IMMUNOCYTOCHEMICAL IDENTIFICATION OF THE PERIPOLAR CELL AND MACULA DENSA CELL TYPES

BY

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SUMMARY

The three "classical" components of the juxtaglomerular apparatus are the myoepithelioid cells, the macula densa cells and the lacis cells, which have been studied for over half a century. This complex multicellular structure is situated in the periglomerular position of every glomerulus, and is responsible for the intrinsic renal control of glomerular filtration rate and tubular reabsorption of sodium, and the regulation of body fluid volume and blood pressure. Complex cellular interactions occur within the juxtaglomerular apparatus. These cells also exhibit physiological relationships with other tissues of the body; but detailed mechanism are still unclear. Ten years ago, a distinctive type of cell, the peripolar cell, was recognised in close anatomical relationship with the juxtaglomerular apparatus. Morphological evidence indicates that the peripolar cell is a secretory type of cell but the substance of the secretory product is unknown. The available data suggests that the peripolar cell might be a previously unrecognised but integral component of the juxtaglomerular apparatus. Its secretory product may be responsible for the hormonal control of renal proximal tubular reabsorption, although this remains speculative.

The aim of this project was to raise monoclonal antibodies to the granules of the peripolar cell. Initially the distribution of peripolar cells in the sheep kidney was studied, and incidence of peripolar cells quantitated. A method has been developed for the isolation of glomeruli retaining a high percentage of peripolar cells. Mice have been immunised with this antigen, and spleen- myeloma cell lines raised from suitable mice. Assessment of antibodies produced by these cells has followed from the development of a range immunocytochemical techniques. The results of five fusions of mice spleen cells with myeloma cells are shown. The possibility, as well as the difficulties of attempting to produce monoclonal antibodies using an antigen in which the molecule of interest is only a small component in a complex mixture is

discussed.

A monoclonal antibody producing cell line has been established during the present study. The antibody reacts specifically with the cytoplasm of the epithelial cells of the macula densa in sheep. Based on immunocytochemical staining patterns, the possibility that there might be more than one type of highly differentiated cell in the macula densa is discussed. Experimental studies using the monoclonal antibody were undertaken to investigate the crossreactivity of this antibody with rat and human kidney. Specific immunostaining patterns, different from those seen with sheep kidney, have been shown in these other species. This monoclonal antibody may be useful in further studies to clarify the function and cellular interactions of the juxtaglomerular apparatus.

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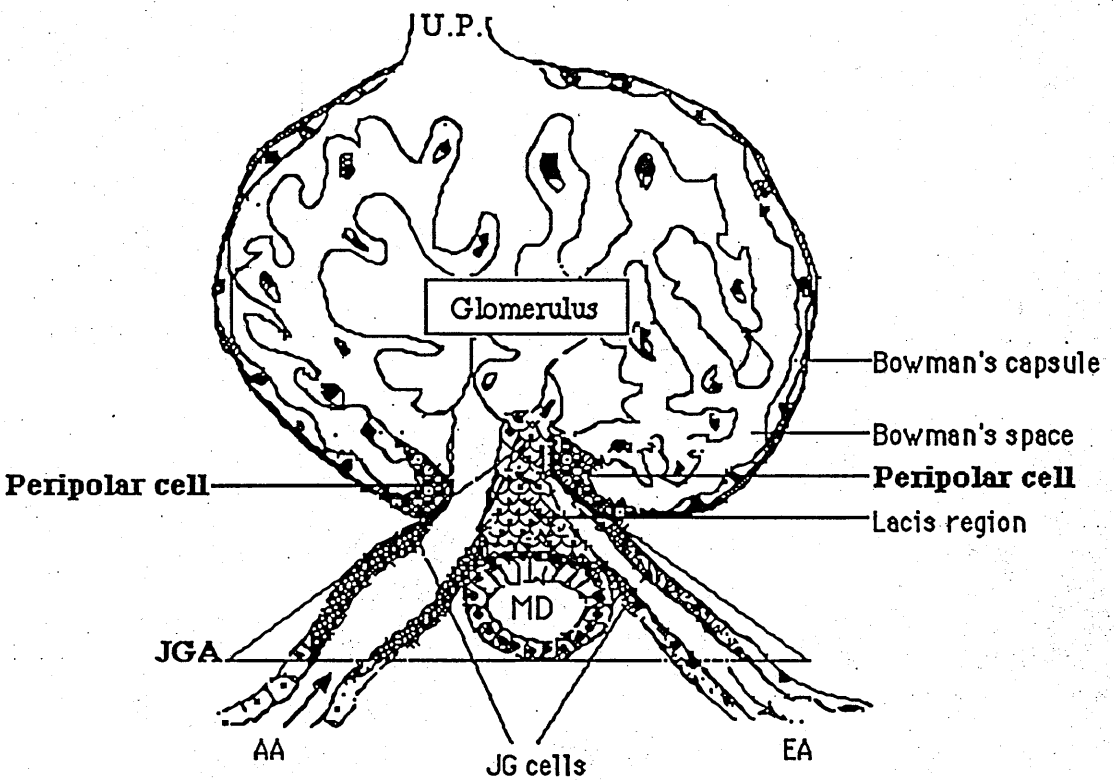


Fig. 1.1

Diagram of renal **juxtaglomerular apparatus** (JGA, triangle area), which includes the JG cells in the afferent arteriole (AA) and efferent arteriole (EA), macula densa (MD) in their close physical relationship, and the lacis cell in between. Granules in the JG cells are renin containing granules. UP: urinary pole. The **peripolar cells** are shown with their typical topographical location within Bowman's capsule of the glomerulus and in contact with the components of the JG complex.

Part 1 INTRODUCTION

The peripolar cell is a newly discovered secretory cell at the vascular pole of vertebrate glomerulus. Since it was first identified in sheep (Ryan et al, 1979), the peripolar cell has been described in many other species (Gall et al, 1986) including man (Gardiner & Lindop, 1985; Gardiner et al, 1986).

For the past decade, valuable evidence indicating the secretory nature of the peripolar cell and, significant experimental information regarding the possible function of the cell have provoked increasing interest and research effort. The peripolar cell attracted attention because of its close anatomic relationship to the cells of juxtaglomerular apparatus (JGA) (Fig. 1.1 & 1.2). This gave rise to the hypothesis that the peripolar cell could be a previously unrecognised component of the JGA (Ryan et al, 1979).

1.1 Juxtaglomerular apparatus (JGA)

In the mammalian renal glomerulus, there is a complex multicellular structure known as the juxtaglomerular apparatus (JGA). It is a functional unit important in the regulation of body fluid volume, blood pressure and in the intrinsic renal control of glomerular filtration rate.

This special structure has been studied for over half a century. Classically, it consists of three groups of cells: 1), the myoepithelioid cells of afferent and efferent glomerular arterioles, 2), the macula densa --- a pad of specialised cells in the distal tubular segment which contact the arteriolar wall, and 3), the polar cushion cells --- the lacis region or extraglomerular mesangium.

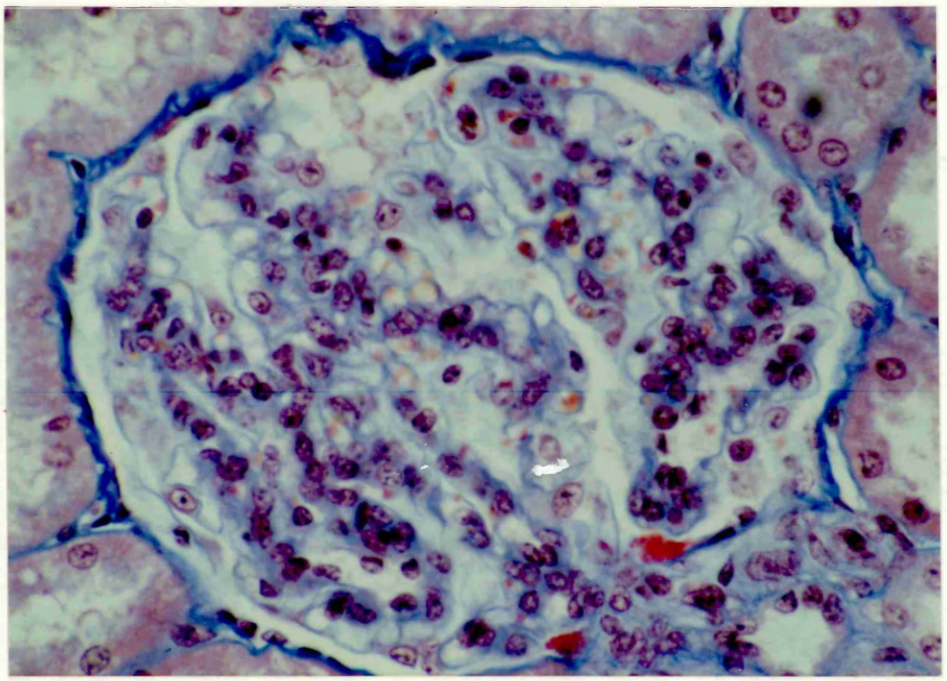


Fig. 1.2
Light micrograph showing 2 granular peripolar cells (red) one at each side of the glomerular vascular pole. Sheep kidney paraffin-embedded tissue section, MSB stain, x250.

1.1.1 The myoepithelioid cell (Juxtaglomerular cell)

Early in 1925, Ruyter described for the first time the differentiation of the smooth muscle cell in the wall of renal arteriole before it penetrated into the glomerulus (Rouiller, & Orci, 1971). Studying the reaction of mitochondrial stains with sections of white mouse kidney, he noted that the common smooth muscle cell in that particular preglomerular arteriole segment was replaced by a granular epithelial like cell which took up the mitochondrial stains. Ruyter called it "epithelioid" and believed that it was derived from the smooth muscle cell of the glomerular arterioles. Two years later, on human kidney sections, Oberling also observed the epithelioid structure of cells in the tunica media of the preglomerular arteriole. He gave it another name 'the glomerular myoepithelioid cell' because of the myoepithelial features of the structure (Rouiller, & Orci,1971). They both contained fuchsinophilic granules in their cytoplasm. Similar cells were then found in a wide range of animals species, but are particularly prominent in mice and rats (Ryan et al, 1982).

By light microscopy, the granules of the myoepithelioid cell can be identified with Bowie's stain which is a method for staining the pepsinogen granules in gastric glands (Bowie, 1936), the periodic acid-Schiff reaction which selectively stains carbohydrate groups, brilliant crystal scarlet in Lendrum's Martius Scarlet Blue stain (Lendrum et al, 1962), and with methylene blue or toluidine blue. Ultrastructurally, the granules are rounded, membrane-bound, and most of them contain amorphous electron-dense material. A regular crystalline lattice substance sometimes presented in the granules which were thought to be newly formed immature protogranules (Barajas, 1966), containing inactive renin (de Senarclens et al, 1977). These granules are secreted by exocytosis (Lindop, 1987) mainly into the interstitium (Morgan et al, 1975).

1.1.2 The macula densa

These cells are a plaque of highly differentiated epithelium located in the specialised segment of the distal tubule which forms the junction between the straight and convoluted portions of the tubule. Its characteristics were first noticed by Peter in 1907. The name "macula densa" was given to the group of these cells by Zimmermann, because of the accumulation of nuclei and the pile of the cells that constituted it. Besides the characteristic accumulation of nuclei, other morphological changes have been described. The most intriguing one is that the Golgi apparatus appears in the basal, rather than the apical part of the cell, close to the vascular component (MacManus, 1943). Ultrastructurally, the macula densa is different from the other segments of the distal tubule in size, shape, and distribution of mitochondria. In the macula densa, mitochondria are mainly ovoid in shape and are scattered throughout the cytoplasm.

1.1.3 The lacis

In addition to the epithelioid cells, Goormaghtigh (1932, cited in Rouiller & Orci, 1971) later described another group of morphologically distinct cells. These cells are arranged in columns, like the tactile cells of Meissner's corpuscles of the skin and similar to the Schwannian elements. He termed them therefore "pseudo - Meissnerian cells". Goormaghtigh also noticed this group of very small cells in the wedge-shape area surrounded by the afferent and efferent arterioles and the macula densa. They had poorly defined cell borders, little cytoplasm and flattened nuclei containing a delicate meshwork of chromatin. The ultrastructural features of this Goormaghtigh's cell were described in detail by Oberling (1944), Oberling and Hatt (1960a,b--cited in Rouiller, & Orci, 1971). The name "lacis" was given to this region by Oberling and Hatt because of the irregular arrangement of cells and basement membrane. The function of the lacis cells is not clear though occasionally some granules sparsely distributed in the cytoplasm were seen in normal kidney (Barajas & Latta, 1963a & b). In some pathological states, they might be

transformed into renin-secreting cells (Dunnihue & Bolosser, 1963).

1.2 The Peripolar cell

1.2.1 Identification and histology

During a study of the influence of salt balance on juxtaglomerular morphology in sheep, Ryan et al (1979) observed a new, distinctive type of cell which contained multiple cytoplasmic granules similar to those of the arteriolar myoepithelioid cell. These granules, like those of the renin-containing cells, also stained positively with periodic acid-Schiff, brilliant crystal scarlet in Lendrum's Martius Scarlet Blue stain , Bowie's stain and with methylene blue or toluidine blue.

Apart from the presence of the cytoplasmic granules, the unique anatomic location is another prominent morphological characteristic of the peripolar cell. These cells are located within Bowman's capsule. They are epithelial cells resting on the basement membrane of Bowman's capsule and interposed between the parietal epithelium of Bowman's capsule and the podocytic epithelium of the glomerular tuft. The cells encircle the glomerular tuft at the vascular pole with long processes, occupying the annular groove formed by reflection of Bowman's capsule onto the glomerular arterioles. The peripolar cell has a free surface exposed directly to the urinary space from which the granules might be secreted into the glomerular filtrate (Ryan et al, 1979; Hanner et al, 1980; and Hill et al, 1983)

Following the discovery of peripolar cells in sheep, the peripolar cells have been described in a wide range of mammals such as rat (Ryan et al, 1982; Gibson et al, in press) and man (Gardiner & Lindop, 1985). It was reported that peripolar cells were found in 17 mammalian species studied (Gall et al, 1986). In addition, peripolar cells were also found in two amphibians, the axolotl and toad (Hanner &

Ryan, 1980); in reptiles, the sand lizard (Koval'chuk, 1987); and in birds (Morild et al, 1988). Of all species examined granular peripolar cells are most prominent in sheep (Alcorn et al, 1984). Variation has been observed among the different species studied, but the main morphological characteristics described are in common.

1.2.2 Ultrastructure

The ultrastructure of the peripolar cell has been described in sheep (Ryan et al, 1979; Kelly et al, in press); in axolotl and toad (Hanner et al, 1980); in the rat (Ryan et al, 1982; Gibson et al, in press) and man (Gardiner et al, 1986). These cells are irregular in shape but often elongated with a curved profile. They varied in size and sometimes had long processes. The peripolar cells have a remarkable bossellated appearance with many intracytoplasmic granules bulging into the cell membrane (Kelly et al, in press). The cytoplasmic granules are the prominent feature of the peripolar cell. These granules have the appearance of secretory granules, which are membrane-bound, mostly round in shape but varying in size and contain homogeneous electron-dense material (Ryan et al, 1979).

In sheep (Ryan et al, 1979), axolotl and toad (Hanner & Ryan, 1980), peripolar cells have been observed occasionally in the process of discharging their cytoplasmic granules at the cell periphery into the glomerular urinary space, providing evidence of granular exocytosis. In man, although granule exocytosis was not observed, some granules appeared to be undergoing dissolution while other granules were pale, the latter feature has also been noted with exocytotic activity in sheep (Ryan et al, 1979, Hill et al, 1983).

In the rat, the proportion of glomeruli which contain peripolar cells was found to be greater when the same kidney was examined by scanning electron-microscopy compared with observations using light microscopy. Under light microscopy only

those peripolar cells which contain granules are readily seen. Therefore, the existence of non-granular peripolar cells was proposed (Gibson et al, in press).

In addition to the granules, the cytoplasm of peripolar cells usually have prominent Golgi apparatus, and rough endoplasmic reticulum segments. In addition, marked pinocytosis at the basal surface of peripolar cells and the presence of membrane invaginations into the cells were noted in human kidney (Gardiner et al, 1986). It was suggested that these changes might relate to the synthesis and release of the secretory granules of the peripolar cell.

The cytoplasm is often relatively sparse and the nuclei are commonly rounded with finely dispersed chromatin. These cells contain mitochondria, free ribosomes, and a few microfilament bundles. Short microvilli are present on the free surface of the peripolar cell.

1.2.3 Morphogenesis

To understand the histological development of the juxtaglomerular and peripolar cells, the metanephric juxtaglomerular region of fetal, newborn and the adult sheep were studied.

By light and electron microscopy, Mitchell et al (1982) found that the JGA was first detectable at 45 days of gestation following the constriction of the edges of Bowman's capsule and the formation of the vascular pole of the renal corpuscle. A mesenchymal cell gave rise to the myoepithelioid cell, the smooth muscle in the juxtaglomerular arteriole, and the lacis cell. The macula densa developed from tubular cells at the junction of the middle and upper limbs of the S-shaped body of the developing nephron. In contrast to the conventional three components of JGA, peripolar cells arose from epithelial cells in the lower limb of the S-shaped body. They were found at the constricting edges of Bowman's capsule and formed a cuff

around the origin of the glomerular tuft. The cytoplasmic granules of peripolar cells were first seen at an early stage of gestation, at 53 days, which was much earlier than those of the myoepithelioid cells first seen at 92 days. These findings support the concept that the peripolar cell may be a special secretory type of cell and it might play an important physiological role in the body.

In the immediate postnatal period of newborn sheep, it was observed that the granular peripolar cells were strikingly prominent and the granules of the cells were very much larger than those found in fetal or adult sheep. Similar peripolar cell hypertrophy could be triggered in fetal lambs treated in utero with intraperitoneal injections of dexamethasone (Alcorn et al, 1984). Alcorn suggested that such peripolar cell hypertrophy might reflect a functional adaptation of the kidney to immediate postnatal life.

1.2.4 Pathophysiological observations

In sheep subjected to rapid sodium depletion in response to parotid cannula drainage, Hill et al (1983) showed that glomerular peripolar cells appear to be active in protein synthesis with increased rough endoplasmic reticulum and well developed Golgi apparatus, and marked hetergeneity of granule density. This was typical of the appearance of active secretory cells and confirmed therefore the secretory nature of peripolar cells. Moreover, one of the major new findings in the study was that during sodium depletion exocytotic release of granule material from peripolar cells into the urinary space was commonly observed, with some pale granules in the periphery of the cells. These features could not be found in normal or sodium loaded sheep. Neither could they be found in normal pregnant sheep (Hill et al, 1983). Although not strong evidence, these observations support the concept that the peripolar cell may play a role in sodium homeostasis.

Similar features were found in peripolar cells in sodium depleted pregnant sheep

(Hill et al, 1984a) and ovine toxaemia (Hill et al, 1984b). In ovine toxaemia, mitotic activity was detected in peripolar cells in association with granular exocytosis and a decrease of granulation index. Hill et al postuated that peripolar cells were being subjected to significant stimulation in ovine toxaemia (Hill et al, 1984b). This provides further support for the view that the peripolar cell could be a functional component of the JGA.

1.2.5 Granule composition

i) Histochemistry

The granules of peripolar cells stain positively with periodic acid-Shiff technique which specifically stains aldehyde groups on carbohydrates (Bancroft, & Cook, 1984). This observation combined with the morphological features of the secretory granules, suggested that these PAS positive granules might contain a zymogen (Gardiner & Lindop, 1985).

ii) Immunohistochemistry

The first detailed discussion about the composition of peripolar cell granules in human kidney based on immunohistochemistry was presented by Gardiner and Lindop (1985). Since the localisation, staining reactions and morphology of the peripolar cell are similar to those of the myoepithelioid cell in the juxtaglomerular apparatus, the peripolar cells was originally thought to be a renin-secreting cell located within the glomerulus (Ryan et al,1982). This would explain why the quantity of renin found in rat glomerular ultrafiltrate, by micropuncture of the glomerular filtration, was more than it would be expected from the serum renin concentration (Leyssac, 1978). Using a pure antibody to human renin and the PAP technique, however, Gardiner and Lindop demonstrated that the human peripolar cell contains no immunostainable renin (Gardiner & Lindop,1985).

Using commercially available antisera to plasma protein components, Trahair and Ryan (1988) demonstrated that the peripolar cell granules in newborn lambs

and adult sheep reacted with antibodies to sheep albumin, and immunoglobulins including specifically IgG $F(ab')_2$. A diffuse immunostaining pattern was also seen in some of the tissue blocks but a granular pattern appeared in the others. Anti-sheep albumin and anti-sheep immunoglobulins labelled with immunogold were used to confirm the granule staining pattern. They showed that the cytoplasmic granules of the peripolar cell were the ultrastructural site of localisation for each antigen.

In a published abstract, the granules of the peripolar cells were also reported to contain immunoreactive kallikrein (Gall et al, 1984; Turner, 1985, Trahair & Ryan, 1987). This was contradicted by a subsequent publication (Trahair et al, 1989).

The substance in the peripolar cell granules remains unknown.

1.2.6 Possible functions of peripolar cell secretory product

i) Hormonal control of proximal tubule reabsorption

In view of its topographical location and secretory nature, Ryan et al (1979) deduced that the peripolar cell might participate in juxtaglomerular complex activity. They postulated that peripolar cells might release their product into the urinary space in response to electrolyte changes in the glomerular ultrafiltrate, to variations in glomerular arteriolar calibre, or to diffusible mediators released from other components of the juxtaglomerular complex.

Renal tubular reabsorption takes place throughout every segment of the tubule. As a key enzyme in the renin-angiotensin-aldosterone system, renin plays a important role in the control of distal tubular reabsorption of sodium by aldosterone. In addition, angiotensin II receptors have been identified on glomerular and brush border membranes (Lindop & Lever, 1986). Despite the fact that most reabsorption occurs in the proximal tubular, mechanisms controlling this activity are still poorly

understood. By analogy with the role of renin, a hypothesis that a factor released from peripolar cells could be involved in the modulation of secretory or reabsorptive functions of proximal tubular cells was suggested (Ryan et al, 1979). This supported an earlier suggestion by Haberle and Shigai, (1978), who postulated the existence of an unrecognised hormone or other compound in proximal tubule fluid able to mediate increased volume reabsorption in response to increased glomerular filtration rate. Such increased volume reabsorption is thought to result from increased proximal tubular sodium reabsorption. Thus, the possibility that the peripolar cell might play this role has been suggested.

ii) Renal reabsorption

The granules of peripolar cells may contain immunoreactive plasma proteins, including albumin and immunoglobulins (Trahair & Ryan, 1988). Based on this observation, Trahair and Ryan suggested that the peripolar cell, in common with the general capacity of renal tubular epithelium and glomerular podocytes (Dick & Kurtz, 1968, Szokol et al, 1979), has the capacity to reabsorb filtered plasma proteins from the ultrafiltrate and incorporate them into granules in its cytoplasm.

However, several observations are not explained by this hypothesis: firstly, the distinctive position of the peripolar cells which seems too specialized for the peripolar cell function to be simply the uptake of glomerular filtered plasma proteins. Secondly, there is evidence indicating the secretory nature of the peripolar cell. Moreover, it is not yet clear whether any other components apart from the reabsorbed plasma proteins exist in the peripolar cell granules.

1.3 Function and cellular interaction of the JGA

1.3.1 Renin-angiotensin-aldosterone system

Since the early observations of Ruyter, Oberling, Goormaghtigh and Zimmermann, many investigators have described the morphology of juxtaglomerular cells, and attempted to elucidate their physiological and biochemical roles and their clinical significance. However, it was not until the finding of proliferation of epithelioid cells in ischemic kidney and the understanding of renin localisation in the glomerular myoepithelioid cells, that the importance of the JGA began to be understood (Hatt, 1967).

Renin was discovered nearly a century prior to the recognition of the characteristic morphological features of the JGA. In 1898, Tigerstedt and Bergman found that extracts of rabbit kidney caused a rise in blood pressure when injected into other rabbits (cited in Hatt, 1967). However, this phenomenon remained unexplained until Goldblatt demonstrated the role of the ischemic kidney in causing hypertension. He produced hypertension in dogs by applying a clamp to one renal artery and leaving the other renal artery untouched. From this observation, he postulated that the ischemic kidney liberates large amounts of renin which is responsible for a rise in blood pressure. This was the foundation of the famous "Goldblatt's humoral theory of hypertension of renal origin" (Goldblatt et al, 1934, cited in Hatt, 1967).

Several years later, Goormaghtigh observed the proliferation of epithelioid cells in the ischemic kidney (Goormaghtigh & Grimson, 1939), and in the kidneys of other pathological conditions such as severe scarlatina, eclampsia, acute glomerulonephritis, crush syndrome. They postulated that the juxtaglomerular myoepithelioid cells could be the site of renin elaboration. Later it became clear that

renin was not a direct pressor, but acted on a substance in plasma to produce a heat stable, short acting vasoconstrictor, which caused vasoconstriction in the body and resulted in increased blood pressure (Page & Helmer, 1940). Page and Helmer named the new substance 'angiotonin' while Braun-Menendez and his group used the term 'hypertensin'. The name 'angiotensin' was agreed between these two schools many years later. In 1951, the first sucessful demonstration of the presence of angiotensin in the blood of animals with experimental hypertension was provided by Skeggs (1951). Thus, the endocrine function of the renal arterioles was established as one of the essential factors in the regulation of arterial blood pressure.

Following the discovery of aldosterone (Davis et al, 1961, 1962), further progress in understanding the functional mechanism of the JGA was made by Hartroft. By measuring the average amount of granulation in any given kidney she showed that, far from always being linked to modifications in arterial tension, the granularity reacts in a manner parallel to the zona glomerulosa of the adrenal cortex. Therefore, it was suggested that angiotensin might also stimulate aldosterone secretion from the adrenal gland, which increases sodium and water reabsorption in renal distal tubule. By this mechanism of increasing volume circulation, renin can also cause an increase in blood pressure (Hartroft, 1953).

It has been proved that the myoepithelioid cells are the major source of renin (Cook & Pickering, 1959; Cook, 1971). Fluorescent-labelled antibodies to partially purified renin stain the granular epithelioid cells of the JGA in rabbit and dog(Edelman and Hartroft, 1961). Later, a similar observation was made with human kidney using antiserum to purified renin (Camilleri et al,1980).

The composition, physiological role, and clinical significance of renin have been investigated in some detail. Renin in human and other animals has been purified (Fisher, 1971). It is a glycoprotein with proteolytic activity. The only known direct function of renin is to cleave the α_2 globulin angiotensinogen at the leu-leu

bond between the 10th and the 11th amino acids. However, following a series of other enzymic actions, the inactive decapeptide angiotensin I is further cleaved to produce an octapeptide angiotensin II and a heptatide angiotensin III. Angiotensins II and III are vasoconstrictors, and in addition they interact with the zona glomerulosa cells of the adrenal gland to release aldosterone, which acts to increase the retention of sodium and water . As a key enzyme in the body renin-angiotension-aldosterone system, renin participates therefore in both blood pressure regulation and electrolyte and water regulation. In addition, through a complex series of intracellular actions, angiotensin II stimulates RNA, DNA and protein synthesis (Khairallah et al 1972; Re, 1982).

1.3.2 Kallikrein-kinin system

Abelous and Bardier (1909, cited in Fisher, 1986) found that injection of a watery dialyzed solution of an alcoholic precipitate of the urine caused a marked mitosis, a profound fall of blood pressure and a series of physiological changes in rabbit or dog. They attributed this to a substance which they called 'Urohypotensin'. Some years later, Frey (1926, cited in Fisher, 1986) recognised that this hypotensive property was due to kallikrein, a enzyme detected in large amounts in the pancreas and also in body fluids.

It is now known that the major function kallikrein is to act on protein substrates-kininogens and produces kinins, kallidin and bradykinin. Also kallikrein can activate inactive renin (Derkx et al, 1979), while the angiotensin-converting enzyme can inactivate bradykinin (Dover et al, 1974). It indicates there are functional interactions between the kallikrein-kinin system and the renin-angiotensin system.

The kinin present in urine is bradykinin. It was thought that kallikrein and bradykinin appearing in the urine were of plasma origin. However, experimental evidence showed that plasma bradykinin does not penetrate into the urine (Carone et

al, 1976). Rich stores of urinary pro-kallikrein have been found in the renal cortex (Nustad et al, 1975) which, after activation by, for example, trypsin or the Hageman factor, gives rise to kallikrein. Renal kallikrein is synthesised and secreted by cells of the distal tubule.

