh size 60 μm), two washes in GBSS and centrifugation through a Nycodenz density gradient at 1700g for 15 minutes. In most experiments, a double layer Nycodenz gradient was used with 17% (w/v) at the bottom and 11.4% above. Using this approach, Kupffer cells and endothelial cells could be retrieved at the interface between 17 and 11.4% Nycodenz while fat-storing cells were found above the 11.4% layer (Figure 2.2). To further purify Kupffer cells and endothelial cells, the cell fraction at the 17%/11.4% Nycodenz interface was centrifuged on a Beckman JE6B elutriation rotor at a speed of 2540 rpm. Endothelial cells were collected using a pump speed of 22 ml/minute and Kupffer cells at a speed of 46 ml/minute. These fractions were then washed in GBSS and resuspended in Dulbecco's medium (DMEM) containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Preliminary studies indicated that for maximal cell viability and *in vitro* protein synthesis, addition of 20% fetal calf serum (Gibco, United Kingdom) was required. As this contains complement components which may be detected by the ELISA (vide infra), the fetal calf serum was heat-inactivated at 56°C for 30 minutes. Cells were cultured in either 24-well plastic tissue culture dishes (Nunc, United Kingdom) or in 3 cm diameter dishes (Falcon, Palo Alto, USA). Initial studies indicated that endothelial cells would not adhere to plastic but could be cultured in dishes coated with rat tail collagen. For fat-storing cell isolations, the fraction retrieved from above the 11.4% Nycodenz was washed in GBSS, resuspended in DMEM as

Figure 2.2 Nycodenz density gradient for isolation of rat liver sinusoidal cells

Cell suspensions were centrifuged over a column comprising 17% Nycodenz in the lowest level, 11.4% Nycodenz in the middle and GBSS at the surface. Parenchymal cell debris and red blood cells are found at the bottom of the column. A fraction comprising a mixture of Kupffer cells (K) and sinusoidal endothelial cells (E) is present at the interface between 17% and 11.4% Nycodenz. Because of their lipid content, fat-storing cells (F) are found at the interface between 11.4% Nycodenz and buffer.


F
11·4
K&E
17

above and cultured at 37°C in 95% O_2/5% CO_2. Non-adherent debris was removed by washing with medium at 18 hours.

All cultures were examined by phase contrast microscopy using a Zeiss inverted microscope IM35 and photographed at defined time intervals; aliquots of culture supernatants were removed, centrifuged on a Beckman microfuge to remove debris, and stored at -70°C at each time point. Cell counts were obtained from photographs of random fields (unit area = 0.0442 mm^2).

2.2.2 Characterization of isolated cells

Cultured cells were further characterized by (i) transmission electron microscopy, with and without enzyme histochemistry, (ii) scanning electron microscopy and (iii) phagocytosis of 0.8 μm latex particles (Difco, United Kingdom). Cultures for electron microscopy were washed in GBSS, fixed in 1.5% glutaraldehyde and post-fixed in 1% osmium tetroxide. For transmission electron microscopy, the cells were subsequently dehydrated in alcohol and embedded in Epon; 100 nm sections were cut using a Reichert Jung microtome, mounted on copper grids, stained with uranyl acetate and Reynold's lead citrate and viewed under a Phillips EM400 microscope at 80kV. Endogenous peroxidase activity in cultured cells was demonstrated using the method outlined in Appendix 1. For scanning electron microscopy, cells were critical point-dried and sputter-coated with gold palladium.

2.2.3 ELISA for measurement of secreted C3

C3 levels in culture supernatants were measured using a double layer antibody sandwich ELISA technique (Gaastra, 1984) (Appendix 6). Horseradish peroxidase-antibody conjugates were prepared using a periodate method (Appendix 7) with goat anti-rat C3 (IgG fraction; Cappell Laboratories, Oxford, England). O-phenylene diamine in phosphate/citrate buffer was used as the chromogen and optical densities were measured using a Titertek Multiscan MC (Flow) reader at 492nm. Standard curves were obtained using dilutions of pooled rat serum of known C3 concentration. Albumin levels in culture supernatants were also measured using a

similar ELISA method. Rabbit anti-rat serum albumin was kindly provided by Dr. R. Anthony. All results were expressed as ng protein/10^6 cells. The level of sensitivity for both C3 and albumin ELISAs was determined to be 2ng/ml.

2.2.4 Protein synthesis inhibition experiments

The protein synthesis inhibitor cycloheximide (Sigma, UK) was added to cultures for a period of 24 hours at concentrations of 0.1, 0.25, 0.5 and 1μg/ml. Control cultures received medium alone. Following exposure, culture supernatants were removed completely and the cells washed in fresh medium. Aliquots were subsequently removed at defined time intervals as outlined above.

2.2.5 Enhancement of C3 secretion by endotoxin (lipopolysaccharide)

Bacterial endotoxin or lipopolysaccharide (LPS) was purified from Salmonella Abortus Equi 1301 by a phenol extraction technique (Galanos *et al*, 1979). Lyophilized endotoxin was resuspended in DMEM, sonicated for 5 minutes and the resultant suspension exposed to ultra violet light for 10 minutes. Measured volumes were then added to sinusoidal cell cultures during the first 24 hours in culture after which time the supernatants were collected, the cells washed in DMEM and endotoxin-free medium added. Aliquots were removed as before for C3 estimations.

2.2.6 Immunolocalization studies

Intracellular C3 was immunolocalized in normal rat liver using an indirect immunofluorescent technique. Livers of adult male Wistar rats (~ 250g; n = 6) were perfused with phosphate buffer for 1 minute followed by 2% paraformaldehyde in 0.1M phosphate buffer for 10 minutes. Tissue was then mounted in OCT and frozen in liquid nitrogen at -70°C. Six μm sections were cut on an American Optical cryotome and mounted on albumin-coated glass slides. Sections were incubated with (i) glycine and (ii) 2% bovine serum albumin in PBS for

one hour at room temperature in a moist box. Excess fluid was then removed and sections were incubated with goat anti-rat C3 (50 μg/ml in 0.1% saponin/PBS) for 3 hours at room temperature. The sections were subsequently washed repeatedly in PBS and incubated with fluorescent isothiocyanate-conjugated anti-goat immunoglobulin (Hoescht, Germany; 1:20 dilution in 0.1% saponin/PBS) for one hour at room temperature. Following extensive washing in PBS, sections were subsequently mounted in Citifluor and viewed under a Leitz Orthoplan microscope with epifluorescence.

2.3 RESULTS

2.3.1 Characterization of isolated cells

By inversion microscopy, cells in the Kupffer cell fraction following elutriation exhibited a pericellular attachment zone within 45 minutes of being incubated in medium (Figure 2.3a). Within four hours there was spreading of the cells with the formation of long cytoplasmic extensions (Figure 2.3b). Using transmission electron microscopy, the cells were seen to have prominent surface pseudopodia, abundant cytoplasmic lysosomes and phagocytosed debris within vacuoles. Endogenous peroxidase activity was observed in over 85% of the cells in this fraction (Figure 2.4a). The principal contaminating cells were endothelial cells. Although occasional parenchymal cell blebs were seen in cell preparations during the first 24 hours in culture, no viable parenchymal cells were identified. By scanning electron microscopy, isolated Kupffer cells could be identified by the presence of abundant surface pseudopodia (Figure 2.5). Over 70% of the cells in the Kupffer cell fraction phagocytosed 0.8 μm latex beads *in vitro* (Figure 2.4b). Kupffer cells could be maintained for up to 7 days after which time the cells began to detach from the culture dishes.

Isolated endothelial cells showed cytoplasmic vacuolation by inversion microscopy (Figure 2.6). The cells were more elongated than isolated Kupffer cells and did not contain cytoplasmic debris. By transmission and scanning electron microscopy, some of the cytoplasmic vacuoles could be identified as fenestrae of similar size to those found *in vivo* (Figure 2.7). Less than 5% of cells in this fraction showed endogenous peroxidase activity or latex phagocytosis.

Figure 2.3 Phase contrast inversion microscopy of isolated rat Kupffer cells.

a. Isolated Kupffer cells after one hour in culture. A clear pericellular attachment zone can be readily identified around most of the cells in this preparation.

b. Primary culture of Kupffer cells at 24 hours. Long cytoplasmic processes can now be identified. Many of the dense cytoplasmic bodies seen by inversion phase microscopy represent lysosomes.

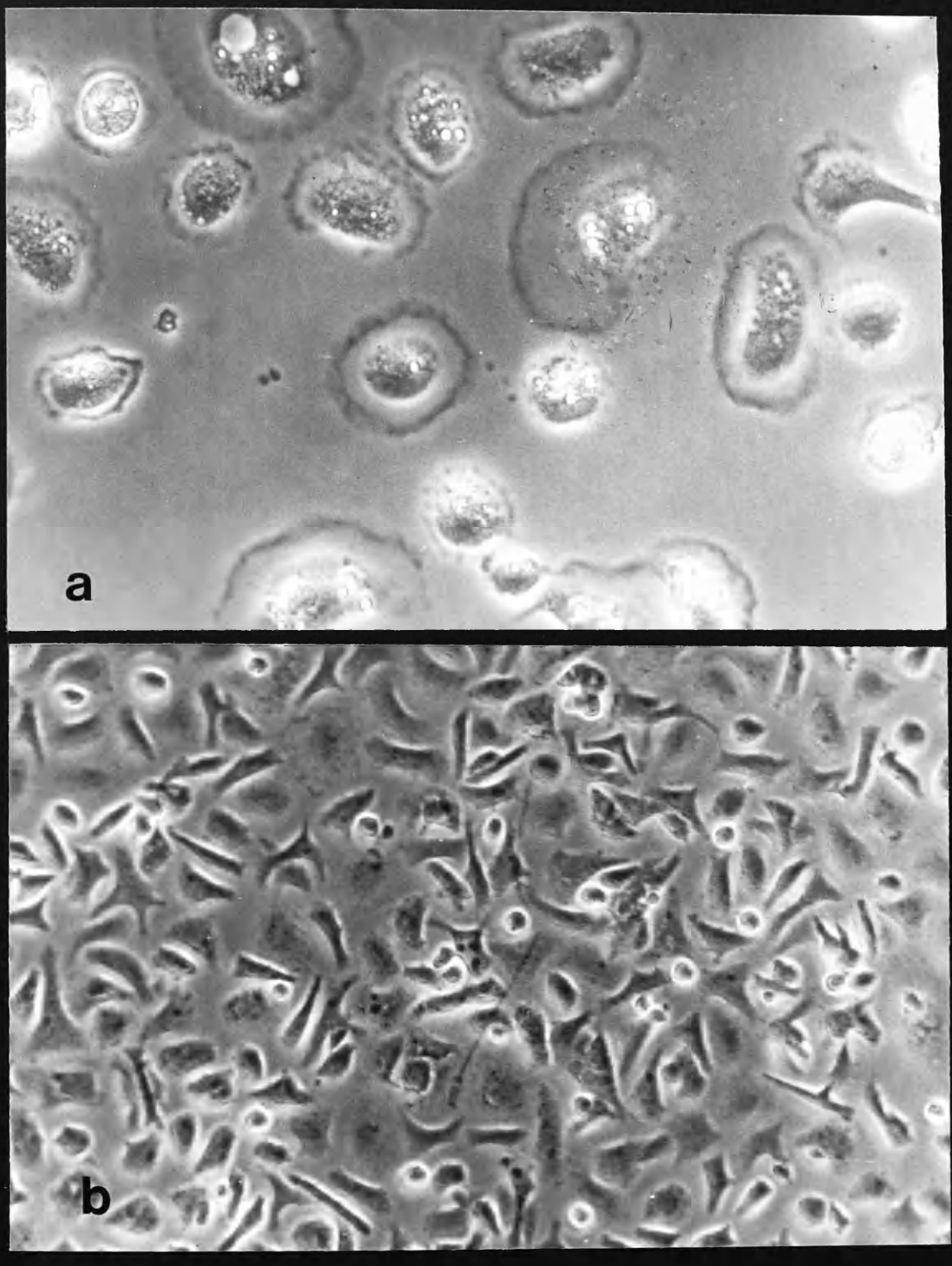
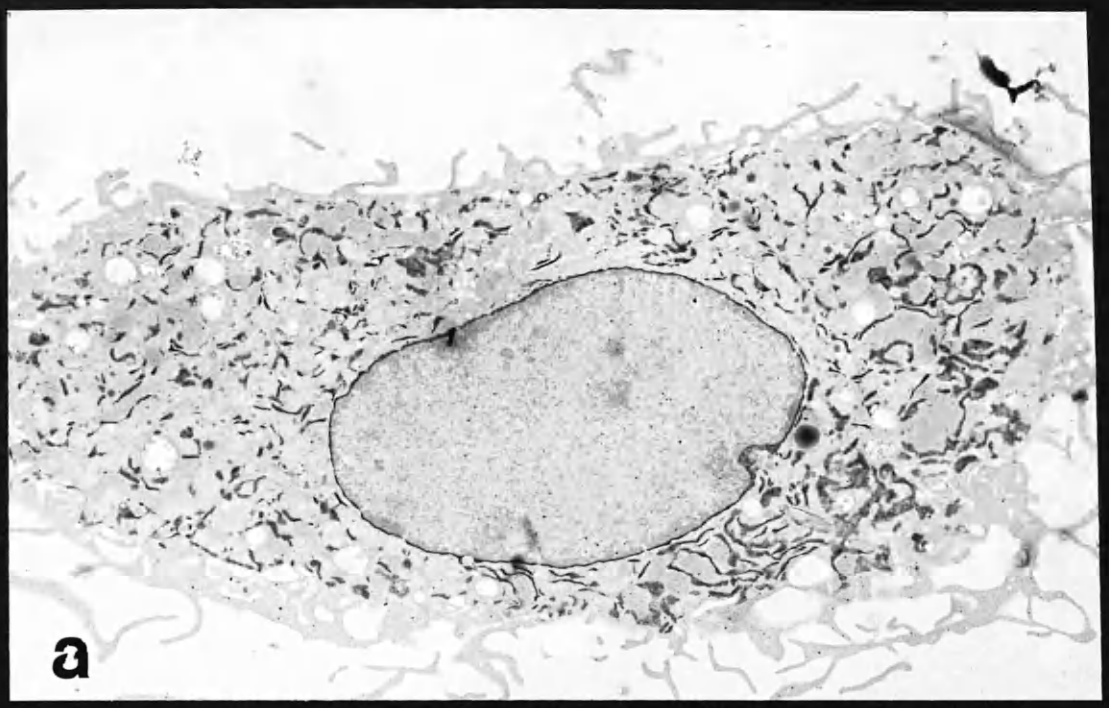

a
b

Figure 2.4 Characterization of isolated rat Kupffer cells using enzyme histochemistry and latex phagocytosis.

a. Enzyme histochemistry/transmission electron microscopy of isolated Kupffer cell. Endogenous peroxidase activity can be identified within the endoplasmic reticulum and nuclear envelope. (Modified Graham and Karnovsky's method (Appendix 1); uranyl acetate/lead citrate; x 12,000)

b. Phase contrast inversion microscopy of isolated rat Kupffer cells incubated with a suspension of 0.8 μm latex particles. Over 70% of this cell fraction showed phagocytosis of latex particles; the presence of phagocytosed particles within the cell cytoplasm was confirmed by transmission electron microscopy (not shown).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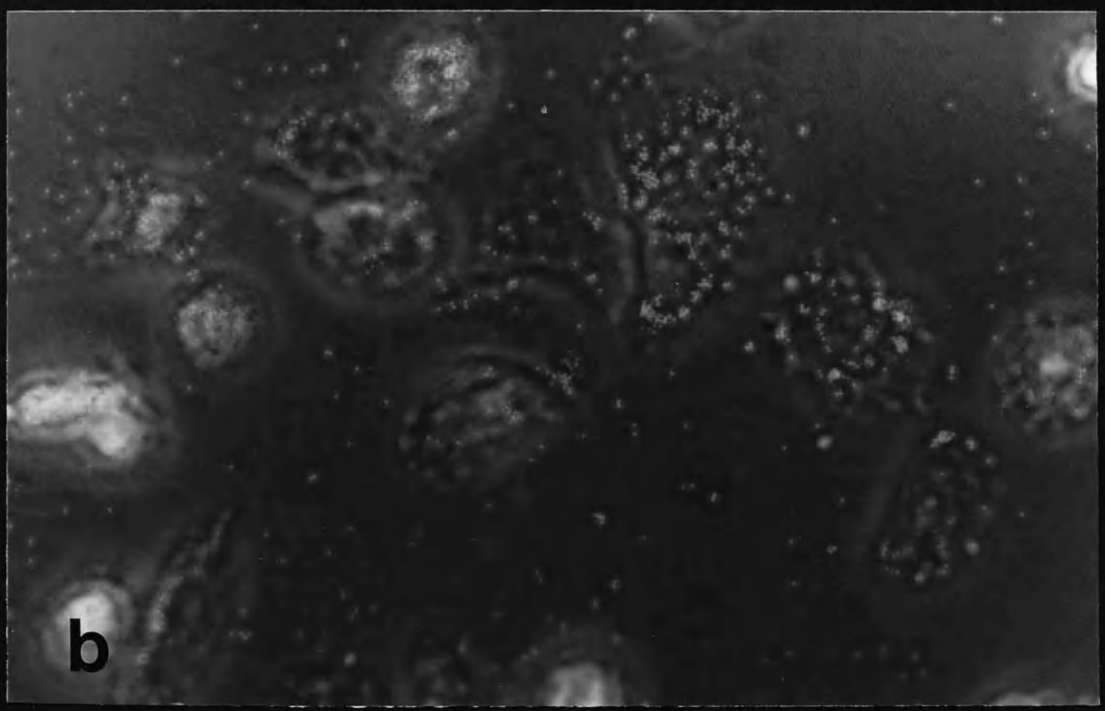
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Figure 2.5 Scanning electron micrograph of isolated rat Kupffer cell.

Rat Kupffer cells after 24 hours in culture were characterized by irregular cell borders and by the presence of prominent surface pseudopodia. (Gold palladium; x 6,000).

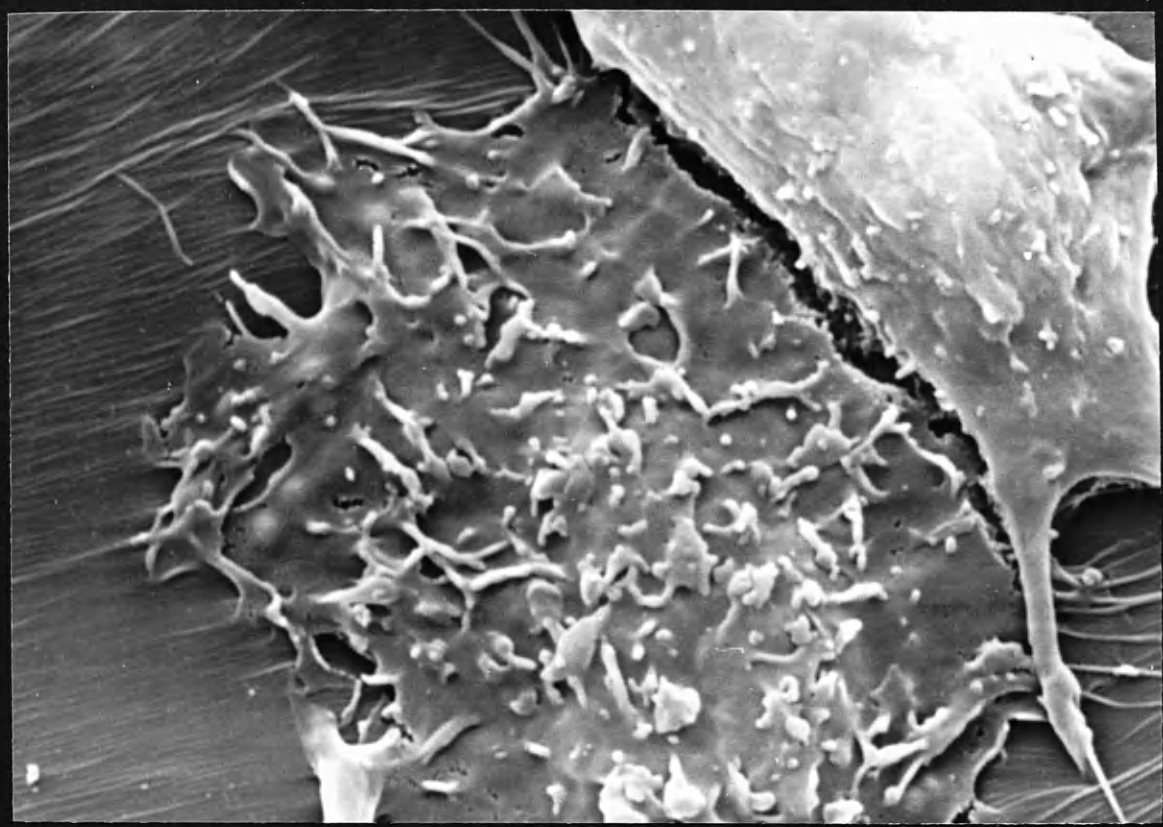
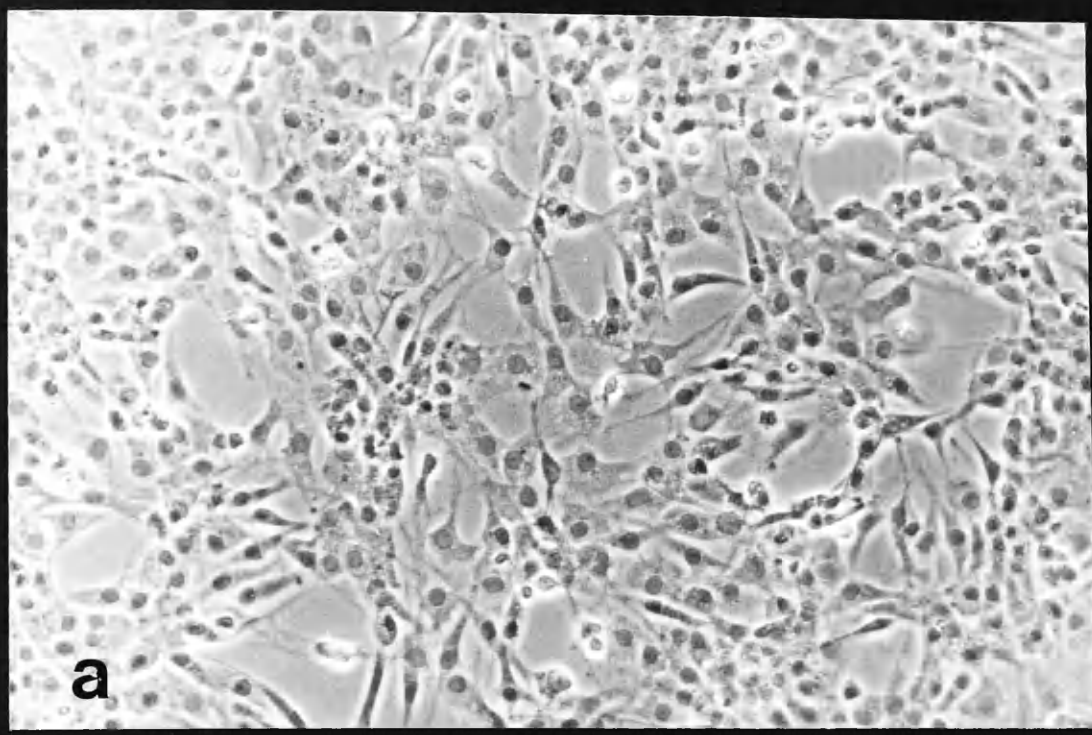


Figure 2.6 Phase contrast inversion microscopy of isolated rat sinusoidal endothelial cells.

a. At a low power of magnification, these cells can be seen to have a different growth pattern to that of Kupffer cells (cf. Figure 2.3b).

b. In 24 hour-old cultures, cytoplasmic vacuoles could be readily identified; these were considered to represent fenestrae.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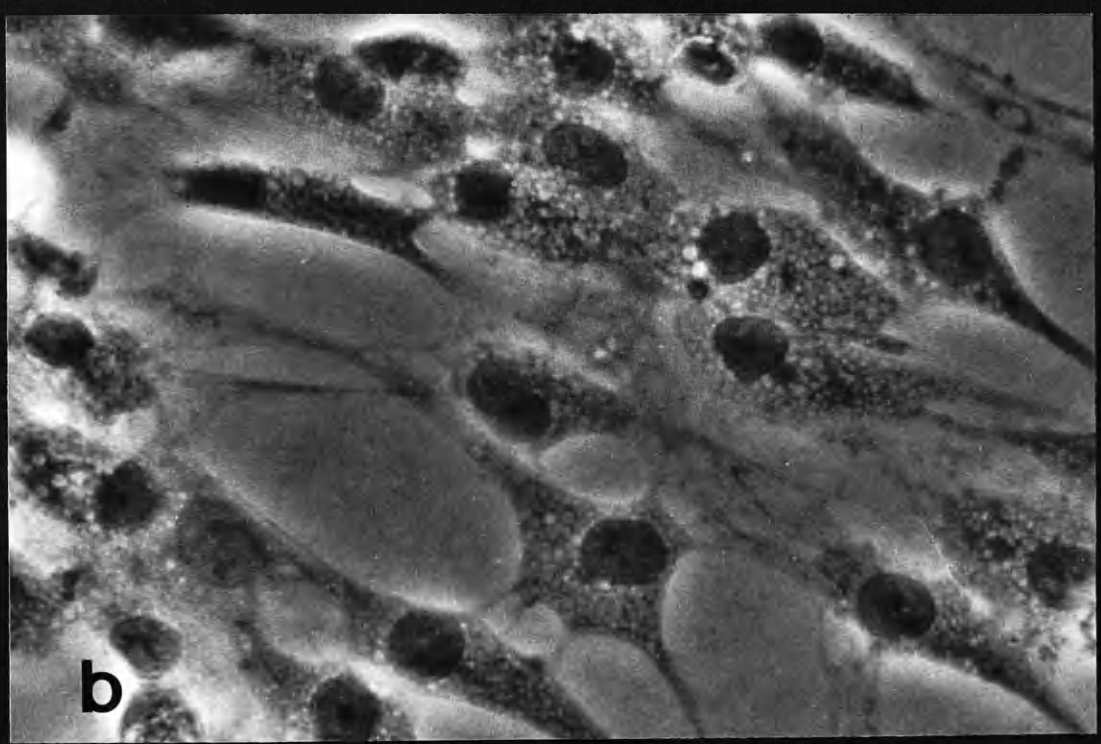
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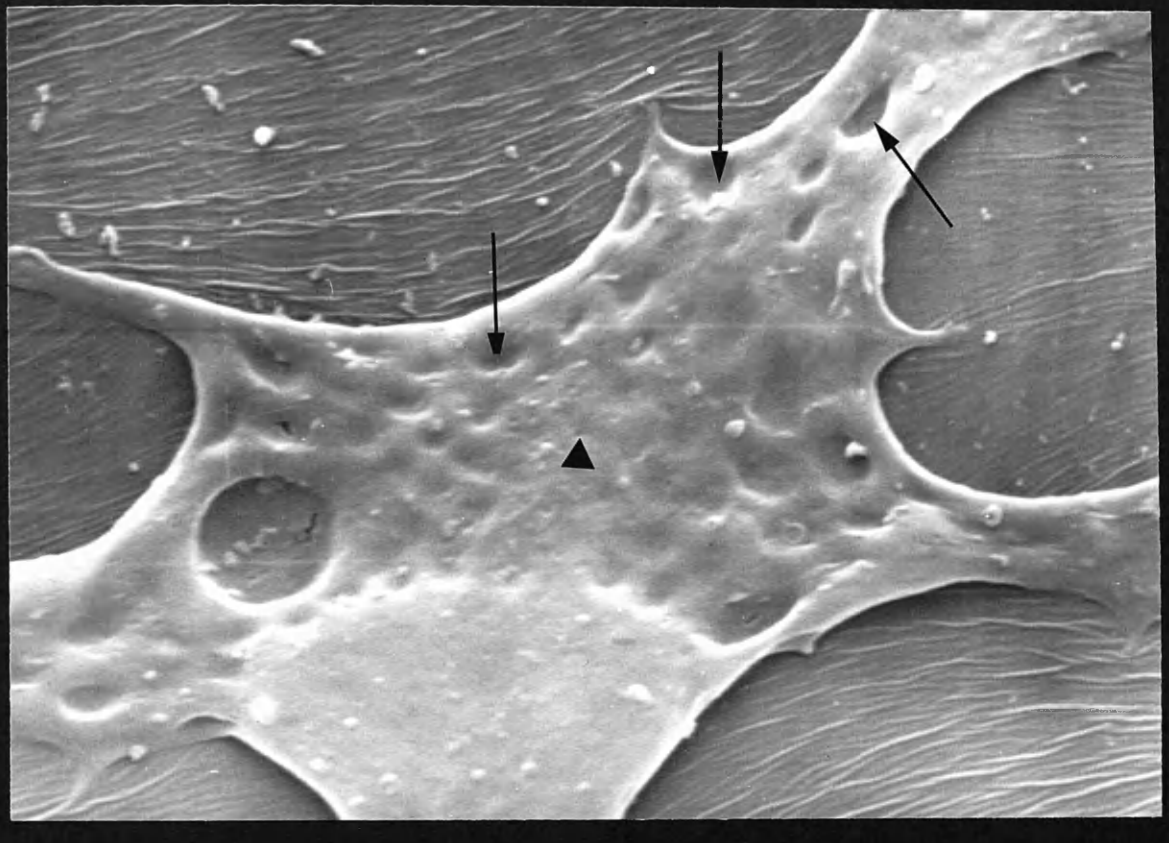
Figure 2.7 Scanning electron micrograph of isolated rat sinusoidal endothelial cell.

In this 48 hour-old cultured endothelial cell, occasional fenestrae are visible (arrowhead). However, in addition larger pores are now seen (arrows) possibly as a result of coalescence of sieve plates. (Gold palladium; x 6,000).



By inversion microscopy, cells in the fat-storing cell fraction initially appeared as round cells with abundant cytoplasmic translucent droplets (Figure 2.8). After 48 hours there was spreading of the cells and evidence of cell division; by day 5 there was near-confluent growth of cells. These cultures could subsequently be passaged. Transmission electron micrographs confirmed the presence of abundant osmiophilic cytoplasmic droplets and showed a well developed rough endoplasmic reticulum. Further description of the ultrastructural appearances of these cells is included in Chapter 4. Five to 10% of cells in this fraction showed endogenous peroxidase activity; these rarely contained lipid and were considered to represent contaminating Kupffer cells.

2.3.2 C3 secretion by isolated sinusoidal cells

C3 was detected in Kupffer cell supernatant samples obtained after the initial 24 hours in culture at a mean level of 26.7 ± 14.1 ng/10^6 cells/24 hours (Figure 2.9). Thereafter, C3 content in Kupffer cell supernatants increased, reaching a peak at 72 hours before falling at 120 hours.

Absolute values obtained showed little variation between triplicate cultures of cells isolated from individual animals. However, considerable variation between levels in supernatants from cells isolated from different animals was noted accounting for the large standard errors illustrated in Figure 2.9. Albumin was detected in the supernatant samples taken at 24 hours at a level of 61 ng ± 5.8/10^6 cells but was not detectable in later cultures.

No C3 could be detected by ELISA in any sinusoidal endothelial cell culture supernatants. It should be noted however that after 72 hours in culture, loss of attachment in many cells was noted and cell viability as assessed by trypan blue exclusion was less than 20%. Albumin was detectable in endothelial cell culture supernatants at level of 40 ng ± 8.9/10^6 cells in samples obtained at 24 hours but was not identified in later cultures.

Finally although C3 was undetectable in early fat-storing cell cultures, low levels were found in day 5 cultures (25.4 ± 4.9 ng/10^6 cells). Albumin was again found in samples obtained at 24 hours (35 ± 6.7 ng/10^6 cells) but was absent from later cultures.

Figure 2.8 Phase contrast inversion microscopy of isolated rat liver fat-storing cells.

In this 24 hour-old culture, the isolated cells can be seen to have regular outlines. Intracytoplasmic lipid droplets are readily identified at the periphery of cells as refractile granules by phase contrast microscopy.

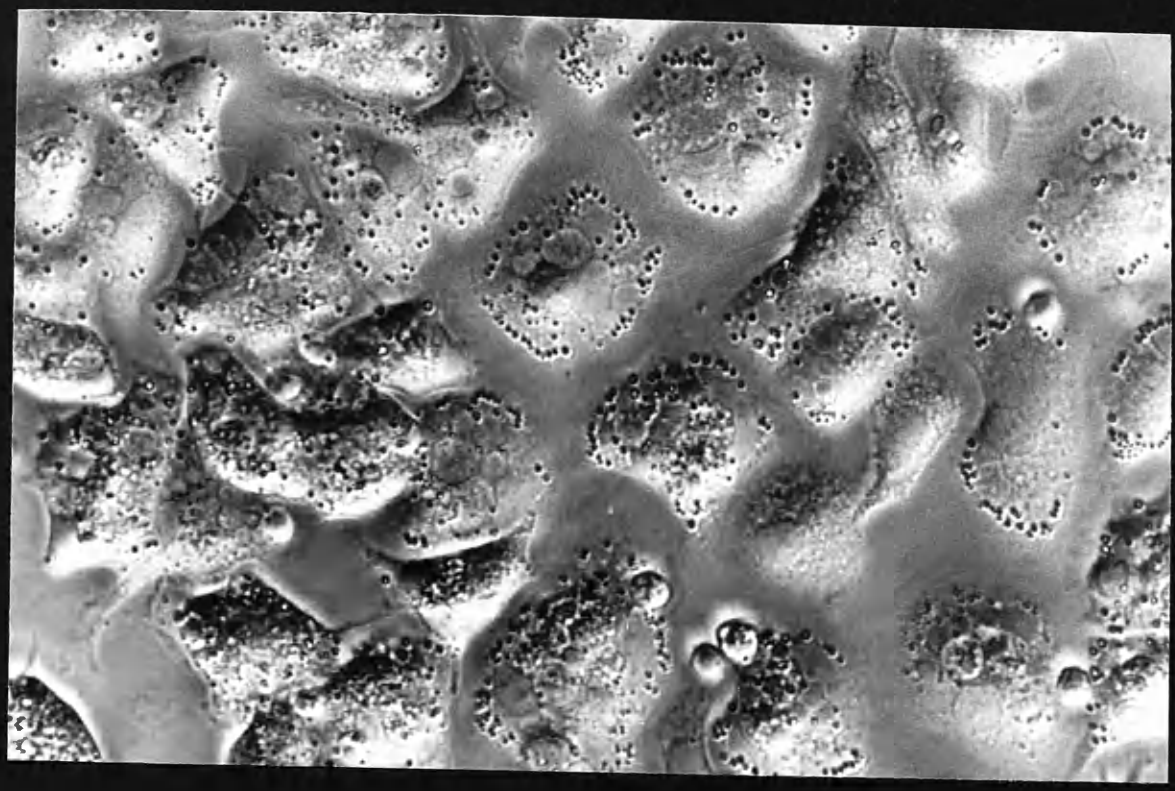
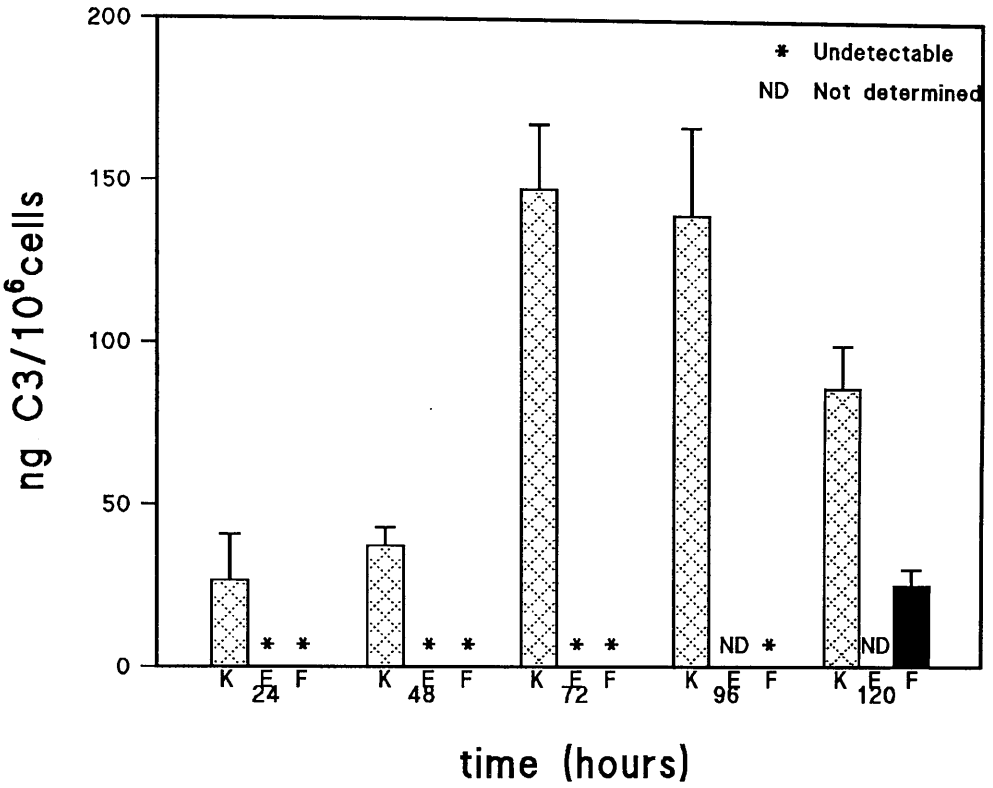


Figure 2.9 C3 secretion by isolated sinusoidal cells : comparison between Kupffer cells, sinusoidal endothelial cells and fat-storing cells.

Each value is the mean value (+ SEM) obtained from cell cultures derived from eight animals. (Triplicate cultures were performed in each animal). Following an initial lag phase, C3 secretion by Kupffer cells can be seen to reach a peak at 72 hours. As supernatants were completely removed at each time point, C3 secretion during the period between 48 and 72 hours was estimated to be 6.16 ng/10^6cells/hour. No C3 was detectable in endothelial cultures.

K: Kupffer cells; E: sinusoidal endothelial cells; F: fat-storing cells.


200
150
100
50
0
ng C3/10^6cells
* Undetectable
ND Not determined
K E F
24
48
72
96
120
ND
time (hours)

The results obtained in all three sinusoidal cell types are illustrated in Figure 2.9. As these indicated that Kupffer cells secreted substantially more C3 than either endothelial cells or fat-storing cells in primary culture, further studies dealing with protein synthesis inhibition and stimulation by endotoxin were limited to Kupffer cells.

2.3.3 Protein synthesis inhibition experiments in Kupffer cell cultures

Addition of the protein synthesis inhibitor cycloheximide to Kupffer cell cultures during the first 24 hours in culture produced a significant reduction in C3 levels in a dose dependant manner (Figure 2.10). However, with doses of 0.25 and 0.5 μg/ml, there was a return to normal levels by 48 hours after removal of cycloheximide.

2.3.4 Stimulation of C3 secretion by endotoxin

Addition of purified endotoxin at levels of 0.05 μg and 0.1 μg/ml to Kupffer cell cultures produced no significant effects on C3 levels in culture supernatants (Figure 2.11). By contrast, in cultures which had been exposed to 0.5 μg/ml or 1 μg/ml endotoxin during the initial 24 hours in culture, there was evidence of stimulation of C3 release. Elevated levels of C3 were noted in culture supernatants at 48 hours (ie. 24 hours after removal of endotoxin). The stimulatory response was more marked at 72 hours at which point C3 levels in cultures treated initially with 1 μg/ml endotoxin were 3.8 times greater than those in untreated cultures ($p < 0.001$).

2.3.5 Immunolocalization studies

Staining for C3 was noted in parenchymal cells. The intensity of immunofluorescent staining varied between cells. In some there was negligible signal while in others there was intense labelling. Punctate staining was noted within the cytoplasm of some cells possibly representing C3 within the endoplasmic reticulum (Figure 2.12). In all preparations, immunoreactivity was greater in perivenular zones than in periportal zones.

Figure 2.10 C3 secretion by isolated Kupffer cells : reversible inhibition following addition of the protein synthesis inhibitor, cycloheximide.

Triplicate cultures of isolated Kupffer cells were incubated with varying concentrations of cycloheximide during the initial 24 hours in culture. C3 levels in culture supernatants were then compared to control cultures. After exposure to 0.25 and 0.5 μg/ml cycloheximide there was an initial decrease in C3 levels compared to control values but levels subsequently returned towards normal by 72 hours. Cycloheximide at a dose of 1 μg/ml appeared to be toxic to the cultured Kupffer cells although viable cells could still be seen by inversion microscopy.

This experiment was repeated with cultures from four different animals and produced similar values to those plotted in this graph.

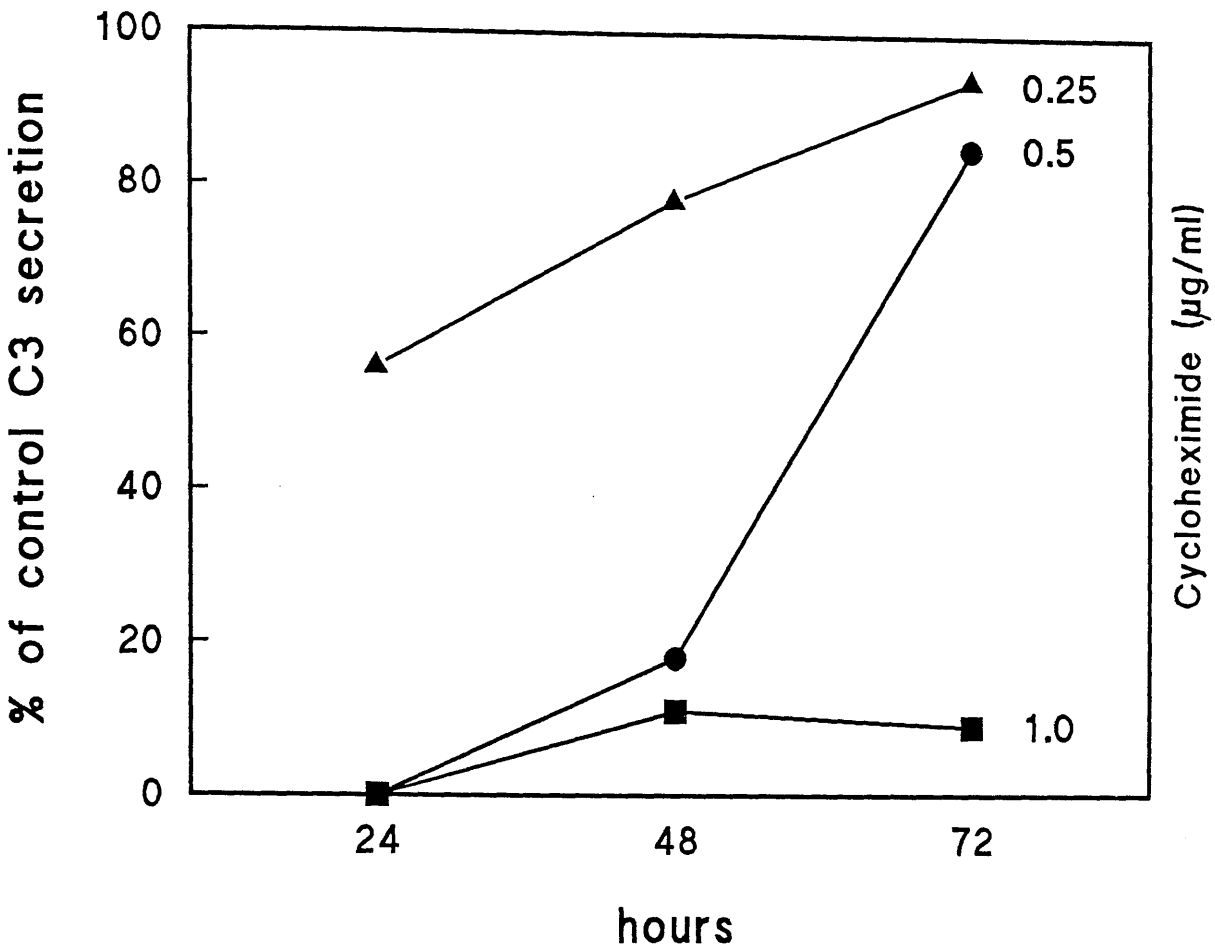

100
80
60
40
20
0
% of control C3 secretion
24
48
72
hours
0.25
0.5
1.0
Cycloheximide (µg/ml)

Figure 2.11 Stimulation of C3 release by rat Kupffer cells following incubation with bacterial endotoxin.

Each point on the graph represents the mean (+ SEM) of triplicate cultures from each of nine animals. The effects of addition of varying concentrations of S. abortus equi endotoxin during the initial 24 hours in culture on C3 release is illustrated. It can be seen that with doses of 0.5 and 1 μg/ml a significant increase in C3 levels compared with controls is found at 48 hours (Student's t test : $p < 0.05$) and that this response is sustained (72, 96 and 120 hours cf. controls $p < 0.001$ using Student's t test). Endotoxin at lower doses had no such stimulatory effect while higher doses (not shown) were toxic for the cells.

Controls (no endotoxins) : ----------

0.05 μg/ml endotoxin : .....

0.1 μg/ml endotoxin : — — —

0.5 μg/ml endotoxin : - - - - -

1.0 μg/ml endotoxin : _____

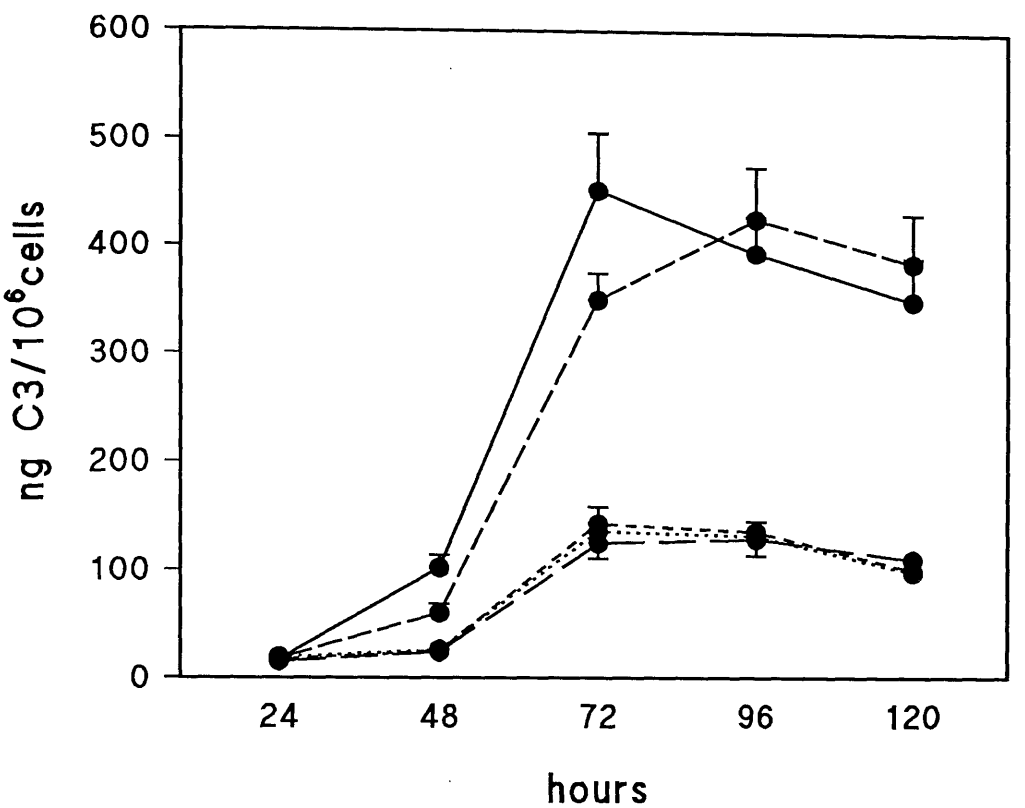

600
500
400
300
200
100
0
ng C3/10^6cells
24
48
72
96
120
hours

Figure 2.12 Immunolocalization of C3 in normal rat liver.

a. Immunofluorescent staining for the protein can be seen here within the cytoplasm of parenchymal cells. In many cells the signal is present diffusely throughout the cytoplasm although in some punctate staining can be identified possibly representing C3 within the endoplasmic reticulum. Immunoreactivity in parenchymal cells was variable although the signal was most intense in perivenular zones.

b. In areas where parenchymal cell staining was weak such as in this micrograph, immunoreactivity for C3 can be identified within a population of sinusoidal cells. (Polyclonal anti-rat C3 (Cappel Laboratories; 1:120); indirect immunofluorescence on 2% paraformaldehyde perfusion-fixed tissue). Magnifications: a - x 850; b - x 600.

a
b

In areas in which parenchymal cell staining was weak, immunoreactivity could also be readily identified within a population of sinusoidal cells. These were generally large cells which protruded into the lumen of the sinusoids. The morphological characteristics of these cells suggested that they were Kupffer cells. The immunofluorescent signal within these cells was present throughout the cytoplasm. In some, intensely staining granules were observed (Figure 2.12b). No staining was observed in control sections in which the primary antibody was replaced by normal goat serum.

2.4 DISCUSSION

By virtue of its central role in the activation cascade, the third component of complement, C3 has probably been more extensively studied than any other complement protein. It is a glycoprotein of molecular weight 183kD (DeBruijn & Fey, 1985) which is present in (human) serum at a concentration of approximately 1.2 mg/ml (Kohler & Muller-Eberhard, 1967). The mature protein is composed of two polypeptide chains (α and β) linked by a thioester bond; this site is of functional importance in opsonization following activation to C3b (Levine & Dodds, 1990). However, it is initially synthesized as a single-chain precursor within which the α and β chains are linked by a tetra-arginine linker (Strunk *et al*, 1988). Post-translational alterations leading to the glycoylated mature two-chain form occur within the Golgi apparatus (Bednarczyk & Capra, 1988).

Synthesis of C3 has previously been described in at least six distinct cell types : (i) liver parenchymal cells (Ramadori *et al*, 1984; Anthony *et al*, 1985), (ii) extrahepatic cells of the mononuclear phagocyte system (Johnson & Hetland, 1988), (iii) fibroblasts and related cell types (Katz & Strunk, 1988; Katz *et al*, 1989), (iv) endothelial cells (Ueki *et al*, 1987), (v) astrocytes (Lévi-Strauss & Mallat, 1987), and (vi) type II pneumocytes (Strunk *et al*, 1989).

The ability of macrophages to synthesize and secrete C3 was described as early as 1965. In a series of experiments, Stecher and co-workers (Stecher *et al*, 1965; Stecher & Thorbecke, 1967) demonstrated that peritoneal and alveolar macrophages from several species (guinea-pig, mouse, rat and rabbit) secreted immunoreactive C3 into culture supernatants; that this secreted C3 was functionally active was not confirmed until 1976 (Bentley *et al*, 1976). Since then a large

number of studies have shown that peritoneal and alveolar macrophages from a number of mammalian species have the capacity to synthesize a wide range of complement components (Brade & Kreuzpainter, 1982; Newell *et al*, 1982). Studies with human material have also shown that circulating monocytes (Whaley, 1980) breast milk macrophages (Cole *et al*, 1982) and synovial tissue macrophages (De Ceulaer *et al*, 1980) share this property. Recent work indicates that some human mononuclear phagocytes may possess the capacity to synthesize all components of the complement system including those of the terminal attack sequence (Hetland *et al*, 1986).

The results presented here indicate that Kupffer cells may also be involved in complement biosynthesis. To my knowledge, there are no previous reports dealing with the production of C3 by these cells. An earlier study by Ilgen & Burkholder (1974) suggested that non-parenchymal cells isolated from guinea-pig liver may have the ability to synthesize C4 but the isolated cells were poorly characterised. The isolation method used in the current study provided a good yield of Kupffer cells of high purity as assessed by transmission and scanning electron microscopy and enzyme histochemistry. The capacity for the isolated cells for latex phagocytosis provides further support although this cannot be used as an absolute discriminant (see Chapter 1). The absence of viable parenchymal cells from these cultures excludes the possibility that C3 detected in supernatants was derived from contaminating parenchymal cells. This is supported by the lack of detectable albumin in supernatants from 24 hours; expression of this protein being restricted to parenchymal cells. It is likely that albumin found in samples taken at 24 hours was released from parenchymal cell blebs; these could be identified by transmission electron microscopy in early cultures. While detection of C3 in supernatants indicates release of the protein by Kupffer cells, it does not demonstrate active synthesis of the protein by cells in culture. However, the reversible inhibition in C3 release observed with the addition of cycloheximide suggests that Kupffer cells do actively synthesise this component *in vitro*.

The results obtained using indirect immunofluorescence provide further support for synthesis of C3 by rat Kupffer cells. Although the predominent signal was noted within parenchymal cells in keeping with the studies of Nagura *et al* (1985) in human liver, intracellular C3 was also found in sinusoidal cells with morphological features of Kupffer cells. It should be

noted however, that demonstration of cytoplasmic staining for a serum protein may only be regarded as supportive evidence for active synthesis by a cell; it is impossible to exclude by light microscopy whether the intracellular signal represents detection of endocytosed protein. Indeed, the intense immunoreactivity noted within large granules in these cells may represent lysosomal protein in endocytosed immune complexes. More conclusive evidence from immuno-histochemistry requires localization at the ultrastructural level with identification of immuno-labelling on the endoplasmic reticulum or Golgi apparatus. Unfortunately, attempts to date using the method outlined in Chapter 4 have resulted in high levels of background labelling, precluding any assessment of ultrastructural localization of C3.

Further confirmation of C3 synthesis by Kupffer cells could be obtained by (i) radio-labelled amino acid incorporation studies with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) analysis of newly synthesised proteins and (ii) identification of mRNA for C3. In preliminary studies, isolated Kupffer cells were labelled with [^{35}S]-methionine and immunoprecipitable C3 in cell lysates and supernatants measured. These studies indicated that C3 accounted for 0.09% total protein secretion by the cells. Application of immunoprecipitated C3 to 5% SDS PAGE gels revealed a very faint band at the 120kD position in keeping with that of the α chain of C3 although most of the radioactivity was seen at the gel front indicating degradation of the protein.

How do Kupffer cells compare with other mononuclear phagocytes in their rate of synthesis and secretion of C3 ? In my preliminary studies, the rate of release of C3 by isolated rat Kupffer cells was found to be significantly less than that of rat peritoneal macrophages (Burt *et al*, 1986a). However, in these earlier experiments Kupffer cells were purified by selective dish adherence rather than the elutriation method described above. Furthermore, measurements were only obtained during the initial 48 hours in culture. From the results presented in this chapter it can be seen that there is an inital lag phase in C3 release by Kupffer cells, possibly resulting from the effects of the pronase and collagenase used during isolation on cell biosynthetic function. The peak level of C3 secretion (147 ng/10^6cells/24 hours) noted in the present study is comparable to that found for guinea-pig macrophages by Zimmer *et al* (1982).

The stimulatory effect of endotoxin on C3 secretion by Kupffer cells has been noted in previous studies using mononuclear phagocytes. Strunk *et al.* (1985) demonstrated a significant

increase in C3 synthesis in human monocytes following exposure to endotoxin and Goodrum (1987) noted two- to four-fold enhancement in mouse macrophage-like cell lines over a range of endotoxin concentrations. In the present study, there was an elevation of C3 levels in samples taken 24 hours after exposure to 0.5 or 1 μg/ml endotoxin. No such stimulatory effect was noted with lower doses and higher concentrations were found to be toxic for the cultured cells. The augmentation of C3 release by these doses of endotoxin was sustained with a peak at 48 hours following exposure when there was a three fold increase in C3 levels in supernatants.

How do Kupffer cells compare with parenchymal cells with respect to C3 synthesis and secretion? No attempt was made in the present study to measure C3 secretion by isolated rat liver parenchymal cells. Although Anthony *et al* (1985) analysed the kinetics of C3 secretion by rat parenchymal cells in primary culture, they used a functional assay to detect the protein. As the ELISA used here detects both cleaved and functional protein, a direct comparison is not possible. However, Ramadori *et al* (1984) demonstrated a secretion rate of 100 ng/10^6 cells/hour for isolated guinea-pig liver parenchymal cells. If we assume that a similar level would be found with rat parenchymal cells, then Kupffer cells produce approximately 16-fold less C3 than parenchymal cells. Given the morphometric data indicating that there are around seven times more parenchymal cells than Kupffer cells in rat liver (Blouin, 1977), it can be concluded that Kupffer cells contribute to less than 1% of total hepatic C3 production. However, synthesis and secretion of this protein may be important in local host defence within the sinusoids. These cells are known to express receptors for activated C3 and to be involved in clearance of endotoxin, which itself can activate the alternative pathway of complement. Augmentation of C3 secretion by endotoxin may be of importance in the hepatic handling of gut-derived bacterial proteins such as endotoxin (Steffan & Kirn, 1986).

Although Ueki *et al* (1987) demonstrated C3 secretion by human capillary endothelial cells obtained from hypertrophic scar tissue, I was unable to demonstrate any C3 release by cultured rat sinusoidal endothelial cells. This may represent a further manifestation of functional differences between sinusoidal endothelial cells and other forms of endothelium. It should be noted, however, that viable cultures could only be maintained for 72 hours and [^{35}S]-methionine incorporation studies indicated that protein synthesis may be impaired as early as 12 hours.

Similar methodological difficulties with sinusoidal endothelial cell culture have been experienced by other groups (Brouwer *et al*; Friedman *et al*; personal communications) although culture on other substrata such as collagen type IV may improve *in vitro* viability.

Low levels of C3 were detectable in fat-storing cell cultures after 120 hours in culture. As discussed in Chapter 4, these cells undergo phenotypic changes *in vitro*; by 120 hours in primary culture they adopt a myofibroblast-like appearance. As skin fibroblasts (Katz *et al*, 1989) and synovial fibroblast-like cells (Katz & Strunk, 1988) have been shown to synthesize C3, release of this protein by fat-storing cells after 5 days may reflect an *in vitro* shift in functional activity towards that of (myo)fibroblasts. It seems likely therefore that fat-storing cells do not contribute to C3 synthesis in normal liver. However, it is possible that in parallel with the phenotypic changes seen in these cells following activation in response to liver injury (Chapter 5) they may begin to express C3, albeit at low levels.

In summary, rat Kupffer cells have the capacity to synthesize and secrete the most abundant complement protein, C3. Although they are likely to contribute less than 1% of total hepatic C3 production in normal liver, the observation that this may be enhanced by exposure to endotoxin suggests that C3 release by Kupffer cells may play an important role in the local intrahepatic host response to bacterial toxins. Furthermore, as discussed in Chapter 9, although the direct contribution to hepatic C3 production may not be substantial, they may, through the release of mediators such as interleukin 6, play an important indirect role in regulating serum levels of the protein by modulating C3 synthesis in parenchymal cells. The role of sinusoidal liver cells in the synthesis and secretion of other complement proteins remains to be determined. Liver parenchymal cells have been shown to synthesize several other components including C2, C4 (Anthony *et al*, 1985), C1q, C1r, C1s (Ramadori *et al*, 1986), C8 (Ng & Sodetz, 1987) C9 (Ramadori *et al*, 1985) and Factor B (Anthony *et al*, 1985). The possiblity that Kupffer cells may also contribute to the hepatic synthesis of these proteins should now be explored. Preliminary studies using ELISA demonstrated immunodetectable C4 in Kupffer cell culture supernatants from the above experiments. However, the antibody used was raised against human C4 and the sensitivty of the ELISA was consequently poor; this should now be repeated with an immunoassay using anti-rat C4. Indirect immunofluorescence using the anti-human C4

antibody did, however, show intracellular labelling of cells which had morphological features of Kupffer cells.

CHAPTER 3. THE EXTRACELLULAR MATRIX OF THE SINUSOIDS: ALTERATIONS IN LIVER DISEASE

3.1 INTRODUCTION

The constituent cells of all tissues are surrounded by a complex assembly of macromolecules which constitute the "connective tissue" or extracellular matrix. The precise composition of the matrix is unique for each tissue; the amount of extracellular matrix, as a proportion of total volume, is less in internal organs such as liver than in other tissues such as bone or cartilage (Schuppan, 1990). It was previously assumed that this connective tissue merely served to provide a structural framework. However, recent studies have indicated that, far from being an inert scaffolding, the extracellular matrix in all tissues exerts important effects on the differentiation, proliferation and metabolic functions of surrounding cells (Bissell & Choun, 1988). Thus, it not only provides cohesiveness within tissue compartments, but induces polarization of cells and modulates gene expression (Ben Ze'ev *et al*, 1988; Bucher *et al*, 1990; Lindblad *et al*, 1991).

3.1.1 Composition of the extracellular matrix

In recent years, there has been substantial progress in our understanding of the composition of the extracellular matrix. It is now recognized to contain a large number of genetically distinct proteins, many of which interact not only with other components of the matrix, but also with surrounding cells via specific membrane receptors, so-called integrins (Hynes, 1987; Albelda & Buck, 1990). Four classes of extracellular matrix proteins are recognized : (i) collagens, (ii) non-collagenous structural glycoproteins, (iii) proteoglycans and (iv) elastin.

Collagens

The collagens, a family of highly conserved molecules, represent the most abundant proteins in mammals, accounting for almost one third of total protein in man. Since the isolation of the most abundant collagen (type I) in 1969 (reviewed in Miller & Gay, 1987), a further twelve collagens have been identified and at least partially characterized (Burgeson, 1988). Collagens share common structural properties which are unique to this family of

molecules. Each is composed, at least in part, of a triple helical structure formed from three protein chains (α chains); in some collagens (eg. type III) the individual α chains are identical whereas in others the molecule is composed of non-identical chains (Miller & Gay, 1987). The helical arrangement provides the molecules with structural stability; it is made possible by the presence of large stretches within the polypeptide that are composed of the repetitive sequence $(Gly\text{-}Xaa\text{-}Yaa)_n$ with a high frequency of proline in positions Xaa or Yaa and 4-hydroxyproline in position Yaa. Collagen-specific post-translational modifications introduced into the triple helix include hydroxylation of lysine and proline residues and the glycosylation of hydroxylysine; these processes occur within the endoplasmic reticulum and require several co-factors including ascorbic acid, ferrous ions and copper (Kivirikko & Myllylä, 1982).

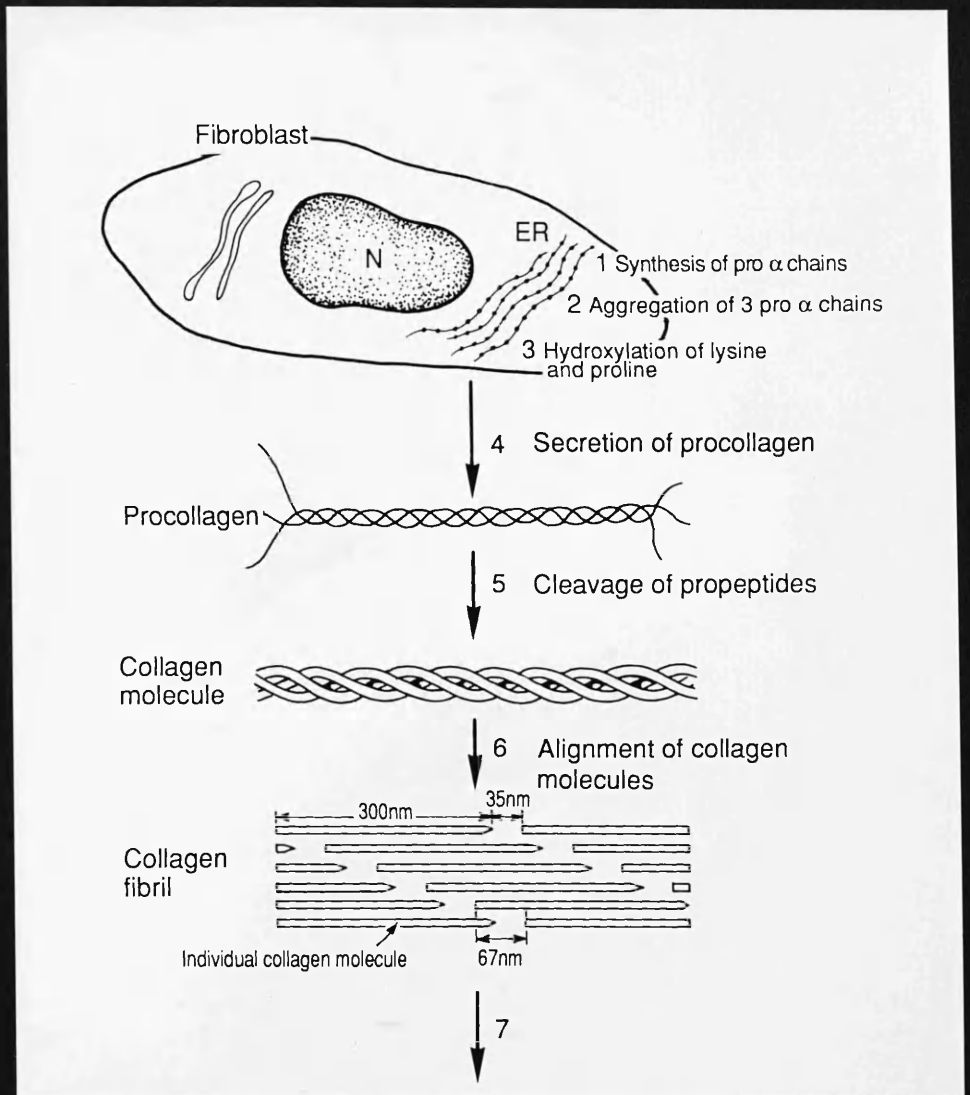
The principal steps in the biosynthesis of the most abundant (fibril-forming) collagens, the so-called interstitial collagens (I, II, III and V), are outlined in Figure 3.1. They are initially synthesized as triple helical precursor molecules (Fessler & Fessler, 1978). At the amino and carboxy-terminal ends of the procollagen molecule are non-helical (globular) domains which are required for intracellular molecular processing. However, following secretion of the procollagen, the globular domains (propeptides) are removed by specific endopeptidases. The cleaved propeptides are soluble and are efficiently removed, although some may remain within the extracellular matrix. They are detectable in serum; quantitation provides a measure of active collagen synthesis (Rohde *et al*, 1979). The so-called tropocollagen molecules thus formed aggregate to form collagen fibrils (Piez, 1984). These have a banded appearance by transmission electron microscopy by virtue of the staggered arrangement of molecules in a fibril. This also contibutes to the tensile strength of the fibrils and is reinforced by cross-linking of lysyl and hydroxlysyl residues. Finally, the fibrils aggregate together to form collagen fibres with diameters of 1 to 10 μm.

Other less abundant collagens, such as type IV collagen which is found in basement membranes, are structurally distinct from the major fibril-forming interstitial collagens. The procollagen molecule of type IV collagen is considerably longer and the terminal propeptides are not removed by endopeptidases. Instead the propeptides are utilized to cross-link the molecule into a three dimensional network (Timpl *et al*, 1981). Four amino terminal propeptides condense to form a tetramer (7-S collagen) while two carboxy-terminal propeptides

Figure 3.1 Outline of principal steps in biosynthesis of interstitial collagens.

1. Synthesis of pro-α chains in rough endoplasmic reticulum.
2. Aggregation of 3 pro-α chains.
3. Hydroxylation of lysine and proline residues.
4. Secretion of procollagen molecule.
5. Cleavage of propeptides to form tropocollagen.
6. Alignment of collagen molecules to form fibrils.
7. Aggregation of fibrils to form collagen fibre. By transmission electron microscopy individual fibrils can be seen to exhibit a banded appearance.

Reproduced from Whaley and Burt (1991) with kind permission of the publishers (Edward Arnold, London).


