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Preface

I am pleased and honored to have been asked to serve as guest editor for this first thematic volume of the *International Review of Experimental Pathology*. This is the thirtieth volume of a highly successful series edited by G. W. Richter, which has been the site of publication for many eminent reviews. Like all other branches of medicine, experimental pathology is becoming more and more specialized. It seemed reasonable to undertake a volume with a single organ theme to more clearly define the audience and to ensure that many individuals would find the entire volume of value, not just one or two contributions. The choice of renal disease as a theme for this volume reflects both my personal interest and the realization that there is a need for such a collection of reviews in this area. There are many new books on renal pathology, but almost all have a clinical rather than experimental orientation. This volume will be of interest to a diverse group of readers interested in renal disease. This broad spectrum of potential readership is reflected in the list of contributors which includes, in addition to pathologists, nephrologists, anatomists, veterinarians, and experimental chemists. Certainly this volume will also be of interest to transplant surgeons and to pediatricians specializing in renal disease. I very much appreciate the efforts of the many scientists from around the world who have contributed to this volume. I would welcome comments or suggestions from any reader regarding this or possible future thematic volumes.

Kim Solez

INTERNATIONAL REVIEW OF EXPERIMENTAL PATHOLOGY, VOL. 30

Experimentally Induced Renal Papillary Necrosis and Upper Urothelial Carcinoma

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I. Introduction

The etiology of renal papillary necrosis (RPN) in humans has been associated with the long-term abuse of analgesics and therapeutic doses of nonsteroidal antiinflammatory drugs (NSAID). However, the lesion has not been clearly defined in terms of the exact causative agent(s), how much (of each) was taken to cause a lesion, and over what period. The primary pathogenesis and the role of other complicating factors are also not clearly understood, nor have the secondary pathophysiological consequences of RPN been adequately interrelated, despite the fact that chronic renal failure and upper urothelial carcinoma are frequently associated with analgesic abuse (Bach and Bridges, 1985).

The understanding of the pathophysiology of a chronically developing renal lesion in humans is a major problem in those conditions where the etiology has been clearly defined, because of the strong likelihood of concurrent and complicating secondary (and unrelated disease) factors. There are important anatomical and functional differences between the kidneys of most animals and humans (Mudge, 1982; Stolte and Alt, 1980). The use of experimental models has generally shown a number of very important clinical and morphological differences; therefore, the use of these models has often limited the understanding of similar conditions in humans.

Although RPN (and upper urothelial carcinoma) are examples of renal disease developing chronically in humans, it has been possible to study a number of chemicals that induce these lesions rapidly in experimental animals. These models (Bach and Hardy, 1985; Bach and Bridges, 1985) all have the important pathophysiological hallmarks of the lesion that has been

described in humans (Burry, 1968; Burry *et al.*, 1977; Rosner, 1976; Bach and Bridges, 1985). The use of these experimental models has therefore fortuitously provided a way to study the development of papillary necrosis and the progression to a series of renal changes similar to those seen in human analgesic abusers. These models are also allowing the interrelationship between the primary lesion and its secondary consequences to be defined in terms of biochemical mechanisms. An understanding of the molecular genesis of this syndrome may be highly relevant to improved clinical management of RPN and upper urothelial carcinoma in humans.

II. Renal Papillary Necrosis and Upper Urothelial Carcinoma in Humans

RPN was first described over 100 years ago (Turner, 1885). It is a lesion that may have a number of different causes (Table I), but most often when encountered in the clinical environment before the 1950s, was due to diabetes mellitus or sickle cell disease (Mandel, 1952). The most frequent cause of RPN since then (and in current clinical experience) is chronic, inappropriate, high-dose analgesic intake, especially the addiction to mixed

TABLE I

CAUSES OF RENAL PAPILLARY NECROSIS IN HUMANS

Frequently reported causes
Diabetes mellitus
Analgesic abuse
High-dose nonsteroidal antiinflammatory drug therapy
Upper urinary tract obstructive uropathy
Recurrent urinary tract infection
Sickle cell hemoglobinopathy
Acute pyelonephritis
Less frequently reported causes
Dehydrated newborn infants (frequently jaundiced)
Renal vein thromboses
Chronic alcoholism
Severe jaundice
Calyceal arthritis
Renal transplant
Systemic candidosis
Trauma
Prolonged hypotension

analgesics over a number of years. Therapeutic doses of NSAID may also induce RPN (Nanra and Kincaid-Smith, 1972; Burry *et al.*, 1977; Prescott, 1979, 1982; Bach and Bridges, 1985).

Initially, all of the mixed analgesics that were associated with the pyelonephritis seen in urology clinics contained phenacetin, and the condition was dubbed "phenacetin kidney" (Spuhler and Zollinger, 1953). Subsequently, however, it become apparent that other analgesics had the potential to cause RPN (Gilman, 1964). The early confusion over the cause of RPN, and the fact that most patients abused, or were prescribed, mixed analgesics and/or a number of different NSAID, also served to obscure case history data that might have provided vital information for the more accurate identification of which analgesics and/or NSAID had the greatest potential to cause the lesion (Cove-Smith and Knapp, 1978; Nanra and Kincaid-Smith, 1975; Nanra *et al.*, 1980). The early failure to realize that phenacetin was *not the sole cause* of RPN shaped the dogma that resulted in the withdrawal of this drug from the market (Shelley, 1967, 1978). This, it was assumed, would remove the major etiological factor in the genesis of the lesion. When acetaminophen (paracetamol) replaced phenacetin in mixed analgesic preparations the incidence of RPN was expected to drop (Gault *et al.*, 1968; Duggin, 1977; Kincaid-Smith, 1979). The occurrence of the lesion did not, however, decrease in those circumstances where the abuse of mixed analgesics continued (Prescott, 1979, 1982), although some decreases have been attributed to the withdrawal of phenacetin and extensive educational programs to discourage the abuse of mixed analgesics (Wilson and Gault, 1982). A variety of indirect evidence (Table II) has now branded acetaminophen as a very important cause of the lesion, but it is still not clear if there is any scientific foundation for this conclusion. By the time it was realized that most (if not all) mixed analgesics (and many on their own) had the potential to cause RPN (Table III), a great deal of the "phenacetin-containing mixed analgesic" dogma had been established in the medical literature. There is, however, strong evidence from several different sources to suggest that therapeutic doses of NSAID may also cause RPN (Prescott, 1979, 1982; Robertson *et al;*, 1980; Shah *et al.*, 1981; Erwin and Boulton-Jones, 1982; Mitchell *et al.*, 1982; Bach and Bridges, 1985). Based on toxicity data, it is obvious that there are other chemical substances (Table III) that have the potential to cause RPN in animals. Many of these chemicals have industrial uses, and some are persistent environmental contaminants. Clinical situations rarely (if ever) focus on these possible environmental causes, or on the potential for substances other than analgesics and NSAID to contribute to RPN in humans.

There is good clinical evidence to show that patients who continue to abuse analgesics (after the condition is diagnosed), have a very poor

TABLE II

CIRCUMSTANTIAL EVIDENCE LINKING ACETAMINOPHEN TO THE ETIOLOGY OF RENAL PAPILLARY NECROSIS

Phenacetin dogma—the belief that phenacetin was the only etiological factor in the genesis of papillary necrosis
Phenacetin is metabolized to acetaminophen
Acetaminophen replaced phenacetin in mixed analgesics, and the incidence of pyelonephritis did not decrease
Urinary acetaminophen was used as a measure of phenacetin abuse in patients known to be taking mixed analgesics
Acetaminophen and aspirin are concentrated in the medulla, but phenacetin is not
The genesis of acute hepatic and proximal tubular necrosis following large doses of acetaminophen has been widely studied, and the conclusions extrapolated to a chronic lesion in the medulla

prognosis and rapidly develop end-stage renal disease (Nanra and Kincaid-Smith, 1972; Kingsley *et al.,* 1972; Murray and Goldberg, 1975; Burry *et al.,* 1977; Cove-Smith and Knapp, 1978), while patients who discontinue the abuse of the offending drugs tend to stabilize, or show improved renal function (Bell *et al.,* 1969; Dubach *et al.,* 1978, 1983). It is these patients, however, who may be at risk of developing upper urothelial carcinoma. There is a very high incidence of epidemiologically associated upper urothelial carcinoma in those countries such as Scandinavia, Switzerland, and Australia where RPN has a high clinical prevalence (Bengtsson *et al.,* 1968, 1978; Dubach *et al.,* 1971; Johansson *et al.,* 1974, 1976; Mihatsch *et al.,* 1979, 1980a–c, 1982a–c; Mahony *et al.,* 1977; McCredie *et al.,* 1982a,b, 1983). There is, however, no proved cause–effect relationship between RPN and upper urothelial carcinoma (Bach and Bridges, 1985).

The diagnosis of RPN (Gault *et al.,* 1968; Duggin, 1977, 1980; Kincaid-Smith, 1979; Bach and Bridges, 1985) and of upper urothelial carcinoma (Bengtsson *et al.,* 1968, 1978; Dubach *et al.,* 1971; Johansson *et al.,* 1974, 1976; Mihatsch *et al.,* 1979, 1980a–c, 1982a–c; Mahony *et al.,* 1977; McCredie *et al.,* 1982a,b, 1983; Bach and Bridges, 1985) is most difficult in the clinical situation, and both progress silently. One early clinical sign of analgesic nephropathy is the loss of urine-concentrating capacity (Bengtsson, 1962; Dubach *et al.,* 1975; Nanra *et al.,* 1978; Nanra, 1980). Polyuria may, however, be a consequence of several nephropathies, and

TABLE III

ANALGESICS, NSAID, AND OTHER DRUGS AND CHEMICALS WITH PAPILLOTOXIC EFFECTS[a]

Acetaminophen	Ibuprofen
Aclofenac	Indomethacin
Aminopyrine	4-Isopropylbiphenyl
5-Aminosalicylic acid	Ketophenbutazone
Antipyrine	Ketoprofen
Aspirin	Meclofenamic acid
Aspirin, phenacetin, and codeine	Mefanamic acid
Aspirin, phenacetin, and caffeine	Mono-*N*-methylaniline
2-Bromoethanamine hydrobromide	Naproxen
3-Bromopropanamine hydrobromide	Niflumic acid
Bucloxic acid	Oxyphenbutazone
2-Chloroethanamine hydrochloride	Phenacetin
2-Chloro-*N*,*N*-dimethylethanamine	Phenothiazine
Cyclophosphamide	Phenylalkanoic acid
Dapsone	*N*-Phenylanthranilic acid
Diphenyl	Phenylbutazone
Diphenylamine	Propyleneimine
Diphenylmethyl alcohol	Sudoxicam
Ethyleneimine	Sulfinpyrazone
Fenoprofen	Tetrahydroxyquinoline
Flufenamic acid	Tolfenamic acid
Glaphenine	Tolmetin

[a]Full references in Bach and Bridges (1985).

loss of the concentrating mechanism may have a number of renal and extrarenal causes. RPN is also associated with electrolyte disturbances. Cove-Smith and Knapp (1978) reported a high incidence of sodium wastage, and Jaeger *et al.*, (1982) showed that patients were hypocalcemic as a result of a urinary Ca^{2+} loss. Patients with analgesic nephropathy have a pronounced defect in the urinary acidification mechanism following NH_4Cl administration (Bengtsson, 1962; Steele *et al.*, 1969; Krishnaswamy *et al.*, 1976; Nanra *et al.*, 1978; Nanra, 1980), suggesting that damage to the medulla might be synonymous with loss of effective urinary acidification and altered electrolyte balance. Other classical clinical biochemical parameters used to diagnose renal disease only identify incipient renal failure, by which time papillary necrosis has long since occurred and the secondary degenerative changes that follow this lesion have progressed toward end-stage renal disease. There are few telltale clinical symptoms (Table IV), none of which are pathognomonic of the condition. Degenerative renal changes may be identified by radiology (Lindvall, 1978), but these are essentially indicative of an advanced lesion, and they may miss early, but

TABLE IV

CLINICAL FEATURES ASSOCIATED WITH RENAL PAPILLARY NECROSIS

Early symptoms
Female predominance 3 : 1 to 8 : 1
Psychiatric dependence, emotional instability, and anxiety
Upper gastrointestinal disease
Anemia
Intermediate symptoms
Bacteriuria, sterile pyuria, nocturia, dysuria, microscopic hematuria, ureteral colic, and lower back pains
Defective concentration and acidification of urine, proteinuria, nocturia, and azotemia
Late symptoms
Hypertension, ischemic heart disease, and peripheral vascular disease
Calculi and bladder stones
Decreased glomerular filtration rate, increased blood urea nitrogen, renal tubular acidosis, and end-stage renal disease
Upper urothelial carcinoma

frank RPN. The most dependable method of assessing analgesic-related disease is by detailed patient histories, but the stigma of analgesic abuse normally leads to patients giving unreliable or misleading data on their drug usage (Murray, 1974, 1978). Similarly, it is difficult to diagnose upper urothelial carcinoma unless cytology and other diagnostic procedures such as computerized tomography (Gatewood *et al.*, 1982) are applied. The knowledge that a patient has been an analgesic abuser can provide a basis for routine cytological monitoring, but this is rarely carried out even in situations where the incidence of the disease is high (Jackson *et al.*, 1978). The prognosis for the patients with upper urothelial carcinoma is poor, due to the advanced stage of renal parenchymal disease and widespread metastases (Hultengren *et al.*, 1965; Mihatsch and Knusli, 1982) when the condition is first diagnosed.

III. Experimentally Induced Renal Papillary Necrosis

Early attempts to study RPN experimentally using analgesics and NSAID have been plagued with irreproducible experiments and conflicting data, so much so that Rosner (1976) was of the opinion that analgesic-associated

RPN was a lesion peculiar to humans and that animals were remarkably resistant to this type of pathology. Analgesics, NSAID, and a variety of other drugs and chemicals can, in fact, be used to induce RPN experimentally. The objectives of this article are to review briefly the different animal models of RPN that may be useful for the experimental pathologists, and highlight their advantages and limitations. The focus of this article will be on the use of chemicals that induce RPN acutely or subacutely, because these compounds (particularly if they affect the kidney only) provide a most useful way of studying the pathogenesis of RPN. Many of the histological changes that we have studied have been based on the use of high-resolution light microscopy, where semithin sections of glycolmethacrylate-embedded tissue has been assessed by a number of conventional histochemical methods. In addition, several enzyme histochemical methods have also been used to study changes that originate in the proximal tubule, the urothelial cells, and the endothelial cells. Our own interest has been in the application of a multidisciplinary approach to elucidating the biochemical mechanisms of RPN and its related changes such as chronic renal failure and upper urothelial carcinoma.

A. Spontaneous and Experimentally Manipulated Models of Renal Papillary Necrosis

RPN occurs in animals as a result of a variety of other conditions. These include age (Gorer, 1940) and amyloid-related (Dunn, 1944; Cornelius, 1970) changes in mice, and changes that are a consequence of medullary bilirubin deposition (and perhaps other biochemical effects) in the Gunn rat (Gomba *et al.*, 1973; Call and Tisher, 1975; Henry and Tange, 1982; Axelsen and Burry, 1972; Axelsen, 1973). In addition, systemic candidosis also causes necrosis of the medulla (Adriano and Schwarz, 1955; Hurley and Winner, 1963; Knepshield *et al.*, 1968; Tomashefski and Abromowsky, 1981). It has previously been reported that vascular occlusion (Muirhead *et al.*, 1950; Sheehan and Davis, 1959a,b; Baum *et al.*, 1969; Beswick and Schatzki, 1960), ureteral obstruction (Sheehan and Davis, 1959b; Dziukas *et al.*, 1982), and the injection of heterologous serum into rats (Patrick *et al.*, 1964; Kroe and Klavins, 1965; Wizgird *et al.*, 1965; Ljungqvist and Richardson, 1966; Ljungqvist *et al.*, 1967; Gullbring *et al.*, 1966) also cause RPN. Critical analysis of these data (Bach and Bridges, 1985), however, suggests that the medullary infarct associated with all of these methods differs from the chemically induced RPN, and is more comparable to the "warm ischemic" renal lesion (Mason and Thiel, 1982; Wolgast *et al.*, 1982). It would still be valuable for these lesions to be more fully studied by

histochemical methods, at both the light and ultrastructural levels, to establish the nature of the changes, and where they may be similar to the chemically induced lesion. The long-term feeding of rats with a diet deficient in essential fatty acids (Burr and Burr, 1929, 1930; Borland and Jackson, 1931; Molland, 1982) also causes RPN, but the widespread degenerative changes in many of the major organs makes this a most complex experimental model.

B. Analgesic and Nonsteroidal Antiinflammatory-Induced Renal Papillary Necrosis

Attempts to induce the RPN using analgesics or NSAID have proved to be difficult (Rosner, 1976). A number of researchers have produced the lesion with mixed analgesics (see Rosner, 1976; Macklin and Szot, 1980), single constituents such as amidopyrine (Brown and Hardy, 1968), acetaminophen (Macklin and Szot, 1980; Furman *et al.*, 1976, 1981), and aspirin (Molland, 1976), and a variety of NSAID, including phenylbutazone and indomethacin (Arnold *et al.*, 1974; Burnett, 1982; Bokelman *et al.*, 1971). Many "second-generation" NSAID also have the potential to cause RPN (Table III).

RPN has been most difficult to study because the renal medulla is inaccessible to investigation, is not well defined biochemically, and consists of a heterogeneous array of cell types. Several problems associated with studying this lesion have been reviewed in detail (Bach and Bridges, 1985; Bach and Hardy, 1985). Three experimental considerations are most essential to the design and interpretation of all investigations into the mechanism of renal papillary necrosis and upper urothelial carcinoma and therefore warrant repeating.

1. There have been no definitive noninvasive criteria by which to diagnose experimentally induced RPN. Polyuria represents one of the early renal functional changes which precede RPN in experimental animals given repeated doses of analgesics (Angervall and Bengtsson, 1968; Brown and Hardy, 1968; Nanra, 1980), NSAID (Booth *et al.*, 1961), and NSAID analogs (Hardy, 1970a,b, 1974), and those chemical probes that cause RPN acutely (see below). Loss of urinary concentrating ability is not, however, a specific functional change peculiar to RPN, but it also accompanies many other renal and extrarenal changes (Berndt, 1975; Piperno, 1981). Similarly, enzymuria (Ellis and Price, 1975; Halman *et al.*, 1986) has been studied in the acutely induced RPN, but this fails to define the location and extent of a renal lesion. It is only once the renal cortex shows degenerative changes that changes are observed in the usual clinical parameters of renal function, such as blood urea nitrogen and serum creatinine. There are therefore no routine clinical biochemical parameters that are pathogno-

monic of the lesion even under the most stringently controlled experimental conditions in laboratory animals, and the identification of this silent lesion is dependent on recourse to histopathology.

2. Several analgesics and NSAID (and other chemicals) cause an apex-limited RPN that can be easily missed if painstaking sectioning is not undertaken through this region to ensure that a focal lesion is not missed (Fig. 1). All histology should therefore include the papilla tip or the ducts of Bellini to ensure that this important technical prerequisite has been met.

3. The appropriate choice of species can profoundly affect the course of a chemically induced lesion. It has been suggested that the rat is particularly susceptible to papillotoxic chemicals, because of the highly concentrated urine that they produce (Consensus Conference, 1984). This is, however, unproven (Bach and Hardy, 1985), and there is a paucity of published comparative data to establish if any species or strain is most appropriate; however, there are well-defined but subtle differences between rat strains (Bach and Hardy, 1985; Bach and Bridges, 1985). More importantly, the rat is very sensitive to the ulcerogenic effects of analgesics and especially the NSAID. Thus, it is not uncommon for rats to die from gastric perforation before frank renal lesions are apparent (Kaump, 1966). In addition, several of the chemicals with papillotoxic potential also cause discrete cortical lesions when given to rats at the dose regimens commonly used. There are, however, also instances where rats have proved to be particularly resistant to the papillotoxic effect of analgesics and NSAID (Rosner, 1976) for reasons that are still not understood (Bach and Bridges, 1985). When RPN has been successfully induced, the intensity of the lesion at each different time point varies from gross (with marked advanced cortical degeneration), to mild and focal, and often there are also animals in which no lesion has been found at the end of a long-term study. Based on this variability, it has been difficult to assign either time courses or dose–response relationships to pathological change when the lesion is induced chronically.

In summary, most analgesics and NSAID have been implicated as causing RPN in the animals, but many of the chemicals have not proved to be useful for inducing the lesion experimentally.

The use of those therapeutic compounds that have been implicated in the induction of RPN in humans (Table III) has not, in general, proved to be useful in inducing papillary damage in animal models. Most of the analgesics and NSAID have at one time or another been reported to cause RPN in several different species, but these have not provided robust systems for studying the time course of RPN and interrelating the different morphological changes that take place. Many of these compounds cause marked extrarenal toxicity and have an ulcerogenic potential far greater than the

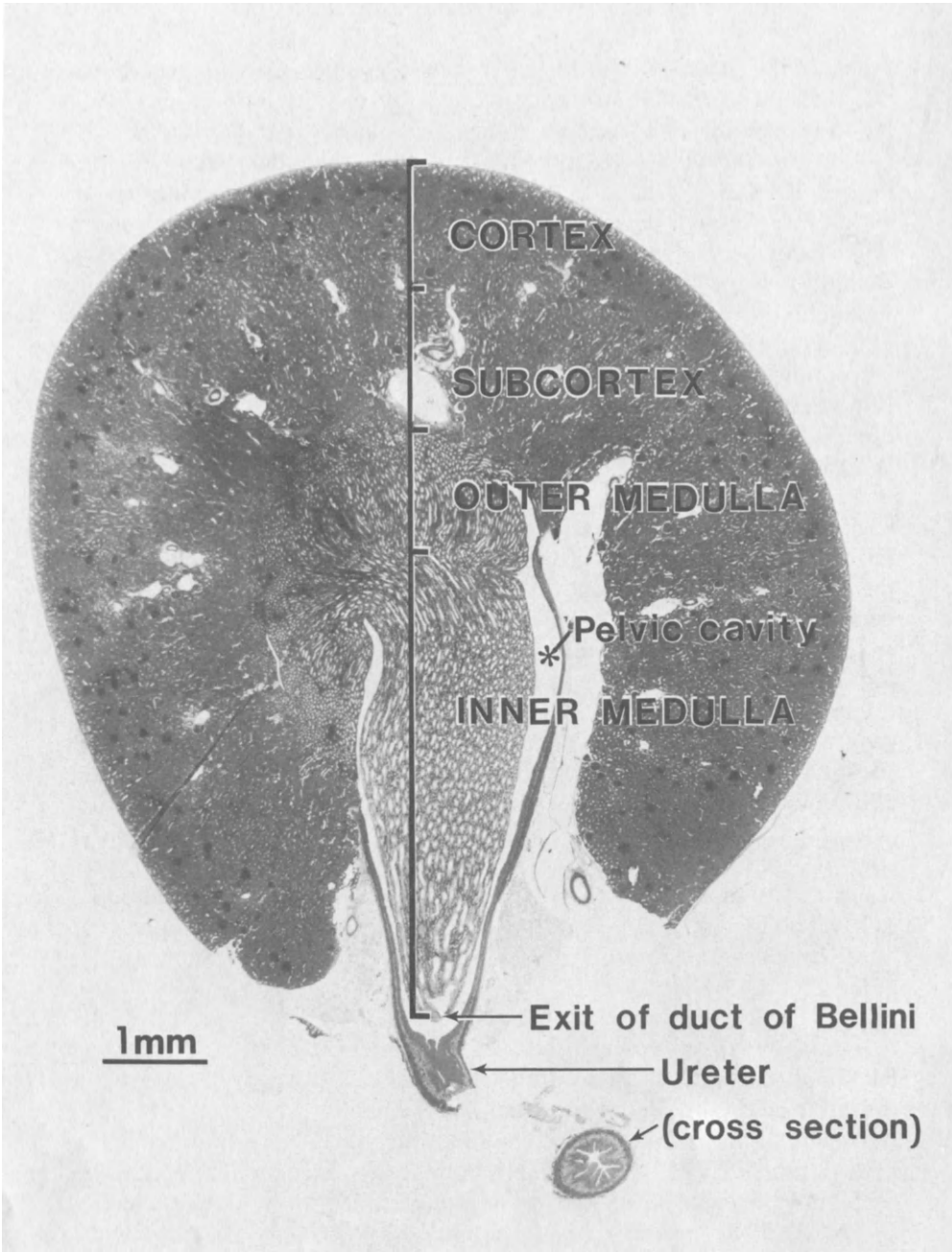


FIG. 1. Transverse semithin kidney section including papilla tip and mouth of ureter. Bar, 1 mm. [From Bach and Bridges (1985).]

nephrotoxic effects (Kaump, 1966). Some of these drugs and their metabolites also have marked toxic effects on the proximal tubule (Green *et al.*, 1969; Calder *et al.*, 1971; Crowe *et al.*, 1979; Newton *et al.*, 1982, 1983a,b). While this may be relevant to the clinical situation, overt cortical damage has not been a prominent feature of RPN in human analgesic abusers. Thus, there is a complicating factor that obfuscates the study of a primary medullary lesion if these compounds are used experimentally. More importantly, there are a number of inadequately identified variables that have meant that successive sets of experiments may not be reproducible. For example, whereas Molland (1976) showed that aspirin caused RPN in hooded rats, there are reports for other species and strains that contradict this toxic effect (Rosner, 1976). Some of the problems associated with using analgesics and NSAID to induce RPN in experimental animals have been reviewed (Bach and Hardy, 1985). In general, the use of analgesics and NSAID has served to confuse rather than to clarify the pathogenesis of chemically induced RPN. There are, however, a number of chemical "probes" that target very selectively for the medulla, and provide model systems that are preferable for studying the development of RPN and its secondary sequelae.

C. Nontherapeutic Chemical Probes for Inducing Renal Papillary Necrosis

The ethos of many studies in experimental pathology has been to use model toxic agents to induce rapidly a lesion of interest. The advantages of inducing lesions over a short time course greatly outweigh the study of chronic lesions (where other factors may obscure the cascade of pathological changes), but there is always the question of validity in extrapolating data from an acute animal model to a chronic lesion that develops in humans. Despite these limitations, most of our understanding on the biochemical mechanisms of carcinogenesis and other toxic lesions in the major organ systems has been built up using this approach.

IV. Use of Model Papillotoxic Probes to Study the Pathogenesis and Secondary Development of Renal Papillary Necrosis

The difficulties that have pervaded the use of therapeutically used compounds for inducing RPN have largely been overcome by the administration of papillotoxins that are chemically unrelated to the analgesics and

NSAID. There are also several NSAID analogs that have very little ulcerogenic effect, and have therefore contributed to our understanding of the pathogenesis of RPN.

A. Ethyleneimine-Induced Renal Papillary Necrosis

The papillotoxicity of ethyleneimine, first described by Levaditi (1901), has been used to study various aspects of RPN (Mandel and Popper, 1951; Davies, 1967, 1968, 1970; Davies *et al.*, 1968; Ham and Tange, 1969; Sherwood *et al.*, 1971; Ellis *et al.*, 1973; Ellis and Price, 1975; Axelsen, 1978a). Ethyleneimine caused a dose-related necrosis (Axelsen, 1978a) that first affected the interstitial cells of the papilla tip, and then other "fine" anatomical elements of the medulla (Ham and Tange, 1969). At subsequent time points (or with higher doses), secondary cortical degenerative changes developed (Davies, 1967, 1968). Using colloidal carbon as a contrasting agent, the microvasculature was shown to be patent up to and beyond the time that necrosis developed (Ham and Tange, 1969). The functional changes associated with the ethyleneimine-induced lesion included marked polyuria, low specific gravity urine, and enzymuria (Mandel and Popper, 1951; Ellis *et al.*, 1973; Ellis and Price, 1975).

There are, however, a number of problems with the use of ethyleneimine as a model papillotoxin. The compound is a powerful alkyating agent and a proved mutagen; it is chemically unstable and may also be explosive (Dermer and Ham, 1969), and it is no longer commercially available. Thus, over the past decade, the use of ethyleneimine as a chemical probe for inducing RPN acutely has declined dramatically.

B. 2-Bromoethanamine Hydrobromide-Induced Renal Papillary Necrosis

2-Bromoethanamine (BEA) hydrobromide has largely replaced ethyleneimine as the model papillotoxin. First shown to cause RPN by Oka (1913), this compound has a number of advantages over ethyleneimine. BEA is commercially available; it is a stable, water-soluble crystalline material, although it is unstable in solution. The BEA-induced lesion is dose related and relatively predictable in its intensity for any dose range in the rat (Bach *et al.*, 1983), and has been characterized in terms of over 35 publications on different renal morphological and functional changes (see Bach and Bridges, 1985, for full reference list). It must, however, be stressed that BEA does cyclize to ethyleneimine *in vitro* under strong alkali conditions (Dermer and Ham, 1969), and this has been proposed as the mechanism of BEA-induced RPN (Murray *et al.*, 1972). There is, however,

no evidence to show that ethyleneimine is excreted in urine following the administration of BEA to rodents (P. H. Bach, unpublished data), although this does not preclude the localized formation of the unstable alkylating molecule extrarenally or in the papilla.

1. *Morphological Changes*

A single 50 mg/kg dose given ip causes RPN acutely in rats (Wyllie *et al.*, 1972; Shimamura, 1972; Bach and Bridges, 1982; Bach *et al.*, 1983; Gregg *et al.*, 1988a,b) and mice (P. H. Bach and N. J. Gregg, unpublished), and higher doses cause a lesion (Fig. 2A) up to but not beyond the cor-

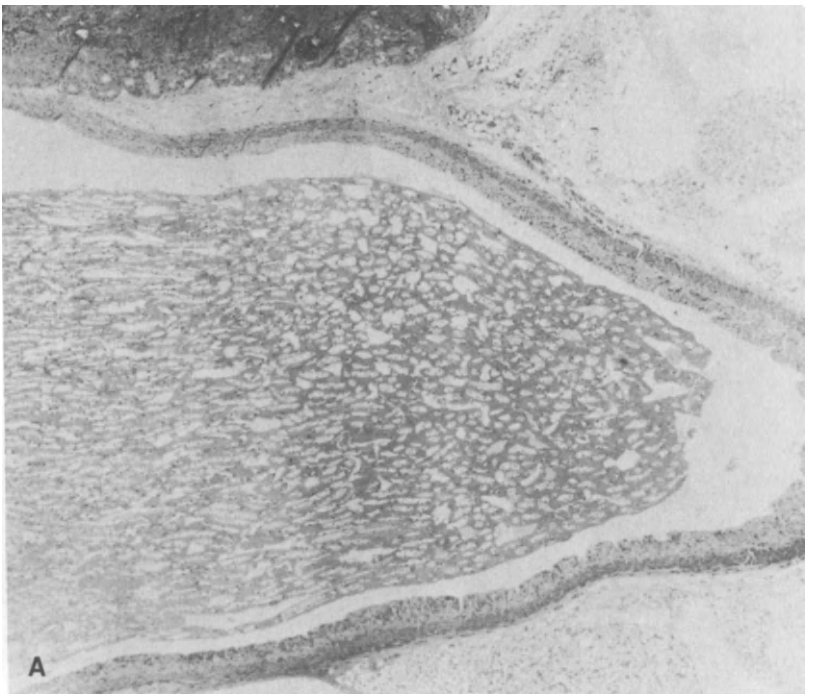
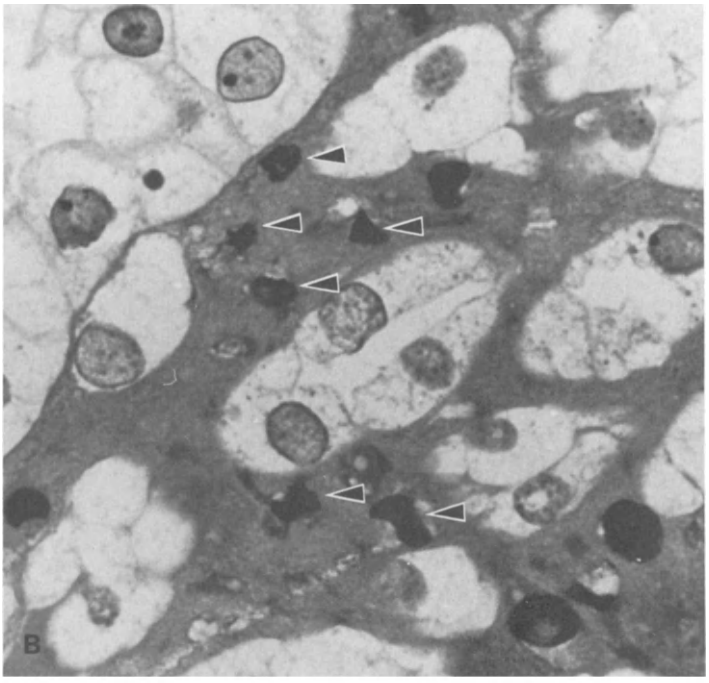


FIG. 2. (A) BEA-induced RPN lesion (100 mg/kg ip after 48 hours) showing limit of necrosis affecting the matrix staining. Giemsa staining, ×4. (B) Medullary interstitial cell necrosis at papilla tip 4 hours after a single ip dose of 100 mg/kg BEA showing pyknotic irregular nuclei (arrowheads). Giemsa, 1-μm resin section, ×100. (C) Dilatation of distal and proximal tubules 48 hours after a single 100 mg/kg ip dose of BEA. Alkaline phosphatase, ×20. (D) Regenerative zone between viable and necrotic tissue in papilla 48 hours after a single ip dose of 100 mg/kg BEA. Note mitotic figures in collecting duct (arrow) and loops of Henle (arrowhead). Giemsa, 1-μm resin section, ×40. (E) Adhesion of platelets to endothelia in area of interstitial cell necrosis, 8 hours after a single ip dose of 100 mg/kg BEA. Giemsa, 1-μm resin section, ×100.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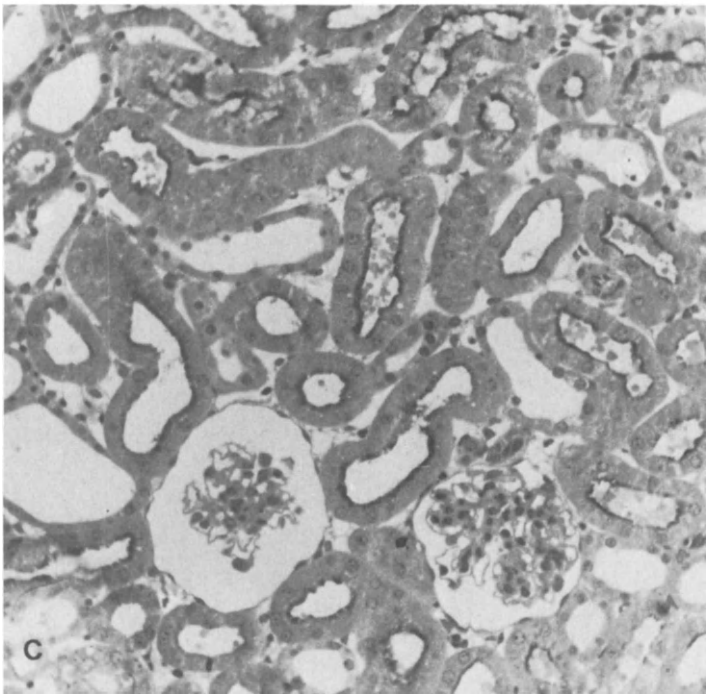
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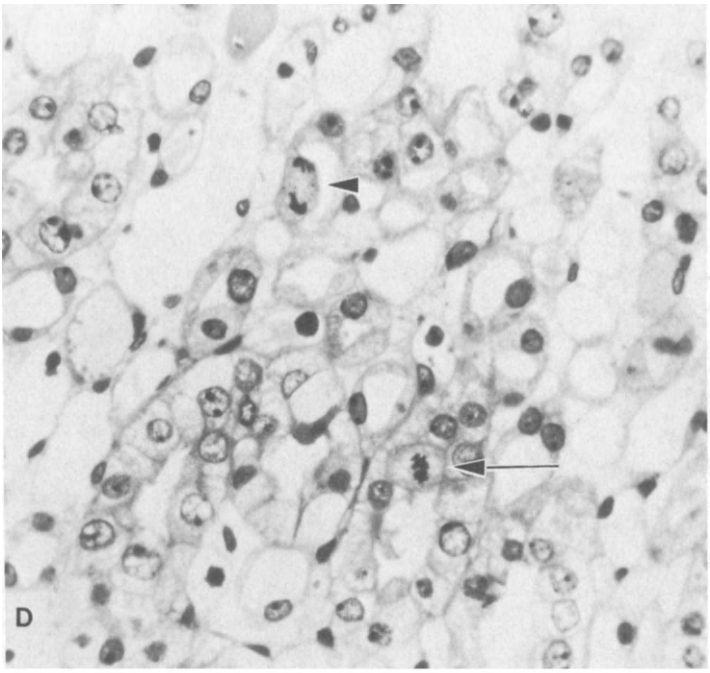
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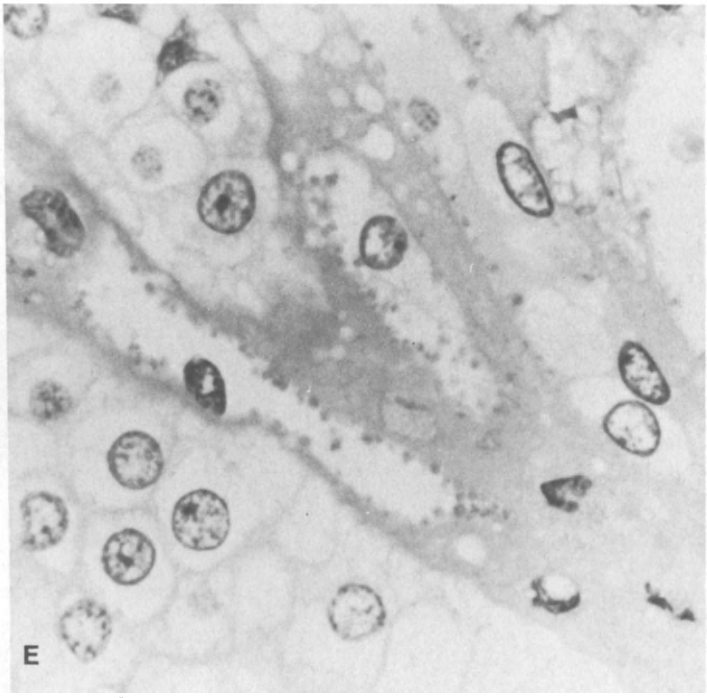
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ticomedullary junction (Bach *et al.*, 1983). Lower doses of BEA do not cause any easily identifiable lesion, and repeated high doses do not exacerbate the degree of RPN. The morphological changes associated with the time course development of BEA-induced RPN have been described in detail elsewhere (Wyllie *et al.*, 1972; Hill *et al.*, 1972; Bach *et al.*, 1983; Gregg *et al.*, 1988a,b) and will only be outlined in brief. Early hydropic changes developed in the proximal tubule 4–6 hours after BEA administration, but these had reverted to normal by 8–12 hours. Within 4 hours of BEA dosing there was a significant collecting duct dilatation which lasted for 24–48 hours.

Medullary interstitial cells had irregular nuclei at 4 hours and lost their cytoplasmic integrity by 8 hours; necrosis spread from the papilla tip to the corticomedullary junction from 12 hours (Fig. 2B). Collecting duct epithelia (and other areas of the distal nephron) showed degenerative changes at 12 hours and cell exfoliation at 18 hours. Cortical changes were confined to PAS-positive casts in the collecting duct and loop of Henle from 8 hours and dilatation of distal and proximal tubules at 8 and 72 hours, respectively (Fig. 2C). There was active repair at the junction between viable tissue and the necrotic papilla from 24 hours with mitoses in the collecting ducts and loops of Henle (Fig. 2D).

Necrotic changes developed as early as 12 hours and had run their course by 24–48 hours. The earliest degenerative changes following low (50 mg/kg) doses of BEA consistently affected the medullary interstitial cells, and the loops of Henle and the microvasculature were damaged later. The urothelial cells covering the papilla and the collecting ducts were left intact with minimal signs of injury. The kidneys taken from animals given higher doses of BEA showed similar early changes, but these were also associated with subsequent total necrosis which included loss of all of the cellular elements which make up the inner medulla.