1.3.3 Mechanisms controlling renin release

In spite of the fact that the renin-angiotensin-aldosterone system has been studied in some detail, it still remains the centre of much experimental and clinical investigation. The focus of attention has been attempts to explained the underlying mechanism controlling the system, especially that controlling renin release. The theories and mechanisms controlling renin release, have been reviewed by Davis and Freeman (1976).

i) The adrenergic nervous system

It is widely accepted that the sympathetic nervous system is capable of modulating renin release both by a direct action through innervation of the juxtaglomerular cells and by indirect actions imposed on other intrarenal and hormonal factors known to control renin. Many studies have demonstrated that renal sympathetic nerves terminate directly on juxtaglomerular cellular membranes (Wagermark et al, 1968). Effort has recently been contributed to characterize the juxtaglomerular membrane receptors, alpha and beta-adrenergic receptors, involved in controlling renin release. Experimental evidence supporting the existence of intrarenal beta-adrenergic receptors has been produced (Vandongen et al, 1973; Johnson et al., 1976). The physiological role of the alpha-adrenergic receptor is still unclear.

ii) The renal arteriolar baroreceptor

Reduced renal perfusion pressure triggers an independent afferent arteriolar receptor, the 'baroreceptor' to stimulate prompt increases in renin release. It was suggested that this receptor is activated by changes in the tensile status of the

afferent arteriolar vascular tree (Blaine, et al, 1970). This increased renin release could be blocked by renal vascular paralysis with papavarine (Witty et al, 1971), but was not influenced by sympathetic and tubular, for example sodium concentration, factors (Blaine et al, 1971). This indicates that the renal arteriolar baroreceptor functions independently and locally. However, the precise cellular events linking altered arteriolar wall tension with release of renin are undefined.

iii) The glomerotubular feedback pathway

In mammals, the macula densa senses changes in the composition or flow of renal tubular fluid, which then influences glomerular filtration rate in the same nephron through a feedback mechanism (macula densa hypothesis).

The relationship between tubular fluid volume, the concentrations of ions and modifications in juxtaglomerular apparatus activity has been recognised for many years. In 1953, Hartroft observed that the activity of the juxtaglomerular apparatus appears to depend primarily on the balance of electrolytes (Hartroft, 1953). It recalled to her the early suggestion of Goormaghtigh that the macula densa is a sensor which could monitor the composition of the fluid in the lumen of the distal tubule and inform the preglomerular arteriole (Goormaghtigh, 1939). With the juxtaglomerular myoepithelioid cells established as a source of renin, the old speculations acquired a new and potentially important significance for the regulation of renin secretion.

In spite of many suggestions, the precise signal perceived by the macula densa remains unclear. The concentration of sodium in the distal tubule was thought to be the factor that supresses renin release by interaction with the macula densa. Thus it was suggested that an excess of sodium had an inhibitory effect, whereas a deficency had a stimulating influence. However, in rat, Kotchen et al showed that plasma renin activity and renal renin content were suppressed by both sodium chloride and potassium chloride, but not by sodium bicarbonate or potassium

bicarbonate. In their view, it was chloride that played the crucial role in the modification of renin release rather than sodium (Kotchen et al, 1976). Although evidence from further investigations indicated that the renin response was not primarily chloride mediated (Stephens et al, 1978), increasing attention has been paid to the important role that chloride plays in the glomerular feedback pathway.

iv) Other suggestions for the control of renin release

Recent studies have revealed more and more possible factors that also correlated with the control of renin:

Calcium:

Ca^{++}, which has been implicated in several secretory processes, could supress renin release in dogs (Kotchen et al, 1974; Watkins et al, 1976), and in human (Kischet et al, 1976). It was suggested that Ca^{++} exerts a direct action on juxtaglomerular cells to modify renin release. Again, the precise cellular site is unclear though the possibility that renin release is affected by some function of membrane Ca^{++} flux or intracellular binding has been considered (Zehr et al, 1980).

Prostaglandins:

There is evidence suggesting that prostaglandins and their synthetic intermediates might modify renin secretion by altering renal hemodynamics, or renal blood flow distribution. It was said that the modification was also possibly related to renal electrolyte handling, or that prostaglandins might act directly on the juxtaglomerular cells (Zehr et al, 1980). The long-term implications of these observations are unclear.

1.3.4 Conclusion

It can be predicted that new insights will continue to evolve. To clarify the precise mechanisms controlling renin release, obviously, a great deal of effort is

needed. A hypothesized "final common signal" responsible for renin release has yet to be identified.

The revelation that a relationship exists between the kidney and the adrenal cortex, between the action of renin and of angiotensin on aldosterone secretion, has stimulated much work on the circulating renin-angiotensin-aldosterone system. The juxtaglomerular apparatus has come to be known as one of the physiological regulatory centres in the kidney and the body endocrine system.

However, the attention of many research workers is now focused on how cells of the JGA interact with each other and with adjacent cells in the kidney. There is special interest in trying to understand whether the glomerular peripolar cell, which might release its product into urine, is another component of the juxtaglomerular complex. If so, the questions arise: what substance does it secrete, what physiological role it plays and how it is functionally related to the other components in the JGA or other body system.

To elucidate the function of the peripolar cell, of couse, great effort is need. Obviously, the identification of the secretory substance in the granules of the perpolar cells will be crucial in solving the puzzle of its role in renal function.

1.4 Monoclonal antibody technology and scope of the project

1.4.1 Monoclonal antibody technology

Specificity and sensitivity are the two major features of immunological methods. The establishment of monoclonal antibody technology (Kohler & Milstein, 1975) is one of the most important contributions to immunological studies. Over the last fourteen years, this new immunological technique has infiltrated into almost every, field of research especially, life science. It has made available reagents specifically

directed against a wide range of substances. The application of these reagents to detect, identify, isolate and purify different molecules in complex biological fluids or cells is rapidly revolutionizing biological, immunological and medical research, with its unique features (Campbell, 1984):

i) Specificity

The antigenic determinant is the binding site of the antigen for the antibody. A polyclonal antiserum preparation contains a family of antibodies directed against the antigen, as well as antibodies which do not react with the antigen of interest. Thus, extensive cross reaction may occur between antibodies and substances which have similar determinants. This can be avoided by the use of monoclonal antibodies which contain a single antibody specificity and one immunoglobulin isotype. Though there are exceptions in which crossreaction could happen if two unrelated tissue or cells or macromolecules possess a shared epitope, the monospecificity reduces non-specific background crossreactivity so that techniques such as immunohistochemical localisation become much more precise.

ii) Affinity

In the applications of immunological techniques, the antibody affinity required depends on the purpose for which it will be used. High affinity will be good for immunohistochemistry and assay systems where maximum sensitivity is required. However, in some cases, it will be difficult to work with if the antibody affinity is too high, such as protein purification by immunoaffinity chromatography where more delicate elution procedures are needed. It is necessary, and also possible to select out an antibody of the required affinity by the monoclonal antibody technology.

iii) Standardisation and unlimited source

Monoclonal antibodies provides the opportunity for standardisation. A monoclonal antibody with its monospecificity, monoaffinity and monosensitivity

can be used as a standard reagent for work between different laboratories. This will very much simplify the experimental procedure of the conventional immunological methods by saving the work required to standardise antisera, which vary between animals and even between different serum samples taken from same animal. In addition, hybridomas which inherit the characteristic of immortality of growth from the parental tumor cell line, are a permenant source of antibody. Therefore, once a clone is established, it will produce an identical antibody in unlimited amounts.

iv) Antigen requirement

The production of conventional antiserum usually requires that the antigen be substantially purified before immunisation of the animal. Purification of antigen may be very complicated and time consuming. In addition some of the minor components of a complex mixture may be impossible to purify by conventional biochemical techniques. One of the major advantages of the monoclonal antibody technique is that, the antigen does not need to be pure for immunisation. In theory, any appropriate antibody, can be selected by cloning the spleen-myeloma hybrids selection being on the basis of a screening assay that distinguishes the antibodies. A good example has been shown in the case of alpha interferon (Secher & Burke, 1980). This feature of the technique should therefore enable the production of monoclonal antibodies against peripolar cells despite the fact that peripolar cells are such a tiny component of kidney.

1.4.2 Aims

To study the juxtaglomerular and peripolar cells, the present project employs the monoclonal antibody immunological technique. The main aim is to produce monoclonal antibodies to the granular peripolar cells. To this end, mice have been immunised with purified sheep glomeruli which contain abundant granular peripolar cells. Spleen cells from these mice have been hybridized with myeloma cells and cloned. Hybrid cell lines which produces monoclonal antibodies to the peripolar cells or other important structure in the glomerulus are selected. The monoclonal

antibodies can be used as a tool to study the juxtaglomerular and peripolar cells. Antibodies specific for the granular peripolar cells can be used not only as a marker to identify, enumerate and localise the peripolar cells, but can also be used, in the long-term as a chromatographic ligand to purify the secretory substance of peripolar cells.

Part 2 MATERIALS & METHODS

2.1 General materials

2.1.1 Animals

Sheep and lambs

Sheep kidney tissue was obtained from 'normal' sheep and lambs freshly killed. The animals, from which the kidneys were removed, were killed for the purposes of: 1). meat for food (The Abattoir, Glasgow).

2). teaching demonstration (The Vet. School, Glasgow).

Mice

9 normal BALB/c strain mice were from the Animal House, Department of Biochemistry, University of Glasgow

2.1.2 Tissue and sections (sheep and lambs)

Frozen tissue

Blocks of full-thickness kidney cortex, measuring 10 x 8 x 3 mm were frozen in liquid nitrogen and kept at -70 ^{0}C. 24-48 hours before each assay, 5 μm sections were cut from the frozen tissue, mounted on washed plain glass slides, and kept at 4 ^{0}C.

Routine processing of paraffin-embedded tissue

Blocks of full-thickness kidney cortex, measuring 10 x 8 x 3 mm, were fixed in neutral buffered formalin and in Bouin's fixative. After being embedded in paraffin wax, 4 μm serial sections were cut and mounted on poly-L-lysine coated glass

slides.

Fresh tissue

After tissue was taken for histological examination, the remaining tissue was stored at 4 ^{0}C for periods of about 3-5 days and used as a source of antigen.

2.1.3 Reagents and chemicals

See Appendices, page A-2 to A-5.

2.2 Morphological Assessments

2.2.1 Quantitation of granular peripolar cells in sheep kidney

i) Preliminary examination

As soon as kidney tissue was obtained, a preliminary histological examination was made to assess the approximate number of peripolar cells in the kidney. Blocks of full-thickness of fresh kidney tissue (mainly cortex), measuring 10 x 8 x 2 mm were put in a histological cassette and immersed in 1000 ml 0.9% (w/v) saline. This was put in a SAMSUNG b30 microwave oven for 5 minutes. The final temperature of the saline in the container was around 70 ^{0}C. The tissue was then dehydrated, through grade alcohols and toluene respectively. After the tissue was embedded, 4 μm paraffin sections, 2 or 3 sections of each case, were cut and mounted on poly-L-lysine coated glass slides. The sections were dewaxed in xylene for 5 minutes, hydrated through graded alcohols and stained with toluidine blue, periodic-acid Schiff technique, and MSB trichrome stain (Lendrum et al, 1962).

By light microscopy, all of the glomeruli (at least 200) in the sections were examined to determine the number of granular peripolar cells. The peripolar cell index (PI) (Alcorn et al, 1984) is the number of glomeruli in which at least one peripolar cell could be identified, expressed as a ratio of the total number of glomeruli examined.

ii) Serial sections

Fifty Bouin's or formalin fixed, paraffin-embedded serial sections were dewaxed in xylene for 5 minutes, hydrated through graded alcohols and stained with the MSB trichrome stain.

Light microscopy was used to examine the entire structure of individual glomeruli by inspection of serial sections. At least 20 glomeruli in the outer one third of the cortex of each kidney were examined in their entirety by following individual glomeruli through the serial sections. The number of glomeruli in which at least one granular peripolar cell could be identified, as a percentage of the glomeruli examined, was expressed as **'PIs'** (peripolar cell index by serial sections). Eight separate kidneys were examined, each from a different animal.

2.2.2 Distribution of granular peripolar cells in sheep kidney

The study of the distribution of peripolar cells in sheep kidney cortex was based on the method of Gardiner and Lindop, used for the study of peripolar cells in human kidneys (1985). Briefly, Bouin's fixative fixed, paraffin wax-embedded and MSB trichrome stained random sections of kidney were studied using light microscopy, for the distribution of granular peripolar cells. The arcuate vein was used as a marker of the cortico-medullary junction. The position of each granular peripolar cell on the sections was recorded by measuring the distance, in fields of a x 40 objective, from the cortico-medullary junction to the peripolar cell ('A', Fig.

2.1). Then, the distance from the cortico-medullary junction to the surface of the kidney was measured along the same straight line ('B', Fig. 2.1). The distribution of peripolar cells in different layers of the cortex was expressed as the ratio, 'A/B x 100%'. Six kidneys from different animals (PIs > 70%) were examined for this purpose. For each kidney, 100 glomeruli which contained granular peripolar cells, examined evenly through all layers of the cortex, were recorded .

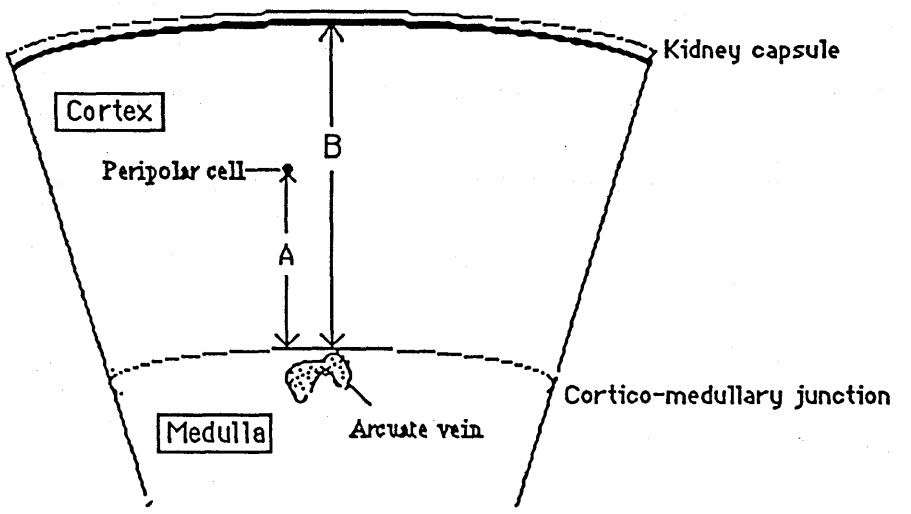


Fig. 2.1 The position of a peripolar cell across the renal cortex was recorded and expressed as a ratio 'A/B x 100%'.

'A': The distance from the cortico-medullary junction to the glomerulus.

'B': The total thickness of cortex from cortico-medullary junction to the capsule.

2.2.3 Examination of the isolated glomeruli

The glomeruli isolated either by enzymic digestion, or mechanical disruption, or microdissection were fixed in Bouin's fixative and embedded in paraffin wax. 5 μm random sections were cut, stained with different stains such as toluidine blue,

periodic-acid-Schiff and MSB trichrome stain. The morphology of the isolated glomeruli was examined.

For a more detailed study of the microdissected glomeruli preparation, fifty to sixty 5 μm serial sections of glomeruli isolated from each of 8 kidneys were cut and mounted on poly-L-lysine coated glass slides for light microscopy. Each glomerulus was examined in its entirety by following the structure of individual glomeruli through the serial sections. Granular peripolar cells in microdissected glomeruli were quantitated by determining the number of glomeruli in which at least one granular peripolar cell could be identified, as a percentage of the total glomeruli examined. This was expressed as '**PIms**' (peripolar cell index of the microdissected glomeruli by serial sections). At least 20 glomeruli from each kidney were examined. In addition, the percentage of glomeruli in which the Bowman's capsule remained intact was recorded and expressed as the 'Bowman's capsule index' (**BcI**).

2.2.4 Detection of antibody

i) Elisa

A goat anti-mouse IgG, heavy and light chains (SERA Lab.), was used to monitor total immunoglobulin (IgG+IgM) secreted by hybrid cells. A rabbit anti-mouse IgG (Fc specific, JACKSON) was used to monitor selectively IgG.

One or other of these "monitoring" antibodies was diluted to 10 μg/ml in coating buffer and 100 μl/well plated on a 96-well Dynatech micro-elisa plate before incubation at 4 ^{0}C overnight. After washing 4 times with tris-tween buffer by immersing the plate, flicking out the wash buffer, and knocking dry, the remaining binding sites on the plate were blocked by adding 150 μl of blocking buffer, 0.5% (w/v) bovine serum albumin (BSA) in PBS, to each well. This was incubated at room temperature for 20 minutes, and then washed 4 times in tris-tween buffer. It

was then ready for applying samples.

The samples tested were the supernatants from hybridoma cultures, which were used without dilution. 100 ul of sample was added to each well and incubated at 4^0C for 2 hours.

Antibodies secreted into the culture supernatants by hybridomas were quantitated by comparison with standard amounts of commercial mouse immunoglobulins, at concentration of: (a) Mouse IgG (chrompurified, JACKSON Lab.): 100, 80, 60, 40, 20, 15, 10, 5, 2 and 1 ng/0.1 ml diluted in RPMI containing 20% FCS, pH 7.2, for the quatitation of IgG; and (b) Mouse IgM (monoclonal antibody, chrompurified, Biochem. Dept. University of Glasgow): 5000, 2500, 1000, 500, 100, 75, 50, 10, 5, 2 and 1 ng/0.1 ml diluted in RPMI containing 20% FCS, pH 7.2, for the quatitation of IgM. Negative controls were provided by using culture medium, the culture supernatants of spleen cells or X63.Ag.653 myeloma cells.

After incubation with standard antibody or hybridoma samples, the plate was washed. 100 ul of rabbit anti-mouse immunoglobulin (Fab_2) horseradish peroxidase conjugated diluted 1:10,000 in 0.05% (w/v) BSA in PBS, was then added to each well and incubated at room temperature for 30 minutes. Following 5 washes in tris-tween buffer, 100 ul of substrate, orthophenylene diamine (OPD) diluted to 0.4 mg/ml in McIlwaine's buffer containing 0.32 ul/ml hydrogen peroxide, was added and incubated for 30 minutes in the dark at room temperature. The reaction was stopped by adding 100 ul per well of 4N sulphuric acid, and the optical density was read on 'Multiscan' spectrophotometer, 492 nm.

ii) Immunofluorescence

Preparation of tissue sections

Cryostat sections: 24-48 hours before screening, 5 μm cryostat tissue sections were cut and stored at 4 ^{0}C. After 30 minutes at room temperature, the sections

were fixed in acetone for 15 minutes.

Paraffin sections: following 8 minutes dewaxed in xylene, the sections were hydrated through grade alcohols and into water.

Procedure

The sections were rinsed in PBS for 30 seconds at room temperature, and treated with pontamine sky blue 0.5% (w/v) in PBS, pH 7.4, for 20 minutes which stains the elastic tissue red and diminishes background. This was washed off by flooding with PBS for 30 seconds. To reduce nonspecific reaction, the sections were blocked with 5% (w/v) BSA in PBS. Excess BSA was drained off, and the sections were treated with the samples to be tested, 100 ul per section, and incubated in a full humidity environment for 1 hour (half an hour extra for paraffin sections) at room temperature. For screening serum, the samples were diluted in 5% (w/v) BSA in PBS as: 1:30, 1:100, 1:200, 1:400, 1:800 and normal mouse serum diluted in the same way was used as the negative controls. For screening hybridoma culture supernatants, medium was used without dilution and the negative control was fresh culture medium, and the culture supernatant in which spleen cells or 653 myeloma cells had been cultured alone.

After the incubation, the excess sample was removed by washing with PBS and the sections were treated with FITC conjugated goat anti-mouse immunoglobulin (polyvalent, Sera-Lab.) diluted 1:40 (or FITC conjugated rabbit anti-mouse immunoglobulinin, DAKO, diluted 1:25) in 5% (w/v) BSA in PBS, and incubated for 30 minutes at room temperature. For antiserum or polyclonal antibody supernatant assays, 1% (v/v) normal sheep serum was added to the FITC preparation to block possible cross reactions. Finally, the sections were washed with PBS, then drained, wiped and mounted in 'Citifluor' mounting fluid.

2.2.5 Localisation of antibody-binding sites on sheep kidney sections

To localise histologically the positive immunofluorescence, the following methods have been developed:

i) Contrast microscopy

Positive immuno-fluorescence on kidney sections was marked on the glass cover slip with a 'Lumocolor 318' marking pen. The section was then examined with a Leitz Orthoplan contrast microscope (Germany, phase and interference, IC7 513406 filter). The results were compared histologically and recorded by photography.

ii) Immunohistochemical staining

Immunofluorescent immunohistochemical staining

Tested on paraffin sections, procedure see section 2.2.4.

Alkaline phosphatase enzyme linked immunohistochemical staining

5 µm thick Bouin's fixed, paraffin-embedded kidney tissue sections, were dewaxed and cleared to water. To reduce nonspecific background staining, the sections were treated with 5% (w/v) BSA in PBS for 2 minutes to block the antigenic binding sites, and the excess was drained. 200 µl of culture supernatant containing the B1-3B3 monoclonal antibodies to be localised, was applied per section. The same volume of control culture medium and a commercial monoclonal IgM antibody (Leu 7) diluted 1:5 (v/v) in 5% (w/v) BSA in PBS were used as the negative and background controls. After 1 hour incubation in a humidity box at room temperature, the samples were washed with 6 changes of PBS over 30 minutes. Then, the sections were treated with 300 µl alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:15 (v/v) in 5%

(w/v) BSA in PBS, and incubated for 90 minutes at room temperature. This was washed in PBS with 6 changes over 30 minutes. At this stage, the reaction was visualised by adding freshly prepared substrate, 0.5 mM levamisole, 1.33 mM fast red violet and 0.54 mM naphthol AS-MX dissolved in 2 drops dimethyl formalide then in veronal acetate buffer (filtered in dark prior to use). After 10 minutes, the sections were washed with PBS, tap water and fixed in buffered formalin for 10 minutes. Following a thorough wash in tap water, the sections were counterstained with haematoxylin for 15 seconds, washed in tap water, STWS tap water and mounted in 'Aquamount' mounting fluid (BDH Ltd.).

PAP immunohistochemical staining (VECTASTAINR ABC Kit)

Paraffin-embedded sections were dewaxed in xylene for 10 minutes, then rehydrated though graded alcohols and water. At this stage, endogenous peroxidase activity was removed by immersing the sections in 1.67% hydrogen peroxide in methanol for 30 minutes. This was washed off with tap water, distilled water and phosphate buffered saline, following which the sections were treated with 2% bovine serum albumin, diluted in PBS for 10 minutes to block the antigen binding sites on the tissue sections. The excess was drained off, and the culture supernatant containing the monoclonal antibodies secreted by B1-3B3 cell hybrids was applied. The slides were then put into a humidity box and left over-night at 4^0C. After the incubation, the excess supernatant was washed off with 3 changes of PBS over 30 minutes. The sections were then treated with secondary antibody, the biotinylated anti-mouse immunoglobulin (VECTASTAIN^R ABC Kit, PK-4002) at a dilution of 0.45% (v/v) in PBS for 30 minutes at room temperature. This was washed off with 3 changes of PBS over 30 minutes. Finally, the sections were treated with VECTASTAIN^R ABC Kit reagents A+B, the avidin DH, biotinylated horseradish peroxidase complex, diluted 0.9% in PBS for 30 minutes at room temperature. After 3 washes with PBS over 30 minutes, the reaction was visualised by immersing the slides in a 0.05 % (w/v) solution of diaminobenzidine tetrahydrochloride in 0.1 M tris buffer, pH 7.2, which also contained 0.01%

hydrogen peroxide. Following a thorough wash in tap water, the sections were counterstained with haematoxalin, before dehydration through graded alcohols and xylene, and were mounted in the Aquamount mounting fluid (BDH Ltd.).

Negative controls were treated in the same way, with the exception that the primary antibody was replaced by culture medium, RPMI containing 20% FCS, from the control well of the culture plate.

2.3 Preparation of Antigen

2.3.1 Isolation of glomeruli

i) Enzymic digestion

Collagenase

The outer 1/3 of the kidney cortex was peeled from the kidneys with a domestic metal "vegetable peeler" and immersed in RPMI 1640 65% (v/v) in Krebs buffer, pH 7.4, at room temperature. The tissue slices were then pressed through a 1.0 mm^2/pore steel mesh with a spatula. The mince was weighed and divided into six portions each 3g in 6 universal flasks. These were treated with freshly prepared collagenase solutions: 0.025%, 0.0083%, 0.0033%, 0.0017%, 0.00083% and 0.00033% (w/v) respectively, dissolved in the RPMI 1640/Krebs buffer, pH7.4. The total volume in each tube was 6 ml. During a period of 30 minutes of incubation in water bath at 37 ^{0}C, the glomeruli were examined every 5 minutes with a light microscope (x 100) to check progress of the digestion. Each of the preparations was mixed 30 times by aspiration with a pasteur pipette, and then centrifuged at 175 g for 5 minutes. The supernatant was removed. To stop the digestion, 20 ml of ice-cold Krebs buffer was added immediately to the sediment remaining in each tube. Resuspended pellets were then filtered through a 350 μm^2/pore nylon gauze. The filtrates were centrifuged at 700 g for 10 minutes. For histological examination, both sediments and the unfiltered mince on the gauze were

fixed in Bouin's fixitive.

Based on the procedure above, experiments were modified and repeated using different mesh pore sizes (1.0 mm^2, 0.8 mm^2, 0.65 mm^2, 0.23 mm^2), incubation periods (5 to 40 minutes), and the size of filter gauze (0.356 mm^2, 0.2 mm^2).

Trypsin

The outer 1/3 of the kidney cortex was peeled at room temperature. The tissue slices (about 25g) were then pressed through a 1.0 mm^2/pore steel mesh with a spatula. The mince was collected and 20ml of the ice-cold RPMI 1640/Krebs buffer was added. The suspension was further forced through a steel mesh (0.5mm^2/pore) attached to a 35 ml syringe. After aspirating 30 times with a 5ml syringe, and allowing the fragments to settle, the excess buffer was removed. The pellets were divided equally into 6 calibrated conical tubes then washed 3 times with small volume of ice-cold RPMI 1640/Krebs buffer. The tissue was treated with freshly prepared trypsin solutions: 0.625%, 0.250%, 0.063%, 0.013%, 0.006% and 0% (w/v) respectively, dissolved in the RPMI 1640/Krebs buffer, pH 7.4. The total volume in each tube was 8 ml. During incubation in water bath at 25 ^{0}C, samples were taken every 5 minutes to check progress of the digestion with a light microscope (X100). When approximately 10% of the glomeruli were free of the tissue in the greatest trypsin concentration, the digestion in all of the samples were stopped by adding soybean trypsin inhibitor (SBTI), 5% (w/v) dissolved in ice-cold RPMI 1640/Krebs buffer, in amounts equal to that of the trypsin added for each tube. After 15 minutes, these were centrifuged at 700 g for 5 minutes and the supernatants were removed. The sediments were then fixed in Bouin's fixative for histological examination.

ii) Mechanical disruption

In this method, several different pore size steel meshes were used to disrupt the tissue mechanically, in an attempt to isolate individual glomeruli.

The outer 1/3 kidney cortex was peeled with the metal peeler. The tissue slices were weighed, and pressed through a 1.0 mm^2/pore steel mesh with a spatula. The mince was collected in a beaker containing 5ml of the ice-cold RPMI 1640/Krebs buffer. This was further squeezed through a 0.6 mm^2/pore mesh and 60 ml of ice-cold RPMI 1640/Krebs buffer was added. The suspension was aspirated 30 times with a 5ml syringe, and filtered through 0.356 mm^2/pore nylon gauze. The filtrate was then centrifuged at 700 g for 5 minutes. Both the sediment and the tissue remained on the gauze were fixed in Bouin's fixative for histological examination. The experiments was repeated using different pore sizes of metal meshes (1 mm^2, 0.8 mm^2, 0.65 mm^2, 0.23 mm^2), and nylon gauzes (0.356 mm^2, 0.2 mm^2).

iii) Microdissection*

Using two dissecting blades which were combined together, the cortex outer 1/3 of the kidney was peeled. The tissue slices between the two blades, about 0.3-0.5 mm in thickness, were put in a white plastic weighing dish set on ice. With a Olympus SZ dissecting microscope, 10 x 2.0 magnification, the glomeruli were dissected individually and carefully by cutting off the tubules, vessels and some connective tissue with a micro-scalpel and micro-forceps. The individual glomeruli were collected into a Eppendorf tube containing ice-cold RPMI 1640/Krebs buffer. This was then pelleted at 175 g for 5 minutes, after which the supernatant was removed with a pasteur pipette. The glomeruli were washed by resuspending them in cool Krebs buffer, and centrifuging at 175 g for 5 minutes. The supernatant was discarded. After 3 washes, the glomeruli were stored at -20 ^{0}C in 50% (v/v) glycerol in Krebs buffer. 50-60 of the microdissected glomeruli from each kidney used were fixed in Bouin's fixative for histological examination.

Kidney tissue from 29 sheep or lambs was used to isolate glomeruli by microdissection.