Fibroblast
N
ER
1 Synthesis of pro α chains
2 Aggregation of 3 pro α chains
3 Hydroxylation of lysine and proline
4 Secretion of procollagen
Procollagen
5 Cleavage of propeptides
Collagen molecule
6 Alignment of collagen molecules
300nm
35nm
Collagen fibril
Individual collagen molecule
67nm
7

form the NC1 domain (Risteli *et al*, 1980). Further cross-linking completes the assembly of collagen type IV. The 7S and NC1 domains can be isolated separately from tissues by limited digestion with pepsin or trypsin; the NC1 domain is resistant to bacterial collagenase.

By convention, the collagens are classified into three groups based on their physico-chemical properties (Burgeson, 1988) (Table 3.1). Group 1 molecules are composed of α chains of molecular weight greater than 95 kD with long, uninterupted helical domains; group 2 molecules also have α chains geater than 95 kD but the helical domains are interspersed with non-helical domains; group 3 molecules are smaller with a molecular weight less than 95kD. The relative abundance of individual collagens varies from tissue to tissue (Table 3.1). Some (eg. collagens II, IX and X) are exclusively found in cartilage. The principal fibril-forming collagens within the connective tissues of internal organs (types I, III and V), are referred to as interstitial collagens. Type VI is less abundant and has been regarded as a "minor" collagen; it is nevertheless a matrix component of a wide variety of tissues. Type VII collagen is present within so-called anchoring fibrils present at the dermal-epidermal junction of skin and other ectodermally-derived epithelia (Leigh *et al*, 1987). Collagen VIII was first detected in endothelial cell cultures but has subsequently been shown to be restricted to specialized extracellular matrices such as Descemets's membrane in the cornea (Kapoor *et al*, 1988).

Non-collagenous structural glycoproteins

The extracellular matrix also contains a number of high molecular weight glycoproteins which function as "adhesion" molecules. The most abundant and best characterized of these is fibronectin. There are at least ten isoforms of this highly conserved protein; each is formed by differential splicing of a common gene (Kornblihtt *et al*, 1985). Fibronectin, which has a molecular weight of 500kD, is a dimer composed of two similar subunits joined by a pair of disulphide bonds. These are folded into a series of globular domains separated by flexible regions (Hynes, 1985). The protein contains binding sites for heparin, fibrin, collagen and cellular integrins; different globular domains are responsible for the binding of these various ligands (Hynes, 1985). The site responsible for binding to cell membranes has been identified as a tripeptide (Arg-Gly-Asp or RGD); this sequence is not confined to fibronectin but is found in many of the non-collagenous glycoprotein matrix molecules (Ruoslahti *et al*, 1987).

Collagen	Chains	Molecular Weight Kd Procollagen/Collagen	Molecular Species	Distribution
Type I	α1 (I)	140/95	α1 (I) $_2$α2 (I)	Skin, tendon, bone, ligaments, cornea, internal organs
	α2 (I)	125/95	α1 $(I)_3$	
Type II	α1 (II)	140/95	α1 $(II)_3$	Cartilage
Type III	α1 (III)	140/95-100	α1 $(III)_3$	Skin, blood vessels, internal organs
Type V	α1 (V)	240/115	α1 (V) $_2$α2 (V)	Skin, blood vessels, internal organs
	α2 (V)	160/?	α1 $(V)_3$	
	α3 (V)	?/?	α1 (V) α2 (V) α3 (V)	
Type IV	α1 (IV)	185/185	α1 (IV) $_2$α2 (IV)	Basement membranes
	α2 (IV)	170/170	α1 $(IV)_3$	
			α2 $(IV)_3$	
Type VI	α1 (VI)	240/140	α1 (VI) α2 (VI) α3 (VI)	See text
	α2 (VI)	240/140	α1 $(VI)_3$	
	α3 (VI)	240/140		
Type VII	α1 (VII)	?/>170	α1 $(VII)_3$	Anchoring fibrils: dermo-epidermal junction
Type VIII	?	180/180	?	Endothelium
Type IX	α1 (IX)	80/70		
	α2 (IX)	80/70	α1 (IX) α2 (IX) α3 (IX)	Cartilage
	α3 (IX)	80/70		
Type X	α1 (X)	59/49	α1 $(X)_3$	Cartilage

Table 3.1 Biochemical properties of collagen molecules.

Three groups of collagen molecules are recognised on the basis of chain structure and molecular weight. The physico-chemical properties of collagens XI - XIII are yet to be fully determined.

Fibronectin is a multi-functional protein. In addition to its role in matrix-matrix and cell-matrix adhesion, it is known to (i) act as an opsonin for macrophage phagocytosis, (ii) serve as a chemotactic factor, (iii) modulate cell migration during embryogenesis and in wound healing and (iv) exhibit growth factor activity for some mesenchymal cells (Furcht, 1983).

Vitronectin (serum spreading factor or S-protein) also binds to cell membranes via an RGD domain (Suzuki *et al*, 1985) and interacts with other matrix proteins such as collagens (Gebb *et al*, 1986). Like fibronectin, it is a multi-functional molecule which may modulate monocyte function (Parker *et al*, 1988); it may also bind to the terminal components of the complement system (Podack & Müller-Eberhard, 1979) and act as a control protein.

Laminin, is found exclusively in basement membranes. This protein interacts with other components of the basement membrane (collagen type IV, nidogen (entactin) and proteoglycans) and with cellular integrins. It is a cross-shaped molecule with a molecular weight of 900kD and is composed of three subunits (the A, B1 and B2 chains) (Martin & Timpl, 1987). Although a modified RGD sequence is contained within the A chain, the domain responsible for binding to cell membranes differs from that of fibronectin and has been identified as the pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR) within the B1 chain (Graf *et al*, 1987). Recent evidence suggests that modified forms of laminin may exist; a novel laminin-like substance (s-laminin), present in basement membranes at neuromuscular junctions, has recently been identified (Hunter *et al*, 1989). Nidogen (entactin), a glycoprotein of molecular weight 150kD interacts specifically with the centre of the laminin molecule (Paulsson *et al*, 1987); this also contains domains with the RGD sequence and is thought to be involved in cell-matrix interactions.

Tenascin, a large (molecular weight 1000kD) glycoprotein with a six-armed structure, is now known to be identical to several other previously described matrix proteins including glioma-mesenchymal extracellular matrix antigen, myotendinous antigen, hexabranchion, J1 and cytotactin (Chiquet-Ehrismann *et al*, 1986). It is most abudantly expressed at sites of epithelial-mesenchymal interaction during embryogenesis but is also known to be expressed in repair processes eg. healing skin wounds (Mackie *et al*, 1988).

Undulin was originally isolated from skin and placenta but has subsequently been shown to be present in a wide range of tissues. It is thought to be composed of three chains (A, B1 and B2) and has a molecular weight of over 1000kD. There is preliminary evidence to suggest

that this protein may bind preferentially to collagen type I (Schuppan *et al*, 1990). The name, derived from the latin "undula" (little wave), was proposed on the basis of initial immunofluorescence studies (Schuppan *et al*, 1990).

Proteoglycans

Proteoglycans are a heterogeneous group of protein-polysaccharide complexes consisting of a core protein linked to so-called glycosaminoglycan molecules (Ruoslahti, 1988). Glycosaminoglycans are composed of unbranched chains of repeating disaccharide units; one of the components of each subunit is either N-acetyl D-glucosamine or N-acetyl/galactosamine. As a result of N- or O- sulphation and acetylation, the glycosaminoglycan chain is a highly charged (anionic) molecule. Proteoglycans can be classified into four sub-groups according to their predominant glycosaminoglycan content: (i) chondroitin sulphate, (ii) dermatan sulphate, (iii) heparan sulphate and heparin and (iv) keratan sulphate. As with collagens, these distinct classes of proteoglycan are found at different concentrations in different tissues. Hyaluronic acid, a related polysaccharide also present in the extracelllular matrix, differs structurally from these proteoglycans by the lack of sulphate groups or association with a core protein.

The glycosaminoglycan chains are strongly hydrophilic. They form so-called random-coil conformations which occupy a large volume relative to their mass. As a result of these properties, proteoglycans attract water into the extracellular matrix forming a hydrated gel. Proteoglycans are also known to interact with cell membrane receptors (Höök *et al*, 1984), interstitial collagens (Scott, 1988) and basement membrane components (Martin & Timpl, 1987).

Elastin

This is the principal component of elastic fibres which are present in tissues such as the dermis and the lung parenchyma, which require the ability to recoil after transient stretching (Cleary & Gibson, 1983). Elastin is a highly charged, non-glycosylated protein rich in proline. The molecules are covalently cross-linked but in the extracellular compartment they form random coils; this structure imparts upon the protein the property of elasticity. Several other molecules (microfibrillar glycoprotein, amyloid P component) are found on the surface of the elastin molecules (Cleary & Gibson, 1983) and form integral parts of elastic fibres.

3.1.2 The hepatic extracellular matrix: biochemical studies

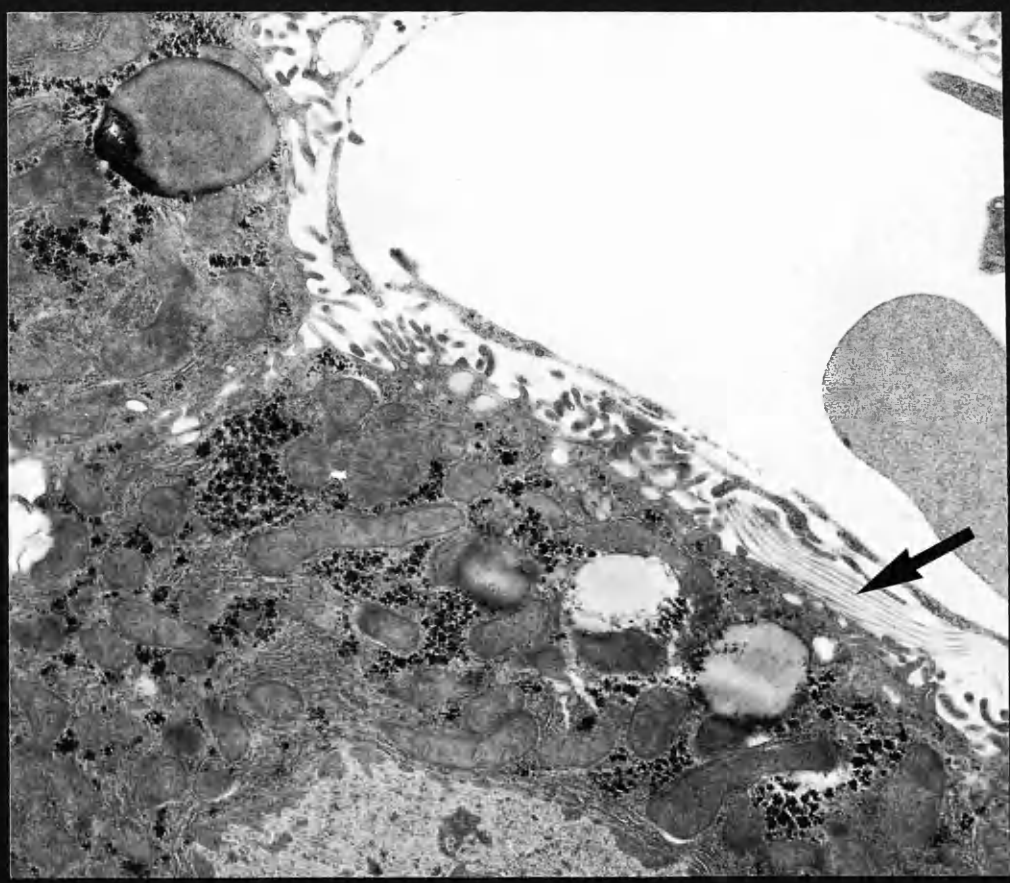
Biochemical studies have identified which of the above extracellular matrix constituents are present in normal liver. Collagen accounts for approximately 5 - 10% of total hepatic protein (Schuppan, 1990); types I, III, IV, V and VI are present. The most abundant are collagen types I and III which are found in approximately equal amounts, together accounting for over 95% of total liver collagen (Seyer *et al*, 1977; Rojkind *et al*, 1979). Fibronectin is the most abundant of the non-collagenous glycoproteins with a normal tissue concentration of approximately 300μg/gm (Gressner & Bachem, 1990). Proteoglycans are also present in significant amounts, the principal component being heparan sulphate proteoglycan (Murata *et al*, 1985).

It has been established that in response to most forms of liver injury, there is accumulation of extracellular matrix proteins. In chronic liver disease, this leads to replacement of the liver parenchyma by scar tissue (fibrosis); this is frequently accompanied by nodular regeneration of remaining parenchymal cells leading to gross distortion of the liver architecture with the development of cirrhosis (MacSween & Burt, 1986a and b; 1989). Biochemical analyses have demonstrated that in established cirrhosis there is not only a several-fold increase in many extracellular matrix proteins (Rojkind *et al*, 1979), but there are also alterations in the ratio of different components. In cirrhosis, type I collagen is more abundant than type III; there are also disproportionate increases in collagen types V and VI (Schuppan, 1990). All of the major proteoglycans are increased but there is a shift in the ratio of dermatan sulphate: heparan sulphate (Gressner & Bachem, 1990).

While such biochemical studies have yielded important data on the composition of the matrix, they rely upon the use of tissue homogenates and therefore tell us nothing about the distribution of extracellular matrix components in either normal or fibrotic liver. The presence of "connective tissue" proteins along sinusoidal walls has long been established using empirical histochemical methods such as silver "reticulin" stains (Figure 1.15). Furthermore, striated collagen fibrils have been identified in the space of Disse using conventional transmission electron microscopy (Figure 3.2). As cell-matrix interactions are thought to be important in

Figure 3.2 Transmission electron micrograph of normal rat liver demonstrating the presence of collagen fibrils within the space of Disse

Striated collagen fibrils (arrow) can be seen in the space of Disse between sinusoidal endothelial cells and parenchymal cells. (Uranyl acetate/lead citrate : x 8,700).



modulating cell function, a detailed knowledge of the composition of the extracellular matrix in the perisinusoidal space of Disse is essential. Furthermore, during the response to liver injury extracellular matrix proteins are thought to be laid down in close proximity to damaged and necrotic parenchymal cells; it is important therefore to document changes occurring in the composition of this perisinusoidal matrix in liver disease. In order to study the precise distribution of matrix proteins in the liver, immunohistochemical studies have been used to specifically localize individual components in tissue sections.

3.1.3 The hepatic extracellular matrix: immunolocalization studies

Initial attempts at investigating the distribution of hepatic extracellular matrix proteins used immunofluorescent methods on unfixed, frozen sections of normal or fibrotic liver in several different mammalian species (Kent *et al*, 1976; Nowack *et al*, 1976; Wick *et al*, 1978; Grimaud *et al*, 1980; Voss *et al*, 1980; Biempica *et al*, 1980; Hahn *et al*, 1980). Although there was general agreement that the two principal interstitial collagens, types I and III were present within portal tract stroma and that type III could be visualized along sinusoidal walls, these studies provided conflicting data on the distribution of collagen types I and IV. This is almost certainly due to two methodological limitations: (i) the antibodies used were poorly characterized and (ii) the immunofluorescent technique used was of relatively low sensitivity. The subsequent application of immunoperoxidase methods provided further information on the distribution of the interstitial collagens (Konomi *et al*, 1981; Geerts *et al*, 1982; Martinez-Hernandez, 1984; Clément *et al*, 1984; 1985) but the expression of basement membrane components within the space of Disse remained controversial (Geerts *et al*, 1982; Bianchi *et al*, 1984), particularly in human liver.

The aims of the studies presented in this chapter were (i) to further define the distribution of the major components of the extracellular matrix within the space of Disse of normal human liver using well-characterized affinity-purified antibodies, (ii) to investigate the distribution of several "minor" matrix components (collagen type VI, undulin, vitronectin) whose precise localization had not hitherto been fully established, (iii) to examine the distribution of major and minor extracellular matrix proteins at the ultrastructural level in normal human liver

and (iv) to study alterations in the ultrastructural distribution of extracellular matrix proteins in different forms of liver disease.

Extracellular matrix proteins are difficult to localize using conventional methods of immuno-electron microscopy because the proteins are readily denatured by standard fixation and embedding procedures. In order to overcome these difficulties, an ultracryomicrotomy/ immuno-gold labelling method was used to localize the proteins ultrastructurally (Burt *et al*, 1990).

3.2 MATERIALS AND METHODS

3.2.1 Liver tissue

Normal human liver was obtained from cadaveric renal transplant donors (n=8). Material was collected fresh at the time of surgical removal of organs and processed immediately. Small wedges of tissue (approximately 4 x 4 x 3cm) were perfusion-fixed through the vascular orifaces of the cut edge using a 16g needle, with one of the following fixatives: (i) Bouin's fixative, (ii) 10% buffered formalin, (iii) formal sublimate, (iv) 2% paraformaldehyde in 0.1M phosphate buffer or (v) 2% paraformaldehyde/ 0.1% glutaraldehyde. Tissue blocks were subsequently processed for paraffin wax-embedding, for light microscopic immunohistochemical studies. In addition, unfixed tissue blocks were mounted in OCT and frozen in liquid nitrogen. Small fragments of 2% paraformaldehyde/0.1% glutaraldehyde-fixed material were immersed in 2.3M sucrose and frozen in liquid nitrogen for ultracryomicrotomy. Sucrose in this concentration acts as a cryopreservative (Griffiths *et al*, 1984).

Material was also obtained from percutaneous liver biopsies performed on patients undergoing investigation for suspected liver disease. In all cases, most of the specimen was fixed in formal sublimate and processed for conventional histopathological assessment. However, small fragments were also puncture perfusion-fixed using microneedles fashioned from Pasteur pipettes, with 2% paraformaldehyde/0.1% glutaraldehyde in 0.1M phosphate buffer. These fragments were subsequently transferred to fresh fixative for one hour, immersed in 2.3M sucrose and frozen in liquid nitrogen for ultracryomicrotomy. The histological diagnosis of the cases used in the study are outlined in Table 3.2.

Mild non specific reactive changes	4
Steatosis	2
Acute hepatitis (HAV; drug induced)	5
Granulomatous hepatitis	3
Intrahepatic cholestasis	2
Extrahepatic biliary obstruction	1
Primary biliary cirrhosis	8
Primary sclerosing cholangitis	3
Chronic active hepatitis (HBV; HCV; autoimmune)	3
Alcoholic hepatitis	2
Alcoholic cirrhosis	7
Inactive cirrhosis	3
Nodular regenerative hyperplasia	1

Table 3.2. Histological diagnoses in liver biopsies from 44 patients used in study.

3.2.2 Antibodies

The primary antibodies used in this study are outlined in Table 3.3. With the exception of antibodies to collagen type V, fibronectin, vitronectin and nidogen, all were generously supplied by Dr. D. Schuppan, Free University of Berlin. Anti-fibronectin was supplied by Dr. B. Voss, University of Munster and anti-nidogen was provided by Dr. G. Ramadori, University of Mainz. Anti-vitronectin antibody was obtained from Seralab, UK. Details of the methods used for the purification of antigen, and generation of antibodies, are given in the appropriate references in Table 3.3. All antibodies were polyclonal and were affinity-purified by repeated passage over Sepharose columns loaded with inappropriate extracellular matrix proteins. Specificities were assessed by radioimmunoassay and Western blotting; these studies confirmed the absence of any cross-reactivity between antibodies and inappropriate matrix proteins.[1]

3.2.3 Immunohistochemical methods: light microscopy

Two methods were used to immunolocalize extracellular matrix proteins at the light microscope level. In initial experiments, an indirect immunofluorescence method was used on 5μm frozen sections. Subsequently, 3μm dewaxed tissue sections were used with a peroxidase-antiperoxidase method (Appendix 2). The effects of trypsinization of sections prior to the application of primary antibodies on immunolabelling was assessed (see Appendix 2). In addition, the effects of treatment with 0.4% pepsin/0.01M HCl for 5 - 15 minutes at 37^{o}C were evaluated with each antibody. 3, 3' diaminobenzidine was used as the chromagen; sections were counterstained with haematoxylin and viewed under a Leitz Orthoplan microscope fitted with Nomarski filters. Negative controls were included in all experiments; these comprised sections treated with (i) normal goat or rabbit serum or (ii) PBS, in place of the primary antibody.

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[1] A minor degree of cross-reactivity for collagen type V was noted by radioimmunoassay with the anti-type VI antibody. However, this was absent at antibody dilutions used in the current study.

Antigen	Source	Species used for antibody production	Working dilution	Reference
Collagen type I	Human	Goat	1:32	Schuppan *et al* (1986a)
Procollagen type I	Monkey	Rabbit	1:160	Becker *et al* (1986a)
Collagen type III	Human	Goat	1:128	Schuppan *et al* (1986a)
Procollagen III N-terminal peptide	Monkey	Rabbit	1:160	Becker *et al* (1986a)
Collagen type V	Human	Rabbit	1:100	*
Collagen type VI	Human	Rabbit	1:160	Schuppan *et al* (1985)
Collagen type IV (7S domain)	Human	Goat	1:160	Schuppan *et al* (1986b)
Collagen type IV (NCl domain)	Human	Rabbit	1:320	Schuppan *et al* (1986b)
Laminin P1	Human	Rabbit	1:100	Becker *et al* (1986a)
Laminin 120kD pepsin fragment	Human	Rabbit	1:100	Schuppan *et al* (in press)
Laminin 60kD pepsin fragment	Human	Rabbit	1:80	Schuppan *et al* (in press)
Nidogen	Human	Rabbit	1:50	Ramadori *et al* (1991)
Fibronectin	Human	Rabbit	1:40	Voss *et al* (1980)
Vitronectin	Human	Rabbit	1:50	Reilly and Nash (1989)
Undulin	Monkey	Rabbit	1:50	Schuppan *et al* (1990)

* Data obtained from Seralab, United Kingdom

Table 3.3 Antibodies used in the study of extracellular matrix proteins

3.2.4 Immuno-electron microscopic methods I: ultracryomicrotomy

Frozen, cryopreserved tissue fragments were mounted on aluminium stubs (Cambridge Biomedical, Cambridge, UK). Ultra-thin sections were then cut on a Reichert Jung FC4D ultracryomicrotome. The microtome chamber temperature was maintained constant at -100°C, the specimen at -80°C and the glass microtomy knife at -90°C. Sections with pale blue/gold interference colours (approximately 50-70 nm thick) were collected on droplets of saturated sucrose solution and transferred to formvar/carbon-coated electron microscopy grids (Agar Aids, United Kingdom).

3.2.5 Immuno-electron microscopic methods II: immuno-gold procedure

Grids were floated on drops of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) to remove sucrose. In some experiments, grids were incubated with leech hyaluronidase (250 IU/ml in PBS; Koch-Light, Boston, USA) at 37°C for 30 minutes to remove glycosaminoglycans masking antigenic sites. The grids were placed on 0.01M glycine/PBS for 10 minutes to quench residual aldehyde groups and subsequently on 2% gelatin/PBS (10 minutes) and 1% BSA/PBS (2 x 1 minute) to reduce non-specific background staining. Each grid was then incubated for one hour at 37°C or overnight with 30 μl of either (i) primary antibody or (ii) normal goat or rabbit IgG (negative controls). Grids were subsequently rinsed five times in 1% BSA/PBS and incubated for one hour at 37°C with protein A/gold complex (10 nm particles; Auroprobe, Janssen Laboratories, Belgium). These were then rinsed with PBS, post-fixed with 2% glutaraldehyde/PBS, rinsed in distilled water (5 x 1 minute) and stained with uranyl acetate/oxalate. After rinsing with distilled water (3 x 20 seconds), they were coated with a 1:1 mixture of 2% aqueous uranyl acetate and 1.5% tylose 300 (Fluka, West Germany). Grids were then examined using a Phillips 300 electron microscope at 80kV.

3.3 RESULTS I : NORMAL LIVER

3.3.1 Ultracryomicrotomy

Although there was variation between specimens, excellent preservation of ultrastructural detail was obtained in ultra-thin frozen sections of most samples (Figure 3.3); membranous structures were particularly well visualized. In some cases, cytoplasmic vacuolation was noted within parenchymal cells but not sinusoidal cells. There was good preservation of the architecture of the sinusoids and the space of Disse.

3.3.2 Effect of enzyme pre-treatment on immunolabelling

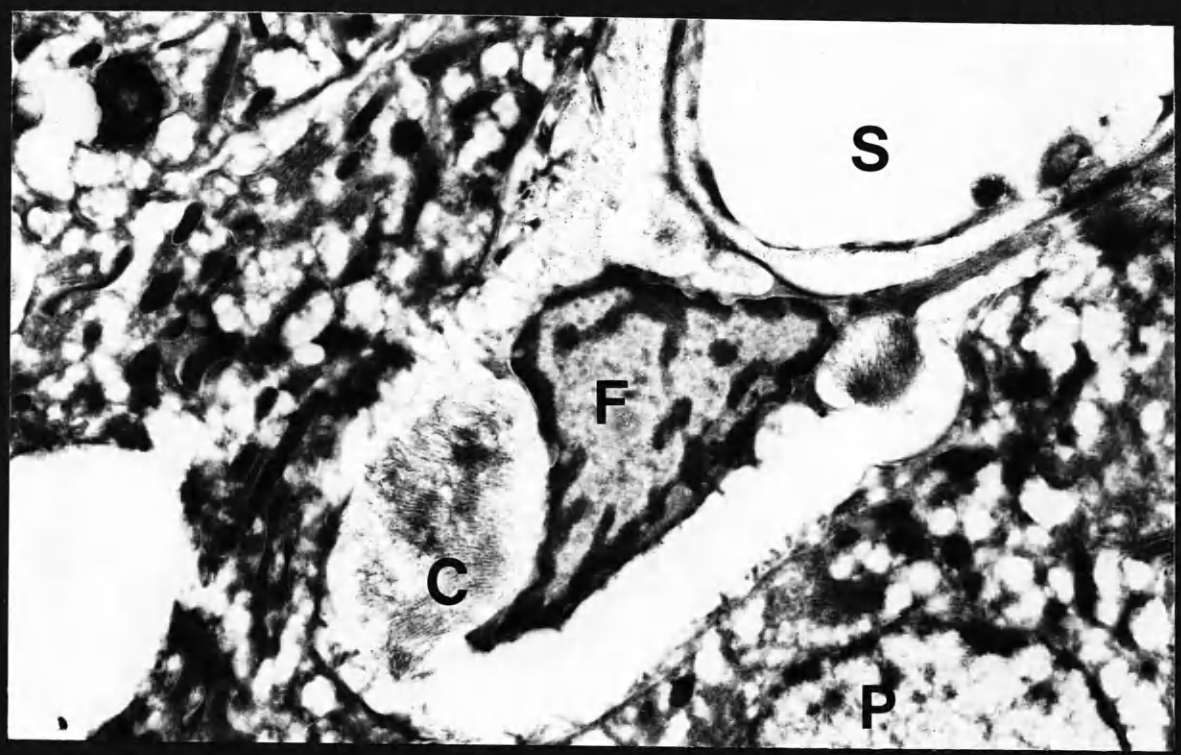
Immunolabelling of paraffin-embedded material using the peroxidase-antiperoxidase method was enhanced with all antibodies when sections were pre-treated with pepsin. Increased intensity of labelling was also noted with some antibodies (anti-type I collagen, anti-type IV (7S) collagen, anti-type VI collagen, anti-fibronectin and anti-laminin 120kD) following trypsinization for 10 minutes. Some loss of morphological detail was noted in pepsin-treated sections but this was not apparent following trypsinization.

Pepsin and trypsin pre-treatment could not be used to enhance labelling at the ultrastructural level as this resulted in serious damage to the ultra-thin sections. As an alternative, hyaluronidase was used and found to improve intensity of labelling with all antibodies to interstitial collagens, anti-type IV collagen (7S) and anti-laminin (120kD). No significant enhancement, however, could be detected when hyaluronidase pre-treatment was used in light microscopic immunolabelling studies.

No staining was seen in negative control sections using the peroxidase-antiperoxidase method. Occasional gold particles were seen in negative control ultra-thin sections in immuno-electron microscopic studies. In experiments where heavy labelling was seen in the controls, test sections were not analysed.

Figure 3.3 Ultracryomicrotomy : normal human liver.

The method used to obtain ultra-thin frozen sections provided good preservation of ultrastructural detail. In this preparation, the normal relationship between parenchymal cells (P) and the sinusoids (S) can be seen to be maintained. A fat-storing cell (F) can be identified within a recess between parenchymal cells, lying adjacent to striated collagen fibrils (C). In many preparations, cytoplasmic vacuolation was seen within parenchymal cells but not sinusoidal cells. This has been noted by other workers and is thought to be related to loss of glycogen during tissue processing. (Uranyl acetate/methyl cellulose; x 7,700).


S
F
C
P

3.3.3 Distribution of interstitial collagens

By immunofluorescence, labelling for both collagen type I and procollagen type I could be detected in portal tract stroma. However, only weak, patchy labelling was found along sinusoidal walls particularly with the anti-procollagen type I antibody. Although more intense immunolabelling was obtained with the peroxidase-antiperoxidase method in Bouin's-fixed and paraformaldehyde-fixed material, only a weak signal was noted along the sinusoids. By immuno-electron microscopy, labelling with both antibodies was found on some striated collagen fibrils within fibres in the space of Disse; labelling was frequently seen at the periphery of such fibres (Figure 3.4).

Strong labelling for collagen type III was found along the sinusoids using both anti-human collagen type III and anti-monkey procollagen type III N-terminal propeptide antibodies with either immunofluorescence or peroxidase-antiperoxidase methods. Staining was obtained in all forms of fixed tissue but was most intense in Bouin's-fixed material. By light microscopy, continuous labelling along sinusoids was found with no zonal heterogeneity (Figure 3.5). At the ultrastructural level, intense labelling was noted along the edges of individual collagen fibrils (Figure 3.6).

A similar distribution to that of collagen type III was found for collagen type V at the light microscope level (Figure 3.7). However, staining was only achieved in Bouin's-fixed tissue; negligible labelling was obtained by immuno-electron microscopy.

3.3.4 Distribution of collagen type VI

Immunoreactivity for type VI collagen was seen using immunofluorescence and the peroxidase-antiperoxidase method; labelling was again most intense in Bouin's-fixed tissue. Staining for this component was seen in portal tract stroma; accentuation of staining was seen around hepatic arteries and portal vein branches. The media of blood vessels were negative. Positivity was also noted around terminal hepatic vein radicles (Figure 3.8a) and along sinusoidal walls. In contrast to other matrix components, there was evidence of zonal heterogeneity with only weak staining in the sinusoids of acinar zone I and more intense labelling in zones II and

Figure 3.4 Immuno-electron microscopic localization of collagen type I in space of Disse : normal human liver.

This micrograph shows positive labelling for collagen type I on striated fibrils within the space of Disse. (Uranyl acetate/methyl cellulose; x 35,000).

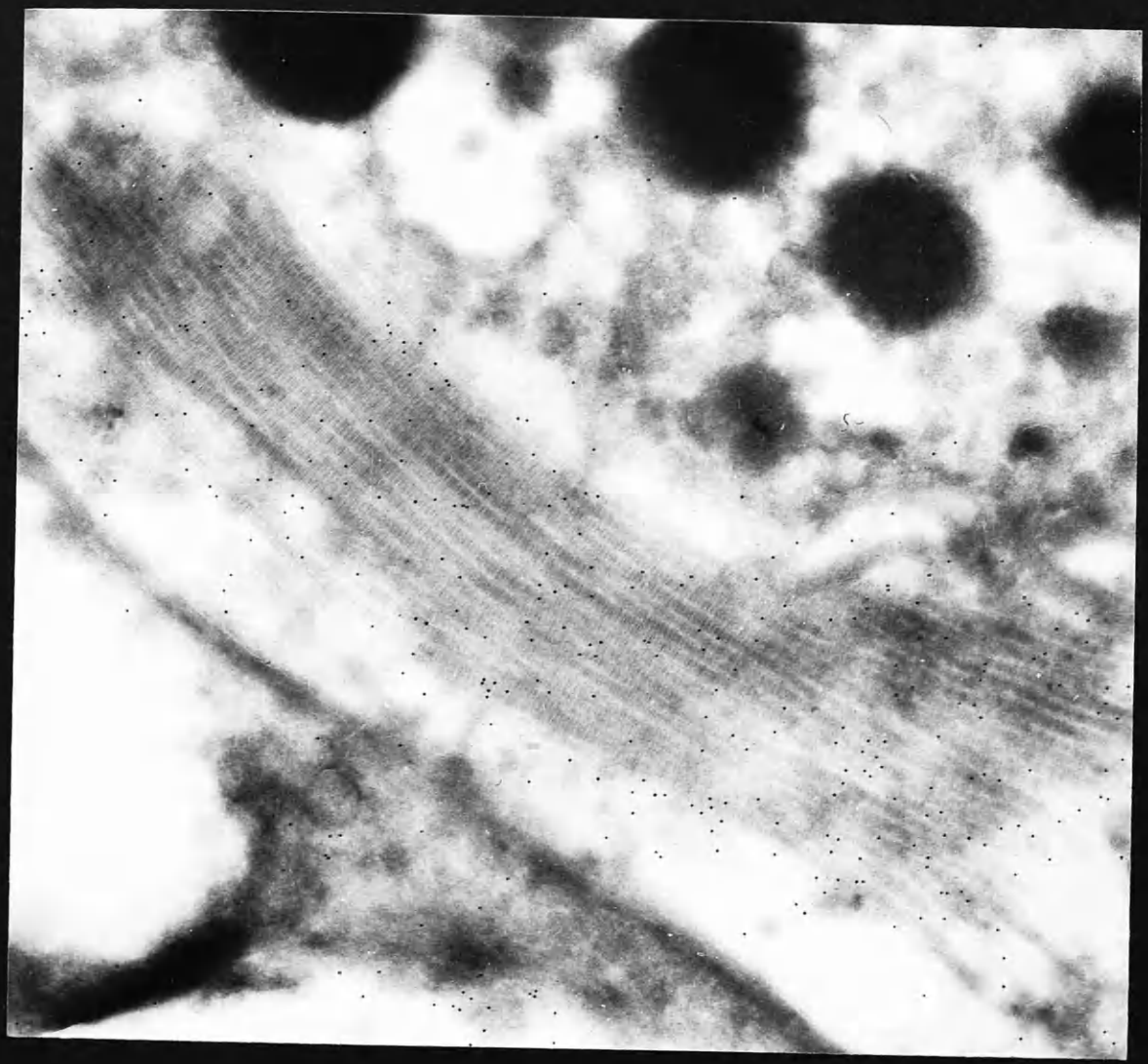


Figure 3.5 Immunolocalization of collagen type III in normal human liver (light microscopy).

Immunoreactivity can be seen within the stroma of a portal tract and surrounding a terminal hepatic vein radicle. In addition, immunoreactivity is present in a continuous manner along the sinusoids with no evidence of zonal heterogeneity. (Peroxidase anti-peroxidase method; haematoxylin counterstain; x 200).

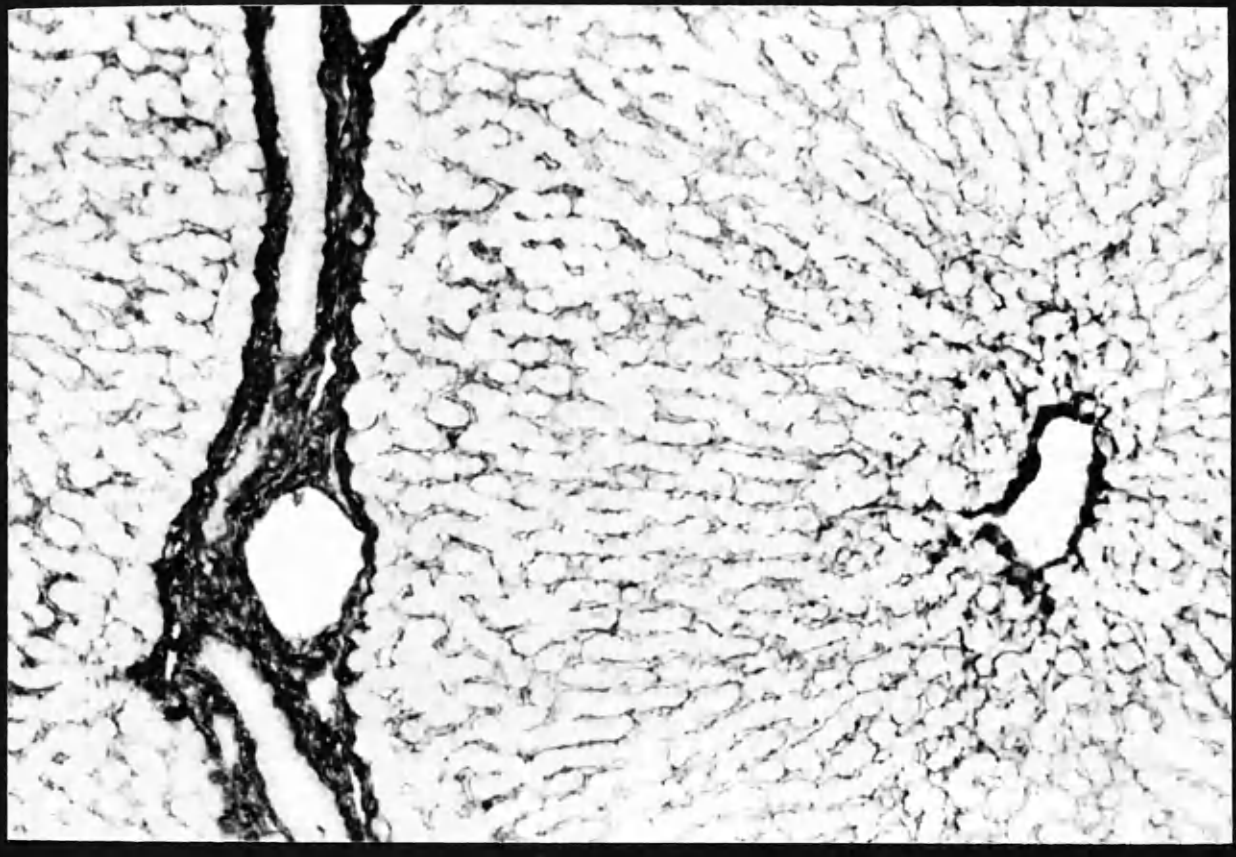


Figure 3.6 Immuno-electron microscopic localization of collagen type III in normal human liver.

Using the affinity-purified polyclonal antibody against the N-terminal peptide of procollagen type III, intense immunolabelling can be identified on striated fibrils within the space of Disse. The intensity of labelling here is greater than that seen for type I collagen. With this antibody staining was maximal along the edges of individual striated fibrils. (Uranyl acetate/methyl cellulose; protein A-gold method; 10nm gold particles; x 65,000).

Figure 3.7 Immunolocalization of collagen type V in normal human liver (light microscopy).

This interstitial collagen could also be localized to the perisinusoidal space of Disse. As with collagens I and III, there is continuous staining with no evidence of significant zonal heterogeneity. Note absence of immunoreactivity within parenchymal cells. (Peroxidase anti-peroxidase method; haematoxylin counterstain; x 320).

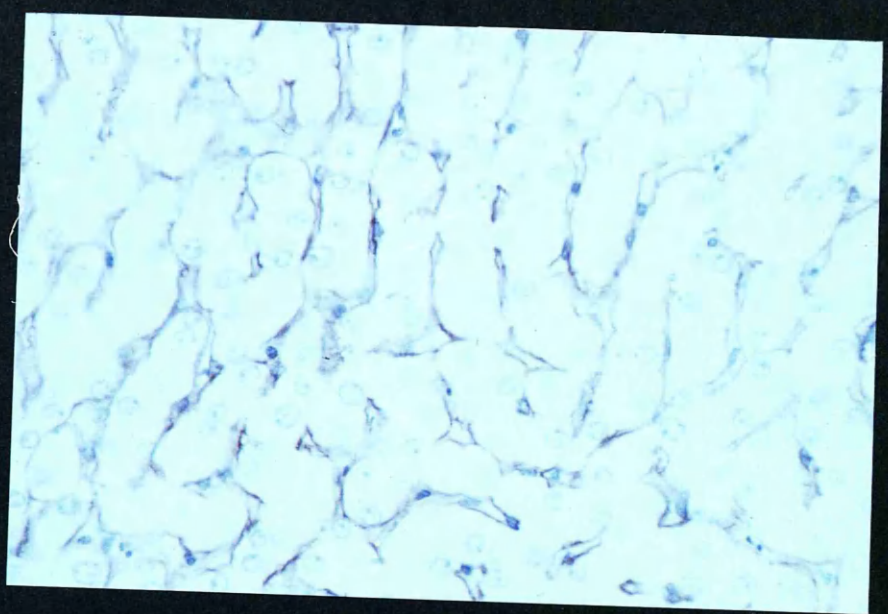
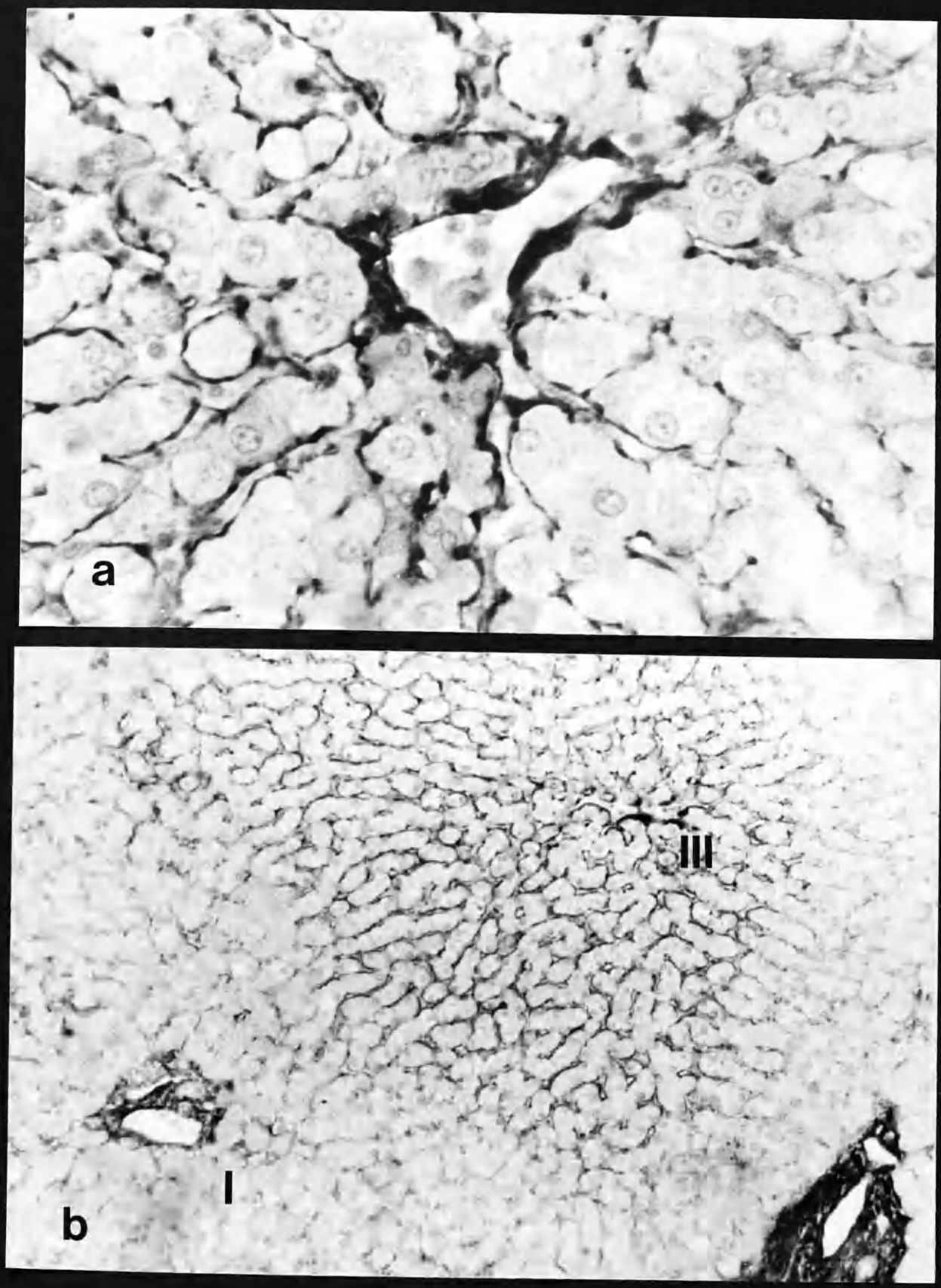


Figure 3.8 Immunolocalization of collagen type VI in normal human liver (light microscopy).

a. Intense immunoreactivity for collagen type VI can be seen around this terminal hepatic vein radicle. In addition, staining can be seen along sinusoidal walls in the perivenular zone. (Peroxidase-antiperoxidase method; haematoxylin counterstain; x 890).

b. At a low power of magnification, evidence of zonal heterogeneity in intensity of staining for type VI collagen can be identified. In periportal zones (I) there is only weak patchy staining for the protein whereas the intensity in mid zones and perivenular zones (III) is much greater. (Peroxidase-antiperoxidase method; haematoxylin counterstain; x 220).


a
III
I
b

III (Figure 3.8b). At the ultrastructural level, labelling was identified within the space of Disse between sinusoidal endothelial cells and parenchymal cells (Figure 3.9). Gold particles were predominantly seen on amorphous and occasional microfilamentous extracellular material lying between striated collagen fibrils; very few gold particles were observed on striated fibrils.

3.3.5 Distribution of basement membrane components

By light microscopy, immunoreactivity for collagen type IV and laminin was demonstrable along sinusoidal walls using immunofluorescence and peroxidase-antiperoxidase methods. The effect of different fixation protocols on immunostaining is summarized in Table 3.4. Immunoreactivity for collagen type IV and laminin was also noted within basement membranes around bile ducts and hepatic arteries in portal tracts (Figures 3.10 and 11). Only two antibodies detected immunoreactivity in material fixed in paraformaldehyde/glutaraldehyde (anti-7S and anti-laminin 120kD); these were used for immuno-gold studies. Although labelling was more intense with anti-7S than anti-laminin 120kD, both components could be localized to the space of Disse beneath sinusoidal endothelial cells and surrounding fat-storing cells (Figure 3.12). No labelling was noted on striated collagen fibrils.

No immunolabelling for nidogen could be seen along sinusoidal walls. However, it should be noted that only weak immunoreactivity was found with this antibody in basement membranes around bile ducts and vessels; staining was limited to Bouin's-fixed material.

3.3.6 Distribution of non-collagenous glycoproteins

Fibronectin could be identified, with a continuous staining pattern, along sinusoidal walls using both immunofluorescence and peroxidase-antiperoxidase methods (Figure 3.13). At the ultrastructural level, labelling could be detected within the space of Disse where it was seen both at the periphery of collagen fibres and running between fibres and parenchymal cells.

Vitronectin could only be detected in frozen section material; in these preparations there was a similar distribution to that noted for fibronectin with no evidence of zonal heterogeneity (Figure 3.14a). As there was no immunoreactivity for this component in tissue fixed in

Figure 3.9 Immuno-electron microscopic localization of collagen type VI in the space of Disse : normal human liver.

Immunogold labelling could be identified within the space of Disse on amorphous material lying adjacent to and running between striated collagen fibrils (arrow). In this micrograph labelling can be seen beneath a sinusoidal endothelial cell (E). (Uranyl acetate/methyl cellulose; protein A-gold method; 10nm gold particles; x 75,000).

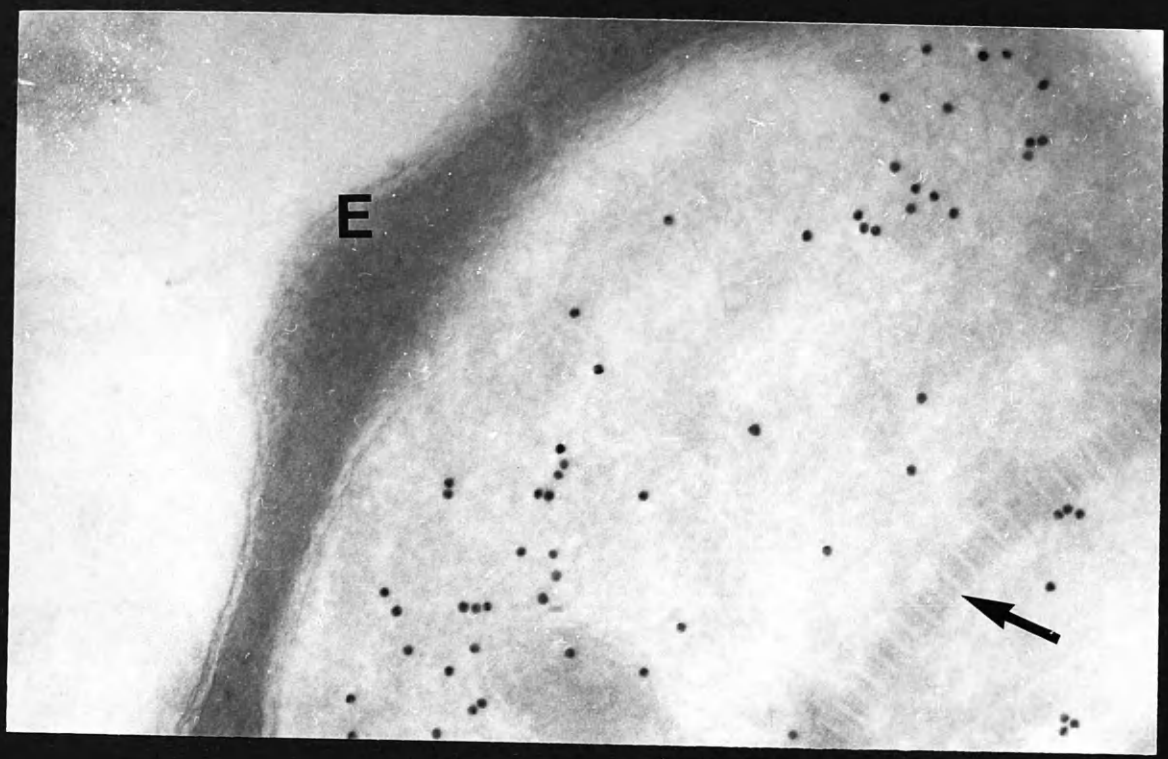
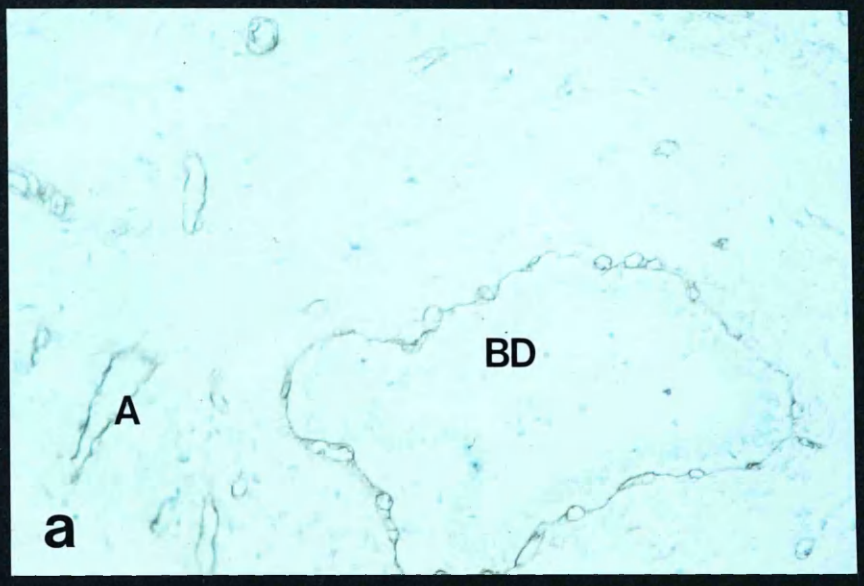

E

Figure 3.10 Immunolocalization of collagen type IV in normal human liver.

a. Immunoreactivity for this basement membrane component can be seen within portal tracts around bile duct radicles (BD) and surrounding hepatic artery branches (A). (Polyclonal anti-7S antibody; peroxidase-antiperoxidase method; haematoxylin counterstain; x 180).

b. Staining for collagen type IV can also be identified along sinusoidal walls. Immunoreactivity is seen between sinusoidal cells (E: probable sinusoidal endothelial cell) and parenchymal cells. (Polyclonal anti-7S antibody; peroxidase-antiperoxidase method; haematoxylin counterstain; photographed under Nomarski filters; x 640).


BD
A
a

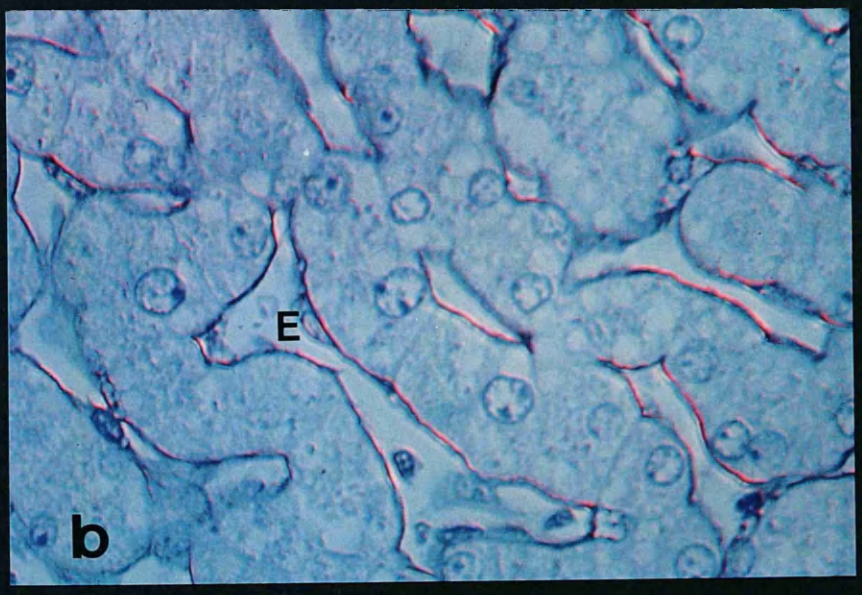

E
b

Figure 3.11 Immunolocalization of laminin (120kD) in normal human liver.

Immunoreactivity for laminin can be seen around this terminal hepatic vein radicle and along sinusoidal walls with a continuous staining pattern. (Peroxidase-antiperoxidase method; haematoxylin counterstain; photographed under Nomarski filters; x 800).

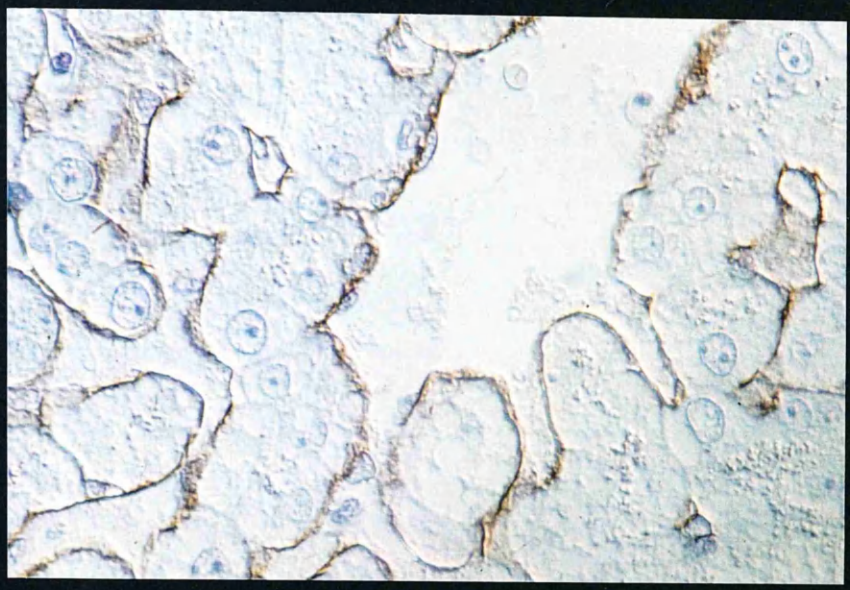


Figure 3.12 Immuno-electron microscopic localization of collagen type IV (7S) in the space of Disse : normal human liver.

a. Immuno-gold labelling for this protein can be identified beneath sinusoidal endothelial cells (arrow), and surrounding fat-storing cells (F) within the space of Disse.

b. At a higher power of magnification, pericellular labelling for collagen type IV (7S) can be seen around the cell process of a fat-storing cell. There is little evidence of labelling on striated collagen fibrils (arrow). (Uranyl acetate/methyl cellulose; protein A-gold method; 10nm gold particles; a: x 10,400; b: x 22,600)

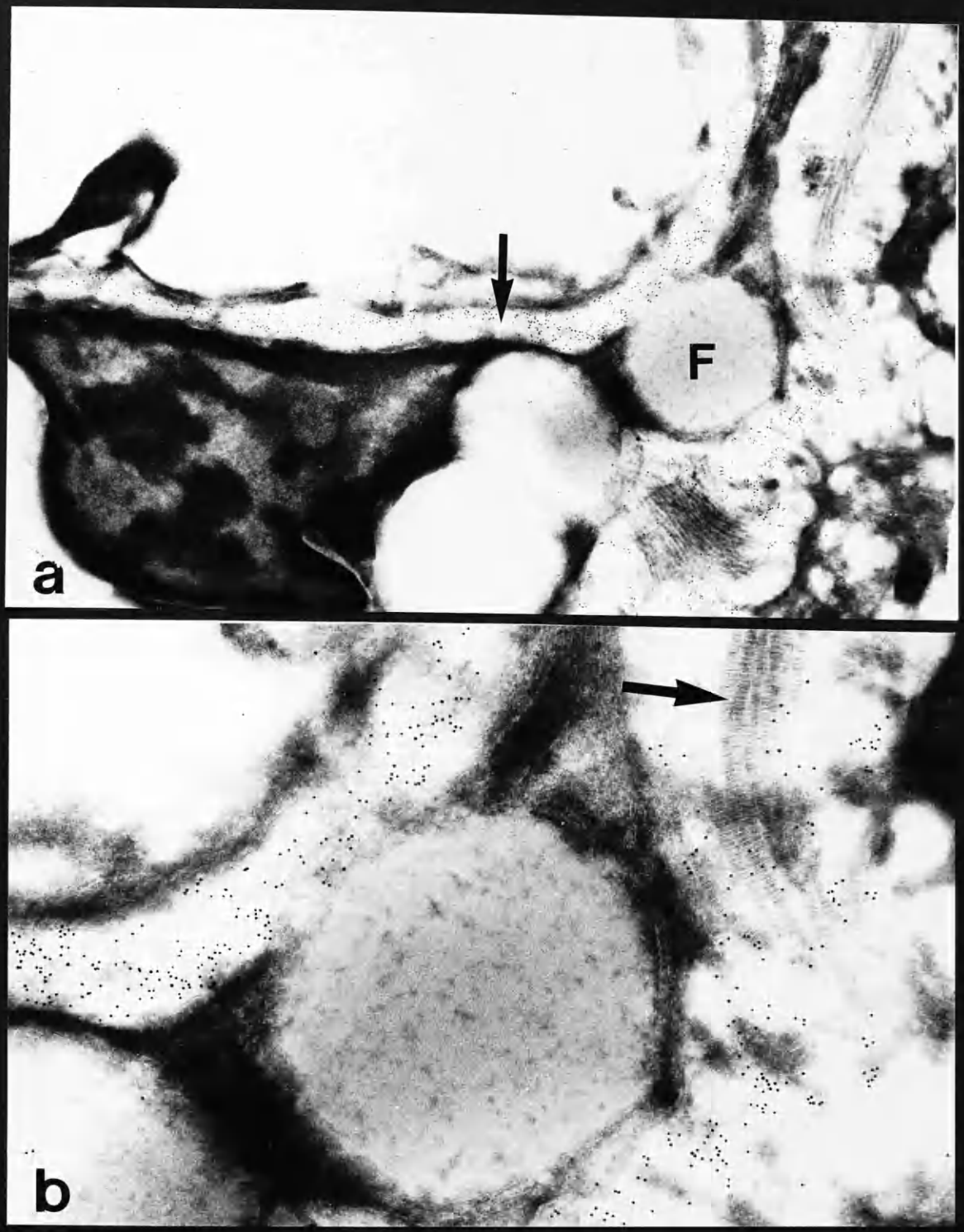

F
a
b

Figure 3.13 Indirect immunofluorescent visualization of fibronectin in normal human liver.

Staining for this structural glycoprotein can be seen along sinusoidal walls with a continuous staining pattern. The orange particles within parenchymal cells represent autofluorescence of lipofucsin. (Indirect immunofluorescence on 4 μm cryostat sections; x 800).

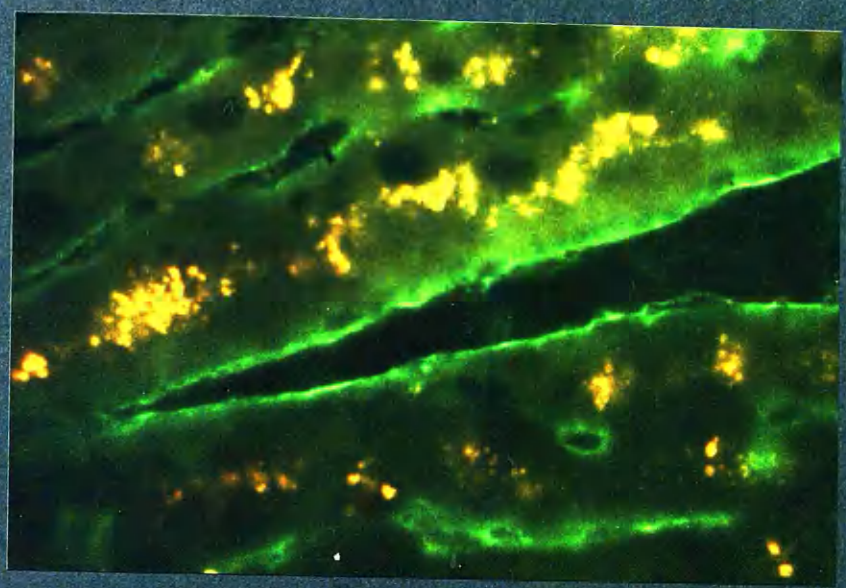
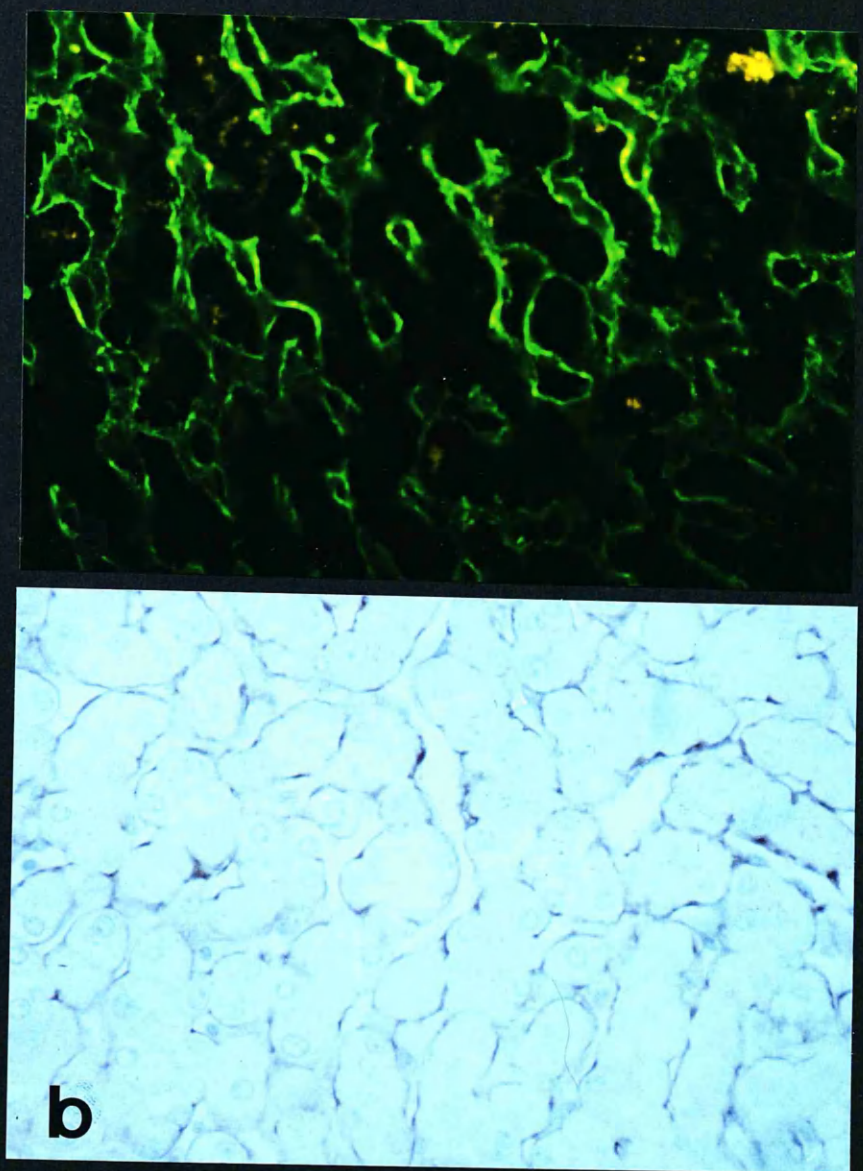


Figure 3.14 Immunolocalization of the structural glycoproteins, vitronectin and undulin in normal human liver.

a. Indirect immunofluorescent visualization of vitronectin. Staining can be identified along the sinusoidal walls with a continuous staining pattern similar to that seen for fibronectin. (Indirect immunofluorescence; x 240).

b. Weak staining can also be identified using the polyclonal antibody to human undulin. Although staining with this antibody was patchy, there was no evidence of any well-defined zonal heterogeneity. (Indirect immunoperoxidase method; haematoxylin counterstain; x 220).


b

paraformaldehyde/glutaraldehyde, it was not possible to localize the protein at the ultrastructural level.

Undulin could be identified within portal tract stroma in Bouin's-fixed material, although the intensity of staining was low. Weak immunoreactivity for this protein was found along sinusoidal walls using the peroxidase-antiperoxidase method (Figure 3.14) in some specimens, although this antibody gave inconsistent results. No labelling for undulin could be detected in the space of Disse by immuno-electron microscopy.

3.4 RESULTS II : ALTERATIONS IN LIVER DISEASE

No differences in either the ultrastructural distribution or intensity of immuno-gold labelling was found for any extracellular matrix protein in biopsy material from patients with non-specific reactive hepatitis, granulomatous hepatitis, nodular regenerative hyperplasia or intrahepatic cholestasis. However, in three cases of acute hepatitis, intense labelling for fibronectin was seen adjacent to areas of parenchymal cell injury.

In the two cases of alcoholic hepatitis studied, marked pericellular type III collagen was noted surrounding damaged parenchymal cells (Burt *et al*, 1990) (Figure 3.15). Strong immuno-gold labelling for collagen types I, III, V and VI was found both within the space of Disse and on fibrous septa in material obtained from patients with stage IV primary biliary cirrhosis, advanced primary sclerosing cholangitis, alcoholic cirrhosis and inactive cryptogenic cirrhosis (Figure 3.16). Heavy labelling was also noted for fibronectin but immunoreactivity for collagen type IV and laminin was not identified in septa. Increased labelling for collagen type IV and laminin was, however, identified within the space of Disse in cirrhotic and pre-cirrhotic primary biliary cirrhosis, alcoholic hepatitis and cirrhosis and in chronic active hepatitis (Figure 3.17). In some cases this was associated with the appearance of basement membrane-like material beneath sinusoidal endothelial cells.

Figure 3.15 Immuno-electron microscopic localization of collagen type III in alcoholic liver disease.

Intense labelling for collagen type III was a prominent feature in cases of alcoholic hepatitis and active alcoholic cirrhosis. Heavy labelling is present on striated collagen fibrils adjacent to a parenchymal cell. (Uranyl acetate/methyl cellulose; protein A-gold method; 10nm gold particles; x 55,000).