Eosinophilic casts were present in the collecting duct from 24 hours, at which time reparative changes were evident at the interface between necrosed and normal areas. There was distal tubular dilatation from 8 hours, but this occurred in the proximal tubules after 72 hours. Endothelial platelet adhesion was first noticeable at 8 hours, was very marked at 18 hours, and continued up to 144 hours; but only the capillaries in necrotic regions were affected, and not those in other parts of the kidney or urothelial tract (Fig. 2E).

2. *Histochemical Changes*

Normally, the renal medullary matrix stains strongly with colloidal iron, Toluidine Blue, and Giemsa (Bach *et al.*, 1983; Gregg *et al.*, 1988a,b).

Following BEA administration there were marked changes in the medullary matrix staining. The earliest changes were an increased staining intensity and a granular appearance around the interstitial cells at the papilla tip 2–4 hours after BEA dosing. The increased staining became diffuse after 8–12 hours, and was progressively lost from those areas where necrotic changes were taking place between 12 and 24 hours. The necrosed areas had totally lost the histochemical staining of the matrix from 24–48 hours (Fig. 3). There was also an increase in PAS-positive material at the tip of the papilla 4–6 hours after BEA, which increased to a maximum at 48 hours, but at this stage the PAS staining in the mid-medulla was decreased. Even when there was reepithelialization of the affected area the mucopolysaccharide matrix was not reestablished, probably due to the absence of medullary interstitial cells.

Changes in the matrix staining have also been associated with RPN in humans, where both increases (Burry *et al.*, 1977; Burry, 1978) and decreases (Gloor, 1978) have been reported. It is tempting to suggest that these are similar to the early and late changes in the acutely induced BEA model. Rats given aspirin chronically also developed RPN and a dense fibrillary network of PAS-positive material, which became irregular with more deeply PAS-staining fibers and bodies in the interstitium (Molland, 1978). Recently, these histochemical changes have been confirmed bio-

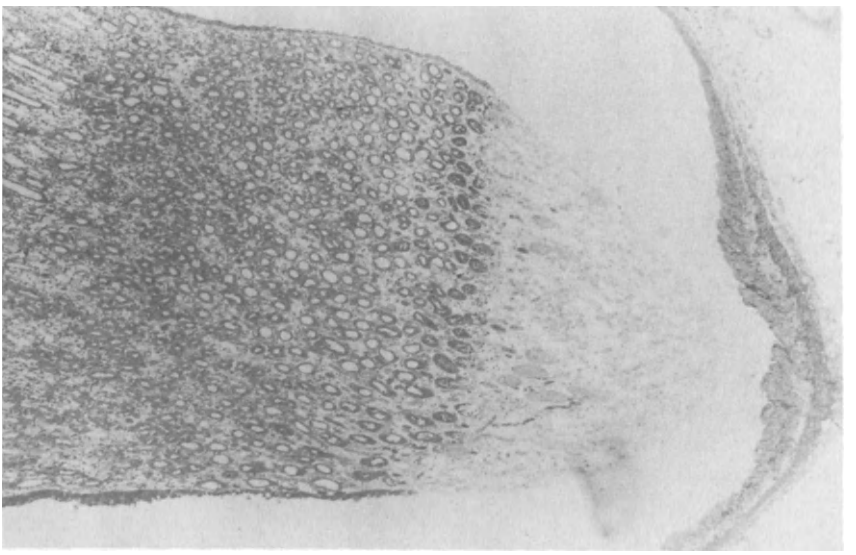


FIG. 3. Necrotic papilla 48 hours after 100 mg/kg BEA, showing loss of matrix staining in extreme tip where tissue integrity has been lost. Giemsa, 1-μm resin section, ×3.2.

chemically as demonstrated by the loss of radiolabeled and covalently bound sulfate from the medulla following BEA administration; in addition, there was a marked perturbation of urinary proteoglycans and glycosaminoglycans (Bach *et al.*, 1988a).

3. *Distal Tubular Changes*

Tamm–Horsfall glycoprotein (THG) is produced by the ascending thick limbs of the loop of Henle and lines the epithelium of that segment and the distal tubule, where it is thought to prevent water reabsorption but still to facilitate Na^+ transport (Lewis *et al.*, 1972). It forms the basic matrix material for tubular casts.

THG staining remained unchanged for 6–8 hours after BEA administration, but during the development of the papillary necrosis this glycoprotein was lost from the distal nephron (Bach *et al.*, 1988b), and small casts were found in the collecting ducts. From 12 hours there were more frequent and marked deposits of heavily stained intraluminal material in the inner medullary collecting ducts, some of which appeared to form aggregates against the epithelial cell walls. Only later, when the medullary mucopolysaccharide staining had been lost, were large casts of THG-positive material deposited in the collecting ducts and ducts of Bellini (Fig. 4), where they were associated with cellular debris (Bach *et al.*, 1988b). The nephrons that appear to feed blocked collecting ducts were generally dilated. Tubular dilatation became more marked at 24 hours, when there were THG-positive casts in the ducts of Bellini. These cast-filled ducts appeared to drain those regions of the cortical nephron where tubular dilatation was most marked. Between 24 and 123 hours the cortical staining pattern was essentially unchanged, but there was more extensive tubular dilatation. The number of THG-positive casts—containing significant quantities of cellular debris—increased, and THG staining in the distal nephrons decreased. Some of the THG-positive material was also extravasated (Bach *et al.*, 1988b). Many of the superficial glomeruli thus affected have THG-positive material in Bowman's space; this finding may be related to glomerular sclerosis (Arruda *et al.*, 1979; Sabatini *et al.*, 1982, 1983) that developed after some weeks. The most marked cystic dilatation of cortical nephrons were associated with the most extensive deposits of THG in the ducts of Bellini, and there were also deposits of THG-positive material around the glomeruli (in Bowman's spaces) of the superficial nephrons, following high-dose BEA.

Perturbation of THG distribution does not appear to play a primary role in the development of RPN, but may be important in the pathogenesis of the related polyuria and the secondary tubular changes that follow the BEA-induced lesion.

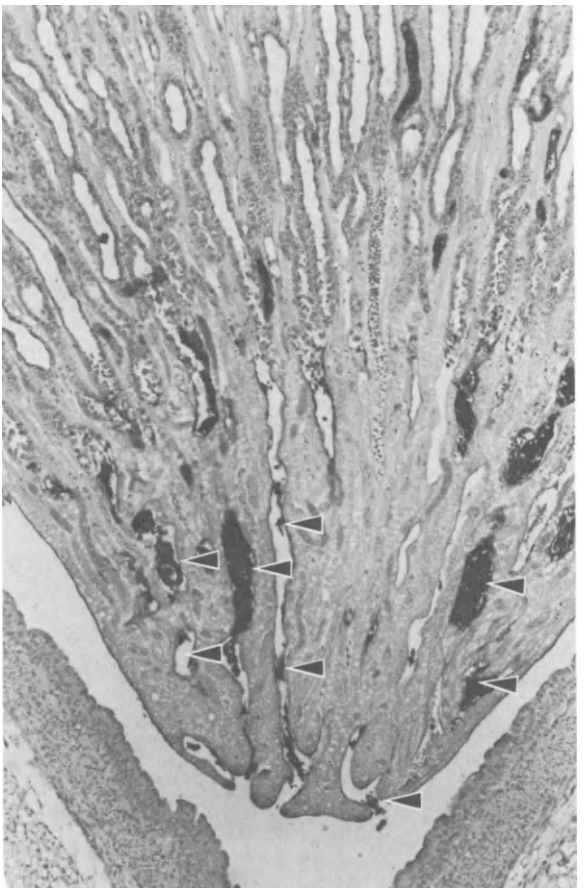
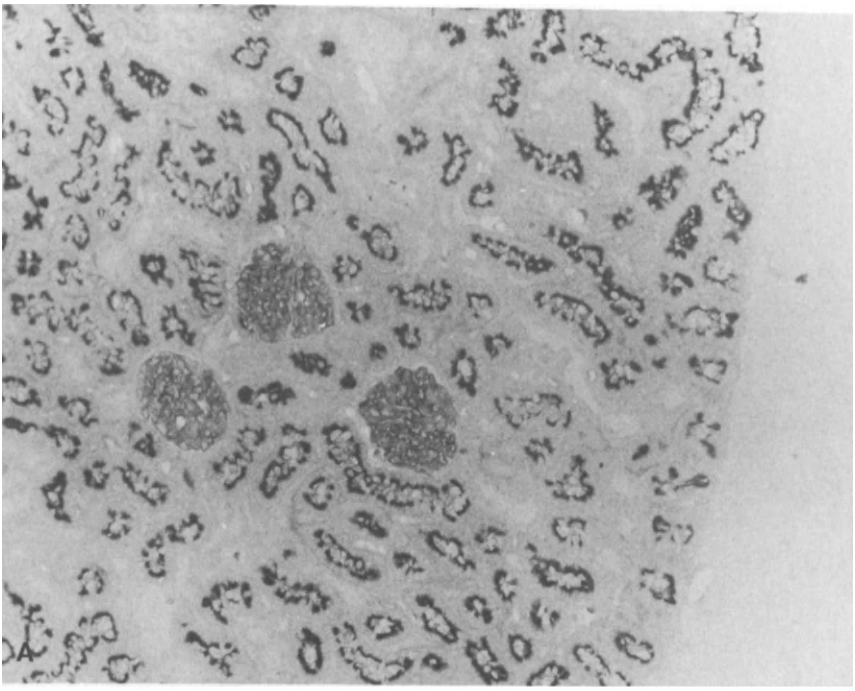


FIG. 4. Casts of Tamm–Horsfall glycoprotein-positive material deposited in collecting ducts (arrowheads). Immunoperoxidase, wax section, ×10. [From Bach and Bridges (1982).]

4. *Enzyme Histochemical Changes in the Proximal Tubule and the Suburothelial Capillaries*

The staining of a number of enzyme markers has been monitored during the development of a BEA-induced RPN. There were no changes in the proximal tubular marker enzymes alkaline phosphatase, γ-glutamyl transpeptidase (GGT), and adenosine triphosphatase (ATPase) before 8 hours, from which time there was a time-related progressive loss of staining up to 144 hours, when GGT was almost undetectable (Fig. 5A). Alkaline phosphatase and GGT (from 12 hours) and ATPase (from 18 hours) staining material occurred in the proteinaceous, PAS-positive casts in the loops of Henle and the collecting ducts (Fig. 5B). Lysosomal acid phospha-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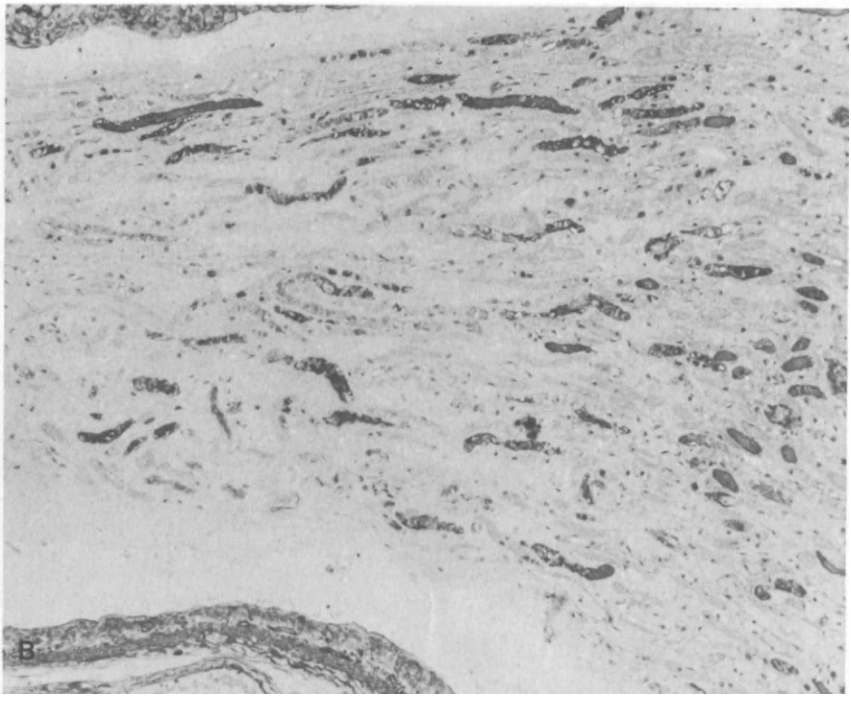
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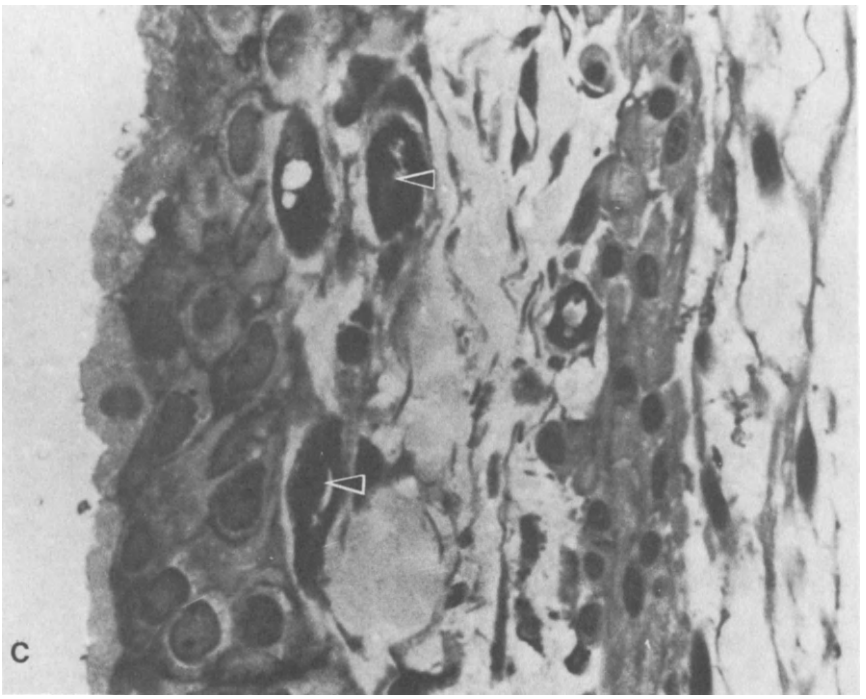


FIG. 5. (A) Alkaline phosphatase staining of proximal tubule brush borders 4 hours after 100 mg/kg BEA ip. 1-μm resin section, ×4. (B) ATPase-positive staining in proteinaceous casts in necrotic region of papilla 24 hours after 100 mg/kg BEA ip. 1-μm resin section, ×10. (C) ATPase-positive staining of endothelial lining in a ureteric capillary showing almost total occlusion of capillary lumen (arrowheads), 144 hours after 100 mg/kg BEA ip. 1-μm resin section, ×100.

tase staining was increased in the pelvic urothelial cells at 12 hours and in the proximal tubules from 12 hours, up to 48 hours.

There was a marked increase in the staining of the pelvic, ureter, and bladder endothelial alkaline phosphatase, and especially ATPase, at 12 hours. The intensity and area of microvascular ATPase staining increased progressively in these regions from 18 hours, and by 144 hours the capillary lumens were almost occluded in the worst affected areas (Fig. 5C). Capillary sclerosis has been described in the kidneys of human analgesic abusers (Mihatsch *et al.*, 1978, 1984) and is thought to be a specific change which has not been described in any other types of renal disease.

5. *Lipid Histochemical Changes*

The medullary interstitial cells have a very high lipogenic potential and contain numerous lipid droplets rich in long-chain polyunsaturated fatty

acids (Bojesen, 1974). Oil Red "O" (ORO) stains the lipid droplets in these cells heavily, but not other parts of the kidney (Bach *et al.*, 1988c). ORO-positive lipid material accumulates in kidneys of analgesic abusers (Munck *et al.*, 1970; Burry *et al.*, 1977; Burry, 1978), and similar changes occur in aspirin-induced (Molland, 1976) and essential fatty acid-deficient diet-induced RPN (Molland, 1982). Recent studies have shown that in an acutely induced papillary necrosis, early lipid changes take place in the capillaries, followed by a marked accumulation (Fig. 6) of lipid in the epithelial cells. Normally there is *no* ORO-positive lipid material in these cells. The epithelial accumulation of lipid material extends into those areas of the outer medulla which were not affected by the papillotoxin and appeared to be normal by routine hematoxylin and eosin staining (Bach *et al.*, 1988c). Other chemically induced lesions, such as those caused by hexachlorobutadiene, aminoglycosides, *cis*-platin, and polybrene, do not produce these ORO lipid changes (Bach *et al.*, 1988c), which suggests that the capillary and epithelial deposits of lipid material may be pathognomonic of RPN.

Biochemically, the lipid changes in the BEA-induced RPN represent a phospholipidosis, in which phosphatidyl- and lysophosphatidyl choline, -inositol, and -ethanolamine were increased (M. J. Duffy and P. H. Bach, unpublished data). The increase in urinary free polyunsaturated fatty acids

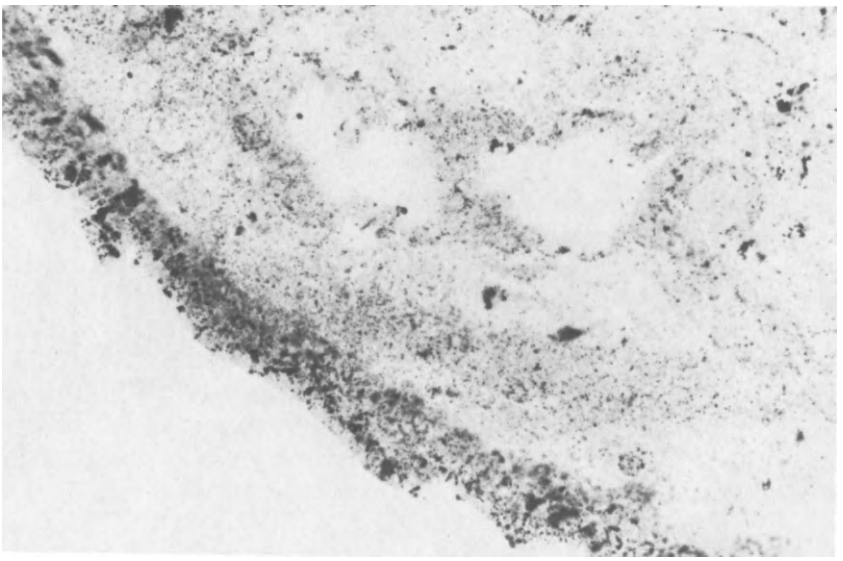


FIG. 6. Lipid droplet accumulation in papilla covering epithelium cells following single ip dose of 100 mg/kg BEA. ORO stain, fixed frozen section, ×32.

with C_{18}, C_{20} and C_{22} suggests a large precursor pool for the synthesis of eicosanoid-related products (D. J. Scholey and P. H. Bach, unpublished data).

6. *Microvascular Changes*

The subtle control of kidney microvasculature and the shunting of blood to (or from) different regions of the medulla and cortex represent a most fundamental process in normal renal function. This may be altered in nephrotoxic lesions that have been linked to ischemic injury. The introduction of exogenous particulate material into the renal microvasculature gives some indication of the patency of the vessels or the presence of occlusion. Colloidal carbon has been used to show the loss of medullary microvascular filling at an advanced stage of ethyleneimine- and aspirin-induced RPN. The introduction of this foreign particulate material for assessing vascular filling may, however, present some difficulties. While India ink has been used as the common source of colloidal carbon, it contains a variety of additives, including phenols and adhesives (Vernon-Booth, 1972), to enhance its drawing properties. In addition, the colloidal nature of this material also imparts a substantial oncotic pressure. Both of these problems (which may cause artifacts in assessing microvascular filling), can be overcome by using India ink that has been dialyzed against isotonic saline. Colloidal carbon prepared thus has been used to follow the time course of microvascular changes in animals treated with BEA. There was an early shift of microvascular filling from the cortex to the outer medulla (2–4 hours after dosing), and later (at 8–26 hours) the filling of the inner medulla was more pronounced (Fig. 7A), but at the expense of the microperfusion of the outer part of the medullary plexus. These changes coincided with the development of RPN. By 48 hours, when necrosis was complete, the damaged medulla was virtually avascular. During the course of development of RPN, however, the microvasculature was patent in the medullary tissue beyond the regions in which necrosis had occurred (Fig. 7B). These data were interpreted as showing that an acute medullary necrosis can occur without capillary occlusion (Bach *et al.*, 1983). These observations have also been confirmed by high-resolution microscopy, where platelet adherence and microvascular changes did not occur until late in the development of RPN.

The colloidal carbon method cannot identify "leaky" capillaries, an endothelial defect that could play a very important role in disrupting renal compartmentalization. Monastral Blue B is a water-insoluble, nontoxic colloid; it has an uniform size distribution, high contrast for thin and thick sections, an obvious appearance under electron microscopy, and is com-

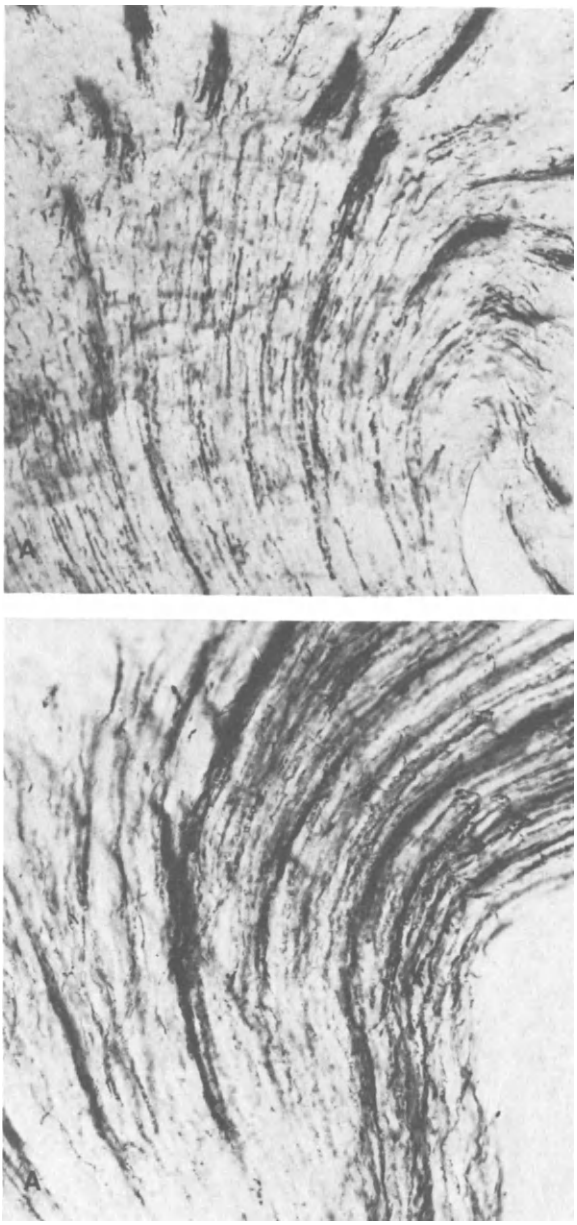


FIG. 7. (A) Microvascular distribution of colloidal carbon in the corticomedullary regions of (top) control and (bottom) BEA-treated rat kidneys (50 mg/kg at 2 hours). Unstained 100-μm celloidin section, ×4. [From Bach *et al.* (1983).] (B) Aggregates of colloidal carbon at the tip of a necrosed papilla (arrows) 26 hours after 50 mg/kg BEA. Hematoxylin–eosin, ×25. [From Bach *et al.* (1983).]

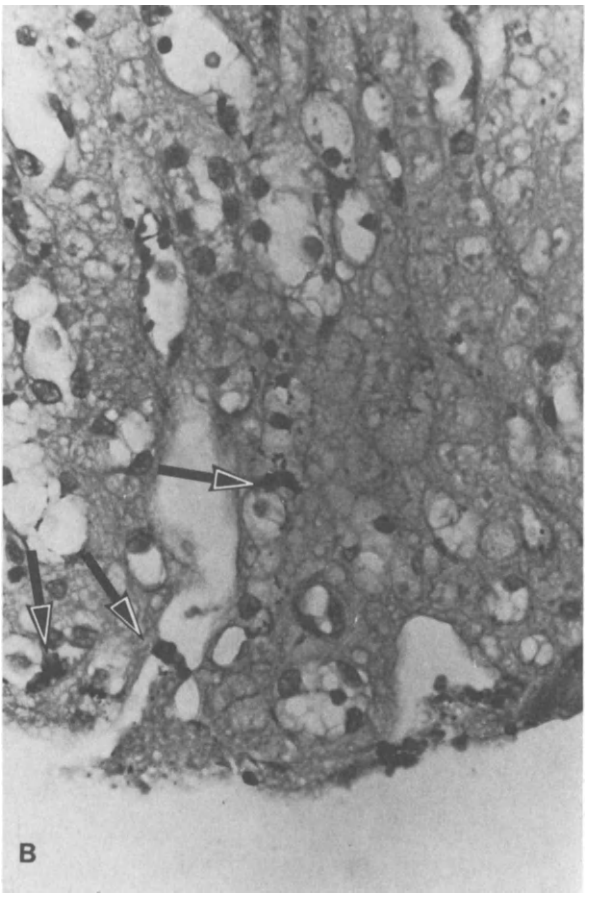


FIG. 7. (B) (*continued*)

mercially available (Joris *et al.*, 1982). Recently, this colloid has been used to assess microvascular integrity in the genesis of RPN, where semithin methacrylate sections showed the vascular labeling of glomeruli and capillaries in the pelvic basal epithelia. No Monastral Blue B was present in the papillary matrix (Fig. 8), suggesting that changes in capillary integrity (and the leakage of material into the interstitium) were not involved in the pathogenesis of RPN (Gregg *et al.*, 1988b).

7. *Interaction between 2-Bromoethanamine and Analgesic and Nonsteroidal Antiinflammatory Drugs*

It has been generally assumed that the mixed analgesics are more papillotoxic than the individual components singly (Nanra and Kincaid-

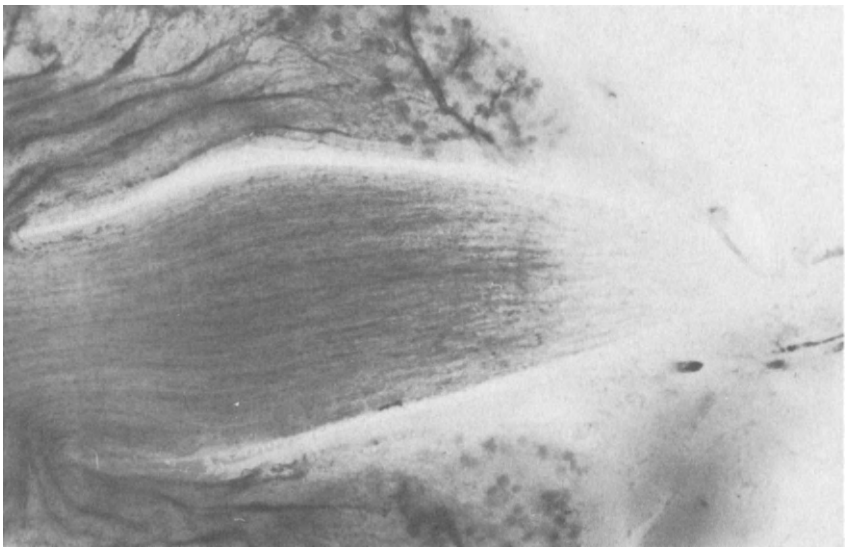


FIG. 8. Monastral Blue-labeled kidney from tissue embedded in glycolmethacrylate resin block. Note lack of label in necrotic papilla tip, 24 hours after 100 mg/kg BEA ip. ×1.6.

Smith, 1972; Duggin, 1977), but there has been very little experimental evidence to substantiate chemical interactions exacerbating RPN. Recently, this question has been addressed using the interactions between a subthreshold dose of BEA <25–35 mg/kg ip, which does not normally cause a discernible lesion), and various analgesics (Bach *et al.*, 1988b). These investigations were undertaken in an attempt to develop a short-term screening method for evaluating and comparing the papillotoxic potential of analgesics and other compounds. Whereas two doses of aspirin and acetaminophen, in the range of 0.1 to 5.0 mmol/kg, had no morphological effect on their own, there were interactions when BEA was given. Aspirin (0.1 mmol/kg and especially 0.5 mmol/kg) plus BEA caused papillary necrosis more severely and frequently, with marked cortical tubular dilatation and very heavy deposits of THG-positive material in the collecting ducts and the remaining interstitium of the medulla. On occasion, THG-positive material was present in Bowman's spaces of the superficial, but not juxtamedullary nephrons, in those kidneys where papillary necrosis was most extensive, and tubular dilatation very marked. By contrast, animals pretreated with 2.0 and 5.0 mmol/kg aspirin, before BEA administration, showed no difference from controls.

Pretreatment of rats with 1.0 mmol/kg of acetaminophen greatly exacerbated the response to BEA. There was more extensive necrosis, marked

tubular dilatation, loss of THG from the distal nephron, and heavy casts in the ducts of Bellini and some in Bowman's spaces of the superficial glomeruli. By contrast, there were very few changes in animals pretreated with 0.5 mmol/kg of acetaminophen. Increasing the pretreatment doses of acetaminophen from 1.0 to 5.0 mmol/kg dramatically exacerbated the necrotic lesion, the tubular dilatation, and the changed THG distribution. There were large casts in collecting ducts (associated with other cellular debris) and loss of THG from the distal nephron. The presence of THG-staining material in Bowman's spaces of superficial glomeruli was most frequently seen in animals pretreated with 1.0 and 2.0 mmol/kg of acetaminophen (Bach *et al.*, 1988b).

These data are difficult to interpret because of the unique pharmacological and toxicological properties of aspirin and acetaminophen (Lovejoy, 1978; Plotz *et al.*, 1981), differences which are further exemplified by the complex dose–response curves that were associated with each compound when BEA was also administered. These data do, however, strongly suggest that significant synergistic interactions may take place between chemicals in the genesis of RPN.

8. *Functional Changes*

There are marked similarities between the renal functional changes and the pathomorphological progression of the lesion following BEA administration and those reported for the analgesic-associated lesion in both experimental animals and humans (Bach and Bridges, 1982, 1985; Bach and Hardy, 1985). Prominent among these is the loss of urinary concentrating ability (Fauwa and Waugh, 1968; Wyllie *et al.*, 1972; Murray *et al.*, 1972; Shimamura, 1976; Sabatini *et al.*, 1981, 1983; Vanholder *et al.*, 1981; Bach *et al.*, 1983), loss of nephron function, and severe cortical degeneration (Sabatini *et al.*, 1981, 1983; Bach and Bridges, 1982). Loss of other urinary electrolytes (Na^+, Cl^-, PO_4^{2-}, and Ca^{2+}) has also been reported following BEA-induced RPN (Arruda *et al.*, 1979; Sabatini *et al.*, 1981). The measurement of urinary acidification capacity and electrolyte handling could therefore offer a simple way of monitoring for RPN in toxicology screening programs. Arruda *et al.*, (1979), however, failed to find any differences between control and BEA-treated rats studied 24 hours after dosing, and Sabatini *et al.*, (1982) found neither an acidosis nor a defective urinary acidification in response to NH_4Cl loading 1 month after dosing with BEA.

Wilks *et al.*, (1986) studied the renal functional changes within 3 hours of BEA administration. Their data showed that the excretion of urea increased (from 30 minutes), osmolality decreased (from 90 minutes), and Na^+

excretion increased at 3 hours, but potassium excretion was unchanged. Glomerular filtration rate and the clearance of the organic anion, *p*-aminohippurate, decreased over the period of investigation. These data reflect early functional abnormalities, and suggest that BEA may be less target selective for the medullary interstitial cells than has previously been thought. This is supported by the enzyme histochemical changes in the proximal tubular brush border that may reflect subtle injury to the cortex. Alternatively, these changes may represent aspects of the complexity in the renal response to injury, and they may reflect a rapid homeostatic consequence caused by perturbation of the medullary cells.

C. Ethyleneimine and 2-Bromoethanamine Analogs for Inducing Renal Papillary Necrosis

Recently, propyleneimine has been shown to cause RPN at doses as low as 20 μl/kg (Halman *et al.,* 1986). Multiple doses of bromopropanamine hydrobromide, 2-chloro-*N,N*-dimethylethanamine hydrochloride, or 2-chloroethanamine hydrochloride (Powell *et al.,* 1985a) also cause this lesion, but each is either less effective and/or more toxic than BEA, and propyleneimine has many of the limitations of ethyleneimine.

D. N-Phenylanthranilic Acid- and Diphenylamine-Induced Renal Papillary Necrosis

The biphenyls are structural analogs of the fenamic acid NSAID, but have the advantage of a lower ulcerogenic potential. Hardy (1970a,b, 1974) has shown that a number of these compounds (Table V) produce RPN, but the most useful experimental models were induced by *N*-phenylanthranilic acid (*N*-PAA) and diphenylamine (DPA). Both compounds did, however, also produce necrosis of the S_3 region of the proximal tubule and marked splenomegaly in the case of DPA (Powell *et al.,* 1983).

1. *Morphological Changes in N-Phenylanthranilic Acid-Induced Renal Papillary Necrosis*

N-Phenylanthranilic acid is a potent papillotoxin which targets very selectively for the medulla and causes a marked papillary urothelial hyperplasia Hardy (1970a,b, 1974; Hardy and Bach, 1984), but has little extrarenal toxicity (Powell *et al.,* 1983).

The administration of *N*-PAA to rats for 14 days caused a dose-related lesion from focal RPN (at low doses) to total RPN, and marked cortical changes follow high doses of the biphenyl (Hardy, 1970a,b). The renal

TABLE V

PAPILLOTOXIC POTENTIAL OF BIPHENYLS AND NSAID AND THE RELATIVE AMOUNT OF PGE INHIBITION[a]

Biphenyl NSAID and their analog	RPN induced in rats	Inhibition of PGE[b] synthesis
Diphenylamine	++++	++++
Flufenamic acid	+++	++++
N-Phenylanthranilic acid	+++	++
Diphenyl	+	++
Diphenylmethyl alcohol	+++	+
Diphenyl-2-carboxylic acid	–	–

[a] From T. L. Hardy (unpublished data).
[b] PGE = prostaglandin E_2.

interstitial cells, the microvasculature, and the loops of Henle at the apex of the papilla were affected at the low doses (0.5–1 mmol/kg); and a progressive destruction of the collecting ducts and epithelium covering the papilla at higher doses (1–2 mmol/kg), and there were also morphological changes in the outer medulla and cortex at the highest dose levels of 3–5 mmol/kg (Hardy and Bach, 1984).

The histochemical changes associated with the *N*-PAA- and DPA-induced RPN have not been fully described, but include loss of the mucopolysaccharide (MPS) staining from the medullary matrix in those areas where necrosis has occurred. THG-positive material was lost from the distal nephron and progressively accumulated in casts in the necrotic collecting ducts (Powell *et al.*, 1983).

There are some important morphological differences between the *N*-PAA-induced lesions, which develop subacutely, compared to an acutely induced RPN (Bach and Hardy, 1985). The lesion caused by *N*-PAA was apex limited and affected no more than 30% of the medulla (i.e., only the papillary tip) for multiple doses of 3–5 mmol/kg of *N*-PAA in Sprague–Dawley rats, but the lesion was diffuse in Wistar rats. Ethyleneimine (Axelsen, 1978a) and BEA (Axelsen, 1978b; Bach *et al.*, 1983) cause a lesion which varied from an apex-limited focal necrosis (at low doses) to total ablation of the medulla.

2. *Functional Changes in N-Phenylanthranilic Acid-Induced Renal Papillary Necrosis*

The *N*-PAA-induced RPN decreased urine osmolality and increased urine volume, but only at dose levels of 2.0 mmol/kg or more. There were

no changes in the urinary acidification after an oral dose of ammonium chloride in animals treated with doses of *N*-PAA less than 5 mmol/kg. If the urinary chloride excretion was studied between 0 and 2 hours after ammonium chloride dosing, there was a dose-related decrease in the concentration of chloride ions that was significantly different from controls at the lowest dose of 0.5 mmol/kg *N*-PAA. The importance of choosing an appropriate window in time through which to monitor the dynamics of the renal response to chemical perturbation is clearly shown by the fact that similar changes were not apparent in urine collected from 2–4 hours (Hardy and Bach, 1984). Increased urinary protein, sodium, potassium, and chloride excretion have also been reported in animals treated with DPA, but *N*-PAA caused a proteinuria only (Powell *et al.*, 1985b).

V. Biochemical Interpretation of the Pathogenesis and Secondary Consequences of Renal Papillary Necrosis

Mechanism of Renal Papillary Necrosis

A variety of mechanisms have been proposed to explain RPN. There is little evidence from studies using animal models and clinical material to support the suggestion that RPN is mediated by an immunological injury (Gault *et al.*, 1971; Murray and Von Stowasser, 1976). Similarly, most of the evidence to suggest that analgesics alter intermediary metabolism (see Shelley, 1978) was based on data using renal cortical tissues, which are biochemically very different from the medulla. A role for anoxic injury and microvascular degeneration is not supported by the acute-model lesion, as assessed by vascular filling data and morphology (Ham and Tange, 1969; Bach *et al.*, 1983; Gregg *et al.*, 1988a,b). Neither the colloidal carbon nor the Monastral Blue method differentiates between stasis and high flow rate areas. Solez *et al.*, (1974) have shown, however, that the clearance of radiolabeled albumin was faster after BEA, thus there is no impaired papillary blood flow or hemostasis as evidence to support a contributing factor in the development of RPN. Vascular changes appear to follow the lesion. While it has been argued that the ubiquity of analgesics in inducing RPN could be linked to depressed prostaglandin (PG) synthesis and vasoocclusion (see Shelley, 1978; Bach and Bridges, 1985), the evidence against this is extensive (Bach and Bridges, 1985). An active increase in the concentration of papillotoxic compounds by countercurrent concentration could militate in favor of the lesion, but the loss of concentrating ability occurs well before a lesion is histologically apparent (Wilks *et al.*, 1986).

None of the theories that have been postulated (Rosner, 1976; Shelley, 1978; Prescott, 1982) to explain the molecular pathogenesis of RPN have

so far defined the factors underlying the development of the primary necrotic lesion, nor have they addressed themselves to the progressive secondary cortical changes which result in the degeneration of nephron function. In the absence of a hypothesis to explain the genesis of the lesion, it has been suggested that the metabolic activation of analgesics, NSAID, and carcinogens could play a very important role in cell injury, and be an essential factor in the genesis of both RPN and upper urothelial carcinoma. The absence of medullary and urothelial cytochrome *P*-450 has precluded this metabolic process from direct involvement in the local formation of putative reactive intermediate(s) that could cause the toxic effect. It is unlikely that a reactive product formed in the cortex (where there is cytochrome *P*-450 activity) would be transported to the papilla and upper urothelial cells and cause injury, because these reactive products are highly labile and their short-lived nature would preclude this "mechanism" (see Bach and Bridges, 1984, 1985).

Recently, the hydroperoxidase activity present in the PG synthase system (and other peroxidative enzymes) have been shown to convert the two major metabolites of phenacetin, *p*-phenetidine (Andersson *et al.*, 1983; Ross *et al.*, 1985) and acetaminophen (Joshi *et al.*, 1978; Zenser *et al.*, 1979a,b; Nelson *et al.*, 1981; Mohandas *et al.*, 1981a,b), to reactive intermediates. This is highly relevant to the medulla and upper ureter, where PG synthetase is *very* active (Mori and Mine, 1981). Only unstable chemical intermediates formed by these enzymes will act locally (at the medulla or ureter) to produce toxic effects, possibly *via* lipid peroxidation (see Bach and Bridges, 1984, 1985). Despite the attractiveness of this hypothesis, neither acetaminophen nor *p*-phenetidine causes RPN, and there are no published data to suggest their role in the development of upper urothelial carcinoma in rodents. Long-term phenacetin feeding (over 24 months) does, however, cause a marked pelvic hyperplasia (see Section VI,C).