2.3.2 Granule fractionation

12.2 g of tissue from the cortex of three fresh sheep kidneys was finely cut with scissors. This was homogenized in a Potter-Elvehjem homogenizer, in 48.8 ml of 0.45M sucrose, 5mM tris, pH 7.4. The homogenate was then centrifuged in a 'MSE18' (8 x 50 rotor), at 480 g for 10 minutes. The supernatant, 50 ml was then layered onto 6 tubes each containing a discontinuous sucrose gradient consisting of 7.5 mls of 1.2 M (1 ml), 1.3 M (1 ml), 1.4 M (1 ml), 1.5 M (1.5 ml), 1.6 M (1.5 ml) and 1.7 M (1.5 ml) sucrose dissolved in 5 mM tris buffer, pH 7.4, and centrifuged at 49640 g for 2 hours, using a SW 40 swing bucket rotor, (Beckman). Material which accumulated at the interfaces was removed. Each band were combined from the six tubes and diluted in distilled H_2O to a total volume of 20 ml. These were then centrifuged at 105,540 g, using a Ti 50 rotor (Beckman). The pellet samples were sent for morphological study by electron-microscopy in the Pathology Department, University of Glasgow.

2.4 Production of Monoclonal Antibody

2.4.1 Immunization

i) Animals

3 BALB/c strain mice were immunised as a group. There have been three groups, A, B and C, totalling 9 mice immunized in this study.

ii) Antigen

Microdissected glomeruli from the kidneys which had been proved from histological evidence (see morphological assesments) to have a rich supply of granular peripolar cells (mostly, the PIs > 70%) were selected as antigen. 50-70 glomeruli (about 100 ug protein) were injected per mouse.

iii) Primary injection

Glycerol was removed from the microdissected glomeruli by washing four times with 1ml cold saline and centrifuging at 8,730 g for 30 seconds. The sample in 0.1 ml of saline was then sonicated in a bath sonicator for 2 minutes and cooled on ice for 2-3 minutes. Then, an equal volume of complete Freund's adjuvant (CFA) was added and the sample was sonicated again for 2 minutes. After thoroughly mixing for several minutes, the glomeruli formed an emulsion in the CFA. 0.2 ml of the emulsion was injected intraperitoneally into each of 3 mice.

iv) Booster injections and protocol for serum analysis

Four weeks after the first immunization, the immune response of the mice was boosted by a further injection of antigen. The procedure was the same as for the first injection, except the adjuvant was replaced with incomplete Freund's adjuvant (IFA).

Four days after the second injection, the immunised mice were bled from the tail vein and the immune sera were screened by immunofluorescence to establish which mice were producing antibodies specific for the glomerular antigen. Mice producing strong staining of glomerular epithelial cells, with little evidence of irrelevant antibodies (i.e. antibodies to tubules or interstitium) were chosen for fusion. Other mice were either given additional injections of antigen and used for a subsequent fusion, or were killed.

By the stage of this study when immune response of C group mice was being assessed, contrast microscopy had been developed as an additional method (section 2.2.5) enabling us to identify more clearly cellular sites showing positive immunostaining. Immunostaining was first identified by fluorescence microscopy, and the precise location at which antibody bound was then examined using contrast microscopy.

2.4.2 Production of spleen-myeloma cell hybrids

The spleen cells from five immunised mice have been fused with myeloma cells in this study.

i) Preparation of mouse spleen cell suspension

All procedures were under sterile conditions at room temperature. Ether anaesthesia was used and, the mouse was bled by cardiac puncture prior to the removal of its spleen. The spleen was removed aseptically and immersed in 5ml serum-free RPMI, then transferred into a plastic bijoux bottle with 5 ml of RPMI containing 20% FCS. For all subsequent procedures, RPMI contained penicillin, streptomycin and amphotericin B with foetal calf serum at the concentrations indicated. The spleen was transferred again into a petri dish containing approximately 5 ml RPMI which contained 20% FCS, and the adherent tissue of the spleen was removed with scissors and forceps. With a 21-gauge needle attached to a 5ml syringe and sterile forceps, the cells were teased from the splenic capsule. Using a syringe without needle, the cell suspension was drawn up and down 2-3 times. Finally, this was expelled through a needle into a centrifuge tube, then centrifuged at 1300 g, for 5 minutes. The pellet was resuspended in residual supernatant, and the spleen cells washed three times in 10ml of RPMI containing 10% foetal calf serum (FCS) and centrifuged at 1300 g, for 5 minutes. Finally the spleen cell pellet was resuspended in the residual supernatant, and 5 ml RPMI containing 20% FCS was added.

The spleen cells were counted after the contaminating red cells were lysed in 0.9% ammonium chloride (w/v) for 10 minutes. It is generally accepted that there are about 10^8 lymphocytes per spleen.

ii) Preparation of mouse myeloma cells

X63.Ag8.653, the HAT sensitive, non-secreting, BALB/c strain mouse myeloma B cell line was chosen for the cell fusions, as the parent neoplastic cell donor. The myeloma cells were recovered from the liquid nitrogen when the project started and the cell line was maintained routinely in RPMI containing 5-10% (v/v) FCS. One week before fusion, the cells were transferred successively into culture medium containing 10%, 15%, and finally 20% FCS immediately before fusion with spleen cells. To ensure exponential growth at the time of fusion, the myeloma cells were suspended at a concentration of between 10^4 and 5 times 10^5 cells per ml in culture, 24 hours before fusion.

iii) Fusion of spleen and myeloma cells

The X63.Ag8.653 myeloma cells were centrifuged at 1300 g for 5 minutes at room temperature. After the supernatant was removed, the cells were resuspended in residual supernatant, 5 ml RPMI containing 20% FCS was added and the cells were counted using a haemocytometer.

10^8 spleen cells suspended in 5ml RPMI containing 20% FCS were combined with $3x10^7$ X63.Ag8.653 myeloma cells in suspension and centrifuged at 550 g for 5 minutes at room temperature. The supernatant was removed. The pellet was resuspended by agitating the tube, and 2ml of 42% (w/v) PEG 4000 in RPMI at 37^0C was added. The same pipette, was used to ensure the cells remained in suspension for 30 seconds. After a further 30 seconds at room temperature, 5 ml culture medium was added dropwise over a period of 90 seconds, while constantly agitating the tube. Following a further rapid addition of 5 ml culture medium, the suspension was mixed by inversion, kept at room temperature for 2.5-3 minutes, then centrifuged immediately at 550 g for 5 minutes. The supernatant was decanted, and the cell pellet was resuspend in residual supernatant. 5ml culture medium, RPMI containing 20% (v/v) FCS, at 37 ^{0}C, was then added and the cell suspension was transferred to a 100 ml sterile bottle. The suspension was diluted

with RPMI containing 20% FCS to approximately 93 ml. 1ml volumes were aliquoted into 4 x 24-well Costar plates. The control wells contained either: spleen cells alone, approximately 50 µl diluted to 1ml; X63.Ag8.653 myeloma cells alone, approximately 50ul diluted to 1ml; or 1 ml RPMI containing 20% FCS free of cells.

After an incubation period of 3 hours at 37 ^{0}C, 5% (w/v) CO_2 in air, 1ml RPMI containing 20% FCS and sufficient hypoxanthine, thymidine and aminopterin to produce final concentrations of 0.1 mM, 0.16 mM and 0.004 mM respectively, was added to each well. The cultures were checked for contamination with micro-organisms after 24 hours, and then left undisturbed in the incubator for 14 days.

2.4.3 Maintenance and propagation of hybrid cells

Two weeks after fusion, the 96 wells of the 4 x 24-well fusion plates were given a quick examination to establish the extent of cell growth, using a inverted light microscope. The culture medium was then replaced with 1ml of RPMI containing 20% FCS and supplemented with hypoxanthine and thymidine but not aminopterin. To avoid disturbing the clones of hybridomas, the culture were handled very carefully when changing the culture medium. The hybridomas were checked twice or three times a week for contamination, pH (color of medium) and colony growth. When the culture medium is yellowing it was replaced with fresh HT medium, and the cells were transferred to new plates or flasks to grow when they were confluent.

2.4.4 Detection and localisation of antibody

Three weeks after fusion, was the earliest time at which hybridoma supernatants were tested for antibody production by immunofluorescence (on cryostat sections, see 2.2.4ii). The subsequent screenings depended upon the extent of cell growth. For the fusion B1 and C1, Elisa immunosorbent assay (see 2.2.4i) had been

developed as a method to detect antibody production prior to the immunofluorescence assay. For fusion B1, only the assay specific for IgG was available. For fusion C1, both the IgG specific assay and assay detecting either IgG or IgM were used. As the hybridoma cell numbers increased, antibody secreted into the culture supernatant was measured by the Elisa periodically. Cultures, in which cells were growing well and secreting antibodies, were tested for specific glomerular antibodies by immunofluorescence on cryostat kidney sections which contained abundant granular peripolar cells (PIs >70%).

For the fusion B1 and C1, contrast microscopy was used to identify those morphological structures reacting with antibody, as indicated by positive immunofluorescence. Those antibody samples reacting with areas of the kidney tissue structure which indicated the presence of antibodies of interest (i.e. the antibodies secreted by B1-3B3 hybridomas), were also tested with paraffin sections by immunofluorescence (section 2.2.4), alkaline phosphatase or peroxidase anti-peroxidase enzyme-linked immunoassays (section 2.2.5). These techniques improved the precision with which antibody-reactive sites on the tissue could be identified. Kidney sections from 6 different sheep or lambs had been used to localise the cellular antigenic binding sites of the antibodies secreted by the B1-3B3 hybridomas, by these methods.

For the negative control, samples were taken from the supernatants of, (1) spleen cells only; (2) X63.Ag8.653 myeloma cells only; and (3) plain culture medium only, and control wells.

2.4.5 Cloning cell hybrids by limiting dilution

i) Cloning cell hybrids B1-3B3

Isolation of hybridoma clones derived from a single spleen cell requires dilution of the culture to a theoretical 1 cell per 200 ul. At this extreme dilution, hybridomas

require a "feeder" layer of spleen cells for growth. A suspension of spleen cells was prepared as described above (section 2.4.2) and diluted to 1 x 10^5 cells per ml in RPMI containing 20% FCS (v/v) and HT.

The hybrid cells of culture B1-3B3 were counted, and diluted to 10 cells per ml. 100 µl aliquots were plated into the wells of a 96-well costar plate each well already containing $1x10^4$ spleen feeder cells in a volume of 100 µl of the same medium. The cells were maintained in a fully humidified incubator at 37 ^{0}C in an atmosphere of 5% (v/v) CO_2 in air.

After approximately 7 days, it is evident whether the wells contain single or multiple discrete clones of hybrid cells. Following a further 7 days in culture, the supernatants were removed from the wells where single hybridoma clones were present, and screened for the specific antibody by immunofluorescence on both cryostat and paraffin sections. Clones producing antibody reacting with the same glomerular structures were subsequently recloned twice. For the second and the third times of cloning these hybrids, the same procedure was used except the cell suspensions were diluted to four different concentrations: 40 cell per ml (4 cells/well), 20 cell per ml (2 cells/well), 10 cells per ml (1 cell/well) and 5 cells per ml (0.5 cell/well). Each of the 4 cell concentrations were plated out into a total of 24 wells in a 96-well plate. This is to ensure that single clones are obtained from some of these dilutions. Cell populations used for cloning were also maintained in 24-well plates and small flasks. Some of these cells were stored in the freezing medium at -120 ^{0}C using a MIC 15 Cryoson and stored in liquid nitrogen (section 2.4.7).

ii) Cloning hybrids C1-2C2

Cells of the C1-2C2 culture were cloned once following the same procedure. There was no further experimentation with this cell line because no antibodies of interest were produced after the first cloning process.

2.4.6 Large scale production of monoclonal antibody (B1-3B3)

After three cloning processes, the selected clone of the B1-3B3 was propagated in a large flasks in RPMI containing firstly 20% then 15% (v/v) FCS. Hypoxanthine and thymidine were omitted from the medium. At this stage, the cell line was monoclonal, producing a single type of antibody. The culture supernatants containing secreted monoclonal antibodies were collected twice a week and stored at -20 ^{0}C for further studies. Samples of the antibody-producing hybrids were frozen periodically and stored in liquid nitrogen (section 2.4.7).

2.4.7 Cryopreservation

Culture hybridomas which produced interesting antibodies were stored in liquid nitrogen:

5 X 10^6 cells were sedimented at 480 g for 5 minutes at 4 ^{0}C. Immediately the cells were resuspended in 1 ml of cold freezing medium, 10% (v/v) dimethyl sulphoxide (DMSO) in inactivated foetal calf serum, and the resulting suspension was transferred into a sterile 2 ml screw-cap vial and frozen down at -120 ^{0}C by a MIC 15 Cryoson. The vial was then transferred to liquid nitrogen for long term storage.

2.4.8 Sterility checks

Components which were used in cell culture were inoculated into Sabouraud fluid medium and into brain heart infusion broth and maintained for 3 weeks at 26^{0}C and 37^{0}C respectively. They were checked microscopically for fungal contamination, and by eye for bacterial infections.

Contaminated samples of fusion A2 and B1 cultures were sent to the Bacteriology Departmant, University of Glasgow, for further examination.

2.5 Applications of the monoclonal antibody-secreting hybrid cell line 'B1-3B3'

The mouse monoclonal antibody (IgM) raised to the cytoplasm of the macula densa cells of sheep were tested on the kidney sections of other species to study the crossreactivity between the species. Kidney tissue of different species included two normal rats (The Animal House, Pathology Deptartment, University of Glasgow), nine human including 3 normal, 3 with renal artery stenosis and 3 foetal human kidneys (Pathology Department, University of Glasgow).

Immunofluorescence, alkaline phosphatase and peroxidase anti-peroxidase immunocytochemical techniques were employed, as described previously (section 2.2) for sheep. In the immunofluorescence assays, the secondary antibody (FITC conjugated) preparation was tested both with and without adding normal sera (1% v/v of normal rat, human serum). In addition, kidney tissue sections of sheep and lambs were used as the positive control and the patterns compared.

Part 3 RESULTS

3.1 The granular peripolar cells in sheep kidney

Granular peripolar cells were identified, by light microscopy, in the hilar position of glomeruli as described originally by Ryan et al (1979). In many cases, the granules of the peripolar cells were closely packed and evenly distributed in the cells. Both small and large granules could be seen in one cell. They stained blue (on cryostat sections, they stained light blue) with toluidine blue, pink with periodic-acid Schiff and red with MSB stain. The MSB trichrome stain gave the best result for the identification of the granular peripolar cells on Bouin's fixed, paraffin embedded tissue sections in the present study (Fig. 3.1 & 3.2).

The structure of the peripolar cells and the granules in the cells were well preserved using routine formalin or Bouin's fixation on paraffin embedded tissue sections. Using microwave-fixed paraffin embedded tissue, the peripolar cells could still be identified satisfactorily for the purpose of preliminary examination in which all the peripolar cells in random sections were quantitated, identified by their cytoplasmic granules and anatomic location. The cellular structure was not as well preserved as that using routine formalin or Bouin's fixation. With cryostat sections, it is difficult to recognise the peripolar cells by ordinary light microscopy using routine histological stains, but they can be readily identified by contrast microscopy with or without histological staining. By phase contrast microscopy, the granules of the peripolar cells appear to be dark while the cytoplasm is light. Especially, the granules of the peripolar cells can be shown clearly on cryostat tissue sections using interference contrast microscopy. Hyaline droplets were sometimes seen in tubular cells and glomerular podocytes in some of the kidneys, and frequently found in those kidneys in which granular peripolar cells were prominent.

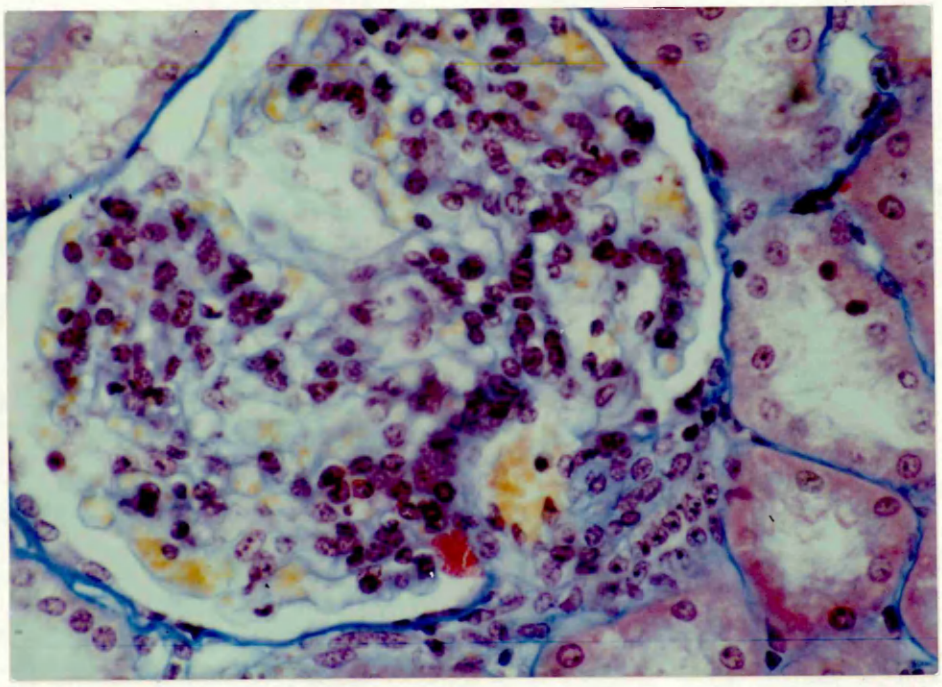


Fig. 3.1
Light micrograph, the granules of peripolar cell of sheep kidney were clearly shown using MSB stain, which were stained red on paraffin embedded tissue section. x250

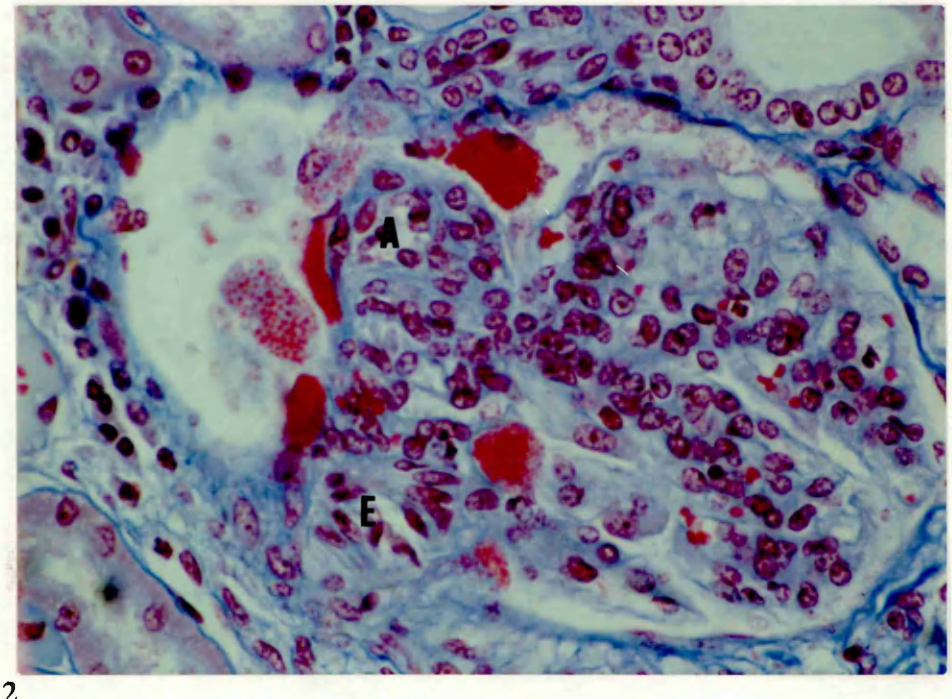


Fig. 3.2
One of the serial sheep kidney sections through the vascular pole, showing at least 7 big, heavily granulated peripolar cells (red, granular) in one glomerulus encircling the vascular pole of both afferent (A) and efferent (E) arterioles. The granular peripolar cell index (PIs) of this kidney is 100%. Paraffin embedded tissue, MSB stained, X250

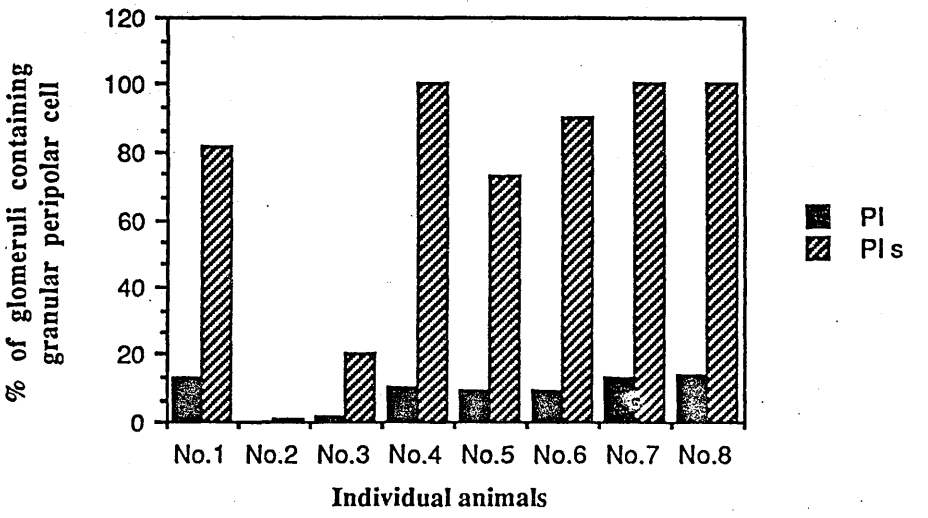
3.1.1 Quantitation

Among 22 kidneys studied, granular peripolar cells varied considerably in number and size. The number and size of cytoplasmic granules also varied. They were prominent in most of the sheep, as many as seven big, heavily granulated peripolar cells were found in one glomerulus encircling the vascular pole of both afferent and efferent arterioles (Fig. 3.2). However, the granules were usually small and sparse in lambs, in which the values of the peripolar indexes could be as low as zero. No significant difference was found between male and female animals. By random sections the peripolar cell indexes (PI) of 22 kidneys ranged from 0 to 14.46%. The mean was 3.85%.

By examining serial sections through an entire glomerulus, the peripolar cell indexes (PIs), which reflects the direct percentage of glomeruli containing the granular peripolar cell, ranged from 0% to 100%. Fig.3.3 shows the 'PIs' of 8 kidneys studied and the comparison with the corresponding 'PI'.

Fig. 3.3

Comparison of peripolar cell indexes from: random section (PI) and serial section (PIs)

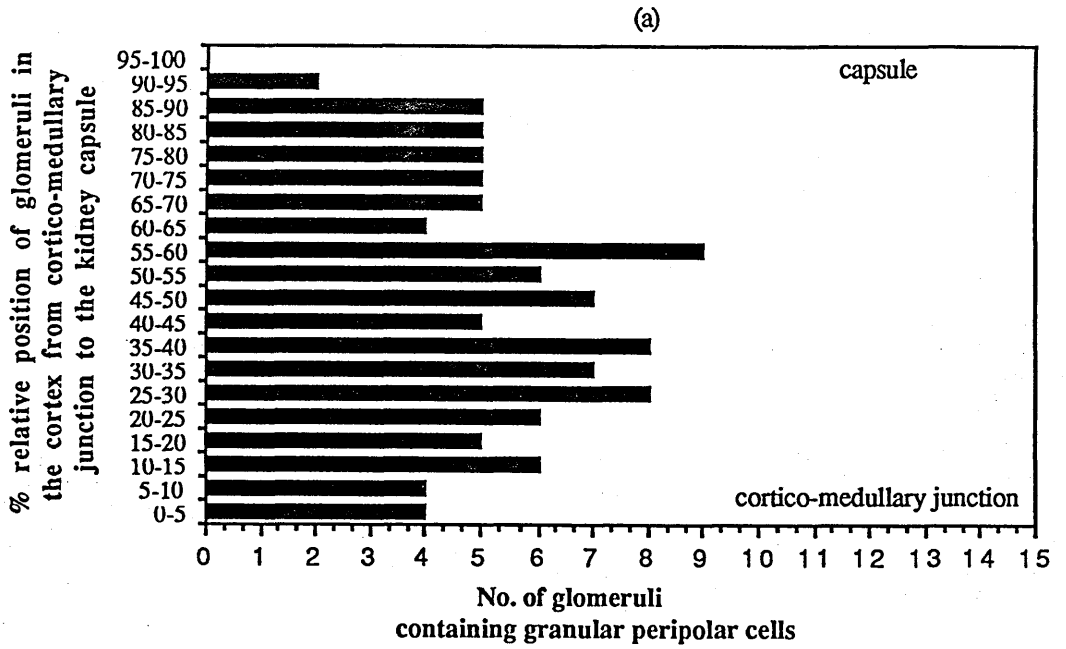


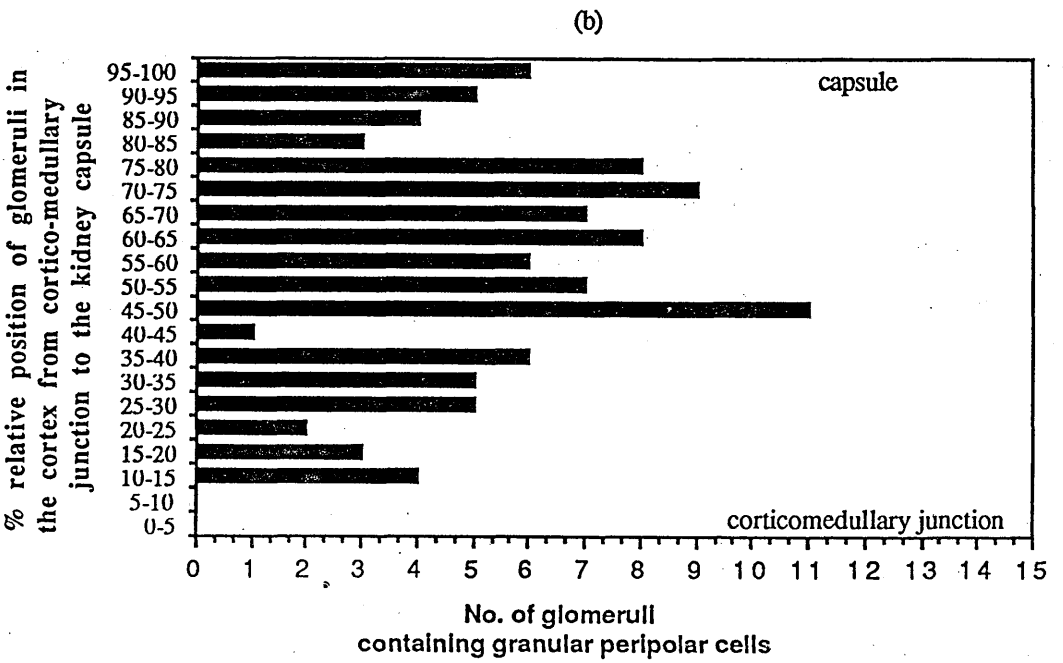
3.1.2 Distribution

The distribution of granular peripolar cells in the cortex of 6 sheep kidneys examined showed considerable variation, in spite of their relative prominence in the middle zone of most of the cases. 3 kidneys showed more peripolar cells in the outer cortex, but 2 had more peripolar cells near the cortico-medullary junction. In kidneys from 3 adult sheep, multiple and heavily granulated peripolar cells were found in the glomeruli near the cortico-medullary junction. Few granular peripolar cells were found in the most superficial zone of the sheep kidney cortex. (Fig. 3.4a-f)