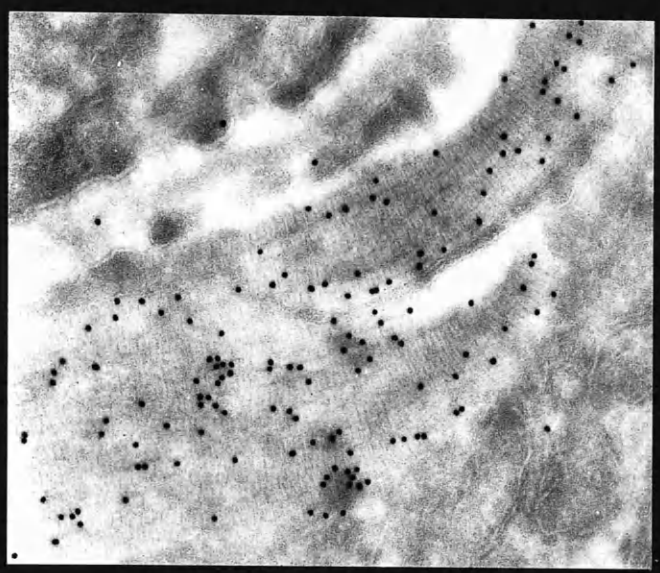


Figure 3.16 Immuno-electron microscopic localization of collagen type VI in chronic active hepatitis.

Heavy labelling is seen on and adjacent to, striated collagen fibrils within the space of Disse in a biopsy from a patient with autoimmune chronic active hepatitis. (E : endothelial cell; P : parenchymal cell). (Uranyl acetate/ methyl cellulose; protein A-gold method; 10nm gold particles; x 25,000).

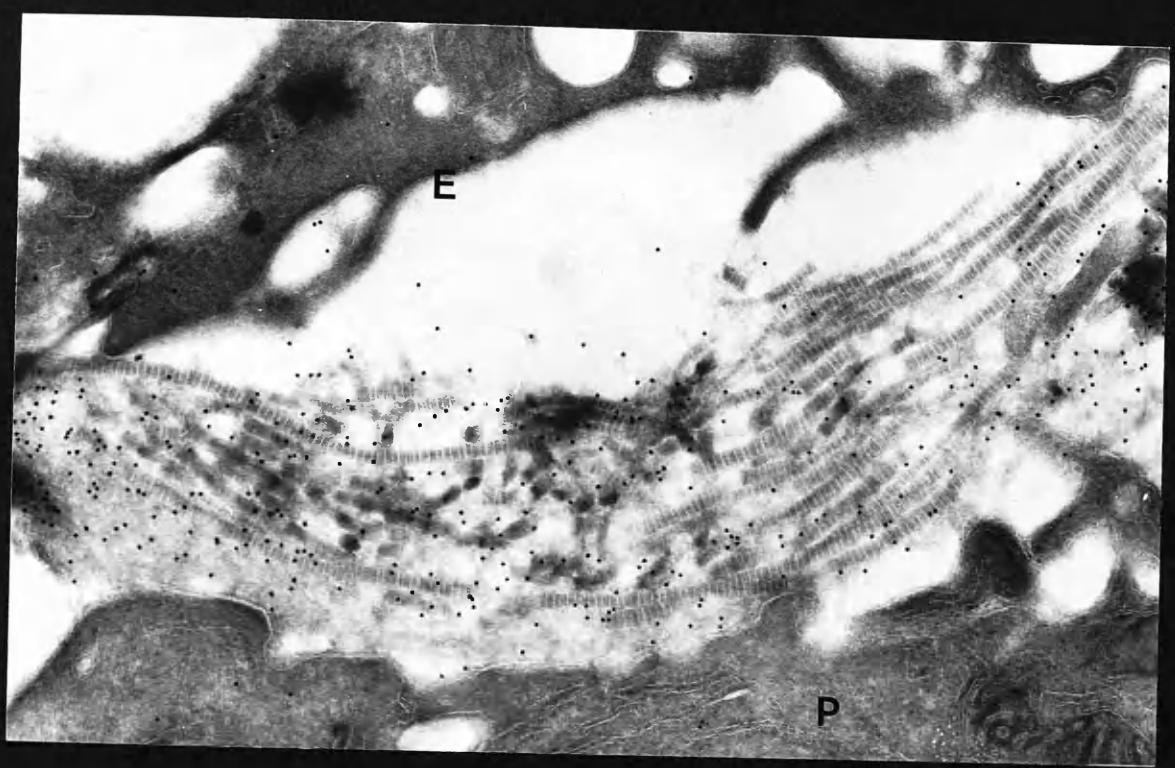
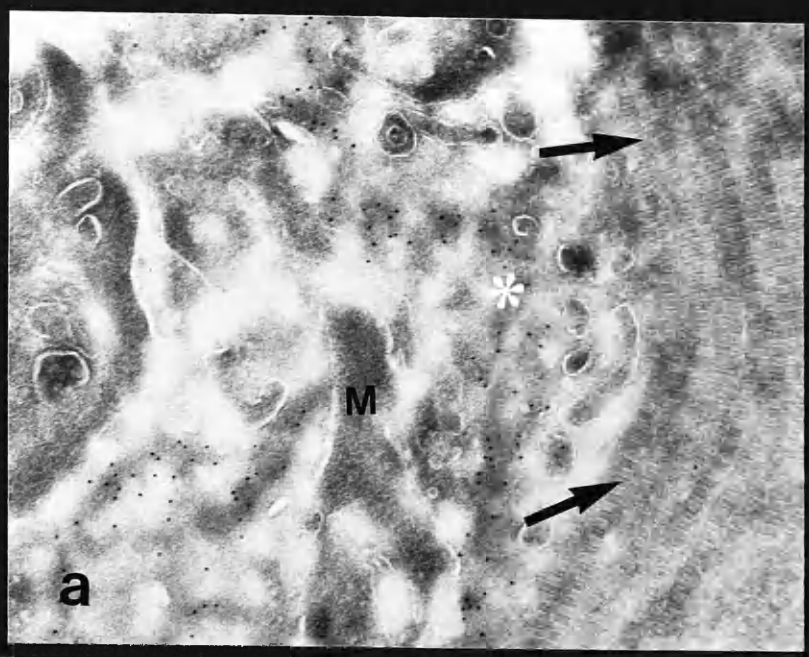
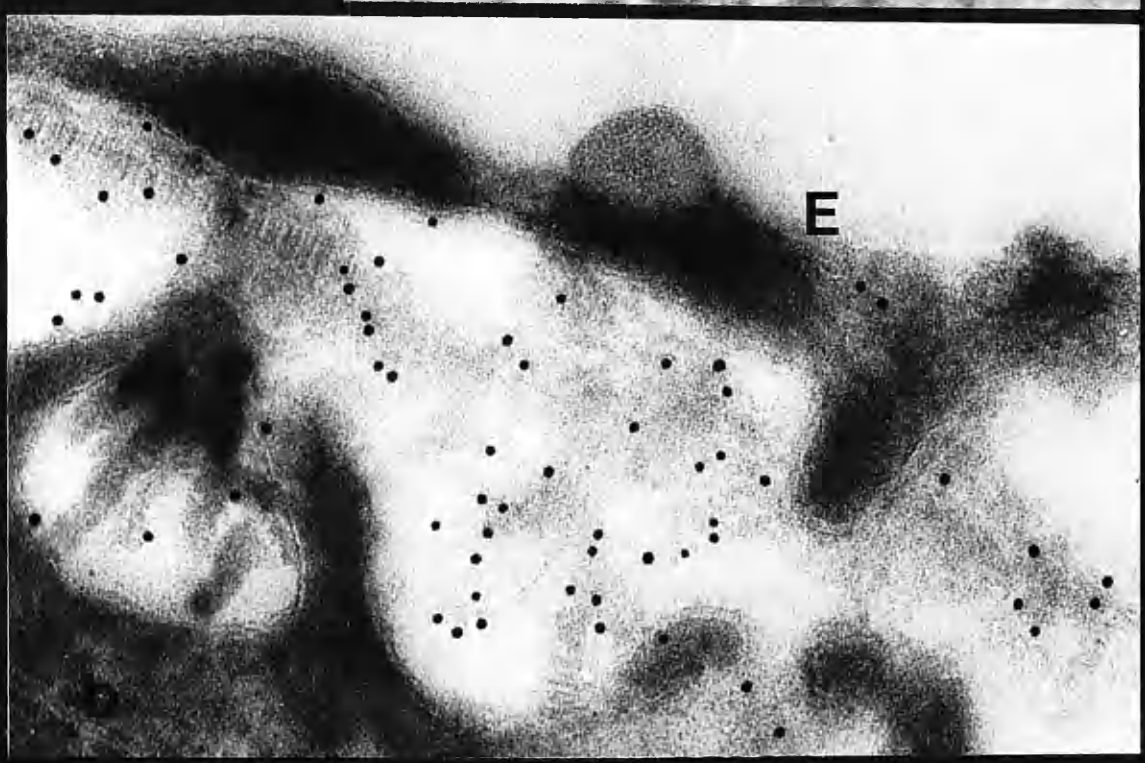

E
P

Figure 3.17 Immuno-electron microscopic visualization of collagen type IV (7S) in chronic liver disease.

a. Heavy labelling for collagen type IV can be seen on flocculent material (*) lying within the space of Disse between parenchymal cell microvilli (M) and striated fibrils (arrows) in a biopsy from a patient with stage III primary biliary cirrhosis. (Uranyl acetate/methyl cellulose; protein A-gold method; x 35,000).

b. In this micrograph, immunogold labelling for collagen type IV (7S) is present on amorphous material lying within the space of Disse in a biopsy from a patient with primary sclerosing cholangitis. (Uranyl acetate/ methyl cellulose; protein A-gold method; x 75,000).


M
a


E

3.5 DISCUSSION

The ultracryomicrotomy method used was originally described by Tokuyasu (1973) and has subsequently been used extensively in cell biology in the study of membranes (Griffiths *et al*, 1982), protein secretion (Slot and Geuze, 1984) and the cytoskeleton (Geiger *et al*, 1981). Such studies have demonstrated that ultracryomicrotomy may provide sections for immuno-electron microscopy in which there is greater preservation of antigenic determinants than can be achieved using traditional resin-embedded material.

In the present study, I have demonstrated that the method may be adapted for use with human biopsy material. The technique used was a modification of the protocol described by Griffiths *et al* (1984) which was subsequently applied to a study of extracellular matrix proteins in perfusion-fixed rat liver (Geerts *et al*, 1986a and b). Although the ultrastructural detail obtained here was less than can be achieved with animal tissues, there was nevertheless good preservation of most structures. The cytoplasmic vacuolations seen in parenchymal cells has been described by other groups (Griffiths *et al*, 1984; Slot *et al*, 1989). While this may in part represent freezing artefact, it is more likely to be the result of loss of cytoplasmic glycogen during tissue processing.

The demonstration of labelling for the interstitial collagen types I and III within the space of Disse in normal human liver confirms the findings of several previous studies (Wick *et al*, 1978, Grimaud *et al*, 1980; Voss *et al*, 1980; Konomi *et al*, 1981; Clément *et al*, 1984, 1985; Sakakibara *et al*, 1986). In keeping with many of the earlier observations, the labelling intensity at both light and electron microscope levels was greater for collagen type III than type I. In the initial immunofluorescence experiments only weak immunoreactivity could be seen along sinusoidal walls using antibodies to the processed collagen type I molecule and procollagen type I. Although the intensity of labelling was enhanced using the peroxidase-antiperoxidase method, staining remained patchy. By contrast, staining for collagen type III using antibodies to the mature protein and to the amino-terminal propeptide of procollagen type III showed intense labelling with a continuous distribution along sinusoidal walls.

Ultrastructurally, labelling for both collagen type III and type I was found on collagen fibres within the space of Disse. The labelling of striated fibrils at the ultrastructural level by

antibodies raised against the amino-terminal propeptide of both procollagen molecules as noted here, has been described by other investigators (Sato *et al*, 1986; Geerts *et al*, 1986a and b; 1990). As the distribution of labelling was identical to that noted using antibodies to the helical domains, this indicates that at least some amino-terminal propeptides are not completely cleaved off when the molecules are incorporated into mature fibrils. Although slight differences were observed in distribution (type I principally being seen at the periphery of collagen fibrils), these results indicate that some fibres (and possibly individual fibrils) within the space of Disse may be composed of both types of interstitial collagen. Classically, type III collagen is considered to be present in thin striated collagen fibrils (20 - 60nm diameter) whereas type I is associated with thicker fibrils (Fleischmajer *et al*, 1981). However, Keene *et al* (1987) showed that type III collagen could be immunolocalized in all striated fibrils in human skin, tendon and amnion, irrespective of diameter. Furthermore, the existence of so-called hybrid fibrils composed of more than one interstitial collagen has been described in the avian cornea (Birk *et al*, 1988). Recently, Geerts *et al* (1990) demonstrated by a double-labelling method using protein A-gold probes of differing sizes that both collagen type I and procollagen type III were present within individual fibrils in the space of Disse in normal rat liver and concluded that most were hybrid. Although their paper contained morphometric data of collagen fibril diameter for rat and human liver, double-labelling experiments were not carried in human tissue. While it seems likely that hybrid fibrils are indeed present in the human space of Disse, it is interesting to note that Geerts and co-workers identified morphometric differences between fibrils of rat and human liver. In the present study, large numbers of fibrils were found which did not label with anti-type I collagen or anti-procollagen I antibodies. This may suggest that in human liver some fibrils are composed exclusively of collagen type III. Although the significance of fibril and fibre composition is at present unclear, there is some evidence from *in vitro* studies of collagen self-assembly that the balance between types I and III may determine the rate of growth of fibrils (Lapiere *et al*, 1977); alterations to this balance may thus be important during fibrogenesis. Further studies to determine fibril composition in human liver are therefore indicated.

Identification of type V collagen as a constituent of the space of Disse in normal liver is in keeping with the observations of Geerts *et al* (1982) and Schuppan *et al* (1986a). Although this protein was originally considered to be a component of basement membranes, its

distribution in other tissues as assessed by immunofluorescence and immuno-electron microscopy indicate that it should be regarded as an interstitial collagen (Schuppan *et al*, 1986a). Although it is frequently found in close proximity to basement membranes, it may also be identified within arterial intima and in buccal submucosa (Schuppan *et al*, 1986a). In keeping with its role as an interstitial collagen is the observation in the present study of immunoreactivity for type V collagen within portal tract stroma with no apparent accentuation around bile ducts or vessels. The study of Birk *et al* (1988) suggests that this collagen may also form part of hybrid fibrils in association with collagen I. Unfortunately, the antibody used in the present study could only detect the antigen in Bouin's-fixed tissue; attempts at localizing collagen type V at the ultrastructural level were unsuccessful. Further studies using other antibodies, including those to the amino-terminal peptide of procollagen type V, are now planned.

Type VI collagen, a group 2 molecule (Table 3.1) has been shown by biochemical analysis and rotary shadowing electron microscopy to have an unusual macromolecular structure (Odermatt *et al*, 1983; Furthmayr *et al*, 1983; Hessle & Engvall, 1984; Engvall *et al*, 1986). Two type VI collagen monomers of length 105nm with large globular domains at each end are aligned to an anti-parallel dimer, which then form a tetramer serving as a building block for a chain-like molecule. The protein was originally isolated from aorta but has subsequently been shown to be present in a wide range of tissues (Linsenmayer *et al*, 1986; Wu *et al*, 1987; Becker *et al*, 1986a and b; Keene *et al*, 1988; Amenta *et al*, 1988). Geerts *et al* (1982) showed by indirect immunoperoxidase labelling that type VI collagen was present in portal tract stroma and along sinusoids in normal rat liver. In the present study, this component was found to be a constituent of the normal perisinusoidal extracellular matrix of human liver. The demonstration of zonal heterogeneity for this protein is a novel observation. As there was no such heterogeneity for other matrix proteins using the same tissue, it is unlikely that this represents any form of fixation artefact. Previous morphometric studies have failed to show quantitative differences in the amount of fibrillar collagen within the space of Disse in different zones of the acinus (Junge *et al*, 1988b). The present study, however, suggests that there may be quantitative differences for some of the "minor" components of the extracellular matrix. It is possible that the observed variations in immunoreactivity may not necessarily reflect different concentrations of type VI collagen itself. Similar observations would be expected if there were differences in

the concentration of other components (such as proteoglycans) which were masking type VI collagen. The significance of such zonal hetergeneity remains to be determined. Intra-acinar zonal heterogeneity in several aspects of parenchymal cell function including carbohydrate, lipid and xenobiotic metabolism is now well documented (Gumucio, 1989; Jungermann & Katz, 1989). It is possible that zonal heterogeneity of extracellular matrix composition may, through the complex cell-matrix interactions discussed in Chapter 9, contribute to this functional heterogeneity within the liver.

At the ultrastructural level, the distribution of this protein in normal liver was seen to differ from that of other components. The demonstration of labelling for type VI collagen on amorphous material between striated collagen fibrils is in keeping with the observations of Von der Mark *et al* (1984) and Bruns *et al* (1986). Although the precise function of type VI collagen remains uncertain, Keene *et al* (1988) have suggested that it may form a flexible network which serves to interconnect structures such as blood vessels, interstitial collagen and cells. The ultra-structural localization noted in the present study suggests that it may form an independent, interconnecting meshwork of microfilaments which reinforce the structural support of the extracellular matrix, lending support to the hypothesis of Keene *et al* (1988). In keeping with this are recent sequence data for the three α chains of type VI collagen which demonstrated the presence of domains with the potential to interact with both cellular integrins and interstitial collagen (Chu *et al*, 1988, 1989; Bonaldo *et al*, 1989).

As discussed in Chapter 1, the normal sinusoids are lined by fenestrated endothelial cells which lack any recognizable basement membrane. Although Voss *et al* (1980) were unable to immunolocalize collagen type IV in human liver sinusoids, several groups subsequently demonstrated its presence at that site in a variety of species including human (Hahn *et al*, 1980; Grimaud *et al*, 1980; Sano *et al*, 1982; Martinez-Hernandez, 1984). In the present study, immunoreactivity for this protein was noted with a continuous staining pattern along sinusoidal walls using antibodies to both 7S and NCl domains. By immuno-electron microscopy, labelling could be seen beneath sinusoidal endothelial cells and surrounding fat-storing cells. If type IV collagen is present in the normal space of Disse, how can we explain the apparent absence of basement membrane at this site? Basement membranes are complex structures which form 3D networks of irregular fuzzy strands referred to as cords. In addition to collagen type IV, these

cords are now known to contain at least three other essential components: laminin, nidogen and heparan sulphate proteoglycan (Leblond & Inoue, 1989). Biochemical studies and rotary shadowing experiments have shown that mixtures of these proteins *in vitro* can result in the assembly of basement membrane material. Although fibronectin is also frequently found within basement membranes, it does not appear to be essential for their formation; it remains unclear whether it is simply absorbed into basement membranes from the plasma.

It is possible that one or more of these other essential components may be absent from the space of Disse. Previous immunohistochemical studies have yielded conflicting results on the presence of laminin in the perisinusoidal space. Geerts *et al* (1982) were unable to demonstrate this protein in rat liver sinusoids. Similarly, in immunofluorescent studies of human liver, Hahn *et al* (1980) and Bianchi *et al* (1984) claimed that laminin was absent from the space of Disse. However, several other investigators have identified this component at that site. Maher *et al* (1988b) immunolocalized laminin in sinusoidal walls of normal rat liver using two affinity-purified antibodies. Clément *et al* (1988) also found labelling in the space of Disse in rat, mouse and human liver but by light microscopy, immunoreactivity was principally noted within a population of sinusoidal cells (see Chapter 4). At the ultrastructural level, faint, discontinuous labelling was detectable. Further support for its presence in the space of Disse comes from the studies of Abrahamson & Caulfield (1985) who demonstrated binding of anti-laminin antibody along sinusoids of rat liver after injection of specific heterologous (sheep) antibody *in vivo*.

In the present study, unequivocal staining for this protein was noted along sinusoidal walls using three different affinity-purified antibodies to distinct regions of the laminin molecule: the P1 domain and two pepsin-derived fragments (120kD and 60kD). With one of these (anti-120kD fragment) the protein could be identified at the ultrastructural level in the space of Disse with a similar distribution to that noted for collagen type IV. However, the results demonstrated that immunoreactivity was highly dependent on the mode of tissue fixation (Table 3.4); this may in part explain the disparate results obtained by previous investigators.

Geerts *et al* (1986a) immunolocalized the core protein of heparan sulphate proteoglycan in normal rat liver. Using indirect immunofluorescence on semi-thin frozen sections (0.5 - 1μm), the protein was identified along sinusoidal walls. Geerts and co-workers were able to

demonstrate by immuno-electron microscopy that it was present in amorphous material apparently connecting parenchymal cells and sinusoidal endothelial cells. In the only other published study of intrahepatic proteoglycan immunolocalization, Voss *et al* (1986) attempted to identify chondroitin sulphate and dermatan sulphate proteoglycan in human liver tissue using antibodies raised against the core protein of an iduronic acid-rich proteoglycan secreted by dermal fibroblasts. Although no staining of sinusoidal walls was found in untreated sections, the core protein was identified within sinusoidal walls after exposure to chondroitinase to remove glycosaminoglycan chains. While this would be in keeping with the presence of proteoglycan in the space of Disse, the antibody used was poorly characterized. Furthermore, the immunofluorescent signal obtained following enzyme treatment was diffuse with marked intracellular staining of all parenchymal and non-parenchymal cells. Until recently, the lack of more rigorously characterized antibodies precluded any further studies of proteoglycan distribution. However, the generation of monoclonal antibodies to several core proteins and to epitopes on specific carbohydrate chains will now permit such investigations (A. Gressner, Marburg; personal communication).

There is evidence therefore that collagen type IV, laminin and possibly heparan sulphate proteoglycan may all be present in the space of Disse of normal liver. Although the paucity of basement membrane material at this site may simply reflect low levels of these individual components, the absence of staining for nidogen in the present study suggests that this may be the "missing link". This observation is in keeping with the recent findings of Ramadori *et al* (1991). However, as both studies used the same antibody and only weak staining of basement membranes around portal tract vessels and bile ducts was noted, further studies using other anti-nidogen antibodies are required to confirm these results.

In the present study, an affinity-purified polyclonal antibody to monkey undulin was used to examine the distribution of this novel matrix protein. Immunoreactivity was demonstrated within portal tract stroma but only weak staining was found along sinusoidal walls. Although staining could be observed in some Bouin's-fixed material, this antibody gave inconsistent results. Undulin is widely distributed in other tissues and has been shown to be closely associated with striated collagen fibrils. It is known to be immunologically unrelated to other extracellular matrix components, although the protein shows some sequence homology with fibronectin and

tenascin (Schuppan *et al*, 1990). The distribution of undulin suggests that it may play a role in the supramolecular organization of striated collagen in the formation of fibres. Unfortunately, it was not possible to identify undulin at the ultrastructural level in my material. Application of the recently developed monoclonal antibody 15IIID6 recognizing an epitope on human undulin, (D. Schuppan, Berlin; personal communication) may provide a more sensitive method for studying the precise localization of this protein.

By light microscopy, vitronectin could be identified along sinusoidal walls and to a lesser extent, within portal tract stroma. The pattern of distribution is therefore similar to that of fibronectin. Although it is considerably smaller than fibronectin (molecular weight 70kD), it is also an adhesion molecule which is present both in plasma and the extracellular matrix. Two previous studies have also shown by indirect immunofluorescence that this protein may be present in the space of Disse (Hayman *et al*, 1983; Reilly & Nash, 1988). Unfortunately as with the antibodies to collagen type V and undulin, no immunolabelling could be obtained in paraformaldehyde/glutaraldehyde-fixed tissue, precluding any assessment of its ultrastructural distribution.

In summary, the present study has demonstrated that the extracellular matrix of the normal space of Disse in human liver is a complex structure which is composed of collagen types I, III, V and VI, the basement membrane proteins type IV collagen and laminin, and the structural glycoproteins fibronectin, vitronectin and possibly undulin; it does not appear to contain nidogen. Van Eyken *et al* (1990) have recently shown that tenascin may also be a constituent. Using an affinity-purified polyclonal antibody to chicken tenascin, they identified immunoreactivity for the protein along sinusoidal walls. A discontinuous staining pattern was noted although the intensity of immunoreactivity was reported to be weak. It is of interest that expression of tenascin in normal liver appeared to be restricted to sinusoidal walls; no staining was identified within portal tract stroma. Van Eyken *et al* did not study the distribution of the protein at the ultrastructural level; such investigations would be of interest to determine the relationship between tenascin and (i) other matrix components such as the interstitial collagens and (ii) cell membranes of sinusoidal and parenchymal cells. Biochemical studies have previously demonstrated that the hexameric structure of the tenascin molecule facilitates binding to other components of the extracellular matrix (Chiquet-Ehrismann *et al*, 1988) and the

presence of an RGD sequence is in keeping with a role in cell-matrix binding through integrin receptors (Bourdon & Ruoslahti, 1989).

Neuronectin is another non-collagenous structural glycoprotein which has also recently been identified within sinusoidal walls (Garin-Chesa *et al*, 1989) The protein, a multimeric glycoprotein composed of disulphide-linked 259 and 180 kD subunits, was identified using a monoclonal antibody raised against SK-MG-10 astrocytoma cells. Sequence data is not yet available, but the distribution of the protein in neural tissues suggests that it is distinct from other known matrix proteins. Although the paper of Garin-Chesa *et al* (1989) does not include illustrations of immunolocalization in liver, they suggest that, like tenascin, neuronectin may be restricted to sinusoidal walls.

The distribution of other extracellular matrix glycoproteins within the human liver remains to be determined; the possibility that proteins such as thrombospondin may also be components of the perisinusoidal matrix should be explored. Furthermore, there is a need for more detailed information on the distribution of elastin in human liver. To date, studies have relied upon histochemical methods such as Shikata's orcein stain for the demonstration of elastic fibres (Scheuer & Maggi, 1980; Bedosa *et al*, 1990). These have suggested that the normal perisinusoidal matrix is devoid of elastin. Further support for this comes from absence of immunoreactivity for amyloid P component along sinusoidal walls in human liver (unpublished observations). However, these require to be confirmed immunohistochemically using antibodies to elastin itself.

The investigations carried out into the distribution of extracellular matrix proteins in disease were limited to ultrastructural studies, as only small pieces of tissue could be obtained for experimental purposes from liver biopsies that were being performed for clinical diagnosis. It is accepted that more information may have been obtained about the deposition of matrix proteins in different diseases if larger pieces of tissue had been available for light microscopic immunolocalization. As most of the antibodies used showed negligible immunoreactivity in routinely processed (formal sublimate-fixed) material, it was not possible to examine this in archival material. Nevertheless, the ultrastructural studies performed did indicate that alterations may occur in the amount of matrix proteins present in different forms of liver injury. In three cases of acute hepatitis, strong labelling was noted for fibronectin in the space of Disse around

damaged parenchymal cells. Although other components could also be identified (collagen types I, III and IV (7S) and laminin 120 kD), no appreciable differences in intensity of labelling were noted compared with normal liver. As the anti-fibronectin antibody used in this study recognizes epitopes common to plasma and extracellular matrix forms of the protein and indeed was raised to the plasma form, it seems likely that some of the protein detected may have been released by surviving parenchymal cells as part of the acute phase response (Pick-Kober *et al*, 1986; Christiansen *et al*, 1988). Nevertheless, irrespective of its origin, deposition of fibronectin is known to be important in the early phases of wound healing where it is thought to act as a scaffold for the deposition of other matrix proteins as part of the repair process (Kurkinen *et al*, 1980; Whaley & Burt, 1991). Ballardini *et al* (1985) have demonstrated that fibronectin is laid down early during the response to experimental liver injury in rats. Two previous studies have also documented enhanced immunolabelling for fibronectin in acute viral hepatitis. Takahashi *et al* (1985) noted intense immunoreactivity for fibronectin by light microscopy in two cases of acute hepatitis. In a more extensive study, Inuzuka *et al* (1990) recently demonstrated accumulation of this protein within areas of necrosis in 15 cases of acute hepatitis A; their study included ultrastructural localization of the protein by immuno-electron microscopy. They also identified increased labelling for collagen types III and V in many of their cases. Although data are not available from my cases on type V collagen, no increased labelling could be demonstrated for type III collagen.

Heavy labelling for fibronectin was also observed in cases of alcoholic liver disease in which there was established fibrosis. This is in keeping with the findings of Junge *et al* (1988a) who used an immunoperoxidase method to study changes in the distribution of this protein at the light microscope level at different stages of alcoholic liver disease. Similarly, Takahashi *et al* (1985) found intense labelling within perivenular sinusoids in alcohol-induced hepatic fibrosis. The most striking finding in the present study, however, was that of intense labelling for type III collagen in the space of Disse around injured parenchymal cells. Striated collagen fibrils in areas of pericellular fibrosis in advanced alcoholic liver disease were almost exclusively labelled by antibodies to collagen type III and the amino-terminal propeptide of procollagen type III. By contrast, in cases in which there was an established cirrhosis, large fibrous septa showed heavy labelling for both type I and type III collagen; this is entirely in accordance with the

findings of previous light microscopic immunohistochemical studies of human cirrhosis (Wick *et al*, 1976; Voss *et al*, 1980; Sakakibara *et al*, 1986; Clément *et al*, 1986; Davis & Madri, 1987b) and carbon tetrachloride-induced cirrhosis in rats (Davis & Madri, 1987a).

The other principal alteration noted in the present study was increased labelling for the basement membrane components, type IV collagen and laminin in chronic active hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis and advanced alcoholic liver disease. Enhanced labelling was more marked for collagen type IV than laminin in most cases. Several other groups have previously documented increased staining intensity for type IV collagen in various forms of chronic liver disease (Voss *et al*, 1980; Hahn *et al*, 1980; Grimaud *et al*, 1980; Van Eyken *et al*, 1990; Babbs *et al*, 1990). Hahn *et al* (1980) and later Bianchi *et al* (1984) suggested that whereas in their experience laminin was absent from the space of Disse in normal liver, perisinusoidal immunoreactivity was detectable in chronic active hepatitis. However, as discussed earlier, with more sensitive immunohistochemical methods laminin can be identified within the normal space of Disse.

The association of intense labelling for collagen type IV and laminin with flocculent material in the space of Disse suggests that accumulation of these proteins may be responsible for the development of basement membranes. It remains to be established whether this is accompanied by neo-expression of nidogen (vide supra). The appearance of a basement membrane within the space of Disse in chronic liver disease was first recognized by Schaffner & Popper (1963) who referred to it as "capillarization" of the sinusoids. Since then, several groups have demonstrated that this is a feature of progressive liver damage both in human disease and in experimental hepatic injury (Martinez-Hernandez, 1985; Bioulac-Sage *et al*, 1988; Le Bail *et al*, 1989). Coincident with the appearance of a basement membrane, the sinusoidal endothelial cells undergo phenotypic changes which lead them to resemble vascular endothelial cells: they become defenestrated (Bioulac-Sage *et al*, 1988) and begin to express Factor VIII related antigen and binding sites for Ulex europaeus (Fukuda *et al*, 1986; Petrovic *et al*, 1989; Babbs *et al*, 1990).

It was initially hoped that the immuno-gold technique could be used to quantify differences in the amounts of extracellular matrix proteins present in different liver diseases. Theoretically, the use of an immunolabelling technique such as this should provide an

opportunity to determine the amount of antigen present by counting the number of gold particles (Posthuma *et al*, 1987). However, it became apparent that the ultracryomicrotomy method used produces sections of variable thickness. As this influences the amount of antigen present, particle counting could not be used to provide accurate measurements of matrix protein concentrations. Even in optimally fixed animal tissues, problems of differential penetration of gold particles into different cell structures has been experienced using ultra-thin frozen sections (Slot *et al*, 1989).

Alterations in the composition of the extracellular matrix of the space of Disse as described in this study may clearly have profound effects on hepatic function. Excess amounts of interstitial collagens and basement membrane proteins at this site in chronic liver disease may produce a physical barrier to the flow of substances (including essential nutrients) between the sinusoidal blood and parenchymal cells leading to hypoxia and metabolic insufficiency. Furthermore, if sufficient quantities of matrix proteins accumulate, they may obliterate the sinusoids, producing a block to the intrahepatic microcirculation; this in turn would contribute to the development of portal hypertension, a clinically important complication of chronic liver disease. Finally, it is likely that in addition to such a "space occupying" effect, alterations in the relative amounts of matrix proteins in the space of Disse may, through cellular integrins, interfere with gene expression in parenchymal cells.

CHAPTER 4: ROLE OF SINUSOIDAL CELLS IN THE SYNTHESIS OF EXTRACELLULAR MATRIX PROTEINS

4.1 INTRODUCTION

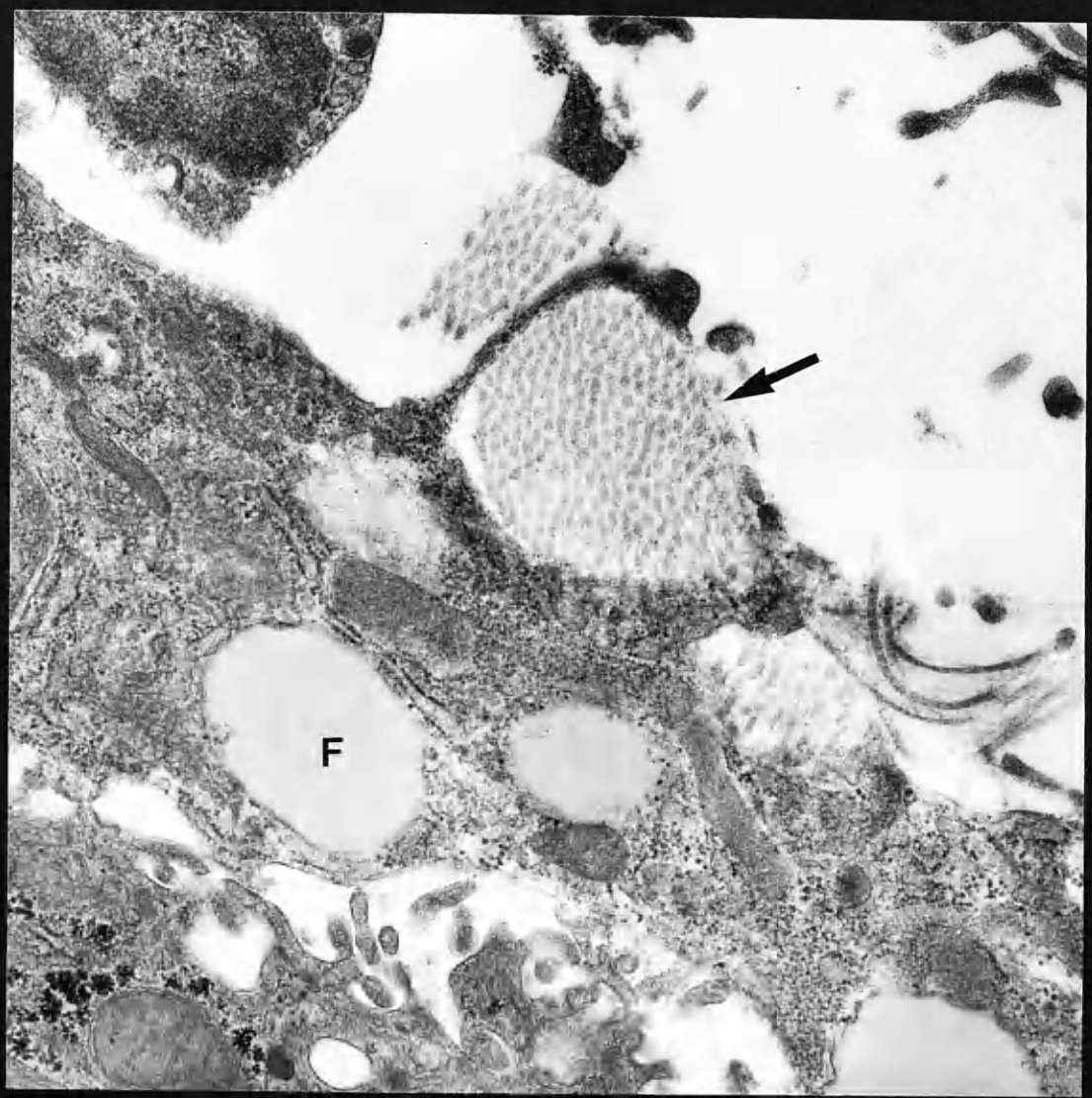
The complex nature of the perisinusoidal extracellular matrix has been described in the preceding chapter. The cellular origin of its constituent proteins is controversial. In most tissues, the predominant source of matrix proteins is the fibroblast. In the liver, however, there are few true fibroblasts; those that are present are limited to portal tract stroma and Glisson's capsule which surrounds the organ. The source of matrix proteins in the normal space of Disse and in hepatic fibrogenesis has therefore been uncertain. The principal contenders are (i) parenchymal cells, (ii) fat-storing cells and (iii) sinusoidal endothelial cells. As macrophages and natural killer cells in other tissues are not major sources of matrix proteins, it has been considered unlikely that Kupffer cells or pit cells play a major role in the production of such proteins. Of the sinusoidal cells, the fat-storing cells have been considered to be the likeliest source of collagens on the basis of ultrastructural observations. These cells can frequently be seen in close apposition to collagen fibres in the space of Disse (Figure 4.1). Furthermore, their well developed rough endoplasmic reticulum indicates that they are active protein synthesizers. The identification of electron microscopic features similar to those noted in smooth muscle cells (see Chapter 1) have suggested to some that fat-storing cells may be related to myofibroblasts. In fibrotic rat liver, so-called transitional cells, with ultrastructural features of both fat-storing cells and fibroblasts have been identified within developing fibrous septa (Kent *et al*, 1976). Similar cells have been noted in human (Callea *et al*, 1982; Minato *et al*, 1983) and baboon liver (Mak *et al*, 1984). The relationship between fat-storing cells and myofibroblasts in considered further in Chapter 5.

The studies described in this chapter were designed to further investigate the cellular origin of hepatic matrix proteins with particular reference to the role of fat-storing cells. As for complement biosynthesis (Chapter 2), two experimental approaches were used (i) immuno-localization of intracellular proteins and (ii) assessment of *in vitro* biosynthesis of matrix proteins by isolated rat sinusoidal cells.

Intracellular matrix proteins were immunolocalized using material obtained during the study of their extracellular distribution as outlined in Chapter 3. It was therefore possible to investigate intracytoplasmic matrix proteins in normal human liver at the light microscope level

Figure 4.1 Transmission electron micrograph of normal rat liver demonstrating close apposition of fat-storing cell to striated collagen fibrils.

The cytoplasm of a fat-storing cell (F) is seen to lie adjacent to striated fibrils (arrow) within the space of Disse. (Uranyl acetate/lead citrate; x 30,000).


F

using the indirect immunofluorescence and peroxidase-antiperoxidase techniques, and at the ultrastructural level using ultracryomicrotomy and immuno-gold labelling; ultrastructural localization was also possible in material obtained from patients with liver disease.

The principal method used for demonstrating *in vitro* synthesis was incorporation of radio-labelled proline and assessment of collagenase-sensitive protein production. Preliminary experiments indicated that negligible amounts of collagenase-sensitive proteins were detectable in rat Kupffer cell or sinusoidal endothelial cell cultures; subsequent studies dealt exclusively with fat-storing cell cultures.

Earlier *in vitro* studies of collagen biosynthesis by isolated liver cells had suggested that parenchymal cells were capable of synthesizing and secreting some collagenous components; this appeared to be enhanced by prolonged culture (Bissell & Guzelian, 1980; Tseng *et al*, 1982; Diegelmann *et al*, 1983). The results of these studies, however, must be interpreted with caution as it has subsequently been shown that fat-storing cells may be contaminants in primary cultures of rat parenchymal cells, particularly after several days in cultures (vide infra). De Leeuw *et al* (1984) identified collagen types I and IV and laminin within rat fat-storing cells in primary culture using immunofluorescence. Furthermore, Friedman *et al* (1985) and Kawase *et al* (1986) demonstrated that supernatants from primary cultures of rat fat-storing cells contained collagen type I and, to a lesser extent, collagen types III and IV. Others were able to show that these cells could synthesize fibronectin (Ramadori *et al*, 1987) and proteoglycans (Schaefer *et al*, 1987). Most of these earlier studies, however, used fat-storing cells isolated from animals that had previously been loaded *in vivo* with vitamin A to improve cell yields. As this may interfere with the biosynthesis of collagenous and non-collagenous proteins by these cells (Davis *et al*, 1987), in the present study only cells obtained from untreated animals were used.

4.2 MATERIALS AND METHODS

4.2.1 Immunohistochemical studies : light microscopy and immuno-electron microscopy

The liver tissue, antibodies and immunohistochemical methods used have been described in Chapter 3.

4.2.2 Isolation, culture and characterization of rat liver fat-storing cells

Fat-storing cells were isolated from livers of male Wistar rats (weight 300g) as described in Chapter 2. Cells harvested from the interface between 11.4% Nycodenz and GBSS were washed in DMEM and subsequently incubated in DMEM containing 10% fetal calf serum (FCS; Gibco, Paisley), 100 U/ml penicillin and 100 ug/ml streptomycin and plated in 24-well culture dishes (for protein synthesis measurements) or in 9.6 cm^2 culture plates (for SDS PAGE studies). After 18 hours incubation at 37°C in 5% CO_2/95% O_2, non-adherent cells and parenchymal cell debris were removed by washing in medium. Purity of cells was assessed at 24 hour intervals after isolation using inversion phase contrast microscopy (Zeiss IM 35). In addition, three and six day-old cell cultures were fixed for 10 minutes at room temperature with 1.5% glutaraldehyde in 0.1M phosphate buffer containing 2% sucrose followed by postfixation with 1% Millonig's osmium tetroxide. For transmission electron microscopy, cells were subsequently dehydrated through graded alcohols and embedded in Epon. For scanning electron microscopy, cells were critical point dried and sputter-coated with gold.

The number of cells per well or per culture dish was counted in at least three randomly selected microscopic fields (0.276 mm^2) in a minimum of three wells. Preliminary experiments established that fat-storing cell numbers remained almost constant during the 24 hour labelling period (vide infra).

4.2.3 [^{3}H]-proline incorporation studies

Cultured fat-storing cells were washed three times with DMEM containing 5% FCS, 100 U/ml penicillin and 50μg/ml ascorbic acid and incubated with this solution containing 20 μCi/ml L-[2,3-^{3}H] proline (20-40 Ci/mmol; New England Nuclear). After 24 hours, culture supernatants were collected and cell layers were washed with 0.5 ml calcium-free GBSS and solubilized using 0.2N sodium hydroxide for 1 hour. Cell layer extracts were then collected. Incorporation of [^{3}H] into protein was measured in supernatants and cell layer extracts using the hot

trichloroacetic acid method; radioactivity was measured in an LKB Wallac liquid scintillation spectrometer.

Incorporation of radiolabelled proline into collagenous protein was measured using the collagenase method described by Peterkofsky & Diegelmann (1971). Following repeated dialysis of supernatants or cell layer extracts against 0.025 M Tris/HCl buffer containing the protease inhibitor N-ethylmaleimide (0.05M), samples were incubated in the presence or absence of bacterial collagenase (column-purified collagenase type III Sigma; 800 U/mg protein) for 150 minutes at 37°C. After precipitation of the remaining protein in the samples by 5% TCA and 0.25% tannic acid, the radioactivity in the sample supernatant was measured. The fraction of collagenous protein, expressed as percentage of total radiolabelled protein was calculated using the formula:

$$\% \text{ collagen} = \frac{100}{5.4 \times \frac{(cpm_t - cpm_c)}{(cpm_c - cpm_b)} + 1}$$

where cpm_t represents total radioactivity in the sample, cpm_c represents radioactivity in the samples treated with collagenase and cpm_b, radioactivity in samples not treated with collagenase (Verbruggen *et al*, 1981).

4.2.4 SDS PAGE analysis of secreted proteins

Cultures were kept in DMEM supplemented with 10% FCS, penicillin and streptomycin for 6 days. The medium was changed for DMEM containing 25 μCi [^{3}H]-proline, 2% FCS, 100 U/ml penicillin and 50 ug/ml ascorbic acid. After 24 hours the medium was removed and either directly processed for SDS-PAGE analysis or subjected to limited pepsin digestion prior to electrophoresis.

(i) Direct processing: After addition of 10% (v/v) protease inhibitor cocktail (1mM phenyl-methylsulphonylfluoride, 100mM N-ethylmaleimide, 10 mM p-OH mercurybenzoate, 200 mM EDTA,) the medium was repeatedly dialysed against 0.1% ammonium carbonate. The radioactivity in the dialysate was counted and samples equivalent to 10^5 cpm were lyophilized.

(ii) Pepsin treatment: After addition of 10% (v/v) inhibitor cocktail, the medium was dialyzed against 0.1% ammonium carbonate and against 0.1% acetic acid. The insoluble material in the dialysate was removed by centrifugation (27000g for 30 minutes). For each 1ml sample of clear supernatant, 100μl of unlabelled rat skin collagen type I solution (0.5 mg/ml), 20 μl formic acid and 50 μl pepsin solution (Worthington; 0.2 mg/ml) was added. The sample was incubated for four hours at 16°C. The pepsin digestion was stopped by adding solid sodium chloride to a final concentration of 12%. A precipitate was allowed to form overnight at 4°C and was centrifuged at 27000g for 30 minutes. The pellet was redissolved in 0.1% acetic acid and dialyzed overnight against this solution. Radioactivity in the dialysate was measured and aliquots equivalent to 10^5 cpm were lyophilized.

Electrophoresis was carried out in 5% polyacrylamide slab gels in the presence of sodium dodecylsulphate (SDS) using the buffer system of Laemmli (1970). Lyophilized samples of medium (vide supra) were dissolved in 50°l sample buffer. The samples were denatured at 80°C for 5 minutes in the presence or absence of 190 mM dithiothreitol. [^{14}C]-labelled pepsin-extracted rat skin collagen type I, plasma fibronectin, ovalbumin albumin, phosphorylase β and myosin (New England Nuclear) were used as molecular weight markers. After electrophoresis, gels were immersed in Enhance (New England Nuclear) for 1 hour, in distilled water for 15 minutes, dried and exposed to X-Omat AR film (Kodak) at -70°C for 3, 7 or 14 days.

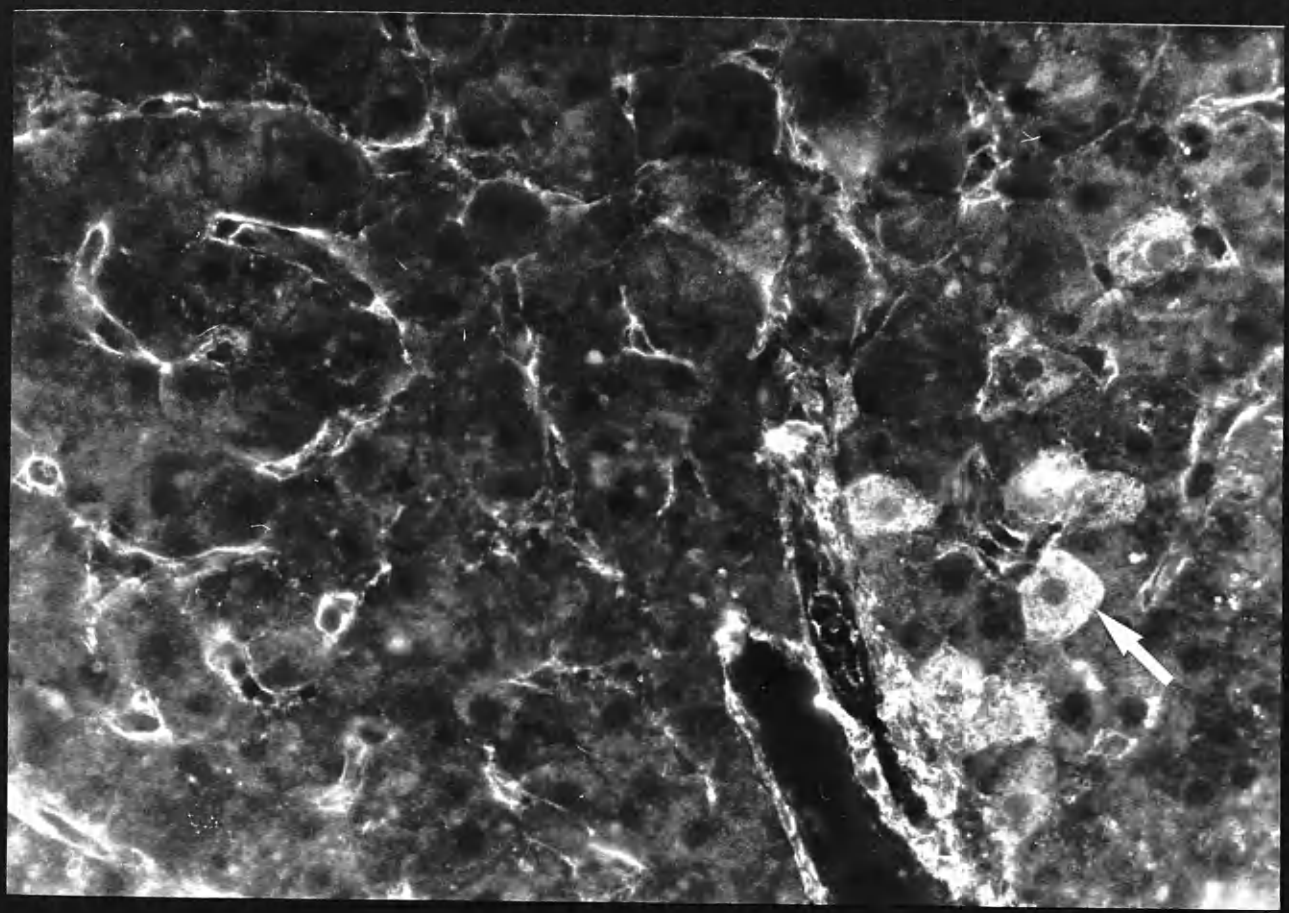
4.3 RESULTS

4.3.1 Immunolocalization of intracellular proteins

In the initial immunofluorescent experiments, strong immunolabelling for fibronectin was seen within some parenchymal cells and occasional non-parenchymal cells (Figure 4.2). However with this technique, reproducible intracellular labelling was not found with any other antibody. By contrast, using the peroxidase-antiperoxidase method on 3μm dewaxed sections, weak cytoplasmic staining for procollagen type I could be detected in occasional parenchymal cells and a smaller number of sinusoidal cells; no intracellular labelling was found using the anti-collagen

Figure 4.2 Indirect immunofluorescent visualization of fibronectin in normal human liver.

In addition to perisinusoidal staining for this structural glycoprotein, occasional parenchymal cells were seen to contain immunoreactive fibronectin (white arrow). (Indirect immunofluorescence; x 400).



I antibody. Similarly, while occasional sinusoidal cells were labelled with the anti-procollagen III N-terminal propeptide, they were negative with the antibody to the processed collagen type III molecule. No intracellular staining could be seen for collagen type V but a population of stellate sinusoidal cells were immunoreactive with anti-type VI collagen (Figure 4.3).

Of the basement membrane components investigated, collagen type IV and laminin could be identified within some sinusoidal cells but were absent from parenchymal cells; no intracellular labelling was found for nidogen. It should be noted that intracellular collagen type IV could only be identified using the anti-7S antibody and not with anti-NC1. Furthermore, staining was seen within only occasional flattened cells (? sinusoidal endothelial cells). Immunoreactivity for laminin (detectable using anti-P1 component and anti-laminin 120kD fragment antibodies) appeared to be restricted to stellate sinusoidal cells (Figure 4.4).

As in the immunofluorescent studies, strong immunolabelling for fibronectin was noted in parenchymal cells and occasional sinusoidal cells using the peroxidase-antiperoxidase method. No intracellular labelling was seen with antibodies to vitronectin or undulin.

At the ultrastructural level, intracellular labelling could be detected using antibodies to the following extracellular matrix components: procollagen types I and III, collagen type IV, fibronectin and laminin. The intensity of signal was enhanced with all antibodies by pre-treatment with hyaluronidase.

The strongest intracellular labelling was found with anti-fibronectin antibody. Gold particles were seen within the cytoplasm of parenchymal cells and some Kupffer and fat-storing cells in normal liver. Increased labelling was noted within parenchymal cells in two cases of acute hepatitis studied. Labelling was present on the endoplasmic reticulum and within cytoplasmic organelles thought to be lysosomes (Figure 4.5).

Intracellular labelling for procollagen type III was restricted to fat-storing cells in which it could be localized to the endoplasmic reticulum (Figure 4.6). In normal liver, only occasional fat-storing cells contained gold particles. By contrast, in material obtained from cases with fibrotic liver disease (alcoholic hepatitis, chronic active hepatitis), greater than 50% of these cells showed significant labelling (ie. above background levels). Labelling for procollagen type I, collagen type VI (Figure 4.7) and laminin (120 kD) was also identified within occasional fat-storing cells although the intensity of labelling was less than that observed for either fibronectin

Figure 4.3 Immunolocalization of collagen type VI in sinusoidal cells : normal human liver.

Intracellular labelling was identified using the anti-collagen type VI antibody. Many of the immunoreactive sinusoidal cells (arrows) had a stellate morphology. (Peroxidase-antiperoxidase method; haematoxylin counterstain; x 1,200).

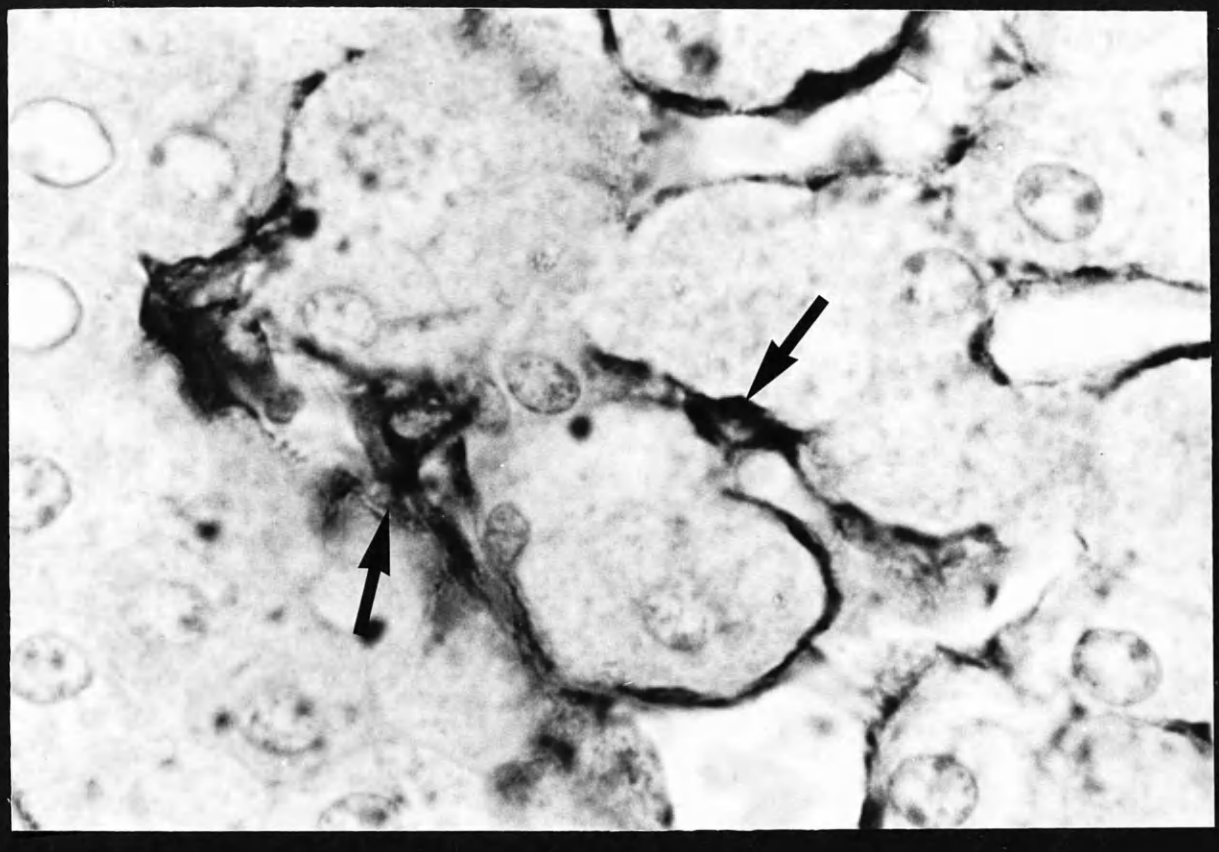


Figure 4.4 Immunolocalization of laminin (120kD) in sinusoidal cells (light microscopy).

Occasional sinusoidal cells can be seen to contain immunoreactive laminin (arrow). Although the limited resolution obtained with light microscopy precluded any definitive identification of these cells, several were seen to contain large numbers of cytoplasmic vacuoles and resembled fat-storing cells. (Peroxidase-antiperoxidase method; haematoxylin counterstain; photographed under Nomarski filters; x 600).

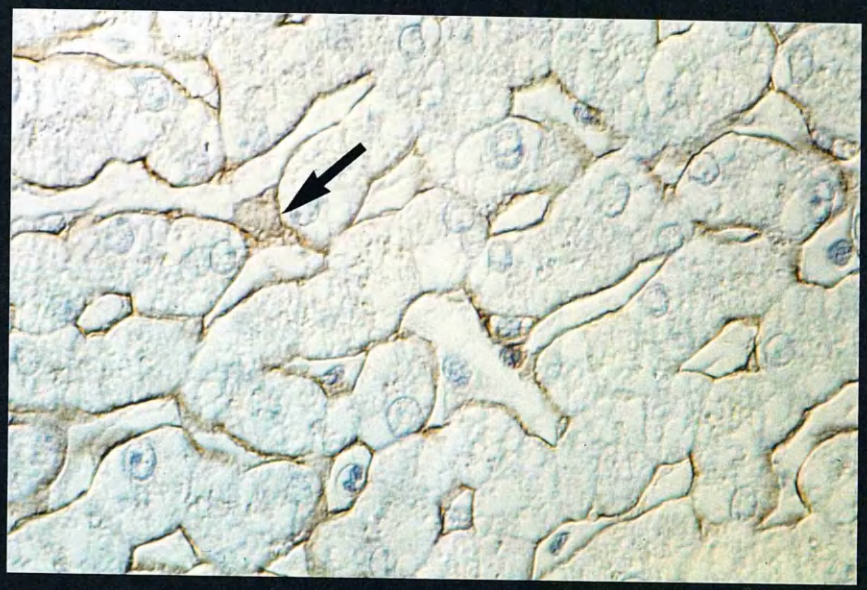


Figure 4.5 Immuno-electron microscopic localization of fibronectin within parenchymal cells : normal human liver.

Heavy labelling can be identified within a lysosome (L) but there is no labelling of an adjacent mitochondrion (M). (Uranyl acetate/methyl cellulose; protein A-gold method; x 89,000).

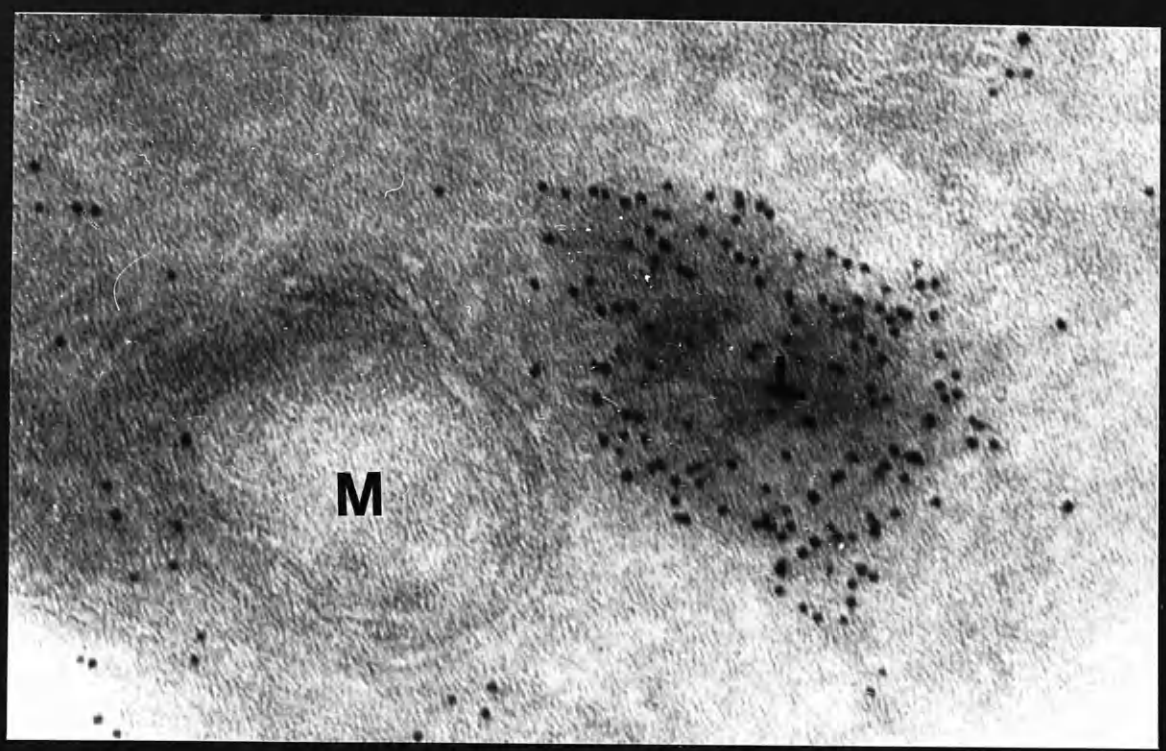

M

Figure 4.6 Immuno-electron microscopic localization of collagen type III within fat-storing cell : normal human liver.

Immuno-gold labelling for this protein could be identified within occasional fat-storing cells using an antibody directed against the N-terminal propeptide of procollagen type III. Gold particles can be seen on and around the endoplasmic reticulum (arrows) but are not present within mitochondria (M). (Uranyl acetate/methyl cellulose; protein A-gold method; x 53,000).

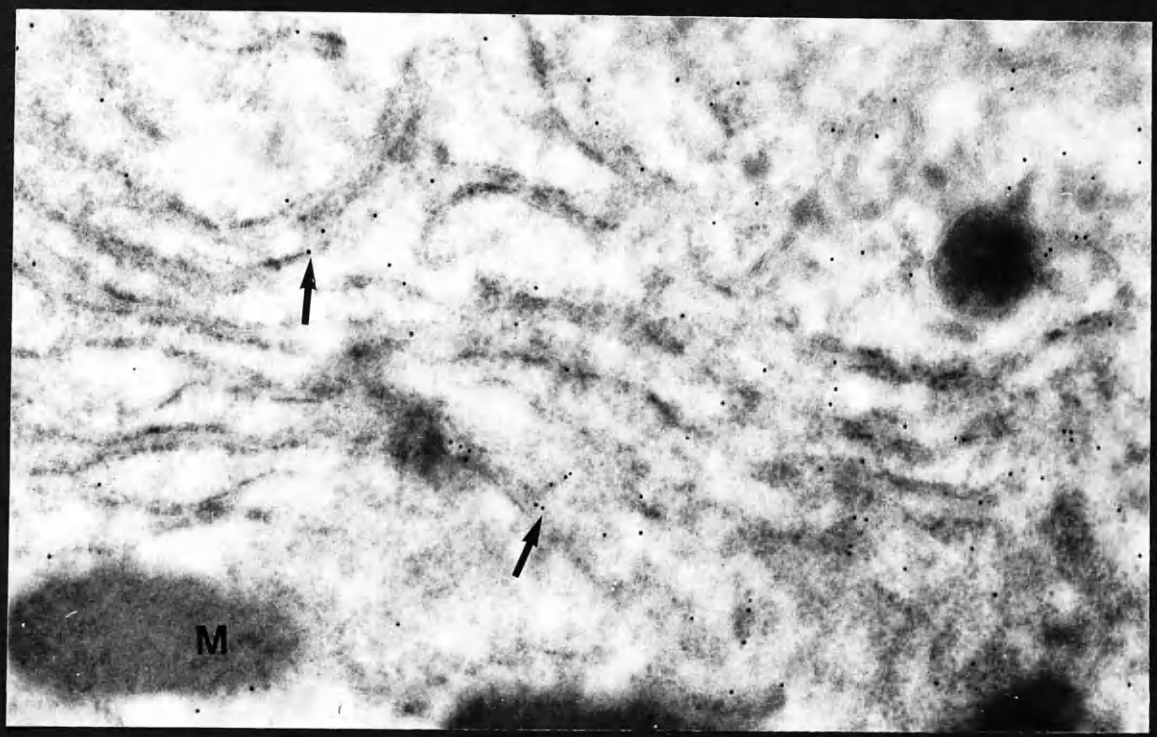
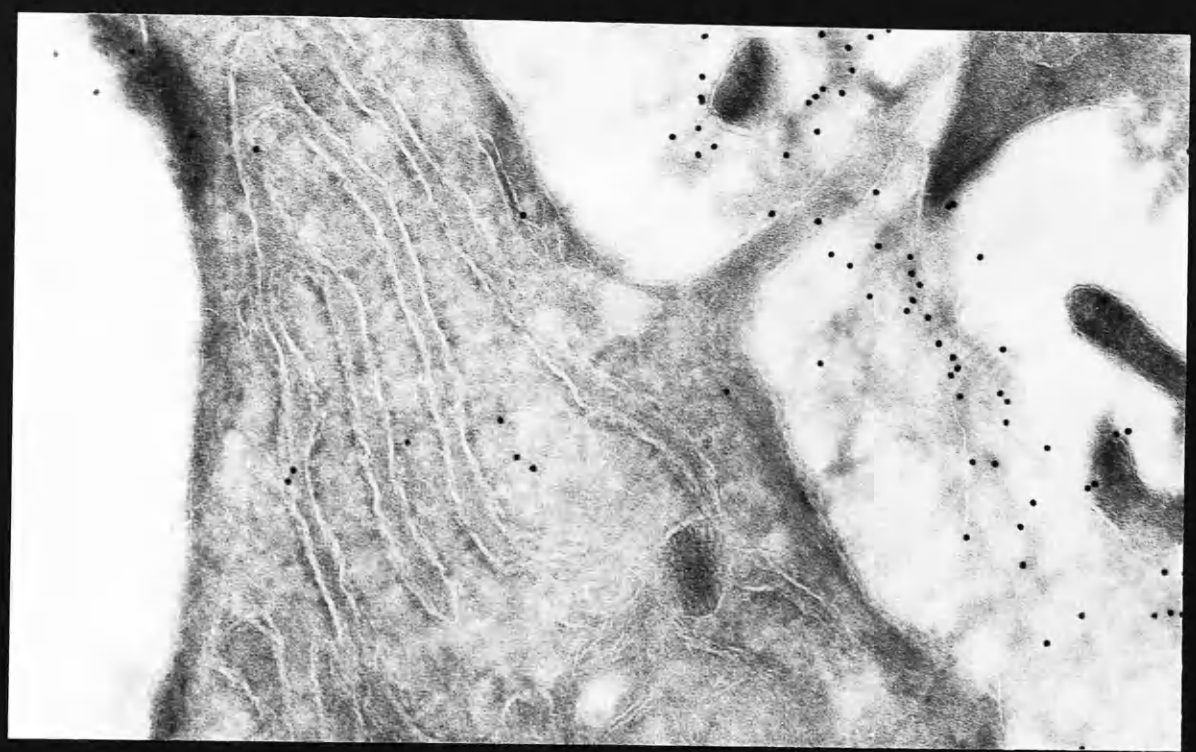

M

Figure 4.7 Immuno-electron microscopic localization of collagen type VI : normal human liver.