Histochemically, the enzymes associated with PG synthesis are distributed in different regions of the kidney. For example, the cyclooxygenase is localized to the medullary interstitial cells, endothelial cells of all arteries and arterioles, the collecting ducts, and glomerular epithelial cells of some species (Smith and Wilkin, 1977a,b; Smith and Bell 1978). Prostaglandin antibodies demonstrated PGE_2 and $PGF_{2\alpha}$ in cortical and medullary collecting ducts, the medullary interstitial cells, both glomerular mesangial and epithelial cells, and endothelial cells of the arteries and arterioles (Mori and Mine, 1981). More PGA_2 has also been shown in the tubular cells of the renal medulla compared to the cortex (Perez and McGuckin, 1972). None of these methods serves to measure other types of peroxidative activity, but total peroxidative enzyme activity can be measured by the diaminobenzidine method. There is more peroxidase activity in the collecting duct compared to the medullary interstitial cells, but the absence of

activity in glomeruli suggests that this method does not demonstrate PG hydroperoxidase (Janszen and Nugteren, 1971, 1973; Litwin, 1977; Al-Ani and Fourman, 1979).

The ubiquitous distribution of the PGs and cyclooxygenase and other peroxidase activities in different parts of the kidney fail to explain why papillotoxic chemicals target selectively for the medullary interstitial cells and do not affect regions such as the collecting ducts or the glomeruli, that have similar enzymes. It has been suggested that the sensitivity of the interstitial cells to these chemicals relates to the presence of both an enzyme-activating system and high levels of polyunsaturated fatty acids. This would predispose to lipid peroxidation and the associated cellular degenerative changes selectively in the interstitial cells (Bach and Bridges, 1984).

The importance of the prostaglandins in the peroxidative activation of potentially papillotoxic chemicals remains to be established. Recent studies have shown that cultured rat medullary interstitial cells are sensitive to BEA (Benns *et al.,* 1985), but not to those nephrotoxins that target for the glomeruli or proximal tubule. These isolated interstitial cells contain lipid droplets and peroxidase activity. Interestingly, 3T3 and MDCK cells have a very high PG synthase activity (Hassid and Levine, 1977), whereas this enzyme has a low level of activity in HaK cells (Hull *et al.,* 1976). The 3T3 cells also contain lipid droplets; the other two cell types are essentially free of lipid material. The 3T3 are very sensitive to BEA and show pronounced cytotoxicity at levels of 0.2 m*M* over 2–4 hours. By contrast, the MDCK and HaK cells are resistant to BEA and appear to be unaffected by exposure to 2.0 m*M* for 24 hours. This observation also points to the increased BEA cytotoxicity in those cells with both peroxidative activity and lipid droplets (Bach *et al.,* 1986). It must, however, be stressed that at present there are no data available to establish if the lipid droplets in the 3T3 cells are polyunsaturated, nor is there any information on differences in the intracellular activities of protective factors such as catalase and superoxide dismutase.

We have shown that *N*-PAA undergoes peroxidative conversion to biologically reactive intermediates (Feldman and Bach, 1988). Horseradish peroxidase and PG synthase from pig seminal vesicle and medullary microsomes, in the presence of hydrogen peroxide or hydroperoxyeicosenoic acid (the precursor of PG formed from exogenous arachidonic acid), activate *N*-PAA. The biologically reactive intermediate(s) bind to protein and nucleic acid, an effect that is quenched by nucleophilic sulfhydryl-protecting agents such as glutathione (Feldman and Bach, 1988). The quantities of *N*-PAA that are bound to protein and nucleic acid (Feldman and Bach, 1988) are of a similar order of magnitude to those of several established carcinogens (Kadlubar *et al.,* 1982).

VI. Renal Papillary Necrosis and Upper Urothelial Carcinoma

The relationship between RPN and upper urothelial carcinoma in human analgesic abusers has been the topic of much debate and continued speculation, although the two are very strongly associated (McCredie *et al.*, 1982a,b, 1983; Bach and Bridges, 1985). Thus, the exact pathogenesis of these malignancies in humans is uncertain; there have been no data on which factors exacerbate or ameliorate the development and/or the progression of the tumors, and under what circumstances they metastasize most readily. Normally the lesion is diagnosed at such an advanced stage, and with such widely disseminated metastases, that surgical resection may only be palliative (Hultengren *et al.*, 1965; Mihatsch and Knusli, 1982).

A. Role of Urothelial Hyperplasia in the Development of Upper Urothelial Carcinoma in Human Analgesic Abusers

The progression of hyperplasia, through dysplasia, to malignancy is now a widely accepted series of changes in the development of carcinoma in epithelial cells in a number of different organs (Faber and Sporn, 1976). Such events may occur in the genesis of analgesic-associated carcinoma. There are well-documented foci of hyperplastic ureteric epithelia, in addition to malignancies, in patients with upper urothelial tumors (Lomax-Smith and Seymour, 1980a,b) and in analgesic abusers with RPN, but no diagnosed malignancies (Blohme and Johansson, 1981). These data, taken in light of the already established very high incidence of upper urothelial carcinoma in analgesic abusers (Bengtsson *et al.*, 1968, 1978) and the strong association between the two (McCredie *et al.*, 1982a,b, 1983), highlights the possibility of these proliferative changes in the pelvic and ureter epithelial cells as being premalignant.

B. Animal Models of Upper Urothelial Carcinoma

There are several animal models of bladder and renal parenchymal carcinoma (Hicks, 1980, 1983; Cohen *et al.*, 1983; Hard, 1987), but to date there has been relatively little research on experimentally induced upper urothelial carcinoma.

Many of the early investigations into the cause of upper urothelial carcinoma concentrated on the role of phenacetin and mixed analgesics (Johansson, 1981; Johansson and Angervall, 1976a,b; National Cancer

Institute, 1978; Isaka *et al.*, 1979; Nakanishi *et al.*, 1982). While several of these studies reported tumors at a variety of renal and extrarenal sites, the only regular and reproducible effect on the kidney was urothelial hyperplasia (Johansson and Angervall, 1976a,b). In the absence of urothelial malignancies, the general conclusion has been adopted that there was sufficient evidence in animals that phenacetin was carcinogenic, but that this was limited for analgesic mixtures containing phenacetin (IARC, 1980, 1982).

The early studies on bladder malignancies caused by feeding animals with 0.188% *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) also showed the presence of upper urothelial malignancies in a few rats after 46 weeks (Erturk *et al.*, 1967). Subsequently, over half the 35 rats fed with 0.188% FANFT for 46 weeks were shown to have severe pelvic hyperplasia, and 4 cases of upper urothelial carcinoma were also reported (Erturk *et al.*, 1969). The FANFT-induced upper urothelial carcinoma has been shown to be exacerbated by phenacetin feeding, by mechanical perforation of the renal pelvis (Anderstrom *et al.*, 1983; Anderstrom and Johansson, 1983), and by sustained *Escherichia coli*-induced urinary tract infection (L. Van Schultz, personal communication). There are also data to show that the administration of phenacetin for a period of 30 weeks, after the urothelia had been initiated with *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (HO-BBN), caused a significantly higher incidence of bladder tumors (Nakanishi *et al.*, 1978).

These data suggest that upper urothelial carcinoma could be induced experimentally with a total carcinogen such as FANFT and/or the combination of initiation and promotion with any agent that causes a hyperplasia of the papilla or pelvic urothelial cells. None of these investigations has, however, adequately addressed the question of the interrelationship between RPN and upper urothelial carcinoma. There is no evidence to suggest that FANFT causes RPN, and where phenacetin was administered after HO-BBN there were no data to suggest that papillary necrosis had been caused by either chemical. In addition, there are several other manipulations such as mechanical injury to the renal pelvis (Anderstrom *et al.*, 1983; Anderstrom and Johansson, 1983), high-salt diets (Lalich *et al.*, 1974), and saccharin feeding (Murasaki *et al.*, 1982) that also cause pelvic hyperplasia in the absence of RPN.

C. Upper Urothelial Hyperplasia in Chemically Induced Renal Papillary Necrosis

Upper urothelial hyperplasia has, in fact, been a common consequence of many papillotoxic chemicals, and varies from mild, following aspirin

(Molland, 1976) or BEA (Bach *et al.*, 1983), to a florid hyperplasia following substituted indeneacetic acid NSAID analog (Bokelman *et al.*, 1971). *N*-PAA often caused hyperplasia in the renal pelvic epithelium adjacent to the necrosed papilla. A group of rats dosed with 5.0 mmol/kg *N*-PAA for 5 days showed significant epithelial changes, which varied from a mild columnar hyperplasia, with minimal inflammatory cell infiltration, to a florid hyperplasia at the papillary apex (Fig. 9), associated with moderate subapical tissue necrosis (Hardy and Bach, 1984).

The protracted period required to induce RPN experimentally with the therapeutically used analgesics or NSAID, and, especially, the variability in

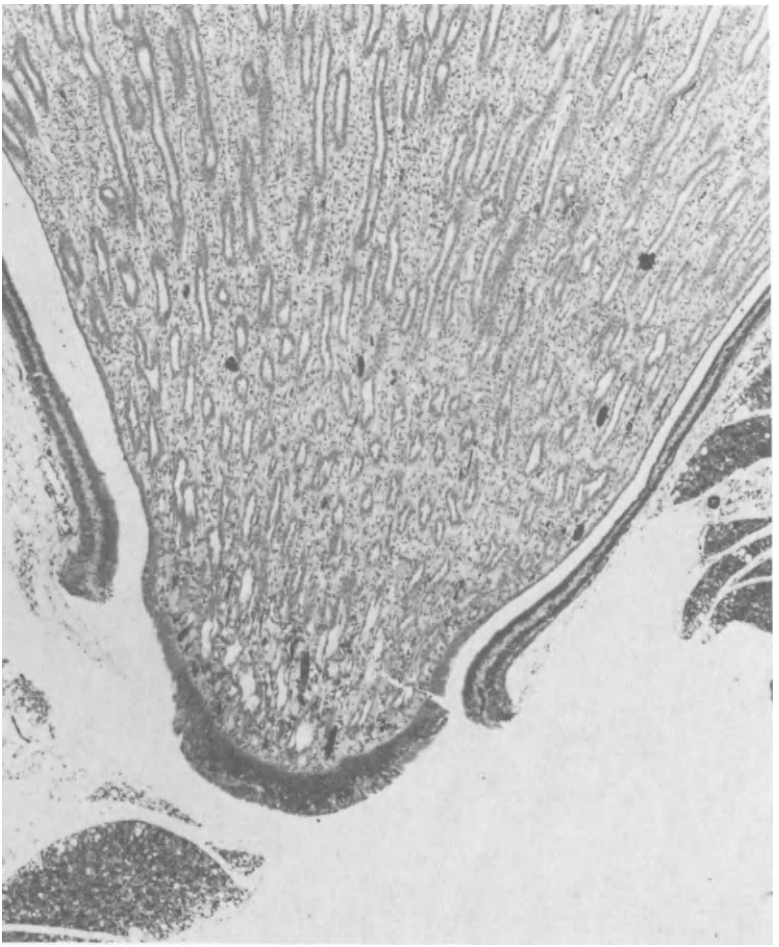
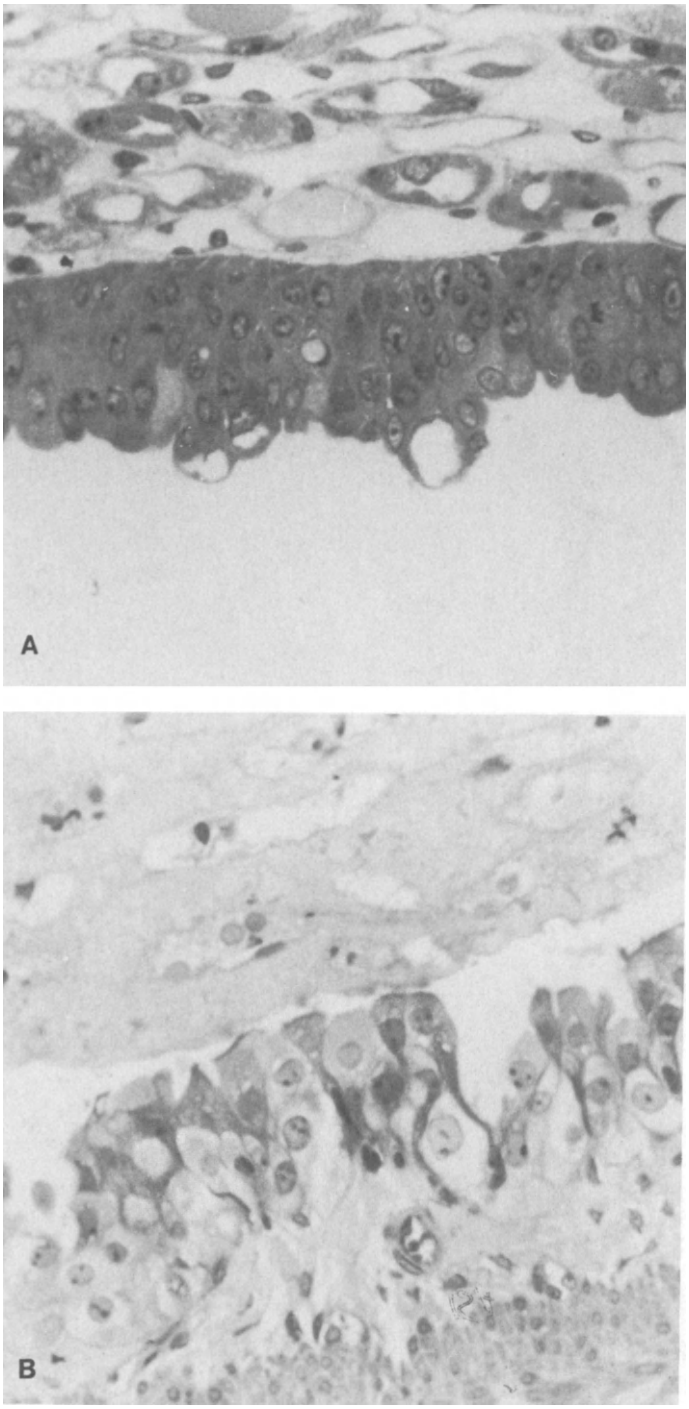


FIG. 9. Apex-limited urothelial hyperplasia following five daily doses of 5 mmol/kg N-PAA. Hematoxylin–eosin, ×5. [From Hardy and Bach, (1984).]


A
B

the extent of the lesion caused by these agents (Rosner, 1976; Bach and Bridges, 1984; Bach and Hardy, 1984), have so far precluded the experimental investigation of the relationship between RPN and upper urothelial carcinoma. The acute nature of the BEA-induced RPN, without extrarenal toxicity and with limited effects on the renal cortex, offers a less complicated model to study pelvic urothelial changes and the factors that may exacerbate the process.

D. Upper Urothelial Hyperplasia following 2-Bromoethanamine-Induced Renal Papillary Necrosis

After BEA treatment the upper urothelia showed marked proliferative changes at the edge of the epithelia covering the papilla (i.e., at the junction between the normal and necrotic part of the papilla). This was five cells thick (whereas it is normally one cell thick in most parts of the urothelia, except for the ureter, where it is four cells thick) at 24–48 hours (Fig. 10A). Hyperplasia was especially marked at the mouth of the ureter (which was seven cells thick) at 18 hours and in the pelvis opposite the region of necrosis, which was seven to eight cells thick at 18 hours (Fig. 10B). This had only partially resolved by 144 hours.

The ureter and the bladder showed minimal histological changes up to 144 hours. There were few morphological changes in the upper urothelia before 24 hours, after which a progressive and very marked hyperplasia was present in the pelvic urothelia (adjacent to the necrosed papilla tip and up to the fornix), in the urothelia covering the papilla (at the junction with the injured area), in the collecting ducts, and at the origin of the ureter. The increased thickness of the urothelia was most marked at 72 hours and continued as such in all of the upper urothelia (except the pelvis opposite the region of papillary injury) for 144 hours after BEA. There were disproportionately fewer mitoses compared to the degree of urothelial thickening (Gregg *et al.*, 1988c).

E. Upper Urothelial Kinetics following 2-Bromoethanamine-Induced Renal Papillary Necrosis

We have used autoradiography to study cell kinetics in different regions of the upper ureter following BEA-induced RPN.

FIG. 10. (A) Marked proliferative changes in papilla covering epithelium at leading-edge junction of normal and necrotic parts of papilla. Toluidine Blue, 1-μm resin section, ×40. (B) Hyperplasia of pelvic epithelia opposite denuded papilla covering epithelium indicative of necrotic region, 24 hours after 100 mg/kg BEA ip. Toluidine Blue, ×40.

The cell kinetics in different parts of the kidney, pelvis, and ureter were based on the incorporation of [^{3}H]thymidine, infused from a subcutaneously implanted miniosmotic pump at a zero-order rate for 144 hours. This method provides data on the total accumulated turnover of epithelial and urothelial cells for the period under investigation. The labeling index for normal cell turnover was highest (and similar) in the proximal and distal tubules, less in the epithelia covering the papilla and the adjacent pelvis, even lower in the ureter and collecting duct, and lowest in the pelvic fornix. Compared to the baseline cell turnover, there was a 2- to 3-fold increase in all the areas 144 hours after BEA, except for the collecting duct (8-fold) and the pelvic fornix, which showed a 16-fold increase (Gregg *et al.*, 1988c). In absolute terms the most active regions of cell division after BEA were the collecting duct, the pelvic fornix (opposite the papilla tip and the margin of papillary injury), and the origins of the ureter. The urothelia at the margin of papillary injury were apparently the least actively dividing cells. Both the proximal and the distal tubules responded to BEA-induced RPN with a markedly increased rate of cell division compared to controls.

These data show that the upper urothelia is very responsive to an acutely induced RPN, particularly as assessed by autoradiography, which was the most sensitive means of demonstrating proliferative changes. Renal tubular epithelia and urothelia each had their own normal cell turnover, and the response of each to the papillary injury differed. The delayed development of urothelial changes for the first 24 hours after the necrosis of the papilla suggests that the upper urothelial hyperplasia may be a consequence of RPN.

F. Changes in the Upper Urothelial Histochemistry following 2-Bromoethanamine-Induced Renal Papillary Necrosis

Carbohydrate granules have been noted in the epithelial cells associated with RPN induced by aspirin (Molland, 1976) and BEA (Gregg *et al.*, 1988a). The aspirin-induced lesion was associated with an accumulation of dense fibrils of PAS-positive material in the interstitial matrix. There was also an accumulation of PAS-positive granules in the cells of the collecting duct and the covering epithelium in BEA-treated animals (Fig. 11). These granules appeared before cell necrosis and may therefore have been part of the autophagic process responding to the release or breakdown of the extracellular matrix. The presence of similar granules in pelvic and upper urothelial cells 21 weeks after the induction of an acute papillary necrosis suggested that this change was a long-term aberration of cellular function,

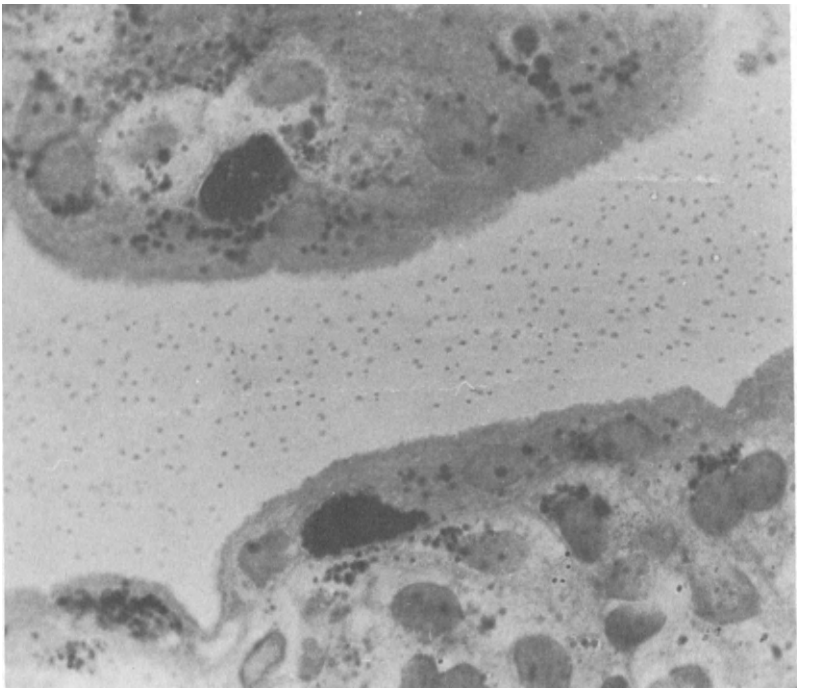


FIG. 11. PAS-positive staining granules in superficial layer of upper ureter urothelium 21 weeks after single ip dose of 100 mg/kg BEA. 1-μm resin section, $\times$100.

especially considering that they were most marked in those regions where the urothelium was most hyperplastic or dysplastic. Similar changes have been described in the upper urothelial carcinoma epithelial cells, and in cells that had metastasized from these regions (Tucker *et al.*, 1959), and in bladder malignancies in humans (Hukill and Vidone, 1965). The presence of these granules could be related to changes in the glycocalyx, which has been linked to tumorigenesis and neoplasia (Iozzo, 1985) and to foreign cell recognition (Smets and Van Beek, 1984). The granules may also represent premalignant changes, although similar staining has been reported in rodents with lithium-induced nephropathy (McAulliffe and Olesen, 1983).

There are a number of enzyme histochemical changes in the urothelial cells following a BEA insult. For example, there was a marked increase in alkaline phosphatase in the pelvic urothelial cells adjacent to the necrosed papilla at 8 hours, that subsequently included other hyperplastic upper urothelial regions (Fig. 12). This pattern was maintained for up to 24 hours and then declined to give a mosaic of staining that was, nevertheless, still strong in the pelvis adjacent to the necrosed papilla at 144 hours.

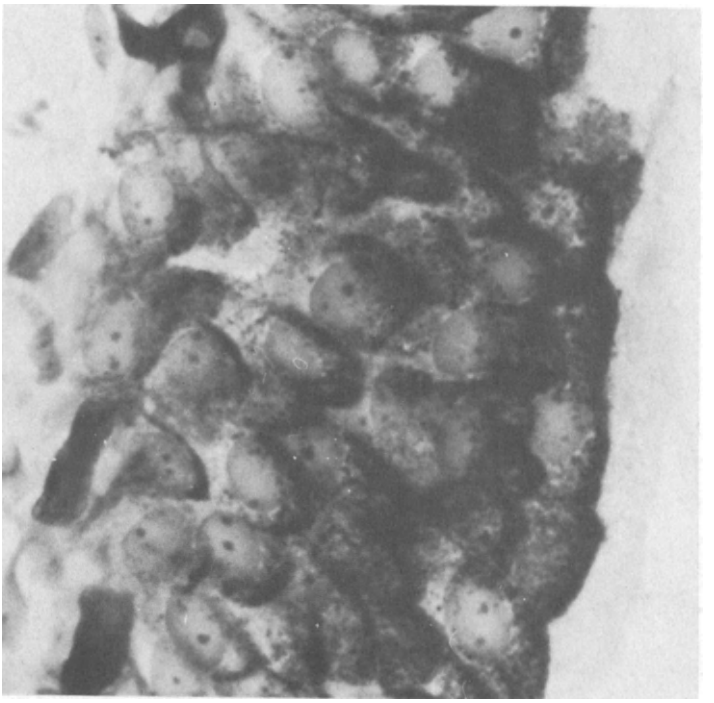


FIG. 12. Marked alkaline phosphatase staining in hyperplastic pelvic urothelium opposite necrotic papilla at 8 hours after 100 mg/kg BEA ip. 1-μm resin section, $\times$100.

G. UROTHELIAL INITIATION WITH HYDROXYDIBUTYLNITROSAMINE (HO-BBN) FOLLOWED BY THE INDUCTION OF RENAL PAPILLARY NECROSIS

Pretreating rats with HO-BBN for 5 weeks, to a toal dose of 800 mg per rat, followed by BEA, produced early diffuse hyperplasia (similar to that described in humans with RPN), which progressed to focal proliferative changes, up to eight cells thick (Fig. 13A) and discrete lesions with pleomorphic, atypical, and disorganized cells (many of which had irregular and bilobular nuclei, and some of which were multinucleated). These also included papillary P1 tumors (Fig. 13B) and several flat carcinomas *in situ* that were invasive P1 into the submucosa (Fig. 13C) or showed early invasion into muscle P2 (N. J. Gregg *et al.*, unpublished). These features are similar to those of the tumors reported in human analgesic abusers (Johansson and Wahlqvist, 1977; Mihatsch *et al.*, 1980c).

Animals treated with HO-BBN followed by BEA and those treated with BEA alone, for up to 21 weeks, showed the same mosaic pattern of alkaline

phosphatase staining in the hyperplastic urothelia. Those areas of the urothelium in the HO-BBN-pretreated animals that appeared to be invasive (P1 stage) lacked alkaline phosphatase staining, and looked similar to foci of "normal" urothelium (Fig. 14A). Some of the hyperplastic cells in both the HO-BBN/BEA-treated and the BEA-treated animals had superficial acid phosphatase staining (Fig. 14B), suggesting an autophagic response. Whereas staining for GGT was effective on the brush border, there were no foci of this activity in the hyperplastic or dysplastic cells at any time point following BEA treatment.

By contrast, however, there are focal and irreversible losses of alkaline phosphatase from otherwise histologically and cytologically normal rat

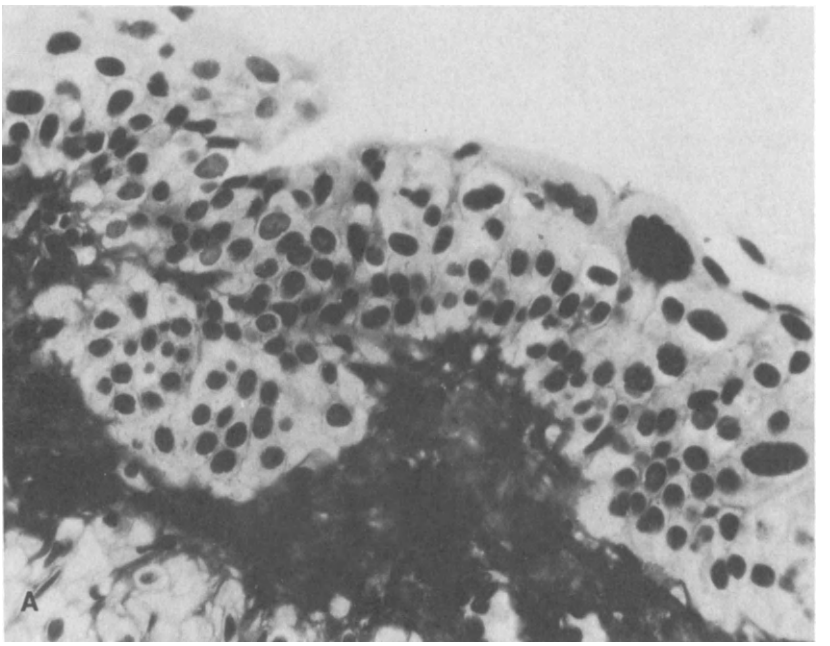
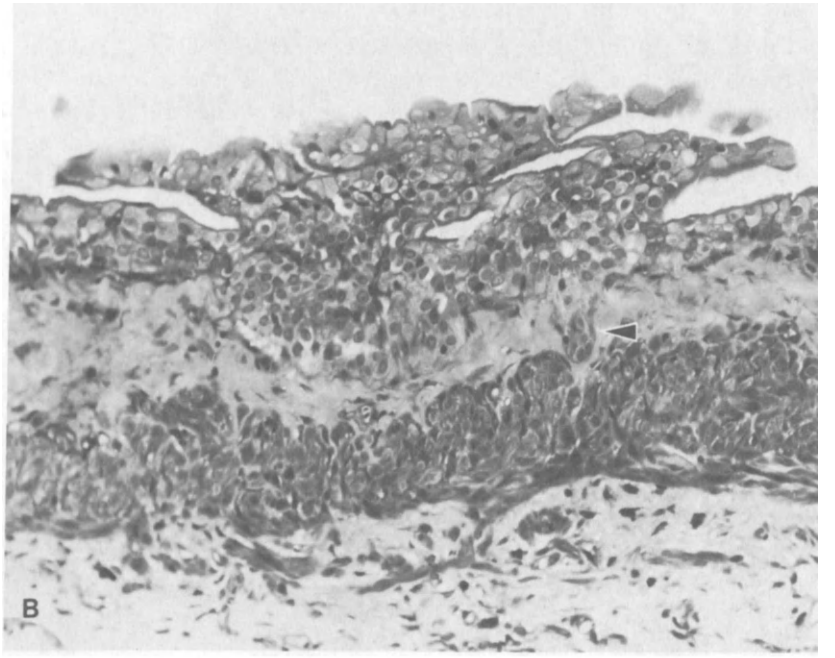


FIG. 13. (A) Atypical, disorganized urothelium with multinucleated cells and potential invasive focal lesions following 5 weeks pretreatment with HO-BBN and 13 weeks after a single dose of 100 mg/kg BEA ip. Giemsa, 1-μm resin section, $\times$40. (B) Papillary P1 tumor in upper ureter following 5 weeks pretreatment with HO-BBN and 13 weeks after a single ip dose of 100 mg/kg BEA. Note invasive finger into submucosa (arrowhead). Toluidine Blue, 1-μm resin section, $\times$20. (C) Early invasion in muscle layer of ureter from flat carcinoma *in situ*, seen as an invasive finger in B. Toluidine Blue, 1-μm resin section, $\times$100.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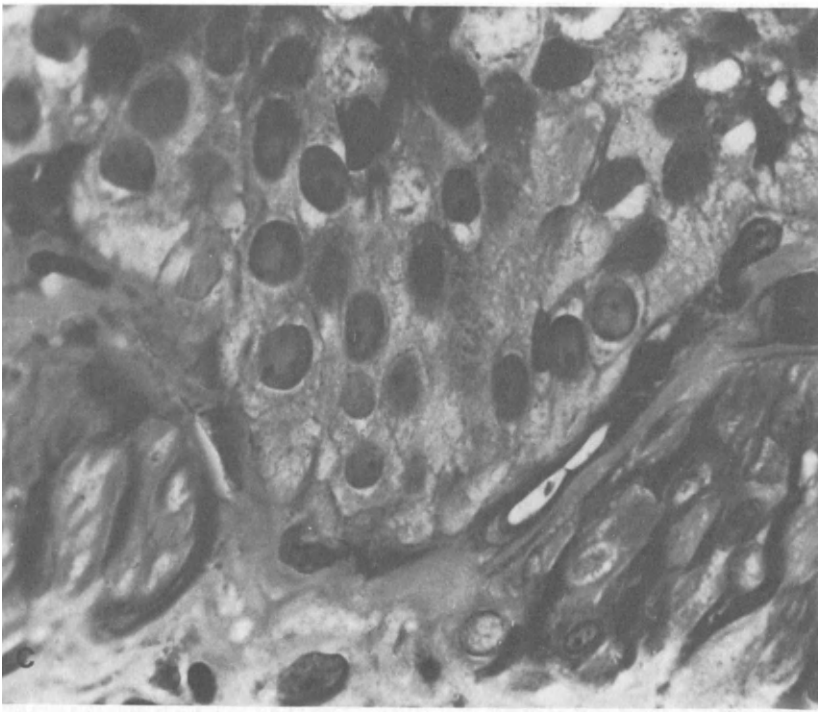

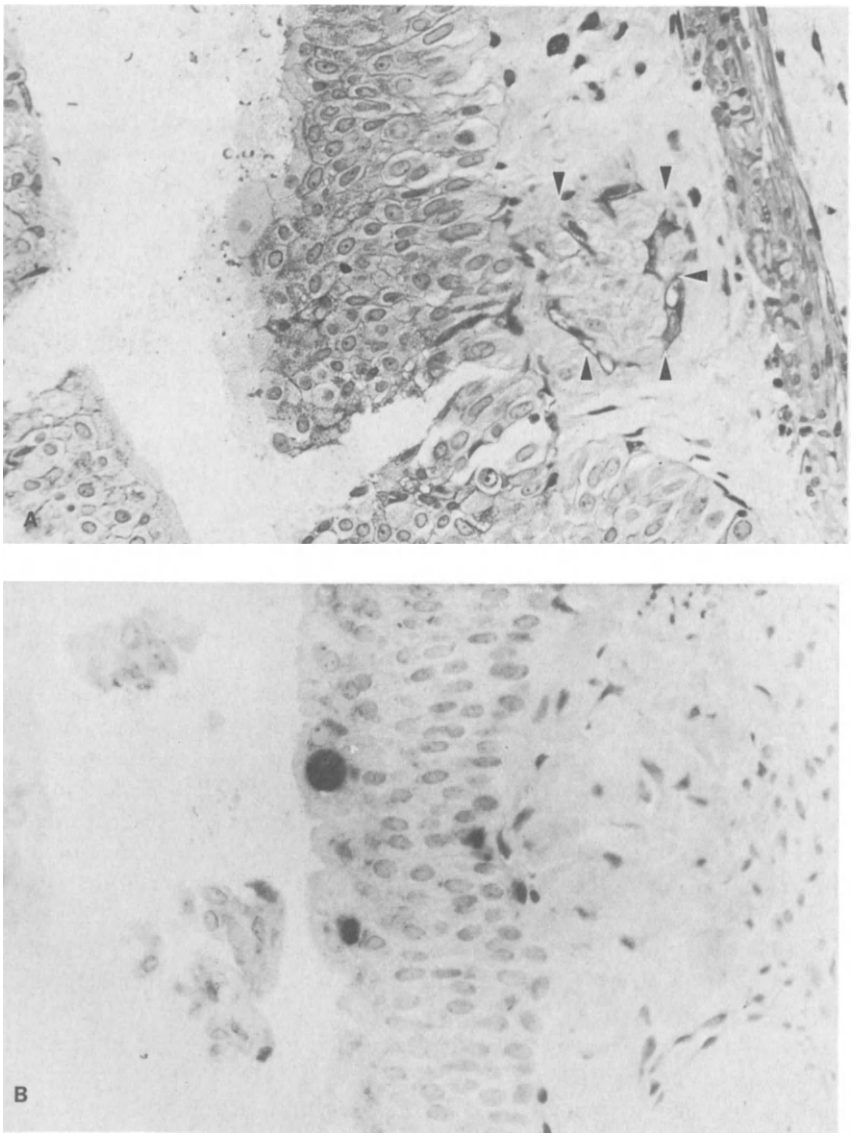

FIG. 14. (A) P1 invasive urothelium showing (arrowheads) negative alkaline phosphatase staining compared to hyperplastic urothelium. Gomori alkaline phosphatase method, 1-μm resin section, $\times$32. (B) Acid phosphatase staining in superficial layer of urothelium following a single BEA 100 mg/kg dose at 30 weeks. Acid phosphatase Napthol AS-B1 method, 1-μm resin section, $\times$32.

bladder urothelial cells following di-*N*-butylnitrosamine, HO-BBN, or FANFT treatment (Kunze, 1979; Kunze *et al.*, 1969, 1973; Kunze and Schauer, 1971; Stiller and Rauscher, 1971; Ito *et al.*, 1973). The alkaline phosphatase-free foci are considered to be preneoplastic cells. GGT-positive cells are a well-established marker for premalignant changes in hepatocarcinoma (Hanigan and Pitot, 1985). Foci of GGT-positive cells are present in otherwise normal urothelia after exposure to HO-BBN (Ozono *et al.*, 1985), a feature that has been present in nodulopapillary hyperplasia and carcinoma that subsequently developed. There is also some evidence to suggest that GGT may not be a pathognomonic indicator of premalignant changes, but rather identifies only advanced carcinoma and large papillomas (Vanderlaan *et al.*, 1982). These data suggest that the histochemical changes that take place in the upper urothelia differ from those that occur in the bladder, and raise the possibility that there may be subtle aspects in the pathomechanism that are unique to lesions in different parts of the urinary tract.

The combination of HO-BBN and BEA did not produce any other obvious gross histopathological changes in the heart, lungs, liver, spleen, or pancreas up to 21 weeks. Similarly, there were no changes in the high-resolution light-microscopic features in the kidney, pelvis, ureter, or bladder associated with the use of HO-BBN only.

Thus the combination of HO-BBN initiation followed by the promotion of hyperplasia in the upper urothelial cells by a BEA-induced lesion appears to offer a very rapid model for the induction of upper urothelial dysplasia. This experimental approach may provide a superior model for studying the genesis and development of RPN-associated malignancy. Furthermore, this system offers the potential to improve the diagnosis and management of similar changes in human analgesic abusers. More importantly, these data suggest that the development of upper urothelial carcinoma may represent a classical two-stage model of cancer. If this is the case, and it is relevant to the condition in humans, there is a very important need to establish which of the carcinogens that humans are exposed to represent the initiating agents. While the elucidation of these agents requires very careful study, and cannot at this stage exclude analgesics or NSAID, it is noteworthy that smoking was also very strongly associated with the development of upper urothelial carcinoma in analgesic abusers in Australia (McCredie *et al.*, 1982a,b, 1983). Benzo[*a*]pyrene is one of the potent carcinogens that is a component of all smokes, and is thought to be a major risk factor in bladder cancer (Mommsen and Aagaard, 1983). Interestingly, the peroxidative enzymes of the type that may be involved in the metabolic activation of analgesics, NSAID, FANFT, and other carcinogens (Rapp *et al.*, 1980; Mattammal *et al.*, 1981; Zenser *et al.*, 1983a,b) also convert benzo[*a*]pyrene to the highly reactive 7,8-diol epoxide (Marnett *et al.*, 1978).

VII. Summary of the Pathogenesis of Experimentally Induced Renal Papillary Necrosis and Upper Urothelial Carcinoma

The morphological, histochemical, and functional data presented above support several distinct series of pathological changes following the administration of BEA. The earliest histochemical changes take place in the medullary matrix, which appears to undergo depolymerization. The renal medullary interstitial cells are the first cell type to undergo degenerative change, which is rapidly followed by damage to the "delicate" elements (endothelium and loops of Henle) of the medulla. The collecting ducts and endothelial changes are late and generally follow the necrosis of other anatomical regions of the medulla. The lipid changes in the medulla are not at present well understood, but they are similar to those already reported in human analgesic abusers.

The early subtle degenerative changes in the proximal tubule do not appear to be central to the development of the papillary lesion, but the subsequent exfoliation of the brush border and proximal tubular cells are important components of the protein casts that begin to form in the distal nephron. These subsequently appear to play at least some role in the development of functional changes that cause marked proximal tubular dilatation.

The intense alkaline phosphatase staining that develops in those regions of the upper urothelia suggests that hyperplasia is a secondary response to papillary injury. Similarly, the increased pelvic, ureter, and bladder endothelial staining for ATPase suggests that there is a progressive suburothelial microangiography, similar to that described in human analgesic abusers, associated with RPN, but also show that this is a consequence of the other changes that are taking place in the kidney.

The proliferative changes in the urinary tract epithelia were assessed by the number of mitoses and the urothelial thickness. The total number of cell divisions that occurred in the epithelia following BEA was measured by the continuous infusion of [^{3}H]thymidine and autoradiography of the semithin sections. Importantly, autoradiography also showed that there is marked regeneration in the proximal and the distal tubules, although routine histology failed to show these changes. Hyperplasia of the upper urothelial cells appears to be a secondary consequence of papillary necrosis in this acute-model lesion.