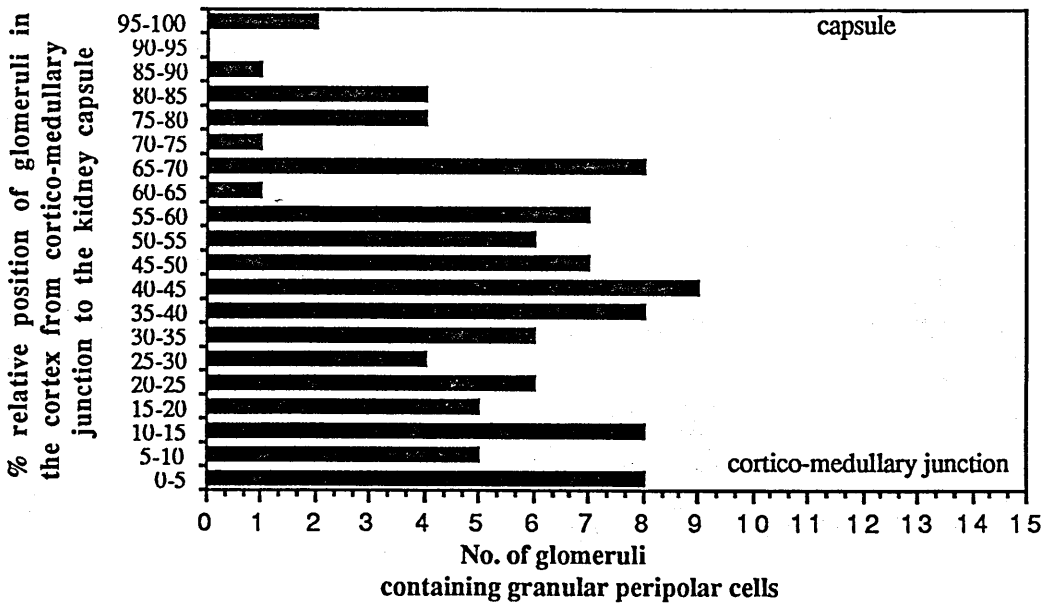
Fig. 3.4a--f

Peripolar cell distribution in different zones of cortex of 6 sheep kidneys

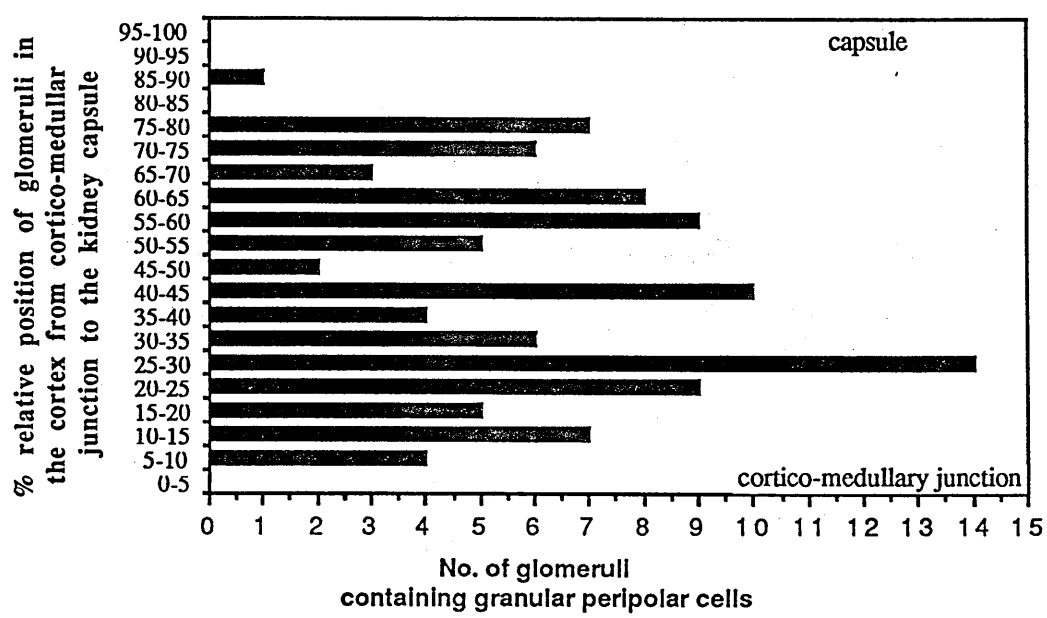




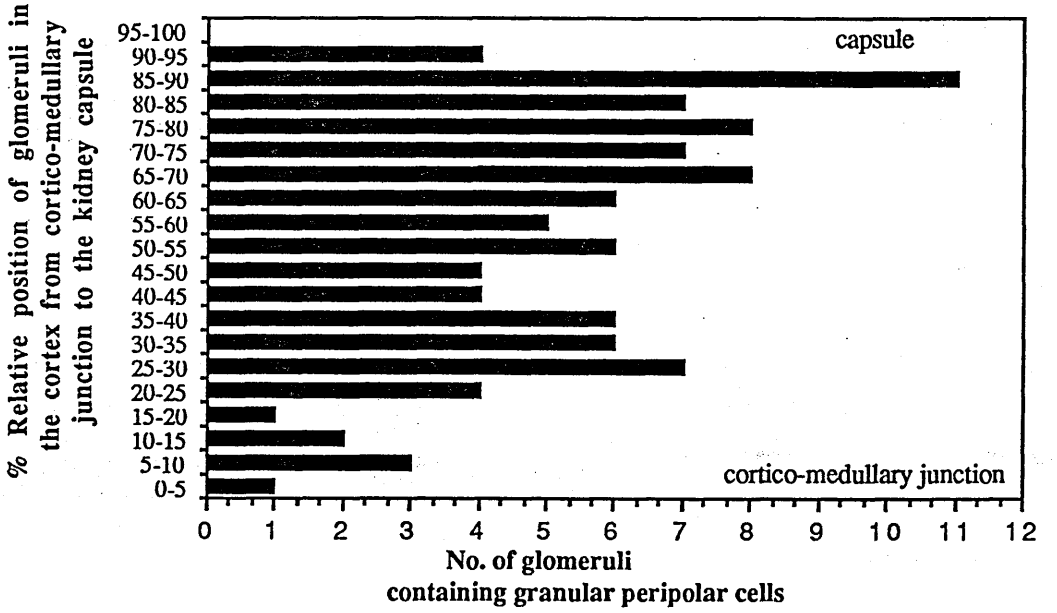
(c)


% relative position of glomeruli in the cortex from cortico-medullary junction to the kidney capsule
95-100
90-95
85-90
80-85
75-80
70-75
65-70
60-65
55-60
50-55
45-50
40-45
35-40
30-35
25-30
20-25
15-20
10-15
5-10
0-5
capsule
cortico-medullary junction
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
No. of glomeruli
containing granular peripolar cells

(d)


% relative position of glomeruli in the cortex from cortico-medullar junction to the kidney capsule
95-100
90-95
85-90
80-85
75-80
70-75
65-70
60-65
55-60
50-55
45-50
40-45
35-40
30-35
25-30
20-25
15-20
10-15
5-10
0-5
capsule
cortico-medullary junction
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
No. of glomeruli
containing granular peripolar cells

(e)


% Relative position of glomeruli in the cortex from cortico-medullary junction to the kidney capsule
95-100
90-95
85-90
80-85
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70-75
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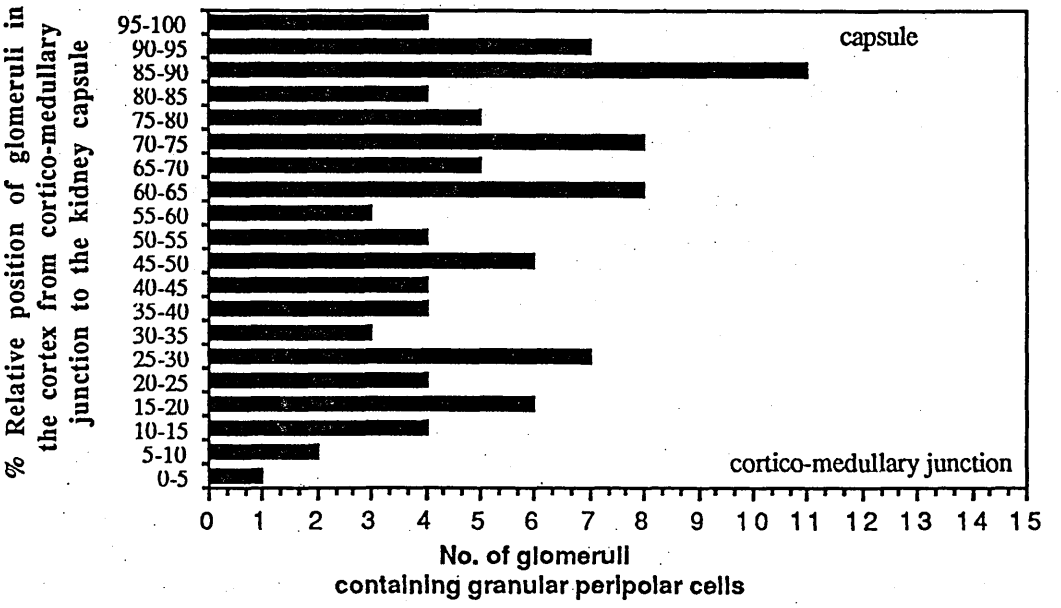
60-65
55-60
50-55
45-50
40-45
35-40
30-35
25-30
20-25
15-20
10-15
5-10
0-5
capsule
cortico-medullary junction
0 1 2 3 4 5 6 7 8 9 10 11 12
No. of glomeruli
containing granular peripolar cells

(f)


% Relative position of glomeruli in the cortex from cortico-medullary junction to the kidney capsule
95-100
90-95
85-90
80-85
75-80
70-75
65-70
60-65
55-60
50-55
45-50
40-45
35-40
30-35
25-30
20-25
15-20
10-15
5-10
0-5
capsule
cortico-medullary junction
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
No. of glomeruli
containing granular peripolar cells

3.2 Preparation of Antigen

3.2.1 Isolation of glomeruli

i) Enzymic digestion and mechanical disruption

The glomeruli were isolated by enzymic digestion of kidney tissue using either collagenase or trypsin. Under conditions of collagenase digestion which released most of the glomeruli from the surrounding tissue, very few retained their Bowman's capsule. No granular peripolar cells were identified in these preparations. There were few free glomeruli isolated by digestion of the tissue with trypsin, none of them retained their Bowman's capsule intact and the glomeruli including those attached to the tissue blocks were all over-digested.

Similarly using mechanical disruption alone, most of the free glomeruli had broken Bowman's capsules. Less than 10% of the isolated glomeruli retained intact Bowman's capsule, but most were contaminated by many other tissue components of the kidney.

ii) Microdissection*

10,220 individual glomeruli have been isolated by microdissection. Histological examinations showed that most of the microdissected glomeruli were relatively pure with their Bowman's capsules intact (Fig. 3.5). Granular peripolar cells were identifiable in those microdissected glomeruli in which Bowman's capsule remained intact (Fig. 3.6). Examination of the serial sections of microdissected glomeruli indicated that, the percentage of the microdissected glomeruli which contain granular peripolar cells was nearly identical to that of the intact cortex tissue for each of the corresponding kidneys (Fig. 3.6a)

The percentages of microdissected glomeruli having intact Boman's capsules (BcI) in glomeruli prepared from 8 different kidneys ranged from 63.16% to

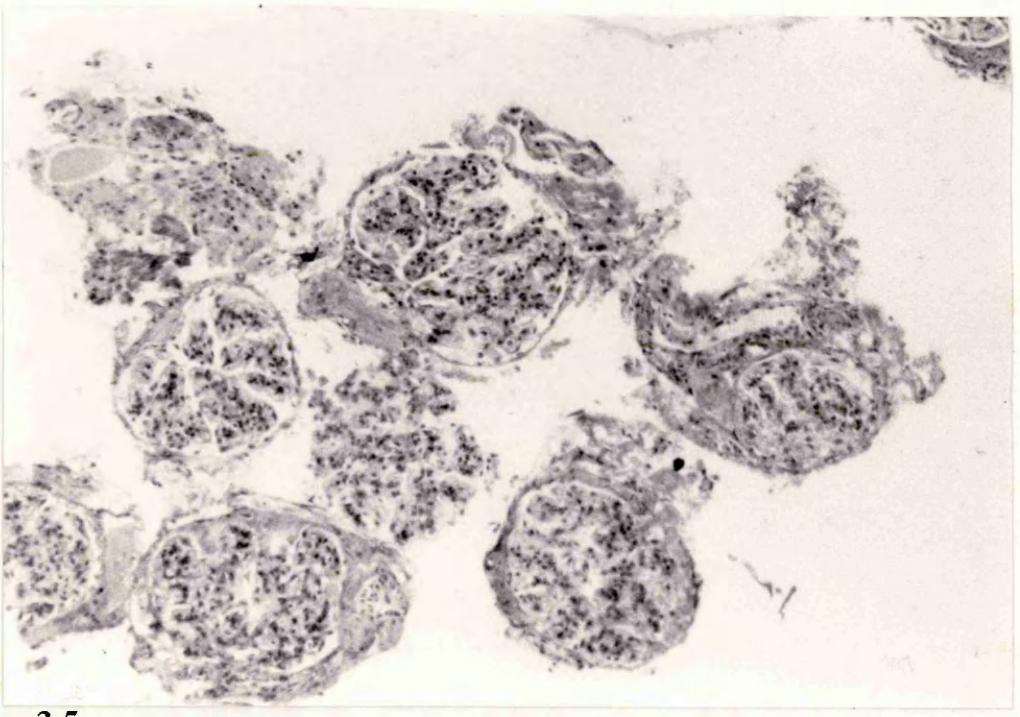


Fig. 3.5
Low power (x 100) micrograph of glomeruli microdissected from sheep kidney showing that most of the glomeruli have intact Bowman's capsules. There is a small amount of tubular tissue attached.

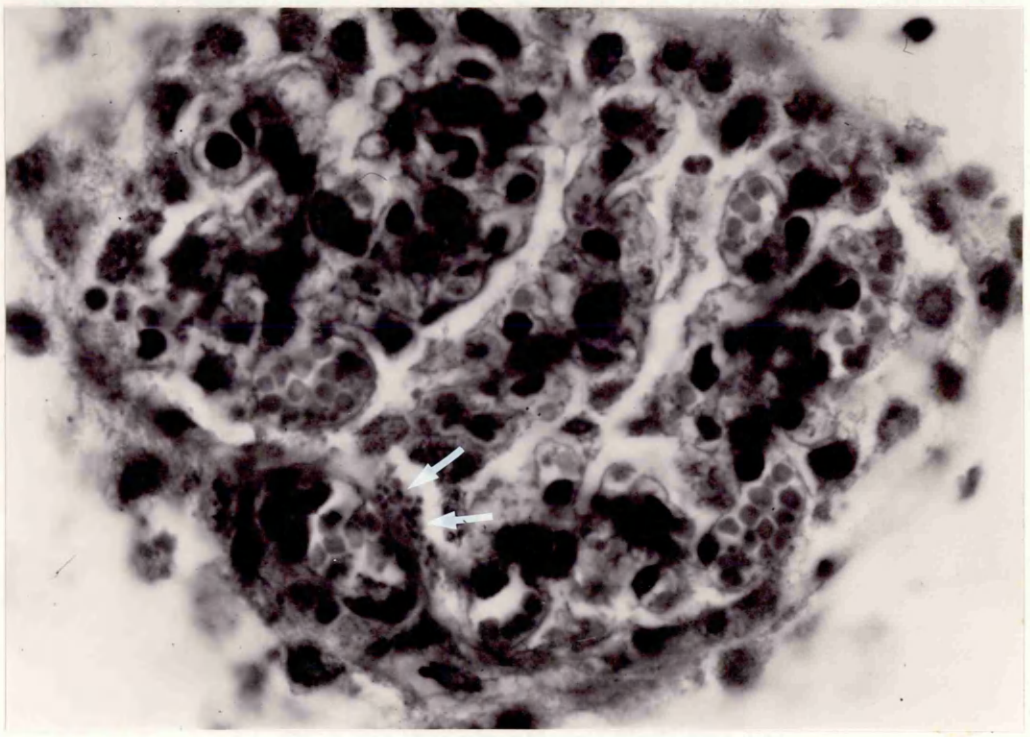


Fig. 3.6
High power (x 400) micrograph of a glomerulus microdissected from sheep kidney showing that granular peripolar cells remained in the glomerulus after microdissection procedure (arrows). Paraffin-embedded section, toluidine blue stained.

87.76%, the mean is 78.05%. (Table 3.1)

Fig. 3.6a

Comparison of peripolar cell indexes in intact cortex (PIs) and microdissected glomeruli (PIms)

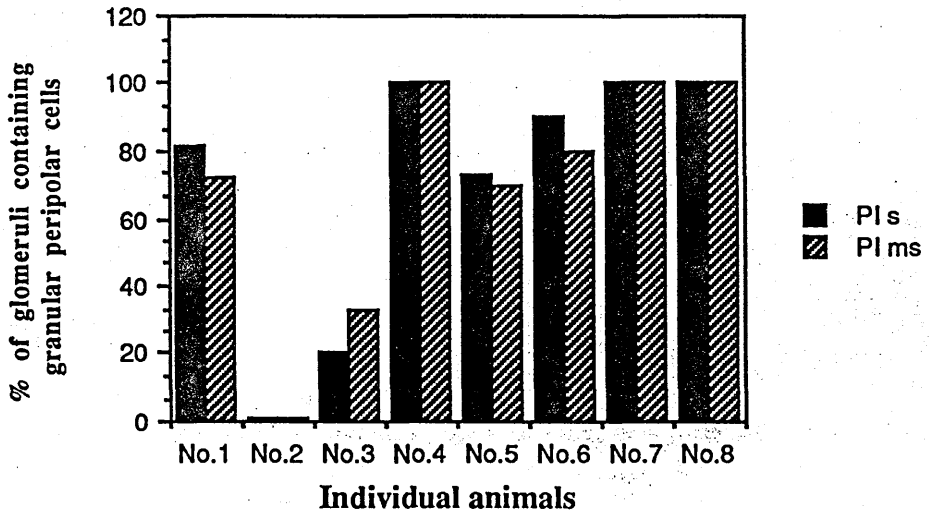


Table 3.1 **Bowman's capsule indexes (BcI) in glomeruli isolated from 8 different kidneys**

Kidney	1	2	3	4	5	6	7	8
BcI(%)	87.8	75.9	63.2	78.6	83.3	75	75	85.7

3.2.2 Granule fractionation

Sucrose density gradient fractionation separated the kidney homogenate into seven distinguishable fractions. Four were bands accumulating at sucrose

interfaces, one fraction sedimented to the bottom of the tubes while the remaining material remained in the upper fraction or formed a lipid layer on the surface of the sucrose. Ultrastructurally, the components in all of the bands were not significantly different; except that relatively more granules accumulated in the second band (between 1.5 M & 1.6 M of sucrose), and the third band (between 1.4 M and 1.5 M of sucrose), all bands were morphologically similar being rich in mitochondria and Golgi apparatus. Similar results were obtained from 3 experiments.

3.3 Production of monoclonal antibody

3.3.1 Immunisation of mice

i) Injections of antigen

At least 3 injections of antigen (microdissected glomeruli) were given before each of the 5 fusions (Table 3.2). The remaining 4 mice which were not used for fusions because their immune responses as detected by the immunostaining patterns indicated they were producing large amounts of antibody directed against kidney components not relevant to this study.

Table 3.2 Numbers of injections and outcome of the 9 mice immunised

Mouse	A1	A2	A3	B1	B2	B3	C1	C2	C3
No. of injection	3	4	3	5	4	3	5	3	5
Outcome	fusion	fusion	killed	fusion	fusion	killed	fusion	killed	killed

ii) Immune response indicated by immunostaining with serum antibodies

Mouse antisera were tested by using immunofluorescence staining on cryostat kidney sections. Under the present assay condition, the background staining was well controlled at a satisfactory level by using 5% BSA to block antigen binding sites on tissue sections. Adding 1% normal sheep serum to the FITC conjugated secondary antibody preparation reduced crossreactivity.

The immunostaining evidence indicated that all of the immunised mice produced antibodies reacting with sheep glomeruli. The immune responses, however, varied considerably from one mouse to another both in terms of antigen specificity and quantitative response. In several of the mice the immune response was directed mainly against glomerular epithelial components, but others appeared to be reacting almost entirely to interstitium contaminating the glomerular preparation.

Immunostaining intensity

First screening of sera was undertaken four days after the second injection of antigen. Serum antibodies were detectable in the sera of all nine mice tested, though the immunostaining intensity for the different components on the sections varied. At 1:100 dilution of antiserum, immunostaining were clearly shown for most of the serum samples tested. Under the same assay condition, following further dilution of the antiserum, the strong immunostaining of some components on the section was still detectable at the dilution up to 1:200 for most of the cases and 1:400 for the cases of serum from mice A1 and B2. At 1:30 dilution, the immunostaining was usually too strong for it to be possible to identify the cellular location of bound antigen, due to the excessive brightness of FITC fluorescence under U.V. light.

The second screening of antisera was carried out one week after the third injection of antigen, and the following screenings were carried out one week after each of the further injections of antigen. In most cases, the immunostaining intensity increased compared with that seen with the previous serum sample, but the

types of antibody, as indicated by immunostaining patterns, were relatively unchanged compared with the first screening for each of the nine mice immunised.

Immunostaining patterns

a) The antigen used for immunisation of mice was complex multi-cellular material, which was the microdissected glomeruli. In response to the antigen injected, 5 out of 9 mice (mice A1, A2, B2, B1, C1) produced antibodies which were mainly directed against components within glomeruli. Meanwhile, antibodies to other components on the kidney sections were also produced in varying amounts, probably reflecting contamination of the microdissected glomeruli with other kidney tissue components. Fig.3.7 shows the immunofluorescent staining of sheep kidney cryostat section by the antiserum from mouse A1 using serum collected immediately before fusion of the slpeen.

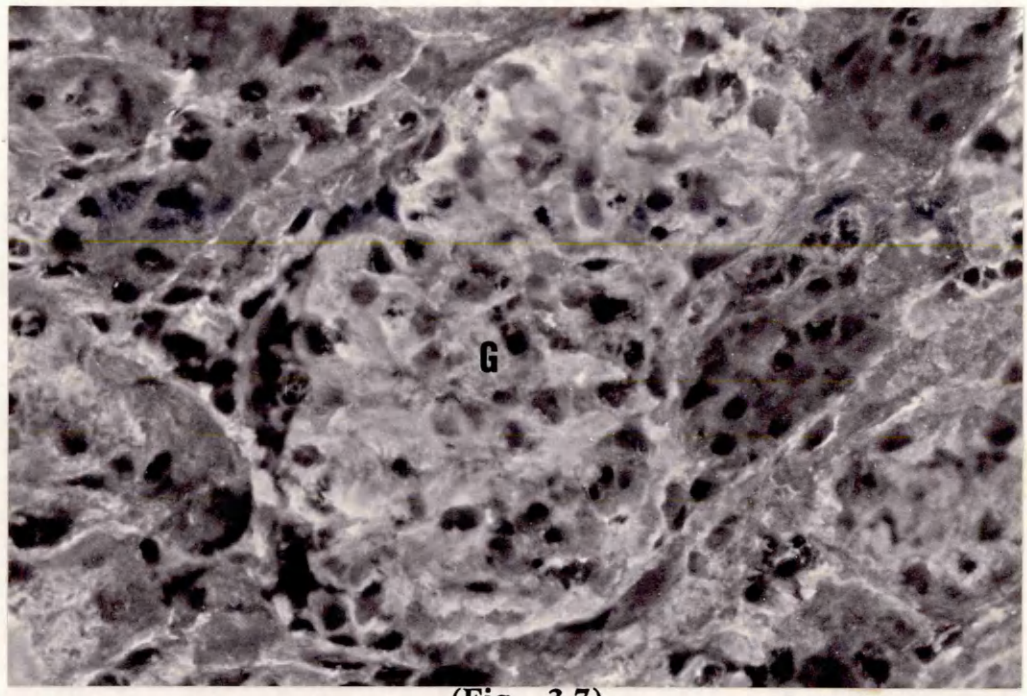
b) A distinctive type of immune response indicated by immunostaining was seen in the remaining 4 mice (mouse A3, B3, C2 and C3). Strong immunostaining indicated that these mice had produced antibodies mainly to the components in the tissue interstium rather than to cellular components of the glomeruli and tubules (Fig. 3.8).

iii) Immunostaining patterns established from the antisera, at 1:100 dilution, of the 5 mice at the time immediately before each of the fusions:

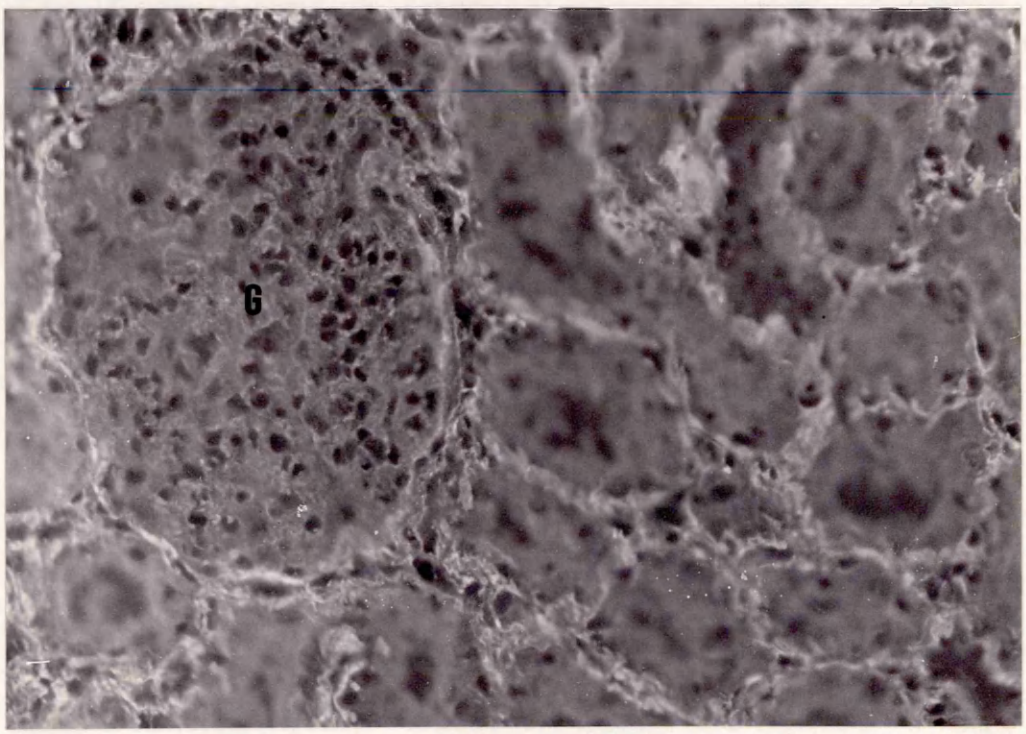
Antiserum A1 (Fig. 3.7)

Strong immunostaining, indicating this mouse had produced antibodies to the cytoplasm of glomerular parietal and visceral epithelial cells, the proximal tubular epithelial cells and the interstitium, was shown by detection of FITC fluorescence under U.V. light, one week after the third injection of antigen. There was also weak staining of the epithelial cells of distal and collecting duct tubules. The vascular smooth muscle and the endothelium were negative.

Peripolar cells were difficult to identify due to the poor preservation of tissue



(Fig. 3.7)



(Fig. 3.8)

Fig 3.7 & 3.8

Immunofluorescent staining of sheep kidney by sera from immunised mice immediately before fusion. **Fig. 3.7**: (Mouse A1) glomerular epithelial cells and connective tissue are strongly positively stained while the epithelial cells of proximal and distal tubules appear negative or only weakly positive. (x 200). **Fig. 3.8**: (Mouse B3) positive immunostaining of interstitium. Glomeruli and tubules are negative. (x 200). The tissue was frozen section and the sera were diluted 1:100. Bound antibodies were located by the detection of FITC fluorescence under U.V. light. G: glomerulus

structure in the cryostat sections visualised by the fluorescence. The assay was repeated and the possibility that peripolar cells might be positively stained was deduced from immunostaining at the peripolar position near the vascular poles.

Mouse serum A2

One week after the fourth injection of antigen, an immunostaining pattern similar to that of the mouse serum A1 was seen. Peripolar cells were again possibly but not convincingly, identified to be positively stained.

Mouse serum B2

Strong immunostaining, of the cytoplasm of glomerular parietal and visceral epithelial cells and the interstitium, was seen one week after the fourth injection. Peripolar cells were not able to be defined due to the poor preservation of tissue structure in the cryostat sections visualised by the fluorescence. The cell fusion was undertaken because of the strong immunostaining of the intraglomerular cells in which the peripolar cell might be involved. There was also staining of the vascular endothelium but not of the smooth muscle. The proximal and distal tubular epithelial cells were seen to be negative.

Mouse serum B1

One week after the fifth injection, the epithelial cells of glomeruli and proximal tubules were positively stained, and strong immunostaining of kidney connective tissue was seen. Either the cytoplasm or the granules membrane of the peripolar cells was positively stained but the granules were negative, which made the peripolar cells easily identifed on the cryostat sections (Fig. 3.9).