Occasional gold particles could be identified within the cytoplasm of fat-storing cells. In this micrograph particles are seen on and adjacent to the endoplasmic reticulum of a fat-storing cell and can also be seen within the space of Disse on amorphous material surrounding a striated collagen fibril. (Uranyl acetate/methyl cellulose; protein A-gold method; x 75,000).



or procollagen type III. Occasional parenchymal cells showed a low level of labelling for procollagen type I but not for laminin or collagen type VI.

4.3.2 Morphology of cultured rat fat-storing cells

As noted in Chapter 2, fat-storing cells attached to culture dish plastic within 18 hours. After removal of non-adherent cells and parenchymal cell debris, the purity of the cultures was found to be 77%, the principal contaminants being Kupffer cells. Viable parenchymal cells were not identified. After 3 days in culture, the cells contained a dense nucleus and abundant cytoplasmic fat droplets (Figures 2.8 and 4.8). By day 6, fat droplets were smaller and less numerous. The cisternae of the rough endoplasmic reticulum was more dilated than in earlier cultures and was frequently filled with floculent material (Geerts *et al*, 1989). In day 6 cells microfilaments and microtubules were prominent. Scanning electron microscopy showed the cultured fat-storing cells to be flattened; fat droplets were visible as small protrusions on the upper surface of the cell (Figure 4.9). No extracellular striated collagen fibrils were identified in any of the cultures by transmission or scanning electron microscopy.

4.3.3 [^{3}H]-proline incorporation

Using the method outlined above, fat-storing cells were radiolabelled with tritiated proline at 3, 4, 5 and 6 days after isolation. The incorporation of radio-labelled proline into culture supernatants and cell layer extracts, expressed per 10^6 cells at each time point, is illustrated in Figure 4.10. A significant increase in incorporation into supernatants (Student's t test: $p < 0.05$) was found when results were compared for day 3 and day 6 cultures. Collagenase-sensitive proteins expressed as a percentage of total radiolabelled proteins also increased significantly ($p < 0.01$) although no change was noted in cell layer extracts (Figure 4.11).

Figure 4.8 Transmission electron micrograph of isolated rat fat-storing cells.

Osmiophilic cytoplasmic fat droplets can be readily identified in these day 3 fat-storing cells. There is a well developed rough endoplasmic reticulum. (Uranyl acetate/lead citrate; x6,700).

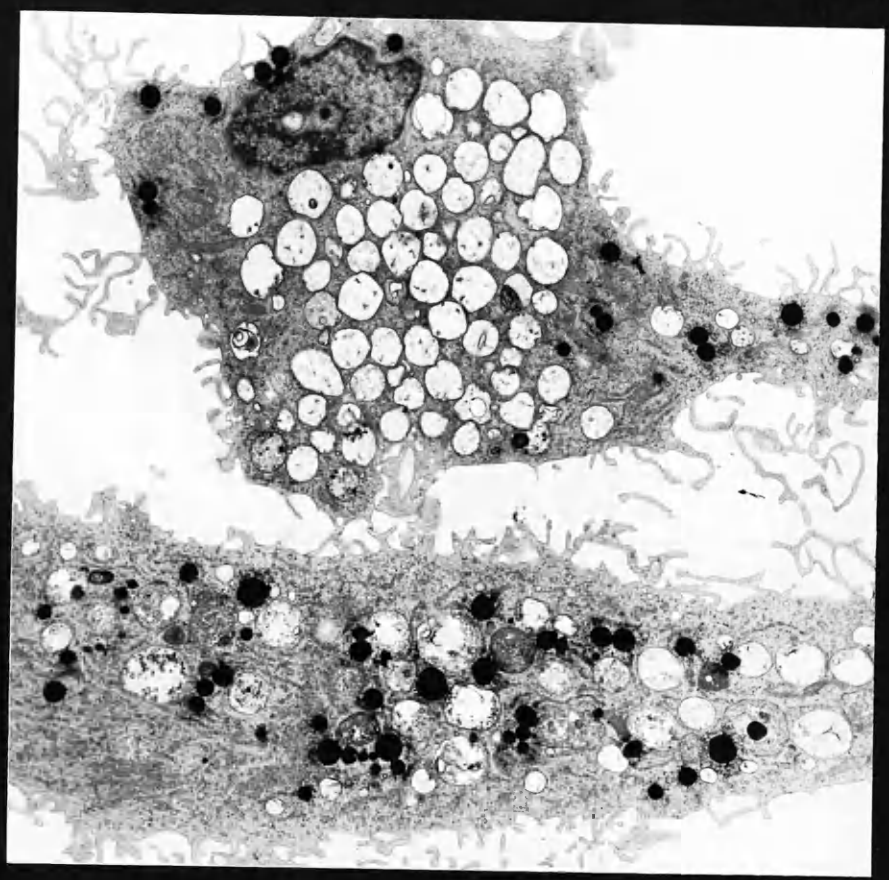


Figure 4.9 Scanning electron micrograph of isolated rat fat-storing cell.

This cell had been cultured for 4 days. By this stage there is flattening of the cell cytoplasm. As a result, small organelles such as nucleoli and cytoplasmic fat droplets (arrows) are visible as local protrusions on the upper surface of the cell. (Gold palladium; x 2,000).

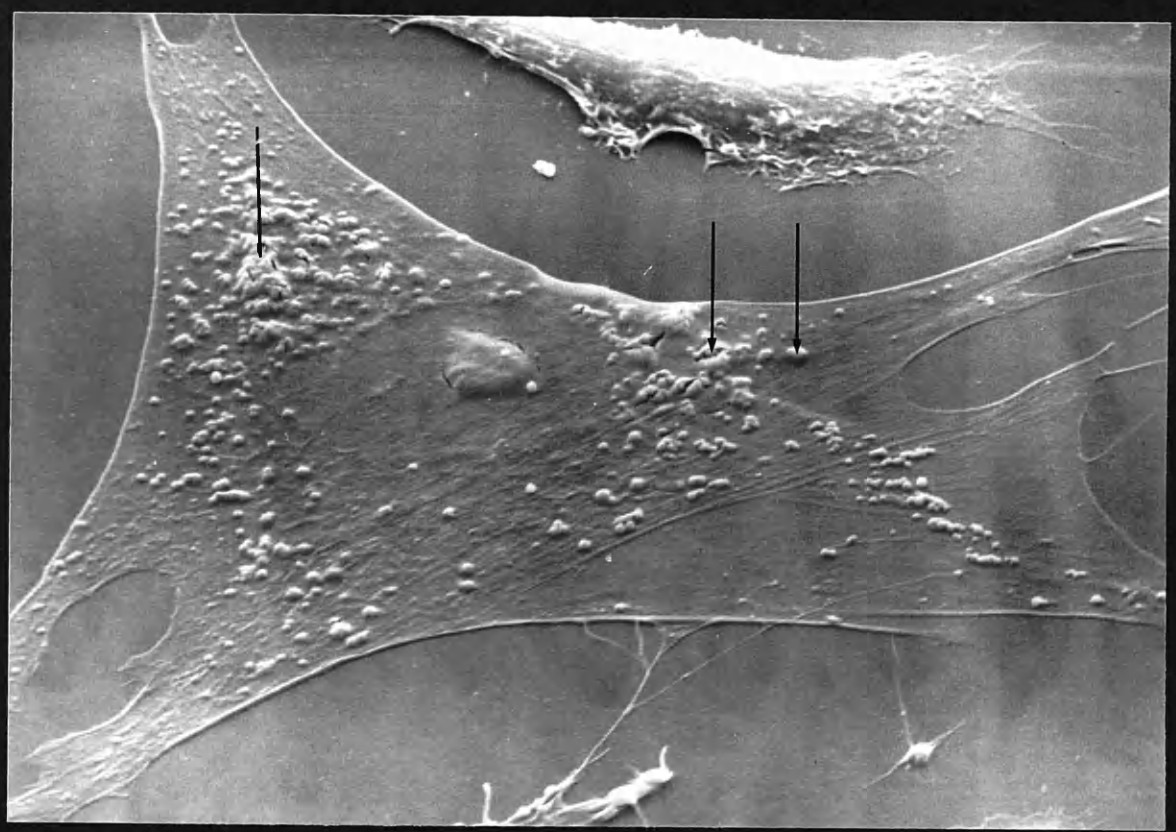


Figure 4.10 Incorporation of [^{3}H]-proline into secreted and cell-associated proteins synthesized by isolated fat-storing cells in primary culture.

The closed bars represent the mean (+ SEM) incorporation into secreted proteins in culture supernatants while the hatched bars represent incorporation into cell-associated proteins (see text). Comparison of day 3 cultures with those at day 6 show a statistically significant increase in incorporation in the medium ($P < 0.05$) although there was no significant difference in incorporation into cell-associated proteins ($P < 0.17$).

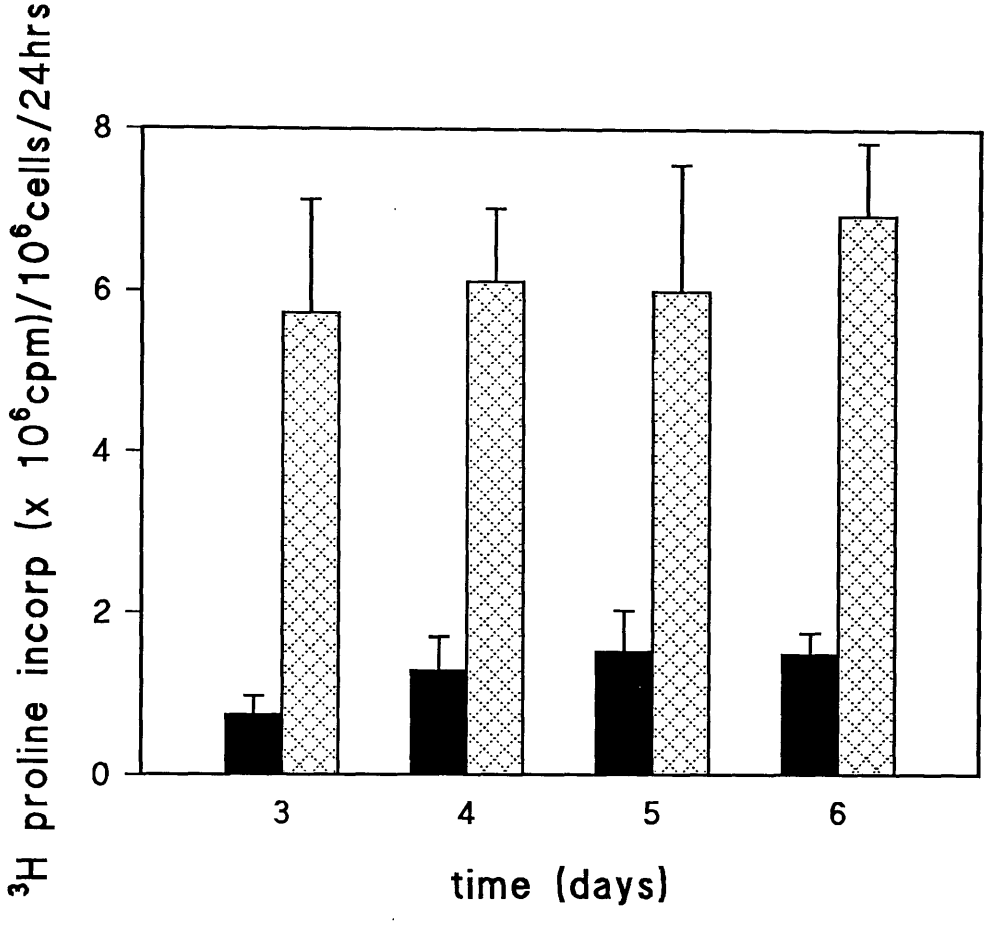
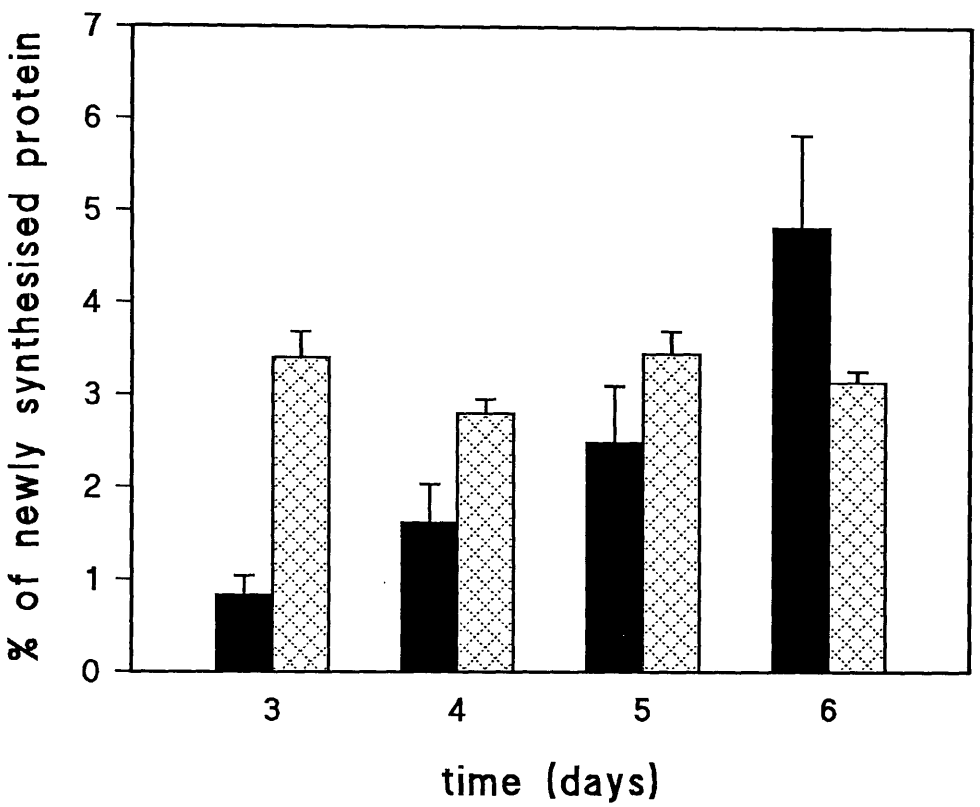

^{3}H proline incorp (x 10^6cpm)/10^6cells/24hrs
8
6
4
2
0
3
4
5
6
time (days)

Figure 4.11 Incorporation of [^{3}H]-proline into collagenase-sensitive proteins synthesised by fat-storing cells in primary culture.

The closed bars represent the mean (+ SEM) of collagenase-sensitive protein expressed as a percentage of total newly synthesized protein in the culture supernatants while the open bars represent the values for cell-associated proteins. It can be seen that the percentage of collagenase-sensitive protein secreted into the medium increases five-fold when day 6 cultures are compared with those at day 3. By contrast, the percentage of cell-associated collagenase-sensitive protein remains constant.


% of newly synthesised protein
0
1
2
3
4
5
6
7
3
4
5
6
time (days)

4.3.4 SDS PAGE analysis of proteins in fat-storing cell culture supernatants

In order to convert the different precursor forms of collagens into tropocollagens (which simplifies interpretation of the SDS gels) some supernatants were subjected to limited pepsin digestion. Electrophoresis of this material under reducing conditions (ie. in presence of dithiothreitol), showed the presence of the $\alpha 1$ and $\alpha 2$ polypeptide chains of collagen type I (Figure 4.12). In non-reduced samples, $[\alpha 1(III)]_3$ was present in the 300 kD region although the intensity of the signal was substantially less than that of α chains for collagen type I. Under reducing conditions, $\alpha 1$(III) chains comigrated with $\alpha 1$(I).

Labelled polypeptides which were not subjected to pepsin treatment were also analysed by SDS PAGE (Figure 4.13). In non-reduced samples the precursor chains of collagen type I were noted. The signal for pro $\alpha 1$ and pro $\alpha 2$ was greater than that for $\alpha 1$(I) and $\alpha 2$(I) indicating that the process of extracellular modification of collagen occurs slowly *in vitro*. In reduced samples, the pro $\alpha 1$ and pro $\alpha 2$ chains of collagen type IV were present as a closely spaced doublet at the 180 kD position. The two chains of fibronectin were noted around the 220 kD position.

4.4 DISCUSSION

4.4.1 Immunohistochemical studies

The identification of intracellular staining for procollagen types I and III, collagen type IV (7S), collagen type VI and laminin within sinusoidal cells by light microscopic immunohistochemistry provides supportive evidence that some of the cellular constituents of the sinusoids are involved in the production of extracellular matrix proteins. The results are similar to those obtained by Clément *et al* (1986) who identified several of these components at the light microscopic level in human sinusoidal cells. It is of interest that of the collagenous components, only procollagen type I could be identified in parenchymal cells. This is in keeping with the results of Malizia *et al* (1987) but is in contrast to the report of Sakakibara *et al* (1986) who suggested that parenchymal cells may also contain immunoreactive procollagen type III.

Figure 4.12 SDS-PAGE analysis of culture supernatants from 6 day old fat-storing cell cultures: pepsin-treated samples.

Prior to electrophoresis the medium was subjected to limited pepsin digestion. Lane A contains the radiolabelled standard collagen type I ([^{14}C]-labelled collagen type I). In this lane the α (100kD), β (200kD) and (300kD) bands are visible. Lane B: reduced sample from fat-storing cell cultures (+ dithiothrietol). Only α bands are present. Lane C: unreduced sample from rat fat-storing cells. α bands are again found but in addition there is evidence of lesser amounts of collagen type III (γ band).

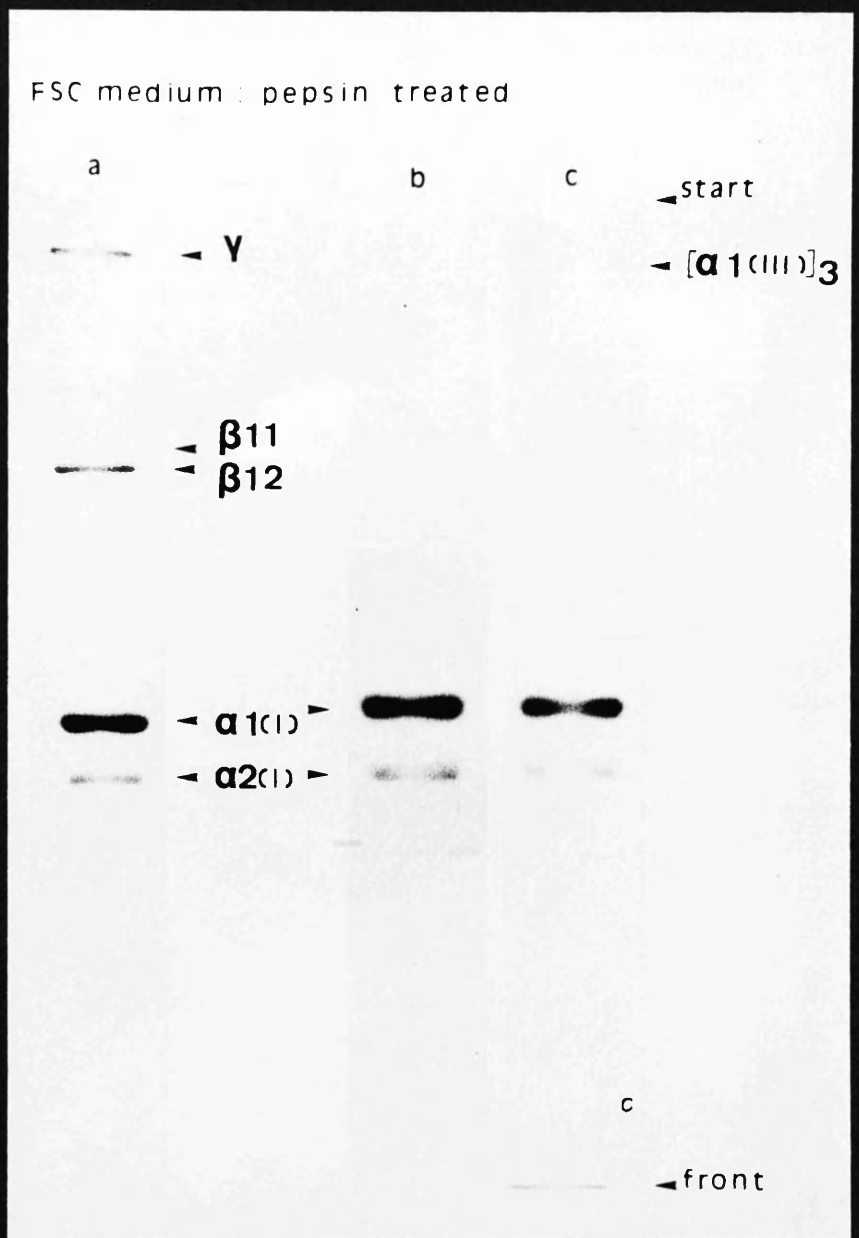
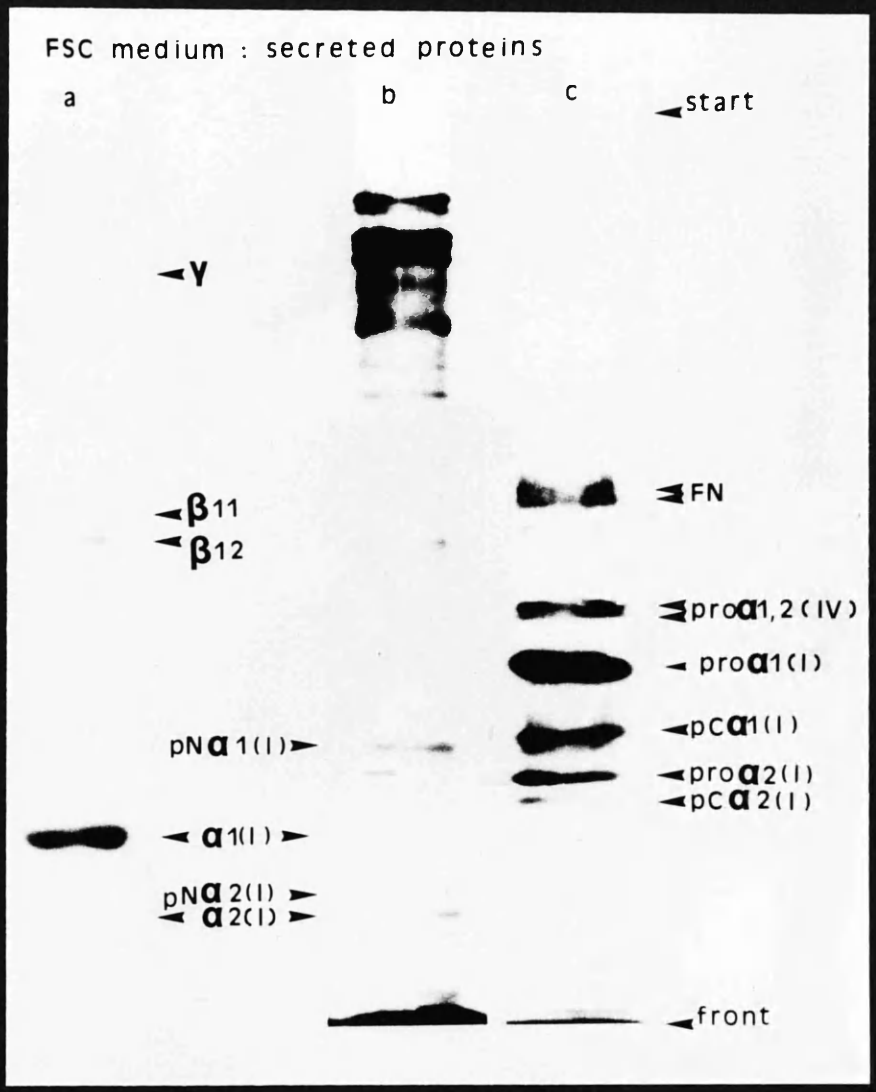

FSC medium : pepsin treated
a
b
c
start
γ
[α1(III)]3
β11
β12
α1(I)
α2(I)
c
front

Figure 4.13 SDS-PAGE analysis of culture supernatants from 6 day old fat-storing cell cultures: no prior pepsin treatment.

In this gel, lane A again contains the radiolabelled collagen type I standard. In this lane the α, β and γ bands are visible although less intense than in figure 4.12. Lane B: unreduced sample from fat-storing cell cultrues. Several precursor chains of collagen type I with molecular weights between 10^5 and 2 x 10^5kD are present. Lane C: reduced sample (+ dithiothreitol). Several precursor forms of collagen type I are again present and, in addition, the pro-α chains of collagen type IV are visible as the closely spaced doublet at 180kD position. The two chains of fibronectin are present around the 220kD - 240kD position. These molecules are pepsin-sensitive and are therefore not present in the gel demonstrated in figure 4.12.


FSC medium : secreted proteins
a
b
c
start
γ
β11
β12
FN
proα1,2(IV)
proα1(I)
pNα1(I)
pCα1(I)
proα2(I)
pCα2(I)
α1(I)
pNα2(I)
α2(I)
front

The lack of resolution obtained at the light microscope level does not permit accurate identification of the specific sinusoidal cell types labelled. Furthermore, as discussed earlier, identification of intracytoplasmic protein *per se* does not prove that a cell is actively synthesizing that protein; endocytosed protein would also be detected using this approach. It has been established in *in vitro* and *in vivo* experiments that sinusoidal endothelial cells may take up hyaluronic acid and collagen (Smersrød *et al*, 1984; 1985); other components may also be endocytosed by these cells and by Kupffer cells. To establish by immunohistochemistry that a cell is synthesizing a protein, it is essential that labelling is found on the rough endoplasmic reticulum and/or Golgi apparatus. The immuno-gold studies outlined above provide support for the production of some matrix components by fat-storing cells both in normal and fibrotic human liver. With the exception of fibronectin (and to a lesser extent procollagen type I), no component could be localized to the protein synthetic apparatus of parenchymal cells. The identification of immunoreactive fibronectin within lysosomes of parenchymal cells and Kupffer cells suggests that these cells may be involved in degradation and turnover of this protein.

It should be noted that in all experiments immuno-gold labelling for intracellular proteins was weak. In the case of some components, such as collagen type VI, the density of gold particles in fat-storing cells was barely greater than background levels. The intensity of labelling could be enhanced by the use of hyaluronidase prior to the application of the primary antibodies, probably by the removal of glycosaminoglycan molecules masking epitopes on procollagen molecules. Nevertheless, it is concluded that, although immuno-gold labelling provides a highly specific method for identification of matrix proteins at the ultrastructural level with little background staining, it does not appear to be of particularly high sensitivity.

Several previous studies have used immunoperoxidase at the ultrastructural level rather than immuno-gold to address the question of the cellular origin of matrix proteins. Although Martinez-Hernandez (1984) was apparently unable to identify intracellular matrix proteins in normal rat liver, he demonstrated immunoreactivity for collagen type I and fibronectin within the endoplasmic reticulum cisternae of parenchymal cells in livers removed from animals with carbon tetrachloride-induced fibrosis. By contrast, labelling for laminin and type IV collagen was restricted to fat-storing cells and endothelial cells (Martinez-Hernandez, 1985). Takahara *et al* (1988) presented conflicting data in a study of carbon tetrachloride-induced injury. The

predominant signal for collagen type I was noted in fat-storing cells with only weak labelling of parenchymal cells. Strong immunoreactivity was also noted within the rough endoplasmic reticulum and Golgi apparatus of fat-storing cells for collagen type III and for prolyl hydroxylase, an essential enzyme for the biosynthesis of interstitial collagens. In contrast to the experience of Martinez-Hernandez, Clément *et al* (1985) found immunoreactivity for fibronectin and collagens I, III and IV within the endoplasmic reticulum of fat-storing cells and sinusoidal endothelial cells of normal rat liver. Weak labelling for collagen type I was also noted in parenchymal cells. The same group later used immunoperoxidase at the ultrastructural level to study matrix protein production in human liver (Clément *et al*, 1986, 1988). In normal liver, fat-storing cells contained collagen types I, III and IV, fibronectin and laminin. These components were also identified in sinusoidal endothelial cells. Parenchymal cells stained positively for fibronectin; weak immunoreactivity was noted for collagen type I but not for collagen types III and IV or laminin. However, in specimens obtained from patients with alcohol-induced fibrosis, some labelling of parenchymal cells for collagen type III was noted. Furthermore, laminin immunoreactivity was noted in parenchymal cells in some fibrotic livers. Clément's group have suggested that laminin (and collagen type IV) expression may also be a feature of parenchymal cells in the developing liver (Rescan *et al*, 1989).

It would appear from these previously published studies that immunoperoxidase may be more sensitive than immuno-gold for the detection of intracellular matrix proteins, although this approach is generally associated with more non-specific background staining and the labelling is more diffuse. Although such immunolocalization studies have provided conflicting data on the expression of matrix proteins by parenchymal cells, they all support a role for fat-storing cells in matrix protein biosynthesis in normal and fibrotic liver. Nevertheless, it should be borne in mind that although demonstration of labelling for a protein on the endoplasmic reticulum of a cell is strong evidence that it is being synthesized, it cannot be assumed that the protein is being actively secreted; *in vitro* studies have indicated that a substantial fraction of newly synthesized collagen in some cell types, undergoes intracellular degradation (Bienowski *et al*, 1978). For this reason, it is important to complement immunolocalization studies with *in vitro* experiments which look at the capacity of different cell types to synthesize and secrete matrix proteins.

4.4.2 *In vitro* biosynthesis experiments

In the present study, fat-storing cells were reproducibly isolated and cultured from adult male Wistar rats. In contrast to many other published studies, vitamin A-pretreated animals were not used(Friedman *et al*, 1985; Kawase *et al*, 1986). This option was taken because pretreatment with retinyl palmitate may interfere with protein synthesis in fat-storing cells isolated from such animals (Shiratori *et al*, 1987). The addition of 10 μM retinol to cultures of passaged fat-storing cells has been shown to alter collagen production and overall protein synthesis by these cells (Davis *et al*, 1987). The purity of the fat-storing cultures in this study was comparable to that reported by others using cells isolated from "old" (ie older than 6 months) animals (De Leeuw *et al*, 1984) but was slightly lower than that described in studies with vitamin A-pretreated animals (Friedman *et al*, 1985)[1].

Morphological changes were noted over time in the cultured fat-storing cells. After 6 days they contained a well developed cytoskeleton consisting of microtubules and of bundles of microfilaments with typical smooth muscle cell-like condensations. These observations suggest that the cells may undergo phenotypic shift *in vitro* towards myofibroblast-like cells. It is possible that culture of these cells on tissue culture plastic (as in this study) may lead to activation of the cells mimicking the situation in injured liver (see Chapter 6). In parallel with the phenotypic alterations, incorporation of [^{3}H]-proline into total protein increased, and more labelled protein was secreted. In early cultures less than 1% of secreted proteins was collagenase-sensitive. By day 6, the amount of secreted collagenous protein had risen to almost 5% of total exported protein.

By SDS-PAGE analysis, it was established that collagen type I was the predominant collagen type secreted by these cells. When the medium was analyzed without prior pepsin treatment, the different precursor chains of collagen type I were identified. Among these bands the pro α1 (I) and pro α2 (I) bands were particularly dense, indicating that processing of secreted procollagen by extracellular proteases occurs slowly in cell culture. As removal of

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1 The greater purity reported by Friedman's group may also relate to the method of density gradient used (Strachtan).

propeptides is required for the organization of interstitial collagen fibrils, such limited propeptidase activity may explain why fibrils were not observed around cultured cells by electron microscopy. In addition to collagen type I, collagen types III and IV and fibronectin were identified, albeit in smaller amounts. No bands corresponding to the α chains of collagen type V were found.

The results therefore demonstrate that isolated rat liver fat-storing cells have the capacity to synthesize several components of the extracellular matrix. These observations are in keeping with several other published studies. De Leeuw *et al* (1984) demonstrated by immunofluorescence, the presence of collagen types I and IV, laminin and fibronectin within cultured rat fat-storing cells. Tsutsumi *et al* (1988) identified collagen types I and IV and laminin in cultured cells but in contrast to De Leeuw and co-workers, also demonstrated collagen type III. Friedman *et al* (1985) obtained similar results using immunofluorescence and also showed, as in the present study, that by biochemical analysis of fat-storing cell culture supernatants, collagen type I was the most abundant collagen secreted. They also demonstrated that collagen accounted for approximately 5% of total secreted protein in one week-old cultures. Although Kawase *et al* (1986) used cells obtained from vitamin A-pretreated animals, they obtained similar values in quantitative studies of matrix protein synthesis with a ratio of collagen type I: collagen type III of about 6:1. It should be noted that in all of these studies, the cells were cultured on a plastic surface; as noted above this may "activate" the cells. Values obtained from cells grown on a matrix similar to that of the normal space of Disse may more accurately reflect the ratio of collagen type I:III synthesized *in vivo*. Friedman *et al* (1989) for example, have shown that isolated fat-storing cells grown on a laminin-rich extract from murine Engelbreth-Holm-Swann (EHS) tumour, which is similar in composition to normal basement membranes, exclusively synthesize collagen type III. The importance of cell-matrix interactions in sinusoidal cell function is discussed further in Chapter 9.

Several groups have demonstrated that isolated rat liver fat-storing cells may also synthesize structural glycoproteins and proteoglycans. In keeping with the results of the present study, Ramadori *et al* (1987) showed that these cells may synthesize and secrete fibronectin. They studied the kinetics of fibronectin production, estimating that following an initial lag phase, they showed a synthetic rate of 2600μg/24 hours/100μg cell protein. Maher *et al* (1988b) also

identified laminin secretion using immunoprecipitation of proteins within the supernatants of fat-storing cell cultures following exposure to radiolabelled methionine. It is of interest that no bands corresponding to laminin were noted in the SDS PAGE analysis in the present study. Arenson *et al* (1988) and Schäefer *et al* (1987) have also shown that these cells synthesize and secrete proteoglycans. The principal type released by the cultured cells is dermatan sulphate but chondroitin sulphate is also produced. The ability of these cells to synthesize and secrete hyaluronic acid has also been documented (Gressner & Haarmann, 1988).

The *in vitro* studies described in this chapter did not attempt to determine the relative capacities of different cell types for matrix protein biosynthesis. In preliminary studies, Kupffer cell and endothelial cell primary cultures were shown to synthesize negligible quantities of collagenase-sensitive proteins (data not shown). However, parenchymal cells were not studied. Earlier reports (Guzelian *et al*, 1981, 1984; Tseng *et al*, 1982, 1983; Diegelmann *et al*, 1983; Hata *et al*, 1985) indicated that parenchymal cells may indeed be a rich source of extracellular matrix proteins. In all of these studies, little collagen was produced by cultured cells during the first 48-72 hours. By 96 hours however, collagen(s) accounted for as much as 8% of all newly synthesized protein. A concomitant shift in the type of collagen produced from types III and IV to type I was noted by Tseng *et al* (1983). These results were interpreted as being changes occurring during adaptation of the isolated parenchymal cells to primary culture. Maher *et al* (1988a) have proposed an alternative explanation, suggesting that the collagen synthesis noted in such cultures is derived not from the parenchymal cells themselves, but from contaminating fat-storing cells. Using several standard methods for the isolation and purification of rat parenchymal cells, she and her colleagues demonstrated that fat-storing cells could be detected in early cultures and, as a result of *in vitro* proliferation, gradually replaced parenchymal cells. By day 8 they accounted for 75% of all cells in culture. Some criticism can be made of at least one of the methods used to characterize fat-storing cells. Maher *et al* claimed that immunolocalization of laminin could be used as a specific marker for these cells. As discussed above, others have suggested that parenchymal cells may also synthesize this protein (Clément *et al*, 1988). Nevertheless, immunolocalization of the intermediate filament protein desmin was also used (see Chapter 5); this is not expressed by parenchymal cells.

An alternative approach to measuring the relative roles of parenchymal and sinusoidal cells in matrix protein synthesis was described by Chojkier and colleagues (Chojkier, 1986; Chojkier *et al*, 1988). Their method relies on the ability of parenchymal cells to convert ornithine to arginine via the urea cycle; none of the sinusoidal cells have this capacity. Rats were injected with both [^{14}C]-ornithine and [^{3}H]-proline and hepatic collagen was isolated. Theoretically, the ratio of radiolabelled arginine (derived from orntithine) to proline should provide a measure of whether the isolated collagen is derived from parenchymal or non-parenchymal cells. Chojkier and co-workers suggested that this method provided evidence that most hepatic collagen is produced by parenchymal cells in both normal and fibrotic rat liver. This work has been criticized by Bissell *et al* (1990) for the methods used to purify newly synthesized collagen from liver for assessment of radiolabelled arginine content. Furthermore, they believe that differences in intracellular RNA turnover between the different cell types might influence the results. Using a modified technique in which an alternative method for collagen isolation was applied, Ogata *et al* (1989) were unable to confirm the findings of Chojkier *et al.* and concluded that parenchymal cells account for only a small fraction of newly synthesized collagen in normal and fibrotic rat liver.

What other methods could be utilized to establish the cellular origin of hepatic extracellular matrix proteins? The availability of cDNA and RNA probes for collagens and other matrix proteins has recently allowed investigators to apply molecular biological techniques in attempts to resolve this issue. Two approaches have been used: (i) localization of specific RNAs by *in situ* hybridization and (ii) quantitation of mRNAs in freshly isolated and cultured cells. Saber *et al* (1983a) identified pro α 2 (I) collagen mRNA transcripts in isolated mouse liver parenchymal cells. Considerable problems of non-specific labelling exist with the method used and to my knowledge their observations have not been repeated. Furthermore, given the findings of Maher *et al* (1988a), it is possible that at least some of their cells may have been fat-storing cells; their demonstration of mRNA for albumin on the other hand, confirms that some were indeed parenchymal cells.

More useful information has been obtained from studies in which probes were used to detect specific RNAs in tissue sections. Saber and co-workers (1983b) used a chicken cDNA probe to localize α 2 (I) gene transcripts in liver tissue from a mouse model of

schistosomiasis-induced hepatic fibrosis. Signal was detected in both non-parenchymal and parenchymal cells. Since their report, substantial progress has been made in the development of *in situ* hybridization methods providing enhanced sensitivity and specificity. For example, the use of cDNA probes (such as that used by Saber *et al*) for *in situ* hybridization has now been superseded by various forms of RNA probes; this overcomes difficulties of non-specific binding of probe to sense genomic DNA. Milani *et al* (1989a) used [^{35}S]-labelled RNA probes to detect α2(I), α1(III) and α1(IV) gene transcripts in normal and fibrotic rat liver. The signal was detected using autoradiography and was found exclusively in non-parenchymal cells in tissue from control animals and from rats with carbon tetrachloride-induced fibrosis. In a subsequent study (Milani *et al*, 1990a), they demonstrated similar findings in a model of chronic cholestatic liver disease (common bile duct ligation). In this model they also noted signal for α1(IV) procollagen RNA in proliferating bile ducts. However, in both studies the signal noted in parenchymal cells was found to be no greater than background levels. These investigators went on to apply a similar approach to normal and fibrotic human liver (Milani *et al*, 1989b, 1990b). Gene transcripts for procollagen I, III and IV and laminin were identified in non-parenchymal cells; α1(IV) procollagen and laminin B1 RNA was also detected in bile duct epithelium and in some vascular endothelial cells.

These results appear to strongly support the concept that fat-storing cells are largely responsible for most matrix protein biosynthesis in normal and injured liver and that parenchymal cells play a negligible role. However, even this approach is not without its problems. The radio-labelled *in situ* hybridization method used by Milani *et al* is undoubtedly a sensitive method for the localization of gene transcripts in tissue sections but the grains detected by autoradiography (indicating a positive signal) are less clearly localized to individual cells than the chromagenic reaction product of immunohistochemistry. The development of non-isotopic methods for *in situ* hybridization (Chapter 9) should overcome such problems.[1] Furthermore, the precise nature of the non-parenchymal cells labelled for the procollagen gene transcripts could not be firmly established. Although immunohistochemical studies performed

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[1] It is of interest that a preliminary study using a non-isotopic method with digoxigenein labelled probes confirmed Milani's observation that non-parenchymal cells in human liver contain α1(III) procollagen RNA but also suggested that signal could detected in parenchymal cells (Yamada *et al*, 1989).

in parallel with *in situ* hybridization in their study of experimental cholestatic injury, indicated that the labelled cells may be fat-storing cells, direct evidence using double labelling was not presented. Although a daunting prospect, application of *in situ* hybridization at the ultrastructural level may provide a more accurate assessment of cellular origin of matrix proteins.

Rather than identifying specific RNAs in tissue sections, Maher and McGuire (1990) used RNA probes to quantify the relative abundance of α1 (I), α1 (III) and α1 (IV) procollagen and laminin B2 gene transcripts in freshly isolated rat parenchymal, fat-storing and sinusoidal endothelial cells by Northern blotting. The cells were isolated form normal animals and from rats with carbon tetrachloride-induced fibrosis or secondary biliary cirrhosis (common bile duct ligation). In isolates obtained from normal animals, mRNA for types III and IV procollagen could be identified in fat-storing cells and, interestingly, in sinusoidal endothelial cells but not parenchymal cells; procollagen type I expression was negligible in all cell types. In fat-storing cells isolated from animals from either experimental model, there was a dramatic increase in the abundance of procollagen type I and III mRNAs although levels of gene transcripts for procollagen type IV and laminin B2 chain were unchanged. By contrast, no alteration in any matrix gene expression was noted in isolated parenchymal cells. Theoretically mRNA levels in freshly isolated cells should reflect the situation *in vivo* (Clayton and Darnell, 1983). It remains to be established whether this is true for cells isolated by the enzyme perfusion method used in Maher and McGuire's study. It should be noted however, that demonstration of RNA in itself does not prove that a cell is actively producing a protein. Differences in RNA stability, translational rates and post-translational modifications have all to be considered when assessing the relative roles of different cell types in the synthesis and secretion of a protein.

In summary, the immunolocalization studies presented here demonstrate that sinusoidal cells in normal and fibrotic human liver contain several components of the extracellular matrix. By electron microscopy labelling appears to be principally found in fat-storing cells. The biosynthetic studies described indicate that these cells, when isolated from normal rat liver, have the capacity to synthesize and secrete such proteins *in vitro*. From this and the other published data reviewed above, it can be concluded that although the relative roles of fat-storing and parenchymal cells have yet to be unequivocally determined, the bulk of evidence suggests that fat-storing cells are the principal matrix-producers of normal and fibrotic liver.

STRUCTURAL AND FUNCTIONAL ASPECTS OF THE HEPATIC SINUSOIDS

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CHAPTER 5. IMMUNOHISTOCHEMICAL LOCALIZATION OF FAT-STORING CELLS IN RAT AND HUMAN LIVER

5.1 INTRODUCTION

Given the importance of fat-storing cells in the production of extracellular matrix proteins, it is essential to establish how these cells respond *in vivo* to various forms of liver injury in order to obtain a greater understanding of the mechanisms of liver fibrogenesis. Using transmission electron microscopy, McGee & Patrick (1972) demonstrated accumulation of fat-storing cells in response to experimental acute liver injury in mice. Their study was the first to highlight the importance of these cells in the early repair process following chemically-induced parenchymal cell necrosis. Unfortunately, quantitative analyses and kinetic studies are difficult to perform at the electron microscope level; a reliable method for the identification of fat-storing cells by light microscopy was therefore required. A subsequent study by Kent *et al* (1976) utilised auto-fluorescence of vitamin A as a marker for the cells in an investigation of their response to chronic carbon tetrachloride exposure in rats. In keeping with McGee and Patrick's ultrastructural data, fat-storing cells were found in areas of parenchymal cell damage within several days of exposure to the toxin. This persisted in material from animals treated for up to 24 weeks; by this stage, vitamin A-containing cells were identified in fibrous septa. A limitation of this approach however, was that the method appeared to be considerably more sensitive if animals were pre-treated with large doses of subcutaneous vitamin A prior to administration of the toxin. Furthermore, the auto-fluorescent signal obtained using the method described by Kent *et al* (1976) fades rapidly, making it unsuitable for light microscopic quantitative studies.

Vitamin A can also be demonstrated histochemically using Kupffer's gold chloride method. A modification of this technique was described by Tozuka *et al* (1985) for the demonstration of fat-storing cells in human liver biopsies. Unfortunately, this is also a capricious method and in keeping with other research groups (A Geerts, Brussels; A Brouwer, Rijswijk; personal communications), I have found it to be associated with considerable background staining. Furthermore, as it relys on the interaction with vitamin A, it may underestimate the number of fat-storing cells in the injured liver. As discussed in Chapter 6, these cells may lose some of their cytoplasmic lipid droplets on activation during the response to injury (Okanue *et al*, 1983; Mak *et al*, 1984). A similar criticism can be made of techniques such as Alsop's method in

which the identification of fat-storing cells relies upon the demonstration of cytoplasmic lipid in semi-thin sections (Bronfenmajer *et al*, 1966; Hopwood & Nyfors, 1976; Ballardini *et al*, 1983; Jézéquel *et al*, 1984).

The possibility that histochemical demonstration of specific patterns of phosphatase activity could be used to identify different sinusoidal cell populations has been explored by several groups of investigators (Tanaka *et al*, 1976; Yamamoto & Ogawa, 1983; De Valck *et al*, 1988). Fat-storing cells of normal rat liver were shown to exhibit 5'nucleotidase activity on their cell membranes and neutral phosphatase activity within their endoplasmic reticulum but were negative for alkaline phosphatase. De Valck *et al* (1988) suggested that a combination of staining for 5'-nucleotidase and endogenous peroxidase (see Chapter 1) could be used to discriminate between the different types of sinusoidal cell. However, as such enzyme activity is best demonstrated at the ultrastructural level, this approach would also seem to have limited scope for application to quantitative studies.

Yokoi *et al* (1984) were the first to use immunohistochemistry for the detection of fat-storing cells in tissue sections. Stimulated by ultrastructural observations that these cells exhibit some properties of smooth muscle cells (see Chapter 1), they raised a polyclonal antibody to the intermediate filament protein desmin (extracted from chicken gizzard muscle) and used this to immunostain cryostat sections of normal rat liver. Expression of desmin, a 55kD protein, had previously been shown to be largely restricted to cells showing muscle-differentiation (Lazarides, 1982). As expected, Yokoi and co-workers demonstrated positive staining for this antigen in the smooth muscle cells of hepatic arteries. However, in addition, a population of desmin-containing non-parenchymal cells was identified. The immunoreactive cells showed morphological features of fat-storing cells and appeared to contain auto-fluorescing vitamin A. Furthermore, they were shown in colloidal carbon-uptake studies to be non-phagocytic, indicating that the immunostained cells were not Kupffer cells. This appeared to be a suitable marker for fat-storing cell identification by light microscopic visualization. However, the antibody used in their study was only partially characterized and the authors did not describe in detail the distribution of desmin-positive cells.

The first aim of the studies outlined in this chapter was to extend the work of Yokoi *et al* (1984) using a panel of polyclonal and monoclonal anti-desmin antibodies to further

investigate whether desmin is expressed by rat liver fat-storing cells. In their original paper, Yokoi *et al* (1984) restricted their immunohistochemical studies to unfixed, frozen sections of rat liver. For accurate quantitative studies the use of fixed, paraffin-embedded material is preferable since there is less variability in section thickness and the tissue is less liable to crush artefact. Furthermore, the enhanced resolution obtained in 3 μm paraffin sections provides a better appreciation of cellular morphology. For these reasons, methods were developed by which desmin-containing sinusoidal cells could be identified in sections of fixed, embedded tissue. Given the ultrastructural observations of smooth-muscle like features in fat-storing cells and the proposed relationship between these cells and myofibroblasts (see Chapater 6), the expression of other muscle-associated proteins by rat liver sinusoidal cells was also studied. A monoclonal antibody to the α (smooth muscle) isoform of actin was used, as was an antibody to the actin-binding protein, filamin. In addition, expression of another intermediate filament protein, vimentin by rat sinusoidal cells was investigated. The monoclonal antibodies ED1 and ED2 were used to detect Kupffer cells; these reagents have previously been shown to detect cytoplasmic proteins in rat tissue macrophages (ED1 and ED2) (Dijkstra *et al*, 1985).

Finally, I wished to examine whether a similar immunohistochemical approach could be used to identify fat-storing cells in human liver. Three monoclonal anti-desmin antibodies were assessed as were anti-α (smooth muscle) actin, anti-filamin and anti-vimentin antibodies. In addition, further antibodies were used which recognize epitopes on (i) other forms of actin, (ii) other intermediate filament proteins and cytoskeletal elements (cytokeratins and tissue polypeptide antigen (TPA)) and (iii) a protein present in human myofibroblasts (PR2D3). Preliminary studies had established that none of this group of antibodies were suitable for use in rat tissues. The monoclonal antibody EBM/11 was used to identify human Kupffer cells as neither ED1 nor ED2 recognize epitopes on human macrophages.

5.2 MATERIALS AND METHODS

5.2.1 Liver tissue

Tissue was obtained from normal male Wistar (n=9) or Sprague Dawley (n=11) rats (weights : 200-250g). In some animals (n=7), the liver was perfusion-fixed using the low pressure method of De Zanger and Wisse (1982) (Chapter 1) with 2% paraformaldehyde in 0.1M phosphate buffer. In the remaining animals (n=13), liver tissue was obtained immediately following death by cervical dislocation. Small tissue blocks were either embedded in OCT and frozen in liquid nitrogen or immersion-fixed in one of the following fixatives: (i) formal sublimate (4% neutral phosphate buffered formaldehyde in saturated aqueous mercuric chloride), (ii) Bouin's solution (75% saturated aqueous picric acid/20% formaldehyde/5% glacial acetic acid), (iii) 10% buffered formal saline. All fixed material was processed through graded alcohols for paraffin wax-embedding. Normal human liver tissue was obtained from cadaveric renal transplant donors (n=8). Details of the fixation and processing of this is given in Chapter 3.

5.2.2 Antibodies

Details of the primary antibodies used in the study of rat liver sinusoidal cells are given in Table 5.1. In initial experiments carried out during 1985-1986, three antibodies were used: a mouse monoclonal anti-porcine desmin antibody obtained from Amersham Int PLC and two rabbit polyclonals (Burt and MacSween, 1986b; Burt *et al*, 1986b). Two other monoclonal anti-desmin antibodies recently became available (DE-B-5 and D33) and were evaluated as for the other reagents. The anti-filamin antibody used (PM6/317) was the generous gift of Dr J M Wilkinson. This had been raised to human platelet filamin and was of IgG_1 subclass. The primary antibodies used in the immunohistochemical study of human liver sinusoidal cells are outlined in Table 5.2. With the exception of anti-TPA B1, all were mouse monoclonals. The antibody PR2D3 was kindly provided by the Director's Laboratory, Imperial Cancer Research Fund, London.

Antibody	Source	Dilution	Reference
Anti-porcine desmin	Amersham Int. PLC; Bucks., England	1:4	-
Anti-chicken * desmin	Dakopatts, Denmark	1:160	-
Anti-chicken * desmin	Eurodiagnostics, BV; Netherlands	1:90	-
Anti-porcine desmin (DE-B-5)	Boehringer Mannheim GmbH; Germany	1:10	Altmannsberger *et al* (1985)
Anti-human desmin (D33)	Dakopatts; Denmark	1:300	Van Muijen *et al* (1987)
Anti-vimentin	Labsystems, Helsinki, Finland	1:50	Osborn & Weber (1983)
Anti-α (smooth muscle) actin (1A4)	Sigma; Poole, England	1:300	Skalli *et al* (1986)
Anti-filamin (PM6/317)	Dr. J.M. Wilkinson, Royal College of Surgeons, London.	1:250	-
Anti-rat monocyte/ macrophages ED1	Serotec; United Kingdom	1:400	Dijkstra *et al* (1985)
Anti-rat macrophage ED2	Serotec; United Kingdom	1:60	Dijkstra *et al* (1985)

Table 5.1 Antibodies used in study of rat liver sinusoidal cells

Two antibodies (*) were rabbit polyclonal; all others were mouse monoclonal

Antibody	Source	Dilution	Reference
Anti-desmin (DE-B-5)	Boehringer Mannheim GmbH; Germany	1:10	Altmannsberger *et al* (1985)
Anti-porcine desmin	Amersham Int. PLC; Bucks., England	1:4	-
Anti-human desmin (D33)	Dakopatts; Denmark	1:300	Van Muijen *et al* (1987)
Anti-actin	Amersham Int. PLC; Bucks., England	1:200	Lin (1981)
Anti-"muscle actin" (HHF35)	Enzo Diagnostics, New York, USA	1:1200	Tsukada *et al* (1986)
Anti-"smooth muscle" actin	Enzo Diagnostics, New York, USA	1:300	Gown *et al* (1985)
Anti-α (smooth muscle) actin (IA4)	Sigma; Poole, England	1:300	Skalli *et al* (1986)
Anti-filamin PM6/317	Dr. J.M. Wilkinson, Royal College of Surgeons, London.	1:700	-
Anti-vimentin	Labsystems, Helsinki, Finland	1:50	Osborn & Weber (1983)
PR 2D3	Dr. P.I. Richman, ICRF, London	Neat	Richman *et al* (1987)
Anti-CD68 (EBM/11)	Dakopatts A/S, Glostrup, Denmark	1:20	Kelly *et al* (1989)
Anti-tissue* polypeptide antigen (TPA B1)	CIS Ltd., England	1:10	Nathrath *et al* (1985)
Anti-cytokeratin (CAM5.2)	Becton Dickinson PLC; England	1:10	Makin *et al* (1984)

Table 5.2 Antibodies used in study of human liver sinusoidal cells (*polyclonal)

5.2.3 Immunohistochemical methods

Several immunohistochemical techniques were used in this study. A peroxidase-antiperoxidase method was used with all polyclonal antibodies. The protocol for this is outlined in Appendix 2. In the initial studies carried out in 1985, a streptavidin-biotin method (see Appendix 8) was used with mouse monoclonal primary antibodies on 3-4μm dewaxed sections of fixed liver tissue and an immuno-alkaline phosphatase method (Appendix 3) on 5μm cryostat sections. In more recent experiments, an indirect immunoperoxidase method was used (Appendix 4) often in combination with nickel/cobalt enhancement (Appendix 5). In all experiments, negative controls were included; these comprised (i) sections incubated with normal mouse immunoglobulin (control for monoclonals) or normal rabbit serum (control for polyclonals) in place of the primary antibody and (ii) sections incubated with PBS in place of secondary antibody. In addition, positive controls were included; this consisted of tissue sections known to contain the antigen of interest. In the case of muscle-associated proteins (such as desmin), normal human myometrium or aortic wall was used.

All immunostained preparations were assessed using either a Leitz Orthoplan microscope or a Nikon Optiphot microscope. Some sections were visualized through Leitz Nomarski interference contrast filters which creates a three-dimensional effect. When 3', 3 diamino-benzidine is used in the immunohistochemical method, this produces enhanced visualization of the chromagenic precipitate. All colour photographs were taken using Kodak ASA 100 Tungston film and black and white photographs using Agfa 100 ASA film.

5.3 RESULTS

5.3.1 Rat liver

(i) Desmin. In the initial studies (Burt & MacSween, 1986b; Burt *et al*, 1986b), two polyclonal antibodies and monoclonal anti-desmin obtained from Amersham PLC were used. With all three antibodies, desmin immunoreactivity could be identified within smooth muscle cells in

the walls of hepatic artery branches and surrounding portal vein and large hepatic vein branches; labelling of these structures was seen irrespective of the mode of fixation or immunohistochemical method used (Figure 5.1). However, the intensity of staining was greatest with the monoclonal antibody in combination with sections of Bouin's-fixed tissue following 15-20 minutes prior trypsinization, or on cryostat sections with the immuno-alkaline phosphatase method. With this antibody, desmin-containing sinusoidal cells were also detected (Figure 5.2). This was, however, highly dependant on the processing protocol; they were found only in trypsinized/Bouin's-fixed or cryostat sections. Length of trypsinization time was crucial, cells being detectable only after at least 15 minutes incubation. Similar desmin-containing sinusoidal cells were also identified in more recent experiments using the monoclonal antibodies DE-B-5 and D33. With both reagents, desmin-containing sinusoidal cells could be identified in cryostat and paraffin-embedded material. However, a greater number of cells was found with D33 than DE-B-5. Furthermore, whereas the latter required prior trypsinization for immunolabelling in sections of fixed, paraffin-embedded tissue, none was required with the D33 antibody. Maximal numbers of desmin-containing sinusoidal cells were found with D33 on formal sublimate-fixed tissue.

The sinusoidal cells detected by the three monoclonal anti-desmin antibodies showed cytoplasmic immunoreactivity only; nuclei were consistently negative. The cells were characterized by the presence of long cytoplasmic processes which could be seen to extend along the sinusoidal walls. Occasional vacuoles could be seen in some of the positively stained cells (Figure 5.2). Immunoreactive cells were seen throughout the acini with no obvious zonal heterogeneity. Cells within acinar zone III in the immediate perivenular area showed more intense labelling than those in zones II or I (periportal). However, all labelled sinusoidal cells showed considerably weaker labelling for desmin than the smooth muscle cells of hepatic artery branches, even in D33-stained preparations. All parenchymal cells were negative as were bile duct epithelial cells. Within portal tract stroma, desmin-positive stellate-shaped cells were identified with morphological features similar to those of cells seen within the sinusoids (Figure 5.1).

A desmin-positive sinusoidal cell index was obtained by counting the number of immunostained cells and the number of parenchymal cells per unit area ($0.635mm^2$) using a

Figure 5.1 Immunolocalization of desmin in a portal tract: normal rat liver.

Immunoreactivity for desmin is identified within the smooth muscle cells of the walls of hepatic artery branches (A) but there is no staining in or around bile ducts (BD). Occasional desmin-positive stellate cells can be identified within the stroma of portal tracts (arrows). (Monoclonal anti-desmin antibody (Amersham); streptavidin-biotin method; haematoxylin counterstain; photographed under Nomarski filters; x 140).

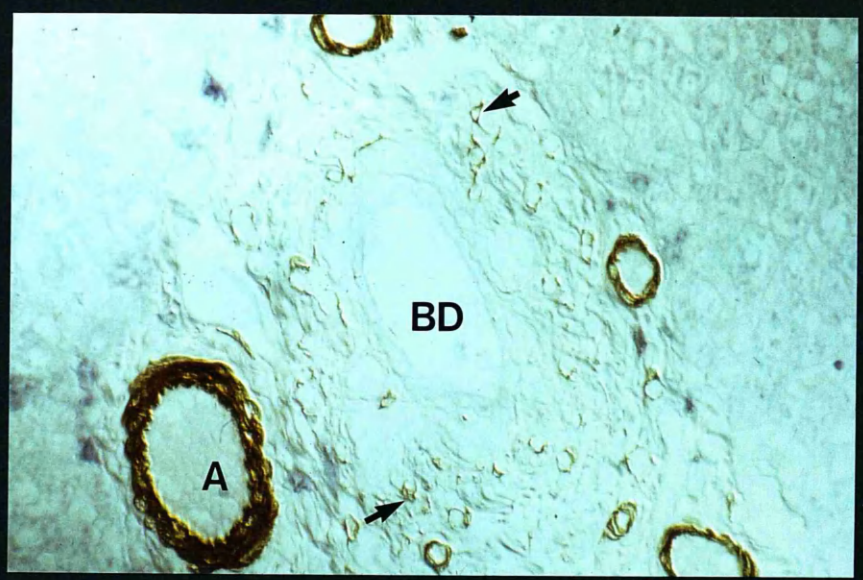
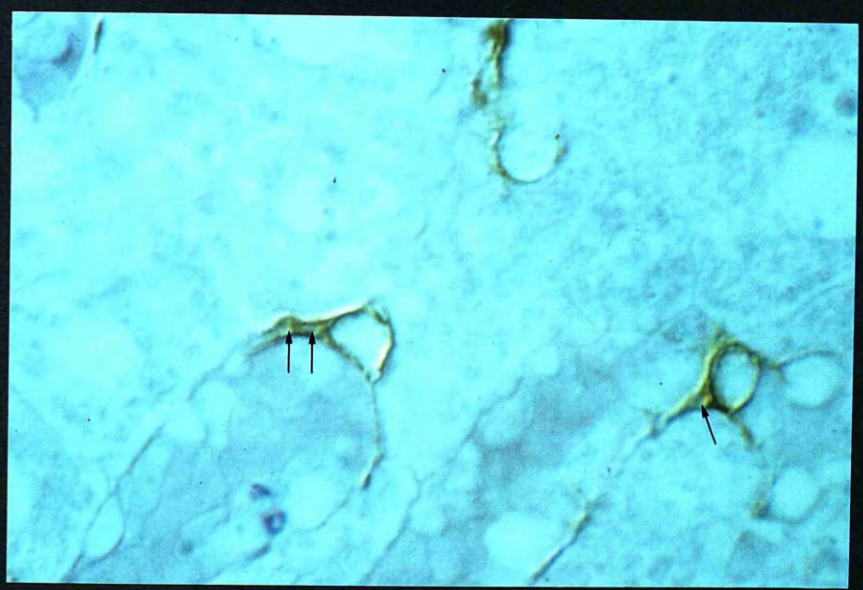

BD
A

Figure 5.2 Immunolocalization of desmin within fat-storing cells.

Immunoreactivity is clearly identified within the cytoplasm of these sinusoidal cells which have a stellate morphology and which are characterized by the presence of long cytoplasmic processes and cytoplasmic vacuoles presumed to be fat droplets (arrows). The cells have the distinctive morphological features of fat-storing cells. In en face-labelling experiments they were found to be negative for the macrophage markers ED1 and ED2. (Monoclonal anti-desmin antibody (Amersham); indirect immunoperoxidase method; light haematoxylin counterstain; photographed under Nomarski filters; x 840).



Nikon Optiphot microscope at a magnification of x400 and an eyepiece graticule, and expressing this a ratio of immunoreactive cells per 100 parenchymal cells. In D33-stained sections (n=15; 3 from each of 5 animals) an index of 4.5 ± 3.3 (mean ± S.D.) was found.

The morphological features of desmin-positive sinusoidal cells were compared to those of ED1 and ED2-immunoreactive cells. Whereas some ED1 and ED2-positive cells were stellate in outline, they had more abundant cytoplasm and did not possess the long cytoplasmic processes noted for desmin-positive cells (Figure 5.3). Examination of en-face preparations[1] stained with anti-desmin and ED1 or ED2 on consecutive sections indicated that there were no cells co-expressing desmin and either macrophage marker. Attempts at double-labelling for desmin and ED1 or ED2 on the same section however, produced inconsistent results.

(ii) Vimentin. All sinusoidal cells appeared to be labelled using the monoclonal anti-vimentin antibody. Parenchymal cells and bile duct epithelial cells were negative. The intensity of immunoreactivity was greatest in cryostat sections or in Bouin's-fixed tissue with 10 minutes prior trypsinization, although in all rat tissue staining was weaker than that seen in human liver sinusoidal cells.

(iii) α-(smooth muscle) actin. Smooth muscle cells within the walls of hepatic arteries and large hepatic vein branches were stained using the antibody 1A4 in cryostat sections and in formal-sublimate fixed tissue but showed only weak immunoreactivity in the other fixed tissue. Prior trypsinization had no apparent effect on the intensity of labelling. Parenchymal and bile duct epithelial cells were consistently negative.

In cryostat and formal sublimate-fixed tissue, strong staining was also noted in cells surrounding terminal hepatic vein branches; many of these were plump and appeared to form an integral part of the vein wall. However, in addition occasional immunoreactive sinusoidal cells were noted with a stellate morphology in acinar zone III (Figure 5.4). In contrast to

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[1] In the preparation of en-face sections, two serial sections are cut and one is reversed. This enables the immunostaining of two cut faces of individual sections on separate slides. This is a useful technique when technical difficulties are encountered in true double-labelling procedures as they were here.

Figure 5.3 ED2 immunoreactivity in sinusoidal cells: normal rat liver.

Immunostaining with the anti-rat macrophage antibody ED2 detects a population of sinusoidal cells. Although many are stellate they are more plump than the desmin-positive cells and lack the long cytoplasmic processes. (ED2; indirect immunoperoxidase; haematoxylin counterstain; x 210).

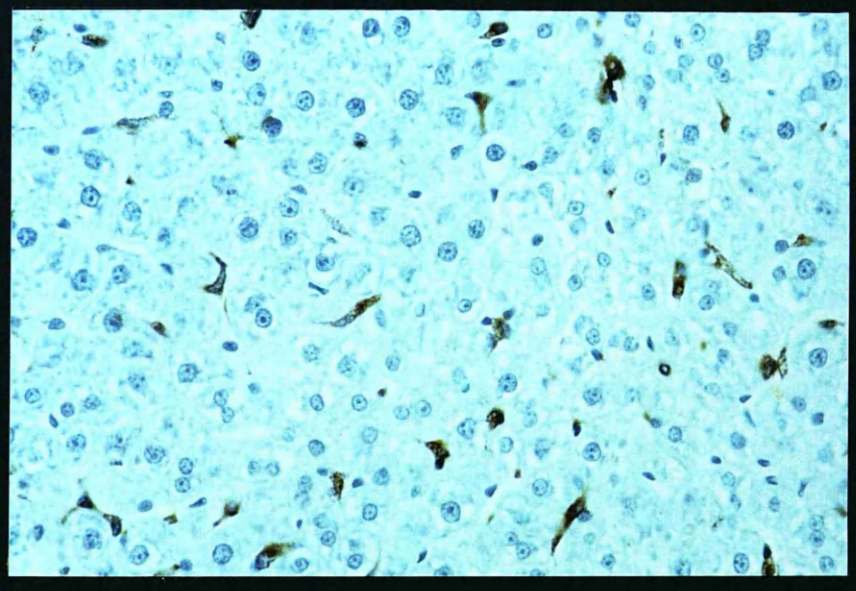
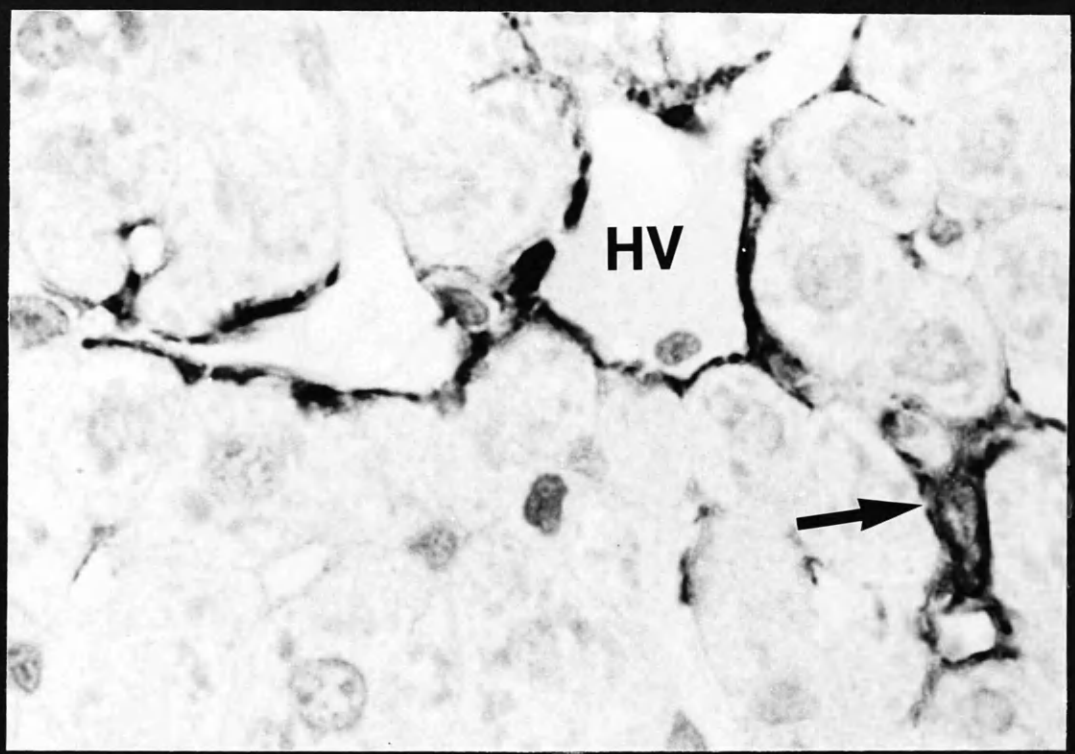


Figure 5.4 Immunolocalization of α (smooth muscle) actin in non-parenchymal stellate cells: normal rat liver.