The time course of the major pathophysiological changes associated with the development of RPN and its secondary consequences of cortical degeneration and upper urothelial hyperplasia are presented schematically in Fig. 15. These data show that there are discrete series of pathological

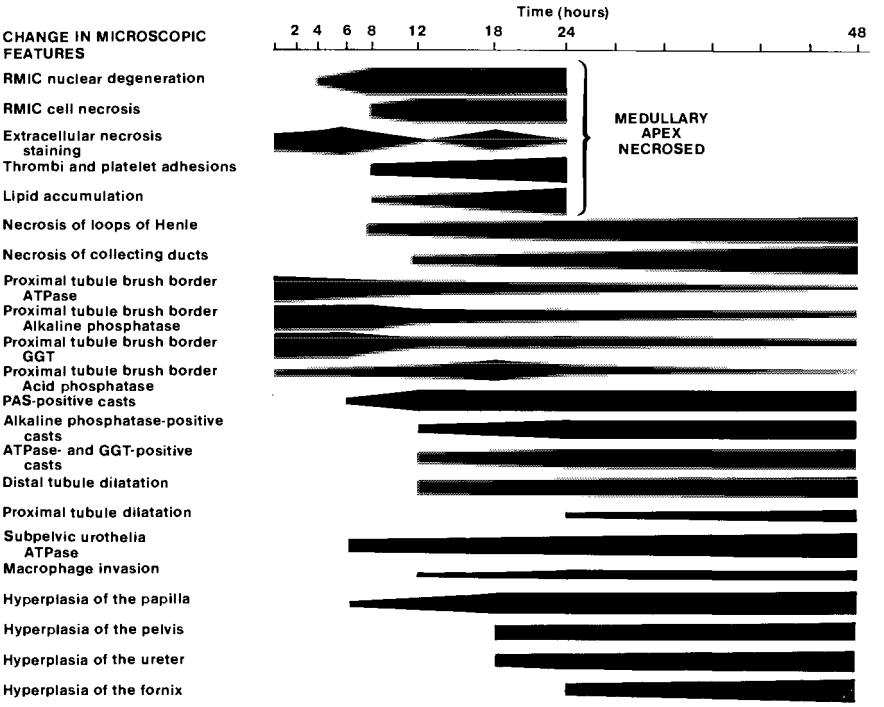

FIG. 15. Schematic representation of the cascade of degenerative morphological and histochemical changes associated with BEA-induced renal papillary necrosis. RMIC, Renal medullary interstitial cells. GGT = Gamma-glutamyltranspeptidase; ATPase = Adenosine triphosphatase.

changes that appear to start on the medullary interstitial cells, and the degenerative changes then go on to affect a variety of other cell types in different parts of the kidney. While much of the effort in understanding nephrotoxicity has been directed at the physiology, morphology, and biochemistry of the primary renal lesion, there is obviously an equally important role for establishing the cascade of degenerative changes that follow a primary lesion. The complexity of the kidney is such that it is unlikely that any one cell type can be damaged without there being repercussions in the rest of the organ and probably extrarenally.

VIII. Remaining Questions

It is now obvious that a variety of chemicals, in addition to the analgesics and NSAID, have the propensity to cause renal papillary necrosis. The study of RPN and upper urothelial carcinoma therefore is no longer to be

regarded as being purely the consequence of long-term analgesic abuse or therapeutic NSAID exposure. In humans RPN may also be a consequence of exposure to industrial and environmental chemicals. There is also some evidence to show that the papillotoxic chemicals and analgesics and/or NSAID may interact synergistically to produce RPN. Despite the large number of pathophysiological similarities between the acute and subchronic models of RPN, and between these and the chronic analgesic disease in animals and in humans, it would not be wise to extrapolate all of the pathophysiological changes reported in the rat model to other species. Instead, there is an important need to develop more subtle criteria to interrelate the cascade of degenerative changes and to develop better experimental techniques to allow a progression from acute, through subacute, to chronic animal models to be followed. Similarly, it is likely that studies on a number of different species can help address the question of anatomical and functional differences between humans and animals. Upper urothelial carcinoma may be a consequence of a total carcinogen, or it may more likely represent an initiation of the upper urothelial cells (due to their endogenous peroxidative activity or medullary enzyme activation), and subsequently the development of RPN and the associated injury to the upper urothelial cells and/or the associated functional changes that act as a promoting factor.

A number of questions still need to be addressed to understand more fully the pathogenesis of RPN and particularly the sequence of events that lead from a primary lesion in the medullary interstitial cells to the secondary degenerative loss of cortical parenchyma and urothelial abnormalities. One area that needs to be addressed in particular is the dichotomy between morphology and cell biochemistry that will describe the lesion in terms of molecular changes, in both animals and humans.

The major questions that still need to be answered can be formulated here:

1. By what mechanism are the degenerative changes in the renal medullary interstitial cells caused, and are the changes in the other cell types (e.g., loops of Henle, urothelial cells) a consequence of chemical injury or a result of the medullary interstitial cell degeneration?
2. If peroxidative metabolism of papillotoxic chemicals is a key factor in the generation of reactive intermediates, what is the role of the lipid droplets in the medullary interstitial cells? While the central role of such peroxidative activation can explain target toxicity associated with acetaminophen, *N*-phenylanthranilic acid, and other analgesics and NSAID, at present there is no known role for this mechanism in the genesis of the BEA-induced lesion.

3. What are the interactions that occur between analgesics and other therapeutic substances (and/or environmental chemicals) that may exacerbate or ameliorate the development of renal papillary necrosis, or affect its progression to chronic renal failure or to upper urothelial carcinoma?

4. What is the clinical significance of chemicals other than analgesics and NSAID in causing renal papillary necrosis and upper urothelial carcinoma in humans? Ethyleneimine is a very good example of a chemical that was used extensively in industry long before there was a focus on occupational health and hygiene.

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The Contribution of Cell Culture to the Study of Renal Diseases

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I. Introduction

Many of the recent advances in the scientific basis of cell biology of the kidney have utilized newly developed cell and molecular biological techniques. For the most part these techniques depend on the availability of large populations of homogeneous cells (i.e., cell lines). Tubular cell lines have recently become available, and the rapid advances in our understanding of tubular physiology and pathophysiology is detailed in the second part of this review. Glomerular cell lines have only recently become available; thus, the data reviewed in the glomerular section will deal with enriched populations, but not clonal lines. It should be noted that the definition of a clone is quite precise, referring to the derivation of a line from a single cell. Some have referred to cells as being clonal using a broader definition.

The use of cell cultures in the study of renal diseases is a relatively new area of investigation. Some have used isolated cells simply as a means of obtaining sufficiently separated cell populations to be able to verify that a population contains different cell types, for example, the presence of macrophages in the mesangium (Camazine *et al.*, 1970; Schreiner and Cotran, 1982; Schreiner *et al.*, 1981; Schreiner and Unanue, 1984). Others have begun to assess the effect of inflammatory cells or their products on renal cells in culture. Recent advances in cell and molecular biology, biochemistry, and cell culture now enable these types of experiments to be more sharply focused. For instance, the availability of cloned cell lines for

the first time makes available studies such as cell–cell interactions, the type and cell biology of receptors, and the contribution of each cell to renal metabolism including biosynthesis of the renal extracellular matrix (ECM). This list represents only a small fraction of those now possible. In this review we will focus on the recently published information and will attempt to define gaps in the data now available.

The statement that an understanding of normal function is necessary in the study of disease processes hardly needs repeating, but it is especially important in the kidney, where relatively modest changes in the filtration barrier or ability to handle fluid and electrolytes can lead to substantial disruptions of body function. Finally the role of the kidney as an endocrine organ is just now becoming a subject of study.

II. Glomerular Cell Culture

A. General Observations

In this section we will perseverate the assumption implicit in current studies that the glomerular cells of any one type are identical. For instance, that population of all cells found to be contractile and to have an elongated, multilayered morphology are assumed to be homogeneous and to represent indigenous contractile mesangial cells (Mahieu *et al.*, 1980). They have a similarity to, and continuity with, the smooth muscle cells of the afferent and efferent arterioles. Smooth muscle cells from the two sites also are contractile and would most likely resemble mesangial cells morphologically (Chamley *et al.*, 1977; Rightsel *et al.*, 1982). Nonetheless, they have markedly different responses or sensitivities to agonists and antagonists of contraction. It seems reasonable to expect that mesangial cells might vary one from another, since the shunting of blood and regulation of filtration surface are finely regulated by a process which could require glomerular vascular channels to be more or less independently regulated. The irregularity of lesions in many acute and chronic glomerular diseases also raises the specter of heterogeneity in individual glomerular cell populations. Finally, one must keep in mind that the current classification of glomerular cell types is based on geographic location, rather than a specific or defined function. As in the adjacent tubular segments of the nephron, this topographic categorization may prove to be too simplistic.

More than 15 years ago glomerular cells were first isolated (Bernik, 1969; Burlington and Cronkite, 1973; Quadracci and Striker, 1970). Subsequently, glomerular cells were isolated from humans and many animal

kidneys (Fish *et al.*, 1975; Holdsworth *et al.*, 1978a–c; Hunt *et al.*, 1981; Ishikawa *et al.*, 1980; Kreisberg and Karnovsky, 1983; Morita *et al.*, 1980). The identification of these cells has been difficult in culture, since their original topography was lost. Thus investigators were left with defining cell types by either phase microscopy or some functional characteristic. These features include cell size and shape, surface markers, growth characteristics, biosynthetic products, or intracellular features. Some of these characteristics are either not agreed upon, or may modulate *in vitro* (Kreisberg and Karnovsky, 1983; Striker and Striker, 1985).

The assumption that the initial outgrowth is composed of epithelial cells because of their flattened cuboidal appearance (Foidart *et al.*, 1979) is certainly partially correct, but this seriously underestimates the contribution of other cell types such as macrophages, endothelial cells, parietal epithelial cells, and possibly even a contaminant from the tubules (Foidart *et al.*, 1979; Schreiner and Cotran, 1982; Schreiner and Unanue, 1984). The considerable heterogeneity of cells in the glomerular outgrowth is also affected by the age of the donor (Foidart *et al.*, 1979). It has been shown that endothelial cells are present at early time points (G. E. Striker *et al.*, 1984) and that all glomerular cell types were synthesizing DNA for the first few passages (Norgaard, 1983). The prevailing assumption that epithelial cells, macrophages, and endothelial cells disappear in late cultures is difficult to prove, as is the assumption that late cultures are composed primarily of mesangial cells. This is particularly problematic, since, in our hands, even "clones" obtained by the dilute-plating technique have been found to have a heterogeneous population of cells when late passages are examined (MacKay *et al.*, 1988).

B. *In Vitro* Conditions

The exact medium requirements for each glomerular cell type are most likely quite different, but this is an area which needs to be further defined (Taub and Sato, 1980). *Epithelial cells* from tubules and other body sites have a facilitated growth in certain types of basal medium (Oberley *et al.*, 1980, 1981c,d, 1982; Oii *et al.*, 1980, 1983). In our hands these requirements are different for human and mouse cells. Some have found that a low concentration of serum favors epithelial cell growth (Harper *et al.*, 1984), but others reported that guinea pig epithelial cells were most easily propagated in medium containing 20% serum (Norgaard, 1983). Optimization of cell growth may be favored by certain conditioned media, platelet factors, and a high concentration of serum (Harper *et al.*, 1984; Kreisberg and Karnovsky, 1983; G. E. Striker *et al.*, 1984). *Mesangial cells* generally

fare best in the presence of high serum concentrations. *Endothelial cells* have only recently been propagated *in vitro*. Their requirements have not been extensively studied, but growth appears to be facilitated by commercially prepared mixtures such as Nu-serum and/or purified platelet-derived growth factor, or PDGF (G. E. Striker *et al.*, 1984) and medium conditioned by HEP-G2 cells (Hoshi and McKeehan, 1984).

The definition of conditioned medium requirements is complex, since this medium may contain cell products which are stimulatory and/or inhibitory for one cell type and leave others unaffected (Castellot *et al.*, 1985; Hoshi and McKeehan, 1984). A factor often neglected is that the basal medium in which the conditioning cells are placed may have a subsequent profound effect on the test cells.

The effect of different *substrates* on multiplication, adhesion, and phenotypic expression is now receiving considerable emphasis (Harper *et al.*, 1984; Killen and Striker, 1979; Oberley *et al.*, 1983; Schlondorff *et al.*, 1985). Surfaces coated with fibronectin (Striker *et al.*, 1980) favor epithelial and endothelial cell attachment, migration, and proliferation, whereas mesangial cells attach and proliferate on plastic surfaces (Scheinman and Fish, 1978a; Striker *et al.*, 1976, 1980) but not on surfaces coated with interstitial collagen. The conditions which favor isolation and/or propagation of cells in the mesangium (macrophages) derived from the bone marrow have not been elucidated. However, macrophages from other sites readily adhere to plastic or glass.

C. Preparation of Glomeruli

The isolation of all four glomerular cell types is more reproducibly obtained in human kidneys that had been previously perfused in preparation for transplantation than in either surgical or autopsy specimens, and from glomeruli of young donors, in agreement with animal studies (Norgaard, 1983). It has been reported that in kidneys subjected to prolonged anoxia the outgrowth contains predominantly mesangial cells (Mahieu *et al.*, 1980).

There have been many modifications of the original isolation procedure (Quadracci and Striker, 1970); however, we have found that the most simple method yields the best results in humans and mice. Briefly, small fragments of tissue are pushed through stainless-steel screens with a glass pestle. The resulting brei, containing glomeruli, is then washed and sedimented several times by gravity in basal medium. These steps result in a preparation of glomeruli virtually free from nonglomerular contaminants. Encapsulated glomeruli are not a source of contamination because they

infrequently adhere to the culture surface (Ausiello *et al.*, 1980a; Foidart *et al.*, 1979; Kreisberg and Karnovsky, 1983; Quadracci and Striker, 1970). Vigorous pipetting and trypsinization of cortical fragments or repeated sieving reduces the viability of epithelial cells from mouse and human glomeruli. Digestion with even mild proteases such as collagenase leads to a substantial loss in the number of epithelial cells (Killen and Striker, 1979). For these reasons we have used other methods to obtain purified cell populations, such as cloning, which is possible only at early passage.

The use of trypsin in the preparation of isolated glomeruli has been carefully studied in the rat (Schreiner and Unanue, 1984). The glomeruli were denuded of epithelial cells after 30 minutes of trypsinization. This study demonstrated that glomeruli isolated in this manner may be nearly devoid of epithelial cells and raises questions about the use of trypsin in isolation of glomerular epithelial cells in the rat. We find similar results in the human.

D. Initial Outgrowth, Isolation of the Different Cell Types

The time of appearance and the cellular composition of the first outgrowth are dependent on the culture conditions, including the brand of plastic ware, the presence and type of substrate, and the addition of supplements. In addition, the interspecies differences appear to be substantial (Striker and Striker, 1985).

Conditions for culturing glomerular cells are far from standard. Although there has been some agreement that in the rat, early cultures tend to contain predominantly epithelium whereas late cultures are more rich in mesangial cells, few investigators have developed well-defined lines allowing definitive conclusions about normal cell functions.

Castellot *et al.* (1985) pointed out that mesangial cell proliferation is inhibited by the heparin species produced by epithelial cells. It is likely that this interaction affects cell functions other than proliferation. Therefore, even a minimal contamination by epithelial cells of a mesangial cell preparation may trigger a substantial shift in their behavior. This has not yet been investigated, but it may explain some of the discrepancies found in the literature.

It is also clear that the isolation of pure populations of cells through the selective toxic effects of certain drugs is restricted to certain species. Mitomycin C is not toxic for mouse or human mesangial cells in our laboratory, whereas it has been used by Mahieu and others (1980) to kill mesangial cells selectively. The same applies to the aminonucleoside of

Puromycin, a well-known toxin for rat epithelium (Fishman and Karnovsky, 1985; Foidart *et al.*, 1980b; Kreisberg *et al.*, 1978; Kreisberg and Karnovsky, 1983), which has failed to be selectively cytotoxic in humans, pigs, and mice (Norgaard, 1983). Finally, the recent finding of Oberley *et al.* (1982) that serum-free medium only allows the growth of epithelial cells in guinea pigs needs to be confirmed and investigated in other species.

The discrepancy between *in vivo* and *in vitro* data on the localization of atrial natriuretic factor (Ballerman *et al.*, 1985; Bianchi *et al.*, 1986) raises the questions of modulation of cell behavior *in vitro*, the need to test multiple cell isolates *in vitro*, and the purity of cell populations. Similar questions are raised by the discrepancies we have noted in prostaglandin (PG) synthesis between rats and humans by what appeared to be morphologically similar populations of contractile mesangial cells (Ardaillou *et al.*, 1983). Finally, the considerable controversy about the phagocytic capability of rat mesangial cells which exists may be based on the same issues (Baud *et al.*, 1983, 1986; Schreiner and Unanue, 1984; Striker *et al.*, 1979). Thus the effect of *in vitro* conditions on each cell type between and within species must be fully considered and ultimately related to and confirmed by *in vivo* observations. These data bring into sharp focus the need to work with defined cell populations derived from single-cell clones.

E. Epithelial Cells

1. *General Observations*

Epithelial cells may be isolated from glomeruli by collagenase digestion in relatively pure populations (Killen and Striker, 1979). However, the yield of cells is very low and this technique has been abandoned in our laboratory. It has been suggested that human cells obtained in this way have a proliferative response to added leukotriene B_4 (LTB_4) and can be isolated in very large numbers (Baud *et al.*, 1985). Epithelial cells have never been previously obtained in large numbers from humans; therefore, this observation should be pursued. We obtain epithelial cells by dilute-plating the first outgrowth. Individual colonies are isolated with a cloning ring and transferred. The resultant cells are brought to confluence and repassaged, resulting in a partially purified population of normal cells.

2. *Identification*

These large cells (diameters between 50 and 150 μm) form a monolayer of cuboidal cells which may have more than one nucleus. They form many shallow domes after confluence is reached. After several passages they became larger and appeared senescent.

Epithelial cells retain a macromolecular antigen which is associated with podocyte cell surface polyanions *in vivo* (Hancock and Atkins, 1983). Antibodies to actin reveal a dense array of microfilaments, visible mainly at the periphery of the cells. Cytokeratin is present in large amounts, with a characteristic array of evenly distributed regular filaments which stretch from the periphery of the nucleus to the cell membrane. It should be noted that antibody to cytokeratin recognizes few podocytes *in vivo* (Striker and Striker, 1985).

The fact that podocytes express cytokeratin in cultures argues that this may be a normal physiological response. This may be of significance for disease processes, since the presence or absence of cytokeratin in cells of glomerular crescents has been used as a means of determining whether podocytes could be involved (Magil, 1985). The *in vitro* data draw into question the conclusions based on this type of data.

Transmission electron microscopy of rat cells reveals a monolayer with large, sometimes multilobed nuclei. The remaining cytoplasm contains very few microfilaments near the plasma membrane and no filament bundles. Adjacent cell processes frequently interdigitate, and the cell junctions are composed of electron-dense condensations of bundles of filaments resembling desmosomes (Killen and Striker, 1979). The cell surface in contact with medium has occasional cilia and a small number of microvilli.

Another feature of podocytes which is retained *in vitro* and may be useful in their identification are the receptors for C3b using sheep erythrocytes (Gelfand *et al.*, 1975; Killen and Striker, 1979). This has been found in both humans and rats (Kasinath *et al.*, 1985). Depending on culture conditions, the cells may lose their C3b receptors (Foidart *et al.*, 1979; Scheinman *et al.*, 1978b). They also express a glycoprotein called Heymann antigen (Camussi *et al.*, 1985). The renal epithelial (Heymann) antigens were demonstrated using polyclonal antibodies produced by immunization with membrane vesicles prepared from proximal tubule brush borders and polyclonal rabbit and monoclonal mouse antibodies to a rat proximal tubule membrane glycoprotein (gp 330).

3. *Podocyte Surface Molecules*

Some of the most exciting work *in vitro* on disease processes concerns the epithelial cell glycoproteins of the outer plasmalemmal membrane, especially those designated as gp 330. The molecules appear to be identical to those in the brush border of the proximal tubule. Significantly, the epithelial cell responds to anti-gp 330 antibody by a process consisting of aggregation of the randomly dispersed molecules to form a structure resembling capping (Camussi *et al.*, 1986). The aggregates are then shed. Since this rat model is considered to resemble closely membranous

nephropathy in humans, these observations may be an important clue to the genesis of the subepithelial aggregates of IgG found in membranous glomerulonephritis.

As noted above, C3b receptors are present on podocytes, but there is little information on their biological significance. The interaction of immune complexes with intact glomeruli has been the subject of several communications (Lavelle, 1982, 1985; Lavelle *et al.*, 1984). These observations are unexplained, but it has been reported that Fc receptors may be present on podocytes *in vitro* (Mancilla-Jimenez *et al.*, 1984).

4. *Proliferation*

Epithelial cell proliferation is thought to be important in the genesis of crescents *in vivo* (Magil, 1985). Several groups have shown that macrophages are an important component of the total number of cells in the crescents of certain types of experimental and clinical diseases (Atkins *et al.*, 1976, 1980; Becker *et al.*, 1982). However, the relation, if any, between these macrophages and epithelial cell proliferation was not clear, since the supernatants from macrophages did not affect epithelial cell growth (Melcion *et al.*, 1982). However, one group has shown that leukotriene C_4, LTC_4 (Baud *et al.*, 1985) induces proliferation of podocytes in cultures derived from human glomeruli. If confirmed, this may be very important, since most others have found a low turnover rate even during early outgrowth conditions (Norgaard, 1983). In addition, since macrophages are a rich source of LTC_4 after stimulation, this observation may help explain the proliferation of epithelial cells in inflammatory glomerulonephritides. Another macrophage product, interleukin 1 (IL-1), does not lead to an increase in epithelial cell turnover (Melcion *et al.*, 1982).

5. *Lipoxygenase and Cyclooxygenase Products*

Human and rat glomerular epithelial cells have been studied. Cyclooxygenase products have not been found in human epithelial cells although in early outgrowing cells from rat glomeruli, PGE_2 has been identified (Ardaillou *et al.*, 1983; Petrulis *et al.*, 1981). In addition, TxA_2 was released after stimulation with either arachidonic acid or A-23187. In contradiction to these findings, when epithelial cells were isolated in relatively pure populations one investigator found that the principal prostaglandin synthesized and released was PGI_2 (Kreisberg *et al.*, 1982), and at nearly 10 times the rate of a comparable culture of mesangial cells. Prostaglandins I_2 and E_2 in rat podocytes were stimulated by arginine vasopressin, AVP (Lieberthal and Levine, 1984). This effect was inhibited by the calcium channel antagonist, nifedipine.

Lipoxygenase products were also examined in rat glomeruli, and 12-hydroxyeicosatetranoic acid was the only biosynthetic product identified (Jim *et al.*, 1982).

6. *Vasoactive Peptides*

Podocytes have been studied for the presence of binding sites for atrial natriuretic peptides. *In vivo* autoradiographic studies suggest that atrin receptors are present on several glomerular cell types in the rat, including podocytes (Bianchi *et al.*, 1986). However, rat podocytes in culture were not found to have such receptors (Ballerman *et al.*, 1985). This is an area of considerable importance and requires further work.

Finally, podocytes do not have a contractile response to the administration of angiotensin II *in vitro.* This would correlate with the apparent absence of binding sites in this region by autoradiographic studies (Osborne *et al.*, 1975).

7. *Disease Processes*

Visceral epithelial cells have been implicated in several diseases both in humans and in experimental animals. They are known to produce the components of basement membrane, some of which may govern the traffic of macromolecules across the glomerular walls. Of special note is the charge barrier. It is thought to be lost in the so-called minimal-change nephrotic syndrome in humans. Experimental diseases resulting in albuminuria such as aminonucleoside nephrosis are assumed to be mainly governed by epithelial cell integrity and particularly their synthesis of basement membrane components.

The study of ECM synthesis by visceral epithelial cells has been hampered by the difficulty of obtaining replicate cultures of these cells of sufficient number and purity. Pulse-labeled cultures of nearly homogeneous, confluent epithelial cells demonstrate a linear incorporation of amino acids into nondialyzable protein for the first 24 hours in culture (Killen and Striker, 1979). Seventy percent of the total radiolabeled hydroxyproline is found in the medium and the ratio of 3-hydroxyproline to total hydroxyproline is 0.177 ± 0.012, suggesting that the collagenous protein consists in large part of type IV collagen, a finding confirmed by polyacrylamide gel electrophoresis.

One of the components of the ECM which have been postulated to play a role in the glomerular charge barrier is the proteoglycans. Epithelial cells synthesize a heparan sulfate-containing proteoglycan (Foidart *et al.*, 1980c; Striker *et al.*, 1978). A small amount of a chondroitin 4- and chondroitin 6-sulfate is found in the medium. As noted above, a heparinlike material

synthesized by rat glomerular epithelial cells has been shown to have an inhibitory effect on mesangial cell proliferation (Castellot *et al.*, 1985). Epithelial cells were also found to synthesize a protease-sensitive material which stimulated mesangial cell proliferation.

Glycoproteins are another important part of the ECM. Fibronectin is found in both the cell layer and medium (Foidart *et al.*, 1980a,c; Killen and Striker, 1979; Oberley *et al.*, 1979).

F. Endothelial Cells

1. *General Observations*

Putative endothelial cells have been recognized in explants of rats with glomerulonephritis (Dubois *et al.*, 1981) and normal pigs (Norgaard, 1983), but they have only recently been isolated and propagated *in vitro* (G. E. Striker *et al.*, 1984). Endothelial cells were obtained from human glomeruli in complete Waymouth's medium supplemented with 20% fetal bovine serum containing 2 ng/ml of PDGF. The first outgrowth was found to contain a mixed population of cells, and endothelial cells were isolated by dilute-plating followed by cloning. Between 10 and 15% of the cells in the initial outgrowth were endothelial cells under these conditions in both human and mouse cultures.

These glomerular endothelial cells contained angiotensin-converting enzyme activity which increased in amount after confluence was reached. They were positive for factor VIII-related antigen. The distribution and the size of the granule varied widely between cells and between species. Those in mouse endothelial cells have been difficult to visualize as distinct granules, rather appearing as a diffuse haze.

Endothelial cells formed confluent monolayers and only required light trypsinization. They could not be distinguished from glomerular epithelial cells by phase microscopy, but they differed from mesangial cells, which were characteristically elongated or stellate.

They formed a monolayer of cells with tight junctions by electron microscopy. Abundant microtubules and intermediate filaments were present throughout the cytoplasm; however, microfilaments were observed only in the vicinity of the cell surface. Small numbers of microvilli were observed on the surface of the endothelial cells and cilia were either absent or very infrequent.

2. *Disease Processes*

The Schwartzman reaction is characterized by glomerular thrombosis. The role of endotoxin in this reaction has long been suspected. In fact, it

was speculated that there was a direct toxic interaction, since depletion of complement or inflammatory cell depletion had no effect on the response (Raghu *et al.*, 1986). It has been found that glomerular endothelial cells exhibit a dose-dependent detachment in response to endotoxin (Raghu *et al.*, 1986), whereas umbilical vein endothelial cells and mesangial cells were unaffected. These data support a direct, specific effect of endotoxin on human glomerular endothelial cells and may help explain the glomerular thrombotic lesion found in some septic patients.

G. Mesangial Cells

There are at least two functionally distinct subgroups of cell types in the mesangial area; one resembles a macrophage and the second a smooth muscle cell. These two cell types can also be identified *in vitro* and they retain many of their functional characteristics.

1. *Contractile Mesangial Cells*

a. General Observations. These cells are derived from the paraglomerular mesoderm and appear in the glomerulus during development at the same time as endothelial cells (Sariola *et al.*, 1983). The identification of mesangial cells *in vitro* depends on their phase microscopic appearance and the absence of markers characteristic of the other glomerular cell types. Thus elongated, multilayered cells which lack factor VIII-related and cytokeratin antigens (Kreisberg and Karnovsky, 1983; Striker and Striker, 1985; Sterzel *et al.*, 1986) and which contract in response to angiotensin II are presumed to be mesangial in origin. In rats some have sought to exclude contamination with interstitial fibroblasts through using *d*-valine in the medium or restricting analyses until late phases of glomerular culture, at which time all cells, save those from the mesangium, are presumed to have disappeared (Kreisberg *et al.*, 1978). Another feature of this population of elongated cells is that they were not found to incorporate [^{3}H]thymidine in the first 2.5 days of culture, but thereafter they rapidly divided to form complex multilayers (Norgaard, 1983) resembling smooth muscle cells (Ross, 1971). We showed that these cells were derived from all segments of the glomerulus and did not arise solely from adherent segments of afferent or efferent arterioles by collagenase digestion of the tufts followed by dilute-plating (Striker *et al.*, 1980). Each of the isolated segments developed outgrowths of cells morphologically identical to those generally considered to be contractile mesangial cells. This study needs to be repeated to assess other parameters of identification as well as function.

One group has reported that rat mesangial cells bear distinct markers *in vitro* (Yaoita *et al.*, 1985). Their intermediate filaments are recognized by

antibodies against vimentin and desmin, but the cells do not contain cytokeratin. They were also found to contain Thy-1 antigen (Yaoita *et al.*, 1985). Human mesangial cells do not contain vimentin and desmin (unpublished). The distribution of actin in end-to-end long filaments differs from that found in other glomerular cells.

b. Mesangial Cell Tone. An important function of the contractile mesangial cell population is to regulate the perfusion of the glomerulus. This may be accomplished by regulating the patency of afferent–efferent intraglomerular shunts or the entire glomerular vascular volume. Mesangial cells possess receptors for several vasoactive peptides, emphasizing their role in the regulation of glomerular hemodynamics.

The first investigator to study whole glomeruli *in vitro* noted that they had contractile properties (Bernik, 1969). Subsequently, glomerular angiotensin receptors were noted by autoradiography in whole kidney (Osborne *et al.*, 1975) in isolated glomerular preparations, and then they were localized to cells within the glomerular outgrowth that morphologically resembled smooth muscle cells (Foidart *et al.*, 1980e). The contractile response to angiotensin II has more recently been found to be calcium dependent, independent of cyclonucleotide generation, inhibited by saralasin, and dependent on the presence of insulin (Kreisberg, 1982). Rat mesangial cells also respond to AVP, platelet-activating factors (PAf), LTC_4, LTD_4, and histamine, with a contractile response *in vitro* (Petrulis *et al.*, 1981; Schlondorff *et al.*, 1984; Sedor and Abboud, 1985). Despite the fact that the cultures were presumed to be homogeneous, the maximum number of cells responding to angiotensin II did not exceed 35%, even when the cells were replaced on synthetic plastic substrates (Venkatchalam and Kreisberg, 1985). The contractile response was dose dependent, peaked at 10 minutes, became maximal 11 days after the appearance of outgrowing cells from glomeruli, and remained until the cultures were terminated (Venkatachalam and Kreisberg, 1985; Mahieu *et al.*, 1980). Mesangial cell tone has also been shown to be affected by eicosanoids (see below).

c. Eicosanoids and Platelet-Activating Factor (PAf). Prostaglandin synthesis in human mesangial cells was initially reported to be principally 6-keto-$PGF_{1\alpha}$. It has been recently shown that butyrate added to the medium markedly enhances the synthesis of 6-keto-PGF_1 and PGE_2 in mesangial cells (Ardaillou *et al.*, 1985). Others had noted that such large concentrations of butyrate stimulated PG synthesis generally. This synthesis was stimulated by angiotensin II, AVP, PAF, ionophore A-23187, and serotonin (Knauss and Abboud, 1986). Rat mesangial cells were found to synthesize a similar array of prostaglandins, but in contrast to humans, angiotensin II and A-23187 stimulated increased release of PGE_2.

These products were both agonists, although the mechanisms differed (Scharschmidt and Dunn, 1983; Schlondorff *et al.*, 1984; Sraer *et al.*, 1982a,b; Knauss and Abboud, 1986). "Cloned" rat mesangial cells were found to have a PG profile similar to human mesangial cells in that PGI_2 represented 14–15 of the total PGs synthesized by mesangial cells (Kreisberg *et al.*, 1982b; Ardaillou *et al.*, 1985; Uglesity *et al.*, 1983) and that they synthesized only 10% of that produced by epithelial cells. There appears to be a modification of the PG profile in experimental conditions mimicking diabetes in rat mesangial cells (Kreisberg and Patel, 1983). These series of observations underscore the potential for artifacts in the study of heterogeneous populations of cells under varying culture conditions. The only lipoxygenase product thus far demonstrated to be synthesized by rat mesangial cells is 12-hydroxyeicosatetranoic acid (Jim *et al.*, 1982).

Rat mesangial cells have been found to release large amounts of PAF following stimulation with A-23187 (Schlondorff *et al.*, 1986). PAF also induced contraction in these semipurified populations (Ardaillou *et al.*, 1985; Barnett *et al.*, 1986).

d. Extracellular Matrix Synthesis. The mesangial region contains an ECM which antigenically resembles the peripheral basement membrane (L. Striker *et al.*, 1984). Fibrils have been demonstrated in this region, but not interstitial connective tissue antigens. Nonetheless, rat mesangial cells, as well as those from humans, synthesize both interstitial and basement membrane collagens with a predominance of the former (Foidart *et al.*, 1980c; Striker *et al.*, 1976, 1978; Sterzel *et al.*, 1986). This would therefore appear to represent an *in vitro* phenotypic alteration. This biosynthetic pattern resembles that of aortic smooth muscle cells *in vitro* with the exception that mesangial cells synthesized a relatively larger amount of type IV collagen (Foidart *et al.*, 1979; Striker *et al.*, 1978). One report demonstrated that the regions of multilayered cells in long-term cultures contained central aggregates of ECM surrounded by mesangial cells (Sterzel *et al.*, 1986).

The glycosaminoglycans synthesized by both human and rat mesangial cells resembled that from aortic smooth muscle cells *in vitro* (Foidart *et al.*, 1979). Again they reflected synthesis of non-basement membrane types of molecules. Chondroitin 4-sulfate and chondroitin 6-sulfate were the principal glycosaminoglycans synthesized, although there was a small amount of heparan sulfate found in the cell layer. The glycoprotein profile of mesangial cells *in vitro* suggested that both fibronectin and GP-2 were present (Killen and Striker, 1979; Oberley *et al.*, 1979, 1981a).

It has been shown that rat mesangial cells synthesized and secreted a neutral proteinase which was capable of degrading basement membrane

components (Lovett *et al.*, 1983c). It was secreted as a proenzyme, as was found true in other types of smooth muscle cells. It has been shown that rat mesangial cells produced erythropoietin (Kurtz *et al.*, 1983).

An isolated report of the expression of renal tubular epithelial antigen synthesis by rat mesangial cells has not been confirmed (Bertani *et al.*, 1979).

e. Disease Processes. i. General observations. Mesangial cells appear to be involved in a number of diseases. Many glomerular diseases are characterized by an increase in the number of mesangial cells, although it is not known whether the increase in cell number is restricted to contractile mesangial cells or also involves the resident macrophages (Atkins *et al.*, 1980; Thompson *et al.*, 1980). There are few diseases in which it has been documented that macrophages make a significant contribution to mesangial hypercellularity. For the most part, contractile mesangial cells account for the mesangial hypercellularity in nonsclerosing diseases such as minimal-change nephrotic syndrome with IgM deposits, IgA disease, infectious proliferative glomerulonephritis, lupus erythematosus, Schonlein–Henoch purpura, and mixed cryoglobulinemia. In other disorders the mesangial hypercellularity is associated with deposition of matrix material, such as glomerulosclerosis of diabetes mellitus, multiple myeloma, and light-chain systemic deposition. Finally, mesangial cells are likely to be one of the main sources of the proliferating cells in membranoproliferative glomerulonephritis types I and II.

In numerous instances mesangial proliferation is associated with immune deposits in the mesangial region as in IgA disease and systemic lupus erythematosus or other connective tissue disorders. In these diseases it has been debated as to whether the presence of immune reactants was linked to selective phagocytosis of circulating immune complexes or to *in situ* formation. The role of mesangial cells in phagocytizing or excreting circulating particles has therefore remained a central issue in the understanding of many immunologically mediated types of glomerulonephritis. Finally, the mesangial cells may undergo localized cytolysis, known as mesangiolysis. This phenomenon has also been explored *in vitro*. Mesangiolysis can occur in response to cyclosporine, cobra venom, and mitomycin C administration. Mitomycin C has been shown to have direct toxicity for rat mesangial cells *in vitro*, a fact which could explain its *in vivo* action. The effect of fibrinogen degradation products on mesangial cell turnover *in vitro* was investigated because of their potential role in mesangiolysis (Tsumagari and Tanaka, 1984). None of those tested had an effect on cell number save for fraction 2 of the low molecular weight products. This fraction depressed cell turnover and led to ^{51}Cr leakage.

In both human disease and experimental animals, an increase in glomerular pressure has been postulated to be an important cause of progressive

glomerulosclerosis (Hostetter *et al.*, 1981). This has been extensively studied in a subtotal-nephrectomy model and in streptozotocin-induced insulin deficiency in rats (Hostetter *et al.*, 1981; Zatz *et al.*, 1986). In both of these experimental models it has been speculated that the intraglomerular hypertension is directly related to the mesangial cell dysfunction which leads to sclerosis.

ii. Proliferation. The proliferation of mesangial cells *in vitro* has been shown to be affected by inflammatory cell products and medium conditioned by epithelial cells. As noted above, epithelial cells synthesized both proliferative and inhibitory substances (Lovett *et al.*, 1983a; Lovett and Sterzel, 1986). Human mesangial cells have been shown to proliferate equally well in plasma and serum (Melcion *et al.*, 1982); however, rabbits were found to have a proliferative response following exposure to whole-platelet lysates (Nakashima *et al.*, 1980). Crude macrophage supernatants increased mesangial cell proliferation (Melcion *et al.*, 1982; Wagner *et al.*, 1983), and when purified preparations of IL-1 were separately assessed they were found to stimulate cell division in confluent cell layers, whereas no additive affect was noted on cells in log-phase growth (Striker *et al.*, 1978). The data were consistent with the conclusion that cells in log phase were already either maximally stimulated or did not possess receptors. Subsequently rat mesangial cells were shown to produce an autocrine which closely resembled IL-1 physiologically and biochemically (Lovett *et al.*, 1983a,b). This material was released by rat mesangial cells in log-phase growth. Mesangial cell supernatants also were shown to contain a factor which stimulated spleen cell proliferation presumably by inducing macrophages to release IL-1-like activity (MacCarthy *et al.*, 1985). The data on the effect of macrophage products or the proliferation of mouse mesangial cells are not uniform, being shown both to decrease (Ooi *et al.*, 1983) and to increase the proliferative rate (Dubois *et al.*, 1981; Wagner *et al.*, 1983). IL-1 stimulated both protein synthesis and collagen synthesis in dense cultures, but no effect could be demonstrated in log-phase cells, an effect similar to that noted on proliferation (Melcion *et al.*, 1982).

iii. Phagocytosis. The role of the two mesangial cell types in the uptake and disposal of macromolecules from the circulation has been the subject of considerable discussion. This issue will probably not be resolved, *in vitro,* until cloned cell populations become available and one can achieve clear separation of the various populations of bone marrow-derived and contractile cells.

Phagocytic cells in cultures described as consisting of mesangial cells developed an oxidative burst during the uptake of opsonized particles (Bernik, 1969). The oxidative burst could be inhibited by dexamethasone, and the inhibition had no effect on phagocytosis (Baud *et al.*, 1986). The complement membrane attack complex (C5–9) stimulates the release of

O_2^- and H_2O_2 (Adler *et al.*, 1986). Similarly identified cells from some of these laboratories have also been shown to contract in response to angiotensin II and to have a PG profile more characteristic of smooth muscle cells than macrophages. Others have found that the response, in freshly isolated populations of rat cells, to a phagocytic stimulus is limited to a small population of cells which closely resemble monocytes (Schreiner *et al.*, 1981). Nonetheless, many cells, including fibroblasts, can ingest particles *in vitro* (Grinnel, 1980; Subba *et al.*, 1983; Werb and Reynolds, 1974).

2. *Bone Marrow-Derived Mesangial Cells*

The presence of a population of macrophages in the glomerulus was suspected because of the uptake of immune reactants by some of the cells in the mesangial region. However, their identification as being derived from the bone marrow awaited studies using crossed bone marrow exchange between syngeneic mice where a marker was available to distinguish between donor and host. The Chediak–Higashi mouse provided this model, since the lysosomes of bone marrow cells are identifiable by light microscopy because of their large size. Thus, if these cells localized in the mesangial region, when injected into irradiated hosts, and if they phagocytized immune complexes in that site, two conclusions could be made: (1) the mesangium contained a population of bone marrow-derived cells; (2) that population was phagocytic, since their identification depended on the *in vitro* formation of giant lysosomes following uptake of macromolecules. The observations were that in normal mice given syngeneic Chediak–Higashi bone marrow, only those cells in the mesangium which had large lysosomes were found to have ingested dense deposits resembling antigen–antibody complexes (Striker *et al.*, 1979). These data were consistent with the hypothesis that the principal phagocytic cell *in vivo* was a bone marrow-derived phagocyte, rather than the resident, contractile smooth muscle cell. These data were confirmed in irradiated, reconstituted rats (Schreiner and Unanue, 1984).