Mouse serum C1

Similar immunostaining pattern to that of the mouse serum A1 and strong immunostaining of glomerular cells was seen, after a total of five injections of antigen. By contrast microscopy, granular peripolar cells were readily identified on

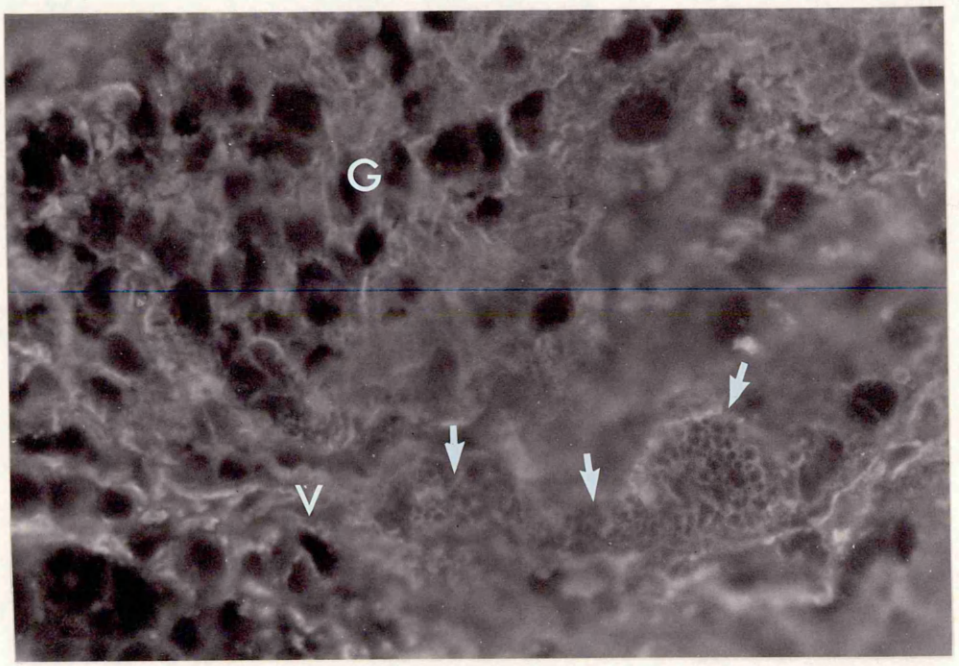


Fig. 3.9

Immunofluorescent staining of sheep kidney by serum from immunised mouse B1 immediately before fusion. The tissue was frozen section and the serum was diluted 1:100. Bound antibody was located by the detection of FITC fluorescence under U.V. light. The cytoplasm or the membrane of the peripolar cell granules stain positively while granules are negative (arrows). (X 500) G: glomerulus; V: vascular pole.

the same cryostat kidney sections which had been tested by immunofluorescent procedure. The granules of the peripolar cells were faintly stained, by the combination of these two techniques.

3.3.2 Production and culture of hybridomas

The spleen cells from five mice (A1, A2, B2, B1 and C1) were fused with X63.Ag8.653 mouse myeloma cells. In each case, small clones of hybrid cells were seen by two weeks after fusion. Except for the fusion A2 and B2 which became contaminated with unidentified micro-organisms, most of the hybridomas had been growing well while a few clones of the hybridomas were unstable and died within the first few weeks. After fusion, the number of cells increased relatively slowly for the first three or four weeks, but the cultures subsequently showed increased rates of cell division. The numbers of cultures in which viable clones of hybrids developed, is shown in Table 3.3, for each of the five fusions.

Table 3.3 Cell hybrid growth of the five fusions

Fusion	A1	A2	B2	B1	C1
No. of wells in which hybridomas developed					
2 weeks after fusion	23	2	16	42	55
Developed in total	26	2	30	46	56

Micro-organisms were found to be growing in the culture medium of fusion A2 two weeks after fusion and, in some of the wells of fusion B2 three weeks after fusion. Sterility checks indicated that the contaminating organisms had been introduced into the foetal calf serum. By light microscopy, the contaminant were

small moving black organisms which were not further characterised.

3.3.3 Detection of antibodies secreted by hybridomas

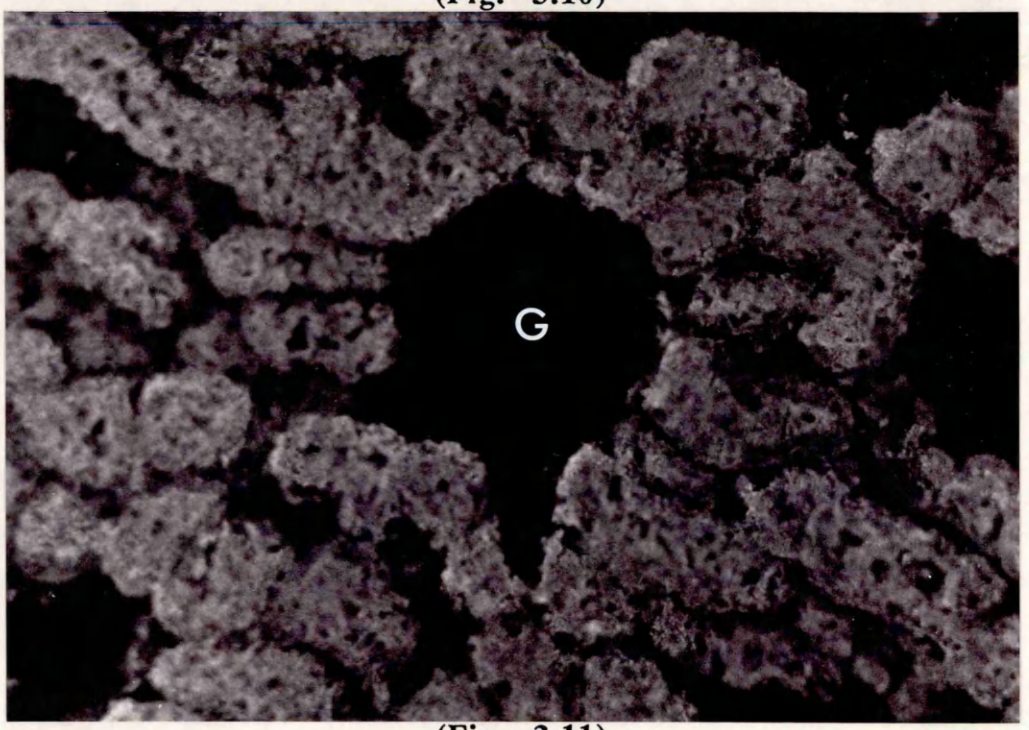
i) Immunofluorescence

Except for the fusion A2 which was abandoned 3 weeks after the fusion because of contamination, the hybridomas derived from all other fusions were shown to secrete antibodies raised against some components of sheep glomeruli, tubules or connective tissue. In each case immunofluorescence assays were used to identify the specific components of the kidney sections which provided the antigenic determinants. These were mainly the intraglomerular epithelial cells, for example the glomerular podocytes (Fig. 3.10); as well as other kidney components, for example the proximal tubular epithelial cells (Fig.3.11).

The immunofluorescence assay indicated that antibodies secreted by hybridomas of the A1-3A3 culture reacted strongly with granules of the sheep peripolar cells (Fig 3.12), and those secreted by C1-2C2 reacted weakly. However, in both cases these cultures failed to thrive --- four weeks after fusion cells ceasing to divide in culture A1-3A3, and in culture C1-2C2 there was no antibody secretion detectable by immunofluorescence and Elisa assays after the first cloning procedure.

Antibody secreted by the hybridomas in culture B1-3B3 was initially judged to react with the cytoplasm of the peripolar cells (Fig. 3.13). The immunostaining was consistently and specifically shown in a location very close to the glomerular vascular poles and sometimes presented a granular appearance. Therefore, the first cloning process was undertaken. After the hybridomas were cloned, however, subsequent investigation revealed that the antibody secreted by the cells reacted specifically with the cytoplasm of the sheep macula densa cells (Fig.3.14 -- 3.20, for detail see section 3.3.4).

(Fig. 3.10)



(Fig. 3.11)

Fig. 3.10 & 3.11

Immunofluorescent staining of sheep kidney by supernatants from fused mice spleen/myeloma cells grown in cultures. **Fig.3.10**: Immunostaining pattern of C1-4D2 showing glomerular podocytes positively stained. **Fig.3.11**: Immunostaining pattern of C_1-1-C_6, showing the cytoplasm of proximal tabular epithelial cell positively stained, glomeruli (G) are negative. The tissue used was frozen sections and the supernatants were used without dilution. Bound antibodies were located by the detection of FITC fluorescence under U.V. light. (X 100)

Fig. 3.12

Immunofluorescent staining of sheep kidney by supernatant from fused mouse spleen/myeloma cells grown in culture of A1-1A3. The tissue was frozen section and the supernatant was used without dilution. Bound antibodies were located by the detection of FITC fluorescence under U.V. light. Positive granules are present in a peripolar cell adjacent to the vascular pole (V) of the glomerulus (G). (X 450)

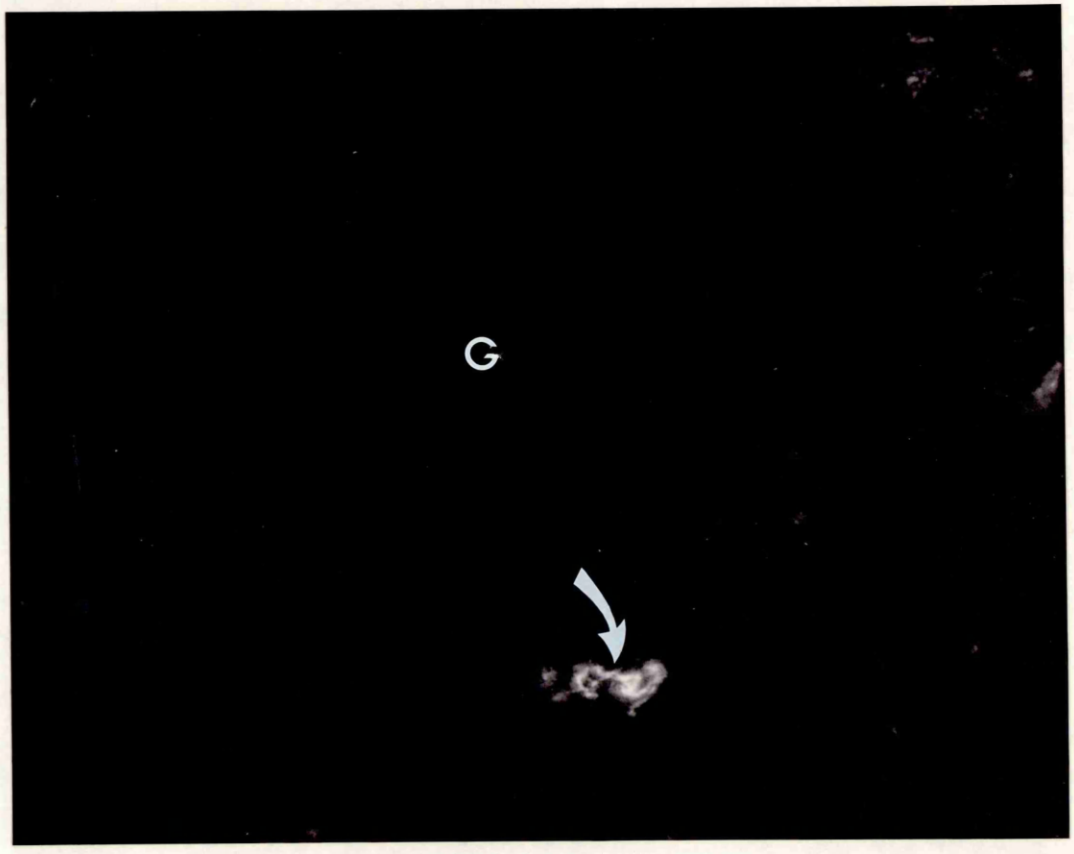
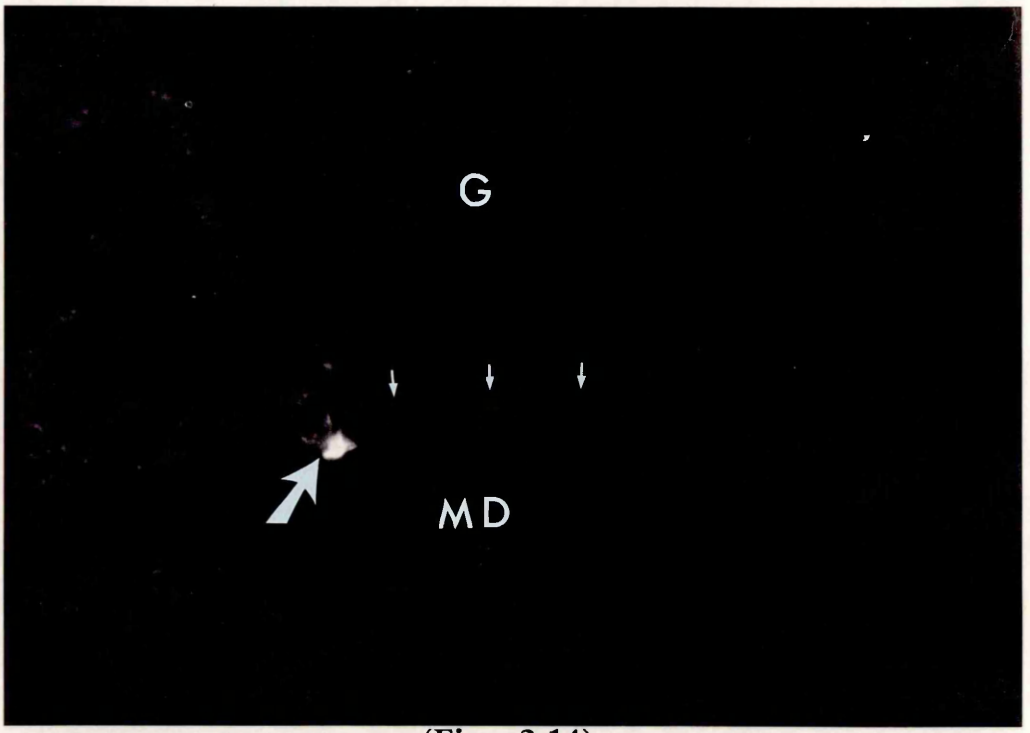
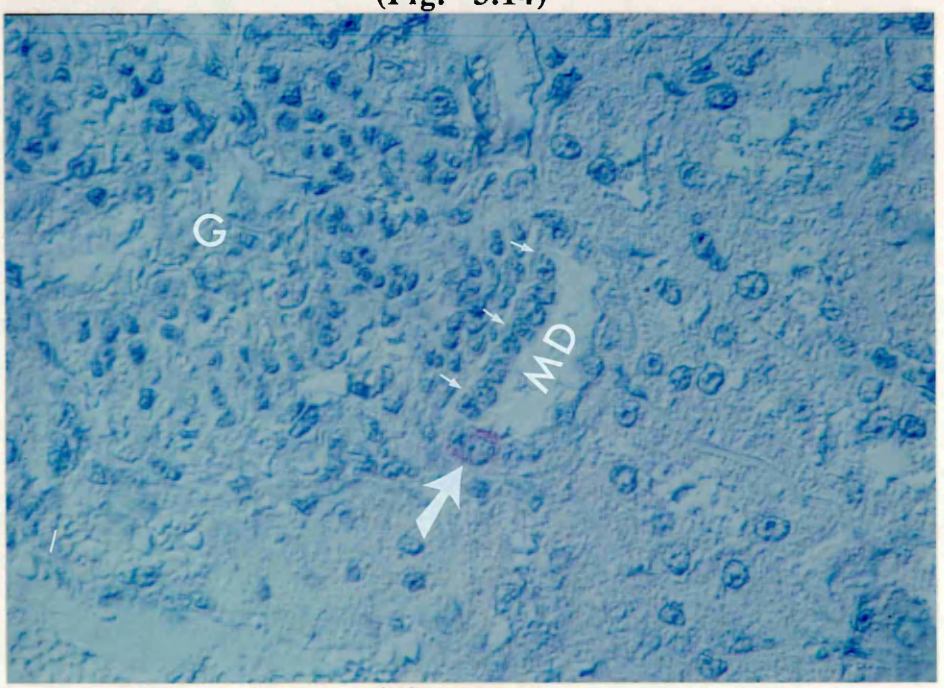


Fig. 3.13

Immunofluorescent staining of sheep kidney by supernatant from fused mouse spleen/myeloma cells grown in culture of B1-3B3 after the first cloning. The tissue was frozen section and the supernatant was used without dilution. Bound antibodies were located by the detection of FITC fluorescence under U.V. light. Positive staining (arrow) is seen at the periphery of the glomerulus (G). Since the staining was close to the vascular pole where peripolar cell is normally present, the sample was thought to be staining the peripolar cell. Later it was proved to be the cytoplasm of the highly differentiated epithelial cell in the macula densa. (x 312.5)



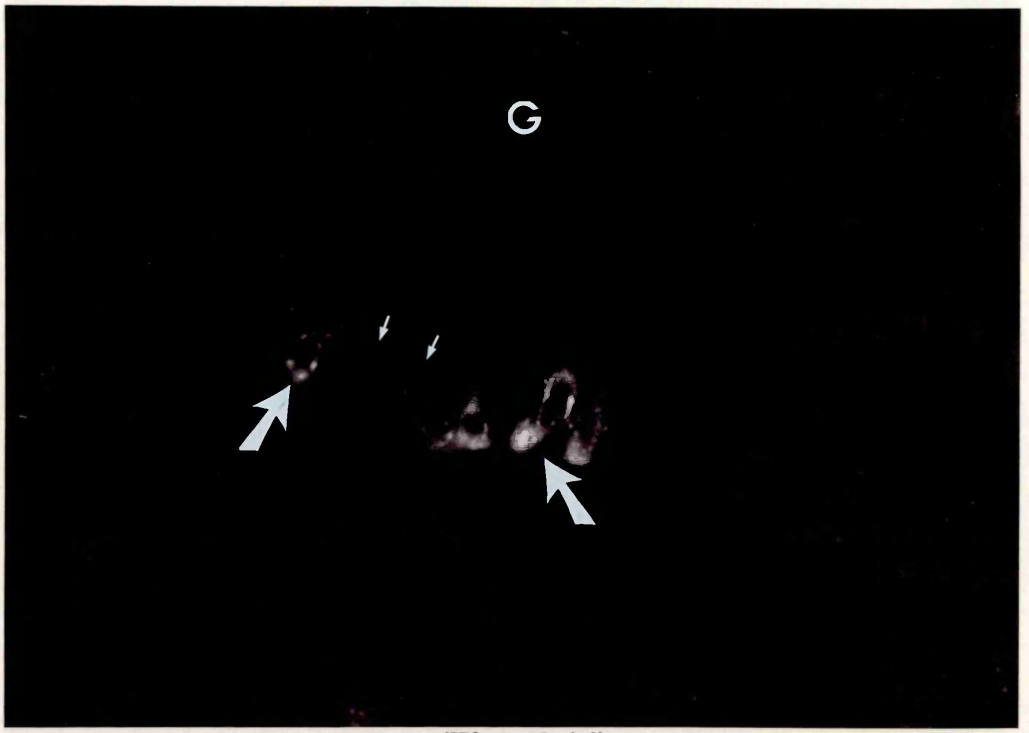
(Fig. 3.14)



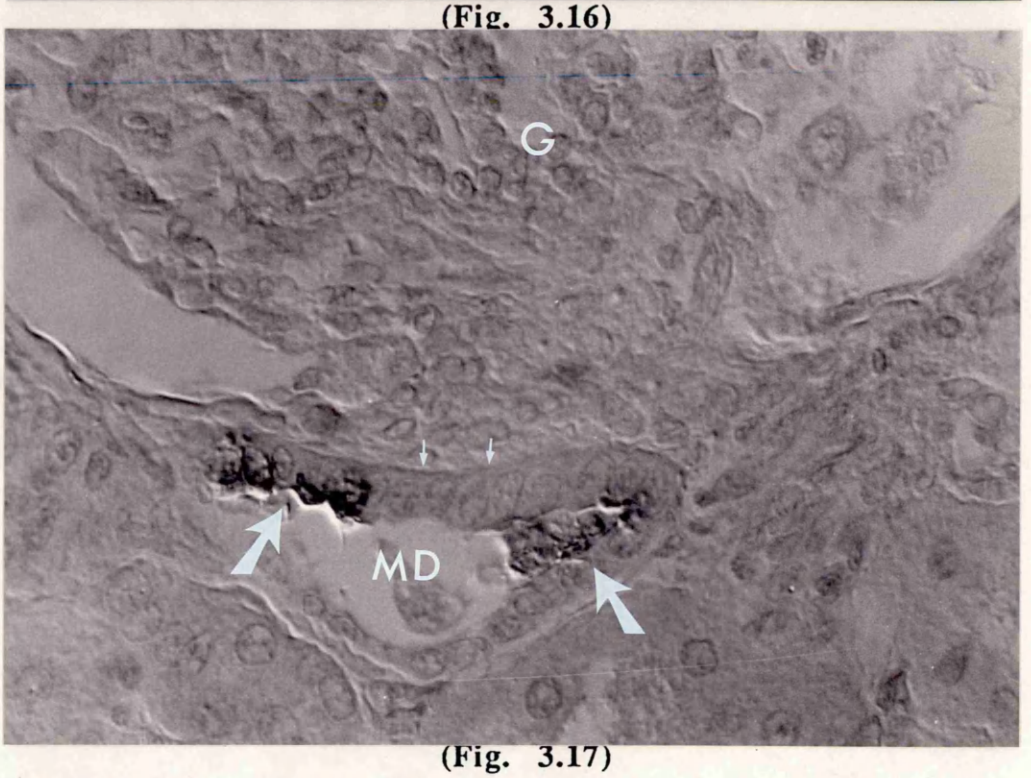
(Fig. 3.15)

Fig. 3.14 & 3.15 ***'B1-3B3' immunostaining pattern 1***

Immunostaining of sheep kidney macula densa cell, by the monoclonal antibody secreted in the culture supernatant of hybridomas B1-3B3, indicates the antibody reacts with the cytoplasm of only a single cell (big arrow) but not the rest of the cells (small arrows) in same macula densa. Note: The cells which were positively stained situated at the corner of the macula densa. G: glomerulus MD: macula densa. Same immunostaining pattern was shown by immunofluorescence (**Fig. 3.14**) and alkaline phosphatase (**Fig. 3.15**), immunohistochemical procedure using the same antibodies on the paraffin-embedded tissue sections from same kidney. (x 500)



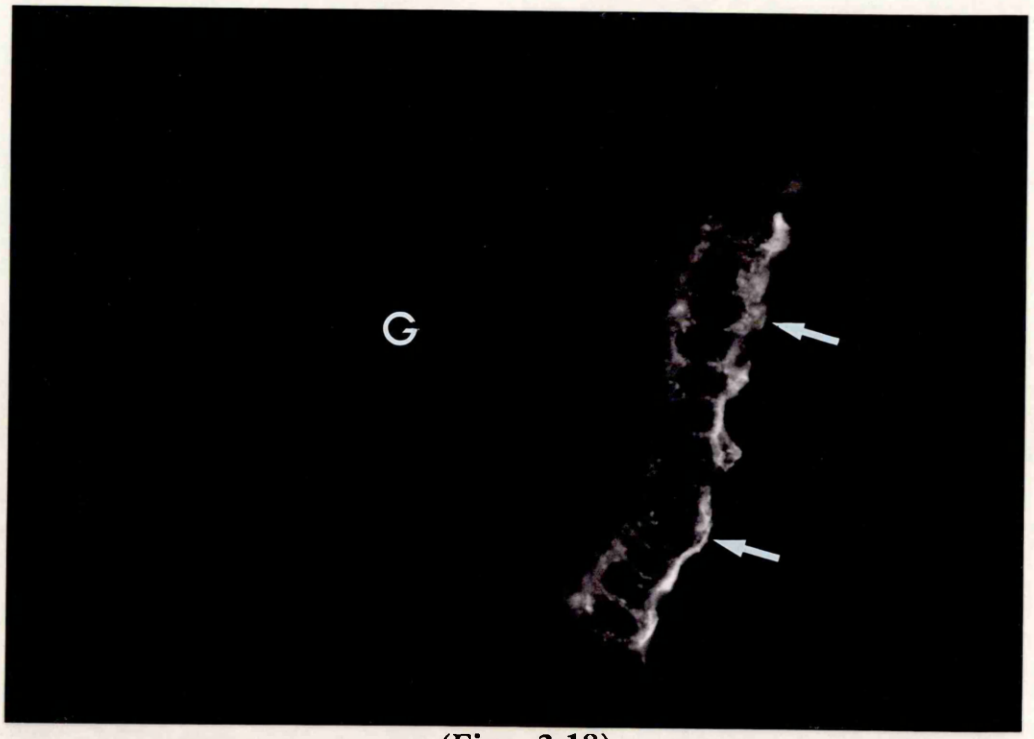
(Fig. 3.16)



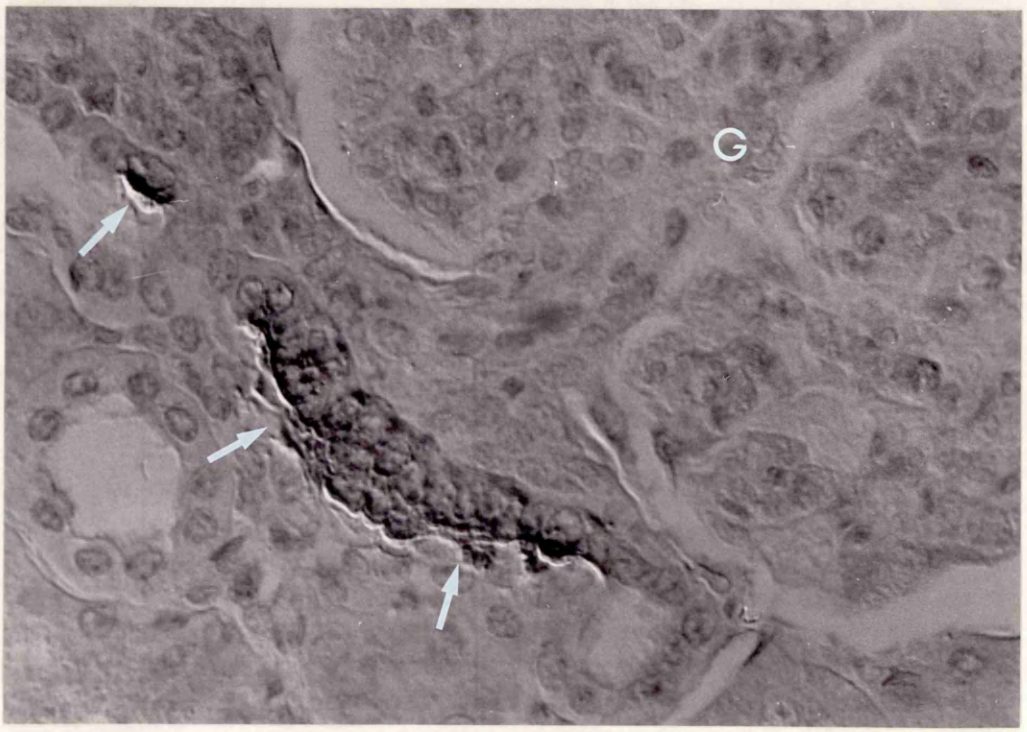
(Fig. 3.17)

Fig. 3.16 & 3.17 ***'B1-3B3 ' immunostaining pattern 2***

Immunostaining of sheep kidney macula densa cells by the monoclonal antibody secreted in the culture supernatant of hybridomas B1-3B3. Multiple cells in the macula densa are shown to react with the antibody. **Fig.3.16** shows an immunofluorescent staining of the cytoplasm of 6 epithelial cells in one macula densa (big arrows) while some other adjacent macula densa cells (small arrows) were negative. **Fig.3.17** shows a similar immunostaining using the same antibody by a PAP procedure. Note: The cells which were positively stained were situated at the corner of the macula densa. Paraffin-embedded tissue sections, x 500. G: glomerulus, MD: macula densa.



(Fig. 3.18)



(Fig. 3.19)

Fig. 3.18 & 3.19 ***'B1-3B3 ' immunostaining pattern 3***

Immunostaining of sheep kidney macula densa cells by the monoclonal antibody secreted in the culture supernatant of hybridomas B1-3B3, showing the cytoplasm of all of a pad of highly differentiated epithelial cells in macula densa (arrows) stained positively with the antibody. Similar immunostaining was shown by immunofluorescence **(Fig.3.18)** and alkaline phosphatase **(Fig.3.19)**, immunohistochemical procedures using the same antibodies on the paraffin-embedded tissue sections from same kidney. (x 500) G: glomerulus,

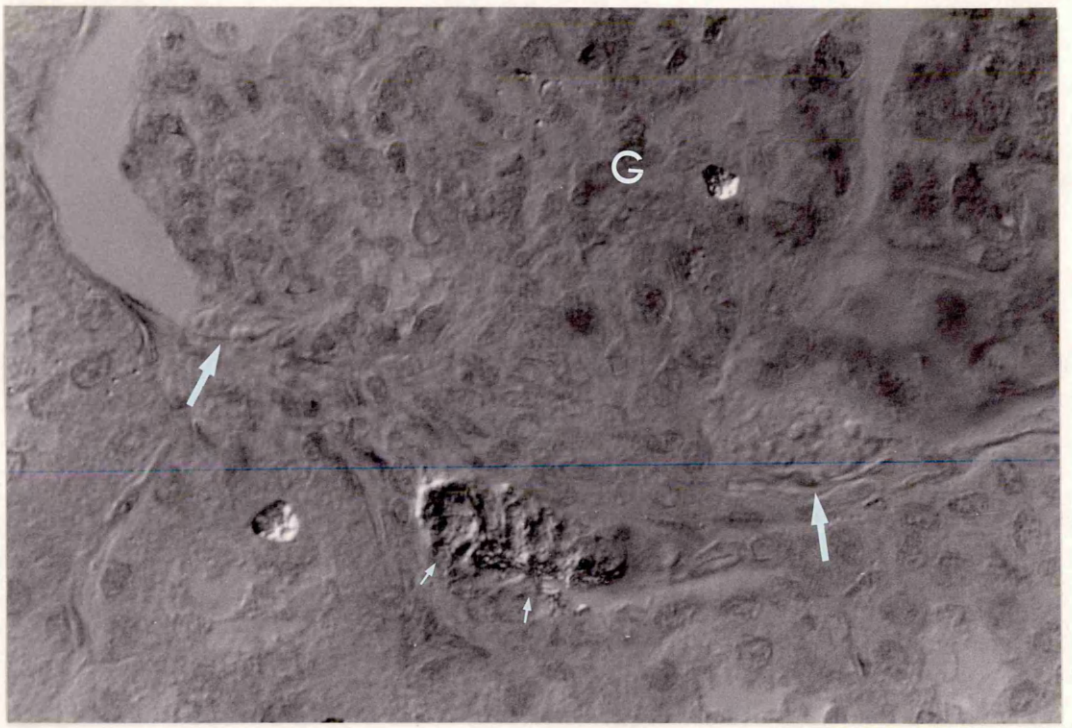


Fig. 3.20

Immunostaining of sheep kidney by the monoclonal antibody secreted in the culture supernatant of hybridomas B1-3B3. The antibody is directed against the antigen of cytoplasm of the highly differentiated epithelial cells in macula densa (small arrows), while the granular peripolar cells (big arrows) which was convincingly shown by interference contrast microscopy appears to be negative. The antigenic binding sites was localised using an immunoperoxidase procedure on paraffin embedded tissue section. G: glomerulus; interference contrast microscopy, x 500.