Immunoreactivity is seen within cells surrounding a terminal hepatic vein radicle (HV). A single stellate shaped sinusoidal cell can also be seen (arrow). (Monoclonal antibody 1A4; indirect immunoperoxidase; haematoxylin counterstain; x 760).


HV

desmin, actin-positive sinusoidal cells were not identified elsewhere in the acini. Some immunoreactive stellate cells were found within portal tract stroma; these were present in similar numbers to desmin-positive cells at that site.

(iv) Filamin. The distribution of filamin was similar to that noted for vimentin with all sinusoidal cells labelled. There was no evidence of expression of this protein in parenchymal or bile duct epithelial cells.

5.3.2 Human liver

(i) Desmin. With all three antibodies used in the initial study (vide supra), staining for this protein could be seen within smooth muscle cells in the walls of hepatic arteries and large hepatic veins. In cryostat sections stained with the Amersham monoclonal antibody, very occasional immunoreactive cells were noted within the sinusoids using the immuno-alkaline phosphatase method. The signal was however, considerably less than that noted for rat liver desmin-positive sinusoidal cells and could not be detected in any fixed tissue, irrespective of mode of fixation or length of trypsinization.

In more recent experiments using D33 and DE-B-5, immunoreactive cells were also identified in plump cells lying beneath vascular endothelial cells of terminal hepatic vein radicles (Figure 5.5b). In addition occasional desmin-positive cells were noted within the sinusoids with a stellate morphology similar to those seen in rat liver. However, these were only readily identifiable in cryostat or 2% paraformaldehyde-fixed tissue with prior trypsinization (15 minutes) and using the indirect immunoperoxidase method with Nickel/Cobalt enhancement (Figure 5.5a).

(ii) Actin. The monoclonal anti-actin antibody obtained from Amersham stained all sinusoidal cells and outlined biliary canaliculi in some parenchymal cells (Figure 5.6). By contrast, the anti-"smooth muscle" actin antibody CGA7 stained only smooth muscle cells in hepatic arteries and vein walls (Figure. 5.5c) and occasional plump cells surrounding terminal hepatic vein radicles. No immunoreactive sinusoidal cells were noted with this antibody. The antibody 1A4 showed a similar distribution of staining but in addition, very occasional stellate-shaped positive

Figure 5.5 Expression of muscle-associated proteins in normal human liver.

a. Immunolocalization of desmin. As in rat liver, immunoreactivity for this intermediate filament protein is seen within the smooth muscle cells of hepatic artery branches and within occasional stellate cells in the stroma of portal tracts. In contrast to rat liver however, only very occasional sinusoidal cells express this protein; these could only be identified in nickel-enhanced preparations (arrow). Monoclonal antibody DE-B-5; indirect immunoperoxidase with Nickel/Cobalt enhancement; x 500).

b. Immunolocalization of desmin in smooth muscle cells around hepatic vein branches. Although fat-storing cells appeared to only weakly express desmin in human liver, cells surrounding the terminal hepatic vein radicles, as seen here, were intensely stained using the anti-desmin antibody; these are likely to be smooth muscle cells. (HV: hepatic vein). (Monoclonal anti-desmin antibody DE-B-5; indirect immunoperoxidase method; photographed under Nomarski filters; x 750).

c. CGA7 immunoreactivity in portal tracts. Positive staining with this antibody is identified within smooth muscle cells of this hepatic artery branch and in occasional cells surrounding the portal vein; immunoreactivity could not be demonstrated within any sinusoidal cells. (Monoclonal antibody CGA7; indirect immunoperoxidase technique method; haematoxylin counterstain; x 300).

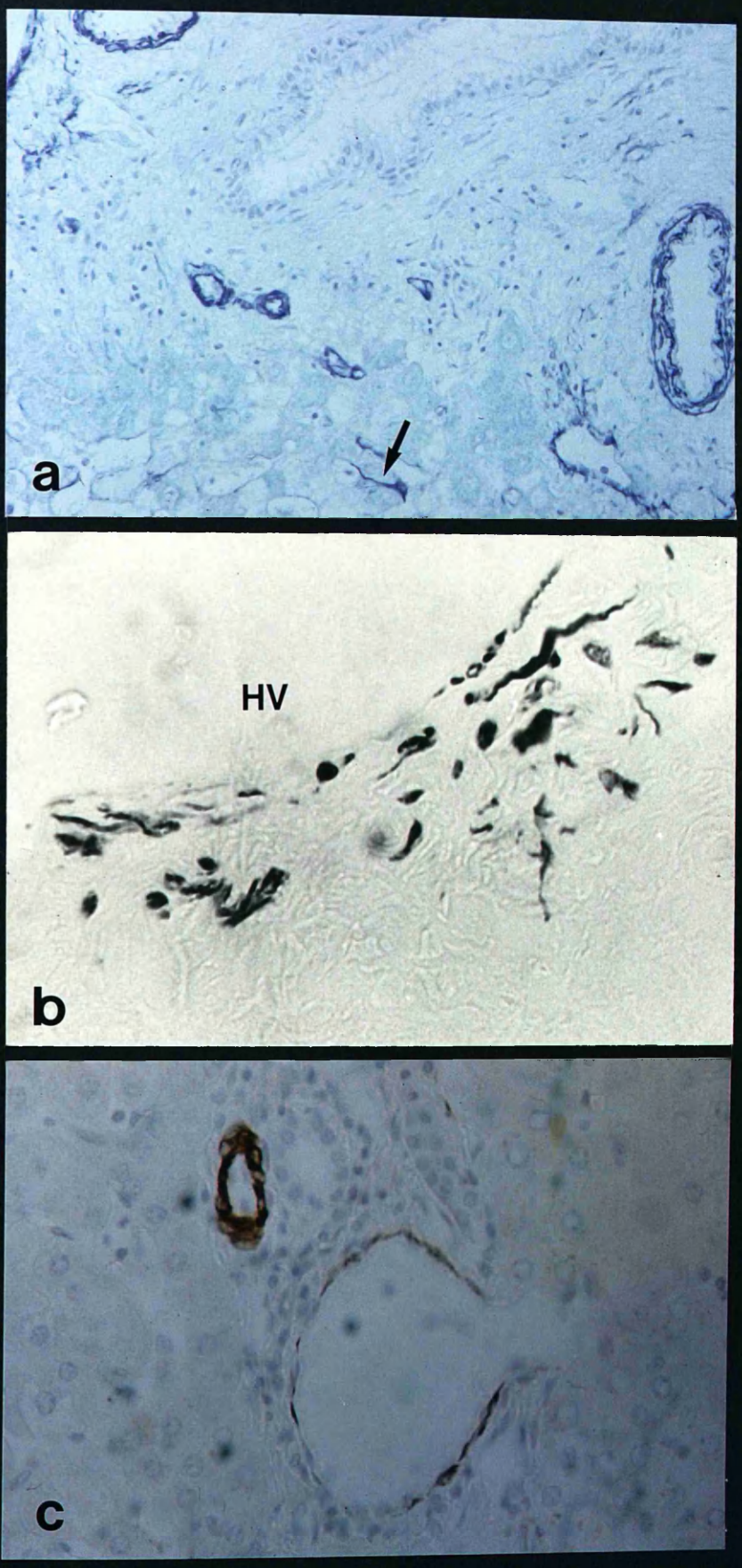
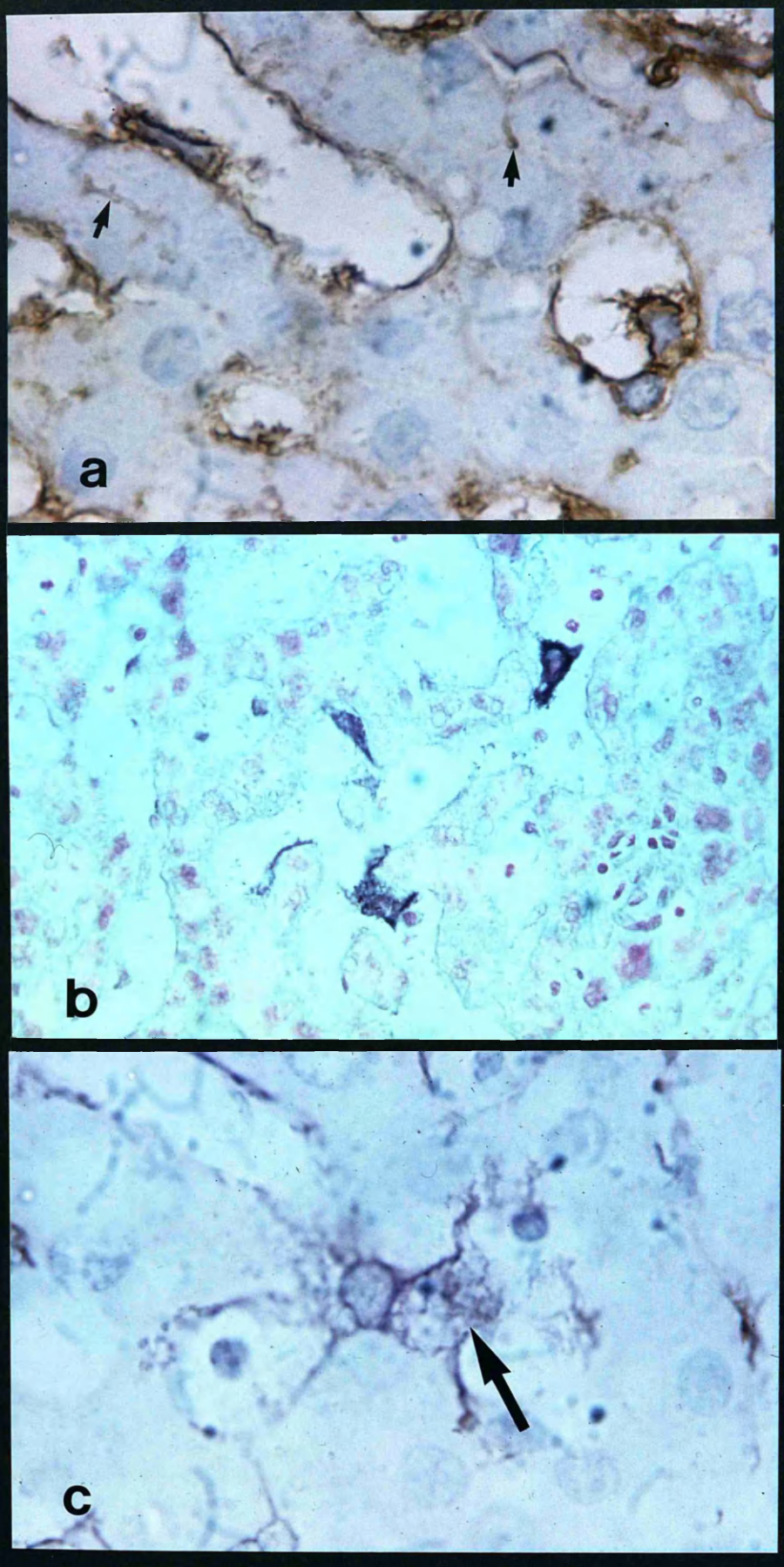

a
HV
b
c

Figure 5.6 Expression of muscle-associated proteins by human sinusoidal cells.

a. Immunolocalization of actin, using a monoclonal antibody which recognises an epitope common to all isoforms of actin. Immunoreactivity is noted within all sinusoidal cells and, in addition, within parenchymal cells at the canalicular domain (arrows). (Monoclonal anti-actin antibody (Amersham); indirect immunoperoxidase method; haematoxylin counterstain; x 850).

b. Immunoreactivity with monoclonal antibody HHF35. A population of sinusoidal cells can be seen to be immunoreactive with this monoclonal antibody. Although some of these cells had morphological features suggestive of fat-storing cells, subsequent double-labelling experiments showed that within the HHF35 positive population there were cells immunoreactive with the monoclonal antibody EBM/11 which detects the macrophage-associated cluster differentiation antigen CD68. (Monoclonal antibody HHF35; indirect immunoperoxidase method with nickel-cobalt enhancement; neutral red counterstain; x 500).

c. Immunolocalization of filamin in sinusoidal cells. This protein was detected in all sinusoidal cells but did not appear to be present in parenchymal cells. Some of the immunoreactive sinusoidal cells such as the one illustrated here had morphological features suggestive of fat-storing cells (arrow). (Monoclonal anti-filamin antibody PM317; indirect immunoperoxidase method; haematoxylin counterstain; x 900).


a
b
c

sinusoidal cells were identified in acinar zone III. α-(smooth muscle) actin-containing cells were also seen in portal tract stroma.

The monoclonal antibody HHF35 stained greater than 50% of sinusoidal cells in 2% paraformaldehyde- and formal sublimate-fixed sections (Figure 5.6b) but not in Bouin's-fixed tissue; labelling intensity was weak in cryostat sections. Most of the immunoreactive cells were of stellate morphology; some were characterized by the presence of long cytoplasmic processes while others appeared to protrude into the sinusoidal lumen. The morphology of some of the larger HHF35-positive cells resembled that of EBM/11-positive cells. Furthermore, immunoreactive cells were more abundant in acinar zone 1 (periportal) than zone III. In double labelling experiments, greater than 60% of the cells were immunoreactive with both HHF35 and EBM/11. However, a population of HHF35-positive/EBM/11-negative cells was identified.

(iii) Filamin. As with rat liver all sinusoidal cells appeared to express this protein but parenchymal cells and bile duct epithelial cells were consistently negative (Figure 5.6c).

(iv) Vimentin, cytokeratin and TPA. Immunolocalization of these other cytoskeletal proteins showed that all sinusoidal cells were positive for vimentin (Figure 5.7) but negative for cytokeratins and TPA B1. By contrast, parenchymal cells were immunoreactive with CAM 5.2 but did not stain with anti-TPA B1 or anti-vimentin. Bile duct epithelial cells were strongly labelled with both CAM 5.2 and anti-TPA B1 but were negative for vimentin (Burt *et al*, 1987b).

(v) PR2D3. This antibody stained approximately 25% of all sinusoidal cells in cryostat sections but not in any fixed tissue. The immunoreactive cells were randomly distributed throughout the acini and were characterized by the presence of a vacuolated cytoplasm; some of the cells were seen to have cytoplasmic extensions (Figure 5.8). Occasional PR2D3-positive cells were also found around terminal hepatic vein radicles and scattered throughout portal tract stroma. Immunoreactivity was also noted in smooth muscle cells within the walls of hepatic arteries. The morphology and distribution of PR2D3-positive sinusoidal cells differed from that of EBM/11-positive cells. Furthermore, in double-labelling experiments two distinct cell populations were identified; no cells were found to be positive with both PR2D3 and EBM/11.

Figure 5.7 Immunolocalization of vimentin in sinusoidal cells.

In this micrograph, positive staining for the intermediate filament protein vimentin is seen within a cell with morphological features of a sinusoidal endothelial cell (E). Note the absence of staining within parenchymal cells (P). (Monoclonal anti-vimentin antibody; indirect immunoperoxidase method; haematoxylin counterstain; x 1,000).

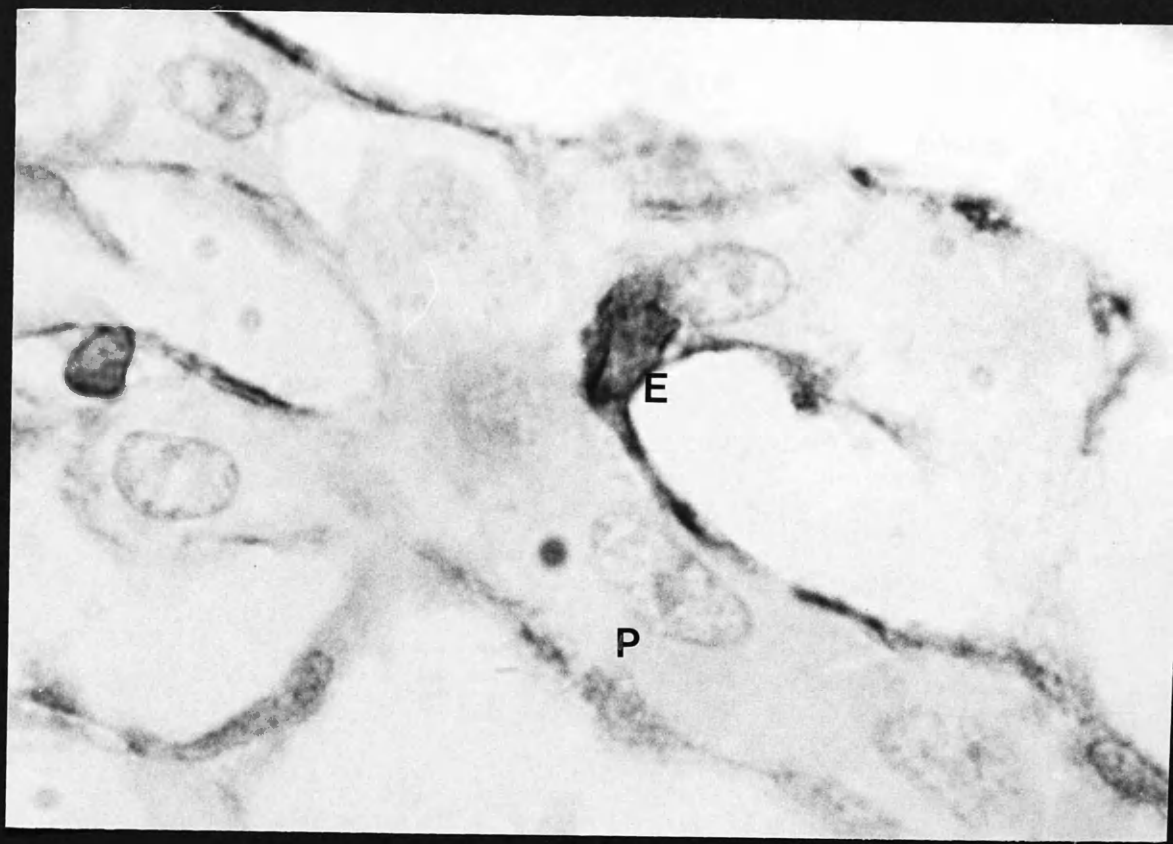
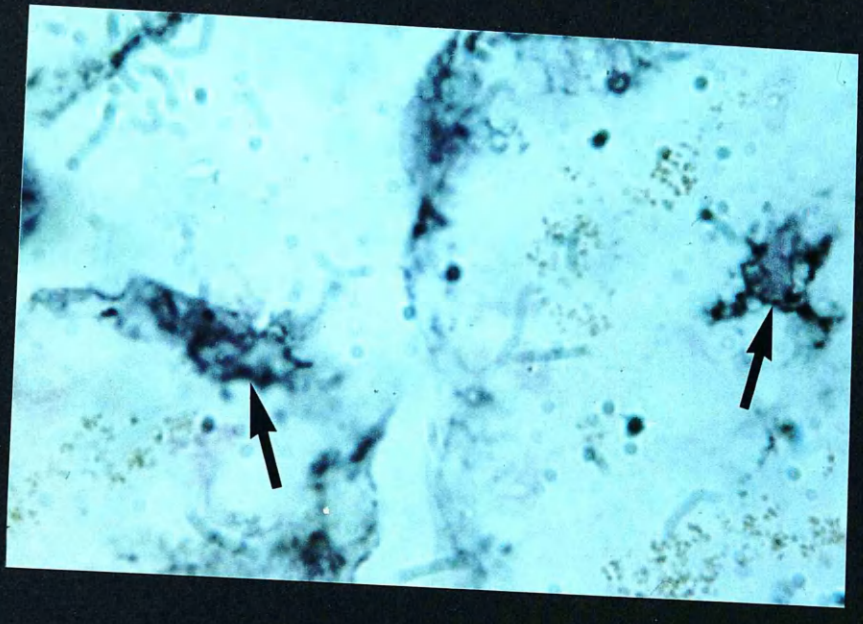

E
P

Figure 5.8 Immunoreactivity of a population of human sinusoidal cells with monoclonal antibody PR2D3.

Immunoreactivity with the antibody PR2D3 can be seen here in two sinusoidal cells in acinar zone III (perivenular zone) (arrows). (PR2D3; indirect immunoperoxidase with nickel/cobalt enhancement; light nuclear red counterstain; x 750).



5.4 DISCUSSION

5.4.1 Fat-storing cells in rat liver

Desmin is a 55kD intermediate filament protein which is characteristically found in muscle cells (Lazarides, 1982; Ip *et al*, 1983). Although it was originally thought to be entirely restricted to cells showing myogenic differentiation, several reports indicate that other cell types may contain desmin, including stromal cells of intestinal submucosa, testis and endometrium, as well as a population of renal glomerular cells (Skalli *et al*, 1986; Glasser & Julian, 1986; Yaoita *et al*, 1990). In the initial studies described above (Burt & MacSween, 1986b; Burt *et al*, 1986b) I confirmed the findings of Yokoi *et al* (1984) that a population of cells is present within the sinusoids of normal rat liver which contain this cytoskeletal protein. Subsequent experiments showed that these results could be further substantiated using different monoclonal anti-desmin antibodies; optimization of the immunohistochemical methods using paraffin-embedded material provided enhanced microscopic visualization of the immunostained cells. They were found in a perisinusoidal location and were characterized by the presence of long cytoplasmic processes and cytoplasmic vacuoles. The morphological features of the cells strongly suggested that these were fat-storing cells. En face staining showed the desmin-positive cells to be negative with ED1 and ED2, monoclonal antibodies which react with epitopes on cytoplasmic proteins of rat macrophages; this is in keeping with the results of Yokoi *et al* (1984) who demonstrated that the desmin-positive cells were non-phagocytic.

It remains to be established what proportion of fat-storing cells in normal rat liver express desmin; is this universal or is there a sub-population of desmin-negative fat-storing cells? The desmin-positive sinusoidal cell index obtained in this study is slightly lower than that reported by Yokoi *et al* (1984). Both values however, are of a similar order of magnitude to that noted in morphometric studies using Alsop's histochemical method on semi-thin sections (Jézéquel *et al*, 1984) suggesting that most (if not all) fat-storing cells contain desmin. Further support for this comes from the cell culture studies of Tsutsumi *et al* (1987). They investigated desmin-immunoreactivity in freshly isolated rat liver fat-storing cell fractions and in primary

cultures. They noted that over 90% of cells in the fat-storing cell fraction were desmin-positive and that this protein continued to be expressed in cultured cells. These results must nevertheless be interpreted with caution. Indirect immunofluorescence for cytoskeletal proteins in cell suspensions can often be associated with non-specific binding of antibodies to cell membranes. Furthermore, we cannot assume that the fat-storing cell fraction obtained from their modified Metrizamide density gradient contained the entire hepatic fat-storing cell population; the possibility of a desmin-negative fat-storing cell population in their other cell fractions was not excluded. Indeed, Ballardini *et al* (1989) found large numbers of vitamin A-positive, desmin-negative fat-storing cells in their isolation experiments and concluded that "desmin cannot be assumed...an absolute marker for rat Ito cells." It is clear that this question can only be fully answered by careful immuno-electron microscopic studies. Although I have attempted to localize this protein in rat liver at the ultrastructural level using the methods outlined in Chapter 4, only weak labelling was obtained, even in smooth muscle cells, and no conclusions could be drawn from the experiments.

In contrast to the study of Yokoi *et al* (1984), I identified desmin-positive cells scattered throughout portal tract stroma. On morphological grounds they appeared similar to those seen within the sinusoids. To my knowledge, typical fat-storing cells have not been identified within portal tracts in any previous ultrastructural study. The precise nature of this cell population needs to be defined; immuno-electron microscopy would again be of considerable value.

Actin is a ubiquitous cytoskeletal protein which has been highly conserved during evolution. Biochemical heterogeneity in the 17 amino acids at the N-terminal end of the protein has been identified in different tissues within an individual species (Vanderkerkhove & Weber, 1978). On the basis of electrophoretic mobilities, three isoforms were initially described: α, β and γ. Sequence data indicated that three distinct forms of α-actin exist, one specific for smooth muscle cells, one for cardiac muscle and the other for striated muscle. Furthermore, two distinct isoforms were identified, one present in smooth muscle cells and the other present in all cell types. Six different isoforms have therefore been described, each encoded by different genes and showing a cell type-restricted distribution. The monoclonal antibody used in the study of rat liver (1A4) detects an epitope on the α isoform specific for smooth muscle (Skalli *et al*, 1986). The cells immunoreactive for this protein detected in perivenular regions of normal rat

liver in close proximity to terminal hepatic vein radicles are likely to be true smooth muscle cells. However, occassional α-actin positive cells were noted to have a stellate morphology and it remains possible that a small sub-population of fat-storing cells in acinar zone III express this muscle-associated protein in normal rat liver; elsewhere, however, fat-storing cells appear to be α (smooth muscle) actin-negative. These observations are in keeping with the recent reports of Nouchi *et al* (1991) and Tanaka *et al* (1991) and are in accordance with the cell isolation studies of Ballardini *et al* (1989), Ramadori *et al* (1989, 1990) and Rockey *et al* (1991) in which α (smooth muscle) actin was not detectable in freshly isolated fat-storing cell fractions or in early cultures by immunohistochemistry or Western blotting to detect the protein or Northern blotting to detect specific RNA. These studies did however, demonstrate that after several days in culture the cells showed immunoreactivity for this protein. The expression of α (smooth muscle) actin in "activated" fat-storing cells is discussed in Chapter 6.

5.4.2 Fat-storing cells in human liver

The desmin-containing cells identified around hepatic vein branches in normal human liver did not have a stellate morphology and it is likely that these represent smooth muscle cells rather than fat-storing cells. Occasional cells within sinusoidal walls were also identified which were immunoreactive for desmin. These were detectable, however, only in sections immunostained using a nickel/cobalt enhancement method and were considerably fewer than in rat liver. These observations suggest that only some human fat-storing cells express this intermediate filament protein and indicate that desmin is unlikely to be a reliable marker for these cells in human liver. Schmitt-Gräff *et al* (1991) presented similar findings in a recently published study of desmin immunoreactivity in normal and fibrotic human liver. Although some desmin-containing sinusoidal cells could be identified in fetal liver, they were sparse in normal adult liver. Immunolocalization of desmin at the ultrastructural level within a fat-storing cell in normal human liver was illustrated in a review article by Peyrol & Grimaud (1988) but no details were provided about the number of immunoreactive cells present or the age of the individual. It is difficult to explain the inter-species difference in the degree of expression of desmin by fat-storing cells but it seems unlikely to reflect any true functional differences. It is

of interest that variable expression of this protein has been described even within an individual species. In their study of desmin-containing cells in rat glomeruli, Yaoita *et al* (1990) found that substantially greater numbers of immunoreactive cells could be seen in Wistar rats compared to SHR rats; Fischer 344 and Lewis rats contained amounts intermediate between these strains. Furthermore, in each strain there was a progressive increase in the number of labelled cells with aging.

Of the other intermediate filament proteins studied, vimentin but not cytokeratin (or the related TPA) appeared to be expressed by sinusoidal cells. Vimentin is the principal intermediate filament protein of mesenchymal cells and it is perhaps not surprising that fat-storing cells should contain this protein. Likewise, expression of cytokeratins and TPA B1 is almost exclusively restricted to epithelial cells and the lack of immunoreactivity for CAM 5.2 and anti-TPA B1 in all sinusoidal cells was expected. The differential expression of these proteins by parenchymal and bile duct epithelial cells is discussed elsewhere (Burt *et al*, 1987b).

Actin is a ubiquitous molecule which is abundantly expressed by most cell types. It is not surprising therefore, that all sinusoidal cells stained positively with the monoclonal anti-actin antibody used which recognizes an epitope common to all isoforms of this cytoskeletal protein (Lin, 1981). The actin-binding protein filamin is also widely distributed, but is more abundantly expressed in some cell types (including smooth muscle cells) than others (Small *et al*, 1986). It is of interest that although I could immunolocalize this protein in all sinusoidal cells, it did not appear to be present in parenchymal cells.

In the present study, a population of cells close to terminal hepatic vein radicles were seen to be immunoreactive for the α (smooth muscle) isoform of actin, similar to those observed in rat liver. These were plump cells without any cytoplasmic extensions, although some stellate shaped cells were present along sinusoidal walls in acinar zone III. However, no immunoreactive cells were found with CGA7, an antibody which is said to react with an epitope common to α and isoforms of smooth muscle actin. The reasons for the discrepancy between the results obtained with 1A4 and CGA7 are uncertain.

A population of sinusoidal cells was immunoreactive with the monoclonal antibody HHF35. This is reported to react with α and γ isoforms common to all types of muscle cell (Tsukada *et al*, 1987; Tsukada & Yokofujita, 1990). However, the distribution and morphology

of the labelled cells suggested that at least some were Kupffer cells. This was confirmed by demonstrating co-localization with the macrophage-associated cluster differentiation antigen CD68 using the monoclonal antibody EBM/11. Although the HHF35-positive/EBM/11-negative cells may represent fat-storing cells, lack of specificity limits the utility of this anti-"muscle actin" antibody.

The monoclonal antibody PR2D3 was originally raised against a crude homogenate of normal human colorectal mucosa and found to react with the so-called peri-crypt sheath cells which surround mucosal glands (Richman *et al*, 1987). The antibody recognizes a membrane protein which is present on smooth muscle cells and myofibroblast-like cells from a variety of sites. In their original publication, Richman and co-workers refer to PR2D3-immunoreactivity in "peri-sinusoidal cells" but provided no other details of their results in human liver. The distribution and morphological features of the PR2D3-positive sinusoidal cells noted in the present study suggest that they are fat-storing cells; this is supported by double-labelling experiments which demonstrated that they were EBM/11-negative. Unfortunately, PR2D3-immunoractivity is completely lost following aldehyde fixation; this precludes immuno-electron microscopic studies which might further define this cell population.

In summary, the studies outlined in this chapter indicate that fat-storing cells in rat liver contain the intermediate filament protein desmin; immunolocalization of this protein therefore provides a useful tool for the study of the *in vivo* response of these cells to liver injury. By contrast, few fat-storing cells in normal human liver contain desmin, suggesting that this could not be regarded as a reliable marker for their identification in studies of human disease. However, the antibody PR2D3 does appear to detect a population of human sinusoidal cells with the morphological features and distribution of fat-storing cells; this may provide a means of monitoring fat-storing cell numbers in human liver disease.

CHAPTER 6. RESPONSE OF FAT-STORING CELLS TO EXPERIMENTAL LIVER INJURY

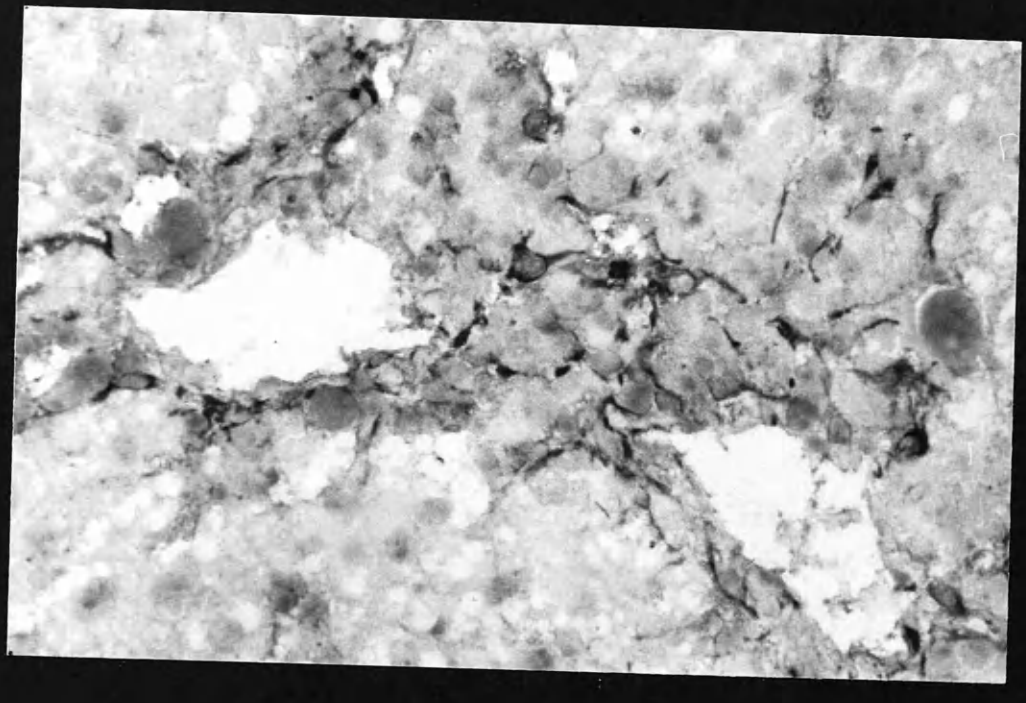
6.1 INTRODUCTION

Development of the desmin-immunolabelling method for identification of fat-storing cells in tissue sections of rat liver provided a means by which the response of these cells to various forms of experimental injury could be quantified. I initially applied this approach to examine their cell kinetics following acute liver injury (Burt *et al*, 1986b). This study demonstrated that following a single, sub-lethal bolus of the toxin carbon tetrachloride, desmin-containing fat-storing cells accumulated in areas of necrosis (Figure 6.1). A significant increase in the number of such cells was seen as early as 48 hours after administration of the toxin, reaching a peak at 72 hours, before returning to control values. The experiments carried out at that time involved the use of the Amersham monoclonal anti-desmin antibody described in Chapter 5. As immunoreactivity with this reagent in fixed tissue was highly dependant on the mode of fixation and trypsinization, cell counts in paraffin-embedded tissue showed poor reproducibility. An immuno-alkaline phosphatase method was therefore used on cryostat sections for quantitative studies. This provided cell counts showing greater consistency between different animals at each time point, and between different experiments. However, the disadvantages of quantitative studies using frozen sections have already been stressed. The first aim of the work described in this chapter was therefore to repeat the experiment, having established the optimal conditions for localization of desmin-containing sinusoidal cells in fixed, paraffin-embedded material. In addition, the response of fat-storing cells to chronic liver injury was studied. A model of chronic cholestatic disease was chosen, using common bile duct ligation to produce secondary biliary cirrhosis. Although prolonged exposure to chemical toxins such as carbon tetrachloride and dimethylnitrosamine can also be used to induce cirrhosis in rats, the cholestatic model provided a form of injury in which the maximal damage occured in acinar zone I (periportal zone). It was therefore possible to compare the distribution of desmin-containing cells in progressive cholestatic injury with that seen in carbon tetrachloride-induced acute injury where necrosis is predominantly seen in acinar zone III (perivenular zone).

There are several possible mechanisms by which fat-storing cells may accumulate in response to injury (vide infra). In order to establish whether local proliferation may contribute to this, a novel double labelling method was developed by which proliferating fat-storing cells

Figure 6.1 Immunolocalization of desmin-positive cells in acute carbon tetrachloride-induced injury: immuno-alkaline phosphatase method.

In the preliminary experiments (Burt *et al*, 1986b), an immuno-alkaline phosphatase method was used on cryostat sections. Accumulation of desmin-positive stellate cells can be identified here in a perivenular zone (acinar zone III). The immunoreactive cells surround injured parenchymal cells. (Monoclonal anti-desmin antibody (Amersham); immuno-alkaline phosphatase method; x 390).



could be identified by the simultaneous demonstration of desmin and incorporated bromodeoxyuridine (BrdU). BrdU is an analogue of thymidine which, when administered to animals *in vivo*, is incorporated into newly synthesized DNA; subsequent immunolocalization of this compound in tissue sections provides an accurate and reproducible means of identifying cells in S phase of the cell cycle.

Finally, I wished to examine whether fat-storing cells undergo phenotypic modulation during the response to acute and chronic liver injury. Kent *et al* (1976), in their study of carbon tetrachloride-induced cirrhosis in rats, described cells which had ultrastructural features intermediate between those of normal fat-storing cells and fibroblasts. Referring to them as "transitional cells", Kent and co-workers suggested that they represented activated forms of fat-storing cells. In a baboon model of alcohol-induced hepatic injury, Mak *et al* (1984) also identified transitional cells in perivenular zones during the development of fibrosis. While retaining many of the ultrastructural features of fat-storing cells, there was a decrease in the volume of intracytoplasmic lipid droplets and a concomitant hypertrophy of the rough endoplasmic reticulum. These investigators noted the presence of microfilaments, dense bodies and pinocytotic vesicles within the cells, structures characteristic of smooth muscle cells and so-called myofibroblasts (Gabbiani *et al*, 1972); their identification led Mak *et al* (1984) to speculate that fat-storing cells, transitional cells and myofibroblasts may belong to a common cell lineage. Similar ultrastructural changes were observed in other experimental models. Leo & Lieber (1983), for example, demonstrated that in rats fed for up to 9 months on a diet containing vitamin A supplementation and 36% of calories as ethanol, there was a progressive reduction in the number of typical fat-storing cells identified by transmission electron microscopy but an increase in myofibroblast-like cells. French *et al* (1988) subsequently showed that this could also be observed if a high fat-low protein diet was instituted instead of vitamin A supplementation. Transitional cells have also been described in human liver disease. Okanue *et al* (1983) identified cells in alcoholic hepatitis which were considered to represent "activated" fat-storing cells in which there was a substantial reduction in cytoplasmic lipid; the cells were found in close apposition to parenchymal cells showing ballooning degeneration. Similar observations were made by Minato *et al* (1983) and in a more detailed morphometric study by Mak & Lieber (1988). Typical myofibroblasts were described in 75% of alcoholic cirrhotic

livers studied by Rudolph *et al* (1979) and in two cases of hypervitaminosis A (Hruban *et al*, 1974).

Some of these changes observed *in vivo* may be mimicked during the culture of rat fat-storing cells *in vitro*. De Leeuw *et al* (1984) noted a progressive increase in microfilament bundles during serial passage of their cells. As outlined in Chapter 4, I also noted ultrastructural changes over a period of six days in primary culture including reduction in lipid droplet number and density, and hypertrophy of endoplasmic reticulum. It is possible that these changes may have resulted from *in vitro* activation of the cells by virtue of exposure to the "foreign" environment of tissue culture plastic rather than the matrix of the normal space of Disse (see Chapter 9). Several groups have also shown that such phenotypic changes can be induced *in vitro* by the addition of certain peptide growth factors. These *in vivo* and *in vitro* observations have led to the suggestion that in response to injury, fat-storing cells may become activated with the adoption of phenotypic characteristics of myofibroblast-like cells and with a concomitant increase in the production of extracellular matrix proteins (Gressner & Bachem, 1990).

Myofibroblasts were originally described in granulation tissue as mesenchymal stromal cells with ultrastructural properties intermediate between true fibroblasts and smooth muscle cells (Gabbiani *et al*, 1971). It was suggested that their well developed cytoskeletal apparatus might impart upon the cells the property of contractility; it was further postulated that this may provide the force for wound contraction in healing skin wounds. Cells with identical ultrastructural features were also recognized in a number of diverse tissues including periodontal ligament (Beertson *et al*, 1974), pulmonary septa (Kapanci *et al*, 1974) and intestinal mucosa (pericryptal sheath cells) (Kaye *et al*, 1968). Several studies carried out by Gabbiani's group established that myofibroblasts may express the α (smooth muscle) isoform of actin (reviewed in Sappino *et al*, 1990). In granulation tissue this appears to be a transient phenomenon (Darby *et al*, 1990) but in chronic fibrotic diseases there may be persistence of α (smooth muscle) actin expression (Skalli and Gabbiani, 1988). In order to test the hypothesis that fat-storing cells undergo phenotypic modulation towards myofibroblast-like cells, I investigated α (smooth muscle) actin expression by non-parenchymal cells using immunohistochemistry on tissue obtained form both animal models of liver injury. If such changes occur *in vivo*, α (smooth

muscle) actin-positive cells should be seen in areas of injury and with morphological features identical to those of desmin-positive fat-storing cells.

6.2 MATERIALS AND METHODS

6.2.1 Animal model of acute liver injury: carbon tetrachloride intoxication

Acute liver injury was induced in male Wistar rats (200-225g) by administation of a single bolus of 40% carbon tetrachloride (CCl_4) in liquid paraffin 1, 2, 3, 4, 7 and 10 days before death (n=4 per time point). Untreated day zero animals served as controls (n=4). An intraperitoneal injection of 50mg/kg body weight 5'-bromo-2'-deoxyuridine (BrdU) was given to each animal one hour prior to death by cervical dislocation. A standard laboratory diet and water were given *ad libitum* throughout the study. Wedges of liver tissue (approximately 10x5x2mm) obtained from these animals were immersion fixed in formal sublimate (4% neutral phosphate buffered formaldehyde in saturated aqueous mercuric chloride) for four hours and processed for paraffin wax-embedding. In addition to immunohistochemical studies, 3 μm sections of tissue from each animal were stained with (i) haematoxylin and eosin, (ii) Picro-Mallory's trichrome stain and (iii) Shikata's orcein.

6.6.2 Animal model of chronic cholestatic liver injury: common bile duct ligation

Thirty-three animals were used in this experiment. Under halothane anaesthesia, fifteen of the animals were subjected to laparotomy with double ligation and division of the common bile duct at a point approximately 15mm from the liver hilum. In another fifteen animals (sham operation group), a laparotomy was performed and the common bile duct mobilized but no ligation was carried out. The remaining three animals remained untreated and served as a second (day zero) control group. Liver tissue removed from bile duct-ligated and sham-operated animals (n=3 per group) on 3, 7, 14, 21 and 28 days following operation, was processed as above.

6.2.3 Immunohistochemistry

An indirect immunoperoxidase method (Appendix 4) was used to detect desmin, α (smooth muscle) actin and incorporated BrdU in 3 μm dewaxed sections of formal sublimate-fixed tissue. Details of the anti-desmin (D33) and anti-α (smooth muscle) actin (1A4) antibodies used are provided in Chapter 5. Incorporated BrdU was identified using the mouse monoclonal antibody B44 (Becton Dickenson, Cowley, UK) at a dilution of 1:250. Optimal immunoreactivity with this antibody was found in sections of formal sublimate-fixed tissue following 10 minutes trypsinization and a preliminary DNA denaturation step using 2M HCl for 30 minutes at 37°C.

6.2.4 Double labelling method for identification of proliferating fat-storing cells

In order to identify proliferating fat-storing cells, 3 μm dewaxed sections were (i) incubated with 0.5% hydrogen peroxide in methanol for 10 minutes; (ii) washed in distilled water; (iii) incubated with 20% normal rabbit serum (NRS) in Tris buffered saline (TBS) for 15 minutes; (iv) following removal of excess NRS, incubated with monoclonal anti-desmin antibody D33 overnight at 4°C; (v) washed extensively in TBS; (vi) incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin, dilution 1:20 in NRS/TBS; (vii) washed extensively in TBS; (viii) incubated with 3', 3 diaminobenzidine in the presence of hydrogen peroxide; (ix) washed in TBS; (x) immersed in 0.1% porcine type II trypsin for 10 minutes at 37°C; (xi); incubated with 2M HCl for 30 minutes at 37°C; (xii) washed in TBS; (xiii) incubated with mouse monoclonal anti-BrdU (B44) for 60 minutes at 37°C; (xiv) washed with TBS; (xv) incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin dilution 1:20 in NRS/TBS for 30 minutes at room temperature; (xvi) incubated with nickel-modified diaminobenzidine followed by Tris/cobalt chloride (Appendix 5); (xvii) dehydrated through graded alcohols and mounted in DPX.

6.2.4 Quantitation

Numbers of desmin- and α (smooth muscle) actin-positive cells were assessed using a Leitz microscope at a magnification of x400 and an eyepiece graticule. In each animal, three sections were assessed. In each section, ten fields were analysed to provide a value for labelled cells per unit area ($0.635mm^2$). A mean value of labelled cells/$0.635mm^2$ was then obtained from the three sections. For assessment of desmin-positive fat-storing cells and α (smooth muscle) actin-positive cells only those with distinct nuclei and at least one cytoplasmic process were counted. Fields were selected either randomly or to include specific acinar zones (perivenular (zone III) and periportal (zone I)). A labelling index for fat-storing cell proliferation was obtained in double-labelled sections by expressing the number of double-labelled fat-storing cells (desmin and BrdU) as a percentage of total desmin-positive fat-storing cells; 200-600 cells were counted in tissue from each animal. Values were expressed as mean ± standard error of the mean (SEM). Cell counts for treated animals were compared to those of controls (day 0 or sham-operated animals) using Student's t test.

6.3 RESULTS

6.3.1. Acute liver injury induced by carbon tetrachloride : morphology

Histological examination of haematoxylin and eosin-stained sections confirmed that the appearances were normal in control group animals. In day 1 animals, perivenular parenchymal cells showed marked ballooning degeneration; this was accompanied by an apparent increase in non-parenchymal cells in acinar zone 1. Degenerative changes in parenchymal cells were more marked in day 2 and day 3 animals; in addition to cells showing hydropic change, numerous apoptotic bodies were seen (Figure 6.2). In these animals there was a further increase in the number of non-parenchymal cells in the area of injury (Figure 6.3). At the point of maximal damage (day 3) there was evidence of injury to parenchymal cells in acinar zone II and in some areas there was early bridging necrosis. In day 4 animals, there was a marked reduction in the number of apoptotic bodies and a concomitant decrease in non-parenchymal cells in acinar zone

Figure 6.2 Photomicrograph of acute carbon tetrachloride-induced liver injury.

This shows the histological appearancnes in rat liver two days after an animal had been given a single dose of carbon tetrachloride by gavage. Within this perivenular zone there are large numbers of parenchymal cells showing ballooning degeneration; apoptotic bodies can also be identified. (Haematoxylin and eosin; x 120).

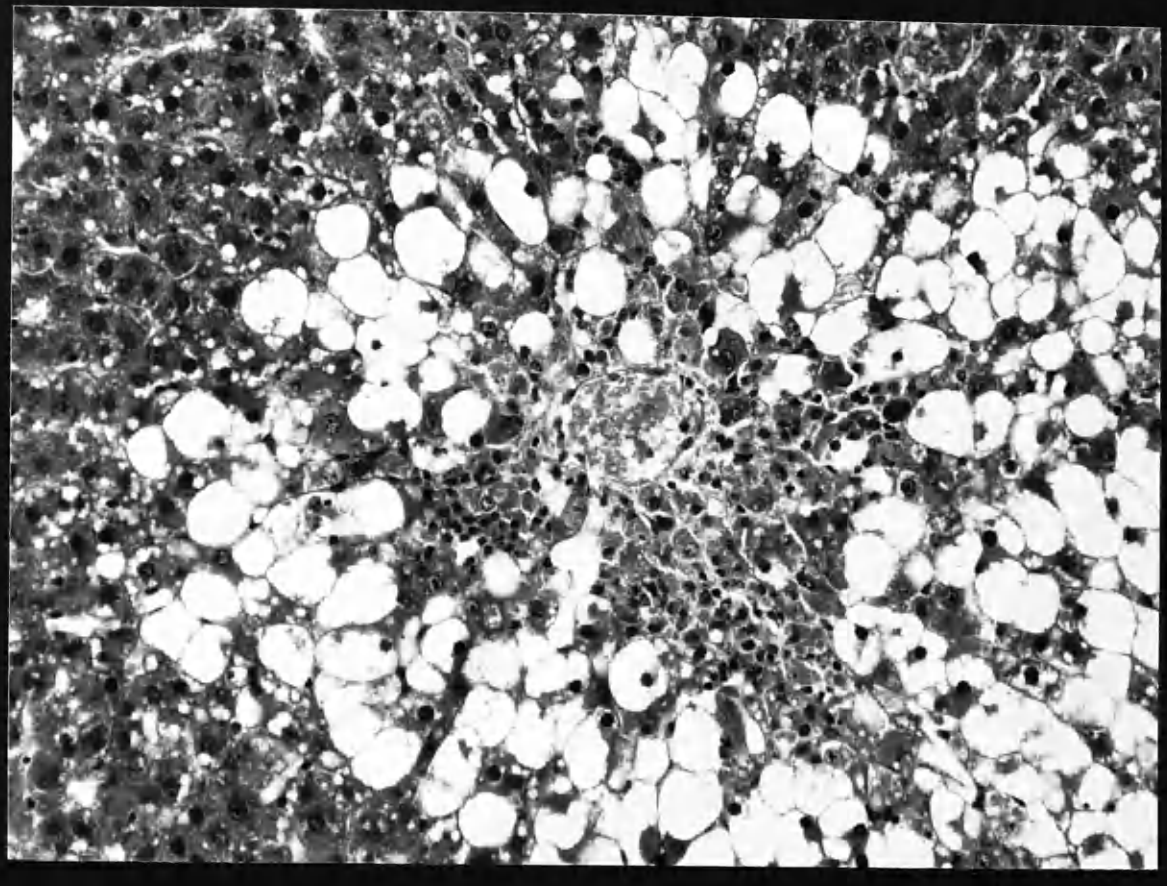
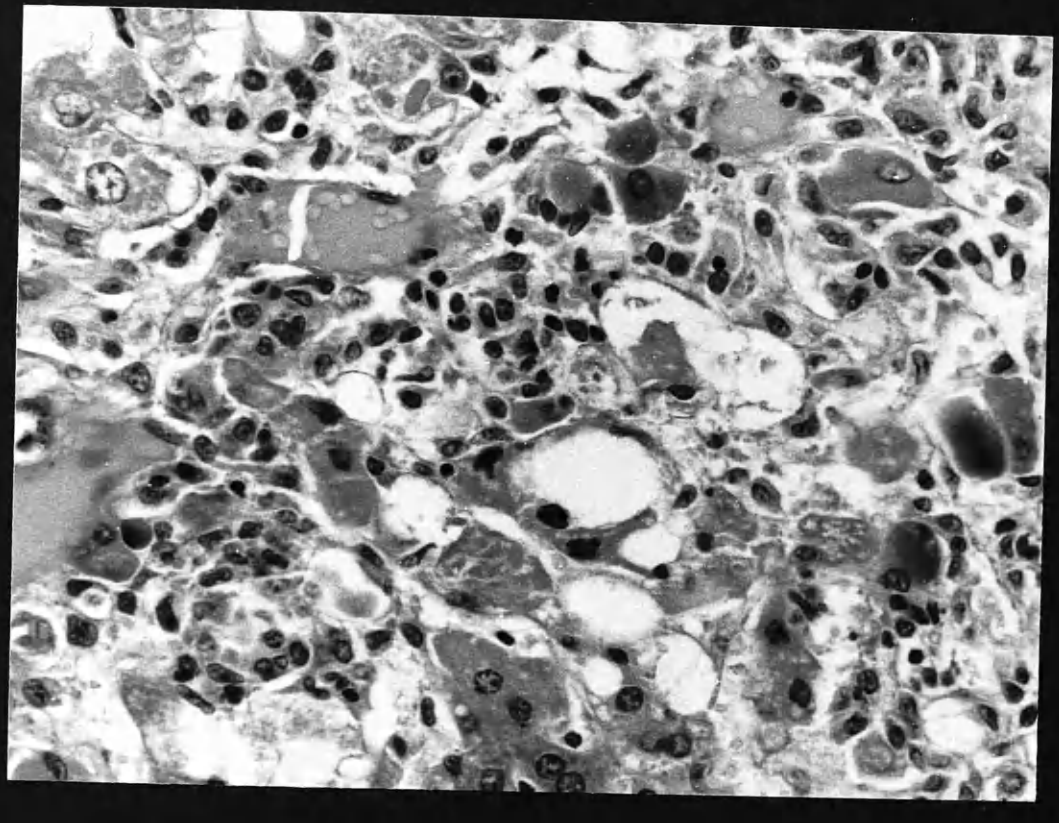


Figure 6.3 Accumulation of non-parenchymal cells following acute carbon tetrachloride-induced liver injury.

In acinar zone III (perivenular zone), large numbers of non-parenchymal cells can be seen adjacent to necrotic parenchymal cells in this section from a day 3 animal. (Haematoxylin and eosin; x 650).



III compared with day 3 animals. The histological appearances had returned essentially to normal by day 7 and remained so in day 10 animals. No parenchymal elastic fibres could be identified using Shikata's orcein stain in liver tissue from any animal in this experimental model.

6.3.2 Acute liver injury : desmin-immunolabelling for quantitation of fat-storing cell population

In control (day 0) animals, desmin-positive sinusoidal cells with the typical stellate morphology of fat-storing cells were identified. In perivenular zones, the mean value of labelled cells was 0.89 ± 0.23 cells/0.635mm^2. In carbon tetrachloride-treated animals there was an increase in the number of immunoreactive cells; this was seen predominantly within acinar zone III (perivenular) (Figure 6.4a). The individual cells generally showed more intense immunoreactivity and were larger than those seen in control animals (Figure 6.4b). A small increase in desmin-positive cells was noted in day 1 animals compared to controls when cell numbers were assessed in perivenular fields but this did not reach statistical significance (Figure 6.5). The increase continued in day 2 animals reaching a peak at day 3 (277.6 ± 30.8 cells/0.635 mm^2) and 4 (279.5 ± 17.7 cells/0.635mm^2). When random fields were assessed, the peak at day 3 represented a 48-fold increase over control values ($p<0.001$). Desmin-positive cell numbers in periportal fields were significantly increased in day 2 animals only ($0.05>p>0.02$). Cell counts obtained in perivenular and random fields in day 7 animals remained significantly elevated (perivenular : $0.05>p>0.02$) but in day 10 animals, values were not significantly different from controls.

6.3.3 Acute liver injury : proliferation of fat-storing cells

In control animals, occasional parenchymal and non-parenchymal cells showed black nuclear staining indicating BrdU incorporation. Desmin-positive sinusoidal cells could be readily identified by brown cytoplasmic immunoreactivity as in single-labelled experiments but in control (day 0) animals, no double-labelled cells could be identified. In day 1, day 2, day 3 and day 4 animals there was a marked increase in the total number of BrdU-labelled parenchymal and non-parenchymal cells. Some of the non-parenchymal cells were seen to show cytoplasmic

Figure 6.4 Immunolocalization of desmin-positive cells following acute carbon tetrachloride-induced injury: indirect immunoperoxidase method.

a. Accumulation of desmin-positive cells can be seen to occur principally in perivenular zones (acinar zone III). (Monoclonal antibody D33; indirect immunoperoxidase; x 250).

b. Desmin-positive cells in CCl_4-treated animals were noted to be larger and to show more intense immunoreactivity than those in normal rat liver. (Monoclonal antibody D33; indirect immunoperoxidase; x 350).

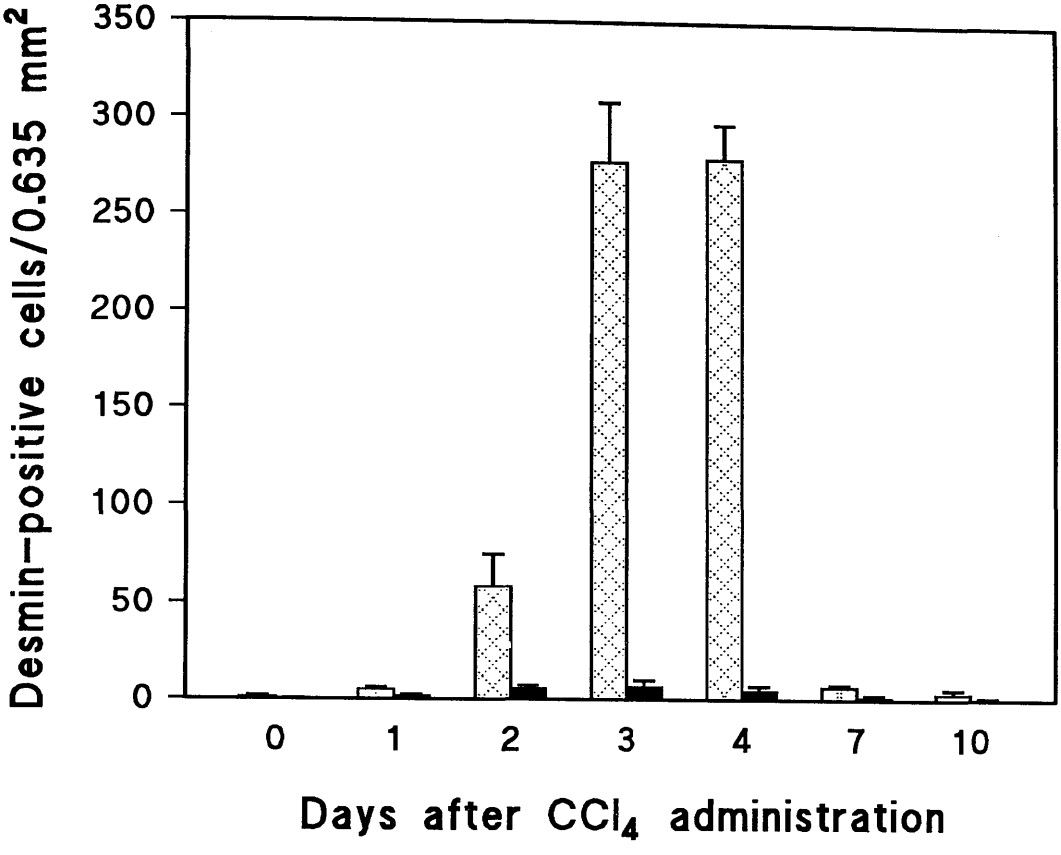
a
b

Figure 6.5 Quantitation of desmin-positive cells following acute liver injury.

Twenty-four animals were given carbon tetrachloride by enterogastric gavage (see text). Four animals were killed at each of the following time points: 1, 2, 3, 4, 7 and 10 days following administration of the toxin. Four untreated animals served as controls (day O). Desmin-positive cells were counted in three sections from each animal and expressed as number of cells per unit area ($0.635mm^2$). This graph plots the mean values (± SEM) obtained for the four animals at each time point. The cross-hatched bars represent the values obtained in perivenular zones and the closed bars represent values for periportal zones.

When compared with control (day O) animals (Student's t test) a significant increase was noted in perivenular zones on day 2 ($0.05 > p > 0.02$), day 3 ($p < 0.001$), day 4 ($p < 0.001$) and day 7 ($0.05 > p > 0.02$).

In periportal zones, a significant increase was only noted in day 2 animals ($0.05 > p > 0.02$).


Desmin-positive cells/0.635 mm²
350
300
250
200
150
100
50
0
0
1
2
3
4
7
10
Days after CCl4 administration

immunoreactivity for desmin (Figure 6.6). These cells were considered to represent fat-storing cells in S phase. The labelling indices for this cell population using random fields for quantitation are shown in Figure 6.7. Proliferative activity showed a peak at day 2 (18.67 ± 0.88%).

6.3.4 Acute liver injury : expression of α (smooth muscle) actin

In control animals, occasional stellate-shaped sinusoidal cells were found to be immunoreactive with the monoclonal anti-α (smooth muscle) actin antibody 1A4, as described in Chapter 5. Increased numbers of immunolabelled cells were seen in day 2 animals. As with desmin-immunoreactive cells, the expansion of this cell population was principally seen in acinar zone III (perivenular) (Figure 6.8). En-face labelling experiments indicated that many α (smooth muscle) actin-positive cells co-expressed desmin (Figure 6.10). The kinetics of the response was similar to that noted for desmin-positive cells with a peak at day 3 representing a 46-fold increase (perivenular fields 226.1 ± 18.1 cells/0.635mm^2; $p<0.001$), therafter falling towards normal. The number of α (smooth muscle) actin positive cells in perivenular zones in day 7 and 10 animals was still significantly greater than that seen in controls ($p<0.05$) (Figure 6.9). It should be noted that the kinetics of this response resembles that of desmin-positive cells although the absolute numbers of immunoreactive cells identified at the peak of the response are less.

6.3.5 Chronic cholestatic injury induced by common bile duct ligation : morphology

Histological examination confirmed the presence of normal histological appearances in the livers of control (unoperated) animals and in all sham-operated animals. In day 3 bile duct-ligated animals, there was expansion of portal tracts with dilatation of large bile duct radicles and surrounding oedema. In addition, there was accentuation of marginal bile ducts and there was a mild accompanying mixed inflammatory cell infiltrate. There was, however, no evidence of a true cholangitis. In day 7 animals, there was a marked increase in the number of ductular structures at the limiting plate and in acinar zone I. Although mitotic figures were identified

Figure 6.6 Immunolocalization of proliferating fat-storing cells following acute liver injury: simultaneous demonstration of desmin and incorporated bromodeoxyuridine.

Proliferating fat-storing cells could be identified by the simultaneous demonstration of cytoplasmic desmin and nuclear-incorporated bromodeoxyuridine. Cells showing double-labelling (white arrow) denote fat-storing cells in S-phase of the cell cycle. In this preparation, desmin-positive cells with no nuclear staining (black arrow) can also be identified; these cells are not in S-phase. Note that this method can also be used to identify proliferation in parenchymal cells (asterisk) and in desmin-negative non-parenchymal cells. (Monoclonal anti-desmin (D33) and monoclonal anti-bromodeoxyuridine antibodies; light haematoxylin counterstain; x 290).

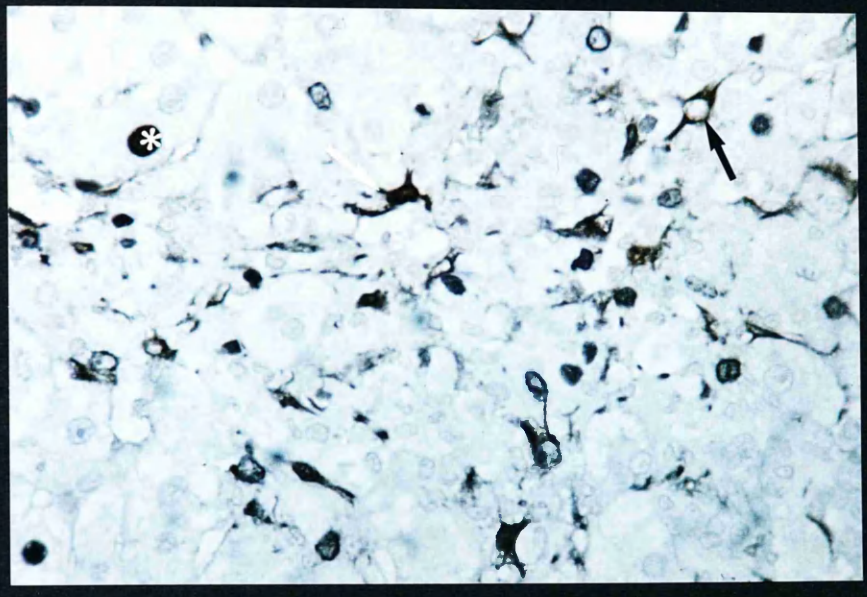


Figure 6.7 Labelling index of proliferating fat-storing cells following acute liver injury.

Material was obtained from 3 animals on each of the following days following carbon tetrachloride administration: 1, 2, 3, 4, 7 and 10. Material was also obtained from 3 untreated animals which served as controls. In each animal between 200-600 desmin-positive cells were counted; the number of desmin-BrdU double labelled cells was assessed and the results expressed as a percentage of total desmin-positive cell numbers to provide a labelling index. In this graph, the results represent the mean (+ SEM) obtained from the 3 animals at each time point. No proliferation could be identified in control animals. However, by day 2 following administration of carbon tetrachloride a labelling index of 18.7% was noted.

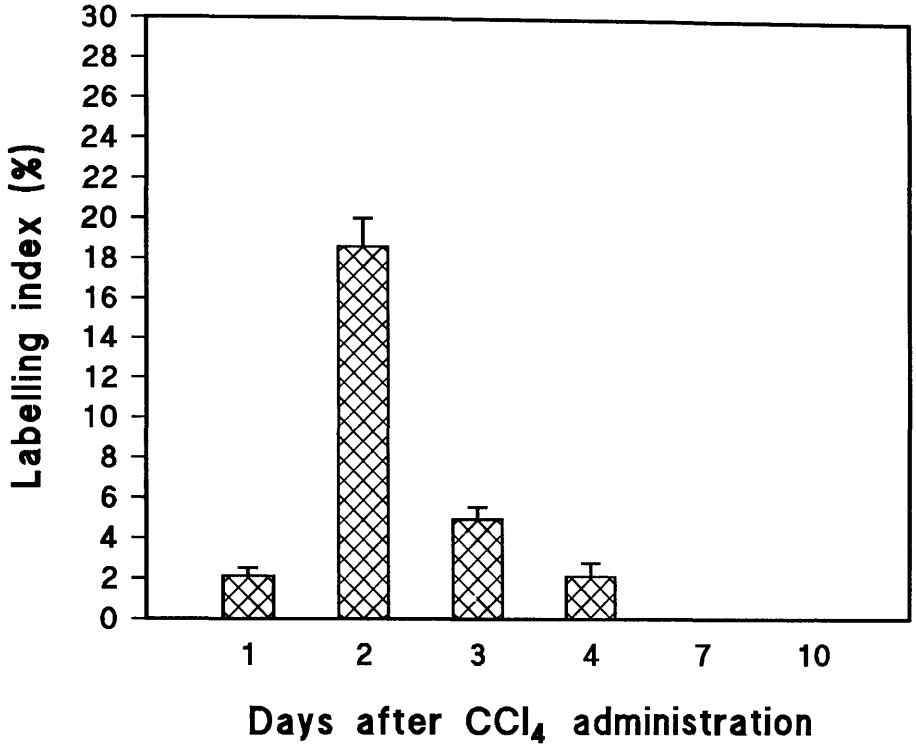

Labelling index (%)
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
7
10
Days after CCl_4 administration

Figure 6.8 Immunolocalization of α (smooth muscle) actin-positive cells following acute liver injury.

Cells immunoreactive for the α isoform of actin are seen here in a perivenular zone, 3 days following administration of the toxin. The morphological features of these cells are similar to those noted for desmin-positive cells. (Monoclonal anti-α (smooth muscle) actin 1A4; indirect immunoperoxidase method; haematoxylin counterstain; x 190).

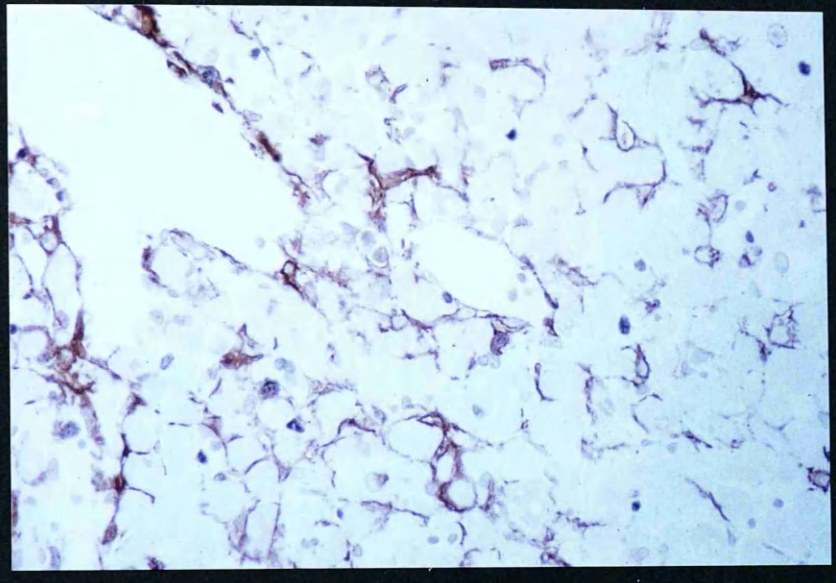


Figure 6.9 Quantitation of α (smooth muscle) actin-positive cells following acute liver injury: perivenular zones.

α (smooth muscle) actin-positive cell numbers were assessed as for desmin-positive cell numbers. In this graph, the values plotted represent the mean (± SEM) of cell counts obtained from 4 animals at each time point in perivenular fields. When compared with control (day O) animals (Student's t test), significant increases were found at days 2, 3 and 4 ($p < 0.001$) and days 7 and 10 ($p < 0.05$).