III. Tubular Cell Culture

A. General Observations

The utilization of cell culture to investigate normal and pathophysiological processes of the renal tubule has increased dramatically in the past decade. This heightened interest is attested to by publication since 1980 of

numerous reviews dealing with this topic (Handler *et al.*, 1980; Horster, 1980a; Sakhrani and Fine, 1983; Taub, 1985; Horster *et al.*, 1985; Salas *et al.*, 1985; Grenier, 1986; Saier *et al.*, 1986; Rodrigues-Boulan, 1986; Lever, 1986; Valentich, 1986; Fine and Sakhrani, 1986; Rabito, 1986a; Wilson, 1986; Horster and Stopp, 1986).

To date, most studies of tubular cell culture have focused on the development of appropriate cell models and on the investigation of normal cell biology. This evolution is anticipated, since the development of model systems and careful elucidation of their normal characteristics is a requirement for the interpretation of studies designed to uncover pathophysiological events. There is, however, a growing body of investigation applying cell culture to study pathophysiology (Wilson *et al.*, 1986), and it can be anticipated that this application, along with the utilization of cell culture to investigate normal function, will burgeon during the next decade.

This section will enumerate the wide variety of issues that have been addressed using tubular cell culture, but will not consider each in exhaustive detail. Rather, it will try to highlight the potential investigative advantages of cell culture, the innovative ways in which this technique has been applied, and also the limitations and pitfalls that must be considered when cell culture is used as an experimental tool.

B. Renal Tubular Cell Culture Models

A variety of established renal cell lines are available and they have been characterized to varying degrees. In addition, primary culture of virtually every segment of the mammalian nephron has proven feasible.

1. *Established Renal Cell Lines*

Table I lists the more commonly employed established cell lines and enumerates some of their defined characteristics.

The two most extensively studied cell lines are the LLC-PK_1 line, derived from the Hampshire pig (Hull *et al.*, 1976), and the MDCK line, derived by Madin and Darby from the cocker spaniel (Gausch *et al.*, 1966). Both of these cell lines possess the major morphological features of renal epithelia, including apical microvilli, tight junctions, and basolateral membrane infoldings, and exhibit vectorial transport of solutes (Gstraunthaler *et al.*, 1985b).

LLC-PK_1 cells predominantly, but not entirely, resemble proximal tubular epithelium. Consistent with a proximal tubular origin, they possess high activities of the brush border enzymes γ-glutamyl transpeptidase, leucine amino peptidase, and alkaline phosphatase (Gstraunthaler *et al.*,

TABLE I

ESTABLISHED RENAL CELL LINES

Cell line	Species	Probable site of origin	Hormone responsiveness[a]				Sodium-coupled transporters	
			PTH	AVP	Calc	Aldo	Glucose	PO_4
$LLC\text{-}PK_1$	Pig	Proximal tubule	−	+	+		+	+
JTC-12	Monkey	Proximal tubule	+	−	−		+	+
OK	Opossum	Proximal tubule	+	−	+		+	+
MDCK	Dog	Distal tubule	−	+	−	?	−	
A_6	Toad	Collecting duct		+		+		
BSC-1	Monkey	?						

[a] Aldo, Aldosterone; AVP, arginine vasopressin; Calc, calcitonin; PTH, parathyroid hormone.

1985b), exhibit both sodium-dependent glucose and phosphate transport (Rabito, 1986a), and manifest pH-modulated ammonia production (Cole *et al.*, 1986). Their hormone profile, however, does not resemble proximal tubular epithelium and is more compatible with a thick ascending limb origin. Vasopressin and calcitonin both stimulate adenylate cyclase, while these cells are unresponsive to parathyroid hormone (PTH) (Meier *et al.*, 1985).

MDCK cells have lower brush border enzyme activities and higher activity of Na^+, K^+-ATPase than $LLC\text{-}PK_1$ cells, consistent with a distal tubular origin (Gstraunthaler *et al.*, 1985b). They do not exhibit Na-dependent glucose uptake (Rindler *et al.*, 1979a), or pH-regulatable ammoniagenesis (R. L. Tannen, unpublished observation). They have the capacity to acidify or alkalinize the solution bathing the apical membrane (Husted *et al.*, 1986). Monoclonal antibodies prepared against MDCK cells localize to the thick ascending limb and distal convoluted tubule of the dog (Herzlinger *et al.*, 1982). Finally, their hormone profile reflects a distal tubular origin, with a cyclic adenosine monophosphate (cAMP) response to vasopressin and not to PTH or calcitonin (Meier and Insel, 1985). The responsiveness of this cell line to aldosterone has not been clarified definitively (Meier and Insel, 1985).

Two other cell lines have been studied less extensively than $LLC\text{-}PK_1$, but also appear to be predominantly of proximal tubule origin. The JTC-12 line derived from the cynomolgus monkey exhibits sodium-dependent glucose and phosphate transport and responsiveness to PTH, but not vasopressin or calcitonin (Malstrom and Murer, 1986; Takuwa and Etsuro,

1985; Ishizuka *et al.*, 1978). The OK cell line, derived from the kidney of the American opossum (Koyama *et al.*, 1978), also transports phosphate and glucose in a sodium-dependent fashion and responds to PTH and calcitonin, but not to vasopressin (Malstrom and Murer, 1986; Pollock *et al.*, 1986). Of interest, this is the only cell line in which PTH increases cAMP levels and concurrently inhibits phosphate transport (Malstrom and Murer, 1986).

The A_6 cell line, derived from the kidney of the South African clawed toad (Rafferty, 1969), resembles collecting duct epithelium. These cells exhibit a high transepithelial electrical resistance ($\sim$5000 ω/cm^2) (Perkins and Handler, 1981), respond to aldosterone with an increase in short-circuit current (SCC) (Perkins and Handler, 1981; Handler *et al.*, 1981), and increase cAMP levels in response to vasopressin, but only when grown on porous collagen-coated filters (Handler *et al.*, 1981; Lang *et al.*, 1986).

It is important to appreciate that these established cell lines are not clonally derived and therefore do not represent a single cell type. For example, LLC-PK_1 cell clones have been isolated which differ in their capacity to form domes and in the response of plasminogen activator synthesis to calcitonin (Wohlwend *et al.*, 1986a). Sublines of MDCK cells have been identified with different PG metabolism, electrophysiological profiles (Husted *et al.*, 1986), responses to chemical inducers of differentiation (Lever, 1986), ultrastructural characteristics, and antigenic markers (Salas *et al.*, 1985; Rodrigues-Boulan, 1986; Valentich, 1981; Richardson *et al.*, 1981). While the existence of more than one cell type can potentially confound interpretation of the data, it also can be used to experimental advantage when clones with different characteristics are isolated. For example, LLC-PK_1 clones which do not form domes and also exhibit low transepithelial electrical resistance might be advantageous to elucidate the mechanisms underlying dome formation (Wohlwend *et al.*, 1986). Similarly, MDCK cells with differences in PG synthesis, differentiation, and electrical resistance might be useful models to generate new insights on hormone action, transport, and cell biology (Meier and Insel, 1985).

An advantage of established cell lines is the ease with which large amounts of cells can be obtained for experimentation. Disadvantages, in addition to the existence of multiple cell types, include the lack of expression of the complete phenotype of the cell of origin and the potential for alterations in the phenotypic pattern after multiple passages. To overcome these limitations, it is recommended that cells be frozen and studied for only a defined number of passages. However, even this approach can be problematic, since storage might alter the phenotypic profile (Husted *et al.*, 1986). Furthermore, extrapolation to the *in vivo* setting must be undertaken cautiously, since an absent or unanticipated

response could reflect a phenotypic modification in the culture system. On the other hand, such phenotypic changes can also be used to advantage toward understanding basic cell biology better.

2. *Primary Cell Cultures*

In order to minimize the alterations in phenotypic expression inherent in established cell lines and to develop models of cellular components from specific regions of the nephron, considerable effort has been expended to develop primary renal tubular cell cultures. As detailed in Table II, virtually every segment of the nephron has been cultured successfully from a variety of mammalian species, including humans.

One critical caveat is the criteria used to validate the specific cell type that has been cultured. With isolated cells the standard identification techniques employed include morphology, antigenic profile, enzymatic profile, metabolic characteristics, hormonal responsiveness, transport characteristics, and other nephron-specific characteristics. These same criteria are applicable to cultured cells; however, because the culture conditions may not permit full expression of the mature cellular phenotype, the results can be misleading. Morphological characteristics, antigenic profiles, enzymatic profiles, metabolic characteristics, hormone responses, and transport characteristics can all be modified in cultured cells (Grenier, 1986; Fine and Sakhrani, 1986, Burg *et al.*, 1982; Chung *et al.*, 1982; Wilson *et al.*, 1985; Spielman *et al.*, 1986; Tang and Tannen, 1986). To overcome this potential pitfall, as many parameters as possible should be assessed to determine the origin of cells grown in primary culture.

The most direct fashion to attain cultured cells from specific portions of the nephron is to initiate the culture with defined, microdissected segments of the nephron. To demonstrate the feasibility of this approach, Horster initially carried out painstaking studies to identify the optimal hormonal, nutritional, and substratum requirements for optimal growth of selected nephron segments (Horster, 1979, 1980b). As indicated in Table II, successful culture of microdissected proximal tubule, cortical and medullary thick ascending limb, and cortical and medullary collecting tubule has been carried out in rabbits, mice, and humans (Burg *et al.*, 1982; Wilson *et al.*, 1985; Horster, 1980b; Currie *et al.*, 1983; Green *et al.*, 1985; Valentich and Stokols, 1986a,b; Wilson and Horster, 1983). The major limitation to this approach is the quantity of material that can be obtained for experimental manipulation. However, recent studies with medullary thick ascending limb cells indicate that it may be feasible to circumvent this issue by establishing permanent cell lines from microdissected material. Rabbit medullary thick ascending limb cells grown on amnion supports have been sustained in

culture beyond 12 passages for more than 2 years and have retained some evidence of differentiated function as reflected by the presence of Tamm–Horsfall antigen and the expression of a lumen positive transepithelial voltage (Green *et al.*, 1985). A continuous epithelial cell line sustained for more than 3 years has also been established from microdissected mouse medullary thick ascending limbs (Valentich and Stokols, 1986a,b). The cells were cultured on a collagen gel substratum and passaged without the use of proteolytic enzymes or cell dispersion by transferring out pieces of the collagen gel. These cells exhibit Tamm–Horsfall antigen, but developed an apical positive transepithelial voltage sensitive to furosemide and chloride removal only after transplantation in a diffusion chamber into allogeneic mice (Valentich and Stokols, 1986a,b). Thus, the cells retain the capacity to express their *in vivo* phenotype, but the culture conditions required to manifest this property have not been identified. Nevertheless, these studies with medullary thick ascending limbs suggest that it should be possible to develop permanent, differentiated cell lines from defined segments of the nephron.

The limitations of cellular material available with cultures from microdissected tubule segments has led to the development of macroseparation techniques for the establishment of primary cell cultures. Rigorous cellular characterization is particularly important in this instance, since the starting material is never entirely from the desired nephron segment. Several innovative approaches have led to the success of this undertaking, and the nephron segments currently amenable to macroculture are catalogued in Table II.

Taub and co-workers employed a combination of tubule separation procedures and use of hormone-supplemented serum-free medium designed to optimize proximal tubule growth to establish primary cell cultures of rabbit proximal tubules (Chung *et al.*, 1982). Modifications of this technique have been utilized successfully in other laboratories for dog as well as rabbit proximal tubules (Grenier, 1986; Fine and Sakhrani, 1986; Tang and Tannen, 1986; Yau *et al.*, 1985; Sakhrani *et al.*, 1984, 1985; Waqar *et al.*, 1985; Hruska *et al.*, 1986).

Smith and co-workers pioneered the use of monoclonal antibodies directed at nephron-specific antigenic sites to isolate and culture selected portions of the tubule (Spielman *et al.*, 1986; Garcia-Perez and Smith, 1983, 1984; Smith and Garcia-Perez, 1985). To date this technique of "immunodissection" has been employed to culture dog and rabbit cortical collecting tubules (Garcia-Perez and Smith, 1983, 1984; Spielman *et al.*, 1986) and later, rat proximal tubules (Harris *et al.*, 1986).

Finally, the unique characteristic of papillary collecting duct cells to resist hypotonic lysis has been utilized to isolate these cells for subsequent

TABLE II

PRIMARY CULTURES OF NEPHRON SEGMENTS

Nephron segment	Species	Hormone profile			Transport			Other characteristics
		PTH	AVP	Calc	Na–Glucose	$Na–PO_4$	Other	
I. Culture established with microdissected segments								
Proximal tubule	Rabbit							
	Human	+	−					Brush border enzymes (gamma GT, alkaline phos)
Cortical thick ascending limb	Rabbit	+	+	++				Na^+,K^+-ATPase
	Human	−	−					Na^+,K^+-ATPase
Medullary thick ascending limb	Rabbit						Furosemide-sensitive lumen + PD	Tamm–Horsfall antigen
	Mouse						Furosemide-sensitive lumen + PD	Tamm–Horsfall antigen

Cortical collecting tubule	Rabbit	+	++	−			
	Human	−	+				
Medullary collecting tubule	Rabbit						
II. Cultures established with macroseparation techniques							
Proximal tubule	Rabbit	+	−	−	+	+	Brush border enzymes (gamma GT, ALK PO_4, LAP)
	Dog	+	−	−	+		
	Chicken				+	+	
	Human						Brush border enzymes (gamma GT, ALK PO_4); glucose-6-phosphatase
Cortical collecting tubule	Rabbit	+	+	−			No succinate dehydrogenase; PGE_2 production with bradykinin
	Dog	−	+	−			No succinate dehydrogenase; PGE_2 production with bradykinin and AVP
Papillary collecting tubule	Rabbit	+	+	−			Bradykinin-modulated phosphatidylinositol turnover and PGE_2 production
	Rat	−	+	+/−			
	Pig						

culture. Rabbit, rat, and pig papillary collecting tubule cells have all been cultured with this approach (Grenier *et al.*, 1981, 1982; Grenier and Smith, 1978; Sato and Dunn, 1984; Shayman and Morrison, 1985; Cuthbert *et al.*, 1985).

Although several groups have reported culturing human proximal tubule cells using either hunks of cortical tissue or separated tubules as the starting material, none of the characterizations have been sufficiently complete to be certain of the cell type (Trifillis *et al.*, 1985; Detrisac *et al.*, 1984; Stater *et al.*, 1986).

3. *Limitations and Pitfalls*

The ability to grow both established cell lines and primary cultures in serum-free, hormonally defined media importantly enhanced the utility of cell culture as a model to investigate normal and pathophysiological phenomena by removing an uncontrolled variable and inhibiting fibroblast growth (Taub, 1985; Taub *et al.*, 1979; Taub and Sato, 1980).

Nevertheless, numerous other factors can influence the phenotypic expression of cells in culture. These include the substratum on which cells are cultured (Land *et al.*, 1986; Green *et al.*, 1985), the access of nutrients to the basal surface (Lang *et al.*, 1986; Valentich and Stokols, 1986a), the availability of sufficient oxygen (Cole *et al.*, 1986), the phase of the growth cycle (Amsler and Cook, 1982), and potentially cell–cell interactions and other factors influencing cell shape (Lever, 1986; Valentich and Stokols, 1986a). A variety of techniques have been used to overcome these limitations. For example, fibronectin, collagen, and amnion membranes have been used as substrata in place of plastic (Green *et al.*, 1985; Valentich and Stokols, 1986a); porous-bottom culture dishes have been developed, which permit nutrient access to the basal surface (Steele *et al.*, 1986); growth on a rocker improves oxygenation and removes the diffusion barrier of unstirred layers (Cole *et al.*, 1986); and culture on a collagen gel substratum has been used to maintain intercellular contacts (Valentich and Stokols, 1986a).

It can be anticipated that continuous progress will be forthcoming to improve differentiated function in cultured cells, but experimental results must be interpreted within the context that cultured cells are not yet precise replicas of their *in vivo* counterparts.

C. Applications of Cell Culture to the Investigation of Normal Function

Cultured cells have been used to investigate transport phenomena, hormonal effects, cell metabolism, and basic cell biology. This section will

consider those parameters that have been examined using cultured cells, but will not focus on the precise experimental findings. Rather, the purpose will be to provide examples of how cell culture can expand the experimental strategies available to yield new insights concerning these processes.

1. *Transport*

Several excellent reviews have considered the current status of transport phenomenon investigated using renal cell culture (Gonzalez-Mariscal *et al.*, 1985; Fernandez-Castelo *et al.*, 1985; Saier, 1985; Mullin and Kleinzeller, 1985; Sepulveda and Pearson, 1985b; Misfeldt and Sanders, 1985; Saier *et al.*, 1986; Rabito, 1986a; Horster and Stopp, 1986). As shown in Table III, the studies have focused on the transport of electrolytes, glucose, phosphate, and amino acids by cultured epithelial cells.

a. Electrolytes. A significant portion of the investigation of electrolyte transport by cultured epithelium has dealt with cellular transport (i.e., membrane transport processes for cellular uptake or extrusion of electrolytes and protons). The specific transport profiles of several established cell lines have been characterized including LLC-PK_1, MDCK, and A_6 cells (see review by Saier in Saier *et al.*, 1986). To date, studies have been directed at, and defined in various cell types, the Na–K pump (Na^+,K^+-ATPase) (Mills *et al.*, 1979); the sodium-proton antiporter (Sakhrani *et al.*, 1985; Rindler *et al.*, 1979b; Haggerty *et al.*, 1985; Kleinman *et al.*, 1986; Harris *et al.*, 1987); the Na–K–Cl cotransporter (Saier *et al.*, 1986; Palmer, 1986; Amsler and Kinne, 1986); and an apical membrane, amiloride-sensitive sodium channel (Palmer, 1986; Sariban-Sohraby *et al.*, 1983). Basolateral K^+-conductive pathways (Husted *et al.*, 1986; Paulmichl *et al.*, 1985) and apical K^+ channels (Palmer, 1986; Guggino *et al.*, 1985) have been defined. An apical Cl channel, as well as a Cl–HCO_3 exchange mechanism, have been delineated (Palmer, 1986; Kolb *et al.*, 1985; Chaillet *et al.*, 1986).

Advantages afforded by cultured cells that have been used to investigate these transport processes include the ability to impale single cells with

TABLE III

TRANSPORT BY CULTURED RENAL EPITHELIUM

Electrolytes	
Na^+	(Na/H antiporter, Na channel, Na–K–Cl symport, Na,K-ATPase)
K^+	(K^+ channel)
Cl^-	(Cl channel, Cl–HCO_3 exchanger)
H^+	(H^+ pump, Na–H antiporter)
Glucose	
Phosphate	
Amino acids	

intracellular electrodes (Husted *et al.*, 1986; Paulmichl *et al.*, 1985), access of the apical membrane to patch–clamp studies of specific ion channels (Palmer, 1986; Kolb *et al.*, 1985; Guggino *et al.*, 1985), and the capacity to obtain membrane vesicles from a defined cell population. Potentially more powerful aspects of the technology include the ability to delineate how alterations in the intracellular environment, such as changes in intracellular calcium, modify transport processes (Sakhrani *et al.*, 1985; Taub *et al.*, 1979). The identification of mutants, which lack a specific transport process, should be particularly advantageous for delineating the functional importance of a given transport process. For example, McRoberts and co-workers have identified a mutant MDCK cell with a defect in the Na–K–Cl symport (McRoberts *et al.*, 1983). Apparently, this mutant grows well in low-K^+ media because the Na–K–Cl symport acts primarily as a mechanism to extrude K^+ under these conditions (Saier and Boyden, 1984).

The formation of domes (hemicysts, blisters) by epithelia cells grown on plastic provided the initial evidence for transepithelial transport of solutes and water (Lever, 1986) and micropuncture analysis of the fluid content of domes has been used to explore transepithelial transport processes (Fernandez-Castelo *et al.*, 1985; Lifschitz, 1986). However, the utility of cultured epithelium to investigate vectorial transport was enhanced significantly by the ability to mount epithelial monolayers grown on porous supports, such as collagen-coated nylon mesh or millipore filters, in Ussing chambers, and to measure transepithelial flux (Steele *et al.*, 1986; Fernandez-Castelo *et al.*, 1985; Misfeldt *et al.*, 1976). A by-product of this technology has been the capacity to examine the sidedness of various responses, as, for example, hormone action (Steele *et al.*, 1986).

Short-circuit current and isotopic flux have been used to investigate transepithelial sodium transport by a variety of established cell lines and primary cultures (Horster and Stopp, 1986; Perkins and Handler, 1981; Handler *et al.*, 1981; Burg *et al.*, 1982; Green *et al.*, 1985; Valentich and Stokols, 1986a,b). Transepithelial proton and bicarbonate flux and chloride secretion have also been assessed (Horster and Stopp, 1986; Husted *et al.*, 1986). To date, vectorial transport studies have served mainly to characterize the properties of established and primary cell lines; however, as discussed subsequently, more recent studies have examined the coupling between hormone action and transport events. One innovative application has been the investigation of the function of tight junctions. Alterations in electrical resistance and ionic permeability of MDCK monolayers were determined in response to disruption and resealing of occluding junctions by modifications in extracellular calcium (Cereijido *et al.*, 1981).

b. Glucose. Sodium-dependent glucose uptake has been demonstrated in several established cell lines with proximal tubular characteristics and also

in primary cultures of proximal tubular cells (Rabito, 1986a; Malstrom and Murer, 1986; Takuwa and Esturo, 1985; Chung *et al.*, 1982; Sakhrani *et al.*, 1984; Hruska *et al.*, 1986). Regulation of the glucose transport has been investigated most extensively in LLC-PK_1 cells (Rabito, 1986a; Mullin and Kleinzeller, 1985; Misfeldt and Sanders, 1985). Both the cellular uptake process, utilizing the nonmetabolizable probe α-methyl-D-glucoside, and transepithelial transport of D-glucose have been examined (Rabito, 1986a; Mullin and Kleinzeller, 1985; Misfeldt and Sanders, 1985). As reviewed by Misfeldt and Sanders, the apical membrane polarity, stoichiometry, and the bioenergetics of sodium-coupled glucose transport have been delineated in cultured cells (Misfeldt and Sanders, 1985). Other studies, focused on the cell biology of sodium-dependent glucose transporters, provide a good example of the experimental strategies that can be employed with cultured cells. Expression of this transport process correlates with the development of cellular differentiation (Amsler and Cook, 1982). Furthermore, the capacity of the transporter is dictated by the ambient glucose environment. Studies with intact cells as well as apical membrane vesicles indicate that a high glucose concentration down-regulates the number of transporters expressed in the cell membrane (Moran *et al.*, 1983, 1984; Handler and Moran, 1985). Down-regulation of the transport capacity was unaltered when ionizing radiation was used to inhibit cell replication, but up-regulation was strongly impaired (Moran *et al.*, 1986). Thus, radiation appears to interfere with the gene expression required to increase the number of transporters when the cells are transferred from a high- to low-glucose environment.

c. Phosphate. Sodium-dependent phosphate transport has been found in all the established cell lines with proximal characteristics as well as in primary cultures of proximal tubular epithelium (Rabito, 1986a; Malstrom and Murer, 1986; Takuwa and Etsuro, 1985; Waqar *et al.*, 1985; Hruska *et al.*, 1986; Sakhrani *et al.*, 1985; Rabito, 1983; Caverzasio *et al.*, 1986). The apical polarity of this process has been demonstrated with cultured cells grown on collagen-coated nucleopore membranes and confirmed with apical membrane vesicles from LLC-PK_1 cells (Rabito, 1983; Brown *et al.*, 1983). The stoichiometry of sodium–phosphate transport as well as the pH characteristics of the transport process have been examined (Takuwa and Etsuro, 1985; Waqar *et al.*, 1985; Rabito, 1983; Brown *et al.*, 1983; Biber *et al.*, 1983).

The different responses of cultured cells to PTH may be advantageous to dissect the mechanisms, whereby the hormone impedes sodium-dependent phosphate transport. In JTC-12 cells PTH increases cAMP levels but has no effect on phosphate transport (Malstrom and Murer, 1986; Takuwa and Etsuro, 1985); in LLC-PK_1 cells the increase in cAMP is either minimal or

absent and the effects on phosphate transport are controversial (Malstrom and Murer, 1986; Caverzasio *et al.*, 1986), whereas in OK cells cAMP is PTH responsive and phosphate transport is inhibited (Caverzasio *et al.*, 1986). Forskolin-induced increases in cAMP also inhibit phosphate transport in OK cells (Caverzasio *et al.*, 1986) but not in LLC-PK_1 or JTC-12 cells (Malstrom and Murer, 1986).

Cell culture has also been used to examine the adaptation of the phosphate transporter. Phosphate deprivation in the media for only 10 minutes increases the activity of sodium-dependent phosphate transport. This activation is not inhibited by cycloheximide, but the K_m value for transport in apical vesicles is unchanged, suggesting that it may be accounted for by exocytosis of transporters into the apical membrane (Biber and Murer, 1985). More prolonged phosphate deprivation also modifies the transporter, but this process is cycloheximide dependent. Thus, protein synthesis appears to be required for this more chronic adaptive process (Takuwa *et al.*, 1986; Coverzasio *et al.*, 1985). Finally, glucocorticoids inhibit sodium-dependent phosphate transport in primary cultures of chick renal cells, and studies with inhibitors indicate that this process is dependent on RNA and protein synthesis (Noronha-Blob and Sacktor, 1986). Cultured cells should be invaluable for dissection of the molecular and genetic mechanisms involved in these adaptive and hormone-mediated process.

d. Amino Acids. Both transepithelial and cellular accumulation of amino acids have been investigated extensively in both LLC-PK_1 and MDCK cells (Saier *et al.*, 1986; Rabito, 1986a; Sepulveda and Pearson, 1985b). The predominant transport mechanisms identified have been localized to the basolateral membrane in both cell types (Saier *et al.*, 1986; Rabito, 1986a; Sepulveda and Pearson, 1985a,b; Misfeldt *et al.*, 1976); however, sodium-dependent amino acid transport has also been localized to the apical membrane using cells grown on permeable supports as well as apical membrane vesicles (Lever *et al.*, 1984; Rabito and Karish, 1983). The transport capacity of the A system, reflected by transport of the nonmetabolizable marker 2-methylaminoisobutyric acid (Me AIB) is up-regulated in both LLC-PK_1 and MDCK cells by amino acid deprivation, and down-regulated when the cells progress from the exponential growth phase to confluence (Saier *et al.*, 1986; Rabito, 1986a; Sepulveda and Pearson, 1985; Lever *et al.*, 1984). These adaptive modifications are subject to hormonal regulation (Boerner and Saier, 1985b). An oncogenic transformed subline of MDCK cells exhibits high basal activity and does not express up-regulation of amino acid transport by the A system in response to an amino acid-deficient medium (Saier *et al.*, 1986; Boerner and Saier, 1985b). This represents an example of how the induction of mutants can be

used as strategy to delineate the biology of regulatable transport functions.

Thus, cultured cells manifest most of the transport properties of the intact kidney; they are uniquely suited to dissection by micromethodology and biochemical techniques; they undergo adaptive modifications and responses to hormonal stimuli; and the different transport characteristics of various cell lines, naturally occurring mutants, and oncogenically transformed mutants can be used to experimental advantage. In addition, cultured cells are particularly suited for studies directed at the genetic regulation of transport processes. There are just some of the features that will be exploited to delineate further the biology of transport processes.

2. *Hormones and Effectors*

Isolated cellular systems are ideal for investigating the biology of hormone and other effector actions at the receptor, intracellular messenger, and response level. Thus, it is not surprising that a rigorous search has ensued for cultured epithelium with hormonal responsiveness and that these systems have been exploited to further the understanding of hormonal action.

A major advance for the study of hormone action in cultured epithelium was the ability to grow cells in serum-free, hormonally defined media (Taub, 1985). This removed the unknown variable that constituents of serum provided. However, cells grown in a particular hormonal environment might modulate their response to those hormones included in and absent from the growth media, so that the data must be evaluated with this caveat in mind (Roy and Ausiello, 1981; Roy *et al.*, 1981; Taub, 1985).

Table IV provides a list of the hormones that have been studied using cultured renal epithelial cells. This has been the subject of several recent reviews (Horster and Stopp, 1986; Meier and Insel, 1985).

a. Parathyroid Hormone. As indicated previously, both the OK and JTC-12 cell lines exhibited an increase in cAMP in response to PTH, but the response in LLC-PK_1 cells is either minimal or absent (Rabito, 1983, 1986a; Malstrom and Murer, 1986; Takuwa and Etsuro, 1985; Caverzasio *et al.*, 1986). Primary cultures of proximal tubules from rabbit, dog, and human also exhibit a cAMP response to PTH (Chung *et al.*, 1982; Wilson *et al.*, 1985; Hruska *et al.*, 1986; Sakhrani *et al.*, 1985). In OK cells the PTH-mediated increase in cAMP is accompanied by inhibition of sodium-dependent phosphate transport (Malstrom and Murer, 1986; Caverzasio *et al.*, 1986); thus, full expression of the hormonal effect appears to be retained in this cell line. PTH also inhibits sodium-proton antiporter activity in OK cells, and this effect also appears to be mediated by cAMP (Pollock *et al.*, 1986). In primary cultures of dog proximal tubule, PTH

TABLE IV

HORMONES/EFFECTORS INVESTIGATED IN CULTURED RENAL EPITHELIUM

Parathyroid hormone
Vasopressin
Calcitonin
Catecholamines
Angiotensin II
Bradykinin
Prostaglandin
Aldosterone
Insulin
Glucagon
Glucocorticoids
Thyroxin
Atrial natriuretic factor
Endoxin
Adenosine

increases intracellular calcium concentration, a response that is not mimicked by cAMP (Hruska *et al.*, 1986). The increase in intracellular calcium was accompanied by transient shortening and rarefaction of the apical microvilli, and this structural change was abolished by use of a calmodulin inhibitor (Goligorsky *et al.*, 1986c).

b. Vasopressin. Vasopressin-mediated increases in cAMP have been characterized in LLC-PK_1, MDCK, and the A_6 established cell lines. In addition, antidiuretic hormone (ADH) responsiveness has been found in primary cultures of both cortical and medullary collecting tubules from several species (Wilson *et al.*, 1985; Spielman *et al.*, 1986; Wilson and Horster, 1983; Garcia-Perez and Smith, 1983, 1984; Sato and Dunn, 1984).

The mechanisms of the vasopressin hormone-receptor-intracellular messenger-effector response have been investigated most extensively using LLC-PK_1 cells (Meier *et al.*, 1985; Skorecki *et al.*, 1986). The binding of vasopressin to its receptor, the relationship between receptor binding and activation of adenylate cyclase, the enhancement of adenylate cyclase activation by calmodulin, and the increase in cAMP-dependent protein kinase have all been investigated in this model cell system (Roy and Ausiello, 1981; Roy *et al.*, 1981; Ausiello and Hall, 1981; Ausiello *et al.*, 1980). More recently, radiation inactivation has been used to delineate subunit interactions leading to the activation of adenylate cyclase (Skorecki *et al.*, 1986). Vasopressin also increases intracellular calcium concentration in LLC-PK_1 cells (Tang and Tannen, 1986), and in primary cultures of rat

inner medullary collecting tubules, an increase in intracellular calcium increases ADH-mediated cAMP production (Teitelbaum and Berl, 1986). Several studies have demonstrated that the culture conditions employed can modulate the number of vasopressin receptors expressed. In LLC-PK_1 subclones both serum and insulin can increase the number of vasopressin receptors expressed (Roy *et al.*, 1980), and a serum-free hormone supplemental media has been described recently, which increases ADH receptors in this subclone by 20- to 40-fold (Roy, 1985). A_6 cells grown on permeable filters express ADH receptors in contrast to cells grown on plastic (Lang *et al.*, 1986).

Primary canine cortical collecting tubule cells grown on Millipore filters increase cAMP when vasopressin is added to the basolateral but not the apical side. By contrast, AVP stimulates PGE_2 production on either side, suggesting that two classes of AVP receptors may exist (Garcia-Perez and Smith, 1984).

The relationship of ADH to transport has undergone less evaluation in cultured cells. ADH increases SCC when applied to the basal surface of primary cultures of pig renal papillary collecting ducts (Cuthbert *et al.*, 1985). It has been shown that PGE_2 inhibits ADH-modulated vectorial H_2O transport by MDCK cells (Martinez and Reyes, 1984). Finally, using whole chick kidney primary cultures on apical membrane, calcium-activated K^+ channel has been identified, which is stimulated by ADH (Guggino *et al.*, 1985).

c. Calcitonin. Calcitonin-mediated increases in cAMP have been delineated in both the LLC-PK_1 and OK cells, but detailed studies have only been carried out in LLC-PK_1. Using a subclone of LLC-PK_1 cells that lack calcitonin receptors, it has been shown that calcitonin binding to its receptor is required to stimulate adenylate cyclase, and this concurrently stimulates plasminogen activator production (Wohlwend *et al.*, 1985, 1986a). Calcitonin gene-related peptide acts in the same fashion as the native hormone (Wohlwend *et al.*, 1985). The activation of adenylate cyclase stimulates production of a cAMP-dependent protein kinase (Ausiello *et al.*, 1980). LLC-PK_1 mutants with defective cAMP protein kinase exhibited reduced plasminogen activator production in response to calcitonin; however, the response to phorbol esters was normal, suggesting that protein kinase C can also stimulate plasminogen activator (Jans and Hemmings, 1986).

Calcitonin also decreases dome formation (Wohlwend *et al.*, 1986b). This inhibition of dome formation is accompanied by a decrease in transepithelial resistance, suggesting that it may result from an increase in the permeability of the paracellular pathway. Calcitonin also inhibits cell multiplication (Wohlwend *et al.*, 1985).

d. Catecholamines. Adrenergic receptors have been identified in cell lines of both proximal and distal tubule origin including LLC-PK_1, JTC-12, MDCK, and A_6 cell lines as well as in primary cultures of proximal and distal tubules (Meier *et al.*, 1985). The most detailed studies of adrenergic mechanisms have been carried out with the MDCK cell line and with its clones that express both α_1 and β_2 receptors (Meier and Insel, 1985; Meier *et al.*, 1985).

The two adrenergic receptors have been solubilized and characterized biochemically as glycoproteins (Meier and Insel, 1985). The β_2 receptor activates adenylate cyclase, while the α_1 receptor increases PGE_2 production and phosphatidylinositol hydrolysis (Meier and Insel, 1985; Meier *et al.*, 1985). Utilizing the MDCK subclone with α_1 and β_2 receptors, incubation in epinephrine down-regulated both the number of receptors and their respective responses, that is, cAMP and PGE_2 production (Meier and Insel, 1985). Of interest, the loss of β_2 receptors occurred more rapidly than the loss of α_1 receptors.

Epinephrine increases SCC and stimulates chloride secretion by MDCK cells (Richardson *et al.*, 1981; Brown and Simmons, 1981; Kurtz *et al.*, 1986), and when applied to the basolateral membrane of primary cultures of pig papillary collecting duct (Cuthbert *et al.*, 1985). It also stimulates K^+ efflux from the cells, via an α-adrenergic-mediated alteration in a calcium-activated K^+ channel (Brown and Simmons, 1982). α-Adrenergic agonists increase intracellular calcium in primary cultures of dog proximal tubule (Goligorsky *et al.*, 1986a).

e. Angiotensin. The response of MDCK cells to angiotensin II is currently unclear (Meier and Insel, 1985). A recent preliminary report with primary cultures of rabbit proximal tubules found that low concentrations of angiotensin II inhibited cAMP production, while higher concentrations were stimulatory secondary to an increase in PGE_2 production (Douglas *et al.*, 1986).

f. Bradykinin. Bradykinin increases PG synthesis in established cell lines (MDCK) and primary tubule cultures of distal nephron origin (cortical and papillary collecting ducts) from several species (Grenier, 1986; Garcia-Perez and Smith, 1983, 1984; Spielman *et al.*, 1986; Grenier *et al.*, 1981; Shayman and Morrison, 1985; Hassid, 1981, 1983a). With primary cultures of canine cortical collecting tubules, it was found that bradykinin stimulates PGE_2 production only when added to the apical and not the basolateral surface (Garcia-Perez and Smith, 1984). Shayman and Morrison, using rabbit papillary collecting tubule cultures, have shown that bradykinin stimulates phosphatidylinositol turnover and increases intracellular calcium concentration, and that a guanine nucleotide regulatory protein is involved in the response (Shayman and Morrison, 1985, 1986; Shayman *et al.*, 1986).

Bradykinin increases SCC when applied to either side of pig papillary collecting tubule cells, and this response was attenuated by inhibition of PG synthesis (Cuthbert *et al.*, 1985). It also was abolished if SCC was increased first by other maneuvers which increase cAMP. The authors speculated that bradykinin stimulates Cl secretion by a PG or a coupled PG–adenylate cyclase mechanism.

g. Prostaglandins. Although LLC-PK_1 cells do not synthesize PGs (Hassid, 1981), PG biosynthesis has been documented in MDCK cells as well as primary cultures of distal tubular origin. Increased synthesis of PGs by MDCK cells activates adenylate cyclase (Hassid, 1983a), but an increase in cAMP, in turn, appears to inhibit PG biosynthesis (Hassid, 1983b).

Considerable attention has focused on the relationships of PGs to both bradykinin and vasopressin using cultured cells as model systems. The relationships to bradykinin are discussed above. Vasopressin, in contrast to bradykinin, does not stimulate PG production in MDCK cells (Hassid, 1981) or rat and rabbit papillary collecting duct cells (Grenier, 1986; Grenier *et al.*, 1981, 1982). However, both ADH and bradykinin stimulate PG biosynthesis by canine and rat cortical collecting tubule cells in culture (Garcia-Perez and Smith, 1983, 1984; Spielman *et al.*, 1986). PG production is stimulated by the addition of ADH to either the apical or basal surface, whereas the effects of ADH on cAMP production are limited to basolateral application of the hormone, suggesting that two different receptors or receptor-mediated complexes may be involved (Garcia-Perez and Smith, 1984). PGE_2 inhibits the vasopressin-induced increase in cAMP by cultured cortical but not papillary collecting cells (Grenier, 1986; Grenier *et al.*, 1981). However, a hypertonic saline environment increases vasopressin-mediated cAMP formation and diminishes PGE_2 production by rat papillary collecting cells (Sato and Dunn, 1984).

PGE_1 applied to the basolateral cell surface increases SCC in MDCK cells (Richardson *et al.*, 1981). Micropuncture of domes formed by MDCK, but not LLC-PK_1 cells, revealed that the Cl concentration was significantly lower than in the culture medium (Lifschitz, 1986). The gradient was abolished by PG synthetase inhibitors, suggesting that PG-mediated Cl transport accounts for this phenomenon. In A_6 cells PGs stimulate Cl secretion as evidenced by a change in SCC equivalent to the increase in chloride flux from the basolateral to apical side (Keeler and Wong, 1986). Kurtz and co-workers have reported that an increase in cAMP-dependent, furosemide-sensitive Cl secretion by MDCK cells, provided by epinephrine, isoproterenol, or forskolin, increases PGE_2 release (Kurtz *et al.*, 1986). Furthermore, the increase in PGE_2 production appears to be the result of increased NaCl transport, since inhibition of chloride secretion by furosemide or ouabain did not prevent stimulation of adenylate cyclase but

abolished the increase in PGE_2 release. In view of the enhanced O_2 consumption induced by increased NaCl transport, the authors speculated that hypoxia might account for the increase in PG production.