Some samples which showed strong immunostaining on cryostat sections were also tested on paraffin embedded tissue sections. Some antibodies (e.g. hybridomas B1-3B3) showed consistent staining, but others (e.g. hybridomas C1-1C6, C1-4D2) showed negative staining.

ii) Elisa

Elisa immunosorbent assay was developed as a method to detect antibody production for the fusion B1 and C1. The reason for employing this assay was to decided which of the cultures were secreting antibodies, in the hope that this would reduce the number of samples needing to be screened by the cytochemical assay. Rather than measuring the precise amount of secreted antibody, the Elisa assay was used qualitatively to detect antibody production. To reduce costs and simplify the procedure, antibody production was assessed by comparing the Elisa results from hybrid cultures with each of the corresponding negative controls.

A rough estimate of the amount of antibody produced was obtained by comparison with standard curves established using known amounts of purified antibodies, mouse IgG (whole molecule, JACKSON-Lab) and mouse IgM (anti-rat myosin, Biochem. Dept., University of Glasgow) for the quantitation of IgG and IgM production, respectively. I am grateful to Mrs Andrea Brown for the data in Fig.3.22 which is included to illustrate the relative insensitivity of IgM measurement by this assay.

Fig.3.21 shows the standard curve established by serial dilutions of known standard antibody, mouse IgG, for detection and estimate of IgG prodution.

Fig. 3.21 Standard assay for quantitation of IgG by Elisa

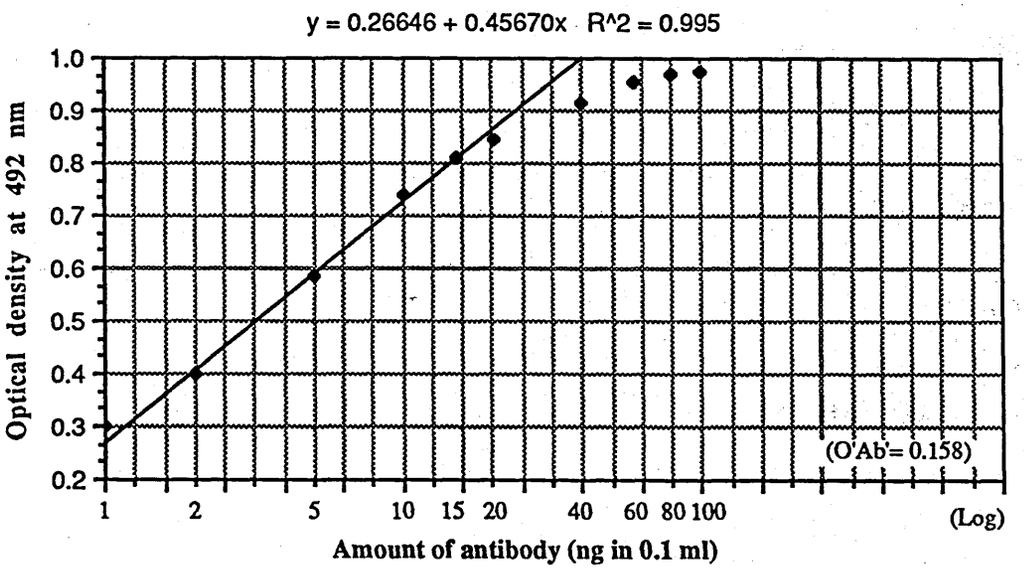
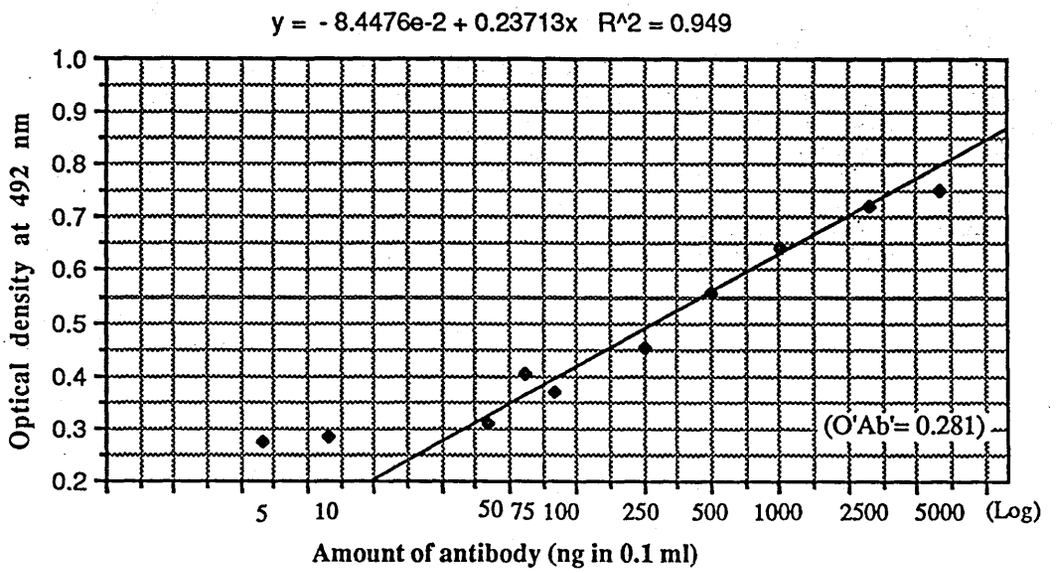


Fig.3.22 shows the standard curve established using known amounts of a standard antibody, mouse IgM (whole molecule), for detection and estimate of IgM prodution.

Fig. 3.22 Standard assay for quantitation of IgM by Elisa

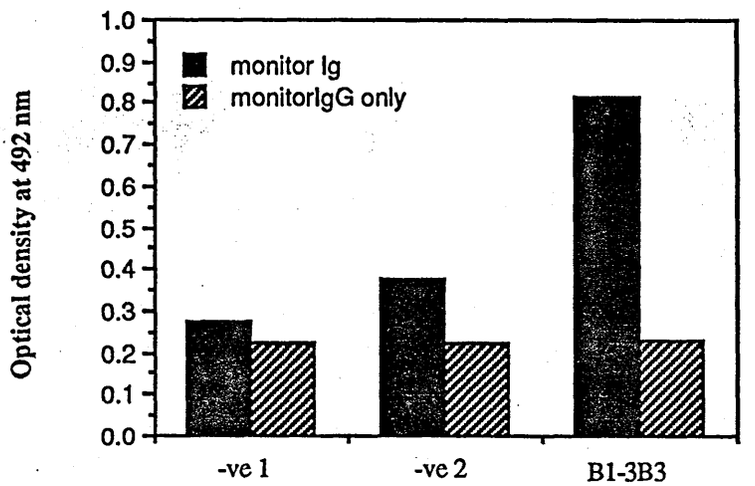


Fusion B1

Seven weeks after fusion, the Elisa assay was first introduced and it was not possible to detect any significant secretion of IgG from the B1 fusion hybridomas in culture. However at eight weeks, one of the cultures, B1-3B3 which was known from cytochemical assay to be secreting antibodies directed against the cytoplasm of sheep macula densa cells was shown to secreting significant amount of IgM (Fig.3.23). According to the standard curve for the detection of IgM, the estimated amount of IgM in the culture of B1-3B3 was approximately 61 μg per ml.

Fig. 3.23

Elisa result for the detection of antibody production in culture of hybridomas B1-3B3 (after the second cloning procedure)



Note. -ve 1: negative control sample from spleen cell culture supernatant

-ve 2: negative control sample of RPMI containing 20% FCS,

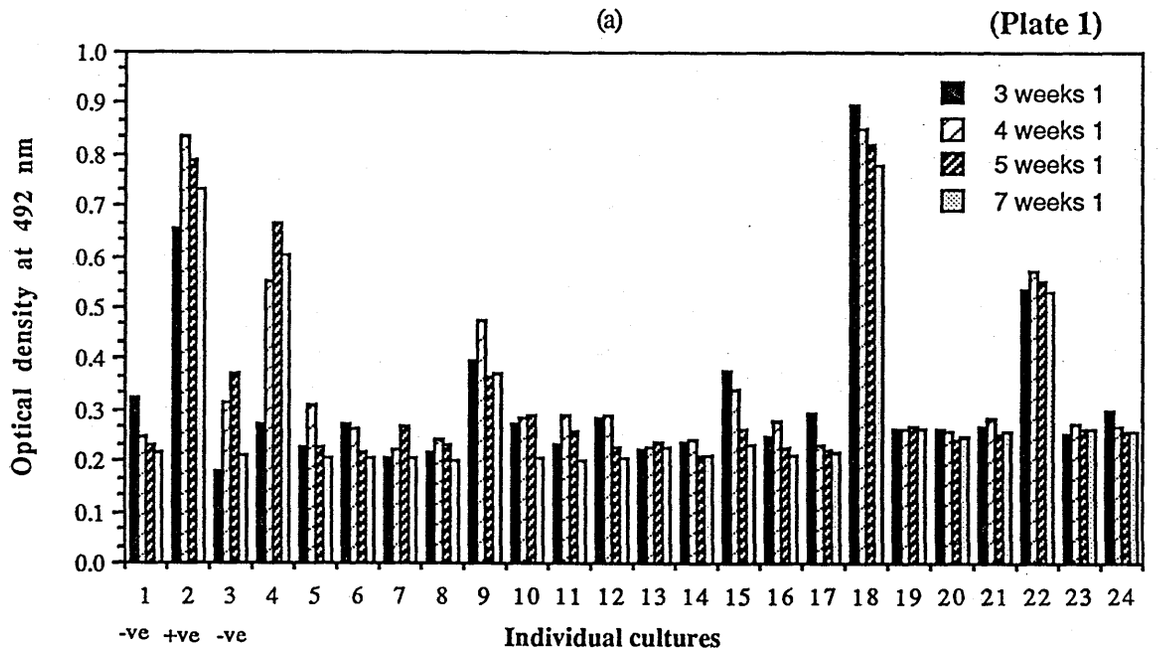
B1-3B3: sample tested from the culture supernatant of B1-3B3 hybridomas

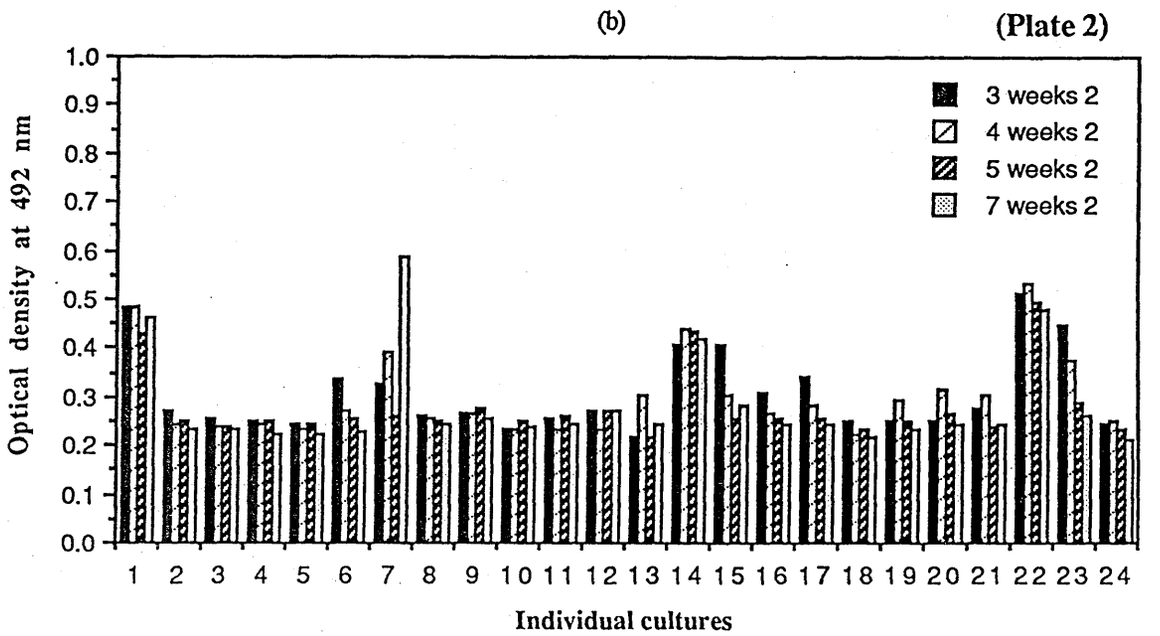
Fusion C1

Results from the Elisa assays for the detection of antibody production by

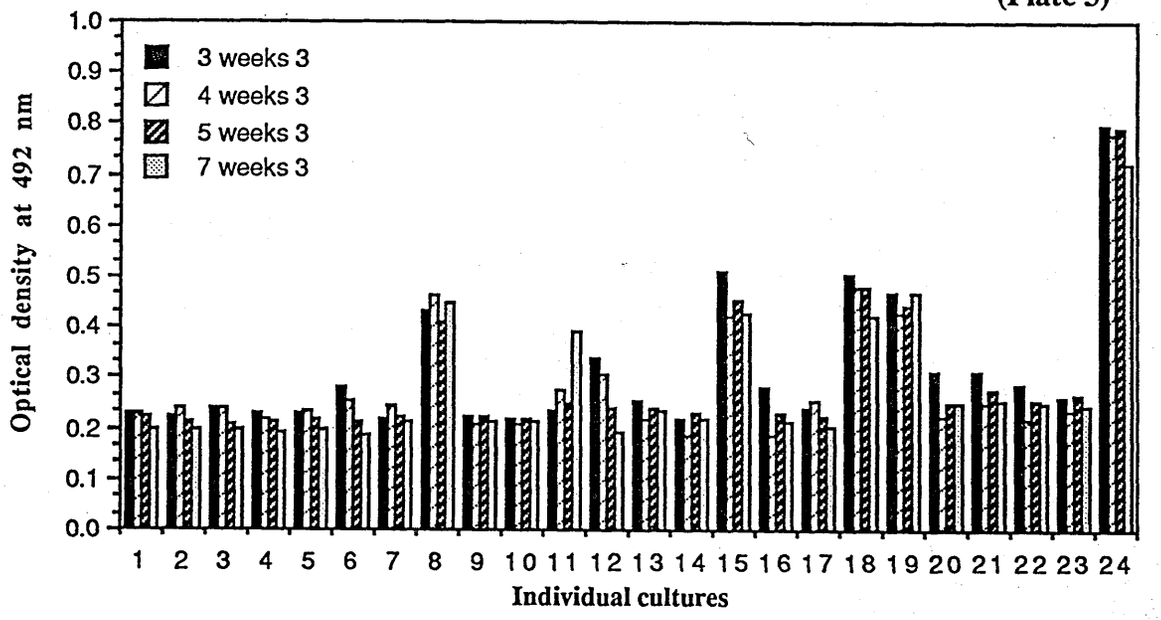
Fig. 3.24a--d

Results from Elisa assays for the fusion C1

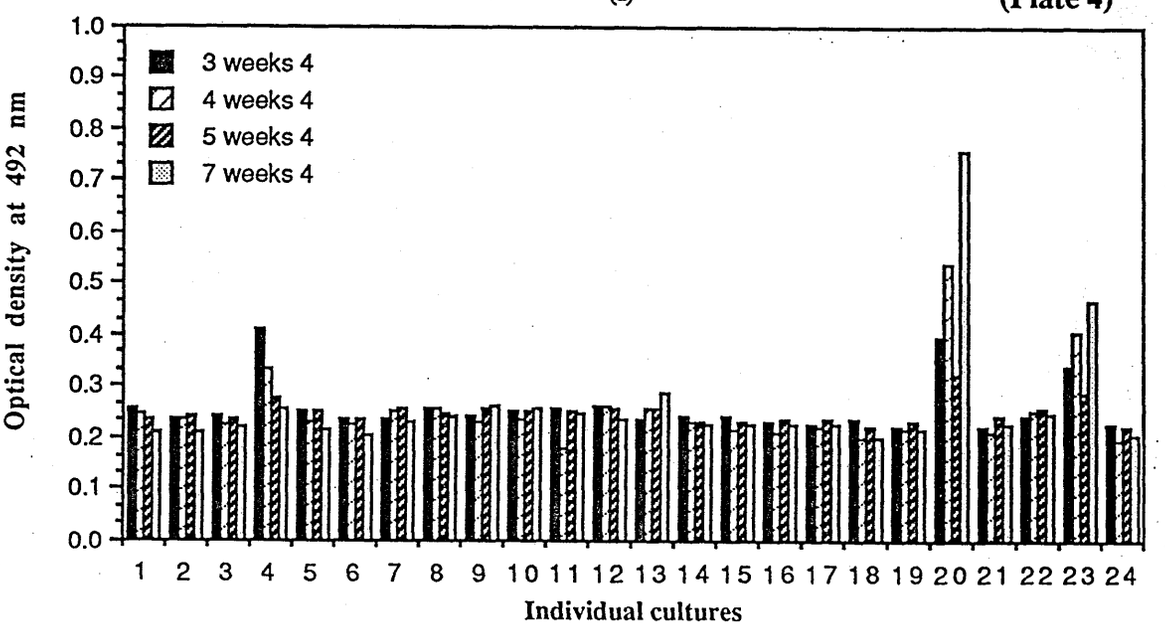




(c) **(Plate 3)**



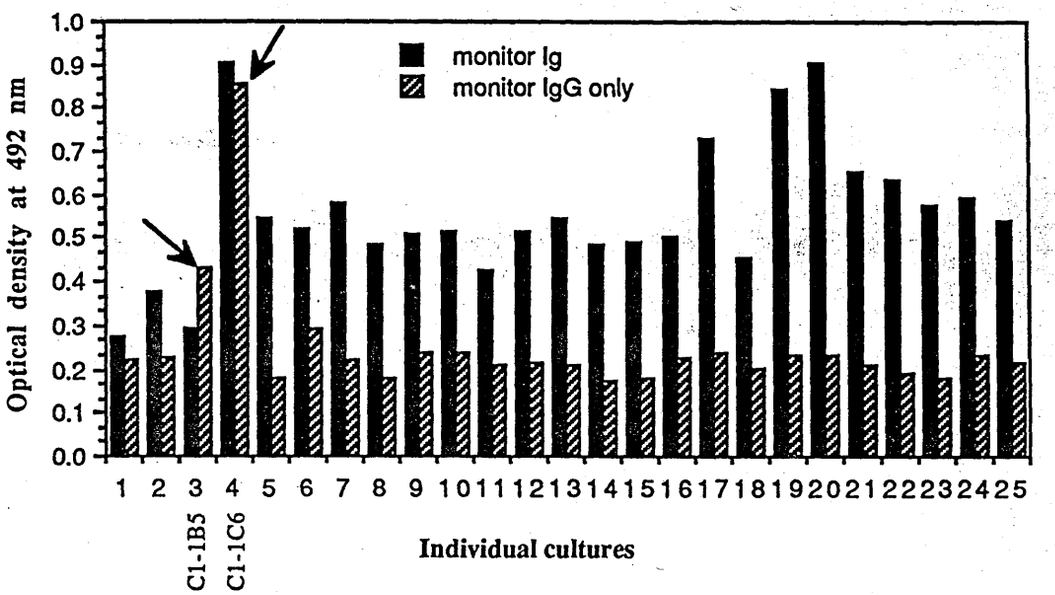
(d) **(Plate 4)**



hybridomas produced from the fusion of the spleen from mouse C1 indicated that, the hybridomas in 19 out of 93 cultures at 3, 4, 5 and 7 weeks, and in 23 out of 93 cultures at 10 weeks after fusion, were secreting at least small amounts of immunoglobulin (Fig.3.24a -- d). However, only in two of the cultures (C1-1B5 &C1-1C6) of the hybridomas secreted signficant amounts of IgG (Fig.3.25).

Fig. 3.25

Elisa results for the detection of antibody production from the 23 immunoglobulin-producing hybridomas produced from the spleen of mouse C1



Note. No. 1: negative control sample from spleen cell culture supernatant

No. 2: negative control sample of RPMI containing 20% FCS

No. 3: sample tested from the culture supernatant of C1-1B5 hybridomas

No. 4: sample tested from the culture supernatant of C1-1C6 hybridomas

Table 3.4 shows the estimated concentration of the immunoglobulin IgG secreted in the culture supernatants of hybridomas C1-1C6 and C1-1B5, at the 3rd

and 10th week after fusion.

Table 3.4 **Estimated concentration of IgG in the hybridomas cultures of C1-1C6 and C1-1B5, at 3th and 10th week after fusion**

	estimated concentration of IgG (ng/ml)	
Culture	3th week	10th week
C1-1C6	165	196
C1-1B5	0	23

iii) Growth of hybridomas and detection of antibody secreted by the hybridomas of five fusions

The successful growth of spleen-myeloma hybrid cells for the five fusions, and the secretion of antibody as indicated by the results of immunofluorescence and Elisa assays are shown on Table 3.5.

Table 3.5

Fusion	Number of culture wells in which cell hybrids developed	Number of culture wells in which antibody detetable		
		IF*	Elisa**	
			Ig	IgG
A1	26	15	--	--
A2	2	0	--	--
B2	30	5	--	--
B1	46	8	--	0
C1	56	8	24	2

* IF: immunofluorescent assay

** Elisa assay was introduced as a method to detect antibody prodution from fusion B1 (detection of specific IgG), and then the fusion C1 (detection of immunoglobulin prodution and specific IgG).

3.3.4 Localisation of antibody secreted by hybridomas B1-3B3

Early investigation with the hybrid cell line B1-3B3 suggested it might be producing an antibody directed against a component of the peripolar cell. There remained however some uncertainty about the precise antigenic localisation of the antibody on sheep kidney cryostat sections. For this reason more attention was given to defining the location. For this, peripolar cells were located under phase contrast microscopy and marked on the sections. By comparison with the immunostaining pattern arising from specific binding of B1-3B3 antibody to the kidney sections, it became apparent that this antibody was interacting with a component of the macula densa. Furthermore, the precise location of the cellular antigenic binding sites of this antibody was confirmed by testing it on paraffin-embedded kidney tissue sections with immunofluorescence (Fig. 3.14, 3.16 & 3.18), the alkaline phosphatase (Fig. 3.15) and the peroxidase anti-peroxidase (Fig. 3.17, 3.19 & 3.20) immunohistochemical assays. The evidence from these studies indicates that the antibody reacts specifically and consistently with the cytoplasm of the highly differentiated epithelial cells in the macula densa, and not with the peripolar cell (Fig. 3.20), in all of the six sheep kidneys studied.

However, the immunostaining patterns indicated that different numbers of cells in the macula densa interacted with the antibody in different sheep and lamb kidneys. In one sheep, the predominate finding was that only a single cell of the macula densa stained positively (Fig. 3.14 & 3.15), although occasionally two or three cells in one macula densa were stained. In three other sheep and two lamb kidneys, multiple cells stained positively in each of the macula densas examined. In these kidneys, glomeruli were found to have macula densas with several cells interacting with the antibody and some apparently not (Fig. 3.16 & 3.17). In one kidney, the whole pad of macula densa cells was strongly positively immunostained in many of the macula densas on the same section (Fig. 3.18 & 3.19).

From the patterns of immunostaining it appears that the antigen may be a cytoplasmic component of the macula densa cells. Strongest staining was seen in cells at the lumenal side of the macula densa and the cells were often found to be situated at both corners where the macula densa was in contact with the afferent and efferent arterioles (Fig.3.14, 3.15 & 3.17). A granular appearance of the staining was sometimes seen in the cytoplasm of the cells and the nuclei were negative. The normal differentiated epithelial cells in the opposite side of macula densa in the same segment of the distal tubule were negative. The cells of other part of tubules, glomeruli and vessels were consistently negative.

3.3.5 Cloning

i) Cloning cell hybrids B1-3B3

The results of the three cloning procedures for the B1-3B3 hybrids, including the percentage of wells in which single clones developed and secreted antibody giving rise to the same immunostaining patterns, are shown in Table 3.6. The immunostaining pattern remained identical throughout the three cloning periods.

ii) Cloning cell hybrids C1-2C2

The cell hybrids of C1-2C2 developed in 4 out of 93 wells but none of the clones secreted antibody detectable by Elisa and immunofluorescence assays after the first cloning process. This culture was then abandoned.

Table 3.6 Data from cloning cell hybrids of B1-3B3

Times of Cloning cells	Cell diluted	No. of wells in which hybrids developed		Single clone		
				N0. of wells		% of the clones tested secreting antibodies (Immunofluorescence)
1st time	1 cell / well	6 2		8		75%(6/8)
2nd time	4 cells / well	7	1 2	6	1 0	71.43%(5/7)
	2 cells / well	1		1		
	1 cell / well	3		2		
	0.5 cel / well	1		1		
3rd time	4 cells / well	15	3 2	7	2 1	100%(5/5)
	2 cells / well	10		7		
	1 cell / well	5		5		
	0.5cell / well	2		2		

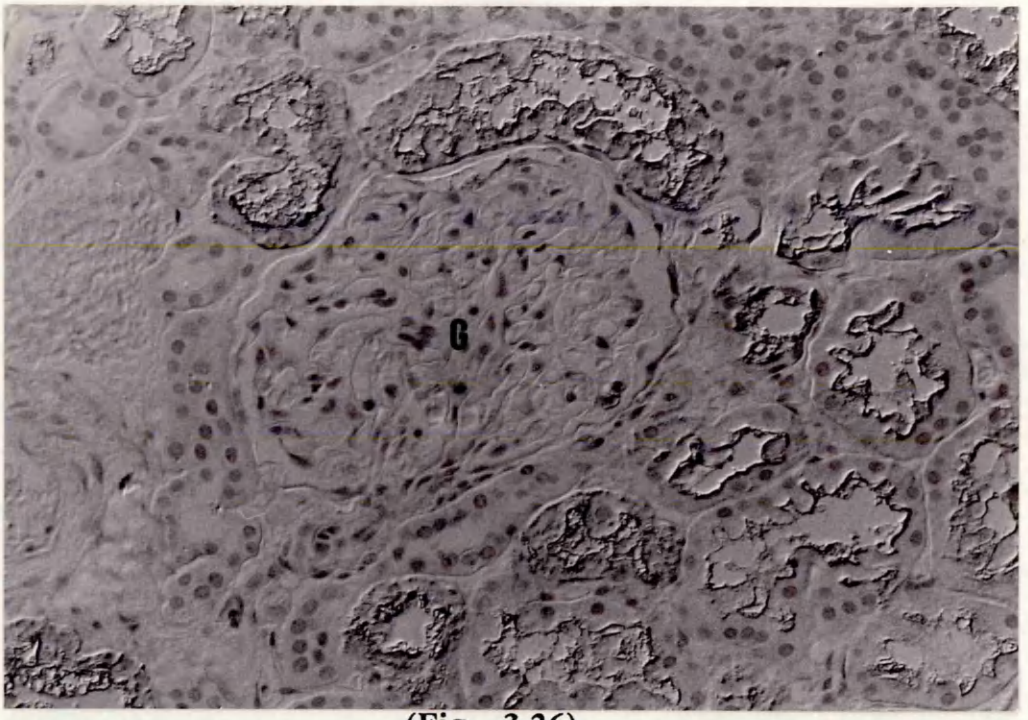
3.4 Applications of the monoclonal antibody-secreting hybrid cell line 'B1-3B3' to elucidation of the structure and function of JGA components

3.4.1 Immunostaining pattern of B1-3B3 McAb on human kidney sections

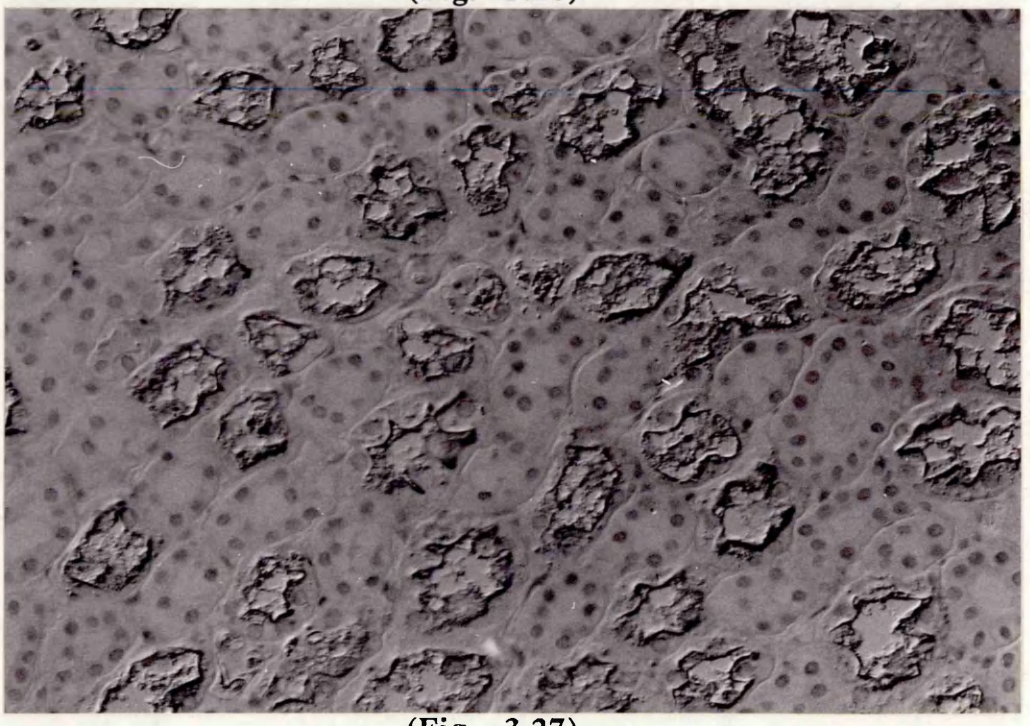
The monoclonal antibodies secreted by the hybridomas of B1-3B3, which were raised in mouse to the cytoplasm of sheep macula densa epithelial cells, crossreact strongly and specifically with the cytoplasm of human renal proximal tubular epithelial cells, including both straight and convoluted parts. However, the antibodies do not crossreact with the macula densa cells and the other components in the human kidney. Similar staining was shown on all of the sections from normal, abnormal (renal artery stenosis) and foetal human kidneys. No immunostaining difference was found between the FITC conjugated secondary antibody preparations with or without preabsorption with normal human serum. Fig. 3.26 and Fig. 3.27 show the immunostaining pattern in the cortex and the medulla of human kidney sections.