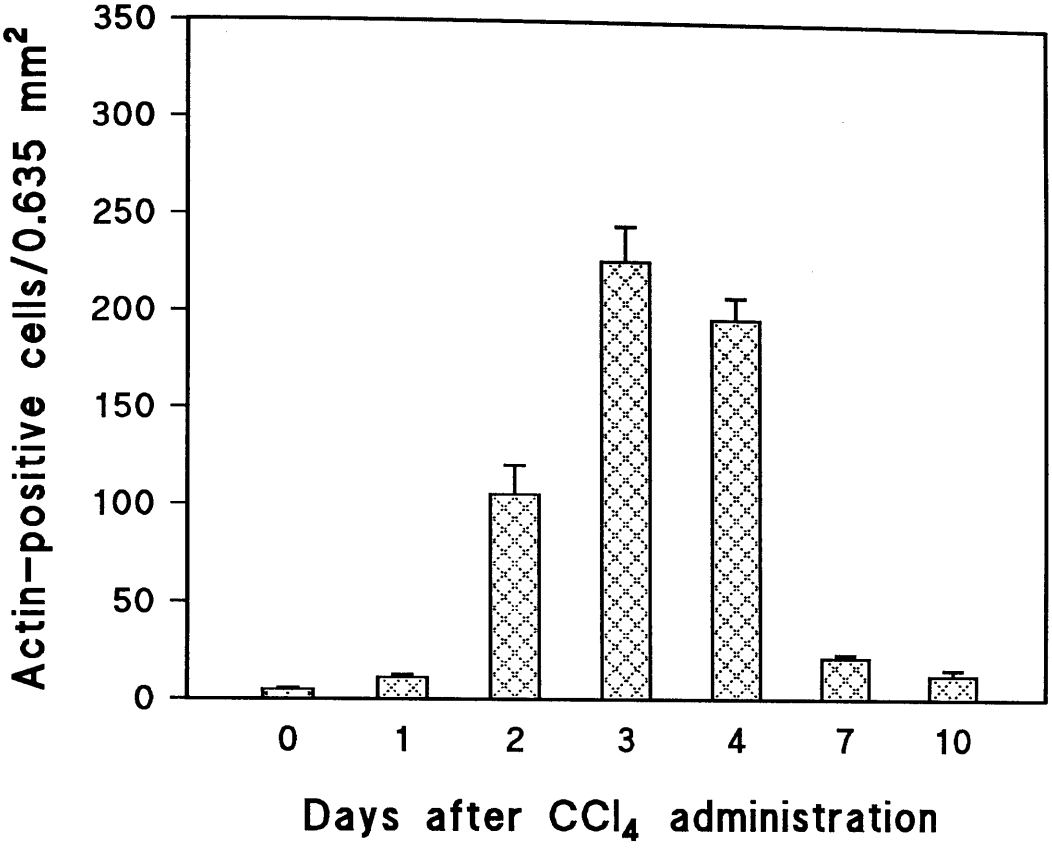

Actin-positive cells/0.635 mm²
350
300
250
200
150
100
50
0
0
1
2
3
4
7
10
Days after CCl4 administration

Figure 6.10 Immunolabelling of en-face preparations: demonstration of co-expression of desmin and α (smooth muscle) actin by stellate cells.

These immunostained en-face sections (see text) demonstrate that many desmin-positive (D) cells are also immunoreactive for α (smooth muscle) actin (A). However some cells (arrow) appear to be desmin-positive/actin-negative. (Monoclonal antibodies D33 and 1A4; indirect immunoperoxidase; light haematoxylin counterstain; x 180).

D
A

within some bile duct epithelial cells, indicating that some of the increase in ductular structures may result from true proliferation, there were morphological changes within zone I parenchymal cells which indicated that ductular metaplasia was also contributing (Figure 6.11a). A progressive increase in the number of ductular structures was noted in material from animals on later days; in day 21 animals these were found throughout acinar zones I and II. In day 14, 21 and 28 animals there was an increase in the amount of fibrous tissue present seen in sections stained with Picro-Mallory's trichrome (Figure 6.12b). Elastic fibres were identified within fibrous septa using Shikata's orcein.

6.3.6 Chronic cholestatic injury : desmin-immunolabelling for quantitation of fat-storing cell population

Increased numbers of desmin-positive cells were identified in bile duct-ligated animals from day 3 on (Figure 6.12), reaching a peak at day 14 (random fields: 127.85 + 5.13 cells/0.635 mm^2); comparison with control or sham-operated animals: $p<0.001$). A subsequent decrease was noted in desmin-positive cell numbers on days 21 and 28 (Figure 6.13). The desmin-positive cells were initially seen to predominate within periportal zones (acinar zone I) and were frequently closely applied to proliferating ductular structures (Figure 6.12). In day 21 and day 28 animals, they were seen within developing fibrous septa and in parenchymal nodules. Because of the marked architectural distortion which resulted in the later stages of this cirrhotic model, all cell counts were performed using random fields. Values obtained in all sham-operated animals did not significantly differ from those noted in day O control animals.

6.3.7 Chronic cholestatic injury : proliferation of fat-storing cells

As in the carbon tetrachloride model, no BrdU/desmin double labelled cells were identified in control animals. Similarly, no proliferating desmin-positive fat-storing cells were seen in sham-operated animals. In bile duct-ligated animals, however, double labelled cells could be identified at all time points in the experiment. The peak of labelling index occured

Figure 6.11 Common bile duct ligation model of chronic cholestatic liver injury: morphological features.

a. Day 14 animal. As documented in previous studies, common bile duct ligation led to increased numbers of ductular structures being present in the liver parenchyma. Although this is in part contributed to by proliferation of pre-existing ducts, transitional cell forms between those of parenchymal and bile duct cells can be seen here indicating that ductular metaplasia may also occur. (Haematoxylin and eosin; x 390).

b. Day 28 animal. In this preparation it can be seen that a cirrhosis is established with regenerative nodules and fibrous septa. (Picro Mallory; x 90).

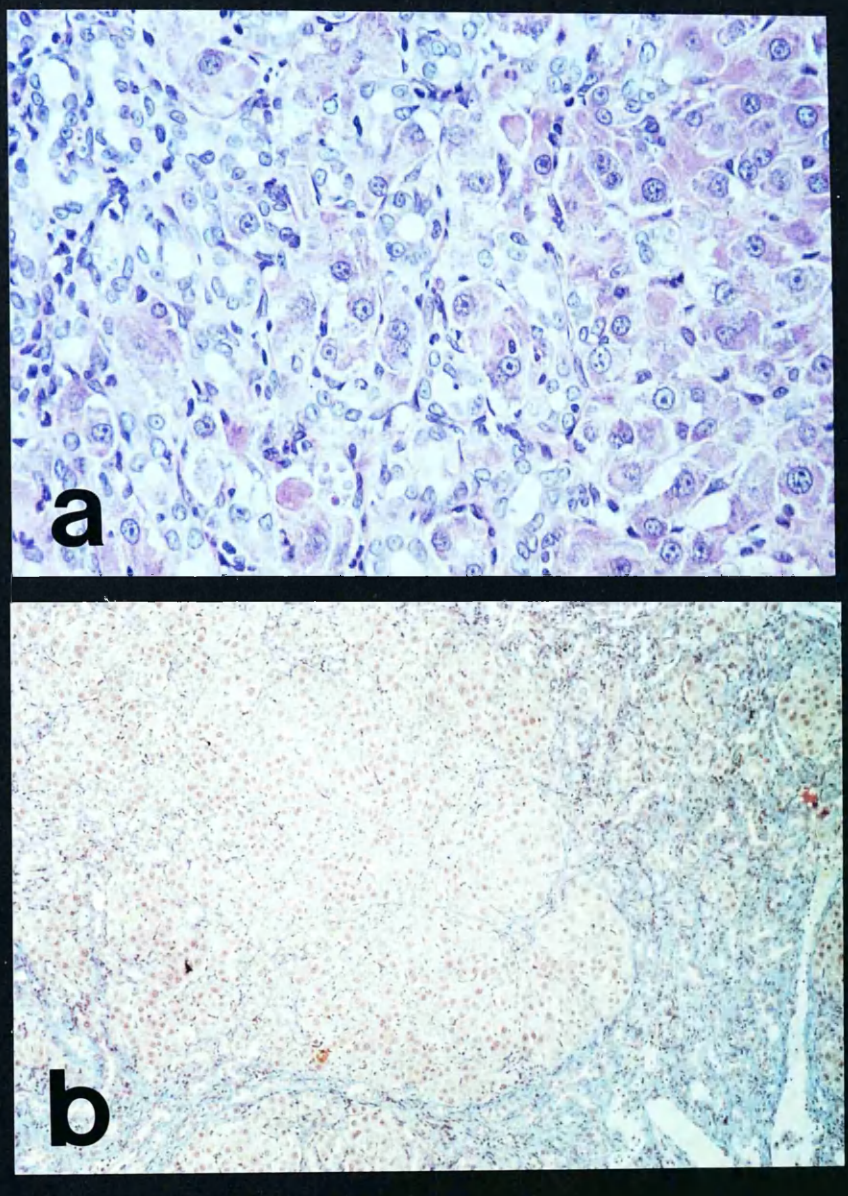

a
b

Figure 6.12 Immunolocalization of desmin-positive cells following common bile duct ligation.

Accumulation of desmin-positive cells can be seen here around ductular structures in acinar zone I (periportal zone) in this section obtained from a day 14 bile duct-ligated animal. (Monoclonal antibody D33; indirect immunoperoxidase; haematoxylin counterstain; x 280).

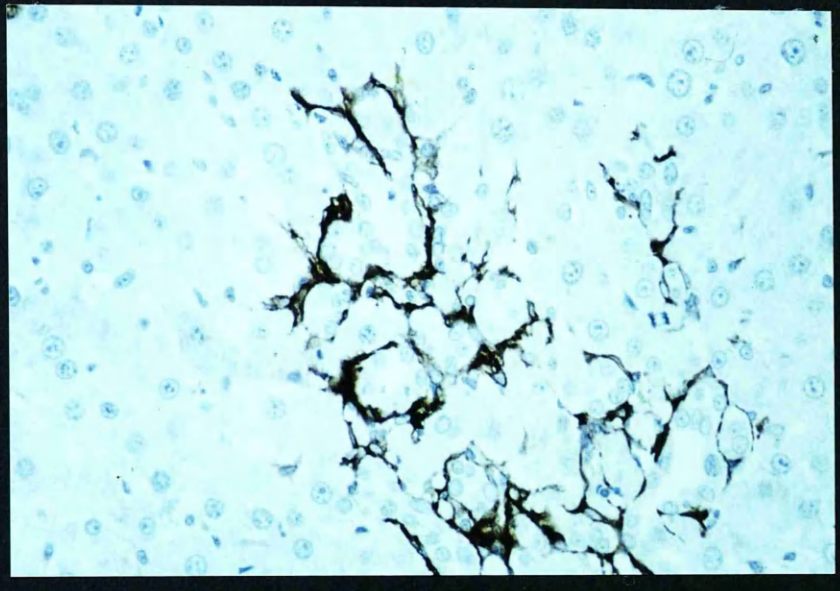
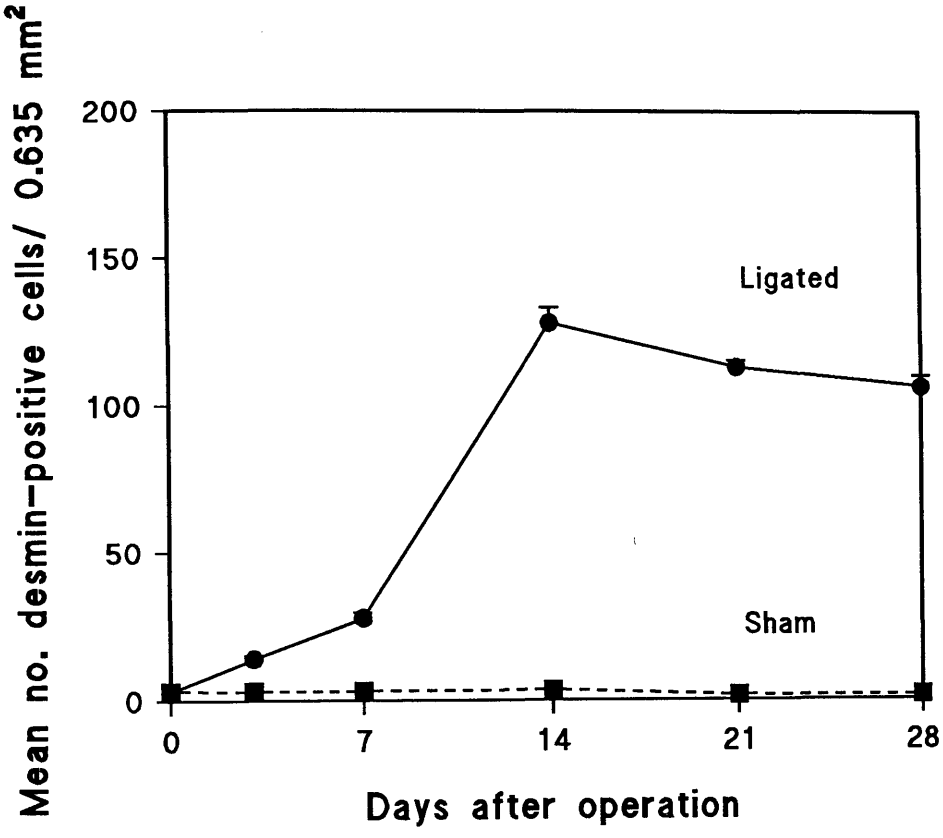


Figure 6.13 Quantitation of desmin-positive cells following common bile duct ligation.

Desmin-positive cell numbers were assessed as in the carbon tetrachloride model. This graph illustrates the values obtained from three animals at each time point following operation with a comparison between results obtained from bile duct-ligated animals and those of sham operated animals. The values in this graph represent those obtained in counts using random fields. Significantly greater numbers of desmin-positive cells were seen in bile duct-ligated animals at day 7 ($0.05 > p > 0.02$) and at all remaining time points ($p < 0.001$).


Mean no. desmin-positive cells/ 0.635 mm²
200
150
100
50
0
Ligated
Sham
0
7
14
21
28
Days after operation

at day 3 (11.7 ± 1.17%). In day 28 animals, only 1.45 ± 0.22% of desmin-positive cells showed simultaneous labelling for incorporated BrdU (figure 6.14).

6.3.8 Chronic cholestatic injury : expression of α (smooth muscle) actin

Stellate cells with identical morphological appearances to those of desmin-positive cells were found which were immunoreactive for α (smooth muscle) actin (Figure 6.15). Increased numbers of these cells were initally seen in periportal zones (acinar zone I) but in day 21 and day 28 animals they were found both within fibrous septa, and in developing parenchymal nodules. In contrast to desmin-positive cell numbers, values continued to rise throughout the study. At all stages of this experimental model, α (smooth muscle) actin-positive cell numbers were greater than those of desmin-positive cells (Figure 6.16).

6.4 DISCUSSION

In the present study, experimental models were used to simulate (i) acute drug-induced liver injury and (ii) chronic cholestatic liver disease. Acute liver injury was produced by a single bolus of CCl_4 by gavage. This has previously been shown to be an effective method for inducing perivenular parenchymal cell necrosis with subsequent healing by regeneration and production of extracellular matrix proteins. As there is no persistence of parenchymal cell injury, there is no net accumulation of matrix proteins and the histological changes return to normal by day 7; fibrosis can be stimulated by repeated doses of the toxin (Geerts *et al*, 1988). This therefore serves as a model of drug or toxin-related reversible injury similar to that seen in humans following parcetamol overdose. As with many hepatotoxins, CCl_4 requires bioactivation by mixed function oxidases to yield the reactive metabolite (CCl_3) which is thought to damage parenchymal cells by lipid peroxidation and by reacting with sulphydryl groups on cytosolic proteins (Reknagel & Glende, 1973).

The model of chronic cholestatic liver injury used in the present study (common bile duct ligation) has previously been shown to produce a biliary cirrhosis (Kountouras *et al*, 1984). Although the morphological changes may not be identical to those seen in human cholestatic

Figure 6.14 Labelling index of fat-storing cells following common bile duct ligation.

Proliferating desmin-positive fat-storing cells were identified in bile duct-ligated animals using the double labelling method outlined in the text. No proliferation of fat-storing cells was found in control (untreated) animals or in sham operated animals at any time point. A labelling index was obtained for bile duct-ligated animals as described for the carbon tetrachloride model. The peak of proliferation can be seen to occur early following the induction of cholestatic injury.

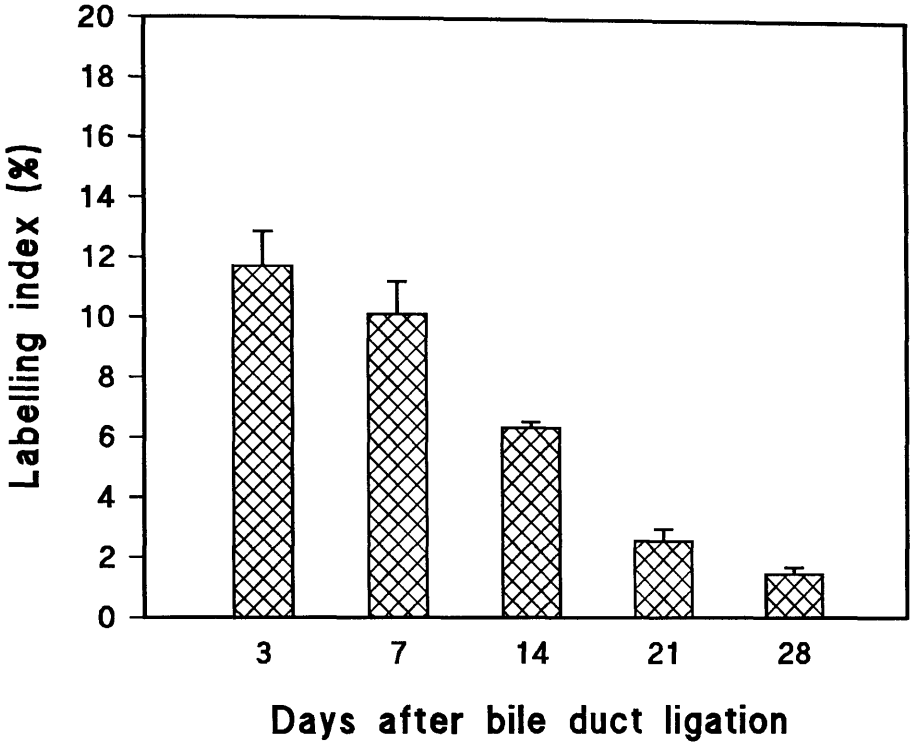

Labelling index (%)
20
18
16
14
12
10
8
6
4
2
0
3
7
14
21
28
Days after bile duct ligation

Figure 6.15 Immunolocalization of α (smooth muscle) actin-positive cells following common bile duct ligation.

Large numbers of immunoreactive cells can be seen around ductular structures in acinar zone I (periportal zone) of this day 21 bile duct-ligated animal. (Monoclonal antibody 1A4; indirect immunoperoxidase; haematoxylin counterstain; x 90).

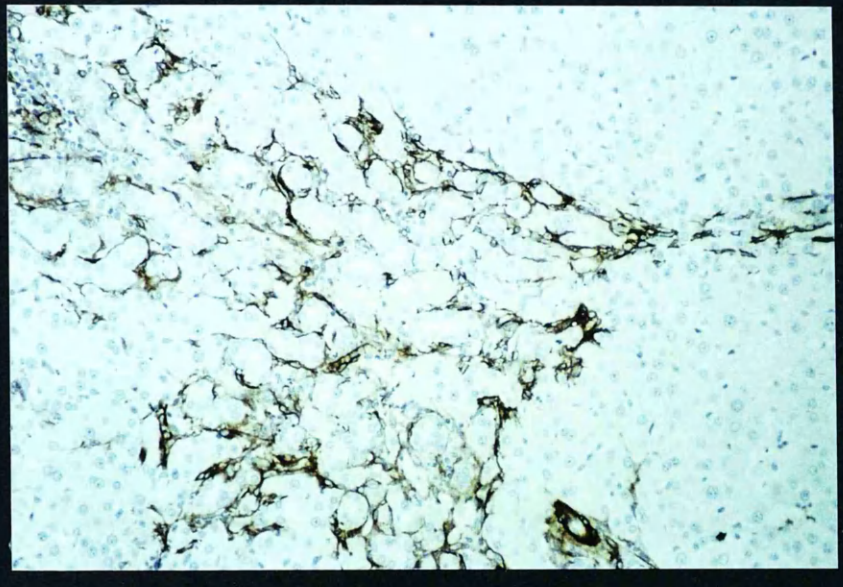
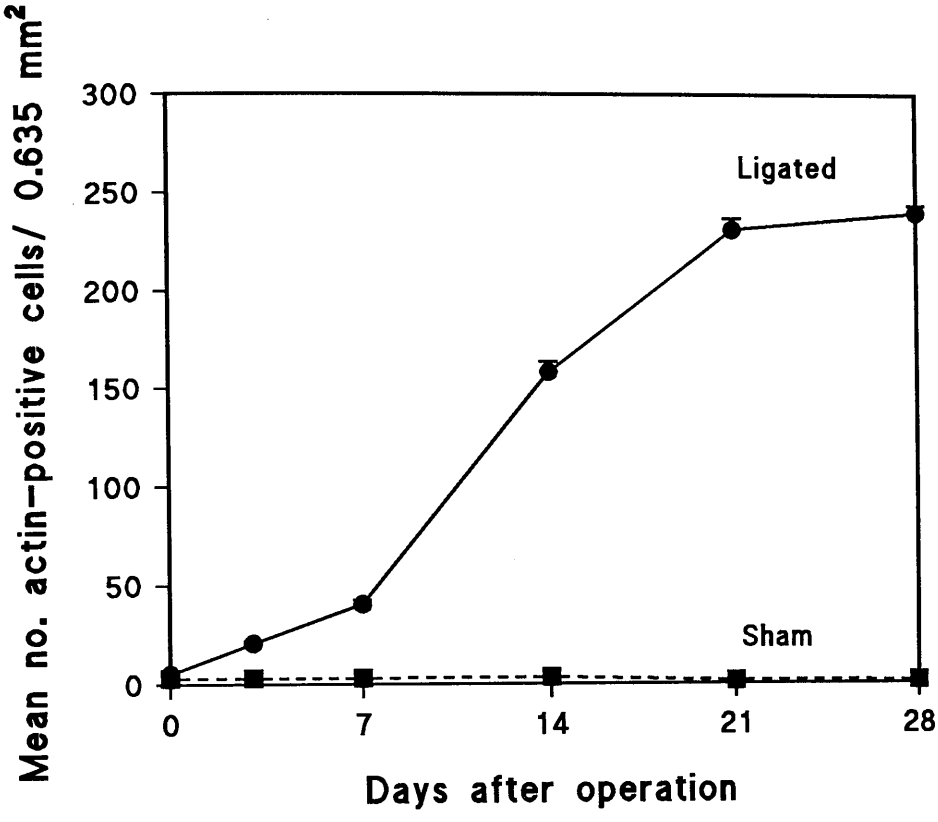


Figure 6.16 Quantitation of α (smooth muscle) actin-positive cells following common bile duct ligation.

Actin-positive cell numbers were assessed as described for desmin-positive cells. This graph illustrates the results obtained in random fields with a comparison between those found in bile duct-ligated animals with those of sham operated rats. Significantly greater actin-positive cell numbers were found in bile duct-ligated animals at day 7 ($p < 0.05$) and at all subsequent time points ($p < 0.001$). It should be noted that in day 21 and 28 bile duct-ligated animals, the absolute numbers of actin-positive cells were greater than that of desmin-positive cells (see Figure 6.13).


Mean no. actin-positive cells/ 0.635 mm²
300
250
200
150
100
50
0
Ligated
Sham
0
7
14
21
28
Days after operation

liver disease (Tsukamoto *et al*, 1990) and may, in the initial phases, be reversible (Abdel-Aziz *et al*, 1990), in the present study the changes were sufficiently advanced to produce portal hypertension in day 28 animals as evidenced by the presence of splenomegaly and ascites. Histologically, there was marked fibrosis and nodular regeneration.

My initial study of CCl_4-induced injury was the first to apply immunolocalization of desmin to quantify the fat-storing cell response to experimental injury. The results presented in this chapter confirm my earlier observations with the demonstration of a rapid expansion of the desmin-positive cell population within 48 hours of administration of the toxin. The absolute values and magnitude of the increase obtained in the present study were both higher than in the earlier report; this is likely to reflect increased sensitivity of the method used. This early increase in desmin-positive cells occurs shortly before collagen gene transcription increases. As in the previous study, comparison of cell counts obtained from perivenular and periportal fields confirmed the visual impression that the fat-storing cell response was centered upon the area of injury, which in the carbon tetrachloride model is acinar zone III (perivenular zone). A significant increase was, however, noted in day 2 animals when periportal fields were assessed; it is possible that this may in part represent overlap of fields contributed to by early bridging necrosis. The peak of fat-storing cell population expansion occured between 72 and 96 hours, thereafter falling towards normal. It is worth noting however, that although the histological appearances had returned to normal by day 7 there were still increased numbers of desmin-positive cells present in perivenular zones. Two other groups have confirmed the results I have presented in the carbon tetrachloride model. Yokoi *et al* (1988) also noted a rapid expansion of this cell population and demonstrated that this was predominantly seen in perivenular zones. Ramadori *et al* (1990) have, more recently, also described fat-storing cell population expansion following a single dose regimen, although these investigators made no attempt to quantify the response.

There have been several other studies which have investigated the fat-storing cell response to acute injury using desmin-immunolabelling. Ogawa *et al* (1986) used anti-desmin antibody to study the role of fat-storing cells in the healing of focal liver injury induced by the insertion of either a heated or liquid nitrogen-cooled 23 gauge needle. They demonstrated an increase in the number of desmin-positive cells in areas of necrosis during the first five days following

injury. Immunoreactivity was noted to be more intense in these cells compared to fat-storing cells in normal animals. This was in keeping with their earlier observations in vitamin A-loaded animals that auto-fluorescent fat-storing cells accumulated in response to focal liver injury (Ogawa *et al*, 1985). Kuroda *et al* (1989) also described accumulation of desmin-positive fat-storing cells following injection of 99.5% ethanol into the liver parenchyma of rats. Increased cell numbers were noted as early as 24 hours after injury with an apparent peak at 72 hours. While these studies are further evidence of a rapid fat-storing cell response to injury, neither Ogawa *et al* (1986) nor Kuroda *et al* (1989) presented quantitative data. Furthermore, such models of localized injury are of questionable relevance to human disease apart, perhaps, from in the healing of partial hepatectomy wounds ! Of greater relevance is the study of Jonker *et al* (1990) who used a single dose of D-galactosamine hydrochloride to produce another form of acute chemically-induced damage. The morphological changes seen after administration of this compound differ from those following carbon tetrachloride. Whereas with carbon tetrachloride there in zonal necrosis, parenchymal cell injury occurs throughout the acinus with galactosamine. Furthermore, there is a substantial lymphocytic infiltrate involving parenchyma and portal tracts, which is not observed after carbon tetrachloride. These changes are similar to those found in some human drug-induced hepatitides in which there are thought to be immunological mechanisms. Male Wistar rats were given 500mg/kg galactosamine hydrochloride intraperitoneally and tissue removed from animals killed at 6 hourly intervals up to 72 hours, and from those killed at 7 and 21 days following administration of the compound. Desmin-positive fat-storing cells were said to increase over time with maximum numbers observed at 48 to 72 hours. Although the authors indicate in their paper that cell counts were performed using a similar protocol to that of the present study, no absolute values were presented. Nevertheless, their illustrations of abundant desmin-positive fat-storing cells within areas of parenchymal cell necrosis at 48 hours closely resemble the appearances noted in the present study. The kinetics of the response to both toxins therefore, appears to be broadly similar.

Several groups have more recently used desmin-immunolocalization to study fat-storing cell population kinetics in models of chronic injury leading to fibrosis and cirrhosis. Yokoi *et al* (1988) used intraperitoneal injections of heterologous (swine) serum to induce hepatic fibrosis

in rats. In this model, desmin-positive cell numbers did not increase until 8 weeks of bi-weekly injections; this coincided with development of early fibrous septa seen histologically. Using a similar model, Ballardini *et al* (1988) also documented progressive expansion in the size of the desmin-positive fat-storing cell population. However, in contrast to Yokoi *et al*, they found this to occur as early as three weeks, following just five injections. It must be noted that as with many of these studies, Ballardini *et al* (1988) failed to provide quantitative data. This is a feature of two other studies in which alternative models of chronic chemically-induced liver injury were used to study the fat-storing cell response during fibrogenesis. Jézéquel *et al* (1990) used low-dose dimethylnitrosamine to induce cirrhosis in Sprague-Dawley rats. This group had previously shown that administration of 10μl/kg body weight by intraperitoneal injection thrice weekly produces a micronodular cirrhosis after only 3 weeks, the histological appearances of which closely resemble end-stage alcoholic cirrhosis in humans (Jézéquel *et al*, 1989). After nine injections, there was a marked increase in hydroxyproline in the liver and a concomitant increase in the amounts of collagen types I and III demonstrated immunohistochemically. The authors indicate that "prominent desmin-positive littoral cells were scattered in the parenchyma" of these cirrhotic animals. Ramadori *et al* (1990) also included in their study, a model of hepatic fibrosis using chronic exposure to carbon tetrachloride. Rats were given low doses of the toxin by gavage weekly for up to ten weeks. Although this produces fibrosis with formation of septa, it is said to be less consistent in inducing cirrhosis than repeated subcutaneous doses of the toxin (Tsukamoto *et al*, 1990). Ramadori *et al* (1990) suggested that desmin-positive fat-storing cells accumulated within septa in fibrotic animals but did not describe the kinetics of this response. Dijkhuis *et al* (1989) also examined the fat-storing cell response in a model of chronic granulomatous liver injury. Rats were given intravenous injections of particulate pig intestinal alkaline phosphatase. After two weeks, this resulted in the appearance of multiple granulomas throughout the liver parenchyma; some of these were associated with surrounding fibrosis. Desmin-positive cells were seen to form part of the developing granulomas. Rather than count desmin-positive cell numbers, this group assessed surface density of immunoreactivity, demonstrating a significant increase in desmin-positivity after 2 weeks with a peak at 12 weeks.

Although Milani *et al* (1990b) described the presence of desmin-positive cells in their model of biliary cirrhosis, the present study is the first to quantify the response of these cells

to cholestatic injury. As with the acute model, expansion occurred early, a significant increase in desmin-positive cell numbers being noted as early as 7 days after bile duct ligation. In contrast to the carbon tetrachloride model, these cells were initially seen within periportal zones. This further supports the concept that these cells accumulate principally within areas of parenchymal cell injury, as it is at this site that there is maximal cholate-stasis-related damage in biliary obstruction (MacSween & Burt, 1989). As there is persistent injury in this chronic model, one might expect continued expansion of the desmin-positive fat-storing cell population. While this was observed until day 14, there was a subsequent fall in cell numbers, albeit not as dramatically as that seen in the acute model. The reasons for this are uncertain but may relate to phenotypic modulation of the cells to desmin-negative cells (vide infra).

There are several possible reasons to explain the expansion of the desmin-positive fat-storing cell population noted in both animal models in the present study, and in the other studies quoted above. First, in response to injury fat-storing cells may show enhanced expression of desmin. It is possible therefore, that fat-storing cells whose desmin content was below the threshold for detection by immunohistochemistry may then become immunoreactive. Second, their accumulation may, in part, reflect parenchmal cell loss with collapse of the perivenular hepatic architecture. Migration of fat-storing cells from elsewhere in the acini is also possible although in my studies of acute liver injury there was no reduction in desmin-positive cells in periportal zones to support this. In fact, statistical analysis showed a significant increase in this zone at day 2, although it is likely that this represents field overlap into perivenular zones resulting from the parenchymal collapse. Finally, it seemed likely that local proliferation of fat-storing cells may contribute to expansion of the cell population. Some support for this comes from the original study of McGee & Patrick (1972) who demonstrated incorporation of radio-labelled thymidine into non-parenchymal cells in areas of necrosis. The autoradiographic method used, however, did not permit accurate identification of the proliferating cells; it could not be excluded therefore that thymidine uptake was occuring exclusively within macrophages (or other inflammatory cells). Enzan (1985) repeated the experiments of McGee & Patrick, confirming their observations in acute carbon tetrachloride-induced injury in mice. In addition, however, localization of incorporated [^{3}H]-thymidine was studied at the ultrastructural level. Many of the labelled cells in areas of injury were found to have electron microscopic features

of fat-storing cells. Further support for their local proliferation in response to injury was presented by Jézéquel *et al* (1989) who identified mitotic figures within fat-storing cells by transmission electron microscopy in rat liver during the development of dimethylnitrosamine-induced fibrosis. None of these studies, however, could be used to quantify the proliferation in these cells.

In the present study, evidence of fat-storing cell proliferation was sought using a novel double labelling method which included the use of BrdU to detect cells in S phase of the cell cycle. Paolucci *et al* (1990) previously used immunolocalization of incorporated BrdU to study proliferation in parenchymal and non-parenchymal cells following chronic exposure to dimethylnitrosamine in rats. The overall numbers of cells in S phase increased up to five-fold during the first week of the experiment and remained elevated thereafter; at all time points non-parenchymal cell labelling indices were greater in non-parenchymal cells than parenchymal cells. In their study, however, no attempt was made to identify proliferative activity within any particular population of sinusoidal cell.

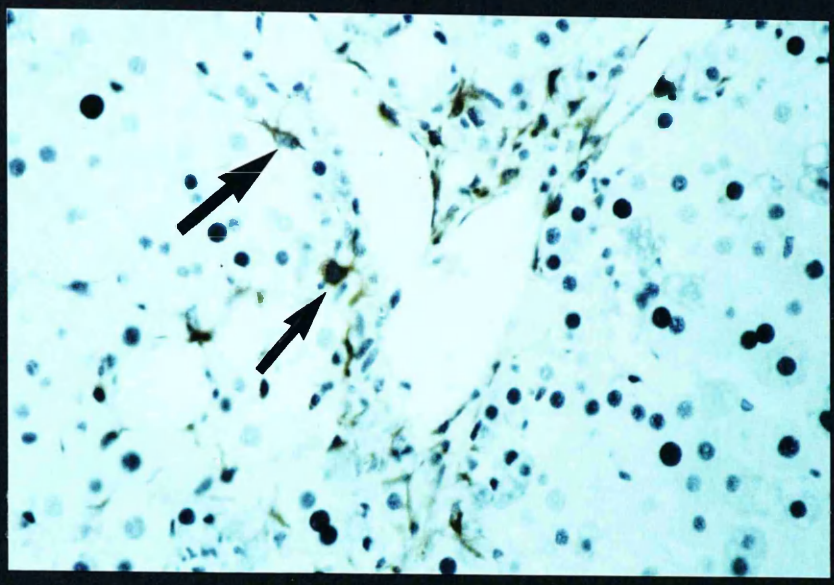
Tanaka *et al* (1990) described a method which combined the immunolocalization of incorporated BrdU with simultaneous demonstration of cytoplasmic desmin to identify proliferating fat-storing cells. This group applied the technique to a study of fat-storing cell kinetics during liver regeneration following partial hepatectomy in rats. Their method, however, could only be applied to unfixed frozen tissue. As noted earlier, cryostat sections are more liable to show crush artefacts, precluding accurate quantitative studies. Furthermore, in my own laboratory we have been unable to reproduce their findings using an identical protocol. For this reason, a method for immunolocalization of proliferating fat-storing cells in fixed, paraffin-embedded material was developed. The protocol outlined above provides a reproducible means of detecting fat-storing cells in S phase. Several different methods were initially assessed including variations of the order of immunostaining for desmin and BrdU, mode of DNA denaturation and choice of chromagen. The method described was found to give the most consistent results. Although there was a theoretical risk that nickel from the nickel-enhanced DAB used for BrdU immunolocaliastion may be released and bind to the DAB reaction product of the desmin-immunolocalization step, this did not appear to occur. The black reaction product of the nickel-enhanced DAB could clearly be distinguished from the russet brown of standard

DAB. Furthermore, immunoreactivity for BrdU and desmin could be further distinguished by their site of localization; staining for BrdU was restricted to the nuclei of cells and desmin to the cytoplasm. The peak of proliferative activity appeared to occur in the acute model at 48 hours; this preceded the peak in desmin-positive cell numbers by 24 hours. In the chronic cholestatic model, an early peak in proliferative activity was also noted, again preceding the peak in cell numbers. These observations strongly suggest that local cell porliferation contributes to expansion of the fat-storing cell population in response to both acute and chronic injury. Further evidence of local proliferation comes from double labelling experiments using an antibody to proliferating cell nuclear antigen (PCNA)/cyclin in combination with anti-desmin (Figure 6.17). PCNA is a 36 kD polypeptide which has been identified as an auxiliary protein of DNA polymerase. It is expressed throughout the cell cycle but is undetectable in quiescent cells, making it another useful marker for proliferating cells in tissue sections (Hall *et al*, 1990). However, the double labelling experiments alone cannot be used to study the rate of cells entering or leaving the cell cycle. Further kinetic studies using stathmokinetic methods (Geerts *et al*, 1988) are therefore required to establish whether local proliferation alone can account for the increased numbers of fat-storing cells following injury. Such studies may also provide information on the fate of desmin-positive cells during resolution of acute injury. As we have seen, cell counts fell dramatically after 4 days. While this may in part result from "deactivation" with reduction in the amount of desmin present in cells to that below the level which is detectable by our immunohistochemical method, it seems likely that there may be dropout of cells by apoptosis as has been described for myofibroblasts in granulation tissue (Darby *et al*, 1990).

In the present study, immunolocalization of α (smooth muscle) actin was used to investigate phenotypic modulation of fat-storing cells in response to injury. In their study of focal liver injury, Ogawa *et al* (1986) described an increased number of actin-positive cells around necrotic areas. It should be noted, however, that their antibody was raised against chicken actin and recognized all isoforms of the protein. In their illustrations it appears that all sinusoidal cells were positive. Ballardini *et al* (1988) used an alternative approach for the demonstration of actin in experimental liver injury. Their method was based on the specific binding of tetramethylrhodinyl-phalloidin to filamentous actin. Actin-positive cells were identified

Figure 6.17 Immunolocalization of proliferating fat-storing cells in rat liver: simultaneous demonstration of proliferating cell nuclear antigen (PCNA)/cyclin and desmin.

In this preparation from a day 2 carbon tetrachloride-injured rat liver, double labelling for PCNA and desmin has been achieved using a similar method to that outlined for BrdU/desmin double labelling. With this approach however, no DNA denaturation step is required. In this micrograph, several cells show nuclear immunoreactivity for PCNA and cytoplasmic positivity for desmin (arrows). Numerous PCNA-positive parenchymal cell nuclei can also be seen. As PCNA/cyclin is expressed throughout the cell cycle rather than being S-phase restricted as is BrdU labelling, a greater proportion of desmin-positive cells show double labelling than in BrdU/desmin labelling studies. (monoclonal antibodies PC10 (Novocastra Laboratories, England) and D33; light haematoxylin counterstain; indirect immunoperoxidase; x 250).



in fibrous septa in rats exposed to repeated intraperitoneal injections of heterologous (swine) serum, although no quantitative data were presented. It should be noted, however, that phalloidin does not specifically bind to muscle-associated forms of actin. Indeed, in their paper they illustrate strong staining of bile canaliculi using this reagent. I have demonstrated that in both acute and chronic injury, α (smooth muscle) actin-positive cells are seen in areas of injury. These cells have identical morphological appearances to those of desmin-positive fat-storing cells. In carbon tetrachloride treated animals, the kinetics of the α (smooth muscle) actin response was very similar to that noted for the desmin-positive cell population. The absolute values obtained were slightly smaller for α (smooth muscle) actin than desmin. In en-face labelling experiments many cells appeared to express both proteins, although some desmin-positive/α (smooth muscle) actin-negative cells were identified. These results strongly suggest that fat-storing cells do undergo phenotypic modulation in response to acute injury. However, given that a small population of α (smooth muscle) actin-positive cells was identified in perivenular zones of normal rat liver, I cannot entirely exclude the possibility that accumulation of desmin-positive/α (smooth muscle) actin-positive cells occurs due to expansion of this population rather than from phenotypic modulation of desmin-positive/α (smooth muscle) actin-negative cells.

The results of the present study are similar to those recently presented by Ramadori *et al* (1990). They also used 1A4 to immunolocalize α (smooth muscle) actin and reported that the protein could not be detected on sinusoidal cells of normal rat liver but α (smooth muscle) actin-positive cells accumulated in areas of necrosis in response to acute CCl_4-induced liver damage. Although absolute values were not provided, they suggested that α (smooth muscle) actin cells were less numerous than desmin-positive cells. However, in rats chronically exposed to the toxin α (smooth muscle) actin-positive cells continued to increase in number and intensity of immunoreactivity in fibrotic septa. In the common bile duct ligation model of the present study, continued expansion of the α (smooth muscle) actin-positive cell population was also seen. The kinetics of this response differed from that of desmin-positive cells in which there was a fall in cell numbers in later stages. Actin-positive cell numbers exceeded those of desmin-positive cells suggesting that there was a population of actin-positive/desmin-negative cells; this is supported by preliminary en-face immunolocalization studies (not shown). This

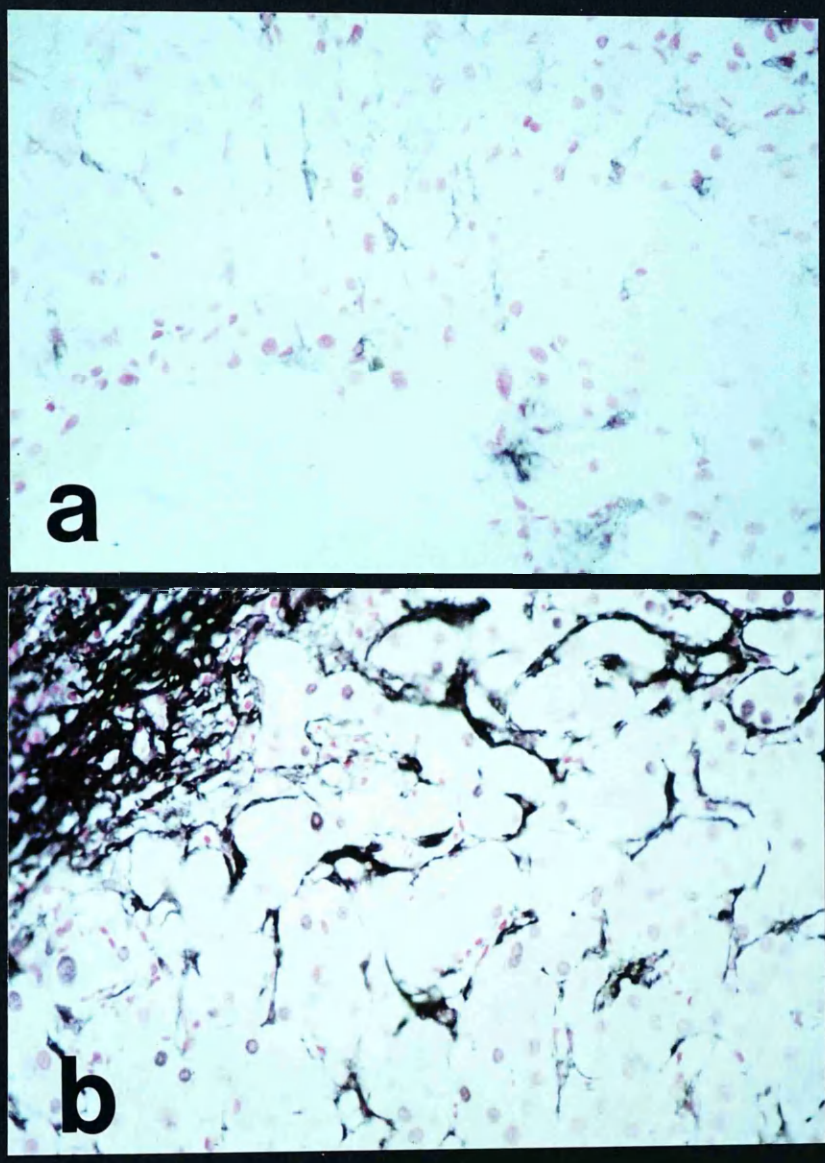
would be in keeping with the hypothesis that in chronic injury the phenotypic modulation involves a series of steps from desmin-positive/actin-negative fat-storing cells through "activated" transitional or myofibroblast-like cells (desmin-positive/actin-positive) to true myofibroblsts which are actin-positive/desmin-negative (Skalli & Gabbiani, 1988). However, again I could not entirely exclude the possibility that the actin-positive cell population expansion had resulted from proliferation of a pre-existing actin-positive/desmin-negative cell population present within portal tracts. In fact, the observation that actin-positive cell numbers exceeded those of desmin-positive cells, even in day 3 bile duct-ligated animals would suggest that this may occur and at least contribute to the findings. Further studies using double labelling for α (smooth muscle) actin and incorporated BrdU may help to resolve this issue.

Can a similar approach be used to study the response of human fat-storing cells to injury? As noted in chapter 5, desmin is only weakly expressed by a small number of these cells in normal human liver. I have recently used the monoclonal antibody D33 to study desmin-immunoreactivity in a small series of biopsies from patients with primary biliary cirrhosis. In those obtained from patients with stage IV disease (ie. established cirrhosis), occasional desmin-positive cells could be identified in fibrous septa. However, no increase in desmin-positive cell numbers could be observed in pre-cirrhotic biopsies. By contrast, in both pre-cirrhotic and cirrhotic cases, there appeared to be accumulation of α (smooth muscle) actin-positive cells (Figure 6.18). In stage II and III disease these were predominantly seen in periportal zones within areas of ductular proliferation. This observation is in accordance with the results of a study recently published by Schmitt-Gräff *et al* (1991) who demonstrated expansion of the α (smooth muscle) actin-positive cell population in various forms of chronic liver disease. Although their immunostained preparations were scored semi-quantitatively, no attempt was made to compare cell numbers in progressive disease. In my own unpublished study, frozen section material was also available from ten cases of primary biliary cirrhosis. This was used to study the response of PR2D3-immunoreactive cells to progressive liver injury. Increased numbers of these cells were seen in periportal zones in 6 cases in which there was established cirrhosis in parallel to an increase in α (smooth muscle) actin-positive cells (Figure 6.18). Further studies using PR2D3 and 1A4 are now planned to quantify the response at different stages of this progressive form of human fibrotic liver disease.

Figure 6.18 Immunohistochemical study of fat-storing cell response in human chronic biliary disease.

a. PR2D3 immunoreactive cells in primary biliary cirrhosis. Increased numbers of these cells were identified in periportal zones in pre-cirrhotic cases and in fibrous septa of cirrhotic livers. Unfortunately the poor resolution obtained in cryostat sections precludes any accurate quantitative analysis. (PR2D3; indirect immunoperoxidase; nickel/cobalt enhancement; nuclear red counterstain; x 200).

b. α (smooth muscle) actin-positive cells in stage 4 (cirrhotic) primary biliary cirrhosis. Abundant actin-positive cells can be seen here within the sinusoids close to a fibrous septa in which there is intense immunoreactivity. As the monoclonal antibody can be used on routinely processed paraffin-embedded tissue, quantitation of the α (smooth muscle) actin-positive cell response is feasible; these studies are currently in progress. (Monoclonal antibody 1A4; indirect immunoperoxidase method with nickel/cobalt enhancement; nuclear red counterstain; x 390).


a
b

In summary, the studies presented in this chapter have shown that in response to experimentally-induced acute and chronic liver injury in rats, there is expansion of the fat-storing cell population as evidenced by increased desmin-positive cell numbers. This in part results from local proliferation. In addition, evidence from both models supports the hypothesis that, in response to injury, fat-storing cells undergo phenotypic modulation towards myofibroblast-like cells with acquisition of α (smooth muscle) actin expression.

CHAPTER 7. INNERVATION OF THE HEPATIC SINUSOIDS : GUINEA-PIG AND RAT LIVER

7.1 INTRODUCTION

7.1.1 The autonomic nervous system: an overview

Control of many visceral functions is mediated by the autonomic nervous system (Appenzeller & Atkinson, 1985). The efferent portion of this system is classically divided into sympathetic and parasympathetic branches, each subserving different (and often antagonistic) functions (Burnstock, 1979). Although both possess pre- and post-ganglionic fibres, they differ in their anatomical origin and in their utilization of neurotransmitter substances. The pre-ganglionic fibres of the sympathetic system arise from the thoraco-lumbar spinal cord and use acetylcholine as neurotransmitter. Such fibres form synapses with post-ganglionic fibres in either the ganglia of the sympathetic chain or, less commonly, in ganglia within viscera. The post-ganglionic fibres (so-called adrenergic nerves) principally use noradrenaline or less commonly dopamine as transmitter. The parasympathetic supply to most internal organs comes via the vagus nerves. In contrast to the sympathetic system, synapses between pre- and post-ganglionic fibres invariably occur within the target organ; all parasympathetic fibres are cholinergic. The afferent (sensory) limb of the autonomic nervous system cannot be classified so readily as the efferent portion into its component parts; it is frequently categorized on the basis of function (eg. osmoreceptor, baroreceptor fibres) rather than anatomical or neurochemical considerations.

During the past decade it has become apparent that many autonomic nerves contain not only their classical neurotransmitters but also regulatory peptides such as neuropeptide tyrosine (NPY) and its C-flanking neuropeptide C-PON, calcitonin gene-related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide (VIP), enkephalin and bombesin (Hökfelt *et al*, 1981; Lundberg *et al*, 1983; Schultzberg & Dalsgaard, 1983; Costa & Furness, 1984; Gibbins *et al*, 1985; Allen *et al*, 1985; Julé *et al*, 1986); some of these peptides may act as co-transmitters. Furthermore, effector nerves that are peptidergic but neither adrenergic nor cholinergic are thought to exist (Sundler *et al*, 1986). NPY and C-PON are mainly found in sympathetic adrenergic fibres (Lundberg *et al*, 1983; Gulbenkian *et al*, 1985) whereas VIP is thought to be associated with parasympathetic cholinergic fibres. Within the peripheral nervous system,

substance P and CGRP are largely restricted to afferent sensory fibres (Gibbins *et al*, 1985; Lee *et al*, 1985; Franco-Cereceda *et al*, 1987).

7.1.2 Innervation of the liver

In most mammalian species, large nerve bundles can be identified at the hilum of the liver forming two separate but intercommunicating plexuses in close apposition to the portal vein and the hepatic artery. These bundles contain sympathetic fibres from T7 - 10 via the coeliac ganglia, and parasympathetic nerves from posterior and anterior vagi; in some species parasympathetic fibres may also be derived from the right phrenic nerve (Alexander, 1940; Lautt, 1980; Friedman, 1988). The nerve plexuses are also thought to contain afferent nerves, the central projections of which have been well documented in the rat using the technique of retrograde tracing (Carobi & Magni, 1981; Magni & Carobi, 1983; Rogers & Hermann, 1983).

A large number of physiological and pharmacological studies have indicated that the efferent innervation may play important roles in intrahepatic haemodynamic regulation (Greenway & Starke, 1971; Carniero & Donald, 1977), control of carbohydrate metabolism (Hartmann *et al*, 1982; Shimazu, 1983; Jungermann, 1988) and parenchymal cell regeneration (Ashrif *et al*, 1974; Kato & Shimazu, 1983) while the afferent supply may be involved in osmoreception, ionoreception and baroreception (Sawchenko & Friedman, 1979; Lautt, 1980). However, the intrahepatic distribution of the different nerve fibre types has been controversial.

Several early investigators used metal impregnation methods (gold, silver or osmium-based histochemical techniques) to study the distribution of intrahepatic fibres. Pflüger (1869) was the first to suggest that nerves may be present within the liver sinusoids, directly innervating parenchymal cells. When Kupffer (1876) stumbled upon his stellate cells using gold chloride, his initial intention had been to identify nerve fibres. In contrast to Pflüger, he and others (Nesterowsky, 1875, Holbrook, 1882) were unable to identify any intra-sinusoidal nerves. However, several investigators subsequently supported the observations of Pflüger (Macallum, 1887; Korolkow, 1893; Berkeley, 1893; Riegele, 1928; Tsai, 1958; Mikhail & Saleh, 1961; Skarring & Bierring, 1977). It is likely that these disparate results were due to lack of specificity of the methods used for the demonstration of nerve fibres; several of the metal

compounds applied also bind to extracellular matrix components. More convincing evidence in favour of the presence of intra-sinusoidal nerve fibres came from scanning (Skarring & Bierring, 1976; Akiyoshi & Ichihara, 1984) and transmission electron microscopic studies (Yamada, 1965; Forssmann & Ito, 1977; Ueno *et al*, 1987; Lafon *et al*, 1989; Bioulac-Sage *et al*, 1990). However, neither metal impregnation methods nor electron microscopy could establish the precise nature of such fibres.

Several groups used fluorescence histochemistry and enzyme histochemistry to identify intrahepatic adrenergic and cholinergic fibres respectively (Sutherland, 1964; Ungváry & Donáth, 1969; 1975; 1980; Anufriev *et al*, 1973; Satler *et al*, 1974; Skaaring & Bierring, 1976, 1977; Forssmann & Ito, 1977; Nobin *et al*, 1978; Mazzanti *et al*, 1977; Uno, 1977; Reilly *et al*, 1978; Metz & Forssmann, 1980; Järhult *et al*, 1980; Amenta *et al*, 1981; Fuller *et al*, 1981, Moghimzadeh *et al*, 1983; Kyösola *et al*, 1985; Akiyoshi, 1989). These approaches demonstrated the presence of both fibre types in a wide range of species but, as with the earlier metal impregnation studies, there was general disagreement about the existence of intra-sinusoidal nerve fibres. While this may in part be due to species differences, methodological problems are also likely to have contributed. In histochemical studies of cholinergic innervation for example, non-specific cholinesterase activity in non-neural structures including bile canaliculi and sinusoidal cells may have led to erroneous interpretations (Friedman, 1988).

The aim of the work presented in this chapter was to use a novel immunohistochemical approach for the identification of intra-sinusoidal nerves. Adrenergic fibres were demonstrated using antibodies to enzymes essential for the biosynthesis of dopamine and noradrenaline (tyrosine hydroxylase (TH) and dopamine β hydroxylase (DβH)) In addition, NPY-ergic fibres were localized using antibodies to synthetic NPY and its C-flanking peptide, C-PON; these peptides are commonly found within neurosecretory vesicles in adrenergic fibres. While these methods had been used to study sympathetic innervation of other tissues (Hartman, 1973; Gu *et al*, 1984; Polak & Bloom, 1984; Joh & Ross, 1986), they had not previously been applied to the investigation of hepatic innervation. Afferent sensory fibres were identified using antibodies to CGRP and substance P (vide supra). As earlier histochemical studies had suggested possible species differences in the intrahepatic distribution of nerves (Metz & Forssmann, 1980; Moghimzadeh *et al*, 1983), this immunohistochemical approach was applied

to both guinea-pig and rat liver. Finally, in order to further characterize nerve fibres identified by immunohistochemistry, the effects of chemical denervation using 6-hydroxydopamine, a compound which selectively destroys sympathetic fibres and the sensory neurotoxin, capsaicin (Buck & Burks, 1986) was assessed in rat liver.

7.2 MATERIALS AND METHODS

7.2.1 Guinea-pig and rat liver

The livers of male Dunkin Hartley guinea-pigs (n = 5; weight 600 to 800g) and male outbred Wistar rats (n = 11; weight 200 to 300g) were perfusion fixed *in situ* with either 2% paraformaldehyde (Fluka, Switzerland) in 0.1M phosphate buffer containing 2% sucrose or 0.4% parabenzoquinone in phosphate-buffered saline (PBS) using the low-pressure perfusion method of De Zanger & Wisse (1982).

The livers were removed and small tissue blocks ($3mm^3$) taken from the hilum, right and left hepatic lobes were further immersion-fixed in fresh fixative for 1 hour, washed extensively in 15% sucrose/PBS and frozen in liquid nitrogen. Five and 12-μm sections were cut on an American Optical microtome and mounted on albumin-coated glass slides.

7.2.2 Antibodies

Details of the primary antibodies used are outlined in Table 7.1; all were rabbit polyclonal antibodies. The anti-TH antibody was obtained from Institut Jacques Boy SA, Reims, France. The anti-DβH was a generous gift of Dr. Robert Rush, Flinders University, South Australia. The remaining antibodies were kindly provided by Dr. J.M. Polak, Royal Postgraduate Medical School, London. Specificity of the anti-regulatory peptide antibodies was assessed by competitive inhibition studies using purified natural or synthetic peptides (Burt *et al*, 1989); no cross-reactivity was noted between antibodies and inappropriate antigen. All antibodies were diluted in PBS containing 0.1% saponin (Sigma, Dorset, England).

	Antigen	Dilution	Reference
Tyrosine hydroxylase (TH)	Natural rat	1:400	Gu *et al* (1984)
Dopamine β-hydroxylase (DβH)	Natural bovine	1:200	Gu *et al* (1984)
Neuropeptide tyrosine (NPY)	Synthetic porcine	1:800	Gu *et al* (1984)
C terminal flanking peptide of NPY (C-PON)	Synthetic human	1:800	Allen *et al* (1985)
Calcitonin gene-related peptide (CGRP)	Synthetic rat	1:400	Gibson *et al* (1988)
Substance P	Synthetic human	1:800	Franco-Cereceda *et al* (1987)

Table 7.1. Details of primary antibodies used in study of innervation in guinea-pig and rat liver.

7.2.3 Immunohistochemistry : indirect immunofluorescence

Sections of guinea-pig and rat liver were (i) washed in PBS, (ii) incubated with 2% bovine serum albumin (Sigma) in PBS for 15 minutes, (iii) incubated with the primary antibodies for 16 hours at 4°C, (iv) washed in PBS, (v) incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Hoechst, United Kingdom; dilution 1:50 in 0.1% saponin/PBS), (vi) washed in PBS and (vii) mounted in glycerol/PBS (8:3 v/v). In all experiments negative controls were included. These consisted of sections incubated with either PBS or normal rabbit serum in place of primary antibody, or sections incubated with antibody which had previously been absorbed with the relevant antigen. The sections were then analyzed using a Leitz microscope with epifluorescence. All photographs were taken using Ilford E400ASA black and white film or Kodak 400 ASA colour film.

7.2.4 Chemical denervation studies

Three adult rats were each given intraperitoneal injections of three doses (50mg per kg body weight; total volume 0.5ml) of a freshly prepared solution of 6-hydroxydopamine (Sigma) in saline on Days 10, 6 and 1 prior to removal of the tissue. Liver blocks were processed from these animals as outlined above.

Neonatal (day 1) rats (n = 5) were given intraperitoneal injections of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide; Fluka) to destroy primary afferent neurons. These animals received a single dose (50mg per kg) of the drug emulsified in 10% ethanol, 10% Tween 80 and 80% saline. Control rats (n = 3) received vehicle alone. Liver tissue obtained from these animals at 12 weeks was processed as outlined above.

7.3 RESULTS

7.3.1 Guinea-pig liver: adrenergic and NPY-ergic fibres

The results of all immunolocalization studies are summarized in Table 7.2. Within all portal tracts from around the liver hilum, large nerve bundles were identified which contained TH-, DBH-, NPY- and C-PON-positive nerve fibres. Fibres were readily identified in both parabenzoquinone- and paraformaldehyde-fixed material, although the intensity of immunoreactivity was greater with the former. All illustrations in this chapter are of parabenzoquinone-fixed tissue. TH-positive fibres were more abundant than those containing DβH, NPY or C-PON (Figure 7.1a). Adrenergic and NPY-ergic fibres could be identified in large and medium sized portal tracts throughout the liver; no significant differences in distribution could be identified between right and left hepatic lobes. Within portal tracts the fibres were noted to be in close apposition to hepatic artery branches. They were identified less frequently around portal vein branches and were rarely seen in contact with bile duct radicles (Figure 7.1b).

Intra-sinusoidal fibres were identified using anti-TH, anti-DβH, anti-NPY and anti-C-PON antibodies (Figure 7.2). TH-positive fibres were again most abundant. The nerves were seen to extend along sinusoidal walls and occasionally interposed between parenchymal cells. Intra-sinusoidal fibres were most abundant in acinar zone I (periportal) and in some sections could be seen to be in continuity with portal tract fibres (Figure 7.2); the continuous nature of these fibres was most readily appreciated in thick (12μm) cryostat sections. However, substantial numbers of fibres were seen throughout the liver acini, including acinar zone III (perivenular zone). Furthermore, TH- and NPY-positive fibres could be identified within the walls of terminal hepatic vein radicles and larger hepatic veins (Figure 7.3a). At high magnifications, intra-sinusoidal fibres had a characteristic varicose appearance with focal bulbous accentuation of immunoreactivity (Figure 7.3b). No fluorescent fibres were seen in negative control sections, although autofluorescence within the elastic lamina of hepatic arteries, mast cells and collagen fibres in portal tracts was noted.

	TH	DβH	NPY	C-PON	CGRP	Sub P
Guinea pig						
Portal tracts	+++	++	++	++	+	+
Sinusoids	++	+	++	+	-	-
Hepatic veins	+	-	+	+	-	-
Rat						
Portal tracts	+++	+	++	++	+	+
Sinusoids	+[a]	-	+[a]	-	-	-
Hepatic veins	-	-	-	-	-	-

Table 7.2. Immunolocalization of nerve fibres in guinea-pig and rat liver

The number of fibres identified with each antibody has been expressed semi-quantitatively from - (none) to +++ (many).
[a] Periportal zones (acinar zone I) only.

Figure 7.1 Immunolocalization of tyrosine hydroxylase (TH)-positive nerve fibres in guinea-pig liver.

a. Immunoreactive fibres can be identified within nerve bundles (arrows) in this large portal tract from the hilum of the liver. (Indirect immunofluorescence; x 250).

b. Tyrosine hydroxylase-positive fibres can also be identified in close proximity to hepatic artery branches (A) within this large portal tract although there is no evidence of direct innervation of bile duct radicles (BD). (Indirect immunofluorescence; x 230).

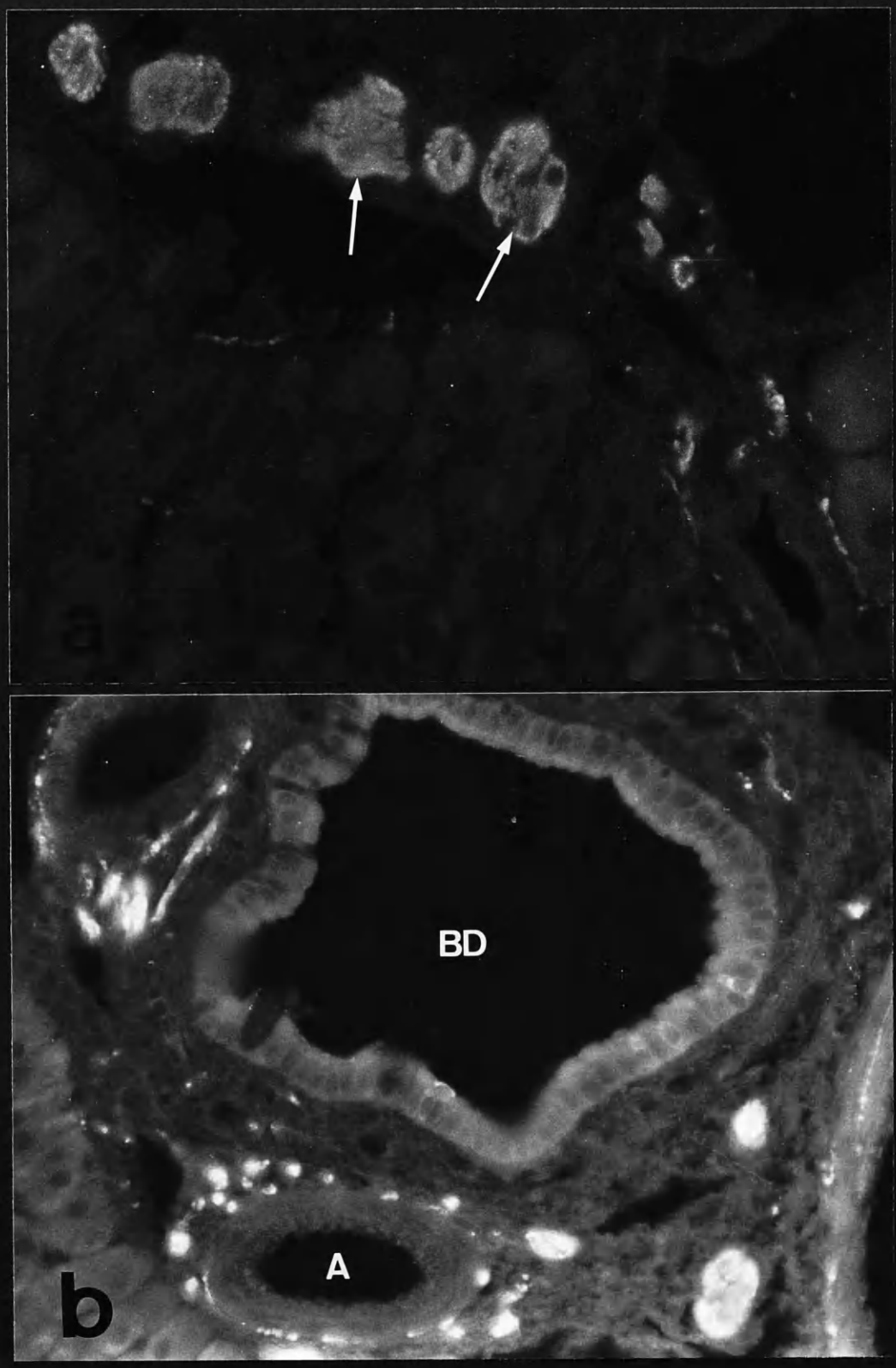

BD
A
b

Figure 7.2 Immunolocalization of neuropeptide tyrosine (NPY)-positive nerve fibres in guinea-pig liver.

NPY-containing fibres can be seen here throughout the liver parenchyma. The fibres were characterized by a varicose appearance (arrows). Fibres can be seen to extend from portal tracts (asterisk) and are most abundant in acinar zone I but can also be identified in acinar zones II and III. (Indirect immunofluorescence; x 130).