Prostaglandins also stimulate both cell growth and dome formation in MDCK cells, but these effects may be secondary to an increase in cAMP levels (Meier and Insel, 1985).

h. Aldosterone. To date, A_6 cells have been the best model to study the effects of aldosterone (Horster and Stopp, 1986; Meier and Insel, 1985). Aldosterone stimulates SCC, which is equivalent to net sodium flux, in these cells (Perkins and Handler, 1981; Handler *et al.*, 1981). The increase in sodium transport is accompanied by an increase in amiloride-inhibitable sodium uptake across the apical membrane (Handler *et al.*, 1981; Sariban-Sohraby *et al.*, 1983), by an increased number of ouabain-binding sites (Meier and Insel, 1985) and an increase in Na^+,K^+-ATPase activity (Johnson *et al.*, 1986). Aldosterone binding to two sites with different affinities has also been described in A_6 cells (Handler *et al.*, 1981; Meier and Insel, 1985). Methylation of apical membrane vesicles from A_6 cells increased amiloride-inhibitable sodium transport; however, there was no additional increase in transport by vesicles from aldosterone-treated cells (Sariban-Sohraby *et al.*, 1984). These studies suggest that lipid and/or protein methylation may be involved in the action of aldosterone on the apical sodium entry step.

MDCK cells may also respond to aldosterone, but the data are rather preliminary (Meier and Insel, 1985).

Preliminary cultures of rabbit cortical collecting tubule exhibit an increase in transepithelial voltage and in Na^+,K;sup+-ATPase activity when grown in the presence of aldosterone. Aldosterone receptors are also expressed in these cultures (Horster *et al.*, 1985; Horster and Stopp, 1986).

i. Insulin. Insulin is a necessary component of serum-free hormonally defined media for essentially all established and primary renal cell cultures (Taub, 1985). It stimulates growth (Horster and Stopp, 1986; Taub, 1985; Meier and Insel, 1985) and may play a role in differentiation, since it increases the number of vasopressin receptors in LLC-PK_1 cells (Roy *et al.*, 1980). As reviewed by Horster, it also stimulates amino acid uptake (Horster and Stopp, 1986).

In A_6 cells insulin increases SCC, and this effect is additive to the aldosterone-induced response (Johnson *et al.*, 1986). Furthermore, although insulin alone has no effect on Na^+,K^+-ATPase activity. it potentiates the effect of aldosterone on activity of the enzyme.

j. Glucagon. Glucagon stimulates adenylate cyclase in MDCK cells (Meier and Insel, 1985). A virally transformed MDCK clone has been identified in which glucagon responsiveness and the appearance of glucagon

receptors is induced by incubation in butyrate (Meier and Insel, 1985; Lin *et al.*, 1982). The reappearance of glucagon sensitivity is blocked by the glycosylation inhibitor, tunicamycin, suggesting that the receptor is a glycoprotein. A nontransformed MDCK clone which lacks glucagon responsiveness has also been described (Meier and Insel, 1985).

k. Other Hormones. Glucocorticoids and thyroid hormones both function as growth factors and can induce differentiation as reflected by dome formation in MDCK cells and Na^+,K^+-ATPase activity in primary cultures, but detailed studies of their actions in renal cells have not been reported (Horster *et al.*, 1985; Horster and Stopp, 1986; Horster, 1980b; Taub, 1985). Atrial natriuretic factor decreases SCC, sodium flux, and transepithelial voltage in LLC-PK_1 cells (Goligorsky *et al.*, 1986d). This effect was replicated by dibutyryl cGMP and reversed by cAMP. The putative natriuretic factor, produced in the hypothalamus, which inhibits Na^+,K^+-ATPase, inhibits the Na–K pump in LLC-PK_1 cells (Haupert *et al.*, 1986). Finally, adenosine has been found to stimulate adenylate cyclase and to increase SCC in A_6 cells (Lang *et al.*, 1985). By contrast, activators of protein kinase C inhibit SCC by inhibiting the amiloride-sensitive sodium channel (Yanase and Handler, 1986).

Thus, cultured cells are useful models to unravel the cell biology of hormone action at the receptor, intracellular mediators, and effector steps. Cells from specific portions of the nephron can be studied and the sidedness of hormone–receptor interactions can be distinguished. The interactions between hormones and the effects of hormones on transport processes can be delineated. The use of various cell lines, clones, and mutants with different receptor and mediator profiles provides powerful models for dissecting the cell biology of hormone action at the nephron level. Finally, cultured cells should be excellent models for determining the genetic regulation of these processes.

3. *Metabolism*

a. Glucose. Relatively few studies have been carried out concerning the metabolism of renal cells in culture. Most cells in culture depend on glycolysis for energy metabolism, and renal tubular epithelium also appears to behave in this fashion. In a detailed study of the enzymatic profiles of LLC-PK_1 and MDCK cells, Gstraunthaler *et al.* found that the activity of the glycolytic enzymes hexokinase, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase was equivalent in both cell lines (Gstraunthaler *et al.*, 1985b). This contrasts with the predominance of gluconeogenic enzymes in proximal tubules and of glycolytic enzymes in distal tubules *in vivo*. The hexose monophosphate shunt enzyme, glucose-6-phosphate dehydroge-

nase, was substantially higher in MDCK cells. When lactate is used as substrate, LLC-PK_1 cells show no evidence for gluconeogenesis (Mullin *et al.*, 1982). Although measurable amounts of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase have been found in LLC-PK_1 cells, fructose-1,6-bisphosphatase (FBPase) is undetectable (Gstraunthaler *et al.*, 1985a). In contrast to these observations, however, a recent preliminary report indicates that a strain of LLC-PK_1 cells has been isolated that contains FBPase activity, and can be grown in glucose- and nucleoside-free media. These cells die when the PEPCK inhibitor mercaptopicolinate is added to the medium, suggesting that they have the capacity for glucose production (Gstraunthaler and Handler, 1985). Although aerobic metabolism can support transport by LLC-PK_1 cells, maximal rates of transport appear to depend on glycolysis for the provision of ATP (Misfeldt *et al.*, 1985).

Utilizing [1-^{14}C]glucose and [6-^{14}C]glucose, primary cultures of rabbit proximal tubules exhibit the capacity to metabolize glucose aerobically and also by the hexose monophosphate shung (Sakhrani *et al.*, 1984). However, studies of net glucose metabolism in our laboratory indicate that, although aerobic metabolism is present, the predominant end product of glucose metabolism is lactate (Tang and Tannen, 1986). Furthermore, when we examined glucose metabolism under conditions where cultures were adequately oxygenated, glycolytic metabolism still predominated. Thus, this metabolic profile is not secondary to the hypoxic conditions that exist with standard culture under layers of still media (McLimans *et al.*, 1968a,b).

Of interest, a recent preliminary report suggests that primary cultures of dog proximal tubule exhibit gluconeogenesis (Goligorsky *et al.*, 1986b). It is not clear what accounts for the difference between canine and rabbit primary cultures.

b. Ammonia. Glutamine is an important substrate for growth in cultured cells (Moreadith and Lehninger, 1984). Recently, glutamine-dependent ammonia formation has been evaluated in cultured cells. Glutamine-dependent ammoniagenesis has been reported in primary cultures of rat papillary collecting duct; however, there was no modulation in response to pH (Margolis and Lifschitz, 1985). Cultured rat kidney cells also produce ammonia from glutamine, and the enzyme γ-glutamyl transpeptidase plays a significant role in the metabolic process (Dass and Wu, 1985). Studies from our laboratory indicate that LLC-PK_1 cells cultured in standard fashion produce ammonia, but that the process is not pH dependent (Cole *et al.*, 1986). However, when the cells are grown on a rocker, which promotes adequate oxygenation and obviates the effects of unstirred layers, the cells exhibit glutamine-dependent ammoniagenesis which responds to acute changes in media pH and adapts to more prolonged acid–base perturba-

tions. In addition to demonstrating that cultured cells can provide a useful model to study ammoniagenesis, these observations demonstrate that alterations in the culture conditions can modify the metabolic profile of cultured epithelium.

c. Vitamin D Metabolism. Primary cultures of both monkey and vitamin D-deficient chicken kidney as well as LLC-PK_1 cells have been used to study the renal metabolism of 25-hydroxyvitamin D_3 and its modulation by calcium, PTH, estradiol, PGs, and cAMP (Handler *et al.*, 1980; Horster, 1980a; Horster and Stopp, 1986; Meier *et al.*, 1985; Juan and DeLuca, 1977; Henry, 1981; Trechsel *et al.*, 1979, 1980).

4. *Cell Biology*

As is the case with all cell types, renal cells in culture provide useful models to study basic cell biology.

a. Growth. The factors influencing growth of kidney cells in culture have been studied most extensively in the BSC-1 established cell lines. These cells produce growth-inhibitory (Holley *et al.*, 1980) and -stimulatory factors (Mordan and Toback, 1984), and also exhibit a mitogenic response to a variety of exogenous factors. Polypeptide growth factors such as epidermal growth factor (EGf); hormones such as vasopressin; the purine nucleotides, AMP and ADP; and electrolyte manipulations of the culture medium stimulate growth (Taback, 1980; Kartha and Toback, 1985; Walsch-Reitz *et al.*, 1984; Mordan and Toback, 1984; Toback *et al.*, 1984). An increase in the media sodium or potassium concentration, as well as a decrease in media K^+ promotes growth in this cell line (Toback, 1980; Walsh-Reitz *et al.*, 1984; Mordan and Toback, 1984; Toback *et al.*, 1984). A decrease in media K^+ is accompanied by a transient increase in intracellular sodium concentration, and the growth-stimulatory effect is abolished by a decrease in sodium content in the media (Walsh-Reitz *et al.*, 1984). Growth in MDCK cells may also depend on sodium uptake (Horster and Stopp, 1986; Reznik *et al.*, 1983). The transient increase in cell sodium could reflect activation of the sodium-proton antiporter, which accompanies mitogenesis in other cells (Fine and Sakhrani, 1986; Paris and Pouyssegur, 1984); however, the primary role of this process in the stimulation of mitogenesis is still controversial (Fine, 1985). A preliminary report in LLC-PK_1 cells suggests that intracellular alkalinization is not a prerequisite for growth (Mohrmann *et al.*, 1986). Of interest, when the growth-inhibitory factor produced by BSC-1 cells, which resembles TGF-β (transforming growth factor β), is combined with the mitogenic hormones insulin and hydrocortisone, primary cultures of proximal tubules do not exhibit increased thymidine incorporation but do increase cellular protein content

(Fine *et al.*, 1985). These provocative findings suggest that growth-stimulatory and -inhibitory factors may interact to promote hypertrophy rather than hyperplasia.

As indicated previously, experiments have defined the hormones required for growth in serum-free culture of the established cell lines, LLC-PK_1 and MDCK, as well as for primary cultures of proximal and distal tubular origin (Horster *et al.*, 1985; Horster and Stopp, 1986).

The factors that control hyperplasia and hypertrophy are pertinent to an understanding of renal hypertrophy *in vivo* and tubular regeneration following injury. For example, the beneficial effects of adenine nucleotides on the course of experimental acute renal failure could be related to their effects on cell proliferation.

b. Differentiation. Differentiation, like growth, is a basic characteristic of all cells, which is particularly amenable to investigation under culture conditions, and has been the subject of investigation in multiple cell types. It provides important information concerning the developmental process *in vivo* as well as new insights into cellular function. Differentiation by renal epithelia has been the subject of two recent detailed reviews by Lever and will only be considered briefly herein (Lever, 1985, 1986).

As discussed in detail by Lever, the formation of domes in both LLC-PK_1 and MDCK cells parallels the development of differentiation and can be induced by a broad variety of compounds that induce differentiated function in other cultured cells (Lever, 1985, 1986). Other markers of differentiation in LLC-PK_1 cells include the induction of the sodium-dependent glucose transporter, and the development of apical microvilli with the associated brush border enzymes. Different factors control cell growth and subsequent differentiation (Lever and Sari, 1983; Devis *et al.*, 1985). The precise signals that commit the cell to undergo differentiation are unknown (Lever, 1985, 1986). It is of interest that Na^+,K^+-ATPase activity declines in MDCK cells when they reach confluence and that this is associated with a factor, dependent on protein synthesis, which inhibits the activity of the pump (Kennedy and Lever, 1985). One potential trigger for differentiation is an increase in intracellular sodium concentration; but as indicated previously, the substratum for growth, access of nutrients to the basal surface, availability of sufficient oxygen, cell structural features, and cell–cell interactions can all contribute to the expression of the cellular phenotype via mechanisms that are not yet elucidated.

c. Intracellular Messengers. With cultured cells it is feasible to use the newly developed fluorescent probes to measure intracellular pH and calcium and to assess the kinetics of changes in these parameters (Chaillet *et al.*, 1986; Bonventre and Cheung, 1986). Mechanisms of cellular calcium accumulation are also amenable to investigation (Parys *et al.*, 1986). Alterations in these key intracellular mediators have been studied in

response to hormone action (Pollock *et al.*, 1986; Hruska *et al.*, 1986; Tang and Tannen, 1986; Goligorsky *et al.*, 1986a; Shayman *et al.*, 1986), and in relationship to growth (Paris and Pouyssegur, 1984; Mohrmann *et al.*, 1986; Fine, 1985). It can be anticipated that cellular pH and calcium will also be examined in relation to transport events, other effector substances, metabolic regulation, and under pathophysiological conditions.

d. Membrane Polarity. One unique feature of epithelial cells is the development of a polarized membrane structure that permits vectorial transport of solutes and water. Some of the most innovative studies utilizing cultured cells have been directed toward understanding the mechanisms involved in the biogenesis of a distinct apical and basolateral membrane structure.

Polarity of the membranes in differentiated renal epithelium has been demonstrated morphologically, by the basolateral distribution of Na^+,K^+-ATPase and apical localization of brush border enzymes, by the apical location of the sodium-glucose transporter, by the distribution of antigenic sites and by the capacity for vectorial transport (Lever, 1985).

The role of tight functions in the maintenance of membrane polarity has been supported by studies utilizing a low calcium concentration to disrupt the occluding junctions in LLC-PK_1 cells (Rabito *et al.*, 1984; Rabito, 1986b). Histochemical studies showed migration of the apical marker enzymes alkaline phosphatase and γ-glutamyl transpeptidase to a basolateral location (Rabito *et al.*, 1984), while measurements of sodium-dependent glucose transport of cells grown on permeable supports demonstrated the appearance of this transporter at a basolateral location (Rabito, 1986b).

In 1978, Rodriguez-Boulan and Sabatini described the novel use of enveloped RNA viruses as a tool to study membrane polarity (Rodriguez-Boulan and Sabatini, 1978). Progress made utilizing this technology in MDCK cells to determine the events that dictate the transport of proteins to the apical or basolateral membrane is described in two reviews (Salas *et al.*, 1985; Rodrigues-Boulan, 1986). Enveloped viruses bud from either the apical or basolateral membrane of the cell, and the glycoproteins which form the envelope are concentrated on the appropriate surface prior to budding. The cell apparently processes and transports these viral glycoproteins in the same fashion as endogenous glycoproteins; hence they can serve as probes which can be followed using immunological markers. Using this approach, the role of the endoplasmic reticulum, Golgi apparatus, exocytic vesicles, and cytoskeleton in the sorting and transport of glycoproteins to specific membrane domains has been assessed. The current data suggest that sorting occurs at a post-Golgi step, but the precise mechanism is still undefined.

e. Cell–Cell Communication. The communication from cell to cell via gap

functions represents another area where cell culture techniques have been invaluable. Movement of electrolytes, cAMP, and other metabolites between cells has been demonstrated (Handler *et al.*, 1980). Cereijedo and his collaborators have used several techniques to demonstrate cell–cell communication to MDCK cells, including morphological demonstration by freeze-fracture microscopy, electrical communication, and the passage of microinjected Lucifer Yellow between cells (Cercijido *et al.*, 1985). Most recently, they have shown that mutant ouabain-resistant MDCK cells protect ouabain-sensitive MDCK cells when they are cocultured (Cercijido *et al.*, 1985). A similar strategy has been employed by Ledbetter *et al.* (1986). They found that hamster kidney (HaK) cells, which are relatively ouabain resistant, sustain higher intracellular potassium levels in MDCK cells when the two are cocultured in ouabain. Furthermore, autoradiography with [^{3}H]leucine showed that only MDCK cells adjacent to HaK cells were able to synthesize protein in a ouabain-treated culture medium.

f. Role of Cytoskeleton. Cells in culture are also being used to determine the biochemistry of cytoskeleton components and their functional role in processes such as the formation of occluding functions and the maintenance of cell volume (Zackroff and Goldman, 1979; Meza *et al.*, 1980; Mills and Lubin, 1986).

D. Application of Cell Culture to Pathophysiology

Although cell culture of renal tubular epithelium has been used for some time to study infectious agents (Wilson *et al.*, 1986), its application toward the pathophysiology of primary renal tubular disease has been limited. It can be expected, however, that the extensive characterization of several established cell lines and the development of primary cultures will lead to increased use of these models to examine abnormalities of the renal tubular epithelium. Wilson has extensively detailed this issue in a review (Wilson *et al.*, 1986).

1. *Renal Tubular Cell Injury*

The effects of anoxia on renal tubular epithelium have been investigated in cultured cells by several investigators (Wilson *et al.*, 1986; Kreisberg *et al.*, 1980; Snowdowne *et al.*, 1985). Utilizing primary cultures of rat tubular epithelium, Kreisberg and co-workers reported that anoxia in combination with substrate depletion caused cell swelling and morphological evidence of plasma membrane blebbing (Kreisberg *et al.*, 1980). Furthermore, cell viability (or growth) was impaired as assessed by the number of cells sustained in culture 18 hours following the anoxic stimulus. The oncotic

agent, 8% polyethylene glycol, prevented cell swelling and maintained cell viability. Wilson and co-workers have subsequently investigated the effects of anoxia and substrate depletion on primary cultures of microdissected rabbit nephrons (Wilson *et al.*, 1986). Using exclusion of Nigrosin dye as a measure of cell viability, they found that the S_3 segment of the proximal tubule and the medullary thick ascending limb were most susceptible to injury. However, all segments studied, including the S_1 and S_2 portions of the proximal tubule and the cortical collecting duct, exhibited a striking decline in viability during the subsequent 5 hours of exposure to normal oxygenation and substrate, an *in vitro* model of the reflow period following the ablation of renal blood flow. The degree of injury during this "reflow period" appeared to be calcium dependent, since it could be abrogated to some degree by the removal of calcium from the media, calcium channel blockers, or the calmodulin inhibitor trifluoperazine. An established monkey cell line, LLC-MK_2 cells, has also been used to investigate the alterations in intracellular calcium that accompany combined anoxia and substrate removal (Snowdowne *et al.*, 1985). These investigations found a striking decrease in ATP content and an accompanying increase in intracellular calcium concentration as determined with the calcium-sensitive photoprotein aequorin, both of which returned promptly to normal upon reexposure to basal conditions. When anoxia was combined with exposure to 20 m*M* glucose, both ATP and intracellular calcium concentration were sustained in the normal range, suggesting that ATP plays a critical role in the maintenance of cellular calcium homeostasis. Additional studies in which intracellular calcium concentration was modified, calcium entry blocked by lanthanum, and mitochondrial function impeded with the uncoupler FCCP suggested that both calcium entry into the cell and disordered mitochondrial calcium accumulation contribute to the increase in intracellular calcium concentration.

All these studies are actually *in vitro* models of ischemia rather than of anoxia, since combined oxygen and substrate removal are utilized. They demonstrate the potential utility of cell culture to investigate the complex biochemical events that induce cell injury during ischemic acute renal failure.

Toxic, as well as ischemic tubular injury is amenable to investigation using cell culture techniques. Several approaches have been employed to investigate mercuric chloride-induced tubular injury (Wilson, 1986; Ash *et al.*, 1975; Inamoto *et al.*, 1976; Troyer *et al.*, 1985a). In early studies carried out before the media requirements for primary culture has been defined, the viability in culture of cells from microdissected proximal tubules of normal and mercuric chloride-treated rabbits was assessed by time-lapse photography (Ash *et al.*, 1975). Mercuric chloride-exposed tubules, like

tubules from fetal rabbits, fared better under culture conditions than tubules from normal animals. Primary cultures of normal rat kidneys have been used to assess RNA and DNA synthesis upon exposure to $HgCl_2$ *in vitro* (Inamoto *et al.*, 1976). LLC-PK_1 cells have since been used to evaluate the alterations in cellular lipid metabolism induced by $HgCl_2$ (Troyer *et al.*, 1985b). $HgCl_2$ added to the culture media resulted in increased accumulation of lysophospholipids and unesterified fatty acids along with the development of plasma membrane blebs and irreversible cell injury. Evidence of lipid peroxidation as reflected by malondialdehyde accumulation was not detected. Addition of unsaturated fatty acids to the culture media also caused plasma membrane blebbing and loss of cell viability as reflected by the exclusion of Nigrosin dye.

Aminoglycoside toxicity has also been evaluated using LLC-PK_1 cells (Hori *et al.*, 1984; Schwertz *et al.*, 1986). *In vitro* exposure to normal and mercuric chloride-treated rabbits was assessed by time-lapse photography (Ash *et al.*, 1975). Mercuric chloride-exposed tubules, like tubules from fetal rabbits, fared better under culture conditions than tubules from normal animals. Primary cultures of normal rat kidneys have been used to assess RNA and DNA synthesis upon exposure to $HgCl_2$ *in vitro* (Inamoto *et al.*, 1976). LLC-PK_1 cells have since been used to evaluate the alterations in cellular lipid metabolism induced by $HgCl_2$ (Troyer *et al.*, 1985b). $HgCl_2$ added to the culture media resulted in increased accumulation of lysophospholipids and unesterfied fatty acids along with the development of plasma membrane blebs and irreversible cell injury. Evidence of lipid peroxidation as reflected by malondialdehyde accumulation was not detected. Addition of unsaturated fatty acids to the culture media also caused plasma membrane blebbing and loss of cell viability as reflected by the exclusion of Nigrosin dye.

Aminoglycoside toxicity has also been evaluated using LLC-PK_1 cells (Hori *et al.*, 1984; Schwertz *et al.*, 1986). *In vitro* exposure to gentamicin caused changes in the lysosomes and the development of myeloid bodies, alterations in phospholipid and free fatty acid metabolism, and alterations in cellular calcium uptake. However, its effects on cell viability are controversial (Hori *et al.*, 1984; Schwertz *et al.*, 1986).

The studies with both gentamicin and $HgCl_2$ indicate the potential application of cell culture techniques to investigate the mechanisms of renal cell injury. Recent studies have also examined potential mediators of cell injury such as oxidants and altered glutathione metabolism on the metabolism, transport, and morphology of cultured epithelial cells (Welsh *et al.*, 1985; Stevens *et al.*, 1986).

Interpretation of these studies of cell injury must be circumspect, however. The altered metabolic profile of cells in culture, the hypoxic

environment of standard cell culture conditions, the modified receptor profile of the plasma membrane, and the relative inaccessibility of toxins to the basolateral surface could all influence the response detected. These issues are not insurmountable, however, and further refinements in cell culture technology should render this technology even more useful to study cell injury. In fact, some of these pitfalls could potentially be turned into advantageous experimental strategies.

2. *Cystic Disease*

Organ cultures of mouse embryo kidney treated with hydrocortisone undergo cyst formation of the proximal tubules, which is preceded by alterations in basement membrane morphology and an increase in Na^+,K^+-ATPase activity (Avner *et al.*, 1984).

Wilson and co-workers have reported the successful culture of individual microdissected cysts from the kidneys of patients with polycystic kidney disease (Wilson *et al.*, 1984). Comparison of these cultured epithelia with cells from proximal tubules and cortical collecting ducts from normal human kidneys revealed accelerated growth; an altered morphology with more flattened epithelia, the development of cystlike areas, and striking abnormalities in the basement membrane; and decreased expression of the apical brush border enzyme γ-glutamyl transpeptidase and decreased reactivity of adenylate cyclase in the polycystic culture. The capacity to culture human polycystic kidney material should provide a powerful new tool to delineate the genetic and cellular abnormalities which underlie this disease process.

3. *Other Genetic Abnormalities of Tubular Function*

A preliminary report described a decrease in phospholipase and cyclooxygenase activity in cultured papillary collecting duct cells from Dahl salt-sensitive rats, which might account for the predisposition to develop hypertension (Reid and Dunn, 1984). It can be anticipated that culture of defined nephron segments will be employed to delineate the biochemical and genetic abnormalities underlying a variety of inherited tubular defects in both experimental animal models and human disease.

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Cyclosporine Nephrotoxicity

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I. Introduction

The new immunosuppressive agent cyclosporine represents not only a major therapeutic and pharmacologic advance, but also a major phenomenon in the medical literature. As depicted in Fig. 1, the number of articles per year devoted to this novel compound, dubbed "the drug of the 1980s," has steadily risen from 3 in 1976 to over 850 in 1986. The estimated number for 1987 is 1100. Many of the articles deal with the nephrotoxicity of cyclosporine, reflecting the fact that the renal toxicity of the drug, which manifests itself clinically in over half of patients given cyclosporine, is the major side effect limiting its use (Table I). It is probable that other drugs of equal potential benefit to humanity have been abandoned early in development because of toxic effects less severe than those associated with cyclosporine use in rats and humans. If the preclinical investigations of cyclosporine had given greater emphasis to studies in rats rather than to studies in dogs and nonhuman primates which are more resistant to its toxic effects, this exciting new agent which has been such a boon to transplantation and related fields might never have become available for clinical use.

This review will discuss data obtained in humans and experimental animals treated with cyclosporine. We will compare nephrotoxic effects in humans and animal systems, review pathological findings, discuss mechanisms of nephrotoxicity and synergism with other agents, and indicate areas of interest for future research on renal effects of cyclosporine.

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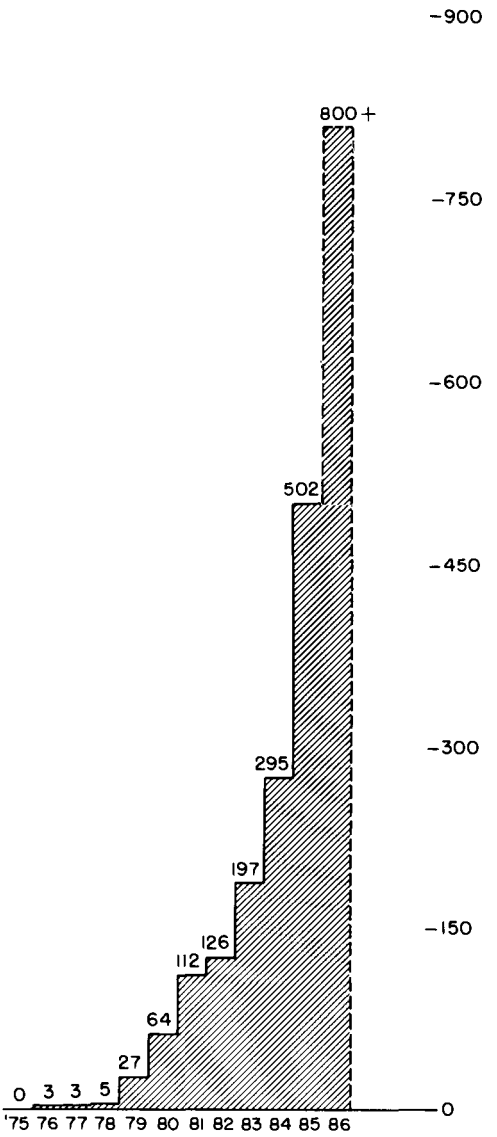


FIG. 1. Number of articles with "cyclosporine" or some variant in the title in the 3000 Medline-indexed journals from 1976 to 1986. By the end of 1986, there were over 1900 total. The estimated number for 1987 is 1100.

Comparison of Nephrotoxicity in Experimental Models and in Human Patients

Progress in the elucidation of the mechanism of cyclosporine nephrotoxicity and in development of strategies to lessen or prevent toxicity requires the use of animal models. However, there is evidence for substantial physiological differences between the animal models and clinical nephrotoxicity. Some of these differences are probably reflections of the larger doses given to animals. While cyclosporine produces *hyper*tension with low or normal plasma renin levels in humans (Adu *et al.,* 1983; Curtis *et al.,* 1987), it causes *hypo*tension when administered in the large doses often given to rats (60–100 mg/kg) and is often associated with a stimulated renin–angiotensin system (Siegl *et al.,* 1982, 1983; Dieperink *et al.,* 1983; Baxter *et al.,* 1982, 1984; Duggin *et al.,* 1986; Lustig *et al.,* 1987; Racusen *et al.,* 1987b). Lower doses (20–25 mg/kg) do augment the hypertension observed in spontaneously hypertensive rats (SHR) (Siegl *et al.,* 1984; Ryffel *et al.,* 1986c; Nahman *et al.,* 1987). Plasma volume depletion is a feature in most animal models (Devarajan *et al.,* 1987), while human

TABLE I

Reported Adverse Effects of CsA in 3518 Renal Transplant Recipients[a]

Adverse effect	Percentage
Renal dysfunction	54.3
Hypertension	42.4
Infections	
Bacterial	15.4
Viral	14.8
Fungal	3.3
Hypertrichosis	32.5
Tremor	22.1
Hepatic dysfunction	18.2
Gum hyperplasia	16.2
Nausea, vomiting	9.0
Paresthesia	5.2
Hyperuricemia	4.1
GI ulcer hemorrhage	3.2
Convulsions	1.8
Hyperkalemia	1.7
Malignancy (including 0.4% lymphoma)	1.3
Joint/muscle pain	1.1
Anemia	1.1

[a] Modified from Krupp *et al.* (1986).

patients manifesting a hypertensive response to cyclosporine are often volume-overloaded (Curtis *et al.,* 1987). Depressed aldosterone levels have been reported in human patients, and this appears to occur without a necessary reduction in plasma renin levels (Stern *et al.,* 1987), while some animal studies have shown elevated aldosterone levels (Siegl *et al.,* 1982, 1983). Seizures and neurologic abnormalities are more prominent in cyclosporine-treated rats (Racusen *et al.,* 1987a) than they are in cyclosporine-treated patients (European Multicentre Trial Group, 1983; Atkinson *et al.,* 1984; Palestine *et al.,* 1984; Kahan *et al.,* 1985). While it remains possible that when comparable doses are used there are many parallels between clinical and experimental nephrotoxicity, in this review we will consider the clinical and experimental situations separately.

II. Experimental Models of Cyclosporine Nephrotoxicity

Over the past 5 years, many investigators have attempted to establish experimental models of cyclosporine nephrotoxicity. It is clear that human beings are the species most susceptible to this cyclosporine side effect. While models of acute cyclosporine toxicity have been developed, the renal dysfunction produced is generally mild. Moreover, there is no published animal model for the so-called chronic lesion, which is characterized by expansion of the interstitium by active fibroblasts producing interstitial fibrosis and renal enlargement. Only one animal model, the SHR, shows overt light microscopic arteriolar changes analogous to those seen in humans, although more subtle arteriolar lesions have been reported recently in other models (Makowka *et al.*, 1986; Fasel *et al.*, 1987).

Development of animal models of cyclosporine nephrotoxicity is particularly important because the clinical settings in which this agent is used are often very complicated, making it difficult to define directly the pathology and pathophysiology of renal injury produced by the drug. Moreover, certain types of studies are not possible in human patients, due to technical or ethical considerations or both, making studies in experimental models even more critical. To date, animal models have been used to assess effects of cyclosporine on renal tubular and glomerular function and renal hemodynamics. The role of the renin–angiotensin system, prostaglandins (PG) and thromboxane (Tx), other mediators, and renal nerves have also been assessed in experimental animals. The potentiation of cyclosporine nephrotoxicity by other factors such as ischemia or concurrent administration of other drugs, and the importance of hepatic metabolism of cyclosporine in the production of toxic renal effects have also been studied. The following

discussion will review these models and will attempt to draw parallels between features of these models and clinical observations.

A. Cyclosporine and the Renal Tubule

While proximal tubular cell vacuolation was one of the earliest reported effects of cyclosporine on the kidney, and has been equated with nephrotoxicity, tubular changes are often disproportionately mild and can be dissociated from alterations in renal function. The nature and extent of injury to the renal tubule and the role of tubular injury in the pathophysiology of cyclosporine nephrotoxicity are under active investigation.

Tubular changes with cyclosporin A (CsA) treatment were an early observation in animal models. Borel (1980) described swelling and vacuolation of tubular cells with occasional tubular cell necrosis and regeneration at "high" doses of the compound in animals. Another early observation of proximal tubular vacuolation in rats treated with CsA, 25 or 50 mg/kg/day po in olive oil, was made by Farthing and Clark (1981); these were associated with very mild elevations in serum urea nitrogen (SUN) and creatinine. Blair *et al.* (1982) described focal proximal tubular cell vacuolation and necrosis by light microscopy in animals treated with high doses of CsA (100 mg/kg/day po) for up to 21 days; electron microscopy (EM) revealed dilatation and vesiculation of the smooth endoplasmic reticulum, with increased lysosomes and the formation of myeloid figures (a finding which is distinctly rare with CsA). Ryffel and co-workers (Ryffel *et al.*, 1983; Siegl *et al.*, 1983) described tubular changes in rat models used for toxicologic evaluation of CsA (20–100 mg/kg/day). Tubular alterations included tubular cell vacuoles, inclusions, and microcalcifications; EM revealed dilatation of endoplasmic reticulum, giant mitochondria, and an increase in lysosomes. In Wistar rats, these tubular changes were detectable after 2 days at 100 mg/kg/day. These tubular changes have since been reported frequently in cyclosporine-treated animals (Thomson *et al.*, 1984) and in human patients (Mihatsch *et al.*, 1985). Interestingly, the eosinophilic cytoplasmic inclusions found in animal models most often represent lysosomes rather than the giant mitochondria found in biopsy samples from cyclosporine-treated patients. Elevations in urinary levels of proximal tubular brush border enzymes, particularly *N*-acetylglucosaminidase (NAG), have also been detected in animal models as well as in humans, presumably reflecting toxic injury to proximal tubular cells.

While alterations in the proximal tubule have been described by many investigators, others have failed to demonstrate histological lesions despite

significant renal dysfunction. Thomson *et al.* (1981) studied Sprague–Dawley rats receiving 100 mg/kg every 48 hours over 21 days. Despite elevations in SUN and urinary NAG and depression of urea clearance, no histological changes were detected in the kidney. Yagisawa *et al.*, (1986) administered CsA (25 mg/kg/day po) to rats for 1–3 weeks and assessed tissue levels of CsA, morphology, and function in a number of organs. Although renal tissue CsA levels were elevated (665 ng/g tissue at 1 week, 910 ng/g tissue at 3 weeks), and creatinine and SUN were mildly elevated, no histological changes in the kidney were detected at this dose. In studies in Fischer rats given CsA, 25–40 mg/kg/day by gavage for 14 days (Schwass *et al.*, 1986), while SUN and serum creatinine were mildly elevated and inulin clearance decreased by 59%, no tubular histological abnormalities were seen. Hamilton *et al.* (1982) and Gerkens *et al.*, (1984) have emphasized the mild degree of histological abnormalities in rats as well as in humans, despite, in some cases, fairly severe renal dysfunction.

It is also clear that tubular vacuolation can also be found, in experimental animals as well as in humans, in the absence of any evidence of renal dysfunction. Of particular interest is the finding by a number of investigators that the polyoxyethylated castor oil derivative used as the vehicle for parenteral cyclosporine can also produce significant tubular vacuolation. Kone *et al.* (1986) described this phenomenon in the Munich-Wistar rat in studies of acute renal failure (ARF) produced by combining cyclosporine with brief renal ischemia. We quantitated the tubular vacuolation as well as the presence of tubular eosinophilic inclusions, tubular necrosis, and tubular regeneration using a ranking method. Analysis revealed that vehicle-treated animals had an extent of tubular vacuolation not different from CsA-treated animals; vehicle-treated animals also were seen to have eosinophilic cytoplasmic inclusions in tubular cells. Chow *et al.* (1986) also found tubular vacuolation in vehicle-treated rats with either ischemic injury or sham surgery, and Dieperink *et al.* (1983) likewise described vacuolation in rats treated with vehicle alone, despite normal renal function in these animals.

Other investigators have also demonstrated dissociation between tubular cell histopathology and renal function. Siegl *et al.* (1983) in their studies in SHR, found that rats treated with CsA, 20 mg/kg/day for 4 weeks, had inulin and *p*-aminohippuric acid (PAH) clearances not different from controls, despite persistent tubular and arteriolar histopathology. In studies of effects of induction of hepatic drug metabolism on CsA-induced nephrotoxicity, Cunningham *et al.* (1984) found that pentobarbitone, an inducer of hepatic enzyme systems, prevented tubular vacuolation but not elevations in SUN. As previously discussed, a number of investigators (Thomson *et al.*, 1981; Yagisawa *et al.*, 1986; Schwass *et al.*, 1986) found no

renal histological abnormalities in their rats treated with high-dose CsA, despite significant elevations in SUN and serum creatinine and/or decrease in inulin clearances.

Do morphological alterations in renal tubules produced by CsA thus have any relationship to functional alterations? Lack of quantitation of histopathological alterations (or failure to assess the histology of the kidney at all!) has made this question somewhat difficult to answer. We have quantitated tubular vacuolation in our Munich-Wistar rat model of CsA-induced nephrotoxicity (Kone *et al.*, 1986). In studies combining cyclosporine (60 mg/kg/day ip) or vehicle administration with brief (20 minutes) renal ischemia, tubular vacuolation was a prominent feature in cyclosporine- and vehicle-treated groups and was significantly correlated with terminal SUN. In later studies from our laboratory (Racusen *et al.*, 1987b) evaluating renal blood flow (RBF) and renal vascular resistance (RVR) in rats given CsA (60 mg/kg/day ip) without ischemia, extent of tubular vacuolation was significantly correlated with RBF (with a suggestive though not significant correlation with inulin clearance) following two daily doses. Clearly, acute RBF and inulin clearance changes occur before evident histopathology develops in experimental animals, but our results suggest that structure and function are correlated in the "maintenance phase" of renal failure produced by CsA, suggesting either that persistent hemodynamic alterations potentiate tubular lesions, or that tubular injury may lead to persistent alterations in hemodynamics and/or function.

In studies designed to define molecular processes which might underlie CsA nephrotoxic effects, Jung and Pergande (1985) exposed isolated rat kidney mitochondria from cortical homogenates to varying doses of CsA *in vitro*. CsA inhibited state 3 and dinitrophenol-stimulated respiration and increased state 4 respiration, suggesting that the drug was both an inhibitor of electron transport and an uncoupler. Effects were seen at concentrations as low as 40 n*M* CsA, which may be attainable in the renal cortex of CsA-treated individuals (Donatsch and Ryffel, 1986). If these studies are confirmed, primary mitochondrial effects may underlie tubular cell alterations.

Tubular *function* in cyclosporine-treated rats has been studied by a number of investigators. Most of the studies designed to assess tubular function directly have utilized doses of CsA which are "nephrotoxic" but are considerably lower than those used in early investigations of CsA nephrotoxicity. While little evidence of tubular dysfunction has been found in these recent investigations, it remains possible that very high doses or more prolonged administration may produce direct tubular injury and tubular functional impairment.

Gnutzmann and co-workers (1986) have examined the effect of cyclo-

sporine on the diluting capacity of the rat kidney. Rats were given either a single iv dose of CsA (5 mg/kg) or were treated with CsA (15 mg/kg po) for 10 days. A pulse perfusion technique was used and the electrolyte concentration of fluid from individual thick ascending limbs was measured by continuous-conductivity monitoring of early distal tubular fluid. Chronic or acute CsA administration produced a significantly higher electrolyte concentration in thick ascending limb fluid. These techniques could not discriminate, however, between an epithelial transport deficit or an increased water permeability in the thick ascending limb.

Micropuncture techniques were also utilized by Muller-Suur and Davis (1986) to examine electrolyte transport in the loop of Henle in rats treated for 5–7 days with cyclosporine (15 mg/kg/day po). Late proximal tubular segments downstream from an immobile wax block were microperfused and the fluid at the earliest distal tubule collected and analyzed. CsA treatment did not produce significant changes in electrolyte transport in Henle's loop or in the whole kidney. The kidneys from CsA-treated rats were also able to increase K^+ excretion acutely after iv KCl loading with only a mild impairment of response compared to controls.