3.4.2 Immunostaining pattern of B1-3B3 McAb on rat kidney sections

There is also crossreaction between sheep and rat using the monoclonal antibodies secreted by the hybridomas B1-3B3. The antibodies crossreact consistently with the epithelial cells of the renal distal tubules apart from the macula densa cells, but not with the cells of glomeruli, proximal tubules and the other tissue components of kidney. No immunostaining difference was found between the FITC conjugated secondary antibody preparations with or without the preabsorption with normal rat serum.



(Fig. 3.26)



(Fig. 3.27)

Fig. 3.26 & 3.27 ***'B1-3B3 ' immunostaining on human kidney sections***
Immunostaining of crossreaction of the monoclonal antibody, which was secreted by fused mouse spleen/myeloma cells grown in culture of 'B1-3B3' to the cytoplasm of the sheep macula densa cells, on human kidney sections (paraffin embedded). Strong crossreaction of this antibody was shown. The antibody were directed specifically against the antigen of cytoplasm, mainly the brush border, of human proximal epithelial cells rather than the macula densa cells as in sheep. **Fig.3.26** shows the immunostaining in the human kidney cortex and **Fig.3.27** in the medullary. The paraffin sections were fixed in Bouin's fixative, antibody was detected using an immunoperoxidase procedure. Interference contrast microscopy, x 200, G: glomerulus.

Part 4 DISCUSSION

4.1 The granular peripolar cell

It has been 10 years now, since Ryan et al (1979) described a previously unrecognised but distinctive type of cell in sheep glomerulus, the granular peripolar cell. Further investigation of the peripolar cells in a wide range of species has provided much evidence indicating that the peripolar cell is a secretory type of cell. The cytoplasm granules, one of the most prominent features of the peripolar cell, have been suggested to contain its secretory product. Morphological evidence has shown that these granules can be released into glomerular filtrate under appropriate physiological or pathological conditions (Hill et al, 1983). A potential role that the peripolar cell might play in the control of renal reabsorptive function and body endocrine system have been suggested and become more and more spectaculative. However, the composition of its secretory product is still unknown.

4.1.1 Quantitation

The granular peripolar cells have been found to vary considerably in terms of cell numbers, size and granularity among a wide range of different species (Gall et al, 1986). Relatively, the granular peripolar cells are prominent in species such as sheep and axolotl (Hanner and Ryan,1980). In human glomeruli, however, the granulated peripolar cells are present in the minority of glomeruli where the peripolar cells are very difficult to find (Gardiner & Lindop, 1985). Of all species studied, the granular peripolar cells have been found to be most prominent in sheep where they are heavily granulated, with as many as ten peripolar cells forming a cuff encircling the vascular pole of glomerulus (Kelly et al, in press).

i) Variation in number of the granular peripolar cells in sheep

From the results of the present quantitative study, the granular peripolar cells have been shown to be prominent in most of the sheep kidneys studied. However, considerable variation in cell numbers, size and granularity were also found among individual animals. In terms of peripolar cell index, significant variation in the numbers of granular peripolar cell was indicated by both 'PI' (0-14.46%) and 'PIs' (0-100%), especially the latter, presented here.

Alcorn et al (1984) found that, although granular peripolar cells were poor in foetal sheep, they were extremely prominent in newborn sheep, where they were large, easily seen and heavily granulated. The sharp changes in the granularity of peripolar cells during different periods of life have lead them to the view that the function of peripolar cells could be related to a functional adaptation of the kidney to immediate postnatal life. Among the 22 animals studied, however, I found that granular peripolar cells were scanty in lambs (under 1 year old), where the cells were small and the granules were sparse or even undetectable. In the contrast, granular peripolar cells were prominent in adult sheep. This morphological difference further indicates that the function of peripolar cells might be related to some appropriate physiological conditions during life.

'Alimentary proteinuria' is a condition in which plasma protein levels rise rapidly in the immediate postnatal period (McDougall, 1965; Bainter, 1986). This is due to the ingestion of colostrum via mechanism in the small intestine after birth (McCarthy & McDougall, 1953; Lecce & Morgan, 1962; McCoy et al, 1970). Based on the finding that immunoreactive plasma proteins were found in the granules of the peripolar cells in sheep, Trahair and Ryan (1988) suggested the possibility of renal reabsorptive function of peripolar cells, and tried to explain the previous finding of peripolar cell hypertrophy in the new born lamb (Alcorn et al, 1984). In Trahair's view, for the first few days after birth the newborn lamb is normally proteinuric, peripolar cell hypertrophy is a physiological phenomenon in

response to the prompt increase of filtered proteins in the renal ultrafiltrate.

The formation of hyalin droplets in the epithelial cells of the renal tubule and sometimes in the glomerular tuft is thought to be correlated with some conditions accompanied by proteinuria. The urine of normal adult sheep has only small amounts of protein. In the present study, hyalin droplets were frequently found in those kidneys of adult sheep in which the granular peripolar cells were prominent. It could be proposed that, in response to some pathological changes where the amount of filtered protein in the renal ultrafiltrate increases, peripolar cells might also become hypertrophic and granulated. What remains unclear is whether peripolar cell hypertrophy occurs simply because the cell itself reabsorbs the filtered proteins and forms granules in its cytoplasm, or because of the increased synthesis of secretory product which might control renal reabsorption.

ii) The peripolar cell indexes (PI & PIs)

'PI' is an index derived from a stochastic process used to quantitate granular peripolar cells by counting 200 glomeruli on random sections (Hill et al, 1983). Studying peripolar cells in human kidney, in which the granular peripolar cells are too scanty to be quantitated by random sections, Gardiner and Lindop (1985) counted granular peripolar cells by examining serial sections through every entire glomerulus and derived a more meaningful peripolar cell index which I called 'PIs' in the present study. Apparently, 'PIs' reflects a direct percentage of glomeruli containing granular peripolar cells. It is not only a meaningful data for the purpose of studying the quantitation of granular peripolar cell in view of accuracy, but also and more significantly it shows the quantitative variation of granular peripolar cells from one kidney to another. However, to examine the peripolar cells by serial sections is very time consuming and relatively technically difficult. It will not be able to satisfy the requirement of quick determination for the isolation of glomeruli containing rich granular peripolar cells from fresh kidney tissue. By examining random sections, 'PI' might represent an approximation of the number of glomeruli

containing granular peripolar cells. As a general assessment, it is a quick way and good enough to estimate the number of glomeruli containing granular peripolar cells in terms of 'PI'.

4.1.2 Distribution of the granular peripolar cells in sheep with high 'PI'

The distribution of granular peripolar cells in kidney cortex has been studied previously in some species. Measuring the distance from the corticomedullary junction to the granular peripolar cells, Gardiner and Lindop (1985) found that both the granular peripolar cells and renin-containing cells in human kidneys were distributed predominantly in the outer 1/3 of the cortex. In agreement with this finding, the same distribution has been reported recently in other mammalian species including sheep (Gall et al, 1986). Moreover, Ryan et al (1979) and Hanner (Hanner and Ryan, 1980) found that the granular peripolar cells were prominent in the species with poorly developed myoepithelioid renin-containing cells, but in contrast, rarely seen in species with highly developed myoepithelioid renin-containing cells. Though the significance of the inverse relationship of their numbers and the similar distribution across the kidney is unclear, possible functional relationship between these two types of cell has been suggested.

However, the results of the present study showed considerable variation, from one kidney to another, in the distribution of glomerular peripolar cells across the cortex. There was a relative prominence of glomerular peripolar cells in the middle zone of most of the kidneys studied. 3 kidneys showed more peripolar cells in the outer cortex, but 2 show more peripolar cells near the cortico-medullary junction, and few were found in the most superficial zone (Fig. 3.4a--f). Multiple and heavily granulated peripolar cells were found in the glomeruli near the cortico-medullary junction. To analyse the results, it must be noted that, all of the 6 kidneys studied were those selected for antigen preparation because they had prominent granular peripolar cells (PIs > 70%). There are more glomeruli

distributed in the middle zone and few in the most superficial zone of the cortex. This indicates in the sheep studied that peripolar cells might exist in all of the glomeruli in the kidney irrespective of their location. Under appropriate physiological conditions or pathological changes they may all have the capacity to become granulated. In these cases, the distribution of granular peripolar cells is reflecting the distribution of glomeruli. Therefore, it supports indirectly the suggestion of existence of non-granulated peripolar cells (Gardiner et al, 1986).

4.2 Immunocytochemical identification of peripolar cells in the sheep kidney using monoclonal antibody technology

4.2.1 Use of multicomponent antigen

Because peripolar cell granules can only be identified by their histological position in the glomerulus, attempts have be made to produce a peripolar cell enriched preparation recognising that the antigen used as immunogen must remain a relatively crude fraction. In spite of the successful example of interferon (Secher and Burke 1980), in practice, it is very often unsatisfactory (Campbell, 1984) to use a complex antigen which contains as only a minor component the molecules of interest. This is because the immune response to the antigen of interest may be suppressed by the presence of other antigens in the immunising mixture. In addition, spleen cells producing antibodies to the major antigens may predominate reducing the probability of other spleen cells fusing with the myeloma cells.

i) Granular peripolar cells in isolated glomeruli

To prepare the antigen, one of the 'best' choices was to isolate pure glomeruli which contain prominent granular peripolar cells. For various purposes, renal glomeruli of many species are commonly isolated from cortical kidney tissue using a variety of methods.

Glomeruli isolated by mechanical disruption and enzymic digestion

Glomeruli may be isolated by mechanical disruption of kidney tissue, using different combinations of meshes (Krackower & Greenspon, 1954; Burlington & Cronkite, 1973; Holdsworth et al, 1978). With this technque, however, it has been reported for the rat that most of the glomeruli isolated (86 ± 6%) are free of Bowman's capsule and only few contain vascular poles. In the present study, similar results were obtained when sheep kidney was subjected to mechanical disruption. Less than 10% of the glomeruli isolated retained intact Bowman's capsule and most were contaminated by many other tissue components of kidney. By enzymic digestion of kidney tissue, large amount of glomeruli were released from sheep kidney tissue. However, the glomeruli which were isolated using collagenase or trypsin digestion were also free of Bowman's capsule and, in the case of trypsin were over digested. While these enzymes released glomeruli by digesting surrounding tissue, they also digested Bowman's capsule which consists of mainly collagen fibres.

Histological examination of the isolated glomeruli indicated that the granular peripolar cells are not present in glomeruli which lack Bowman's capsule. This result agrees with the previous ultrastructural observations for rat (Gibson et al, 1988) and for sheep (Kelly et al, in press). These authors found that in no instance could peripolar cells be identified at the vascular poles of any glomerular tufts when broken out of Bowman's capsule, but were instead found in the Bowman's capsule from which the glomeruli had been released. Thus, it was concluded that peripolar cells were very unlikely to be retained in the isolated glomerular tufts, and that such a preparation would therefore not be suitable as an antigen for this project.

Isolation of intact glomeruli by microdissection*

A technique for isolating sheep glomeruli with intact Bowman's capsule by microdissection has been developed in the present study. With this technique, most of the glomeruli isolated had intact Bowman's capsule, BcI 78.05% (mean), and were relatively free of other kidney components. Evidence from histological

examination by serial sections indicated that the granular peripolar cells are present in the microdissected glomeruli and the peripolar cell index (PIms) is almost identical to that of glomeruli in the intact cortex for each of the corresponding kidneys (Fig. 3.6a). This technique has provided a dependable method for the preparation of granular peripolar cells to be used as antigen for the purpose of monoclonal antibody production.

ii) Isolation of kidney granules by zonal centrifugation

In order to enrich the antigen of interest, an attempt was made to partially purify peripolar cell granules from kidney tissue homogenate.

Gross and Barajas (1975) were able to enrich renin-containing granules from rabbit kidney 5-fold over whole homogenate levels using discontinuous sucrose gradient zonal centrifugation. Based on their method, though relatively more granules were seen in the material accumulating between 1.5 and 1.6 M, and between 1.6 and 1.7M sucrose, 3 replicate experiments of the present study failed to produce a fractionation in which granules were sufficiently enriched to make this the preparation of choice for immunisation. Preparations of enriched renin granules can be assessed by measuring the enzymic activity of renin (Gross and Barajas, 1975), because renin is a well established enzyme. However, what peripolar cell granules contains is unknown. There is no direct assay for the assessment of peripolar cell granule content. Moreover, kidney contains other types of granules which will appear in a whole tissue homogenate. It has never been possible microscopically to distinguish peripolar cell granules from others by this method. Alternatively, an approach attempting to enrich granules from microdissected glomeruli rather than whole kidney tissue is recommended.

Zonal centrifugation separations are based upon the parameters of size (S) and density (P) of the subcellular particles and organelles, also the concentrations of the discoutinuous sucrose gradient and the speed of centrifugation. Modification of the

conditions used in this study might improve the enrichment of granules. Therefore, it is still an approach worth trying.

4.2.2 Immune response of mice immunised with microdissected glomeruli

Successful immunisation of the animals is the first stage in the production of hybridomas and determines whether or not the further effort, which is very expensive and time consuming, is worthwhile.

Most of work to date has been done with mice which is one of the three major systems (mice, rat and human, either in vivo or in vitro) currently used for monocloual antibody production (Campbell, 1984). In general, the myelomas used for fusion are mostly from the BALB/c strain of mice, and it is thus convenient to use this strain for immunsation. In addition, one of the advantages of using this strain of mice is that the myelomas from this strain do not themselve make antibody. The 3 series of 9 mice immunised in the present study were all of BALB/c strain which have shown reasonable good immune response after immunisation.

i) Immunoassay of serum antibody

As a guide to successful immunisation, assessment of antibodies in the serum of the immunised mouse is necessary, particularly since the immunoresponse to a antigen can differ greatly in individual mice. On the other hand, since the antigen used for immunisation was only a minor component of a complex mixture, detection of peripolar cell antibody amongst the many other serum antibodies, might be impossible. The antibody could be detected, however, morphologically using immunocytochemical techniques.

In theory, the immune system of any animal has the totipotency to make antibody in response to any antigen injected. With a broad and sensitive enough screening system it should be possible to detect antibodies to any antigen which has

the potential to elicit a response (Campbell, 1984). In the present study, however, significant variation of immunoreaction both in terms of antigen specificity and strength was seen from one mouse to another. In response to the antigen injected, the microdissected glomeruli, large amounts of antibody mainly to the components of glomeruli were produced by 5 of the total 9 mice immmunised (Fig. 3.7). This immuno-screening of serum antibodies was used as the major indicator determining which mice were used for hybridoma production. Those mice which were producing significant amounts of antibodies to some other components of the kidney were deemed to be an unsuitable source of spleen cells for fusion.

What remains unexplained is the significant difference of immune response of the remaining 4 mice, which had produced large amount of antibodies mainly to the components in the tissue interstium but not to the cell surface or intracellular components of glomeruli and tubules (Fig. 3.8).

ii) Antibody isotype

There are situations in which one or other antibody isotype is preferred depending upon the purpose for which the monoclonal antibody is to be used. One of the reasons for producing monoclonal antibody specifically to the components of peripolar cells, especially its cytoplasmic granules, is that the antibody could be used as an immunoaffinity chromatographic ligand to aid the purification of the secretory product of the peripolar cells. For this purpose, where purification of antibody is firstly required, IgG is preferable to IgM because it is easier to purify and, the affinity of IgG for antigen is normally higher than that of IgM.

The immune response is adaptive. When an animal is injected with an antigen which it has never encountered before, it will make a small amount of antibody which is mainly IgM. This is called the primary response. If some time later, when the same antigen is reinjected a secondary response will not only be much stronger and faster, but also different types of antibody molecules, mainly IgG, will be

produced. At least 3 injections of antigen were given to the mice in this study. However, as indicated by the Elisa results, only a minority of immunoglobulin-secreting hybridomas developed from the fusions of spleens from mice B1 and C1 produced IgG. The reason for that is unclear. It might be possibly due to the use of incomplete Freund's adjuvant after the first injection of antigen.

Since completion of this study, Mrs Andrea Brown has confirmed by establising the molecular weights of antibody light and heavy chains, that the antibody secreted by B1-3B3 is of the IgM class. This was established by growing B1-3B3 hybrids in the presence of 14C-leucine. The secretory product was subjected to electrophoresis on SDS-polyacrylamide gel and the radio-labelled antibody chains identified by fluorography.

4.2.3 Hybridisation, cell development and antibody production

Using the standard procedure for hybridisation, hybridomas were derived from each of the 5 fusions of mice spleen cells, and most of them were well developed after fusion. For most of the cultures by two weeks after fusion, clones were detectable. However, during the first few weeks, the rates of cell division were low, and some of the hybridomas were lost. Hybridomas of culture A1-3A3 had been shown to be secreting antibodies directed to the granules of the sheep peripolar cell, but the culture failed to thrive at 4 weeks after fusion. Hybridomas of culture C2-2C2 were also thought to be possibly secreting antibodies to the granules of peripolar cell at the early stage, but non-secretors then became dominant in the culture.

In theory, myeloma cells die within the first few days because of the aminopterin block. This is because the myeloma cells used for fusion are deficient in either enzymes of TK (thymidine kinase) or HPRT (hypoxanthine phosphoribosyl transferase), which are the essential enzymes of the salvage

pathway leading to nucleic acid synthesis (Campbell, 1984). After hybridisation, cells are cultured in the selective medium in the presence of hypoxanthine, aminopterin and thymidine (HAT). Since aminopterin blocks the main pathway of nucleotide synthesis, unfused myeloma cells can not survive. With adequate supplement of HT (hypoxanthine and thymidine) in culture, hybrids may survive by synthesising nucleotides through the 'salvage pathway', since they adapt this capacity from normal spleen cells. The majority of the spleen cells die because they do not have the inherent capacity to divide or stay alive in culture without exogenous stimuli.

However, many previous studies have also indicated that the majority of fusion products detectable immediately after fusion die in the next few days (Zola et al, 1984). This is probably because, at the early stage after fusion, the new hybrid cells, formed with combinations of the chromosome complement of the parent cells, have an unstable profile. These hybrids may lack the chromosomes required for cell division or protein synthesis, and will therefore not survive in culture. Absence of the chromosomes involved in the synthesis or secretion of immunoglobulin would result in non-secretor hybrids which might otherwise be viable. Therefore, maintanence of hybridoma culture is no less importance than the fusion process itself and great attention should be paid in this period.

Few hybridomas developed in the cultures of fusion A2 and fusion B2. This was due to the contamination of the culture medium with micro-organisms which have not been characterised.

4.2.4 Detection of antibody-secreting hybridomas

In the present study, great efforts were made to detect antibody-secreting hybridomas of interest from the secretors and the non-secretors of the 5 fusions, and to identify the antigenic cellular binding sites of the antibodies secreted.

i) Evaluation of the assay system

The assay system is very important in the selection and generation of hybridomas of interest. It should be capable of satisfying the following several factors: (1) sensitive enough to detect the emerging clones which secrete small amounts of antibody in the early stage; (2) capable of handling a large panel of samples at a time; (3) simple and rapid for the frequent screening required to identify positive samples for early cloning hybridomas; and (4) to morphological identify peripolar cells and other tissue components of interest. A combination of immunofluorescence, Elisa immunoenzymic assays and contrast microscopy has been developed as a practicable assay system, for the purposes of present project.

Immunocytochemical staining

Immunocytochemical assay are suited to the detection of antibodies to rare cell types and particularly useful when tissue localisation of the antigen is an important criterion in whether the antibody is directed against the antigen of interest. Several methods have been used for the detection of antibody-secreting hybridomas in the present study. It was noticed that, under the same assay condition, different results were obtained when different types of tissue section were used. Samples in which antibodies were detectable on cryostat sections were very often undetectable on paraffin embedded tissue sections. Apparent examples have been shown for the antibodies secreted by hybridomas of C1-1C6 (IgG) and C1-4D2 (IgM) in the present study. It has been suggested that tissue fixatives or the series of steps necessary for the processing and embedding tissue might variably destroy a proportion of antigenic determinants (Zola, 1988). Therefore, there will be risk of failing to detect a specific antibody if only paraffin sections are used thoughout the screening of hybridoma secretion products. In some instances, however, antibodies did bind effectively to paraffin sections which is particularly useful for the purpose of localisation of antigenic binding sites of the antibodies. The immunoreaction of the B1-3B3 monoconal antibody with the antigen on paraffin sections, has shown strong immunostaining and excellent preservation of

morphology (Fig. 3.14--3.20).

Phase and interference contrast microscopy

The difference in immunostaining between cryostat and paraffin-embedded tissue sections indicated that antigenicity might be better preserved in cryostat sections. The use of cryostat sections is less likely to lead to false negative results and was therefore routinely used to detect antibodies in the early stages of hybridoma culture. However, the use of cryostat sections has a disadvantage that morphology is relatively difficult to observe. The initial inaccurate conclusion that hybridomas B1-3B3 (section 3.3.3) were producing a peripolar cell antibody reflected the difficulty of cryostat sections where tissue preservation is often inadequate for detailed examination. This caused great difficulty in identification of precise cellular antibody-binding sites on the sections under U.V. light. To solve this problem, phase and interference contrast microscopy have been successfully developed as an additional technique to the immunofluorescence assay for the identification of specific binding on cryostat sections. This is particularly useful for the identification of granular peripolar cells which can be shown clearly on the cryostat sections without any counterstain.

Elisa immunoenzymic assay

To detect antibody production and determine positive samples for early cloning hybrids, the sensitivity of an assay system is also critical. For the detection of antibody production of fusion C1, the results showed that the sensitivity of the Elisa assay was much higher than that of the immunofluorescence assay used. The assays used in this study can detect as little as 20 ng IgG per ml secreted into the culture medium, but required more than 500 ng IgM per ml (Fig. 3.21 and Fig. 3.22). Establishing the specificity of the antibody still depends on immunocytochemical assays, Elisa assay serves as an early sensitive indicator of antibody-secretion by hybridomas. It may also simplify the screening procedure of the cytochemical assay by reducing the numbers of samples to be screened through

elimination of those not secreting antibody. In addition, it can identify the isotypes of the antibodies secreted.

Routinely, a standard curve established by serial dilutions of known antibody is usually necessary for setting up each individual Elisa assay, if measurement of precise amounts of antibody is required. In the present study, however, the Elisa assay was used simply to distinguish between antibody secretors and non-secretors. Therefore, to reduce the costs and simplify the procedure, antibody production was assessed by comparing the Elisa results from hybrid cultures with each of the corresponding negative controls. The approximate amount of antibody produced was then estimated by comparison with an Elisa standard curve for each of the antibody isotype (e.g. IgG and IgM), and based on each of the corresponding negative control samples.

ii) Types of antibody derived from hybridomas

In response to the complex antigen used for immunisation, hybridomas secreting a variety of antibodies were derived from 4 out of 5 fusions of mice spleen cells. The antibodies secreted by the hybridomas were mainly directed against the intraglomerular components, especially the glomerular epithelial cells which were the dominant component of the complex antigen. This result might reflect that the percentage of different antigenic substances is one of the factors to be considered for immunisation. Relative purification of antigen might be therefore necessary.

Antigenicity of antigen is probably another factor which concerns the effect of immunisation. The microdissected glomeruli selected as antigen for immunisation all contained many peripolar cells with relatively prominent granules. However, the granular peripolar cells are still a minor component in such a complex antigen. Nevertheless, antibodies to the granules of peripolar cells secreted have been produced by hybridomas established in the present study. This indicates that the granules of peripolar cells, which have been suggested to contain a glycoprotein

(Gardiner and Lindop, 1985), might have reasonable high antigenic characteristic.

The antibodies secreted by the hybridomas in the culture B1-3B3 were eventually found to interact with antigen located in the cytoplasm of the macula densa cells, rather than with peripolar cells (Fig.3.20). The immunostaining was consistently and specifically shown to be located very close to the vascular poles of the glomerulus, and sometimes presented a granular appearance. A monoclonal antibody binding specifically to macula densa could be a potentially important tool contributing to the developing understanding of the role of these important cells in the juxtaglomerular complex.

4.2.5 Cloning hybridomas by limiting dilution

The purpose of cloning hybrids is to ensure that the cells producing the antibody of interest comprise a monoclonal population secreting a single type of antibody, and free from non-secreting mutants generated during fusion. Cloning efficiency, in practice, is always variable (Zola, 1988). According to Poisson statistics, if the most probable cell number per well is 1, then 37% of wells will have no colonies at all. In order to maximise the chances of obtaining single-cell clones with unknown cloning efficiency, cultures were set up at several theoretical concentrations, 4, 2, 1 and 0.5 cells per well.

i) Cloning the hybridomas of B1-B3

The results presented (table 3.6) show that single-cell clones were obtained from different dilutions of cell suspension used for the cloning process. On the first occassion hybridomas of B1-3B3 were cloned, the number of multiple clones was very high. This might have been due to inaccurate assessment of the numbers of viable cells in the population cloned. Most of the clones were antibody secretors. After the cells were cloned for the first and the second times, a few of them were non-secreting clones. This might due to the gradual loss of chromosomes after

fusion generating additional heterogeneity.

ii) Cloning the hybridomas of C1-2C2

Hybridomas in this culture produce antibodies which generated immunostaining patterns apparently associated with the granules of peripolar cells. However the cloning efficiency was low for cloning the hybridomas C1-2C2, with only 4 out of 93 wells developing clones. These cultures were abandoned eventually because they ceased to secrete detectable antibody. This might have been due to the gradual loss of chromosomes from hybrids with an unstable chromosome complement or to rapidly growing but non-secreting hybridomas eventually dominating the culture .

4.3 Applications of the monoclonal antibody-secreting hybrid cell line 'B1-3B3' to elucidation of the structure and function of JGA components

The mouse monoclonal antibody producing cell line (B1-3B3) has been successfully established during the present study. It has been shown to be secreting large amount of antibodies (IgM) reacting specifically with an antigen in the cytoplasm of the highly differentiated epithelial cells of the macula densa in the sheep kidney. The use of this antibody as a new tool to study JGA has shown its potential significance.

4.3.1 Immunocytochemical identification of cell types within the macula densa

i) Evidence for different cell types within the macula densa

A description of the special topographical relationship between the distal tubule and the glomerular arterioles preceded good physiological evidence of its function by nearly a century. Understanding of the cellular interactions between the highly differentiated epithelial cells of the macula densa and other components of the JGA, is crucial to the elucidation of functional mechanism of the complex structure as a

whole. Since interpretation of current physiological data is based on the anatomical evidence, efforts have been made to study the morphology of the specialised cells in the JGA.