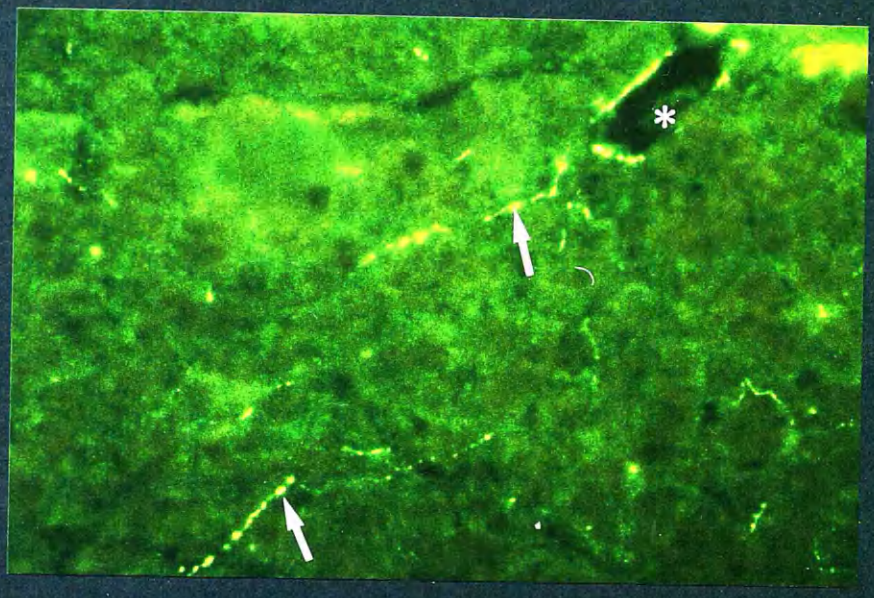
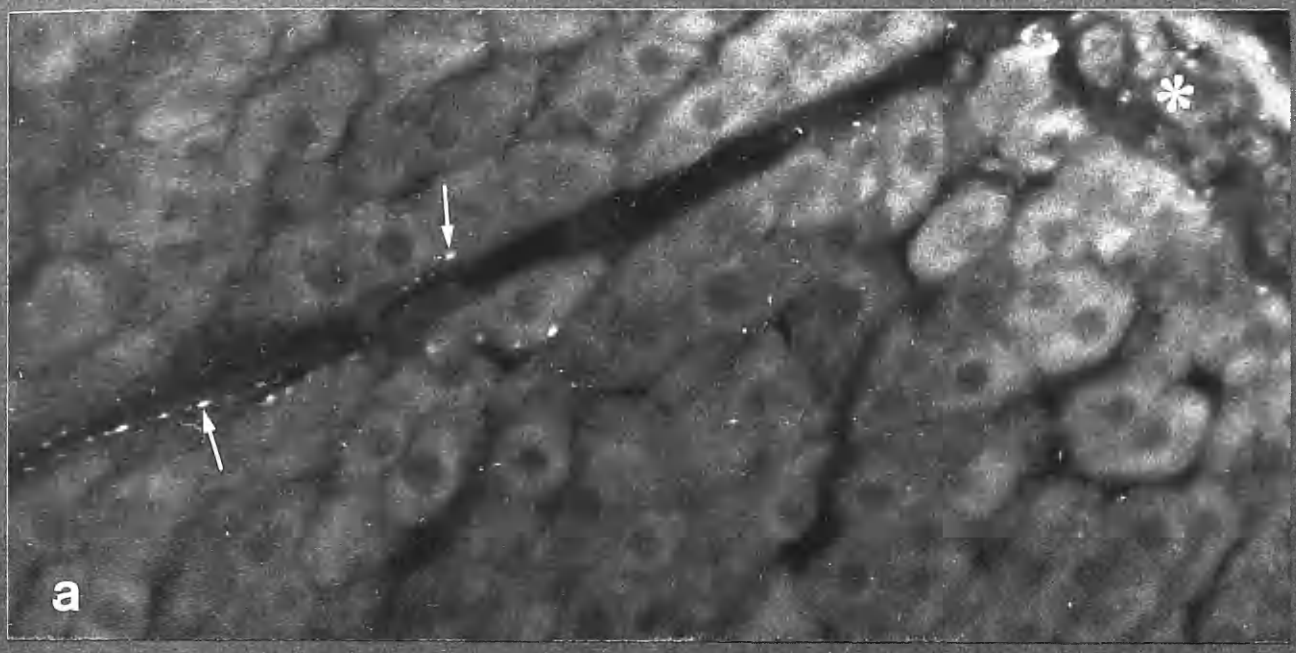
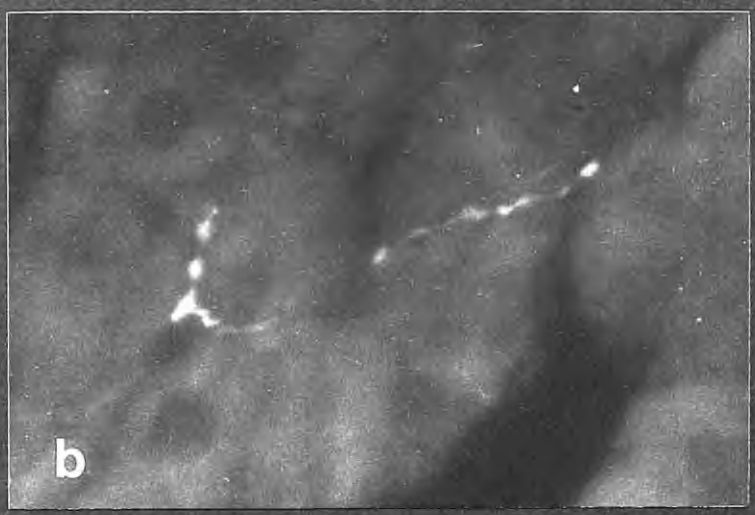


Figure 7.3 Immunolocalization of TH- and C-PON-positive nerve fibres in guinea-pig liver.

a. Fine fibres containing tyrosine hydroxylase can be identified within the walls of this terminal hepatic vein radicle (arrows). In addition, occasional immunoreactive fibres can be identified within small bundles in the walls of larger hepatic vein radicles (asterisk). (Indirect immunofluorescence; x 250).

b. Immunoreactive C-PON-containing fibres are present along sinusoidal walls and between parenchymal cells. This illustrates the typical varicose appearance. (Indirect immunofluorescence; x 500).


a


b

7.3.2 Guinea-pig liver: CGRP- and substance P-positive nerves.

CGRP- and substance P-containing fibres were also identified in portal tracts of all orders of magnitude (Figure 7.4). Although there was variation in the number of fibres present between different portal tracts, no consistent differences could be observed between those of right and left hepatic lobes. The distribution of the two fibre types was similar; examination of serial sections suggested co-localization of the two peptides within individual nerve fibres. These nerves were found in greatest abundance in blocks taken from around the hilum. However, within large nerve bundles they appeared to constitute a small proportion of total fibres (less than 15%) and were considerably less abundant than adrenergic/NPY-ergic fibres. Within portal tracts, the fibres were seen in close apposition to hepatic artery and portal vein branches; occasional CGRP- and substance P-positive fibres were noted around bile duct radicles.

In contrast to adrenergic/NPY-ergic fibres, no CGRP- or substance P-immunoreactive nerves could be identified within the sinusoids. No staining for either of these peptides was noted in hepatic vein walls.

7.3.3 Rat liver: adrenergic and NPY-ergic nerves

TH-, NPY- and C-PON-positive fibres could be identified within portal tracts of all orders of magnitude (Figure 7.5) where they were seen in close apposition to hepatic artery branches but only rarely in contact with portal vein branches or bile duct radicles. Occasional DβH-positive fibres were also noted but the intensity of immunoreactivity was less than that seen in guinea-pig liver; in many portal tracts DβH-positive fibres could not be identified. In contrast to guinea-pig liver, intra-sinusoidal adrenergic/NPY-ergic fibres were sparse and when present, were limited to acinar zone I (periportal). No fibres were seen within hepatic vein walls.

In livers from all animals treated with 6-hydroxydopamine, TH-, DβH-, NPY- and C-PON-immunoreactive nerves were entirely absent. However, in rats treated with neonatal

Figure 7.4 Immunolocalization of substance P- and CGRP-positive nerve fibres in guinea-pig liver.

a. Substance P-containing fibres can be identified within this portal tract around a hepatic artery branch (A). There are no fibres in apposition to bile duct radicles (BD). No sinusoidal substance P-positive fibres could be identified. (Indirect immunofluorescence; x 150).

b. CGRP-positive fibres, as shown here, were characterized by a varicose appearance and, as with substance P-positive fibres, were predominantly seen around hepatic artery branches. No CGRP-positive fibres were seen within the sinusoids. (Indirect immunofluorescence; x 450).

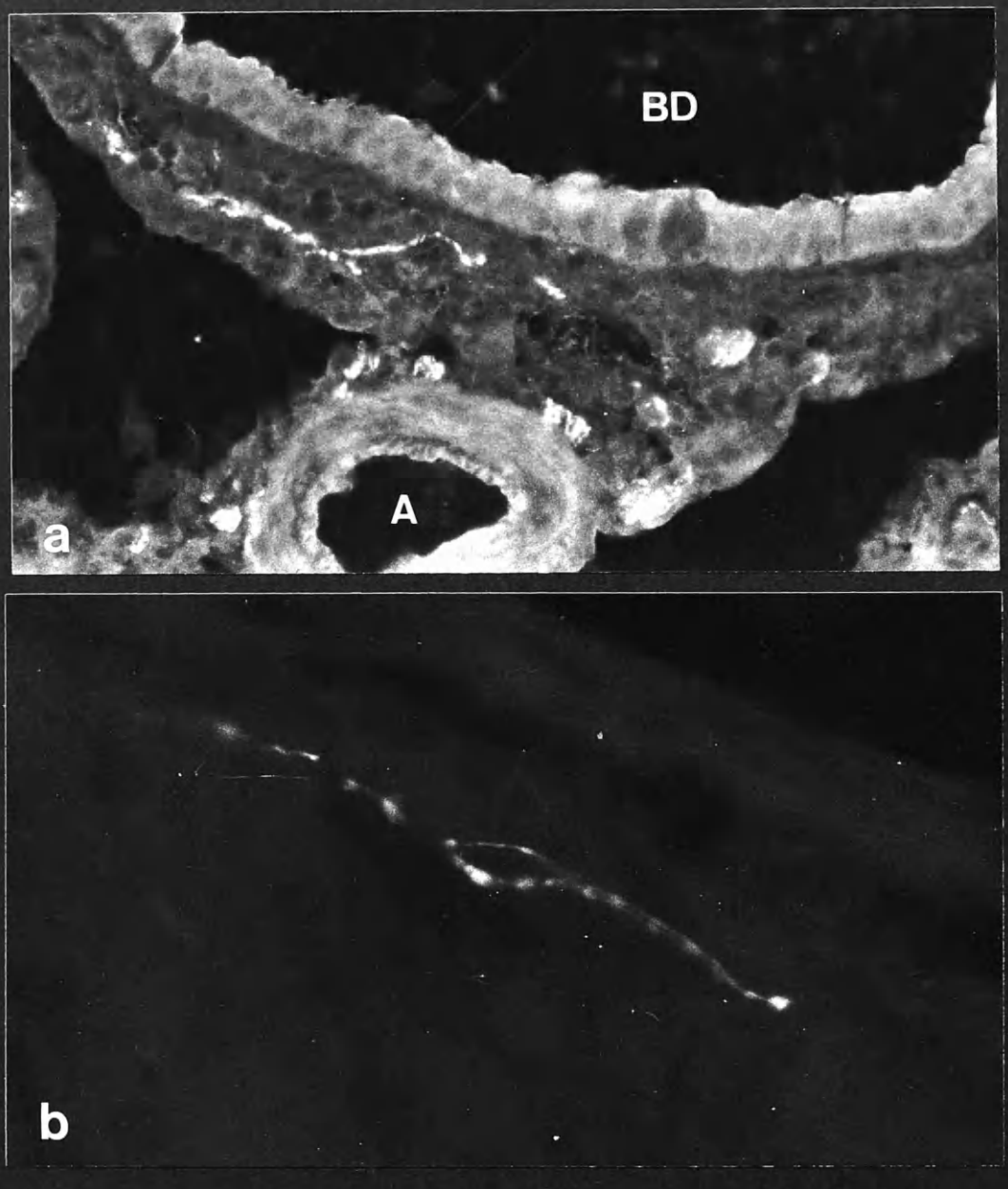

BD
A
a
b

Figure 7.5 Immunolocalization of TH- and NPY-positive nerve fibres in rat liver.

a. Tyrosine hydroxylase-positive fibres can be seen here within a portal tract. Fibres are found predominantly around hepatic artery branches (A). In contrast to guinea-pig liver, few fibres can be identified within the sinusoids; when present they were restricted to acinar zone I.

b. NPY-positive fibres are also present within this portal tract where again they are seen in close apposition to hepatic artery branches (A). (Indirect immunofluorescence; x 180).

A
a
A
b

capsaicin, adrenergic/NPY-ergic fibres were identified with a similar density and distribution to that seen in control animals.

7.3.4 Rat liver: CGRP- and substance P-positive nerves

Fibres immunoreactive for these regulatory peptides were identified in large and medium-sized portal tracts. The density of CGRP- and substance P-positive fibres was similar to that seen in guinea-pig liver. No intra-sinusoidal fibres could be identified. In three of the five animals treated with capsaicin, there was almost total abrogation of staining for CGRP and substance P. In the remaining two animals, there was a reduction in the number of fibres identified to less than approximately 40% of that seen in livers from normal animals or control rats treated with vehicle alone.

7.4 DISCUSSION

Most previous studies dealing with topography of sympathetic innervation in mammalian liver have used one of two fluorescence histochemical methods to identify adrenergic nerves: (i) the Falck-Hillarp technique (Falck *et al*, 1962) and (ii) the glyoxylic-acid technique (Lindvall & Bjorklund, 1974). Both methods are based on the condensation reaction between the aldehyde fixative and primary catecholamines (noradrenaline or dopamine) with the generation of highly fluorescent derivatives (6,7-dihydroxy-3,4-dihydroquinolines). Although it is possible to distinguish between noradrenaline and dopamine after treatment of sections with hydrochloric acid (Livett, 1971), none of the previous studies of hepatic innervation attempted to make this distinction. Both methods are accepted as being capricious (Kyösola *et al*, 1985); the signal obtained is evanescent. The catecholamine neurotransmitters are present within dense core vesicles which can be seen along the length of post-ganglionic sympathetic axons but which are found in greatest abundance within varicosities close to nerve terminals. The essential steps in catecholamine biosynthesis have long been established (Blaschko, 1973). Three enzymes are responsible for the synthesis of noradrenaline from L-tyrosine. The first of these, tyrosine hydroxylase (TH) is the rate-limiting enzyme which converts L-tyrosine to L-DOPA. Dopamine

is then formed from this molecule by the action of DOPA decarboxylase. Finally, dopamine β hydroxylase (DβH) catalyses the formation of noradrenaline from dopamine. DβH is the only biosynthetic enzyme present in the dense core granules (Blaschko, 1973). All three enzymes however, are synthesized within the neuronal cell bodies and transported to terminal varicosities by axoplasmic flow (Livett *et al*, 1969).

Ungváry and Donáth (1969) were the first to apply fluorescence histochemistry to study hepatic innervation[1]. Using the Falck-Hillarp technique, they identified catecholamine-containing nerve fibres within portal tracts of dogs, cats, guinea-pigs, rats and mice. However, they failed to demonstrate any intra-sinusoidal fibres even in animals pre-treated with the mono-amine oxidase inhibitor nialamide to enhance catecholamine accumulation in peripheral nerves. Skarring & Bierring (1976) were also unable to identify intra-sinusoidal sympathetic nerves in rat liver using both the Falck-Hillarp and glyoxylic acid methods. This was also the experience of Reilly *et al* (1978). However, Anufriev *et al* (1973) demonstrated adrenergic fibres along sinusoidal walls in guinea-pig liver, an observation subsequently confirmed by others (Metz & Forssmann, 1980; Fuller *et al*, 1981; Moghimzadeh *et al*, 1983; Akiyoshi, 1989). Intra-sinusoidal fibres were not identified in the livers of cats, dogs or rats. Such inter-species variation in the distribution of intrahepatic nerves was also confirmed by others (Metz & Forssmann, 1980; Moghimzadeh *et al*, 1983), although some discrepancies exist between the results of the various studies. Further evidence in favour of inter-species differences was provided by Moghimzadeh *et al* (1983) who demonstrated considerable variation in the levels of chemically detectable noradrenaline between different animals; the biochemical results correlated well with their histochemical obervations. It is likely that the different results obtained by various groups are in part due to inherent technical difficulties with the fluorescence histochemical methods. The specificity and sensitivity of the histochemical methods used in the previous studies have been questioned by some authors (Kyösola *et al*, 1985). In order to overcome such difficulties, I used an immunohistochemical technique to identify adrenergic nerves by the demonstration of fibres containing the catecholamine synthesis enzymes, TH and DβH. The results indicate that in both guinea-pig and rat liver, abundant adrenergic fibres are present within portal tracts. The

[1]Studies dealing with innervation of human liver are discussed in detail in Chapter 8.

observation that the majority of fibres within large nerve bundles of hilar portal tracts are TH-positive suggests that adrenergic nerves constitute the principal fibre type in mammalian liver. Adrenergic nerves were frequently observed in close apposition to hepatic artery branches but rarely around portal vein branches or bile ducts. This is in keeping with their proposed role in vasomotor control, but does not support the concept that adrenergic nerves may modulate the function of intrahepatic bile ducts (Knutz, 1953). The paucity of TH- or DβH-positive fibres around bile duct radicles is in accordance with the earlier fluorescence histochemical studies (Ungváry and Donáth, 1969; Anufriev *et al*, 1973; Reilly *et al*, 1978).

DβH-positive fibres were less numerous than TH-positive fibres in both species. Although I cannot exclude the possibility that these observations are related to differences in the characteristics of the two antibodies used, this may indicate that some fibres which contain TH do not contain DβH. Such fibres would be unable to synthesize noradrenaline but could be dopaminergic. Some support for this has come from a recent study by Bell and Mann (1990) who demonstrated fibres showing intense immunoreactivity for DOPA decarboxylase around hepatic arteries in human liver. Although this enzyme is required for the biosynthesis of both dopamine and noradrenaline, it is found most abundantly in dopaminergic fibres.

In keeping with the results of previous histochemical studies, I have shown that there is a difference between rat and guinea-pig in the degree of intra-sinusoidal innervation. In rat liver, only occasional periportal fibres could be identified. By contrast, in guinea-pig liver, TH-positive and DβH-positive fibres extended from the portal tracts into the parenchyma. Although they were most abundant in the periportal zone, they could also be identified in perivenular areas. The intra-sinusoidal fibres that were identified in the present study were morphologically similar to those described in fluorescence histochemical studies, being characterized by the presence of prominent varicosities along their lengths. These are thought to relate to bulbous swellings in which the neurosecretory granules are located. In guinea-pig liver, TH-positive fibres were also identified within the walls of terminal hepatic vein radicles. Furthermore, TH-positive nerve bundles were seen within the walls of larger interlobular hepatic vein branches. This observation may indicate that some adrenergic nerves enter the liver along with the vena cava and hepatic veins. In keeping with the experience of others (Priestley, 1988), the nerve

fibres were most readily identified in thick (12 μm) sections; this, however, has the disadvantage of rendering photomicroscopy difficult because of the varied plane of section.

Although NPY-positive nerves were identified in the extrahepatic biliary tract of guinea-pigs by Allen *et al* (1984), the present study was first to demonstrate the presence of intrahepatic nerves containing this peptide and the related C-flanking neuropeptide, C-PON (Burt *et al*, 1986c & d; 1989). NPY is a 36 amino acid peptide which was originally isolated from porcine brain using a novel method for identifying peptides with an amidated C-terminal tyrosine residue (Tatemoto *et al*, 1982) and subsequently found to be widely distributed in both central and peripheral nervous systems (Polak & Bloom, 1984; Gray & Morley, 1986). The molecule shows considerable homology with members of the pancreatic polypeptide family of regulatory peptides (Tatemoto, 1982). NPY has been shown to have direct vasoconstrictor properties but it may also act as a co-transmitter, potentiating the effects of other compounds such as noradrenaline (Gray & Morley, 1986). Previous immunohistochemical studies indicated that NPY is closely associated with the sympathetic branch of the autonomic nervous system and may be co-localized with noradrenaline in peripheral nerves (Lundberg *et al*, 1983). However, the possible existence of NPY-ergic fibres which do not contain TH, and are therefore not adrenergic, has been described (Carlei *et al*, 1985). The distribution of NPY-positive and C-PON-positive fibres in the liver closely resembled that of TH-positive fibres. Furthermore, preliminary experiments with immunofluorescence of step sections have indicated that TH and NPY can be co-localized within individual intra-sinusoidal fibres.

Two groups have now confirmed some of the findings of this study. Carlei *et al* (1988) demonstrated NPY-ergic nerves around vessels at the hilum of rat liver. However, no attempt was made to investigate intrahepatic innervation. Inone *et al* (1989) identified NPY-ergic fibres in and around the walls of vessels in portal tracts of rats but in accordance with my findings, found only limited numbers of intra-sinusoidal fibres in this species. They further demonstrated that immunoreactivity for NPY could be partially abolished by transection of splanchnic nerves just proximal to the coeliac ganglion although occasional NPY-ergic fibres remained. In my own experiments, NPY-ergic fibres were completely abolished by chemical sympathectomy (6-hydroxydopamine). Taking these observations together, this raises the possibility that some intrahepatic NPY-ergic fibres may be short post-ganglionic sympathetic fibres whose ganglia are

present at the liver hilum. In keeping with this hypothesis, Carlei *et al* (1988) identified NPY-positive ganglia in rat liver hilar tissue.

What is the functional significance of the intra-sinusoidal adrenergic and NPY-ergic fibres demonstrated in this study? It is possible that they may be involved in the regulation of intra-sinusoidal blood flow. Nerve stimulation experiments in a variety of animal species have demonstrated that sympathetic activation leads to vasoconstriction within the hepatic arterial and portal venous systems (Greenway & Stark, 1971; Carneiro & Donald, 1977); cholinergic innervation does not appear to play a role in control of hepatic vascular tone. Release of NPY from adrenergic/NPYergic nerves may contribute to the pressor response. Corder & Withrington (1988) have demonstrated that on a molar basis, NPY is a more potent vasoconstrictor of the dog hepatic arterial bed than noradrenaline although the maximal pressor effect produced is less than one-third of that seen with noradrenaline. There is evidence that NPY may potentiate the pressor effects of noradrenaline (Edvinsson *et al*, 1984).

In addition to effects on large vessels, electrical impulse experiments have also indicated that adrenergic nerve stimulation may constrict the sinusoids with resultant slowing of intra-sinusoidal blood flow. Reilly *et al* (1981) demonstrated using *in vivo* microscopy, that stimulation of rat coeliac ganglia led to constriction of hepatic arterioles and sinusoids, an effect which could be blocked pharmacologically by α-adrenergic antagonists. Others have demonstrated that vagal stimulation may have the opposite effect (Koo & Liang, 1977), indicating that sinusoidal dilatation may be mediated through parasympathetic nerves.

Physiological studies have also identified an important role for adrenergic nerves in the control of carbohydrate and lipid metabolism in parenchymal cells (reviewed by Jungermann, 1988). Electrical stimulation of splanchnic nerves in several species leads to hyperglycaemia (Edwards, 1972, Sannemann *et al*, 1986). Studies performed in the isolated perfused rat liver indicate that this is mediated through α-adrenergic control (Hartmann *et al*, 1982). The parasympathetic system provides a reciprocal innervation; in contrast to the sympathetic nerves, stimulation of the vagus nerves promotes glucose uptake and gluconeogenesis (Shimazu, 1983). Hansson *et al* (1985) demonstrated that surgical denervation (stripping of perivascular nerves at the hilum) of rat liver led to a reduction in hepatic lipase activity. Furthermore,

Jungermann's group have shown that stimulation of nerves at the liver hilum can inhibit fatty acid metabolism probably through an α-adrenergic effect (Beuers *et al*, 1986).

The afferent nerve supply to the liver is thought to subserve osmoreceptor, chemoreceptor, ionoreceptor, baroreceptor and "metabolic" receptor functions (Sawchenco & Friedman, 1979; Lautt, 1980). By communicating such information to the central nervous system, these nerves indirectly control glandular secretions, gastric function and renal function. For example, infusion of hypertonic solutions into the portal vein leads to a diuresis even when plasma osmolarity remains constant (Haberich, 1968). This effect appears to be mediated through stimulation of release of vasopressin by the posterior pituitary (Baertschi *et al*, 1985).

Carobi's group have demonstrated using retrograde tracing of horseradish peroxidase that hepatic afferent fibres travel with the vagus and splanchnic nerves and project centrally to the medial portion of the left solitary nucleus (Magni & Carobi, 1983). However, the intrahepatic distribution of sensory nerve terminals remains uncertain. In the present study, I have shown that in both guinea-pig and rat liver, nerve fibres containing the regulatory peptides CGRP and substance P are present around vessels within portal tracts but are not detectable within the sinusoids (Burt *et al*, 1987a). The observations in rat liver are in accordance with those of Sasaki *et al* (1984; 1986). In their initial study, this group (Sasaki *et al*, 1984) identified substance P-positive fibres in portal tracts of rat liver in close apposition to hepatic artery and portal vein branches; no immunoreactive fibres were found in the sinusoids. In a subsequent report (Sasaki *et al*, 1986), CGRP-positive fibres were identified with a similar distribution. Occasional branches however, were identified in acinar zone I sinusoids. As both peptides are commonly found in primary afferent neurons in the peripheral nervous system, the demonstration of CGRP- and substance P-positive fibres can be taken as presumptive evidence of the presence of sensory nerves in guinea-pig and rat liver. However, Gibson *et al* (1988) have recently shown that CGRP may also be present in a subpopulation of motor neurons, albeit at lower levels than in sensory nerves. Nevertheless, the chemical denervation studies performed in rats in the present study provide further evidence for the sensory nature of the at least some intrahepatic CGRP- and substance P-positive fibres. Prior treatment of the animals with 6-hydroxydopamine had no effect whereas administration of capsaicin, a specific sensory neurotoxin, resulted in almost total abrogation of immunoreactivity for CGRP and

substance P in three animals. The less marked response noted in the remaining animals may represent an inadequate dosage schedule.

Goehler *et al* (1988) have also investigated the distribution of CGRP- and substance P-containing fibres in guinea-pig liver. In accordance with the results of the present study, fibres were identified in portal tracts around hepatic artery and portal vein branches. However, these investigators also described close contact with bile duct radicles. No intra-sinusoidal nerve fibres were identified. Double labelling experiments using a monoclonal anti-substance P antibody and a polyclonal anti-CGRP antibody with FITC and rhodamine-conjugated second layer antibodies, demonstrated co-localization of these peptides within almost all fibres in the liver. As in the present study, chemical denervation studies were not performed in this species. Although protocols are described for capsaicin treatment of guinea-pigs, adminstration is associated with severe systemic effects (hypotension, convulsions) and must be given under controlled anaesthesia. Surgical denervation studies could yield similar information and seems more feasible for future study.

In summary, this study has demonstrated that in guinea-pig liver but not rat liver the sinusoids contain a rich adrenergic/NPY-ergic nerve supply. There is no evidence however, that afferent fibres are present at this site.

CHAPTER 8. INNERVATION OF THE HUMAN HEPATIC SINUSOIDS: ALTERATIONS IN DISEASE

8.1 INTRODUCTION

There have been relatively few previous studies dealing with innervation of the human liver. Intra-sinusoidal nerves have been described in several transmission electron microscopic studies (Nobin *et al*, 1978; Ueno *et al*, 1987; Lafon *et al*, 1989; Bioulac-Sage *et al*, 1990). Fibres were identified within the space of Disse and within recesses between parenchymal cells; some were accompanied by a surrounding Schwann cell. In all of these studies, close contact was noted between nerve fibres and fat-storing cells or parenchymal cells; points of contact frequently involved nerve varicosities within which dense-core vesicles could be seen. Bioulac-Sage *et al* (1990) have suggested than indentations of fat-storing cells or parenchymal cells at these sites represent synaptic clefts, although no membrane specializations have been identified on either fat-storing cells or parenchymal cells. Nobin *et al* (1978) also reported close contacts between nerves and Kupffer cells but this was refuted by others (Bioulac-Sage *et al*, 1990).

Fluorescence histochemistry has been used in a few studies to identify adrenergic nerves in human liver (Falck *et al*, 1975; Forssmann & Ito, 1977; Moghimzadeh *et al*, 1983; Kyösola *et al*, 1985). Falck *et al* (1975) were the first to suggest that there may be a rich sympathetic innervation of the human sinusoids. This was substantiated in further studies by the same group (Nobin *et al*, 1978; Moghimzadeh *et al*, 1983) in which the histochemical data were supported by chemical measurements of noradrenaline showing high concentrations in human liver. In addition, they demonstrated uptake of the dopamine analogue, 5-hydroxydopamine in nerve fibres at the ultrastructural level, a property previously shown to be restricted to sympathetic nerves. In their comparative study, Moghimzadeh *et al* (1983) showed that the sinusoids of humans and other primates were as densely innervated as those of the guinea-pig. In contrast to the results of these studies, however, Kyösola *et al* (1985) were unable to identify intra-sinusoidal nerves in human liver using the Falck-Hillarp technique. Amenta *et al* (1981) demonstrated acetylcholinesterase-containing fibres in human liver using the method of Karnovsky & Roots. Fibres were identified within portal tracts and thoughout the parenchyma, along sinusoidal walls. The distribution of fibres was unaltered after *in vitro* incubation with 6-hydroxydopamine leading to the suggestion that these represented cholinergic parasympathetic fibres.

In view of the lack of specificity of these histochemical procedures (vide supra), a preliminary study was carried out using an immunohistochemical approach to identify intrahepatic nerves in normal human liver. TH-, DBH-, NPY-, C-PON-, CGRP- and substance P-positive fibres could be identified using the indirect immunofluorescence method described in Chapter 7. However, difficulties were experienced with autofluorescence of lipofuscin in 12μm cryostat sections and fibres were less easily identified than in either guinea-pig or rat liver. Nerves could also be identified using peroxidase-antiperoxidase (Burt *et al*, 1986c) but this appeared to detect only nerve varicosites (Figure 8.1) and could not be used to reliably assess the extent of intra-sinusoidal innveration.

An alternative approach was therefore used to examine the distribution of intrahepatic nerve fibres. Instead of using antibodies to either cathecholamine-synthesizing enzymes or neuropeptides, both of which are predominantly localized to the granules of nerve fibres, antibodies to constitutive cytosolic proteins of nerve fibres (neurofilament (Bishop *et al*, 1985), neuron specific enolase (NSE) (Kato *et al*, 1982), protein gene product 9.5 (PGP 9.5) (Wilson *et al*, 1988)) and their supporting Schwann cells (S-100 protein) (Vanstapel *et al*, 1986) were applied. The antibodies were used initially to examine the distribution of fibres in normal adult human liver. These studies indicated that intrahepatic nerves could be more readily identified using anti-PGP 9.5 and anti-S-100 antibodies than with the remaining two. Anti-PGP 9.5 and anti-S-100 were subsequently used to study (i) ontogenesis of human liver innervation and (ii) the distribution of intrahepatic nerves in various forms of liver disease.

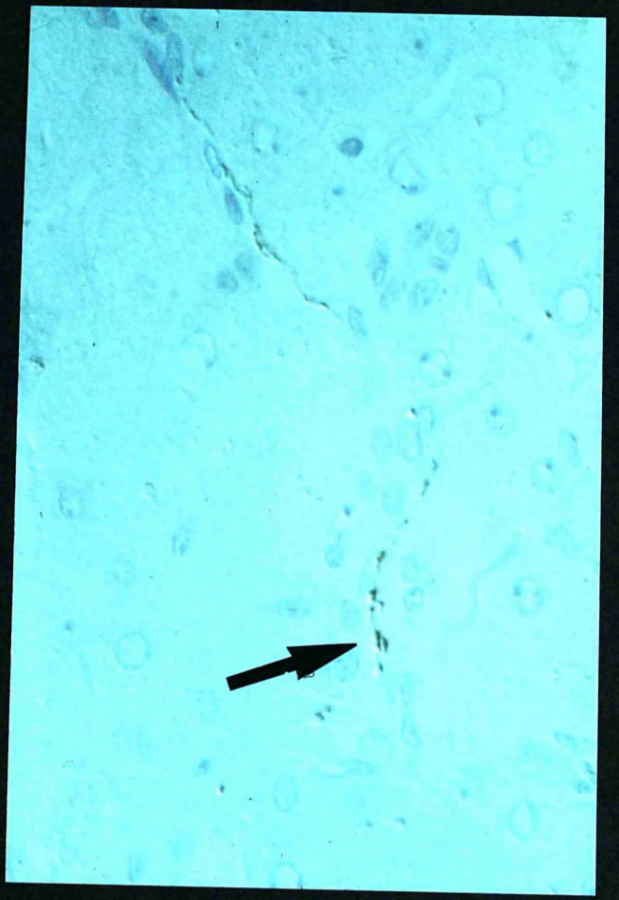
8.2 MATERIALS AND METHODS

8.2.1 Liver tissue

Normal human liver was obtained from cadaveric renal transplant donors as outlined in Chapter 3. Wedges were perfusion-fixed through vascular orifaces at the cut surface and processed for paraffin wax-embedding. Material was also obtained at laparotomy from patients (n=59) with suspected liver disease. This tissue was fixed in 10% buffered formalin and processed for paraffin wax-embedding. Of this group, eight were considered to have either

Figure 8.1 Immunolocalization of NPY-positive nerves in human liver.

Occasional NPY-containing fibres can be identified at the periphery of this small portal tract (arrow) and within the sinusoids of acinar zone I. (Indirect immunoperoxidase method; haematoxylin counterstain; photographed under Nomarski filters; x 450).



normal liver or non-specific reactive changes only. The histological diagnoses in the remaining cases are outlined in Table 8.1.

In order to study ontogenesis of hepatic innervation, formalin-fixed paraffin-embedded liver tissue collected from fetal, perinatal and neonatal autopsies were obtained from the files of the Division of Pathology, Royal Victoria Infirmary (n=20; gestational ages 14 weeks - term and term - 6 months).

8.2.2 Antibodies and immunohistochemical methods

Details of the primary antibodies used in this study are given in Table 8.2. An indirect immunoperoxidase method (Appendix 4) was used with the monoclonal anti-neurofilament antibody and a peroxidase-antiperoxidase technique (Appendix 2) with all other antibodies. 3,3' diaminobenzidine was used as the chromagen; in some experiments nickel/cobalt enhancement was also used (Appendix 5). In all experiments, negative control sections were included in which normal mouse or rabbit serum was used in place of the primary antibody or PBS in place of the second layer antibody. No staining was observed in these negative controls.

8.2.3 Quantitative analysis

PGP 9.5-immunostained sections from cases showing normal or non-specific reactive changes (n=5), acute liver injury (intrahepatic cholestasis or extrahepatic biliary obstruction (n=8)) and established cirrhosis were used to assess intra-sinusoidal nerve fibre density by image analysis (Joyce Loebl Genias Magiscan, version 3.2). Images were made of representative parenchymal fields and a light pen was used to highlight intra-sinusoidal fibres. The total length of fibres (μm) was calculated by the image analyser and divided by the area of the image (μm^2) to give intra-sinusoidal nerve fibre density (μm^{-1}). Results illustrated in Figure 8.10 represent the means of ten or more representative fields from each case.

Diagnosis	Number of Cases
Normal/non-specific reactive changes	8
Acute liver injury	
Extrahepatic biliary obstruction	6
Acute hepatitis	2
Intrahepatic cholestasis	5
Pre-cirrhotic chronic liver disease	
Alcoholic hepatitis with perivenular fibrosis	6
Primary biliary cirrhosis stage I or II	5
Established cirrhosis	
Alcoholic cirrhosis	6
Primary biliary cirrhosis	7
Haemochromatosis	3
Autoimmune liver disease	2
Cryptogenic cirrhosis	4
Malignant tumour (hepatocellular carcinoma; cholangiocarcinoma)	4
Amyloidosis	1

Table 8.1 Histological diagnosis in wedge biopsies used in study.

	Antibody type	Dilution	Source	Reference
Neuron-specific enolase (NSE)	Rabbit polyclonal	1:200	Dakopatts, Denmark	Bishop *et al* (1985)
S-100 protein	Rabbit polyclonal	1:500	Dakopatts, Denmark	Lauriola *et al* (1984)
Protein gene product 9.5 (PGP 9.5)	Rabbit polyclonal	1:20	Ultraclone, UK	Wilson *et al* (1988)
Neurofilament	Mouse monoclonal	1:250	Labsystems, Helsinki	Trojanowski *et al* (1984)

Table 8.2 Details of primary antibodies used in study of innervation of human liver.

8.3 RESULTS

8.3.1 Distribution of nerve fibres in normal liver

Immunoreactive nerve fibres were identified within portal tracts using all four antibodies (Figures 8.2, 8.3 and 8.5). They were most frequently seen in close apposition to hepatic artery branches and portal vein branches and only rarely around bile duct radicles. The intensity of staining was considerably greater with anti-PGP 9.5 and anti-S-100 than with anti-NSE or anti-neurofilament. PGP 9.5-positive and S-100-positive fibres were also seen within the parenchyma and were frequently observed along sinusoidal walls (Figures 8.4 and 8.5). Intra-sinusoidal fibres could not, however, be identified using either anti-NSE or anti-neurofilament antibodies. Intra-sinusoidal PGP 9.5-positive fibres were more abundant than S-100-positive nerves. At high power magnifications, PGP 9.5 and S-100-positive fibres could be seen within the space of Disse; branches were also observed within recesses between hepatocytes. Although the intra-sinusoidal fibres were more abundant in acinar zone I (periportal), they were also frequently observed in zones II and III, and within the walls of terminal hepatic veins and larger order hepatic vein radicles.

8.3.2 Ontogenesis of hepatic innervation

Nerves could be identified in fetal liver as early as 14 weeks gestation. Fibres immunoreactive for NSE, PGP 9.5 and S-100 protein were present in portal tracts (Figure 8.6). No neurofilament-positive fibres could be found. In fetuses of less than 30 weeks, nerves could only be identified within large portal tracts. However, in those of later gestational age, occasional fibres could also be seen in smaller portal tracts. As in adult liver, fibres were mainly noted in close proximity to vessels; no evidence was found for innervation of bile ducts or ductal plate structures (MacSween & Burt, 1989). In three pre-term perinatal autopsy cases (33, 35 and 36 weeks gestation), occasional PGP 9.5-positive fibres were found in acinar zone I. However, intra-sinusoidal fibres could not be identified with the remaining antibodies and were not seen in any other fetuses. Intra-sinusoidal PGP 9.5-positive fibres were seen in three

Figure 8.2 Immunohistochemical demonstration of nerves within normal human liver.

a. Immunoreactivity for neuron specific enolase (NSE). Strong labelling can be identified in fibres within large nerve bundles in this portal tract. Few NSE-positive fibres could be identified within the sinusoids. (Peroxidase-antiperoxidase method; haematoxylin counterstain; x 250).

b. Immunolocalization of neurofilament. Only occasional fibres could be identified within portal tracts using the anti-neurofilament monoclonal antibody. These were principally seen in nickel-enhanced immunohistochemical preparations and were noted within nerve bundles in large portal tracts (arrows). (Indirect immunoperoxidase method with nickel/cobalt enhancement; haematoxylin counterstain; x 180).

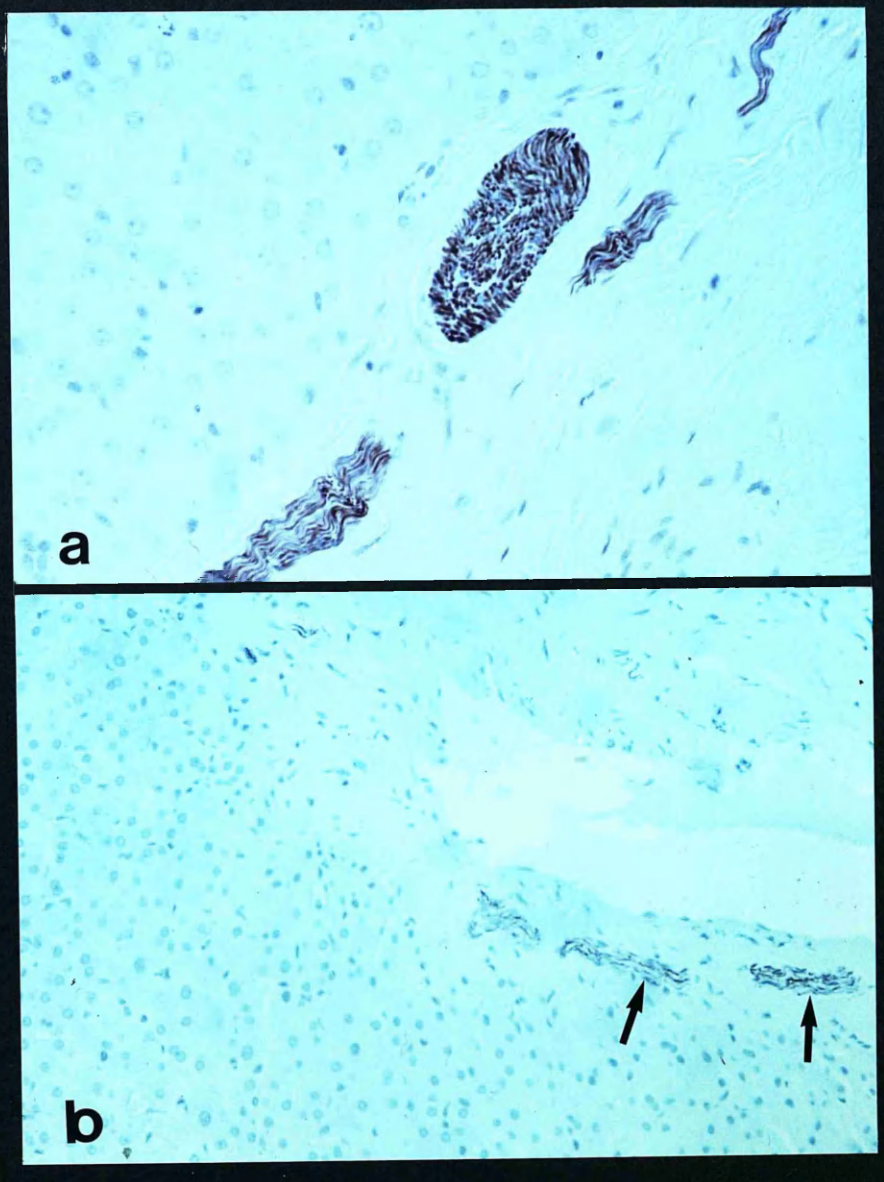

a
b

Figure 8.3 Immunolocalization of PGP 9.5-positive nerves in human liver.

a. PGP 9.5-immunoreactive fibres can be seen in medium and large nerve bundles within this portal tract in a normal human liver. (Peroxidase-antiperoxidase method with nickel/cobalt enhancement; haematoxylin counterstain; x130).

b. Immunoreactivity for PGP 9.5 can also be seen within smaller fibres in portal tracts. Some of these fibres extend into the periportal sinusoids (acinar zone I). (Peroxidase-antiperoxidase method with nickel/cobalt enhancement; haematoxylin counterstain; x 240).

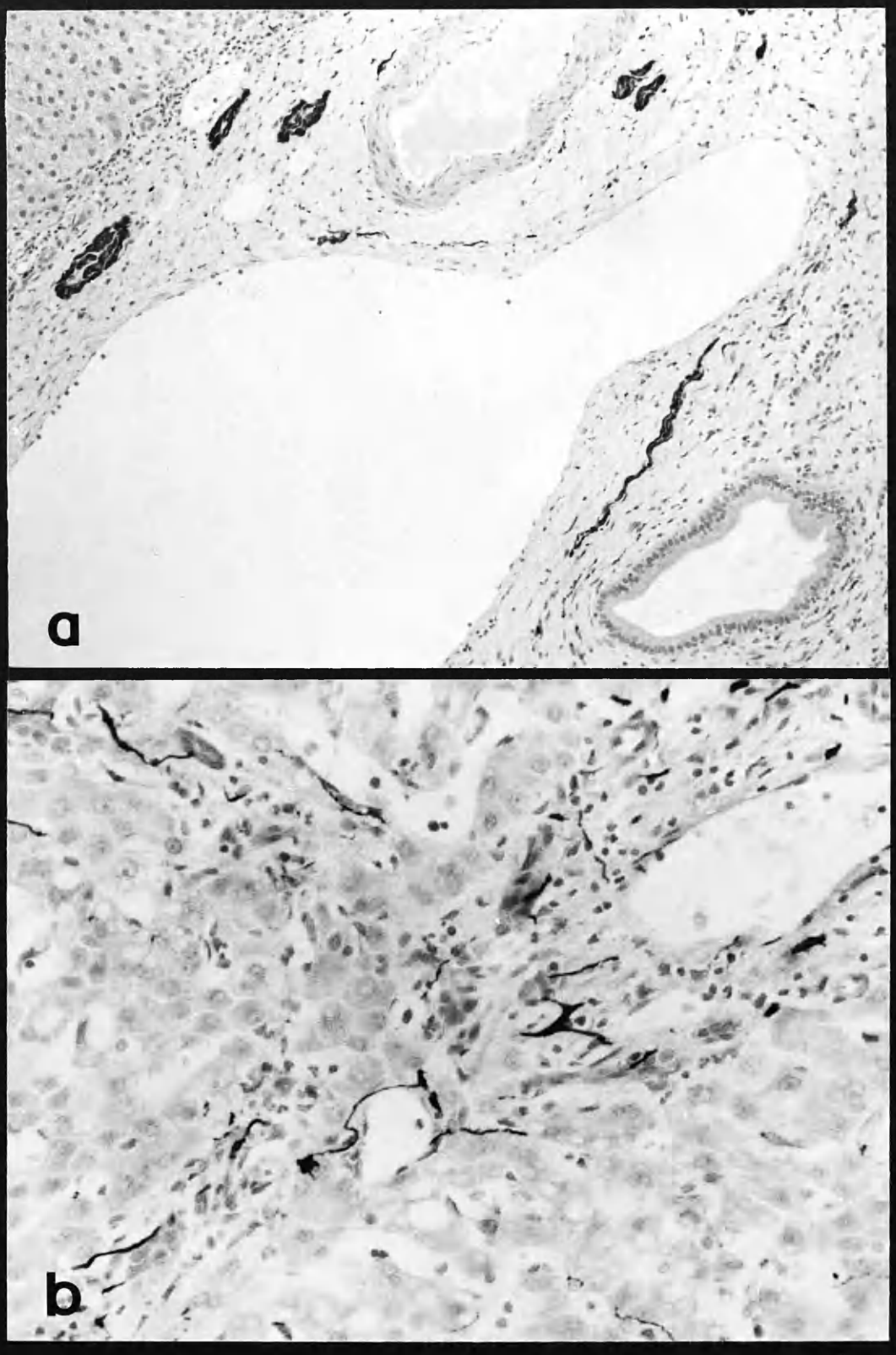

a
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Figure 8.4 Immunolocalization of PGP 9.5-positive nerves within sinusoids of normal human liver.

a. Intense labelling for PGP 9.5 can be identified within nerve fibres which course along the hepatic sinusoids; occasional branches interdigitate between parenchymal cells. (Peroxidase-antiperoxidase method with nickel/cobalt enhancement; haematoxylin counterstain; x 600).

b. PGP 9.5 immunoreactive fibres were predominantly seen in acinar zone I but were also observed in acinar zones II and III. A fibre can be seen here within the sinusoids adjacent to a terminal hepatic vein radicle (HV). (Peroxidase-antiperoxidase method with nickel/cobalt enhancement; haematoxylin counterstain; x 720).

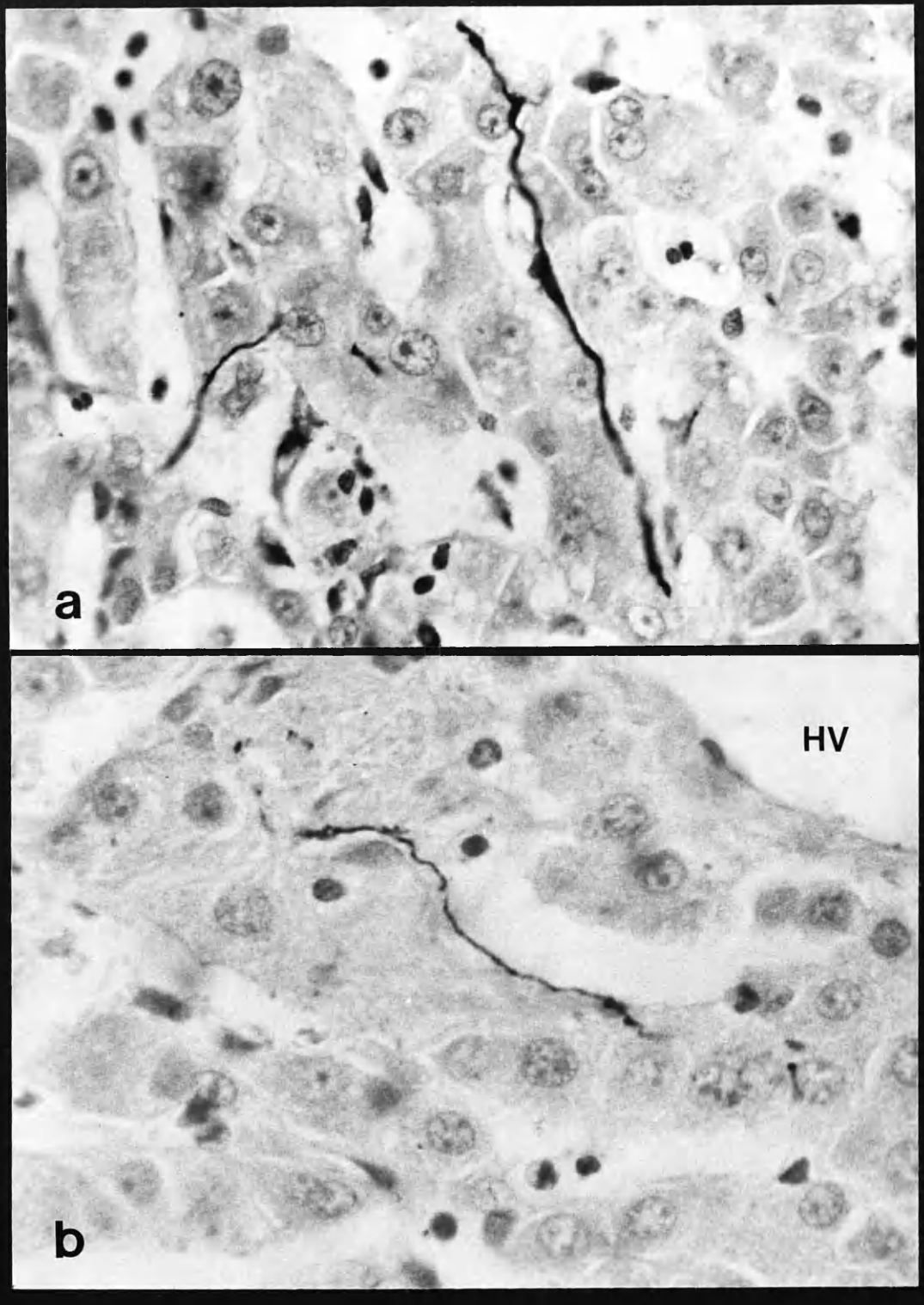

a
HV
b

Figure 8.5 Immunolocalization of S-100 protein-positive nerve fibres in normal human liver.

Fibres containing S-100 protein were identified within portal tracts and within sinusoids in normal human liver. The number of S-100-positive fibres was less than that seen for PGP 9.5. Immunoreactive fibres were generally thicker than those seen with anti-PGP 9.5 and occasional cell bodies could be seen to be positive with this antibody. (Peroxidase-antiperoxidase method; haematoxylin counterstain; x 550).

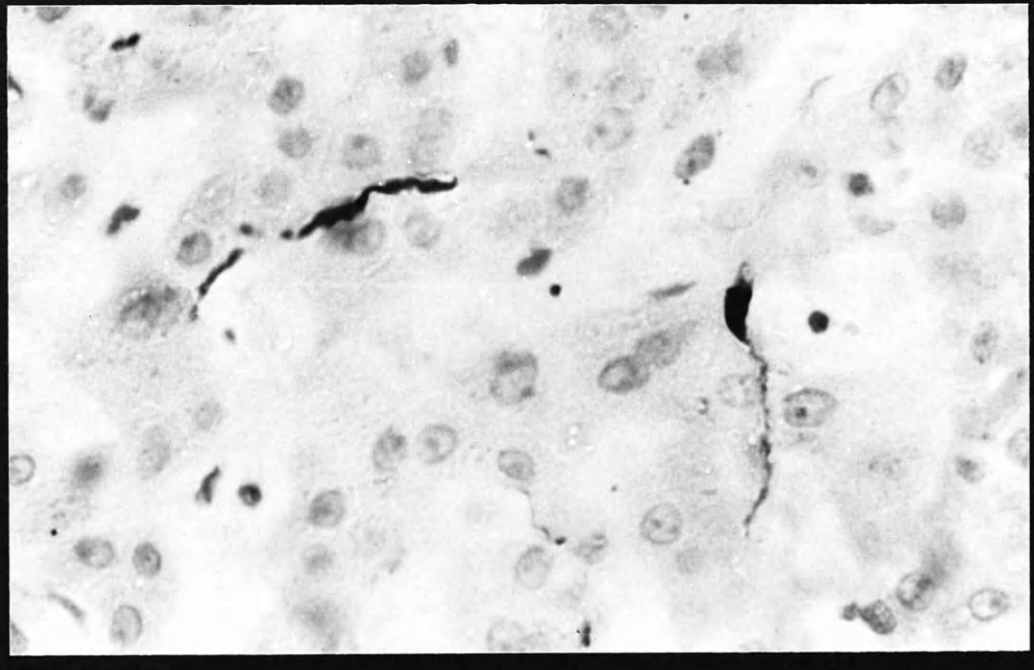
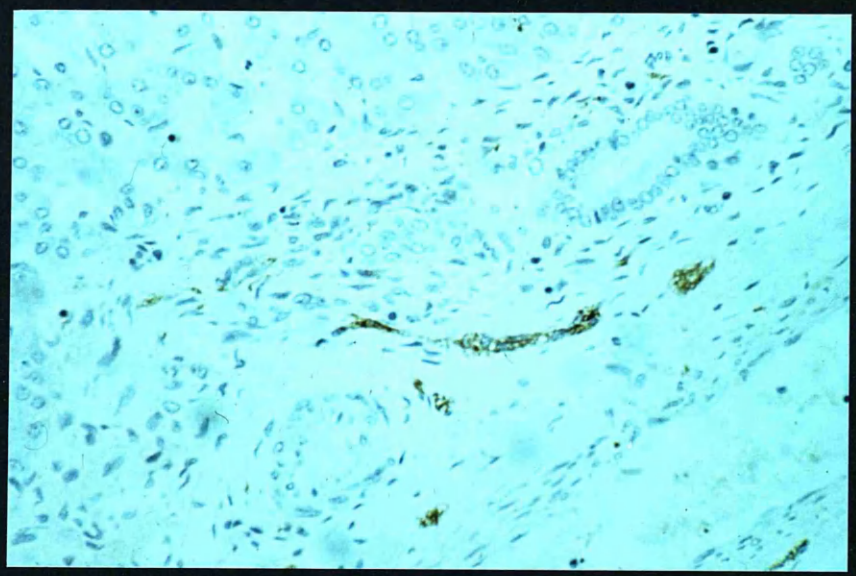


Figure 8.6 Identification of nerve fibres in the developing human liver.

In this photomicrograph, PGP 9.5-positive fibres can be identified within a portal tract in the liver of a 32 week old fetus. Only very occasional PGP 9.5-immunoreactive fibres could be identified within the sinusoids in fetal livers and when present were entirely restricted to the immediate periportal zones (acinar zone I). (Peroxidase-antiperoxidase method; haematoxylin counterstain; x 200).



neonatal autopsy specimens and in one 6 month-old baby, although the density was less than that observed in adult liver.

8.3.3 Alterations of hepatic innervation in liver disease

In wedge biopsies showing either non-specific reactive changes or acute cholestatic liver injury, the density and distribution of PGP 9.5- and S-100-immunoreactive fibres was indistiguishable from that seen in normal liver (Figure 8.7). In the cases of acute hepatitis studied, there was an apparent increase in the density of fibres but in each of these there was significant bridging hepatic necrosis.

In pre-cirrhotic alcoholic liver disease and in stage I or II (ie. pre-cirrhotic) primary biliary cirrhosis, there was a reduction in the number of intra-sinusoidal nerve fibres identified; in two cases of alcoholic hepatitis and three cases of early primary biliary cirrhosis, only occasional fibres could be identified and these were restricted to acinar zone I (periportal zone). In the remaining six cases of pre-cirrhotic chronic liver disease, parenchymal fibres could be identified but were much less abundant than in normal liver. However, the most dramatic change was seen in cases with established cirrhosis. Irrespective of the aetiology of cirrhosis, whether due to alcohol, primary biliary cirrhosis, haemochromatosis or autoimmune chronic liver disease, nerve fibres were absent from regenerative nodules in all but three cases. However, in each case, PGP 9.5- and S-100-immunoreactive fibres could be identified within fibrous septa. Indeed, in many cases nerve fibres were particularly prominent in areas of fibrosis (Figures 8.8 and 8.9).

Of the four cases studied in which there was intrahepatic malignant tumour, two had primary hepatocellular carcinoma; the remaining two had cholangiocarcinoma. In none of these cases was there any evidence of innervation of tumour tissue. However, fibres could be seen in the adjacent liver, both within portal tracts and along sinusoidal walls. In the single case of amyloidosis studied, PGP 9.5-positive fibres could be identified between amyloid deposits in the space of Disse although fibres could not be identified using anti-S-100 antibody (Figure 8.11).

Results of the preliminary quantitative analysis of intra-sinusoidal nerve fibre density in 25 cases stained with anti-PGP 9.5 are illustrated in Figure 8.10. It can be seen that there is

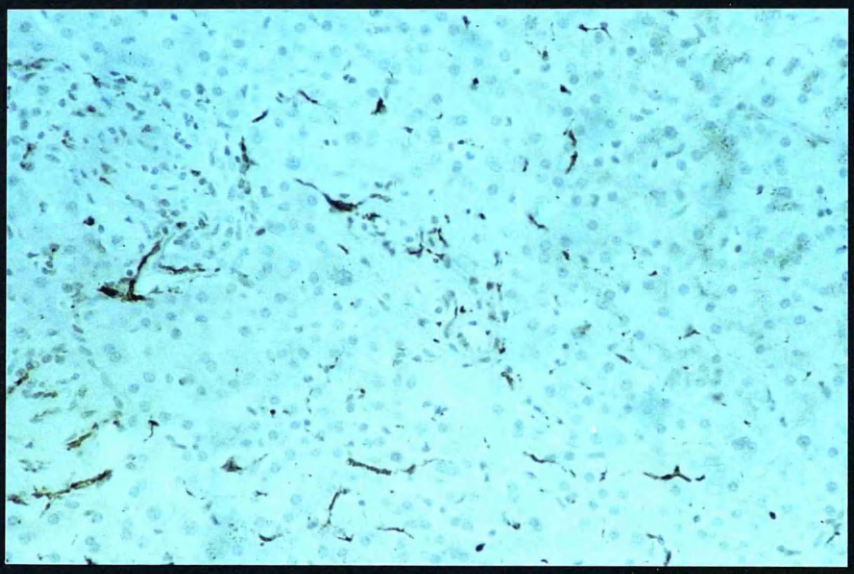


Figure 8.8 Immunolocalization of PGP 9.5-positive nerve fibres in chronic liver disease.

In this case of established cryptogenic cirrhosis, PGP 9.5-immunoreactive fibres can be seen within a residual portal tract surrounding hepatic artery branches. However no fibres are present within a regenerative nodule (N). (Peroxidase-antiperoxidase method with nickel/cobalt enhancement; haematoxylin counterstain; x 180).

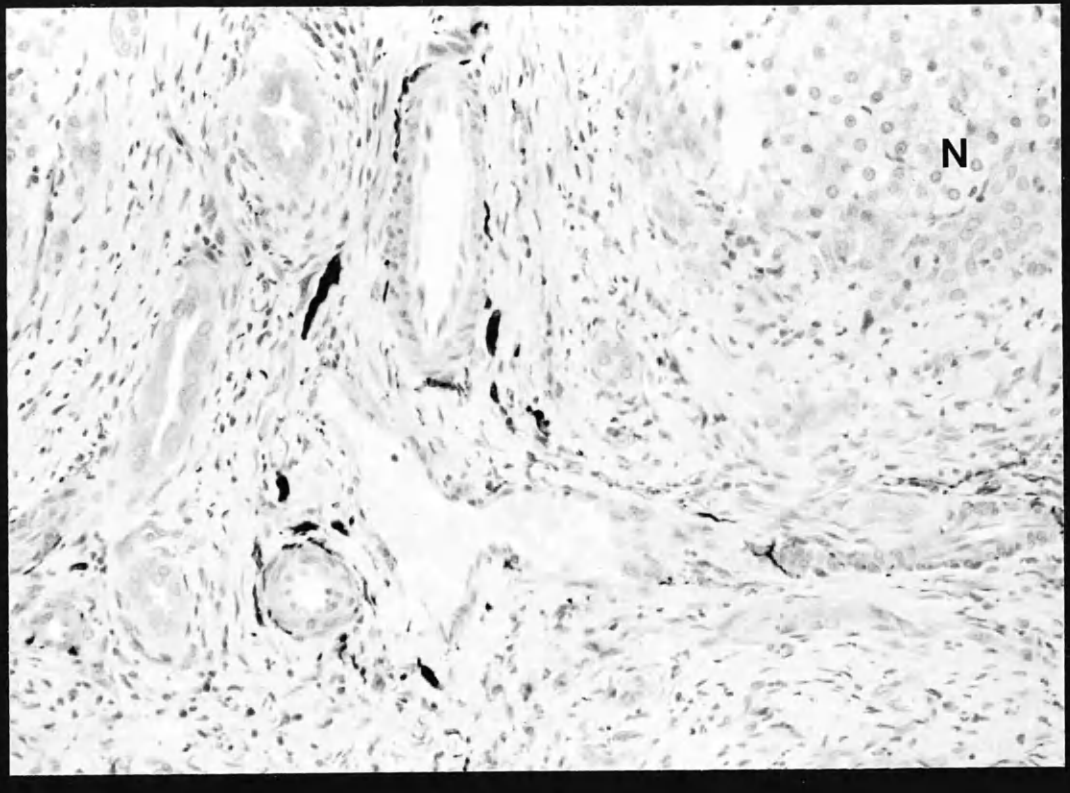

N

Figure 8.9 Immunolocalization of PGP 9.5-positive nerve fibres in established cirrhosis.

a. PGP 9.5-immunoreactive fibres were readily identified within fibrous septa (arrows) but, as can be seen in this case of established cirrhosis, fibres were absent from regenerative nodules (N). (Peroxidase-antiperoxidase method with nickel/cobalt enhancement; haematoxylin counterstain; x 150).

b. PGP 9.5-immunoreactive fibres can be identified within fibrous septa (arrow). (Peroxidase-antiperoxidase method with nickel/cobalt enhancement; haematoxylin counterstain; x 400).

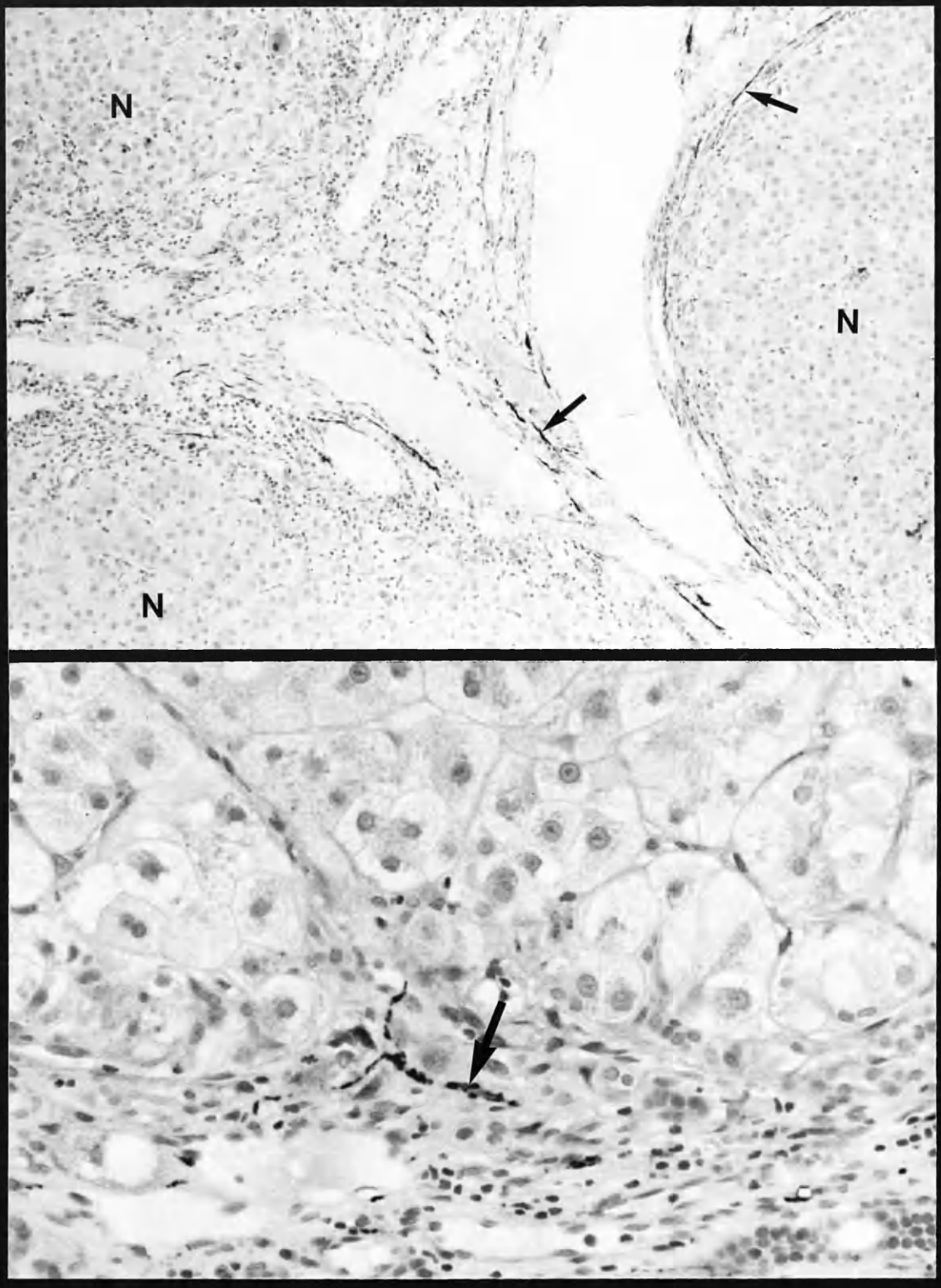

N
N
N

Figure 8.10 Quantitative analysis of intra-sinusoidal nerve fibre density in normal liver, acute liver injury and cirrhosis.

In a preliminary quantitative study, nerve fibre density (see text) was similar in tissue from patients with either normal histology or non-specific reactive changes to that seen in most cases of acute liver injury (extrahepatic biliary obstruction, intrahepatic cholestasis). Intra-sinusoidal fibres were identified in only one of twelve cases of established cirrhosis.