In studies of experimental cyclosporine nephrotoxicity in rats, Schwass *et al.* (1986) found no evidence of tubular toxicity in animals treated with 25 or 40 mg/kg/day or gavage. In addition to normal renal histology, tubular function was normal, as assessed by fractional excretion of sodium and cortical slice transport of tetraethylammonium, reflecting activity of the active organic base transport system.

Dieperink *et al.* (1986c) have reported a lithium clearance (C_{Li}) and micropuncture study in rats designed to assess proximal tubular function in CsA nephrotoxicity. Conscious catheterized rats were studied after 13 days of treatment with CsA (25 mg/kg/day po). Glomerular filtration rate (GFR), absolute proximal reabsorption, urine volume, and electrolyte excretion were decreased with CsA, while proximal fractional reabsorption increased markedly. Absolute and fractional distal reabsorption of water and absolute distal reabsorption of sodium increased, while distal fractional sodium reabsorption was not changed. Acute infusion of 12.5 mg/kg CsA following the 13-day protocol depressed GFR and absolute proximal reabsorption, while fractional proximal reabsorption increased. Micropuncture studies in anesthetized rats revealed prolonged proximal transit times. Proximal intratubular pressures were low-normal, ruling out distal tubular obstruction. In these studies, neither serum nor blood CsA levels were correlated with renal function. Renal morphology, as noted above, was normal in these studies. Thus, there was no evidence morphologically or functionally of toxic proximal tubular injury in this model. In addition, acute isotonic volume expansion significantly and immediately increased

urine volume and K and Li clearances and essentially normalized GFR, absolute proximal reabsorption, and Na clearance militating against direct tubular damage. Acute volume expansion, however, failed to normalize the increase in the fractional proximal reabsorption, suggesting a persistent drug-induced alteration in the tubuloglomerular feedback mechanism. The authors pointed out the similarities between the pattern of functional response to CsA and to acute partial renal artery occlusion, and suggested that CsA has its primary effect on renal vasculature.

B. Effects of Cyclosporine on Organ Blood Flow and Glomerular Hemodynamics

It has become clear that cyclosporine has effects on organ blood flow and renal hemodynamics. McKenzie *et al.* (1986), using radiolabeled microspheres in dogs, found decreased blood flow in the kidneys as well as heart, lungs, liver, and spleen following a 2-hour infusion of CsA, 25 mg/kg. These investigators also found a decrease in urine flow and an increase in release of tubular brush border enzymes.

In studies in conscious rats, Murray *et al.* (1985) used radiolabeled microspheres to examine effects of cyclosporine administration on renal hemodynamics. Acute infusion of 20 mg/kg cyclosporine (in 10% fat emulsion) produced a decrease in RBF and an increase in RVR. In addition, there were significant increases in plasma renin activity (PRA) and in urinary 6-keto-$PGF_{1\alpha}$. Administration of phenoxybenzamine, an α-adrenergic blocker, or renal denervation largely prevented the fall in RBF. A 10 mg/kg infusion produced no change in RBF or RVR in normal rats, despite increase in PRA and increase in urinary PG. However, following administration of meclofenamate, a cyclooxygenase inhibitor, there was a significant decrease in RBF at 10 mg/kg CsA, suggesting that endogenous PG normally protect against the vasoconstrictor effect of cyclosporine. Pretreatment with captopril did not prevent the decrease in RBF.

These investigators also looked at short-term "chronic" effects of CsA given at 20 mg/kg/day. After 7 days, as in acute infusion studies, they found decreased RBF, increased RVR, and an increase in PRA and urinary $PGF_{1\alpha}$.

Sullivan *et al.* (1985), in acute infusion studies in rats using a dose of 10 mg/kg, found that infusion rate is an important variable determining renal hemodynamic effects. Infused over 3 minutes, cyclosporine reduced RBF transiently by more than 50% of controls. If infused over 10 minutes, however, the maximum decrese in RBF was 10%, but was more sustained. Following 7 days of CsA, 10 mg/kg/day administered ip or iv, clearance

studies were performed. In cyclosporine-treated animals, there was a 15% decrease in RBF, a 13% increase in RVR, but a 43% decrease in inulin clearance, results which suggested a decrease in ultrafiltration coefficient. These results also stress the importance of directly assessing clearances rather than relying on SUN or creatinine to assess nephrotoxicity.

We (Racusen *et al.*, 1987b) have used our high-dose Munich-Wistar rat model to look at RBF and RVR following administration of CsA, polyoxyethylated castor oil parenteral vehicle, or olive oil or mineral oil. In initial studies, naive rats were monitored for two baseline periods, and were then given parenteral CsA, oral CsA (60 mg/kg ip), or control solutions. RBF, measured by renal artery flow probe, fell significantly from baseline with parenteral CsA (−24%), oral CsA in olive oil (−25%), or parenteral vehicle (−23%), but remained at baseline in olive oil- or mineral oil-injected animals (−4%). RVR rose significantly in groups with depression of RBF but actually decreased in controls. Thiel *et al.* (1986) reported similar acute effects of the parenteral vehicle, indicating that acute studies using CsA in this parenteral cremophore should be interpreted cautiously. Beserab *et al.* (1987) have recently reported marked renal vasoconstriction in an isolated perfused kidney model when cremophore was added to the perfusate. Additional animals in our studies (Racusen *et al.*, 1987b) were given two daily doses of parenteral CsA (60 mg/kg/day ip) cremophore, or mineral oil, and RBF and GFR were measured 22 hours after the second dose. RBF remained significantly lower in CsA-treated rats (5.1 ml/min) but was normal in cremophore-treated (8.5 ml/min) as well as mineral oil control rats (7.3 ml/min). There was a significant correlation between tubular vacuolation (assessed semiquantitatively) and RBF and RVR. As discussed above, these results could be due to potentiation of tubular changes by altered hemodynamics, or direct/indirect augmentation of RBF and RVR changes by tubular cell alterations.

Dieperink *et al.* (1986b) assessed the effect of a range of vasodilating agents on cyclosporine nephrotoxicity in a rat model. Preexisting cyclosporine nephrotoxicity, induced by administration of CsA, 25 mg/kg/day po for 13 days, was not reversed by acute infusion of phenoxybenzamine, prostacyclin, captopril, nifedipine, or indomethacin in doses sufficient to cause a fall in mean arterial pressure. In addition, concomitant treatment with captopril and CsA did not prevent renal dysfunction. However, the calcium channel blocker nifedipine, given concomitantly with CsA by oral gavage, increased inulin clearance, and absolute proximal and distal tubular reabsorption, and mean arterial pressure while decreasing fractional proximal reabsorption. RBF was also increased compared to CsA-treated animals. The most likely explanation for this effect is that nifedipine is decreasing preglomerular vascular resistance and thereby improving glo-

merular blood flow and GFR. Nifedipine also inhibits platelet-aggregating and thromboxane release factors which may be involved in CsA toxicity (Grace *et al.*, 1987; Mikhailidis *et al.*, 1987). Although the numbers in the study of Dieperink *et al.* (1986b) are small, these results are intriguing and suggest that pharmacological intervention may prevent CsA nephrotoxicity. Solez *et al.* (1988) have found a beneficial effect of nifedipine on function and outcome of renal transplants in retrospective studies of cyclosporine-treated patients.

Micropuncture techniques have recently been utilized to directly assess glomerular hemodynamics following acute CsA administration (50 mg/kg iv) in the Munich-Wistar rat (Barros *et al.*, 1987). Both afferent and efferent arteriolar resistance increased, with a 55% reduction in glomerular plasma flow and approximately 50% reduction in whole kidney and single nephron GFR. Mean glomerular capillary hydraulic pressure rose and K_f decreased. The effects of CsA were ameliorated by captopril and were significantly less in Brattleboro (ADH-deficient) rats, suggesting that both the renin–angiotensin system and ADH may be involved in the effect. Verapamil pretreatment also prevented these changes.

C. Tubuloglomerular Feedback and the Renin–Angiotensin System

The role of tubuloglomerular feedback and the renin–angiotensin system in the pathogenesis of cyclosporine-induced nephrotoxicity has been assessed by a number of investigators. Gnutzmann *et al.* (1986) examined the effect of cyclosporine on proximal tubular function of the rat kidney using free-flow and pulse stop-flow micropuncture in rats treated with 15 mg cyclosporine by oral gavage for 8–10 days. They found increased conductivity of tubular fluid in early distal and diluting segments, consistent with increased distal salt delivery and transport impairment within the proximal tubule and the loop of Henle. The authors postulated that tubuloglomerular feedback operation might be involved in the decreased GFR seen with cyclosporine.

A Wistar rat model was used to assess the effect of salt intake on cyclosporine-induced impairment of renal function (Gerkens *et al.*, 1984). Three groups of rats were placed on low-salt diet and were given water to drink plus oral vehicle or oral cyclosporine (100 mg/kg every other day), or normal saline to drink plus oral cyclosporine. Animals were studied after 3 weeks on protocol and 3 weeks of recovery. The low-salt cyclosporine-treated animals had significantly elevated SUN and serum creatinine and decreased creatinine clearance, and histological changes were also more

severe in this group. High-salt cyclosporine-treated animals were protected from these effects. CsA plasma levels were all within the toxic range. These authors point out that histological tubular abnormalities were mild and suggested that these primary toxic effects on the proximal tubule led to a decrease in absorption and increased delivery of chloride ions to the distal tubules, activating tubuloglomerular feedback and decreasing the GFR, an effect potentiated in Na-depleted animals. However, no change in PRA was detected.

Using an SHR model, Siegl *et al.* (1982, 1983) assessed the renin-angiotensin-aldosterone system during treatment with 0, 20, 50, or 100 mg/kg/day administered by oral gavage. Inulin and PAH clearances and PRA, aldosterone levels, and SUN were assessed weekly. Results of these studies were reported in semiquantitative terms. At 20 mg/kg/day, after 2 weeks, blood pressure (BP) and PRA were slightly increased, GFR and renal plasma flow (RPf) marginally decreased; by 4 weeks, PRA was "distinctly increased," while GFR and RPF were not different from controls despite development of tubular and vascular pathology. At 100 mg/kg/day, after 2 weeks BP was decreased, PRA was slightly increased, and GFR and RPF were "decreased"; by 4 weeks BP remained low, PRA was not different from controls, and GFR and RPF were only marginally decreased. At all doses, in comparing results at 2 and 4 weeks, there was a tendency for functional changes to normalize. PRA was significantly increased only at low doses of cyclosporine, while there was a dose-dependent increase in measured aldosterone. A decrease in plasma Na was seen at all doses, and an increase in K at 50 and 100 mg/kg/day. The authors noted increased heart rate and hyperglycemia as well as increased BP, and speculated that stimulation of the renin-angiotensin-aldosterone system was initiated by stimulation of the sympathetic nervous system. Siegl and collaborators also found elevated plasma renin levels in beagle dogs given oral cyclosporine (25 mg/kg/day) for 5 days (Siegl and Ryffel, 1982; Siegl *et al.*, 1983).

Dieperink *et al.* (1983) assessed overall renal and tubular function in conscious rats treated with 0, 12.5, 25, and 50 mg/kg/day orally for 13 days. Inulin and lithium clearances were assessed. C_{inulin} and C_{Li} and C_{Li}/C_{inulin} were decreased in all cyclosporine-treated animals, and proximal tubular fluid reabsorption, assessed as $C_{inulin} - C_{Li}$, was decreased. C_{Na}, C_K, and urine flow were also decreased, although net Na and K delivered to the distal nephron was increased. Serum CsA levels did not correlate with other parameters. The increased proximal fractional reabsorption was ascribed to a decrease in net ultrafiltration pressure due to CsA and an inadequate adaptive reduction in absolute proximal reabsorption. A glomerular rather than a tubular pathogenetic mechanism was proposed by these authors, an observation reinforced by the fact that tubular vacuolation was seen histologically in all groups, including controls. PRA was increased at 12.5

and 25 mg/kg/day, similar to findings by Siegl *et al.* (1982, 1983), but was not correlated with C_{inulin}, C_{Na}, or C_{Na}/C_{Li}.

Baxter *et al.* have shown increased storage and release of renin from rat kidney *in vivo* and *in vitro* in response to cyclosporine. These investigators have previously demonstrated that renal cortical (and plasma) renin levels are increased in rats treated with CsA (50 mg/kg/day for 3 or 7 days) (Baxter *et al.*, 1982, 1984; Duggin *et al.*, 1986). *In vitro* studies using rat renal slices suggest that renin release into the medium from rat cortical tissues is increased above controls in tissues incubated with cyclosporine, with a peak increase averaging approximately 17% at 8×10^{-6} *M* CsA. Interestingly, outside the concentration range of 10^{-6} to 10^{-5} *M*, CsA had very little effect on slice renin release. While numbers of observations were small, these results indicated that the possible role of renin in cyclosporine nephrotoxicity should be further assessed.

While some investigators have found evidence of increased activity of the renin-angiotensin-aldosterone axis in cyclosporine-treated animals or tissues, results of recent studies suggest that angiotensin II and adenosine are not involved as mediators in the nephrotoxic effects of cyclosporine. As noted previously, Murray *et al.* (1985) found that pretreatment with the angiotensin-converting enzyme inhibitor captopril did not prevent the fall in RBF in conscious rats following CsA infusion (20 mg/kg). Similarly, Dieperink *et al.* (1986b) found that treatment with captopril did not improve renal function in CsA-treated conscious catheterized rats. Gerkens and Smith (1985) also used a rat model to assess the effect of captopril and theophylline treatment on cyclosporine-induced nephrotoxicity in rats. Male Wistar rats (maintained on a low-sodium diet for 2 weeks to potentiate nephrotoxicity) were given CsA 100 mg/kg po every 48 hours. Pretreatment with theophylline (40 mg/kg bid) or captopril 10 mg/kg/day for 3 days did not prevent rises in SUN and creatinine equivalent to those seen in animals pretreated with cyclosporine alone. The adequacy of the pretreatment regimens were confirmed in separate groups of rats injected with angiotensin II or adenosine and monitored for BP alterations. These results suggest that if tubuloglomerular feedback is involved in experimental cyclosporine nephrotoxicity, neither angiotensin II nor adenosine are the principal mediators of such feedback.

D. Cyclosporine and the Renal Nerves

The role of renal nerves and the sympathetic nervous system in producing changes in RBF and renal function with cyclosporine administration has been assessed both directly and indirectly in animal models.

Siegl *et al.* (1983), in studying the role of the renin-angiotensin-

aldosterone system in cyclosporine nephrotoxicity in their SHR model, found that the rats developed increased BP and heart rate as well as hyperglycemia, changes consistent with stimulation of the sympathetic nervous system. They postulated that the stimulation of the sympathetic nervous system in cyclosporine-treated animals might be responsible for the activation of the renin-angiotensin-aldosterone system demonstrated in their rat model.

In studies of RBF and renal function in conscious rats, Murray *et al.* (1985) found that the fall in RBF following an acute infusion of 20 mg/kg cyclosporine could be prevented by administration of phenoxybenzamine, an α-adrenergic blocker. Renal denervation also largely prevented the fall in RBF, suggesting a role for renal nerves in cyclosporine-induced renal vasoconstriction. In additional studies (Murray and Paller, 1986) in which rats underwent unilateral renal denervation (using 10% phenol in ethanol) 3 days before infusion, 20 mg/kg CsA produced a 43% decrease in inulin clearance in the innervated kidney, with clearance remaining normal in the denervated kidney. Prazosin, an α-adrenergic antagonist, also prevented the fall in RBF and rise in serum creatinine seen following "chronic" CsA administration (20 mg/kg/day ip for 7 days).

Recently, the effect of intravenous cyclosporine on renal nerve activity has been directly assessed by Moss and co-workers (1985) in rats. Cyclosporine was administered over 30 minutes at a dose of 10 mg/kg in animals which had undergone unilateral renal denervation and extracellular fluid volume expansion. In the innervated kidney, urine flow rate, sodium excretion, fractional excretion of sodium, and GFR were all decreased by cyclosporine, while RBF remained unchanged. Sodium retention appeared to be due to increased efferent nerve activity. Renal efferent nerve activity was increased 69% and stabilized at that level, while afferent activity increased by 82% but tended to decline. The authors postulated that afferent excitation was the primary neurological event, followed by reflex excitation of efferent sympathetic activity. The increase in renal nerve activity appeared to be part of a generalized sympathetic activation, as genitofemoral efferent nerve activity also increased 60% and BP increased transiently.

In the denervated kidney, Moss *et al.* (1985) found that basal GFR and RPF were increased and were not altered by cyclosporine. Cyclosporine actually produced an increase in sodium excretion and fractional excretion of sodium in the denervated organ. Thus, the sodium retention effect of cyclosporine seen clinically in patients with denervated transplant kidneys must be mediated via other mechanisms, although the authors point out that there would be hypersensitivity to circulating catecholamine in a chronically denervated kidney. Others have pointed out that transplanted

kidneys are significantly reinnervated within months (Gazdar and Dammin, 1970), making it possible that these experimental observations might be relevant to transplanted kidneys as well. Moss also pointed out potential interactions between the sympathetic nervous system and the immune system, and suggested that CsA effects on the immune system may be mediated at least in part by the sympathetic nervous system.

E. Arachidonic Acid Metabolites and Cyclosporine Nephrotoxicity

Recently, there has been considerable interest in the role of vasodilatory and vasoconstrictor arachidonic acid metabolites in producing the renal hemodynamic and functional changes seen following cyclosporine administration. CsA has been shown to increase platelet aggregation and thromboxane release in clinical studies (Grace *et al.*, 1987).

A number of investigators have examined effects of CsA on renal PG production in animal models. As noted previously, Murray *et al.* (1985) have demonstrated that acute and chronic administration of CsA increases urinary excretion of 6-keto-$PGF_{1\alpha}$ and that cyclooxygenase inhibitors exacerbate the decrease in RBF and increase in RVR following cyclosporine administration. These results suggest that vasodilatory PG may ameliorate the vasoconstrictor effects of cyclosporine, and that the use of cyclooxygenase inhibitors in cyclosporine-treated patients could enhance the nephrotoxic effects of this agent. Acute intravenous administration of CsA (10 mg/kg) to conscious rabbits also increased excretion of 6-keto-$PGF_{1\alpha}$ associated with a significant decrease in GFR from baseline values (Caterson *et al.*, 1986). In contrast, Duggin *et al.* (1986) found no change in renal slice release of 6-keto-$PGF_{1\alpha}$, PGE_2, or $PGF_{2\alpha}$ in rats treated with CsA, 100 mg/kg/day, for 3–7 days. Similarly, Perico *et al.* (1986) found no significant change in urinary excretion of PGI_2 and PGE_2 assessed at monthly intervals in rats receiving CsA, 40 mg/kg every 48 hours, for 3 months. Stahl and Kudelka (1986) found significantly *lower* PGE_2 formation by renal papillary slices and isolated glomeruli in rats treated with 25 or 50 mg/kg/day po for 3–6 weeks. *In vitro* incubation of papillary slices with CsA (up to 10^{-6} *M*) for 30 minutes produced no effect on PGE_2 formation or release of ^{14}C-labeled arachidonic acid metabolites, indicating that CsA has no direct effect *in vitro* on cyclooxygenase activity, or arachidonic acid metabolism or release. Bunke *et al.* (1987) have reported significant decreases in synthesis of the vasodilatory prostaglandins PGE_2 and PGF_1 by glomeruli isolated from rats treated with CsA (20 mg/kg) for 7 days. TxB synthesis was unchanged. These discrepancies may be related in part to

differences in duration of CsA treatment. In addition, there are a number of possible sites of PG synthesis, so that assays on isolated glomeruli, for example, may give different results from studies on renal slices or whole kidney.

Effects of administration of exogenous vasodilatory PG on development of CsA nephrotoxicity have been assessed in rat models. Makowka *et al.* (1986) used a high-dose Wistar-Furth rat model in which CsA was given for 7 days and demonstrated that rats given 16,16-dimethyl PGE_2 (a synthetic prostaglandin) (10 μ/kg/day) concomitantly with 100 mg/kg/day CsA had a significant improvement in survival, and near-normal renal function and histology by the eighth day after CsA treatment was discontinued. However, improved survival here is probably not due to prevention of renal effects, since deaths at high doses of CsA are generally due to CNS toxicity (Racusen *et al.*, 1987a). After 5 days of dosing, CsA-treated rats had significant elevations in serum creatinine and marked depression of creatinine clearance; 16,16-dimethyl PGE_2 significantly improved—though did not normalize—these functional effects of CsA. CsA-Induced renal histological changes, consisting of proximal tubular swelling, vacuolation, and necrosis associated with arteriolar endothelial cell swelling, showed "striking improvement" with 16,16-dimethyl PGE_2 administration, although these alterations were not quantitated. Similar protection has been observed in a rat isograft model (Pomer *et al.*, 1988). Ryffel *et al.* (1986a) also reported reduction of CsA-induced nephrotoxicity by exogenous PG in SHR receiving CsA 20 mg/kg/day po, and followed for 28 days. The mild elevation in serum creatinine and mild depression of creatinine clearance seen at this low dose after 4 weeks were prevented by PGE_2, and exudative changes in arterioles, assessed with a grading system, were normalized. (No significant tubular changes were seen at this dose.) However, PGE_2 also reduced the bioavailability of CsA, apparently by inhibiting enteral absorption, and abolished CsA immunosuppression, as assessed by hemagglutinin assay and local graft-versus-host reaction, probably precluding clinical use of PG to protect against nephrotoxicity.

Kawaguchi *et al.* (1985) have demonstrated an increase in urinary TxB_2 caused by cyclosporine in a cardiac isograft model in Fischer rats. Rats undergoing either sham surgery or isografting were given 0, 1.5, or 15 mg/kg/day CsA for 2 weeks. From the fifth postoperative day, daily excretion of urinary TxB_2 was significantly higher in the 15 mg/kg/day animals compared to all other groups. Levels of urinary TxB_2 were tightly correlated with serum CsA levels, and tended to return toward normal within 1–2 weeks. No changes in serum creatinine, SUN, or urine flow rates were found.

Stimulation of PG and Tx production *was* associated with nephrotoxic

alterations in renal function in a postischemic denervated rat model (Coffman *et al.*, 1978b). In these studies, rats underwent uninephrectomy followed by denervation and renal artery clamping for 30 minutes, following which they received parenteral CsA or vehicle ip (50 mg/kg/day) for 12–14 days. At the end of the study period, PAH and inulin clearances were determined, and the kidneys were then removed and the perfused *ex vivo* to allow determination of renal eicosanoid production. Both GFR and RBF were reduced to less than 50% of controls in CsA-treated animals, and unstimulated production of TxB_2, 6-keto-$PGF_{1\alpha}$, and PGE_2 were increased in CsA-toxic rats, while PGF_2 excretion was not.

Using a rat model, Perico *et al.* (1986) have assessed the functional significance of exaggerated renal TxA_2 synthesis induced by CsA. Two groups of rats, one group receiving CsA orally at 40 mg/kg every 48 hours for 3 months followed by discontinuation of the drug for 2 additional months, and the other group given vehicle po for 5 months, were studied. Urinary arachidonic acid metabolites were assessed and inulin clearance studies were performed at monthly intervals. A decrease in GFR was observed in CsA-treated rats which reached significance compared to controls after 3 months (0.41 vs 0.96 ml/min per 100 g). GFR in controls remained stable, and GFR in CsA-treated rats normalized following discontinuation of the drug. RPF, measured by PAH extraction, did not decrease significantly in CsA-treated animals. CsA-Treated animals had significantly higher urinary TxB_2 excretion after 1 month, which remained elevated at 3 months, returning to normal following withdrawal of the drug. There was a significant inverse correlation between urinary TxB_2 excretion and GFR, but none between urinary TxB_2 excretion and RPF. Serum TxB_2 concentrations were not different in cyclosporine-treated rats during and after treatment with the drug.

In additional groups of animals, these investigators assessed the effect of the selective Tx synthetase inhibitor UK-38,485 on TxB_2 urinary excretion and renal function. Following treatment with CsA for 6 weeks, there was a 4-fold increase in urinary TxB_2 excretion and a 3.5-fold decrease in GFR. Treatment with UK-38,485, at a dose causing 98% inhibition of platelet Tx generation in normal rats, produced a marked reduction in urinary TxB_2 and a significant increase in GFR in cyclosporine-treated rats. No effects on RPF were seen. Since TxB_2 is a by-product of TxA_2 which is a potent vasoconstrictor, likely mechanisms for the glomerular effects seen in these studies are an increase in efferent and afferent arteriolar tone, or, alternatively, a reduction in glomerular filtration area due to mesangial contraction. Lack of correlation between enhanced Tx synthesis and RPF in these studies suggests reduction in K_f due to reduced glomerular surface area. Interestingly, GFR increased but did not normalize following suppression

of TxA_2 synthesis, suggesting that other factors may be involved in reducing GFR following CsA treatment.

Elzinga *et al.* (1987) have reported modification of experimental CsA nephrotoxicity in rats using fish oil as vehicle. Rats were pretreated with standard olive oil oral vehicle or fish oil, rich in eicosapentaenoic acid, an inhibitor of cyclooxygenase metabolites, by gavage. After 14 days, CsA (50 mg/kg/day) was added to vehicles of some rats from each group; controls received only vehicle and were pair-fed. GFR was reduced by 60% in animals receiving CsA–olive oil compared to controls (olive oil or fish oil) but by only 36% in CsA–fish oil animals; trough whole-blood CsA concentrations were not different. Tubular cell vacuolation was also less severe in the CsA–fish oil group. CsA–olive oil produced elevated renal cortical content of TxB_2 compared to olive oil controls, while TxB_2 was reduced in both fish oil groups. The authors point out that effects of this vehicle on CsA pharmacokinetics and immunosuppressive properties, other possible mechanisms of action such as depression of lipoxygenase metabolites, and optimal protocol for vehicle administration require further study.

In recent studies, prostacyclin (PGI_2) synthesis by vascular endothelium has been shown to be inhibited by cyclosporine (Neild *et al.*, 1983b; Brown and Neild, 1987), leading us to a general discussion of cyclosporine-induced arteriolar and endothelial injury.

F. Experimental Arteriolar Lesions and Endothelial Injury

It has been difficult to produce an experimental model in which light microscopic lesions analogous to the arteriolotoxic lesions described in humans (Shulman *et al.*, 1981; Mihatsch *et al.*, 1985; Williams *et al.*, 1986) occur. Ryffel and co-workers (1983) screened a variety of species and strains for susceptibility to nephrotoxic effects of cyclosporine, defined by elevations in SUN or morphological changes. While tubular changes including vacuoles, inclusions, and microcalcification were seen in all rat strains, only the SHR developed arteriolopathic lesions following CsA treatment. These probably represented an acceleration of arteriolar lesions which occur spontaneously in these rats once persistent hypertension develops. At 20 mg/kg/day, SUN and serum creatinine were increased and clearances decreased at 4 and 8 weeks in CsA-treated rats; arteriolopathic lesions were greater than in controls after 4 weeks of CsA, but not different at 8 weeks. PRA was increased at 4 weeks, but not at 8 weeks. Arteriolar changes consisted of fibrinoid necrosis at low doses, and proliferative changes at high doses in this model. Makowka *et al.* (1986) have described arteriolar endothelial cell "swelling" following high doses of CsA in

Wistar-Furth rats. Fasel *et al.* (1987) reported necrosis of individual medial smooth muscle cells by electron microscopy in male Wistar rats treated with CsA (100 mg/kg/day po for 10 days).

An acute "arteriopathy" similar to hemolytic-uremic syndrome has been reported in a number of patients receiving cyclosporine (Shulman *et al.*, 1981). A possibly analogous phenomenon has been described in a serum sickness model in the rabbit. Neild *et al.* (1983a) treated rabbits with CsA (15 or 25 mg/kg/day im), vehicle, or no treatment. Serum sickness was produced by giving an iv injection of bovine serum albumin (250 mg/kg) with or without *Escherichia coli* endotoxin. In the kidney, glomerular capillary thrombosis and cortical infarction were seen in cyclosporine-treated animals, although necrotizing arterial injury detected in other organs did not involve the intrarenal arteries.

These investigators have examined PGI_2 synthesis by vascular tissue in rabbits (Neild *et al.*, 1983b). Rabbits were given CsA (25 mg/kg im) or vehicle for 5 days, bled on alternate days, and sacrificed on day 12. Vascular PGI_2 synthesis was measured using aortic rings from CsA-treated or control rabbits and no differences were found; direct effects of CsA assessed by incubating aortas from normal rabbits in CsA-containing buffer revealed no effect on PGI_2 generation. However, plasma from CsA-treated rabbits had profoundly decreased levels of PGI_2-stimulating factor (PSF), as assessed by the ability of PGI-depleted aortas from normal rabbits to convert exogenous arachidonic acid into PGI_2, and this effect persisted despite discontinuing CsA. CsA added to normal plasma *in vitro* had no such effect. CsA appeared to act by preventing production or release of PSF (perhaps as part of a general inhibition of lymphokine release by CsA?), rather than acting as a direct inhibitor of PGI_2 generation. However, Gordon *et al.* (1987), using cultured human umbilical vein endothelial cells, have found that addition of CsA to the medium reduced PGI_2 formation (by 25–30% at 10 μM and 75–80% at 100 μM), even when exogenous arachidonate was added. These cells recovered their ability to synthesize PGI_2 within 24 hours, and the effect appeared to be due to a reversible inhibition of cyclooxygenase.

Using a bovine aortic endothelial cell culture system, Zoja, Remuzzi, and co-workers (1986) have directly assessed cyclosporine-induced endothelial cell injury. They demonstrated a time- and dose-dependent cell injury manifested by early cell detachment and cell lysis when exposed to CsA *in vitro* at concentrations of 10 and 50 μM CsA. Production and release of PGI_2 (as measured by release of 6-keto-$PGF_{1\alpha}$) and TxA_2 (as measured by TxB_2 release) were increased at 10 and 50 μM CsA. Concentrations of CsA used in these *in vitro* experiments were higher than those reached in plasma, but comparable to tissue levels detected in kidney and liver. The

authors suggested that a direct cytotoxic effect of CsA on endothelial cells may contribute to the pathogenesis of CsA-induced vascular damage. Brown and Neild (1987) found no effect of up to 2 μg/ml (1.7 μM) CsA on viability or morphology of umbilical vein endothelial cells in culture, although PGI_2 synthesis was inhibited.

G. Cyclosporine and the Ischemic Kidney

Effects of cyclosporine on ischemically damaged kidneys have been examined in a number of animal models. Such models are felt to be reasonably analogous to the renal transplant setting.

Jablonski *et al.* (1986) have recently assessed effects of varying doses of cyclosporine on ischemic rat kidneys. Oral cyclosporine at doses of 0, 5, 10, and 25 mg/kg/day were administered over 2 weeks following 30 or 60 minutes of warm ischemia plus contralateral nephrectomy, or uninephrectomy alone. SUN and serum creatinine were assessed at intervals. Unilateral nephrectomy with or without 30 minutes of warm ischemia produced no change in SUN and serum creatinine. Unilateral nephrectomy with or without 30 minutes of warm ischemia followed by 25 mg/kg/day CsA resulted in elevations in SUN but no detectable change in serum creatinine. Following 60 minutes of warm ischemia, there were marked elevations of SUN and creatinine, which were normalizing but not normal after 2 weeks; addition of CsA, at 10 or 25 mg/kg/day resulted in significantly higher SUN and creatinine levels at 4, 6, 8, and 15 days. Histologically, uninephrectomy with or without warm ischemia resulted in hypertrophy and interstitial inflammation; cyclosporine (25 mg/kg/day) reduced this hypertrophic response. These studies suggest that cyclosporine does exacerbate renal dysfunction seen after an ischemic insult and that kidneys undergoing compensatory hypertrophy may be more susceptible to the toxic effects of CsA. A number of investigators have used mild ischemic injury to potentiate renal effects of cyclosporine (Iaina *et al.*, 1984; Kone *et al.* 1986).

Chow *et al.* (1986) have reported that ischemia and CsA do not appear to have a synergistic effect on renal function in the rat. Following bilateral renal artery clamping (30–40 minutes) or sham surgery, parenteral CsA was given sc at doses of 5, 25, and 50 mg/kg/day; controls were given the cremophore plus ethanol or saline. Inulin clearances were measured and blood and tissue were obtained at day 2, 4, and 7 after ischemia. Animals undergoing sham surgery with CsA had a 2-fold rise in SUN, and inulin clearance was reduced to 50% of those levels in sham animals receiving vehicle. CsA combined with ischemia resulted in a delay in recovery from

the ischemic insult, with an increase in SUN and inulin clearance reduced to 50% of controls at day 7. Serum creatinine and fractional excretion of sodium were not different in cyclosporine-treated animals and controls. Histological examination revealed tubular necrosis in all ischemic kidneys with similar changes in cyclosporine- and vehicle-treated animals. Tubular vacuolation was present in both CsA- and vehicle-treated animals, and was more extensive in kidneys undergoing ischemia. Trough levels of CsA were 400–500 ng/ml (by RIA). Kidney cyclosporine levels were not increased in animals manifesting nephrotoxicity. Comparable levels of renal function at day 7 in cyclosporine-treated animals with sham surgery or ischemia suggests a superimposition of CsA nephrotoxicity on the kidneys recovering from ischemic injury rather than a synergistic effect.

Effects of CsA in ischemically damaged kidneys have also been assessed in rabbits and dogs, two species relatively resistant to CsA nephrotoxicity (Aeder *et al.*, 1984; Ryffel *et al.*, 1983). Dieperink and Starklint (1983) studied the effect of CsA on ischemically damaged kidneys in the French lop-eared rabbit. Animals underwent right nephrectomy and left renal artery clamping for 75 minutes, followed by daily po doses of vehicle or CsA, 25 mg/kg/day. No differences in serum creatinine at 24 hours or creatinine clearance at 12 days were found. Homan *et al.* (1980) used a dog model to assess effects of CsA on the function of ischemically damaged renal autografts. Animals for study were bilaterally nephrectomized and one kidney was retained in the abdominal cavity to create 60 minutes of warm ischemia, following which the ischemic organ was transplanted as an autograft. One group of autografted dogs received CsA, 20 mg/kg/day po for 2 weeks; a second group received no treatment, and a third group received allografts and CsA. There was no consistent difference in renal function (evaluated by serum creatinine) between dogs that received CsA and those that did not. The kidneys without deliberate ischemic injury (allograft group) had consistently lower serum creatinine levels, indicating that the elevation in creatinine levels were attributable to ischemic injury.

H. Cyclosporine, Reduced Renal Mass, and the Transplanted Kidney

It is commonly observed that renal transplant recipients receiving CsA have higher serum creatinine levels than those on conventional therapy (Canadian Multicentre Transplant Study Group, 1983; European Multicentre Trial Group, 1983; Calne and Wood, 1985), and that conversion to conventional therapy in the posttransplant period often results in rapid reductions in serum creatinine (Henny *et al.*, 1985). It has been suggested

that CsA may interfere with adaptive changes in renal hemodynamics and GFR which normally occur in transplanted kidneys, and may suppress adaptive hypertrophy in these organs. Effects of CsA on compensatory renal adaptation have been studied in animals using models of reduced renal mass, which are not complicated by ischemia or rejection.

Schurek *et al.* (1986) examined the influence of CsA on adaptive hypertrophy after unilateral nephrectomy in juvenile rats. Following left nephrectomy, rats were kept on an 18% protein diet and given 10 or 15 mg/kg/day CsA by gavage, or an equivalent volume of olive oil vehicle. After 4 weeks on this regimen, the remaining kidney from uninephrectomized oil-treated control rats showed a significant increase in kidney weight, *in vivo* creatinine clearance, and inulin clearance (as measured *in vitro* in isolated, perfused kidneys) compared to two-kidney controls. Micropuncture studies on the isolated perfused kidneys removed from uninephrectomized control rats revealed significant increases in single-nephron GFR, K_f (estimated from stop-flow intratubular pressures), glomerular basement membrane surface area (determined by morphometry), and effective hydraulic permeability compared to two-kidney controls. CsA suppressed all of these compensatory changes except the increase in glomerular hydraulic permeability.

Whiting *et al.* (1986) reported studies in rats assessing renal function in animals treated with cyclosporine following unilateral nephrectomy. DA rats were divided into three groups—no surgery, laparotomy only, and uninephrectomy—and given CsA, 25 mg/kg/day for 4 weeks. Renal function in uninephrectomized rats was significantly worse than in other groups, and there was extensive tubular vacuolation in this group. All four animals in the uninephrectomized group showed increased vacuolation and frank nuclear pyknosis in proximal straight tubules histologically. Kidney CsA levels were similar in all groups. These studies suggest that the kidney undergoing compensatory hemodynamic changes and hypertrophy may be more susceptible to toxic effects of CsA.

In contrast to these studies, Brunner *et al.* (1986b) studied effects of CsA begun in Wistar rats 5 weeks after partial infarction of the left kidney and 3 weeks after contralateral nephrectomy (total renal ablation of 4/5 to 5/6). Animals were fed CsA (approximately 5, 10, and 20 mg/kg/day). Every 5 weeks, proteinuria, and creatinine clearance were assessed; surviving animals were sacrificed at 20 weeks. Body weight, serum creatinine, and creatinine clearance were not different from controls. Proteinuria rose progressively up to 15 weeks in all groups except the 20 mg/kg/day group. Groups receiving 10 or 20 mg/kg/day CsA had significantly *less* glomerular sclerosis and hypertensive vasculopathy than low-dose and control groups. Results in this model, in which administration of CsA was delayed until

several weeks after ablation was completed, suggest that CsA may not be deleterious once adaptive changes in the remnant renal mass are established. Indeed, CsA may be beneficial in this setting, perhaps related to a decrease in BP and possibly glomerular capillary pressure in these animals.

I. Nephrotoxicity and Hepatic Drug Metabolism

Cyclosporine is metabolized in the liver by the microsomal *P*-450 mixed-function oxidase enzymes. There have been clinical reports that rifampicin and phenytoin, which induce this enzyme system, decrease CsA blood levels and decrease the drug's immunosuppressive action (Wood and Lemaire, 1986; Langhoff and Madsen, 1983). Conversely, ketaconazole, an antifungal agent which inhibits this enzyme system, increases blood levels and increases nephrotoxicity (Ferguson *et al.*, 1982). Similarly, it has been proposed that cimetidine enhances CsA nephrotoxicity by enzyme inhibition (Wood, 1983).

In several studies, Cunningham *et al.* (1984, 1985) and Whiting *et al.* (1986) have evaluated effects of hepatic drug metabolism on CsA-induced nephrotoxicity in rats. "Nephrotoxicity," as assessed by enzymuria and light microscopic changes, was assessed over 7 weeks in animals given CsA, 25 mg/kg/day po, with groups of animals sacrificed at 0, 4, 7, 10, and 14 days, and weekly thereafter. Serum CsA levels (using RIA, which detects parent drug plus some metabolites), and hepatic microsomal monooxygenase enzyme systems were also monitored. Enzymuria and tubular vacuolation were significantly increased in 10 days and continued for 4 weeks, followed by a period of remission of renal changes, followed by return of renal injury at 6 weeks. Aminopyrine *N*-dimethylase activity was reciprocally related to observed renal changes, and activity of NADPH cytochrome *c* reductase tended to parallel those of *N*-dimethylase. However, cytochrome *P*-450 concentration did not change. Serum CsA levels fell during the period of remission. These results led the authors to suggest that spontaneous remission of CsA-induced nephrotoxicity is due to reduction in circulating drug levels caused by increased hepatic CsA metabolism.