Morphological differences between the cells of macula densa has been mentioned in the literature (Kriz et al, 1978). The possibility of two types of macula densa cells was previously, however unconvincingly, proposed. The difference noticed was that one type of cell appeared to be 'dark' while the other was 'light'. Since the 'dark' macula densa cell had only been found after immersion fixation methods, it was thought that the difference might be due to the fixation procedure, and both 'light' and 'dark' macula densa cells belonged to one homogenous population of cells. However, immunocytochemical evidence has been provided here showing a significant difference between the macula densa cells in the sheep (Fig. 3.14--3.17).

A molecule which is expressed selectively by a particular cell type can serve as a marker for the cell type. As cells differentiate to carry out their specific functions, different genes are expressed, and the molecular composition of a cell, therefore, reflects its differentiation state. The macula densa of a single glomerulus contains cells which reacts with the monoclonal antibody secreted by hybridomas of B1-3B3 (defined here as 'type A'), as well as cells which do not react with the antibody (defined here as 'type B'). This distinction supports the previous suggestion that in sheep the macula densa may consist of two different classes of cell, which might carry out different functions. It might be that this unique monoclonal antibody will provide the basis for experiments to define the possibly different functions of the two cell types, and to establish whether the immunostaining differences relate to the 'light' and 'dark' morphological variation of macula densa cells.

ii) Types of cellular contact of macula densa and other JGA cells

By means of electron microscopy of serial sections and three-dimentional

reconstruction of the juxtaglomerular apparatus, a quantitative study has been made of the areas of contact between the tubular and vascular components (Barajas and Latta, 1963a, 1963b, Faarup, 1964). Two morphologically distinctive types of contact were described. Type 1 is characterized by the presence of cytoplasmic projections from the base of the cells of the distal tubule and the formation of a network by the basement membranes of the tubular and vascular components. This was thought to be permanent, anchoring the distal tubule at the mesangial and hilar arteriole. Type 2 is a simple adjacency between the basement membranes which is thought to be reversible. These anatomical findings have led to the concept that variations in contact between the elements of JGA may be responsible for the control of renin secretion. A phenomenon noted in the present study is in agreement to this view. The cells of macula densa which reacted with B1-3B3 antibodies are mainly located at both corners of the macula densa where the cells of macula densa usually have close contact with the renin-containing cells in the glomerular arterioles. We propose therefore that the cellular component to which the monoclonal antibody bound, could be related to the communicating process of the glomerular feedback pathway with renin-containing myoepithelioid cells. Possibly, through the 'type 1 contact', it could be the 'type A' macula densa cell which senses changes in renal tubular fluid, and informs the preglomerular arteriole to control renin release.

4.3.2 Interspecies immunocrossreaction

Species cross reaction of the monoclonal antibodies of B1-3B3, which were raised in mouse and react with the cytoplasm of sheep macula densa epithelial cells, has been shown also to interact specifically with the cytoplasm of human renal proximal tubule but not with the macula densa cells. In rat this antibody interact with the distal tubular epithelial cells apart from the macula densa cells. One of the explanations could be that some of the functions taking place in the epithelial cells of macula densa of sheep could be taken over by the epithelial cells of proximal tubules in human, and by the epithelial cells of other segment of distal tubule in rat.

However, many cell surface antigens are carbohydrates in nature and the same antigenic sites may be present on same cell types which have similar structure but play different functional roles in the body (Campbell, 1984). Therefore, monoclonal antibody can react with unrelated tissue, cells or macromolecules if they possess a shared epitope. In this case, it is possible to have 100% crossreactivity. For this reason, the application of monoclonal antibody, especially for the use of clinical diagnosis or treatment, should be treated with caution. To assess whether the monoclonal antibody secreted by the B1-3B3 hybridomas detects the same protein in the three species or three different proteins, characterisation of molecules to which this antibody binds in the different species, is required. As an initial step the westen blotting technique could be used to establish the electrophoretic characteristics of the antigen in each species.

4.4 Conclusion, further questions and recommendation

1. The quantitation and the distribution of the granular peripolar cells in the sheep kidney vary considerably between individual animals. The peripolar cell is a distinctive type of cell which might exist in every glomerulus and become granulated or non-granulated under appropriate physiological conditions which occur during different periods of life, or possibly in some pathological conditions. The questions arising from these observations include: whether peripolar cells become granulated because they store their secretory product due to a decrease of secretory activity; or because of the cell hypertrophy due to an increasing synthetic process and secretory activity; or simply because of the storing of renal reabsorptive substance by peripolar cell itself.

2. A monoclonal antibody producing cell line, the hybrids of B1-3B3, has been successfully established in the present study. The value of the monoclonal antibody in the identification of cell types of macula densa of sheep has been

shown. Immunostaining indicating differences in the macula densa cells has provided evidence that, there might be at least 2 types cell, type A and B, existing in macula densa, which might be responsible for different functions. It is also possible that the morphological difference might represent different physiological conditions of some renal function, particularly the physiological condition concerning renin release. Further study of the ultrastructure of the macula densa using this monoclonal antibody is strongly recommended, from which more information can be expected in understanding the cell types of macula densa, the underlying mechanism controlling renin release and the function of JGA as a whole.

3. Attempting to raise monoclonal antibody producing cell lines to an antigen which is a minor component in a complex mixture is a very difficult but still practical approach. To increase the efficiency, relative antigen purification is necessary. Effort should be also made to improve the method of immunisation in order to stimulate an IgG response. Attention must be always paid to the process of screening for detection of antibody and early cloning of cell hybrids while their secreted antibodies are tested. The establishment of this hybrid cell line producing monoclonal antibodies to the macula densa cells is a good example which points optimistically to the future of the production of monoclonal antibody to the granular peripolar cells. To reach this aim, success will depend upon a continuous effort.

APPENDICES

I. Abbreviations

AA	afferent arteriole
BcI	Bowman's capsule index
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
DMSO	dimethyl sulphoxide
EA	efferent arteriole
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
HAT	hypoxanthine, aminopterin and thymidine
HPRT	hypoxanthine phosphoribosyl transferase
HT	hypoxanthine and thymidine
IFA	incomplete Freund's adjuvant
JGA	juxtaglomerular apparatus
MD	macula densa
MSB	Martius scarlet blue stain
NSS	normal sheep serum
OPD	orthophenylene diamine
PAP	peroxidase anti-peroxidase
PBS	phosphate buffered saline
PEG	polyethylene glycol
PI	peripolar cell index
PIs	peripolar cell index by serial sections
PIms	peripolar cell index of microdissected glomeruli by serial sections
SBTI	soybean trypsin inhibitor
STWS	Scotts tap water substitute
TK	Thymidine kinase

II. Reagents and chemicals

i) Buffers

Phosphate buffered saline

contained 0.15 M sodium chloride, 1.98 mM potassium dihydrogen orthophosphate, 7.96 mM disodium hydrogen orthophosphate, pH 7.3.

Krebs buffer

contained 0.12 M sodium chloride, 2.54 mM calcium chloride, 1.15 mM sodium dihydrogen orthophosphate dihydrate, 1.15 mM magnesium sulphate heptahydrate, 24 mM sodium hydrogen carbonate, pH 7.4.

Coating buffer

contained 14.15 mM sodium carbonate anhydrous, 34.88 mM sodium hydrogen carbonate, 3.08 mM sodium azide, pH 9.6.

Blocking buffers

were 5%, 2% and 0.5% (w/v) BSA in PBS, for different assays of immunofluorescence, PAP and Elisa respectively.

Tris-tween washing buffer

contained 0.2 M tris-HCl, 0.19 M sodium chloride, 0.05% (v/v) tween 20, pH 7.4.

McIlwaine's buffer

contained 17.9 mM citric acid, 64.2 mM disodium hydrogen orthophosphate dihydrate, pH 6.0.

Veronal acetate buffer

contained 31mM sodium acetate trihydrate, 31mM sodium barbitone, pH 9.2, adjusted with HCL.

ii) Media

RPMI 1640 medium

with 2 mM L-Glutamine, (GIBCO)

For fusion and culture of hybrids, the culture medium was RPMI 1640 containing 2.5 ug/ml amphotericin B (fungizone), 100 units/ml penicillin, 100 ug/ml streptomycin, and foetal calf serum at 5%, 10%, 15% or 20%. (stored at 4 ^{0}C)

HT culture medium

was the complete culture medium containing 0.1 mM hypoxanthine, 0.016 mM thymidine. (stored at 4 ^{0}C)

HAT culture medium

was the complete culture medium containing 0.1 mM hypoxanthine, 0.016 mM thymidine, 0.004 mM aminopterin. (stored at 4 ^{0}C)

Medium for freezing cells

contained 10% (v/v) DMSO (Koch-Light Ltd.) in inactivated foetal calf serum.

Scotts Tap Water Substitute

contained 42 mM sodium hydrogen carbonate, 150 mM magnesium sulfate heptahydrate, pH 8.0.

iii) Serum product

Foetal calf serum

inactivated by heating at 56 ^{0}C for 45 minutes (Biological industries)

Bovine serum albumin

Fraction V (Miles Laboratory)

Normal sheep serum

(SAPU)

iv) Antibodies

Mouse IgM monoclonal antibody

anti-rat myosin (cardiac), chrompurified (Biochem. Dept., University of Glasgow)

Mouse IgG (whole molecule)

chrompurified (JACKSON-Laboratory)

Goat anti-mouse immunoglobulin

affinity purified (SERA-Laboratory)

Rabbit anti-mouse IgG (Fc specific)

affinity purified (JACKSON-Laboratory)

Rabbit anti-mouse immunoglobulin (Fab')2 fragment

peroxidase conjugated, affinity purified (JACKSON-Laboratory)

Goat anti-mouse immunoglobulin (polyvalent)

FITC labelled, affinity purified (SERA Laboratory)

Rabbit anti-mouse immunoglobulin

FITC labelled, affinity purified (DAKO Laboratory)

Rabbit anti-mouse immunoglobulin

alkaline phosphatase conjugated, affinity purified (DAKO Laboratory)

VECTASTAIN^{R} ABC Kits

1. Biotinylated, anti-mouse immunoglobulin (Mouse IgG, PK-4002)
2. Reagent A (Avidin DH)
3. Reagent B (Biotinylated horseradish peroxidase H)

v) Enzyme substrates

Elisa immunocytochemical assay

The substrate contained 2.21 mM orthophenylene diamine dissolved in McIlwaine's buffer, and 0.032% (v/v) hydrogen peroxide (added immediately before use, kept in dark)

Alkaline phosphatase immunocytochemical assay

The substrate contained 0.5 mM Levamisole, 1.33 mM Fast red violet and 0.54 mM naphthol AS-MX (dissolved in 2 drops dimethyl formalide). These were dissolved in veronal acetate buffer and filtered in dark prior to use.

PAP immunohistochemical assay (VECTASTAIN^{R} ABC Kit)

The substrate contained 1.26 mM diaminobenzidine tetrahydrochloride (DAB) in 0.1 M tris buffer (pH 7.2) and 0.1% (v/v) hydrogen peroxide (added immediately before use, kept in dark).

vi) Enzymes and inhibitor

Collagenase

(WORTHINGTON)

Trypsin

from Bovine Pancreas TYPE I (SIGMA)

Trypsin inhibitor

from soybean TYPE I-S (SIGMA)

vii) Others

Complete Freund's Adjuvant

(DIFCO Laboratories)

Incomplete Freund's Adjuvant

(DIFCO Laboratories)

Polyethylene glycol (PEG 4000)

(MERCK)

Amphotericin B (Fungizone)

(GIBCO)

Penicillin/Streptomycin

(GIBCO)

Polyoxyethylene sorbitan (Tween 20)

(SIGMA)

All other reagents were analytical grade

REFERENCES

Alcorn, D., Cheshire, G. R., Coghlan ,J. P. & Ryan ,G. B. (1984): Peripolar cell hypertrophy in the renal juxtaglomerular region of newbown sheep.
Cell Tissue Res., 236: 197-202.

Baintner, K. (1986): Intestinal absorption of macromolecules of immune transmission from mother to young.
Florida, CRC Press. 57-102.

Bancroft, J.D. & Cook, H.C. (1984): Manual of histological techniques.
Churchill Linvinstone, Edinburough, London, Melbourne & New York.
Printed in Singapore. 102-103.

Barajas, L. and Latta, H. (1963a): A three-dimensional study of the juxtaglomerular apparatus in the rat. Light and electron microscopic observations.
Lab. Invest., 12: 257-269.

Barajas, L. and Latta, H. (1963b): The juxtaglomerular apparatus in adrenalectomized rats. Light and electron microscopic observations.
Lab. Invest., 12: 1046-1059.

Barajas, L. (1966): The development and ultrastructure of the juxtaglomerular cell granules.
J. Ultrastruct. Res., 15: 400-413.

Blaine, E. H., Davis, J. O. & Witty, R. T. (1970): Renin release after hemorrhage and after suprarenal arotic constriction in dogs without sodium delivery to the macula densa.
Circ. Res., 27: 1081-1089.

Blaine, E. H., Davis, J. O. & Prewitt, R. L. (1971): Evidence for a renal vascular receptor in control of renin secretion.
Am. J. Physiol., 220: 1593-1597.

Bowie, D. J. (1936): A method for staining the pepsinogen granules in gastric glands.
Anat. Rec. 64: 357-367.

Burlington H., Cronkite E.P. (1973): Characteristics of cell cultures derived from renal glomeruli.
Proc. Soc. Exp. Biol. Med. 142:143-149.

Camilleri, J. P., Phat, V. N., Bariety, J., Corvol, P. & Menard, J. (1980): Use of a specific antiserum for renin detection in human kidney.
J. Histochem. Cytochem, 28: 1343-1346.

Campbell, A.M. (1984) : Monoclonal antibody technology
In 'Laboratory techniques in biochemistry and molecular biology'.
Amsterdam; Oxford Press.

Carone, F. A., Pullman, T. N., Oparil, S. & Nakamura, S. (1976): Micropuncture evidence of rapid hydrolysis of bradykinin by rat proximal tubule.

Am. J. Physiol., 230: 1420-1424.
Cook, W. F. & Pickering, G.W. (1959): The location of renin in rabbit kidney.
J. Physiol. (London), 149, 526.
Cook, W. F. (1971): Cellular localisation of renin.
In: 'Hormones and the Kidney' (Ed: J.W. Fisher), 117-128.
New York: Academic press.
Davis, J. O., Carpenter, C. C. J., Ayers, C. R., Holman, J. E. & Bahn, R. C. (1961): Evidence for secretion of an aldosterone-stimulating hormone by the kidney.
J. Clin. Invest., 40: 684-696.
Davis, J .O., Hartroft, P. M., Titus, E. O.,Carpenter, C. C. J., Ayers, C. R. & Spiegel, H. E. (1962): The role of the renin-angiotensin system in the control of aldosterone secretion.
J. Clin. Invest., 41:, 378-389.
Davis, J. O. & Freeman, R. H. (1976): Mechanisms controlling renin release.
Physiol. Rev., 56: 1-56.
Derkx, F. H. M., Tan-Tjiong, H. L., Man in't Veld, A. J., Schalekamp, M. P. A. & Schalekamp, M. A. D. H. (1979): Activation of inactive plasma renin by plasma and tissue kallikreins.
Clin. Sci. Mol. Med., 57: 351-357.
de Senarclens, C. F., Pricam, C. E., Banichahi, F. D. & Vallotton M.B. (1977): Renin synthesis, storage and release in the rat: A morphological and biochemical study.
Kidney Int., 11: 161-169.
Dick, B W. & Kurtz, S. M.(1968): Protein absorption from the urinary space by glomerular visceral epithelium.
Laboratory Investigation, 19: 412-420.
Dover, F. E., Kahn, J. R., Lentz, K. E., Levine, M. & Skeggs, L. T. (1974): Hydrolysis of bradykinin by angiotensin converting enzyme.
Circ. Res., 34: 824-7
Dunihue, F. W. & Boldosser, W. G. (1963): Observations on the similarity of mesangial to juxtaglomerular cells.
Lab. Invest., 12: 1228-1240.
Edelman, R. & Hartroft, P. M. (1961): Localization of renin in juxtaglomerular cells of rabbit and dog through the use of fluorescent-antibody technique.
Circ. Res., 9: 1069-1077.
Farrup, P. (1964): Morphology of the juxtaglomerular apparatus.
In: 'Proceedings of the Second International Congress of Nephrology' (Ed.Vortil, Y., & Richet, G.) Excerpta Media Foundation, New York. 427.
Fisher, J.W. (1971): Kidney hormones
Academic Press, London, New York. Vol. I, 59-91.
Fisher, J.W. (1986): Kidney hormones

Academic Press, London, Orlando, San Diego, New York, Austin, Montereal, Sydney, Tokyo, Torondo. Vol. III, 20-22

Gall, J. A. M., Alcorn, D., Coghlan, J. P., Johnston, C. I. & Ryan, G. B. (1984): Immunohistochemical detection of urinary kallikrein-like material in renal juxtaglomerular peripolar cells in sheep.
Proceedings of the IXth international Congress of Nephology, Los angeles. June.

Gall, J. A. M., Alcorn, D., Butkus, A., Coghlan, J. P. & Ryan, G. B. (1986): Distribution of glomerular peripolar cells in different mammalian species.
Cell Tissue Res., 244: 203-208.

Gardiner D. S., Lindop G. B. M. (1985): The granular peripolar cell of the human glomerulus: an new component of the juxtaglomerular apparatus?
Histopathology, 9: 675-685.

Gardiner, D. S., More, I. A. R. & Lindop, G.B.M..(1986): The granular peripolar cell of the human glomerulus: an ultrastructural study.
J. Anat,146: 31-43.

Gibson, I., More, I. A. R., Lindop, G. B. M. (1988): The peripolar cell of the renal glomerulus: a scanning electron micrographic study in the rat.
Cell and Tissue Research (in press).

Goldblatt, H., Lynch, J., Hanzal, R. F. & Summerville, W. (1934): Studies on experimental hypertension. 1: The production of persistent elevation of systolic blood presure by means of renal ischemia.
J. Exptl. Med., 59: 347-380.

Goormaghtigh, N. & Grimson, K. S. (1939): Vascular changes in renal ischemia: Cell mitosis in the media of the renal arterioles.
Proc. Soc. Exptl. Biol. Med., 42: 227-228.

Gross, D.M., & Barajas, L. (1975): The large-scale isolation of renin-containing granules from rabbit renal cortex by zonal centrifugation.
J. Lab. Clin. Med. 85: 467-477.

Haberle, D. A. & Shigai, T. (1978): Flow-dependent volume reabsorption in the proximal convolution of the rat kidney - the glomerular-born tubular fluid for the maintence of glomerulotubular banlance.
In: 'New aspects of renal function' (Ed. Vogel, H. G. & Ullrich, K. J.).
Amsterdam. Excerpta Medica: 198-207

Hanner, R. H. & Ryan, G. B. (1980): Ultrastructure of the renal juxtaglomerular complex and peripolar cells in the axolotl (Ambystoma mexicanum) and toad (Bufo marinus)
J. Anat.,130, 3: 445-455.

Hartroft, P. M. & Hartroft, W. S. (1953): Studies on renal juxtaglomerular cells. I: variations produced by sodium chloride and desoxycorticosterone acetate.
J. Experl. Med., 97: 415-427.

Hatt, P.Y. (1967): The juxtaglomerular apparatus.
In: Ultrature stucture of the kidney. Ed. Dalton, A.J. & Haguenau, F.

Academic Press, New York, London. 101-141.

Hill, P. A., Coghlan, J. P., Scoggins, B. A. & Ryan, G. B.(1983): Ultrastructural changes in the sheep renal juxtaglomerular apparatus in response to sodium depleption or loading.
Pathology , 15: 463-473.

Hill, P. A., Coghlan, J. P., Scoggins, B. A. & Ryan, G. B. (1984a): Functional and morphologic studies of the adrenal cortex and kidney in ovine toxaemia of pregnancy.
J. Pathology, vol.144: 1-13.

Hill, P. A., Coghlan, J. P., Scoggins, B. A. & Ryan, G. B. (1984b): Structural and functional studies of the adrenal cortex and renal juxtaglomerular apparatus in pregnant sheep subjected to sodium depletion or loading.
Pathology, 16: 285-290.

Holdsworth S.R., Glasgow E.F., Atkins R.C., Thomson N.H. (1978): Cell characteristics of cultured glomeruli from different animal species.
Nephron 22: 454-459.

Johnson, J. A., Davis, J. O., Gotshall, R. W., Lohmeier, T. E., Davis, J. L., Braverman, B. & Tempek, G. E. (1976): Evidence for an intrarenal beta receptor in control of renin release.
Am. J. Physiol., 230: 410-418.

Kelly, G., Downie, I., Gardiner D.S., More, I.A.N. and Lindop, G.B.M. (1989): The peripolar cell: a distinctive cell type in the mammalian glomerulus, morphological evidence from a study of sheep.
J. of Anatomy (in press).

Khairallah, P. A., Robertson, A. L. & Davila, D.(1972): Effects of angiotensin II on DNA, RNA and protein synthesis.
In: 'Hypertension' (Ed.Genest J., Koiw E.)
Berlin, Heidelberg, New York: Springer, 212-220.

Kischet, E. S., Dluhy, R. G. & Willams G. H. (1976): Regulation of renin release by calcium and ammonium ions in normal man.
J. Clin. Endocrinol. Metab.,43: 1343-1350.

Kohler, G. and Milstein, C. (1975): Continuous cultures of fused cells secreting antibody of predefined specificity.
Nature 256, 495.

Kotchen, T. A., Galla, J. H. & Luke, R. G. (1976): Failure of NaHCO3 and KHCO3 to inhibit renin in the rat.
Am. J. Physiol., 231: 1050-1056.

Kotchen, T. A., Maull, K. I. Luke, R., Rees, D. & Flamenbaum, W. (1974): Effect of acute and chronic calcium administration on plasma renin.
J. Clin. Invest., 54: 1279-1286.

Kovalchuk, L. E. (1987): Ultrastructure of the juxtaglomerular apparatus of the kidney and of the peripolar cells in the sand lizard (Lacerta agilis)

Arkh Anat Gistol Embriol [8NS] Nov; 93(11): P93-8

Krackower C.A., Greenspon S.A. (1954): Factors leading to variation in concentration of "nephrotoxic" antigens of glomerular basement membranes.
Arch Pathol., 58: 401-423.

Kriz, W., Kaissling, B., and Pszolla, M. (1978): Morphological characterisation of the cells in Henle's loop and the distal tubule.
In: New aspects of renal function (Ed. Vogel, H. G., Ullrich, K. J.).
Amsterdam. Excerpta Medica: 67-79.

Lecce, J. G., & Morgan, D. O.(1962): Effect of dietary regimen on cessation of intestinal absorption of large molecules (colostrum) in the neonatal piglet and lamb.
Journal of Nutrition , 78: 263-268.

Lendrum, A. C. Fraser, D. S., Sliders, W., Henderson, R. (1962): Studies on the character and staining of fibrin.
J. Clin. Pathol., 15: 401- 413.

Leyssac, P. P. (1978): A micropuncture study of glomerular filtration and tubular reabsorbtion of endogenous renin in the rat.
Renal Physiol. Basel, 1:181-188.

Lindop, G. B. M., & Lever, A. F. (1986): Anatomy of the renin-angiotensin system in the normal and pathological kidney.
Histopathology 10: 335-362.

Lindop, G. B. M. (1987): Morphological aspects of renin synthesis, processing, storage, and secretion.
Kidney International, vol. 31, suppl. 20: S-18-S-24

McCarthy, E. F. & McDougall, E. I. (1953): Absorption of immune globulin by the young lamb after ingestion of colostrum.
Biochemical Journal, 55: 177-182.

McCoy, G. C., Reneau, J. K., Hunter, A. G. & Williams, J. B. (1970): Effects of diet and time on blood serum proteins in the new born calf.
Journal of Dairy Science, 53: 358-362.

McDougall, E. I. (1965): Proteinuria of suckling ruminant.
Biochemical Journal, 94: 101-105.

MacManus, J. F. A. (1943): Apparent reversal of position of the Golgi element in the renal tubule.
Nature; 152: 417.

Mitchell, G. M.,Stratford, B. F. & Ryan, G. B.(1982): Morphogenesis of the renal juxtaglomerular apparatus and peripolar cells in the sheep.
Cell tissue Res., 222:101-111.

Morgan, T. & Davis, J. M. (1975): Renin secretion at the individual nephon level.
Pfluegers Archiv. Eur. J. Physiol., 359: 23-31.

Morild, I., Christensen, J. A., Mikeler, E. & Bohle, A. (1988): Peripolar cells in the avian kidney.
Virchows Arch [A] [XD1], 412 (5): P471-7.

Nustad, K., Vaaje, K. & Pierce, J. V. (1975): Synthesis of kallikreins by rat kidney slices.
Br. J. Pharmacol., 53: 229-234.

Oberling, C. (1944): Further studies on the preglomerular apparatus.
Am. J. Pathol., 20: 155-171.

Page, I. H. & Helmer, O. M. (1940): A crystalline pressor substance (angiotensin) resulting from the reaction between renin and renin activator.
J. Exp. Med., 71: 29-42.

Re, R. N. (1982): Changes in nuclear initiation sites after treatment of isolated nuclei with angiotensin II.
Clin.Sci., 63: 191s-193s.

Rouiller, C. & Orci, L. (1971): The structure of the juxtaglomerular complex.
In. The kidney. Ed. Rouiller, C. & Mullar, A.F. Vol. IV, 1-80.
Academic Press, New York, London.

Ryan, G. B. (1979): The granulated peripolar epithelial cell: A potential secretory component of the renal juxtaglomerular complex.
Nature, 277: 655-656.

Ryan, G. B., Alcorn, D., Coghlan, J. P., Hill, P. A. & Jacobs, R.(1982): Ultrastructural morphology of granule release from juxtaglomerular myopethelioid and peripolar cells.
Kidney Int., 22(suppl 12): S3-S8.

Secher, D.S. and Burke, D.C. (1980): A monoclonal antibody for large-scale purification of human leukocyte interferon.
Nature 285, 446.

Skeggs, L. T., Kahn, J. R. & Shumway, N. P. (1951): The isolation of hypertensin from the circulating blood of normal dogs with experimental renal hypertension by dialysis in an artificial kidney.
Circulation 3: 384-389.

Stephens, G. A., Davis, J. O., Freeman, R. H. & Watkins, B. E. (1978): Effects of sodium and potassium salts with anions other than chloride on renin secretion in the dog.
Am. J. Physiol., 234: F10-F15.

Szokol, M., Soltesz, M. & Varga, S.(1979): Ultrastructural changes of the rat renal glomeruli in the early phase renovascular hypertension.
Acta morphologica hangarica, 27: 117-132.

Trahair, J. F., & Ryan, G. B. (1987): Neuron specific enolase and kallikrein-like immunoreactivity on the kidney and salivary gland of the lamb.
Proceedings of the Xth international Congress of Nephology, London.

Trahair, J. F. & Ryan, G. B.(1988): Immunohistochemical identification of plasma proteins in cytoplasmic granules of peripolar cells of the sheep.
J. Anat., 160: 109-115.

Trahair, J. F., Gall, J. A. M., Alcorn, D., Coghlan, J. P., Fernley, R., Penschow,

J., Grattage, L. P., Johnston, C. I. & Ryan, G. B. (1989): Immunohistochemical study of peripolar cells of the sheep.
J. Anat. 162: 125-132.

Turner, D. R. (1985): Granular peripolar cells, renin and the juxtaglomerular apparatus.
Histopathology, 9: 783-785.

Vandongen, R., Peart, W. S. & Boyd, G. W. (1973): Adrenergic stimulation of renin secretion in the isolated perfused rat kidney.
Circ. Res., 32: 290-296.

Wagermark, J., Ungerstedt, W. & Ljungqvist, A. (1968): Sympathetic innervation of the juxtaglomerular cells of the kidney.
Circ. Res., 22: 149-153.

Watkins, B.E., Davis, J O., Lohmeier, T. E. & Freeman, R. H. (1976): Intrarenal site of action of calcium on renin secretion in dogs.
Circ. Res.; 39: 847- 853.

Witty, R. T., Davis, J. O. Johnson, J. A. & Prewitt, R. L. (1971): Effects of papaverine and hemorrhsge on renin secretion in the nonfiltering kidney.
Am. J. Physiol., 221: 1666-1671.

Zehr, J. E., Kurz, K. D., Seymour, A. A. & Schultz, H. D. (1980): Mechanisms controlling renin release.
In: 'Advances in Experimental Medicine and Biology' (Ed. Johnson. J. A. & Anderson, R. R.).
Plenum Press, New York, vol. 130:135-165

Zola, H. (1988): Monoclonal antibodies: A manual of techniques.
CRC Press, Inc. Boca Raton, Florida.

Zola, H., Gardner, I., Hohmann, A. and Bradley, J. (1984): Analytical flow cytometry in the study of hybridisation and hybridomas.
In: Proc. 6th Australian Biotecnology Conf. Doelle, H.W., Ed., University of Queensland, Brisbane, Australia, 405.

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