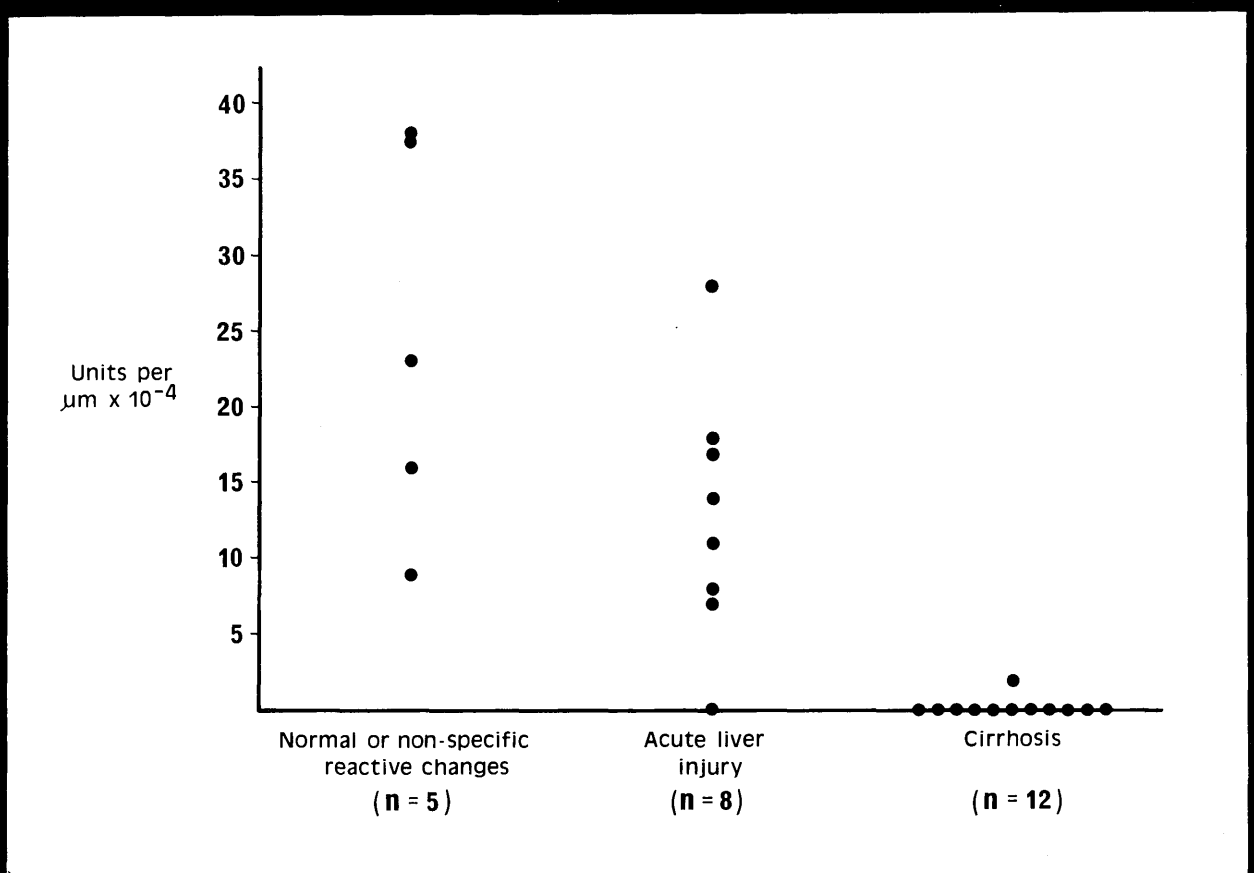
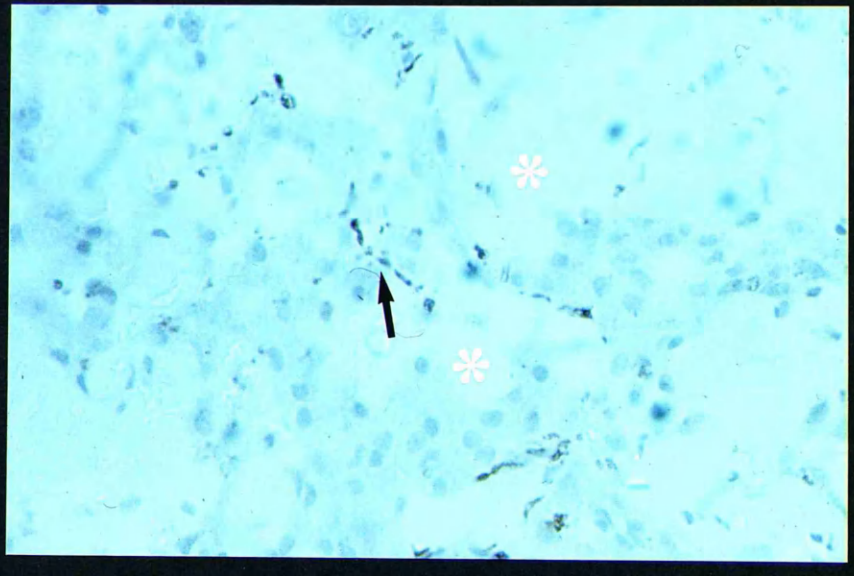

Units per
μm x 10⁻⁴
40
35
30
25
20
15
10
5
Normal or non-specific reactive changes
(n = 5)
Acute liver injury
(n = 8)
Cirrhosis
(n = 12)

Figure 8.11 Immunolocalization of PGP 9.5-positive nerve fibres in hepatic amyloidosis.

PGP 9.5-immunoreactive fibres could still be identified within the sinusoids in this case of hepatic amyloidosis in which the space of Disse was largely obliterated by amyloid deposition (asterisk). The nerve fibres (arrow) are of a similar distribution and density to those seen in normal liver. (Peroxidase-antiperoxidase method; haematoxylin counterstain; x 200).



considerable spread of nerve fibre density in cases with normal or non-specific changes and in acute liver injury. In the acute liver injury group there was complete absence of PGP 9.5 immunoreactive intra-sinusoidal fibres in a single case; this patient had extrahepatic biliary obstruction but on review of the histology, there was evidence of perivenular fibrosis which was attributed to alcoholic liver disease.

8.4 DISCUSSION

Several other groups have previously used immunohistochemical methods to study the distribution of intrahepatic nerves in human liver. Miyazawa *et al* (1988) demonstrated intra-sinusoidal fibres using antibodies to S-100 protein and NSE; fibres immunoreactive for NPY and vasoactive intestinal polypeptide (VIP) were also observed but in keeping with my own data, such peptidergic nerves were found in lower density. No intra-sinusoidal VIP-positive fibres were found. Terada & Nakamura (1989) also demonstrated S-100- and NSE-positive intra-sinusoidal fibres but were unable to immunolocalize peptidergic nerves in their material. By contrast, Ueno *et al* (1988) identified substance P-positive fibres and provided evidence of their presence within sinusoidal walls. The antibody used in their study, however, was poorly characterized. Carlei *et al* (1988) demonstrated NPY-, substance P- and VIP-positive fibres around vessels from the liver hilum but as with their animal studies, no attempts were made to investigate the intrahepatic distribution of these peptidergic nerves.

In the present study, antibodies to four proteins abundantly expressed by peripheral nerves and their supporting cells were assessed for their ability to identify intra-sinusoidal nerve fibres in human liver. Although fibres could be identified using a polyclonal anti-S-100 protein antibody, intra-sinusoidal nerves were more readily demonstrable using an anti-PGP 9.5 antibody. PGP 9.5[1] was originally detected in brain extracts using high resolution two-dimensional electrophoresis (Doran *et al*, 1983). It has recently been shown that the protein is a ubiquitin carboxy-terminal hydrolase (Wilkinson *et al*, 1989) which is abundantly expressed by neuroendocrine cells and neurons of the central and peripheral nervous systems and is present

[1] The name is derived from the proteins mobility in polyacrylamide gel electrophoresis.

within the axoplasm of nerve fibres; it is present in all types of efferent and afferent fibre. Anti-PGP 9.5 antibodies have now been extensively used in the detection of nerve fibres within a wide range of tissues (Wilson *et al*, 1988; Dalsgaard *et al*, 1989).

Unlike PGP 9.5, S-100 protein is not present within the axoplasm of nerves but is a component of surrounding Schwann cells (Vanstapel *et al*, 1986). Transmission electron microscopic studies have previously demonstrated that intra-sinusoidal fibres in human liver may only partly be surrounded by Schwann cell processes (Lafon *et al*, 1989). It is not surprising therefore, that the density of PGP 9.5-positive fibres in this study was greater than that of S-100-positive fibres. In contrast to the findings of Miyazawa *et al* (1988) and Terada & Nakamura (1989), I was unable to identify intra-sinusoidal fibres using an anti-NSE antibody. The reasons for this are uncertain, but may be a result of our fixation protocol; fibres within nerve bundles in portal tracts stained much less intensely with anti-NSE than either anti-S-100 or anti-PGP 9.5. A similar explanation may also be proposed for the results with anti-neurofilament, although it has been shown by others that not all nerves express this intermediate filament protein (Lawson *et al*, 1984; Dalsgaard *et al*, 1989).

The distribution of PGP 9.5- and S100-positive fibres in human liver is similar to that noted in Chapter 7 for adrenergic fibres in guinea-pig liver. Intra-sinusoidal fibres were noted throughout the acini although they were most abundant in acinar zone I. The observation of PGP 9.5-immunoreactive fibres within the walls of terminal hepatic vein radicles suggest that, as in guinea-pig liver, some nerves may enter the liver along with the vena cava and major hepatic veins.

Although the mechanisms involved in neural development have been extensively investigated in lower animals (Crossin, 1989), comparatively little is known about the ontogeny of nerves in mammalian tissues. The development of peptidergic innervation of rat stomach has been studied (Larsson, 1977; Ito *et al*, 1988); VIP-ergic and substance P-containing nerves were found only in late gestation (day 18) and did not reach the density found in adult rat stomach until day 7 of the neonatal period. To my knowledge, however, there are no previous reports of developmental studies of hepatic innervation. In the present study, nerve fibres could be identified in human liver as early as 14 weeks gestation. However, all immunoreactive nerves were restricted to portal tracts until 34 weeks when occasional fibres could be identified in

acinar zone I. Even at 6 months the density of intra-sinusoidal nerves was less than that observed in adult liver. I cannot entirely exclude the possibility that the paucity of immunoreactive nerves may in part have resulted from inadequate fixation and/or autolytic changes in the post-mortem material. The ability to immunolocalize fibres within portal tracts, however, makes this unlikely. Additional information may be obtained by investigating the development of intra-sinusoidal nerves in a species such as guinea-pig where there is also a rich intra-sinusoidal innervation in adult animals.

Alterations to the distribution of intrahepatic nerves in rat liver following CCl_4-induced acute necrosis (Ungváry & Donáth, 1980) and partial hepatectomy (Pietroletti *et al*, 1987) have previously been recorded. However, as we have seen, the distribution of intrahepatic nerves in rat liver differs from the human and these studies are therefore of only limited relevance to our understanding of changes occuring in human liver disease. Using fluorescence and enzyme histochemistry, Ungváry & Donáth (1975) and Akiyoshi (1989) examined changes occuring to intrahepatic innervation in guinea-pig liver during the development of biliary cirrhosis following surgical ligation of the common bile duct. Although Ungváry & Donáth (1975) demonstrated an increase in both adrenergic and cholinergic fibres in portal tracts and in fibrous septa, Akiyoshi (1989) found the changes to predominantly affect cholinergic nerves. Abundant acetyl-cholinesterase-positive fibres could be seen in fibrous septa six weeks after operation. However, no alterations in the pattern of innervation within the parenchymal nodules was detected.

In keeping with these animal studies, Honjo and Hasebe (1965) demonstrated increased numbers of myelinated nerve fibres in areas of fibrosis in human cirrhosis. As this involved the application of a silver impregnation technique, the question of specificity must again be raised. The authors provided little information on the pattern of innervation in regenerating nodules. In their immunohistochemical study, Miyazawa *et al* (1988) showed that in chronic active hepatitis, there was an apparent proliferation of S-100/NSE-positive fibres within developing fibrous septa but, in contrast to the animal models, there was noted to be a concomitant decrease in the density of fibres within developing nodules in cirrhotic liver.

The present study has further demonstrated that there is virtually a complete absence of intra-sinusoidal nerve fibres in established cirrhosis. The results suggest that this is true

irrespective of the aetiology of the liver disease, although a more detailed study with a wider range of types of cirrhosis is now being carried out to confirm this. Observations in pre-cirrhotic chronic liver disease suggested that intra-sinusoidal fibres may be reduced in density prior to the development of estblished cirrhosis. Detailed quantitative analysis of larger numbers of pre-cirrhotic cases is now being performed using the image analysis approach outlined above to examine the progression of these changes during the development of cirrhosis.

The mechanisms responsible for the loss of intra-sinusoidal fibres in cirrhosis are uncertain. Miyazawa *et al* (1988) suggested that in evolving cirrhosis, nerve fibres may degenerate, possibly as a result of the primary liver insult. The observation of either normal or apparently increased nerve fibre density in various forms of acute liver injury suggests that other mechanisms are likely to be involved. The increased fibre density noted in the two cases of acute hepatitis is likely to reflect parenchymal collapse in bridging necrosis rather than true nerve proliferation. It is possible that in chronic liver disease, nerves may be damaged by progressive fibrosis within the space of Disse. However, it seems unlikely that such an obliterative process can fully explain the paucity of nerves, given the ease with which fibres could be identified in the case of amyloidosis studied where there was massive obliteration of the space of Disse by amyloid deposition. It seems likely therefore, that nodular regeneration, an essential feature for the development of established cirrhosis, must contribute towards sinusoidal denervation. One may speculate that although there may be a continued source of growth factors stimulating parenchymal and sinusoidal cell proliferation (Chapter 9), there may be insufficient nerve growth factors to stimulate ingrowth of Schwann cells and neurons into regenerative nodules. The relative roles of fibrosis and nodular regeneration are now being studied by quantifying nerve fibre density at different stages of one form of progressive liver disease, primary biliary cirrhosis. In the early stages of this relatively common disease, there is little fibrosis although there may be marked nodular regenation. As the disease progresses, there is a continuous increase in the amount of fibrosis, leading in time to the development of an established micronodular cirrhosis (MacSween & Burt, 1986a; 1989).

The clinical effects of sinusoidal denervation in cirrhosis are yet to be determined. As noted in Chapter 7, intra-sinusoidal fibres may play an important role in carbohydrate and lipid metabolism. Disruption to the normal nerve supply to the sinusoids may therefore contribute

to the metabolic disturbances that are well documented in end stage liver disease. The postulated role of such fibres in control of microvascular tone (Chapter 9) also raises the possibility that sinusoidal denervation may contribute to alterations in intrahepatic blood flow in cirrhosis and may be associated with the development of portal hypertension.

CHAPTER 9. DISCUSSION : INTERACTIONS BETWEEN COMPONENTS OF THE HEPATIC SINUSOIDS

9.1 INTRODUCTION

In the preceding chapters, the biosynthetic capabilities of the sinusoidal cells were described with reference to complement and extracellular matrix protein production. In addition, the complex structure of the perisinusoidal space of Disse was delineated with the identification of the composition of its extracellular matrix and the nature of intra-sinusoidal nerve fibres. In common with most published works on the hepatic sinusoids, the properties of the individual components were considered separately. However, in multi-cellular organisms the functional integrity of all tissues is dependant upon multifarious interactions between various cell types and with non-cellular components such as the extracellular matrix. The principal aim of this concluding chapter therefore, is to examine the importance of possible inter-relationships between the various constituents of the sinusoids and with adjacent parenchymal cells. First, cell-cell interactions will be considered; particular attention will be paid to the importance of interactions between sinusoidal cells and parenchymal cells in the biosynthesis of complement. The role of interactions between different sinusoidal cells in the control of matrix protein production and the fat-storing cell response to injury will also be discussed. Next, the nature and effects of cell-matrix interactions will be described. Finally, the functional importance of nerve-cell interactions will be considered.

9.2 CELL-CELL INTERACTIONS I: KUPFFER CELLS AND CONTROL OF COMPLEMENT BIOSYNTHESIS BY PARENCHYMAL CELLS

The studies described in Chapter 2 indicated that although Kupffer cells (and to lesser extent fat-storing cells) have the capacity *in vitro* to synthesize and secrete the third component of complement, sinusoidal cells are likely to play only a minor role in total hepatic complement production in the normal animal. These experiments however, dealt solely with the direct contribution of sinusoidal cells; recent evidence suggests that they may also have an indirect role by releasing factors which control complement protein synthesis in parenchymal cells.

Several early studies demonstrated that a population of white blood cells secreted factors which, when administered to animals intravenously, altered the rate of hepatic synthesis of acute

phase proteins such as fibrinogen, haptoglobin and C-reactive protein (Eddington *et al*, 1971). This effect was subsequently reproduced *in vitro* by incubating rat parenchymal cells with white blood cell supernatants or extracts and demonstrating enhanced fibrinogen synthesis (Rupp & Fuller, 1979). The factor responsible for the up-regulation of acute phase protein synthesis was later identified as a product of monocytes and macrophages and termed hepatocyte stimulating factor (HSF) (Ritchie & Fuller, 1982). HSF, which has a molecular weight of 25-30 kD could be identified in rat Kupffer cell culture supernatants; addition of conditioned media from Kupffer cell cultures to rat parenchymal cells resulted in a 5 to 8-fold increase in fibrinogen synthesis (Sanders & Fuller, 1983).

Since these observations, progress in the field of molecular immunobiology has led to the identification and characterization of several low molecular weight peptides which can modulate acute phase protein synthesis in parenchymal cells (Perlmutter *et al*, 1986; Dinarello, 1989; Heinrich *et al*, 1990). These so-called cytokines act at picomolar concentrations through specific high affinity membrane receptors. In contrast to classical hormones, they principally act in a local paracrine manner; they may also feedback on the monocyte/macrophage itself, resulting in autocrine positive feedback loops. However, they may also have effects at distant sites. Cytokines produced by macrophages in the peritoneum for example, in response to chemically-induced inflammation, may alter hepatic acute phase protein synthesis (Anthony *et al*, 1989). Studies in intact animals have identified three major effector molecules which regulate the acute phase response *in vivo*: (i) interleukin-1 (IL-1), (ii) tumour necrosis factor-α (TNF-α), and (iii) interleukin-6 (IL-6) (Geiger *et al*, 1988; Klapproth *et al*, 1989; Delers *et al*, 1989). *In vitro* studies using primary cultures of rat and human liver parenchymal cells, as well as liver tumour cell lines, indicate that although IL-1 and TNF-α may affect the production of some acute phase proteins (Perlmutter *et al*, 1986; Moshage, 1987), IL-6[1] has the broadest spectrum of activity and is likely to be the most important cytokine in the control of acute phase responses (Andus *et al*, 1988; Ganapathi *et al*, 1988; Ramadori *et al*, 1988; Castell *et al*, 1989; Hagiwara *et al*, 1990). IL-1, TNF-α and IL-6 have all been shown to be produced by isolated Kupffer cells (reviewed by Decker, 1990). IL-6 release by isolated rat Kupffer cells can be

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[1] This peptide is now known to be identical to Fuller's HSF.

stimulated *in vitro* by exposure to low concentrations of endotoxin; this may be enhanced by the synergistic activity of IL-1 and TNF-α (Busam *et al*, 1990). Thus, the acute phase response, which occurs as a host reaction to insults such as gram negative septicaemia, may be mediated by Kupffer-cell derived IL-6 in association with IL-1 and TNF-α. It should be noted, however, that IL-6 production is not restricted to monocyte/macrophages. Release of this cytokine has been documented from fibroblasts, endothelial cells, lymphocytes, keratinocytes, synovial cells and a number of tumour cell lines (Heinrich *et al*, 1990). In this respect, it is of interest that Greenwel & Rojkind (1989) presented preliminary data suggesting that fat-storing cell culture supernatants may also show IL-6-like activity.

As several complement components are themselves acute-phase proteins, it is not surprising that stimulation of their synthesis in parenchymal cells may be mediated *in vitro* by IL-6, TNF-α and IL-1. Ramadori *et al* (1988) demonstrated that all three cytokines stimulated C3 synthesis in PLC/PRF5 cells (a human liver tumour cell line), and in *in vivo* experiments showed that each could increase expression of the gene for complement factor B in liver tissue. The study of Anthony *et al* (1989) demonstrated that recombinant IL-6 was more effective in stimulating C3 synthesis by rat liver parenchymal cells than the other two cytokines. However, up-regulation of parenchymal cell C4 synthesis by supernatants of activated peritoneal macrophages appeared to be mediated through a novel cytokine, which they referred to as C4-HSF. It is possible that the observed increase in C3 release by Kupffer cells exposed to endotoxin in my own experiments may be controlled by IL-6 produced by the activated cells themselves as part of an autocrine stimulatory loop; this may also explain the prolonged effect noted after removal of the endotoxin.

In summary, although Kupffer cells probably play a minor direct role in normal hepatic complement production, synthesis of C3 by these cells is increased in response to insults such as endotoxaemia. Furthermore, they may play an essential indirect role by stimulating parenchymal cell complement synthesis through cytokines such as IL-6. Finally, it should be noted that although much of the work on control of acute phase response has dealt with peptide mediators, there is evidence that some eicosanoids such as PGD_2, released by activated Kupffer cells and sinusoidal endothelial cells may also be involved in mediating some of the features of the host response to endotoxin (Brouwer *et al*, 1990). At present, this is largely limited to

studies of the hepatic and plasma glucose response (Casteleijn *et al*, 1988); the possible role of sinusoidal cell-derived eicosanoids in the control of acute phase protein synthesis remains to be fully elucidated.

9.3 CELL-CELL INTERACTIONS II: KUPFFER CELLS AND CONTROL OF THE FAT-STORING CELL RESPONSE TO INJURY

From the experiments outlined in Chapter 4 it can be seen that isolated fat-storing cells have the capacity to synthesize and secrete several extracellular matrix proteins. The immunohistochemical studies presented provide some evidence to suggest that this also occurs *in vivo* in normal and fibrotic human liver. Together with other published data on the cellular origin of hepatic extracellular matrix proteins, it can be concluded that the fat-storing cell is the principal cell type involved in their production. The development of immunolocalization of desmin as a marker for these cells, permitted an assessment of the response of fat-storing cells to one form of acute liver injury (single bolus carbon tetrachloride) and one model of chronic disease (secondary biliary cirrhosis induced by common bile duct ligation). In both forms of injury, there was expansion of the fat-storing cell population which could in part be explained by local cell proliferation. In addition, there was evidence of activation with phenotypic modulation towards myofibroblast-like cells with expression of α-smooth muscle actin. Others have shown that co-incident with these changes there is increased expression of the genes for procollagen types I, III and IV in the liver (Nakatsukasa *et al*, 1990a & b). If we are to design novel molecular approaches for the treatment of hepatic fibrosis, an understanding of the mechanisms which control this fat-storing cell response would appear essential.

Is this process mediated by cytokines similar to those involved in control of the acute phase response ? Probably the earliest study to suggest that the healing response in the liver may be mediated by low molecular weight peptides was that of McGee *et al* (1973) in which fractions obtained from homogenized carbon tetrachloride-injured mouse liver were shown to stimulate collagen synthesis in skin fibroblasts. Four factors were identified (referred to as collagen stimulating factors F1-F4). All were of low molecular weight (F4 being as small as 1000 daltons) and could stimulate collagen synthesis and prolyl hydroxylase activity in human and

mouse fibroblast cultures but had no effect on total protein synthesis or DNA synthesis (Fallon *et al*, 1984). F1-F4 could be isolated from fibrotic human liver by molecular sieve chromatography. Hatahara & Seyer (1982) also identified a low molecular weight compound in injured rat liver which could stimulate collagen production *in vitro*. This group later demonstrated that a factor they referred to as hepatic fibrogenic factor, may stimulate synthesis of collagen types I, III and V (Choe *et al*, 1987). However, the precise nature of such "collagen stimulating factors" or "hepatic fibrogenic factors" remains to be fully characterized.

The development of methods for sinusoidal cell isolation and culture permitted an alternative approach. Shiratori *et al* (1986) demonstrated that a non-dialysable, soluble factor present in the conditioned media of Kupffer cells isolated from carbon tetrachloride-treated rats stimulated a 75% increase in thymidine incorporation into cultured fat-storing cells. A similar effect could be obtained when the Kupffer cells were co-cultured with the fat-storing cells but was not observed with Kupffer cells (or their supernatants) obtained from normal animals. Similar results were obtained by Friedman & Arthur (1988, 1989) and Zerbe and Gressner (1988) although in these studies, activity could be detected in conditioned media from normal Kupffer cell cultures. Friedman & Arthur showed that in addition to stimulating cell proliferation, collagen synthesis was increased up to three-fold. Gressner and Zerbe (1987) also demonstrated that Kupffer cell supernatants obtained from normal, D-galactosamine- or thioacetamide-treated rats could enhance proteoglycan synthesis and secretion by cultured fat-storing cells. These studies provided strong evidence that Kupffer cell products may mediate the fat-storing cell response but, as with the earlier studies using tissue homogenates, the precise nature of factors has not yet been determined.

The factors which control the repair process in other tissues have been intensively studied. Although two of the cytokines discussed above (IL-1, TNF-α) have been shown to be involved in the stimulation of fibroblasts during healing and in the development of fibrosis (Piguet *et al*, 1990), attention has focussed on other low molecular weight effector molecules collectively termed peptide growth factors, of which the best characterized are transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF). TGF-α is predominantly expressed by epithelial cells. Both TGF-β and PDGF are found in greatest abundance in the α granules of platelets but

have been shown to be secreted by a wide range of cell types including macrophages; bFGF has also been shown to be a product of monocyte/macrophage cells (Burgess and Maciag 1989; Ross, 1989; Barnard *et al*, 1990). Based on these observations, several groups of investigators have recently examined the effect of such peptide growth factors *in vitro* on fat-storing cell proliferation and matrix protein biosynthesis. DNA synthesis was shown to be increased by TGF-α. The related epidermal growth factor (EGF), which shares a common receptor with TGF-α, had a similar effect (Pinzani *et al*, 1989, Bachem *et al*, 1989b). PDGF and bFGF also induced a dose-dependant stimulation of DNA synthesis (Pinzani *et al*, 1989). Of these, PDGF was the most potent mitogen on a molar basis. The stimulatory response may be enhanced by the prior addition of Kupffer cell-derived media, suggesting that several factors may interact to mediate fat-storing cell proliferation; this observation may indicate that a factor(s) released by Kupffer cells may up-regulate PDGF receptors on fat-storing cells (Friedman & Arthur, 1988, 1989). The cytokines TNF-α and IL-1 were also shown to stimulate fat-storing cell replication *in vitro* (Matsuoka *et al*, 1989) but to a lesser extent than PDGF.

Studies of the effects of TGF-β on fat-storing cell proliferation have yielded conflicting data. Thus, while Pinzani *et al* (1989) reported no effect, several others have demonstrated an inhibitory effect (Davis, 1988; Matsuoka *et al*, 1989; Czaja *et al*, 1989). On the other hand, there is general agreement that this peptide markedly enhances extracellular matrix protein synthesis by isolated fat-storing cells (Davis, 1988; Matsuoka *et al*, 1989; Weiner *et al*, 1990). Although TNF-α may also enhance collagen production by cultured fat-storing cells (Weiner *et al*, 1990), TGF-β appears to be more important in this respect. Furthermore, there is some evidence that TGF β itself may induce TGF β mRNA in these cells providing an autocrine amplification of collagen synthesis (Weiner *et al*, 1990). On the basis of molecular weight, none of these peptide growth factors are likely to correspond to McGee's collagen stimulating factors or Seyer's hepatic fibrogenic factor.

While these *in vitro* studies have been useful in demonstrating that TGF α/EGF, TGF β, PDGF, bFGF, IL-1 and TNF-α may all potentially be involved in controlling the fat-storing cell response, they cannot be used to assess their role *in vivo*. Czaja *et al* (1989) investigated TGF β expression *in vivo* in rats with schistosomiasis- and carbon tetrachloride-induced fibrosis. In both models, increased levels of TGF β_1 mRNA were identified prior to the noted increase

in collagen synthesis. In order to characterize the origin of this TGF β, Nakatsukasa *et al* (1990a, 1990b) used *in situ* hybridization with a [^{35}S]-labelled RNA probe to detect TGF β_1 gene transcripts in tissue sections. Labelling was seen after 24 hours of carbon tetrachloride exposure, reaching a peak at day 2. TGF β_1 transcripts were initially identified in "inflammatory cells" within necrotic perivenular zones. The resolution obtained with their isotopic *in situ* hybridization method was limited, precluding more detailed characterization of the cells expressing the gene. In collaboration with Dr. K.J. Hillan we have recently demonstrated TGF β_1 RNA in tissue sections from the carbon tetrachloride model described in Chapter 6 using a digoxigenin non-isotopic method. The kinetics of the TGF β response was similar to that noted by Nakatsukasa *et al*; the labelled cells in areas of injury exhibited morphological features of macrophages (Figure 9.1).

On the basis of these observations one may construct a working hypothesis to explain the cellular events occurring following liver injury (Figure 9.2). In response to parenchymal cell necrosis, irrespective of aetiology, peptide growth factors may be initially released by platelets and later by activated Kupffer cells and recruited monocyte/macrophages, which lead to proliferation of fat-storing cells (PDGF) and enhanced extracellular matrix protein synthesis (TGF β). In acute liver injury, such as in the single bolus carbon tetrachloride model, the stimulus for platelet degranulation and macrophage activation is transitory. After sufficient matrix proteins have been laid down for an adequate repair process, fat-storing cell activation ceases. In situations of persistent injury, however, such as in secondary biliary cirrhosis in rats and in many forms of human chronic liver disease, there is a continuing Kupffer cell/macrophage response leading to persistent fat-storing cell activation with the consequent accumulation of extracellular matrix proteins associated with fibrosis. In keeping with this hypothesis is the demonstration that macrophages accumulate in areas of injury prior to the expansion of the fat-storing cell population (Geerts *et al*, 1988). One of my current research staff has recently confirmed this using the antibodies ED1 and ED2. In double labelling experiments similar to those described in Chapter 6, she has shown that this macrophage response is associated with local cell proliferation with a peak in the labelling index at 24 hours, one day before the peak in fat-storing cell proliferation. Support for the role of platelet-derived peptides has also been presented by Bachem *et al* (1989a). Human platelet lysate was shown

Figure 9.1 ***In situ*** **hybridisation for transforming growth factor β mRNA in acute carbon tetrachloride-induced liver injury.**

A non-isotopic digoxigenin-labelled *in situ* hybridization method has been used here on a section of Bouin's-fixed tissue from a day 2 carbon tetrachloride-injured rat to identify TGF β_1 RNA. An anti-sense RNA probe was used; this was derived from the pGEM4 plasmid containing the 582 base pair Sma 1 fragment from the human TGF β_1 gene. Signal can be seen within non-parenchymal cells in a perivenular zone (arrows). The morphological characteristics of these cells indicate that they are likely to be Kupffer cells.

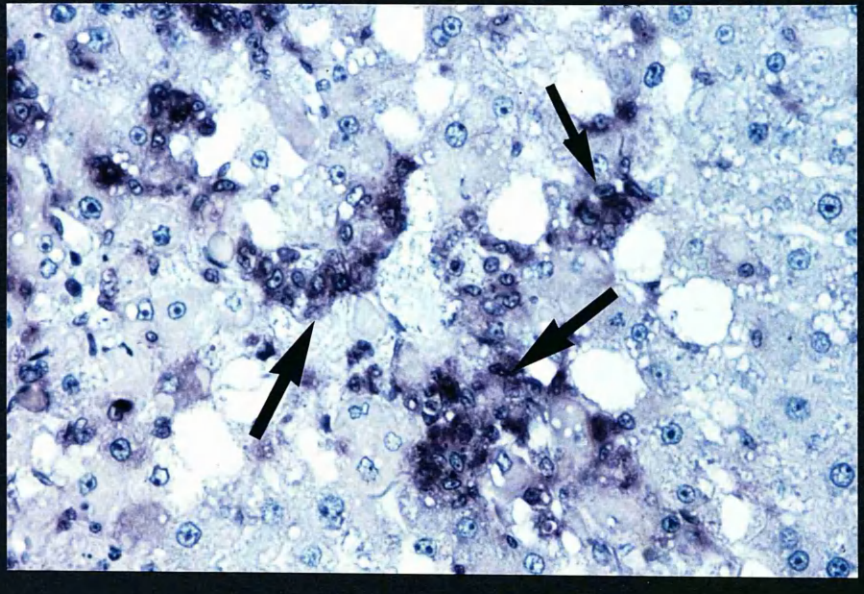
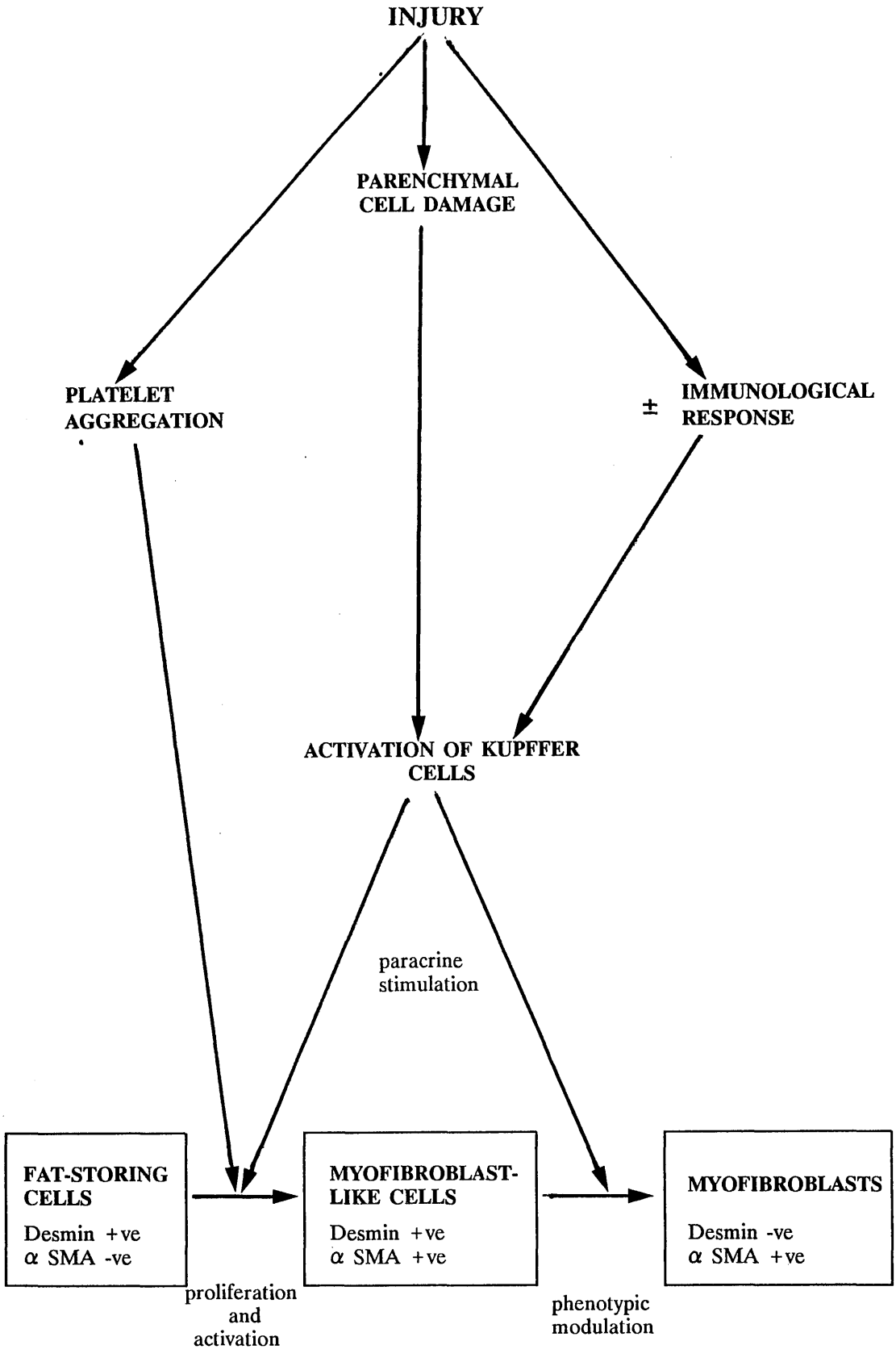


Figure 9.2 Control of fat-storing cell response to injury: a hypothesis.

(SMA : α (smooth muscle actin).


INJURY
PARENCHYMAL CELL DAMAGE
PLATELET AGGREGATION
±
IMMUNOLOGICAL RESPONSE
ACTIVATION OF KUPFFER CELLS
paracrine stimulation
FAT-STORING CELLS
Desmin +ve
α SMA -ve
MYOFIBROBLAST-LIKE CELLS
Desmin +ve
α SMA +ve
MYOFIBROBLASTS
Desmin -ve
α SMA +ve
proliferation and activation
phenotypic modulation

to stimulate, in a dose dependant manner, both the proliferation and glycosaminoglycan synthesis of cultured rat fat-storing cells. The temporal association between TGF β expression and the fat-storing cell response is also in keeping with this hypothesis (Nakatsukasa *et al*, 1990a and b); similar methods should now be used to further examine the role of other peptide growth factors in the *in vivo* response to injury.

As noted above, TGF β may act as a autocrine stimulator of fat-storing cells by switching on TGF β expression in these cells (Weiner *et al*, 1990). In accordance with this data is the suggestion by Nakatsukasa *et al* (1990b) that some TGF β RNA can be localized to desmin-positive cells several days after induction of injury. The illustrations in their paper are unconvincing and we are now attempting to repeat their observations using the digoxigenin-labelled method in conjunction with immunohistochemistry for desmin.

Interactions between Kupffer cells and fat-storing cells appear therefore, to be important in the response to injury and in fibrogenesis. It should be noted however, that sinusoidal cell-parenchymal cell interactions are also likely to be operative in the healing process. There is thus accumulating evidence that parenchymal cell regeneration is in part mediated, by a further peptide growth factor released by activated Kupffer cells, hepatocyte growth factor (Noji *et al*, 1990). Furthermore, TGF β may play a role in controlling parenchymal cell proliferation, serving as a negative growth factor (Fausto & Mead, 1989; Meyer *et al*, 1990).

9.4 CELL-MATRIX INTERACTIONS

The immunohistochemical studies outlined in Chapter 3 demonstrated that the extracellular matrix of the space of Disse in normal liver is a complex mixture of interstitial collagens, "minor" collagens and structural glycoproteins. It is now accepted that these proteins are not static; in normal liver, there is significant turnover of extracellular matrix components. Considerably less is known of matrix degradation than of matrix protein production. Nevertheless, evidence is emerging that sinusoidal cells may also be involved in this process. Bhatnagar *et al* (1982) demonstrated that Kupffer cells isolated from normal rat liver produced collagenase(s) which could degrade collagen type I; release of the enzyme was enhanced by the prior exposure of cells to endotoxin. More recently, Arthur and colleagues have studied the

capacity of isolated sinusoidal cells for the synthesis of neutral metalloproteinases active against collagen type IV. They identified a 65kD proteinase produced by fat-storing cells (Arthur *et al*, 1989) which was active against mature collagen type IV and gelatin but not against interstitial collagens. This group have also identified a distinct proteinase product of Kupffer cells which has a similar spectrum of degradative activity. The precise role(s) of these sinusoidal cell-derived proteinases in control of hepatic matrix composition in normal and diseased liver remains to be determined.

The importance of establishing the nature of the perisinusoidal matrix has already been stressed. Rather than acting as a passive supporting structure, there is now considerable evidence that the connective tissue components may directly influence the function of parenchymal and non-parenchymal cells. Several studies have demonstrated major phenotypic differences between liver parenchymal cells grown on different extracellular matrices. Liver specific gene expression is maintained to a greater degree if cells are plated on reconstituted basement membrane such as EHS matrix rather than on plastic or rat tail collagen (predominantly type I) (Bucher *et al*, 1990; Lindblad *et al*, 1991); proliferation of cultured cells may also be affected. As noted in Chapter 4, the nature of the substrata may also influence the type of collagen synthesized by cultured fat-storing cells. Clearly, these observations have implications for the alterations of parenchymal and non-parenchymal cell function in liver fibrosis when there is a change in the composition of the perisinusoidal extracellular matrix.

How does the extracellular matrix influence function in surrounding cells ? Recent work has identified a number of cell-surface glycoproteins which act as receptors for extracellular matrix components, through which the matrix may exert its effects on cell function. A large family of closely related molecules, collectively termed integrins (Hynes, 1987; Ruoslahti *et al*, 1987), bind to matrix proteins such as fibronectin, vitronectin, laminin and collagens. Each integrin is a non-covalently linked heterodimeric molecule comprising one α chain and one β chain which spans the cell membrane with both intra- and extracellular domains. Several distinct groups of integrin have been described (Albelda & Bucks, 1990); each sub-family is characterized by a common β chain with variable α chains. The largest subfamily is that of the β_1 integrins or very late activation (VLA) antigens. Within this group, structural differences in the α chain impart different ligand specificities on the molecule (Table 9.1). Although many

Integrin	Chain Structure	Extracellular matrix ligand(s)
VLA 1	$(\alpha_1\beta_1)$	Laminin, collagen
VLA 2	$(\alpha_2\beta_1)$	Collagen
VLA 3	$(\alpha_3\beta_1)$	Collagen, laminin fibronectin
VLA 4	$(\alpha_4\beta_1)$	Fibronectin
VLA 5	$(\alpha_5\beta_1)$	Fibronectin
VLA 6	$(\alpha_6\beta_1)$	Laminin
Vitronectin Receptor	$(\alpha_v\beta_1)$ $(\alpha_v\beta_3)$ $(\alpha_v\beta_5)$	Fibronectin Vitronectin, fibrinogen Vitronectin
Platelet gp IIIa	$(\alpha_{11b}\beta_3)$	Fibronectin, fibrinogen

Table 9.1. Molecular structure and ligand specificities of integrin molecules.

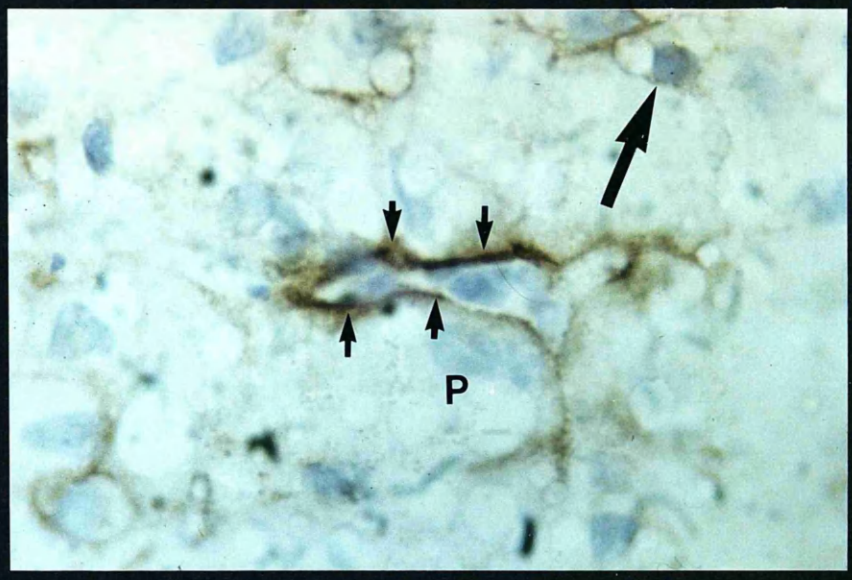
of the proteins were initially identified on lymphoid and myeloid cells (Hemler *et al*, 1985), they are now known to be expressed by many cells (Choy *et al*, 1990; Korhonen *et al*, 1990). More than one integrin may be expressed by an individual cell; profiles of integrin expression vary between cell types (Korhonen *et al*, 1990). Within cells, integrins are co-distributed with components of the cytoskeleton including actin, talin and vinculin (Stamatoglu *et al*, 1990). These receptors may therefore represent transmembrane linkers between the extracellular matrix and the cytoskeleton through which the matrix can influence cell polarity, movement and gene expression (Horwitz *et al*, 1986).

For an understanding of how the matrix may affect parenchymal and sinusoidal cell function in normal and injured liver, a knowledge of intrahepatic integrin expression is therefore essential. *In vitro* experiments have established the presence of a fibronectin receptor ($\alpha_5\beta_1$) (Stamatoglu *et al*, 1990) and a laminin receptor ($\alpha_1\beta_1$) (Forsberg *et al*, 1990) on isolated rat liver parenchymal cells. However, although the expression of a related group of receptors involved in cell-cell adhesion (intercellular-adhesion molecule 1 (I-CAM 1) and leukocyte function-associated antigen-3 (LFA 3)) has been studied in human liver (Volpes *et al*, 1990; Smith & Thomas, 1990), the distribution of integrins has not previously been documented.

In preliminary experiments using the monoclonal antibodies TS2/7.1.1, Fi9 and HP2/1 to immunolocalize VLA 1, VLA 2 and VLA 4 respectively, I have demonstrated that VLA 1 is present along the sinusoidal domain of parenchymal cells (Figure 9.3). VLA 4 appears to be only weakly expressed by parenchymal cells but was found on stellate sinusoidal cells whose position indicated that they may be fat-storing cells; VLA 2 was not present on parenchymal or sinusoidal cells but was localized to bile duct epithelium. These studies clearly need to be extended to include an investigation of the distribution of other integrins in normal liver and examination of alterations to integrin expression by both parenchymal cells and sinusoidal cells in the response to liver injury. Furthermore, in order to overcome the difficulties of limited resolution obtained with light microscopic immunostaining, the proteins should be identified at the ultrastructural level. Although immunoreactivity with monoclonal anti-integrin antibodies is optimal in unfixed tissue, Stomatoglu *et al* (1990) have demonstrated that some integrins may be localized by immuno-electron microscopy after brief aldehyde fixation.

Figure 9.3 Immunolocalization of VLA 1 in normal human liver.

Immunoreactivity for VLA 1 can be seen here along the sinusoidal wall. Labelling (small arrows) is present on the sinusoidal domain of parenchymal cells (P) and within the cytoplasm of occasional non-parenchymal cells with cytoplasmic vacuoles (?fat-storing cells) (large arrow). Further studies using immuno-electron microscopy will be necessary to fully define the precise distribution of the integrin molecules. (Monoclonal antibody TS2/7.1.1; indirect immunoperoxidase; haematoxylin counterstain; x 600).


P

9.5 NERVE-CELL INTERACTIONS

The studies outlined in Chapter 7 illustrate that in some species, such as guinea-pig, there is an abundant adrenergic innervation of the hepatic sinusoids. At least some of the PGP 9.5-positive fibres identified within the sinusoids of normal human liver are likely to be adrenergic and NPY-ergic (Miyazawa *et al*, 1988). What other nerve fibres may be present in mammalian liver sinusoids ? As discussed earlier, several histochemical studies have suggested that post-ganglionic parasympathetic cholinergic fibres may innervate the sinusoids. Sutherland (1964) was the first to demonstrate intrahepatic cholinergic fibres in monkeys, guinea-pigs and rats. Two different cholinesterase methods were applied and intra-sinusoidal fibres were identified in all three species. The author however, drew attention to the potential lack of specificity with this approach ("Attempts to distinguish clearly between acetyl-cholinesterase and (non-specific) cholinesterase activities by the use of inhibitors were not completely successful." (Sutherland, 1964)). Her findings in rat liver were later confirmed by Skarring & Bierring (1976) and Reilly *et al* (1978) although Satler *et al* (1974) were unable to demonstrate intra-sinusoidal cholinergic fibres using a similar histochemical method. As discussed in Chapter 8, cholinergic fibres have also been detected within human sinusoids (Amenta *et al*, 1981) but were reported to be sparse or absent from normal guinea-pig sinusoids (Ungváry & Donáth, 1969; Akiyoshi, 1989). As with fluorescence histochemistry, the technical difficulties associated with the methods used to demonstrate cholinergic fibres are such that the validity of these earlier studies must be questioned. An alternative approach would again be to demonstrate immunohistochemically, peptides which co-localize with acetyl-choline in parasympathetic fibres. Although studies have demonstrated that VIP may be present in some cholinergic fibres (Lundberg *et al*, 1979), it does not appear to be present in all. Furthermore, VIP-ergic, non-adrenergic, non-cholinergic fibres are known to exist in some tissues. Nevertheless, it is of interest that VIP-immunoreactive nerves could not be identified within the sinusoids of either rat (Sasaki *et al*, 1984) or human liver (Miyazawa *et al*, 1988). The question of a sinusoidal cholinergic supply, therefore, remains unresolved. Finally, the possibility of other peptidergic non-adrenergic, non-cholinergic fibres including bombesin- and enkephalin-containing nerves within the sinusoids should now be investigated.

How do hepatic nerves influence liver function? It is reasonable to speculate that in species such as guinea-pig and human, where we have identified abundant intra-sinusoidal nerves, the metabolic functions of parenchymal cells may be controlled by direct innervation. Several groups have identified α_1 and β_2 adrenergic receptors in cell membrane fractions from human liver (Kawai *et al*, 1986; Bevilacqua *et al*, 1987). Although it is likely that these receptors are present on parenchymal cell membranes, their expression at the sinusoidal domain of parenchymal cells must now be confirmed using either radio-labelled ligand studies with autoradiography or immunolocalization of adrenoreceptors using monoclonal antibodies such as DARPP-32 (Priestley, 1987). Furthermore, although ultrastructural studies have identified close contact between dense core-rich nerve varicosities and parenchymal cell membranes, there are no descriptions of membrane densifications to suggest sites of synaptic transmission. More detailed electron microscopic studies of sinusoidal innervation are now indicated. Furthermore, the ability to identify NPY receptors using [^{125}I]-labelled NPY autoradiography (Harfstrand *et al*, 1987) offers a means of identifying binding sites on parenchymal cell membranes.

The paucity of intra-sinusoidal fibres in rat liver means that mechanisms other than direct innervation of parenchymal cells throughout the acinus must operate in some species since the overall effect of sympathetic activation in guinea-pig and rat liver on carbohydrate metabolism is identical (Jungermann, 1988). Efferent nerves could exert their effects on liver metabolic function through at least three other mechanisms: (i) by direct innervation of parenchymal cells in the immediate periportal zone with subsequent transmission of information throughout the liver acinus via gap junctions between parenchymal cells, (ii) by release of transmitters from nerve terminals in portal tracts with diffusion along the sinusoids and (iii) indirectly by their effects on intrahepatic blood flow. The hypothesis that release of neurotransmitters from nerve endings in portal tracts plays a major role has been disproved by the observation that nerve stimulation in rat liver produces identical changes in glucose metabolism whether the liver is perfused anterogradely or retrogradely. Diffusion of transmitters along the sinusoids is therefore unlikely to play a role in the sympathetic control of intermediary metabolism in the rat. Jungermann's group have also previously shown that the effects of hepatic nerve stimulation are independent of flow; sympathetic activation in the presence of a smooth muscle relaxant still resulted in enhanced glycogenolysis (Hartmann *et al*, 1982).

It has been suggested that in species such as the rat in which there is a paucity of intrasinusoidal nerves, transmission of stimulatory signals may occur through direct innervation of parenchymal cells at the limiting plate (ie. the immediate periportal zone) and the subsequent propagation of signal along the acinus via gap junctions between parenchymal cells (Jungermann, 1988). In keeping with this is the observation that there is an inverse relationship between the density of intra-sinusoidal adrenergic nerves and the number of gap junctions in different mammalian species (Forssmann & Ito, 1977).

As noted earlier, intrahepatic nerves may also influence intra-sinusoidal blood flow (Reilly *et al*, 1981). While this may be related to their effects on large intrahepatic blood vessels, it is possible that it may, in part, be mediated through interactions between the intra-sinusoidal nerves and sinusoidal cells. Given the similarities between fat-storing cells and pericytes and the evidence that the former contain muscle-associated cytoskeletal antigens, it is possible that neural stimulation of fat-storing cells may control intra-sinusoidal microvascular tone. That these cells possess contractile properties and/or adrenergic receptors remains to be determined. It is also possible that intra-sinusoidal nerves may affect flow of materials between the sinusoidal lumen and the space of Disse. It has been demonstrated for example, that noradrenaline may induce contraction of fenestrae in sinusoidal endothelial cells, thus altering porosity (Tsukada *et al*, 1983; Wisse *et al*, 1980).

In addition to their effects on metabolic function and blood flow, intrahepatic nerves may play a role in the control of parenchymal cell regeneration. Ashrif *et al* (1974) demonstrated that in rats treated with chemical sympathectomy using 6-hydroxydopamine or subjected to surgical hepatic denervation, there was altered proliferative activity in response to subsequent partial hepatectomy. Based on their studies using total hepatic thymidine uptake, they concluded that hepatic adrenergic nerves facilitate liver regeneration. Although this was later refuted by Kato & Shimazu (1983), who suggested that it was parasympathetic rather than sympathetic innervation which controlled the proliferative response, several groups have subsequently confirmed that parenchymal cell proliferation may in part be regulated via adrenergic receptors. Cruise *et al* (1987) for example, showed that treatment of animals with the α 1-adrenergic blocking agent prazosin, significantly reduced the proliferative response to partial hepatectomy and that this may be mediated by alteration in the binding of peptide growth factors.

Finally, although physiological experiments have shown that hepatic nerves may alter metabolic function and blood flow in the isolated perfused liver, how important is hepatic innervation, in particular that of the sinusoids, in the normal intact animal? It is clear from the ontogenesis studies in Chapter 8 that intra-sinusoidal fibres do not appear to be essential for the function of the normal fetal or neonatal human liver. Similarly, the observation that human orthotopic liver transplants, which are effectively denervated at the time of removal from the donor, apparently function effectively, suggests that hepatic nerves are not essential for normal hepatic function in adult liver. It should be noted however, that metabolic abnormalities do occur in some patients following transplantation. Furthermore, abnormalities of intrahepatic blood flow have also been described; these may contribute to the development of ischaemic injury leading to massive haemorrhagic necrosis, a serious complication arising in up to 10% of patients during the first three months following transplantation (Hübscher *et al*, 1989). Furthermore, the transplanted liver may not remain denervated. In a recent collaborative study with Hübscher's group in Birmingham, we have examined sequential liver biopsies from fifteen liver transplant patients. PGP 9.5- and S-100-positive fibres could be identified in time-zero biopsies taken during the insertion of the donor organ. In subsequent biopsies taken one week after transplantation, no fibres could be identified immunohistochemically. However, in eight of the cases, occasional PGP 9.5- and S-100-immunoreactive nerves reappeared in later biopsies taken after several months. Although these were principally seen in large portal tracts occasional intra-sinusoidal fibres could be visualized.

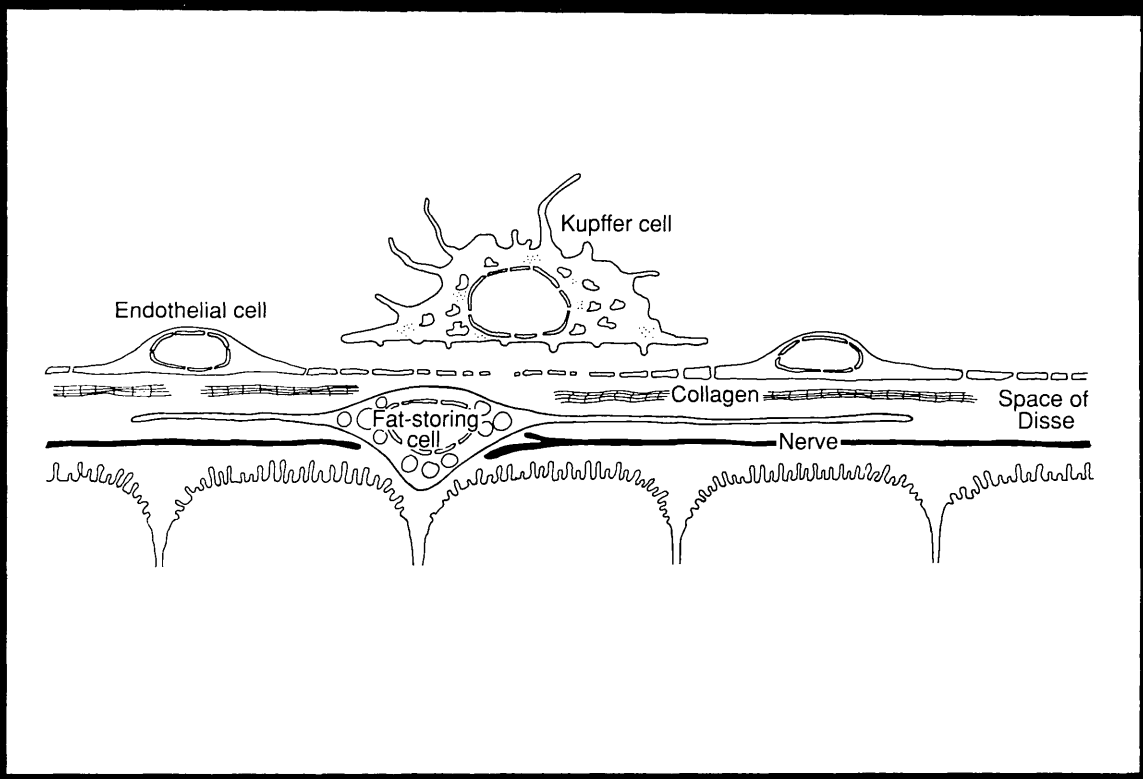
9.6 CONCEPT OF A "PERISINUSOIDAL FUNCTIONAL UNIT"

In this chapter, I have drawn attention to the possible importance of interactions between the individual components of the sinusoids and with adjacent parenchymal cells. The examples that have been chosen were principally related to hepatic functions dealt with earlier in the text such as complement protein synthesis and extracellular matrix biosynthesis. It is however, becoming increasingly evident that interactions between sinusoidal cells and parenchymal cells are also crucial in other physiological processes including vitamin A metabolism (Blomhoff *et al*, 1987), lipid metabolism (Fraser *et al*, 1986), and iron metabolism (Sibille *et al*, 1987). Based

on such data David & Reinke (1987) have proposed the concept of the "Perisinusoidal Functional Unit" to focus attention on the interactions between Kupffer cells, sinusoidal endothelial cells, fat-storing cells and the sinusoidal domain ("vascular pole") of parenchymal cells in the study of liver pathophysiology. On the basis of the observations presented in this thesis, I would suggest that this concept should be extended to include the perisinusoidal extracellular matrix and intra-sinusoidal nerve fibres (Figure 9.4). I believe that intensive investigation of the components of the sinusoids, and of their interactions, will lead to a greater understanding of many other physiological processes in the normal liver and elucidation of the pathogenesis of most forms of liver disease. The sinusoids shall no longer be overlooked !

Figure 9.4 Composition of the hepatic sinusoids: concept of a "perisinusoidal functional unit".

David and Reinke's (1987) concept of a perisinusoidal functional unit is based on evidence of interactions between the different sinusoidal cells and with the sinusoidal domain of parenchymal cells. From the data presented in this thesis, their concept could be modified to take into account interactions of the extracellular matrix and intra-sinusoidal nerves with sinusoidal and parenchymal cells. For simplicity, pit cells have not been included in this diagram.


Kupffer cell
Endothelial cell
Collagen
Space of Disse
Fat-storing cell
Nerve

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APPENDIX 1 : METHOD FOR DEMONSTRATION OF ENDOGENOUS PEROXIDASE ACTIVITY IN TISSUE SECTIONS OF NORMAL RAT LIVER (Modified Graham and Karnovsky's procedure: after Fahimi (1970) and Wisse (1974)

Under ether anaesthesia, a laparotomy was performed on adult male Wistar rats. The portal vein was cannulated with a 14g needle and the liver perfused with (i) 0.14M cacodylate buffer at a pressure of 10 cm H_2O for approximately 30 seconds to remove blood from the hepatic vessels and sinusoids and (ii) 1.5% glutaraldehyde in 0.1M cacodylate buffer for 10 minutes. The inferior vena cava was transected immediately following commencement of the perfusion to ensure a free flow of buffer and fixative through the sinusoids. The liver was subsequently removed and washed in ice-cold cacodylate buffer. Fifty to seventy μm sections were then cut on a Vibratome and floated onto ice-cold cacodylate buffer. The sections were then removed and (i) incubated in 0.1% 3', 3 diaminobenzidine (Sigma, Poole, UK) in 0.14M cacodylate buffer containing 0.01% hydrogen peroxide for 60 minutes at 25°C, (ii) washed extensively in 0.14M cacodylate buffer, (iii) post-fixed in 1% osmium tetroxide and (iv) processed through alcohols for embedding in Epon resin. Ultra-thin sections were then cut on a Reichert-Jung ultramicrotome and mounted onto formvar coated grids and counterstained with uranyl acetate/lead citrate.

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APPENDIX 2 : PEROXIDASE-ANTIPEROXIDASE METHOD FOR USE WITH POLYCLONAL ANTIBODIES

3 μm dewaxed sections of fixed tissue were (i) incubated with 0.5% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity; (ii) washed in distilled water; (iii) incubated with 0.1% porcine type II trypsin (Sigma, Poole, UK) for 5, 10, 15 or 20 minutes (this step was not necessary with all polyclonal antibodies); (iv) washed in distilled water; (v) rinsed in Tris buffered saline (TBS); (vi) incubated with 20% normal swine serum (NSS) in TBS; (vii) following removal of excess NSS/TBS, incubated with the primary antibody

for 90 minutes at room temperature or overnight at 4°C; (viii) washed in TBS; (ix) incubated with swine anti-rabbit immunoglobulin (Dako, High Wycombe, UK) dilution 1:20 in NSS/TBS for 30 minutes at room temperature; (x) washed in TBS; (xi) incubated with rabbit peroxidase-antiperoxidase complex (Dako, High Wycombe, UK) dilution 1:50 in NSS/TBS for 30 minutes at room temperature; (xi) washed in TBS; (xii) incubated in 3', 3 diaminobenzidine in presence of 0.1% hydrogen peroxide for approximately 10 minutes; (xii) washed in distilled water; (xiii) counterstained in haematoxylin; (xiv) dehydrated and mounted in DPX.

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APPENDIX 3 : IMMUNO-ALKALINE PHOSPHATASE METHOD USING MONOCLONAL ANTIBODIES

Five micron cryostat sections were cut and mounted on glass slides coated with poly-L-lysine. The sections were then briefly fixed in ice-cold acetone for 3 minutes and processed as follows: (i) incubated with 2% BSA/PBS, (ii) washed in PBS; (iii) incubated with primary mouse monoclonal antibody diluted in BSA/PBS for 90 minutes at room temperature or at 4°C overnight; (iv) washed extensively in PBS; (v) incubated with alkaline phosphatase-conjugated rabbit polyclonal anti-mouse immunoglobulin (Amersham Int. PLC, UK) dilution 1:100 for 90 minutes at room temperature; (vi) washed three times in PBS; (vii) incubated with the substrate mixture (naphthol AS-TR phosphate in dimethyl formamide/hexanitrogenated new fuchsin/levamisole made up in Tris buffer); (viii) washed in PBS and mounted in Aquamount.

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APPENDIX 4 : INDIRECT IMMUNOPEROXIDASE METHOD USING MONOCLONAL ANTIBODIES

3 μm dewaxed sections of fixed tissue or 4-5 μm frozen sections were mounted on poly-L-lysine coated slides and (i) incubated in 0.5% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity; (ii) washed in distilled water; (iii) incubated in 0.1% porcine type II trypsin (Sigma, Poole, UK) in 0.1% calcium chloride (only for fixed tissue in some experiments, for 5, 10, 15 or 20 minutes); (iv) washed in distilled water; (v) rinsed in Tris buffered saline (TBS) pH 7.6 for 5 minutes; (v) covered with 20% normal rabbit serum (NRS) in TBS for 10 minutes to block non-specific binding; (vi) following removal of excess NRS, incubated with primary antibody diluted in NRS for 90 minutes at room temperature or overnight at 4^{o}C; (vi) washed extensively in TBS; (vii) incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin (Dako, High Wycombe, UK) dilution 1:20 in 20%NRS/TBS for 30 minutes at room temperature; (viii) washed extensively in 3', 3 diaminobenzidine in 0.1% hydrogen peroxidase for approximately 10 minutes; (ix) washed in tap water; (x) counterstained with haematoxylin; (xi) processed through graded alcohols, mounted in DPX and covered with glass coverslips.

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APPENDIX 5 : NICKEL/COBALT ENHANCEMENT OF IMMUNOPEROXIDASE LABELLING

(After Green et al (1989)

In order to enhance the signal obtained by immunoperoxidase, nickel-modified diaminobenzidine was used in place of 3', 3 diaminobenzidine with post-development enhancement using tris/cobalt. The nickel-DAB solution was prepared by dissolving 95mg 3', 3 diaminobenzidine, 1.6g sodium chloride, 0.136g imidazole and 1.5g nickel sulphate in 200 mls 0.1M acetate buffer (pH 6.0). Hydrogen peroxide was then added to a concentration of 0.01% and the solution filtered. Immunostained sections were (i) incubated in this solution for 3 to 5 minutes at room temperature; (ii) washed briefly in TBS; (iii) incubated in Tris/cobalt solution

(0.6g tris and 0.5g cobalt chloride in 100ml distilled water) for 4 minutes at room temperature; (iv) washed briefly in distilled water; (v) counterstained in 0.1% Nuclear Fast Red in 5% aluminium sulphate for 2 minutes; (vi) washed briefly in running tap water; (vii) dehydrated in graded alcohols and mounted in DPX. The reaction product with this technique is a dense black colour.

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APPENDIX 6 : DOUBLE LAYER ANTIBODY SANDWICH ELISA FOR MEASUREMENT OF C3 OR ALBUMIN IN SINUSOIDAL CELL CULTURE SUPERNATANTS

Titretek 96 well ELISA plates were coated with 100μl antibody per well overnight at 4^{o}C in a moist box. Antibodies were diluted in coating buffer (0.1M carbonate/bicarbonate pH 9.6) at the following concentrations : anti-rat C3 : 5μg/ml; anti-rat albumin 10μg/ml. The plates were subsequently washed ten times with PBS containing 0.05% Tween 20. Following removal of any residual buffer, plates were incubated with blocking buffer (0.2% BSA in PBS/Tween 20) for two hours at room temperature in a moist box (250 μl/well). The plates were subsequently : (i) washed ten times in PBS/Tween 20; (ii) incubated with 100μl/ well supernatant samples (or control samples of known C3 or albumin concentration) for two hours at room temperature; (iii) washed ten times in PBS/Tween 20; (iv) incubated with 100μl/well horseradish peroxidase-conjugated antibody (see Appendix 7) for one hour at room temperature; (v) washed ten times in PBS/Tween 20; (vi) incubated with 100μl/well O-phenyldiamine (Sigma. Poole, UK) in phosphate/citrate buffer (pH 5.6) containing hydrogen peroxide for 30 minutes in dark, moist box. The reactions was then stopped by the addition of 25μl 4N sulphuric acid to each well. The colour density of the reaction product was then analysed using an automated Microtiter reader at an optical density of 492 nm.

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APPENDIX 7 : PERIODATE METHOD FOR PRODUCTION OF HORSERADISH PEROXIDASE-CONJUGATED ANTIBODIES FOR ELISA

Twenty mg horseradish peroxidase (Sigma, Poole, UK) was dissolved in 1ml distilled water and added to 0.2ml of 0.1M sodium periodate. The solution was then dialysed against 1mM acetate buffer (pH 4.4) at 4°C overnight. The dialysate was transferred to a glass vial and 3ml ELISA coating buffer (see Appendix 6) was added. 40mg of antibody (either anti-rat albumin (Dr R Anthony, Western Infirmary, Glasgow) or anti-rat C3 (Cappel Laboratories)) was dissolved in this solution and 0.1ml sodium borohydrate solution was then added for two hours at 4°C. The solution was subsequently dialysed against 1N saline and then PBS. The dialysate was dissolved in 50 ml PBS containing 0.1M thimersol as preservative and stored at 4°C in a light-shielded container.

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APPENDIX 8 : STREPTAVIDIN-BIOTIN IMMUNOHISTOCHEMICAL METHOD USING MONOCLONAL ANTIBODIES

3-4 μm sections were cut, processed through graded alcohols to remove paraffin wax and treated as follows: (i) incubated for 30 minutes with 0.5% hydrogen peroxide in methanol; (ii) washed briefly in distilled water, (iii) washed in phosphate buffered saline (PBS); (iv) incubated in 0.1% porcine type II trypsin (Sigma, Poole, UK) in 0.1% calcium chloride (this step was not used in all experiments and when applied was for 5, 10, 15 or 20 minutes); (iv) incubated with 2% bovine serum albumin (Sigma, Poole, UK) in PBS for 10 minutes to block non specific binding; (v) following removal of excess BSA/PBS, covered with primary mouse monoclonal antibody diluted in 2% BSA/PBS overnight at 4°C; (vi) washed extensively in PBS; (vii) incubated with biotinylated sheep anti-mouse immunoglobulin (Amersham Int. PLC, UK) dilution 1:50 in BSA/PBS for 45 minutes at room temperature; (viii) washed three times in PBS and incubated with streptavidin-biotin-horseradish peroxidase complex (Amersham Int. PLC, UK) dilution 1:350 at room temperature; (ix) washed three times in PBS; (x) incubated in 3', 3

diaminobenzidine in 0.1% hydrogen peroxide for approximately 10 minutes; (x) washed in PBS and counterstained with haematoxylin.