Later studies by this group of investigators have utilized a more thorough assessment of renal toxicity, assessing both tubular cell histopathology and functional changes as measured by SUN and serum creatinine (Cunningham *et al.*, 1985). In these studies, a range of hepatic enzyme inducers, including Aroclor 1254, 3-methylcholanthrene, and sodium phenobarbitone were utilized. Rats were given CsA, 50 mg/kg/day po, for 14 days. Serum CsA levels were determined using RIA, and microsomal

P-450 concentration and monooxygenase activity determined. At this dose of CsA, an elevation in SUN and tubular vacuolation were seen. With concomitant administration of phenobarbitone, trough serum CsA levels were decreased, renal histology was normal, and the rise in SUN, though still present, was lower. Aroclor 1254 administered with CsA normalized SUN and renal histology (although it did not prevent an increase in urinary NAG levels), but did not significantly reduce serum CsA levels. 3-Methylcholanthrene alone produced a slight but significant increase in SUN, but did not prevent the rise in SUN and urinary NAG or the tubular vacuolation produced by CsA. These results suggest either primary involvement of the cytochrome *P*-450-dependent monooxygenase enzyme system or induction of UDP-glucuronyl transferase and an induction of CsA conjugation.

Results of these animal studies, as well as results of studies examining effects of concomitant administration of other drugs, as discussed below, which are inhibitors or inducers of microsomal enzymes in both animals and humans, illustrate potential dangers of altering hepatic metabolism of cyclosporine.

J. Potentiation of Cyclosporine Nephrotoxicity by Other Drugs

Both clinically and in experimental animals, nephrotoxic effects of cyclosporine are potentiated by concurrent administration of other drugs, a number of which are commonly used in the transplant setting. The topic of drug interactions with cyclosporine in animal models has been reviewed by Whiting *et al.* (1986).

Whiting *et al.* (1984) described enhancement of high-dose CsA nephrotoxicity by furosemide, a commonly used diuretic. CsA was administered to rats at a dose of 50 mg/kg/day for 14 days, with or without concomitant administration of furosemide, 5 mg/kg/day. Furosemide alone was not nephrotoxic. CsA alone produced relatively mild elevations in SUN and creatinine and a progressive decline in urea nitrogen (UN) and creatinine clearances, with enzymuria and vacuoles in the straight segment of the proximal tubule. When CsA and furosemide were given together, there was a marked reduction in renal function, vacuoles in both straight and convoluted segments of the proximal tubule, and a doubling of serum CsA levels. There was a linear correlation between serum CsA (measured by RIA) and SUN and serum creatinine. These results were ascribed to enhanced toxicity due to Na^+ depletion produced by the diuretic and/or a decrease in hepatic drug metabolism due to suppression of hepatic enzymes by furosemide, resulting in persistence of the nephrotoxic parent compound. We (Racusen *et al.*, 1985) confirmed furosemide's adverse effects on

cyclosporine nephrotoxicity in rats and showed that the effect could not be attributed to dehydration alone.

Assessment of renal function in rats treated with CsA (25 mg/kg/day) alone or in combination with gentamicin (50 mg/kg/day) or cephalosporin (100 mg/kg/day) for 14 days revealed significant potentiation of nephrotoxicity with the combination of CsA and gentamicin (Whiting *et al.*, 1983). CsA or gentamicin alone at these doses produced only mild renal impairment, and cephalosporin produced no significant changes. The combination of CsA and cephalosporin produced a mild increase in UN and slight decrease in UN clearances, with little or no change in creatinine or creatinine clearance. However, CsA and gentamicin given together resulted in frank ARF with vacuolation of the proximal straight segment and necrosis of the proximal convoluted tubule.

Because of clinical reports that ketoconazole, an antifungal agent, potentiates renal toxicity of CsA (Ferguson *et al.*, 1982; Gluckman *et al.*, 1981), Dieperink *et al.* (1986a) examined effects of concomitant ketoconazole and CsA on rat renal function, and serum and tissue CsA levels. CsA (12.5 mg/kg/day by gavage) was given with ketoconazole (20 mg/kg/day) or vehicle for 14 days. Ketoconazole significantly increased serum, liver, and kidney CsA concentrations. Nephrotoxicity was also significantly increased, as measured by decreased inulin and lithium clearances, decreased absolute proximal tubular absorption, and increase in fractional proximal reabsorption. Serum and kidney CsA levels (determined by RIA) correlated with lithium clearance and fractional proximal reabsorption. Hepatic cytochrome *P*-450 *O*-diethylase activity was not decreased in the ketoconazole plus CsA group compared to vehicle plus CsA, but *N*-demethylase activity, which may be more relevant to CsA metabolism, was not assessed. Ketoconazole is known to inhibit microsomal enzyme metabolism in the rat at high doses (>50 mg/kg) (Niemegeers *et al.*, 1981).

In contrast to the above studies, Ryffel *et al.* (1986b) found no exacerbation of CsA-induced nephrotoxicity by supratherapeutic but nontoxic doses of gentamicin (20 mg/kg/day sc), ketoconazole (20 mg/kg/day po), amphotericin B (5 mg/kg/day ip), or acyclovir (20 mg/kg/day po) in rats. They gave 20 mg/kg/day CsA for 4 weeks, producing slight increases in SUN and creatinine, and slight reduction in creatinine clearance, associated with proximal tubular vacuolation, inclusion bodies, and microcalcification. None of these agents at the doses used produced renal dysfunction or morphological changes or exacerbated CsA-induced changes, although ketoconazole did cause a marked increase in plasma CsA levels. Gentamicin at toxic doses of 50 and 100 mg/kg/day produced functional impairment and extensive tubular necrosis and regeneration. Combined treatment with CsA and high doses of gentamicin produced a marked increase in functional and structural changes.

Brunner *et al.* (1986a) noted a possible deleterious interaction between CsA and mannitol in a kidney transplant recipient, and pursued this interaction in a rat model. Rats received CsA 40–50 mg/kg in either 20% mannitol or normal saline, or 20% mannitol alone by continuous infusion via a jugular cannula for 3 or 4 days. Sixty percent of the CsA–mannitol rats developed acute oligoanuric renal failure, with "massive" vacuolation in proximal tubules of rats with ARF and "mild-to-moderate" vacuolation in rats without ARF. In the two animals with severe ARF that could be studied, RBF appeared to be well maintained and proximal tubular pressure was decreased, ruling out tubular obstruction as a cause of filtration failure. No vacuolation was seen in CsA–saline or mannitol-infused rats; "moderate" amounts of tubular cell inclusions and microcalcifications were detected in CsA–mannitol-infused rats without ARF and in CsA–saline-infused rats. The mechanism of this potentiating effect of mannitol on CsA nephrotoxicity is unclear.

Schwass *et al.* (1986) have examined effects of two widely used drugs—phenobarbital, a microsomal enzyme inducer, and cimetidine, a microsomal enzyme inhibitor—on CsA-induced nephrotoxicity in a Fischer 344 rat model. CsA (25 or 40 mg/kg/day) or vehicle was administered by gavage for 14 days with concurrent ip administration of phenobarbital (75 mg/kg), cimetidine (75 mg/kg), or saline. Cimetidine had no effect on CsA-induced nephrotoxicity, but also had no measured effect on cytochrome *P*-450 levels. Animals receiving CsA plus phenobarbital had significantly higher inulin clearance and lower serum creatinine at 25 mg/kg/day, with suggestive but not significant protection at 40 mg/kg/day CsA. Despite the protective effect of phenobarbital, however, no induction of hepatic cytochrome *P*-450 could be demonstrated in CsA–phenobarbital rats, so it remains possible that phenobarbital may provide protection via other mechanisms.

Some of the newer calcium channel blockers including nicardipine (Cantarovich *et al.*, 1987) and diltiazein but not nifedipine (Wagner *et al.*, 1988) lead to a marked increase in CsA blood levels sometimes associated with clinical nephrotoxicity. This interaction has not been studied experimentally but may relate to a change in distribution volume of CsA rather than changes in absorption, bioavailability, or elimination.

K. Cyclosporin G and the Kidney

Hiestand *et al.* (1985) reported results in a Wistar rat model using 100 mg/kg/day cyclosporin G (CsG) for 14 days. These authors found no evidence of nephrotoxicity or hepatotoxicity. In contrast, Duncan *et al.*

(1985) administered 50 mg/kg/day CsA or CsG po for 14 days and found comparable (though significantly milder) elevations in SUN and serum creatinine, comparable decreases in UN and creatinine clearances, and comparable increases in NAG enzymuria in animals treated with the G cogener compared with CsA.

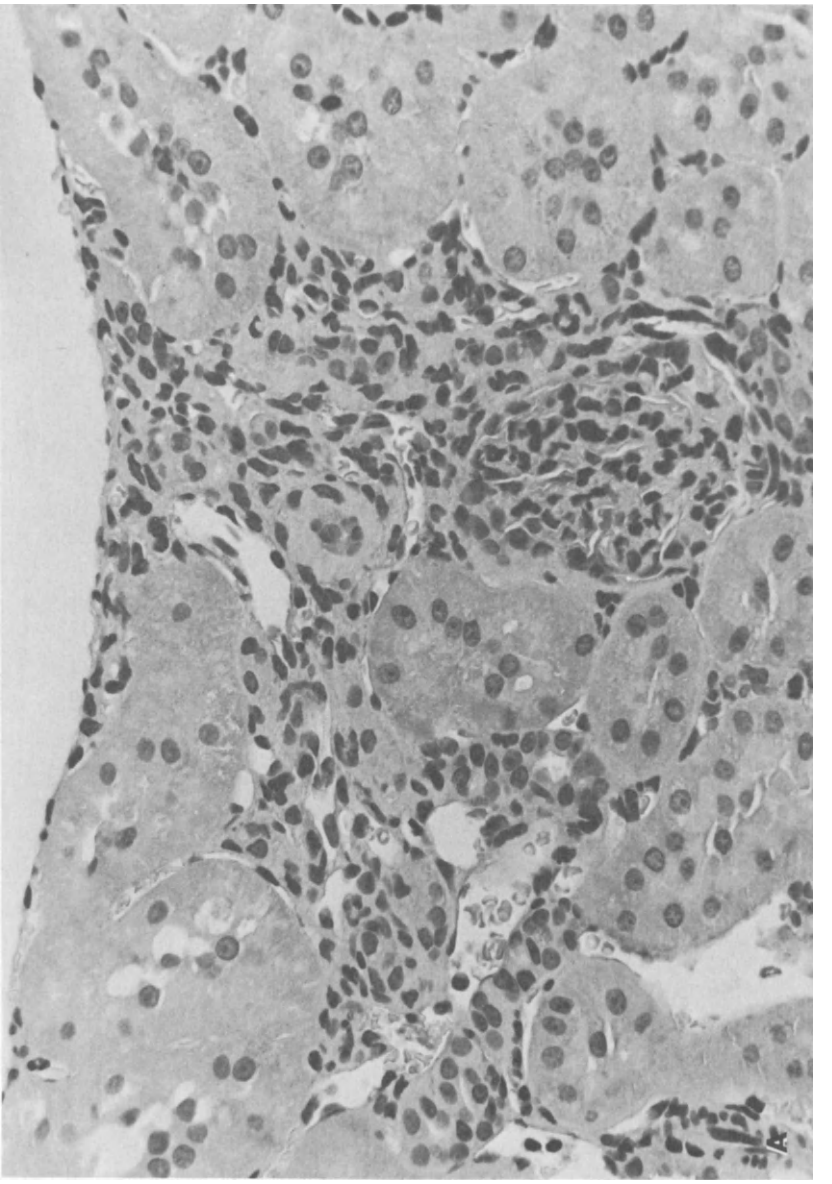
In our laboratory we have observed significantly less nephrotoxicity, neurotoxicity, and mortality with CsG compared to CsA when 60 mg/kd were given ip to Munich-Wistar rats for 4 days (L. C. Racusen, B. Kone, and K. Solez, unpublished).

Paller and Ferris (1987) have studied renal hemodynamic effects of CsG in rats. In acute infusion studies, CsG (10 or 20 mg/kg) reduced GFR, RPF, and RBF by approximately 50%. Daily ip injection of CsG (20 mg/kg/day) for 7 days reduced creatinine clearance by 19%. In contrast, Lancman *et al.* (1987) treated rats with CsA or CsG (25 mg/kg/day) in olive oil sc for 21 days, and found that while CsA-treated animals had significant reductions in RPF (40%) and GFR (58%), CsG-treated rats had RPF and GFR identical to controls. The studies of Ogunnaike *et al.* (1987) in a primate cardiac transplant model suggest that CsG is a substantially less effective immunosuppressive compound than CsA.

L. Chronic Cyclosporine Nephrotoxicity

Most of the studies of effects of cyclosporine on renal structure and function in experimental animals have been carried out over brief periods, with so-called chronic effects assessed after 1–4 weeks. Since chronic administration of cyclosporine over months-to-years in patients has resulted in late renal dysfunction and fibrotic changes in the kidney (Myers *et al.*, 1984; Palestine *et al.*, 1986), assessment of chronic cyclosporine nephrotoxicity in animal models has considerable clinical relevance.

Ryffel *et al.* (1983), in summarizing extensive toxicological evaluations of CsA in a variety of animal models, reported results of "chronic" administration of CsA from 3 months to 1 year. In OFA rats given CsA at doses up to 90 mg/kg/day po for 13 weeks, proximal tubular epithelial cell alterations and "moderate" elevations in SUN were reported, without glomerular or interstitial pathology. Rhesus monkeys given CsA at doses up to 300 mg/kg/day po for 13 weeks had no evidence of nephrotoxicity. Similarly, there was no evidence of nephrotoxicity in beagle dogs given up to 45 mg/kg/day for 1 year. OFA albino rats studied for 2 years at doses of up to 8 mg/kg/day po developed increases in SUN and creatinine at the highest dose, with morphological changes (not specified) seen at 2 and 8 mg/kg/day. The authors reported an increase in extent and severity



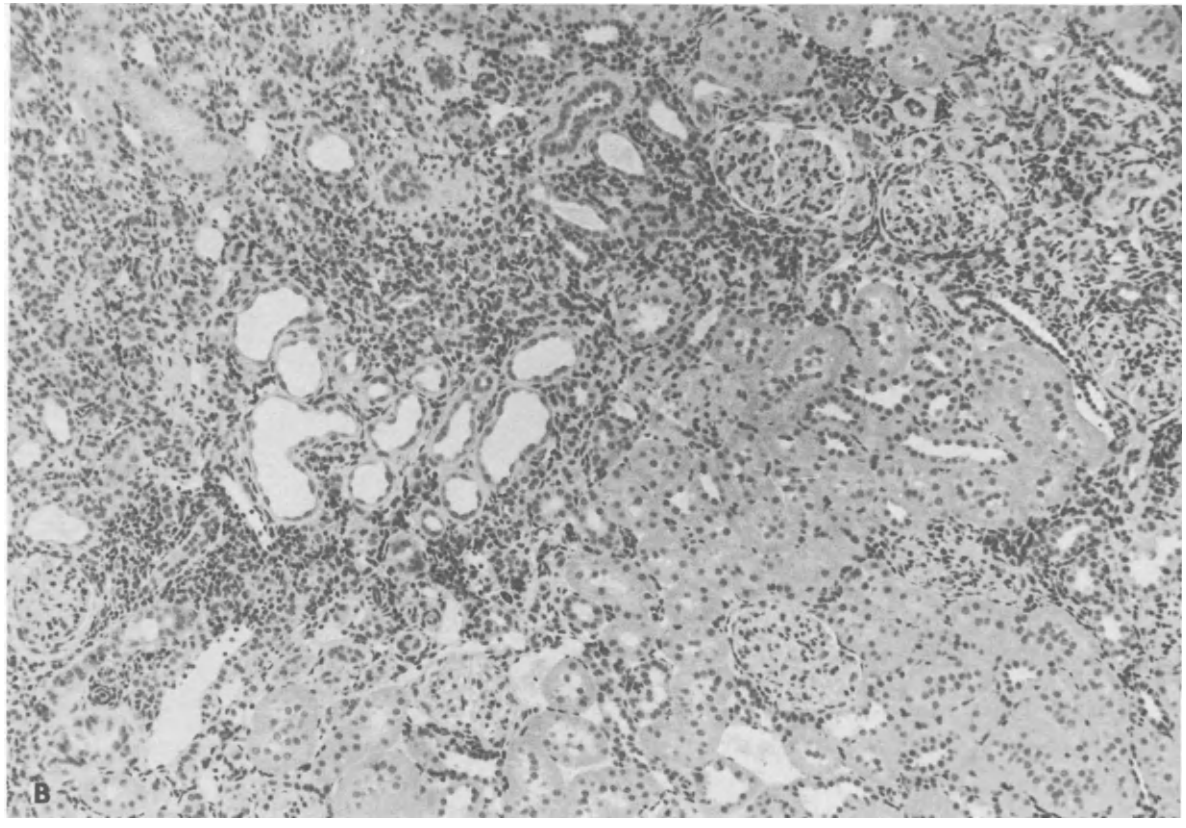


FIG. 2. (A) Triangular area of subcapsular fibrosis in a rat given cyclosporine 12.5 mg/kg/day po for 8 weeks. (B) Area of parenchymal atrophy and scarring in a rat treated with cyclosporine 12.5 mg/kg/day po and sacrificed 8 weeks after cessation of cyclosporine treatment. Although the mononuclear cell inflammatory infiltrate appears prominent in this picture, this feature was not significantly different in control and experimental animals. On the other hand, the subcapsular fibrosis was found in 71% of cyclosporine-treated rats compared to 3.4% of controls ($p < 0.001$), and the striped interstitial fibrosis in a deeper cortex was found in 24% of the cyclosporine-treated rats and in none of the controls ($p < 0.02$). [From the studies of Starklint *et al.* (1987). Used by permission.]

of a strain-specific "chronic progressive nephropathy" as well as an aggravation of naturally occurring polyarteritis nodosa in these animals as well. This "chronic progressive nephropathy" consists of tubular degeneration and regeneration of tubules and interstitial inflammation and fibrosis (Richardson and Luginbuhl, 1976).

Bertani *et al.* (1987) studied Sprague–Dawley rats receiving vehicle or CsA, 40 mg/kg po every other day, for up to 5 months. An additional group received CsA as above for 3 months, and were then studied 2 months after withdrawal. After 1 month, pathological changes were confined to the proximal tubule, with cytoplasmic vacuoles and focal loss of brush border. After 3 months, proximal tubular changes with CsA were more pronounced, and in addition there was increased mesangial matrix and intracapillary inflammatory cells in the glomeruli, dilatation and congestion of peritubular capillaries, and limited areas of interstitial fibrosis which were mainly perivascular. At 5 months, while proximal tubular vacuolization remained stable, brush border loss increased. In addition, there was deposition of glycogen in early distal tubular cells, possibly related to the diabetogenic effect of CsA, and evidence of mild glomerular capillary endothelial cell damage by EM. Associated with these morphological changes, there was a significant increase in urine volume and fractional excretion of sodium, and a significant decrease in GFR by 3 months, but RPF remained unchanged. GFR did not correlate with proximal tubular vacuolation, but *did* correlate with brush border loss.

In animals in which CsA had been discontinued for 2 months, there was normalization of tubular and glomerular changes, but mild interstitial fibrosis persisted. A few of these rats also had glycogen in the distal tubular cells, and these animals had significant polyuria; no other functional changes were detectable in this group 2 months after CsA withdrawal. No vascular changes were seen in any experimental group in these studies. The "striped" interstitial fibrosis seen in humans (Mihatsch *et al.*, 1985; Keown *et al.*, 1986) was not described.

H. Starklint *et al.* (1987) studied the effect of CsA up to 25 mg/kg/day given for 16 weeks to Sprague–Dawley rats and found focal striped interstitial fibrosis (Fig. 2) with a granular renal cortical surface and normal or increased kidney size. Severity of fibrosis correlated with accumulated CsA dose and did not appear to be reversible. Functional alterations in GFR and lithium clearance were also dose related and were only partially reversible (H. Dieperink and H. Starklint, personal communication, 1986). Gillam *et al.* (1988) have recently reported a similar model.

A group of investigators from Stanford (Ogunnaike *et al.*, 1986) has reported on studies comparing CsG and CsA in a cardiac transplant model

in cynomolgus monkeys, a strain previous shown to be sensitive to the nephrotoxic effects of CsA (Ryffel *et al.*, 1983). Following orthotopic cardiac transplantation, animals were immunosuppressed with 16 mg/kg im of Norvalene-2-cyclosporine (CsG) or CsA, given with a tapered dose of methylprednisolone. Animals were studied weekly for up to 1 year; a control group receiving cyclosporine–methylprednisolone with no transplant were also followed for 1 year. Heart and kidneys were taken at the time of death for histologic examination. Animals treated with CsG survived an average of 17 days, with all monkeys dying of cardiac rejection; trough CsG levels averaged 119 ng/ml compared to 208 ng/ml for CsA in transplanted animal groups. CsA animals survived a mean of 75 days, with 6 of 10 animals dying of cardiac rejection. Kidneys from both groups showed mononuclear interstitial infiltrate, proximal tubular damage, and interstitial fibrosis, with fibrosis more common in CsA-treated animals, probably due to relatively prolonged survivals in these animals. Details of renal functional parameters and the nature of the interstitial fibrosis were not available.

Jackson and Humes (1986) have recently reported findings that may have relevance to the development of chronic renal injury following cyclosporine administration. They investigated the incorporation of [^{3}H]thymidine into renal cortical DNA in salt-depleted rats treated with CsA, 100 mg/kg/day sc for 8 days. There was a significant incorporation into renal tissue, greatest in medulla and papilla. Morphometric analysis of autoradiographic specimens revealed that thymidine was being incorporated into cells in the interstitium, perhaps indicating a primary proliferative process. These cells most likely represent fibroblasts (Jackson *et al.*, 1987).

M. Summary of Studies in Experimental Models of CsA Nephrotoxicity

While early studies at high doses of CsA suggested that CsA produced direct tubular injury, it has become clear that the more striking alterations in renal tubules can be dissociated from changes in renal and tubular function and that the parenteral vehicle for cyclosporine can produce morphologically similar changes, although tubular cell alterations do appear to correlate with renal dysfunction in the "maintenance phase" of cyclosporine nephrotoxicity. Alterations in RBF and RVR appear to be very early pathophysiological events, but can be disproportionate to or dissociated from alterations in GFR, suggesting a possible alteration in K_f as an additional factor in pathogenesis. α-Adrenergic blockers prevent changes in RBF and GFR, and CsA has been shown to activate renal and genitofemoral sympathetic nerves, suggesting a neural mechanism, although the relevance

of these observations to the denervated renal transplant is unclear. Endogenous PG appear to ameliorate renal effects of cyclosporine; exogenous PG have similar effects but also seem to reduce immunosuppressive efficacy of the drug. In recent studies, treatment with a Tx synthetase inhibitor prevented renal dysfunction, although the drug did not restore RBF to normal. The calcium channel blocker nifedipine also appears to prevent nephrotoxicity if given concurrently with CsA, presumably by preventing increases in vascular resistance, although other mechanisms are possible. While tubuloglomerular feedback has been proposed as a pathogenic mechanism in cyclosporine-treated animals and renin and/or aldosterone levels are frequently elevated in animal models, recent inhibitor studies suggest that if tubuloglomerular feedback is involved, it is not mediated by angiotensin II or adenosine.

While there are a number of models of tubular morphological changes and/or renal function, only one animal model, the SHR, develops arteriolotoxic lesions comparable to those described in humans by light microscopy. Vascular prostacyclin (PGI_2) production was shown to be depressed by cyclosporine in a rabbit model, probably due to decreased production of PSF. *In vitro,* relatively high concentrations of CsA have been shown to produce endothelial cell injuries in a bovine aortic endothelial cell culture system. Similarly, there are few reports in experimental animals of the type of chronic toxic lesions seen in humans, with "striped" interstitial fibrosis and renal enlargement. However, studies of thymidine incorporation in rats treated with high doses of CsA suggest a possible primary fibroblast proliferative process.

While there are no published studies of effects of cyclosporine on renal transplants in animal models, studies on ischemically injured kidneys and on renal function in the setting of reduced renal mass are reasonably analogous. Ischemic kidneys may be more susceptible to CsA toxic effects. With reduction in renal mass, CsA given soon after ablation appears to interfere with adaptive hemodynamic alterations and compensatory hypertrophy. A recent study in a rat model with ischemic denervated kidneys suggests a role for vasoconstrictor PG in the pathogenesis of CsA toxicity in this setting.

III. Clinical Cyclosporine Nephrotoxicity

It is not clear that the nephrotoxic and immunosuppressive effects of cyclosporine can be separated, that is, that there is a real "therapeutic window" of completely nontoxic but effective doses of the drug. It is likely that almost all patients treated with adequate immunosuppressive doses of

the drug develop at least mild impairment of renal function. If sensitive and accurate serial measurements of GFR were employed in all cyclosporine-treated patients, it is likely that the rate of detected renal dysfunction would approach 100%. Most transplant patients have demonstrated an improvement in renal function when they are converted from cyclosporine to azathioprine immunosuppression, as long as rejection does not occur (Morris *et al.*, 1987; Hoitsma *et al.*, 1987). This finding supports the idea that cyclosporine reduces renal function in most patients in whom it is used. In many cases, however, the renal dysfunction produced by cyclosporine is mild and probably clinically unimportant, at least over the short term.

Four discrete syndromes of clinically significant renal dysfunction caused by cyclosporine in the native or grafted kidney may be discerned (Keown *et al.*, 1986; Greenberg *et al.*, 1987): (1) acute reversible renal functional impairment, usually occurring early in the course of treatment, (2) delayed renal allograft function, (3) acute vasculopathy (thrombotic microangiopathy), and (4) chronic nephropathy with interstitial fibrosis. Various combinations of these syndromes may occur in a given patient, and of course, they can be combined with transplant rejection or other types of renal disease not directly related to cyclosporine therapy.

A. Acute Reversible Renal Functional Impairment

This syndrome is probably most similar to acute models of nephrotoxicity in animals. The earliest reports of this clinical syndrome were in patients receiving cyclosporine following bone marrow transplantation (Powles *et al.*, 1978). Cyclosporine was often administered parenterally in high doses (20 mg/kg/day), and acute impairment of renal function was characteristically observed in the presence of very high serum levels of cyclosporine, exceeding 1000 ng/ml (Powles *et al.*, 1978; Gluckman *et al.*, 1981). Renal functional impairment often occurred in patients who were also receiving other known nephrotoxic drugs, and was sometimes dramatic in onset and protracted.

In cardiac allograft recipients treated with cyclosporine, acute oliguric renal failure frequently occurs within the first 4 days posttransplant. Reporting on the University of Pittsburg experience, Greenberg *et al.* (1987) observed significant azotemia in 25 of 43 patients, with full-blown ARF in 5 of these. Urine sodium is characteristically less than 10 mEq/liter, and the oliguria is normally self-limiting with normal urine output returning by day 5 or 6 (Keown *et al.*, 1986; Devineni *et al.*, 1985; Greenberg *et al.*, 1987). Although peak serum creatinine levels tend to coincide with the highest trough serum cyclosporine levels during the first week posttrans-

plant, the absolute magnitude of the renal functional impairment correlates more closely with the degree of impaired renal function prior to cardiac transplantation than with serum cyclosporine levels, suggesting that patients with inadequate renal reserve are more susceptible to acute reversible renal functional impairment brought about by cyclosporine (Keown *et al.*, 1986). Similar episodes of ARF have been reported following liver transplantation, particularly when intravenous cyclosporine is used (Powell-Jackson *et al.*, 1983).

Although vasopressors were often in use during this period of acute reversible renal functional impairment in cyclosporine-treated cardiac allograft recipients, the patients did not appear to be volume-depleted or in a severe low-output state by clinical examination or cardiac-output measurements (Greenberg *et al.*, 1987). In fact, most patients had a significant persistent weight gain during this early posttransplant period. Nonetheless, the renal dysfunction observed does have features suggestive of a volume depletion "prerenal azotemia." SUN rises proportionately more than serum creatinine, and the kidney retains its ability to reabsorb sodium and to produce a concentrated urine. In these respects the renal dysfunction is like that reported in nonhypotensive sepsis in which azotemia occurs despite good hydration (Walker *et al.*, 1986). In the study of Greenberg *et al.* (1987), slight azotemia also occurred in the azathioprine-treated cardiac allograft group (mean BUN of 32 mg/dl on day 4), suggesting that a small but significant portion of the azotemia observed in the cyclosporine-treated group relates to the post-cardiac transplant status of the patients rather than to cyclosporine toxicity.

Characteristic of this syndrome of acute reversible renal functional impairment brought about by cyclosporine is the very rapid return of renal function when cyclosporine levels are reduced (Henny *et al.*, 1985). As is shown in Fig. 3, taken from the review by Myers (1986), the return of renal function tends to coincide with the normalization of serum cyclosporine levels, with the slope of declining levels and declining serum creatinine concentrations being approximately equal.

Patients treated with a lower dose of cyclosporine have a less dramatic onset of renal failure and a lower incidence of oliguria. The relationship between acute cyclosporine nephrotoxicity and circulating levels of the drug is controversial. However, nephrotoxicity is generally observed in association with rising or elevated serum levels exceeding 200 ng/ml and is common in patients with levels exceeding 400 ng/ml. Toxicity due to cyclosporine alone is rarely observed with serum cyclosporine levels of less than 200 ng/ml (Kahan *et al.*, 1983; Keown *et al.*, 1984).

The distinction between acute cyclosporine nephrotoxicity and acute renal transplant rejection is frequently difficult. Klintmalm *et al.* (1984) and

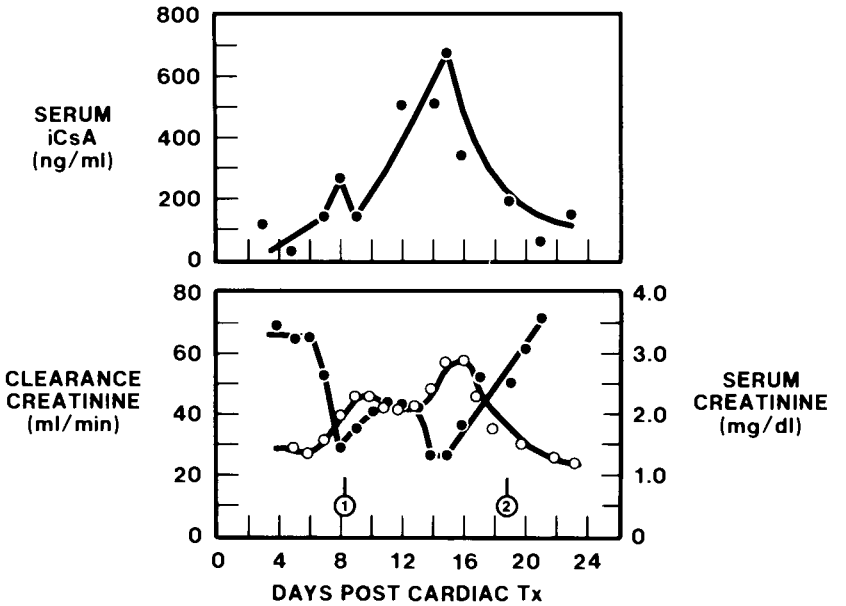


FIG. 3. Serum-immunoreactive cyclosporine, creatinine clearance, and serum creatinine in a heart allograft recipient undergoing an episode of reversible ARF induced by cyclosporine. [From Myers (1986). Used by permission.]

Flechner *et al.* (1984) have compared the clinical characteristics of these two situations. Acute rejection is rare after 4 months posttransplant. In renal allograft recipients, the increase in serum creatinine accompanying cyclosporine toxicity is generally slower, rising to 25% above baseline by approximately 1 week. Oliguria is less common than in acute rejection, and fever is highly unusual. In contrast, acute rejection is usually characterized by at least one of the following findings: oliguria, an increase in weight of more than 0.5 kg, fever exceeding 37.5°C, or a rapidly increasing serum creatinine level. Serum cyclosporine levels are generally higher during acute nephrotoxicity (>200 ng/ml) and have frequently risen just prior to the deterioration of renal function. In contrast, cyclosporine levels tend to be lower during acute rejection (<150 ng/ml), and often have fallen preceding the onset of rejection. In Keown's series (1984), a mean trough serum level during acute rejection within the first 3 months posttransplant was 165 ng/ml compared with 210 ng/ml during periods of stable allograft function.

Percutaneous needle biopsies of the grafted kidney as well as fine-needle aspirates have been used to distinguish between cyclosporine nephrotoxicity and acute rejection. Some typical appearances obtained through these

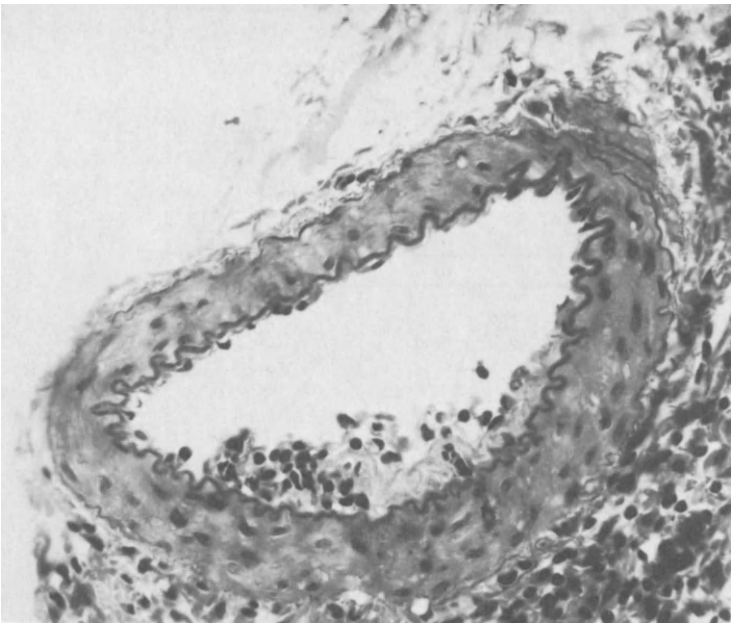


FIG. 4. Typical intimal arteritis of acute rejection. Note lymphocytes in the thickened intima. Hematoxylin and eosin, ×340. [Modified from Burdick *et al.* (1984).]

two techniques are illustrated in color plates in Williams *et al.* (1986). The presence of an intimal arteritis (Burdick *et al.*, 1984; Solez *et al.*, 1985) (Fig. 4) or of $Leu7^+$ intratubular lymphocytes (Beschorner *et al.*, 1985) are reliable indicators of the presence of acute rejection. Although tubular vacuolization and the presence of cytoplasmic inclusions are characteristic of cyclosporine therapy, they are not specific for cyclosporine nephrotoxicity. A substantial interstitial infiltrate of inflammatory cells can occur in the absence of rejection, and it is frequently present even in stable, well-functioning renal allografts (Solez *et al.*, 1985). On a histological basis, cyclosporine nephrotoxicity is often a diagnosis of exclusion made when definite signs of acute rejection are not present. Salaman and Griffin (1983) have described the use of fine-needle intrarenal manometry to distinguish rejection from cyclosporine nephrotoxicity. Acute rejection is associated with intense inflammation and interstitial edema, and therefore the renal interstitial pressure is elevated. Cyclosporine nephrotoxicity is associated with renal vasoconstriction without interstitial edema, and therefore interstitial pressure is low. It is certainly possible for rejection and cyclosporine nephrotoxicity to coexist, and the distinction between these two entities often depends on the response to therapy. If a reduction in the dose of

cyclosporine results in a fall of serum creatinine, this suggests cyclosporine nephrotoxicity. If, on the other hand, there is resolution of renal function following the administration of steroids or antilymphocyte globulin, this suggests the presence of acute rejection.

Kolbeck *et al.* (1987) have suggested that cyclosporine nephrotoxicity and rejection can be differentiated histologically using polyclonal sheep anticyclosporine antiserum to detect cyclosporine in tissues by morphometric techniques. However, there is significant nonspecificity in the staining patterns observed.

B. Delayed Renal Allograft Function

It is now well established that cyclosporine prolongs the duration of ischemic posttransplant ARF in renal allografts. Belitzsky *et al.* (1985) found that delayed function in patients receiving cyclosporine is related to the duration of cold storage of the graft. Countries such as Australia that do not use beating-heart donors have a significantly higher incidence of initial nonfunction. Shiel *et al.* (1984) showed that the incidence of initial nonfunction rose from 60 to 83% with cyclosporine therapy, and the mean duration of posttransplant failure which had been 13 days on steroid–azathioprine immunosuppression nearly doubled to 23 days.

In the Canadian Multicentre Trial, initial nonfunction did not appear to be an entirely benign condition even though most grafts eventually did regain function. Patients with initial nonfunction had a significantly longer hospital stay and increased number of treated "rejection episodes," a higher cumulative steroid dose, a higher serum creatinine 6 months posttransplant, and a lower graft survival (Belitzsky *et al.*, 1985; Stiller *et al.*, 1984). However, it is likely that factors such as early rejection and surgical complications were responsible for some of the nonfunction observed; thus, it cannot be concluded that uncomplicated ischemic injury was associated with an adverse outcome.

C. Acute Vasculopathy (Thrombotic Microangiopathy)

There has been a great deal of confusion between the changes of cyclosporine-induced arteriolopathy and the vascular changes of acute rejection. Differences between these two are clearly depicted in Figs. 4 and 5 and in color Figs. 2A and B in Williams *et al.* (1986). The intimal arteritis of acute rejection (Fig. 4) involves arcuate and interlobular arteries, in which there is a cellular intimal thickening with lymphocytes incorporated into the intimal mass. Cyclosporine arteriolopathy (Fig. 5), on the other

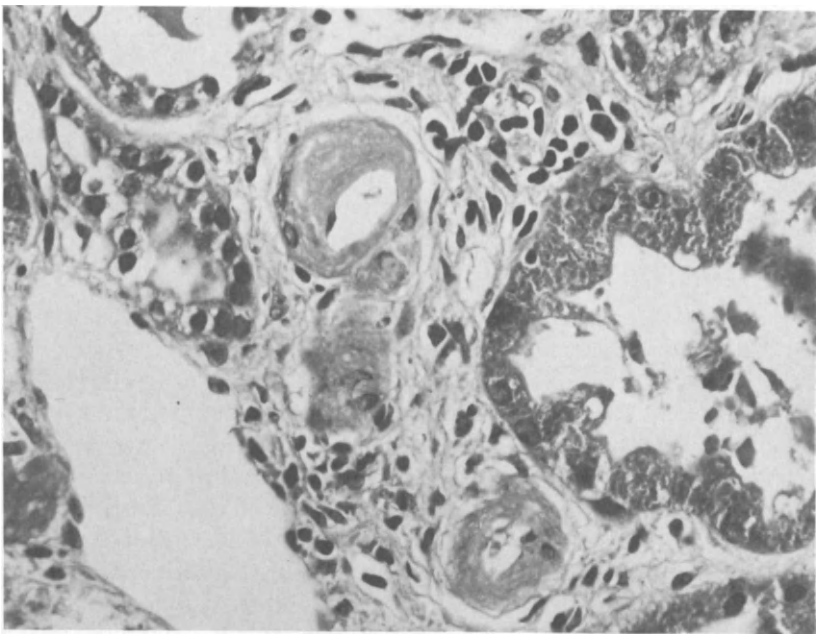


FIG. 5. Hyaline arteriolar thickening from the kidney of a patient manifesting cyclosporine nephrotoxicity. Hematoxylin and eosin, ×400.

hand, affects arterioles and glomerular capillaries and has no intrinsic inflammatory cell component. Arterioles show various combinations of hyaline and fibrinoid change, often with identifiable fibrinogen deposition by immunofluorescence. Glomerular capillaries show thrombosis with endothelial cell desquamation or nuclear pyknosis. Certainly, vascular lesions can occur which represent a combination of rejection and cyclosporine toxicity, and this may be the situation with the lesions described by Sommer *et al.* (1985). We have observed glomerular capillary thrombosis in biopsy specimens taken 1 hour after anastomoses from renal transplant patients pretreated with parenteral cyclosporine. For the first 80 Johns Hopkins Hospital renal transplant patients pretreated with cyclosporine, 7 have shown glomerular capillary thrombosis and/or endothelial injury in the 1-hour posttransplant biopsy (Fig. 6). In these cases, we excluded not only hyperacute rejection and perfusion injury as possible causes of the changes seen, but also such phenomena as the presence of cold-reactive IgM antibodies (Lobo *et al.*, 1984; Sturgill *et al.*, 1984) or red cell cold agglutinins (Schweizer *et al.*, 1982). By the process of exclusion, therefore, since unexplained capillary thrombosis had not occurred in our experience in the precyclosporine era, we concluded that cyclosporine was the likely

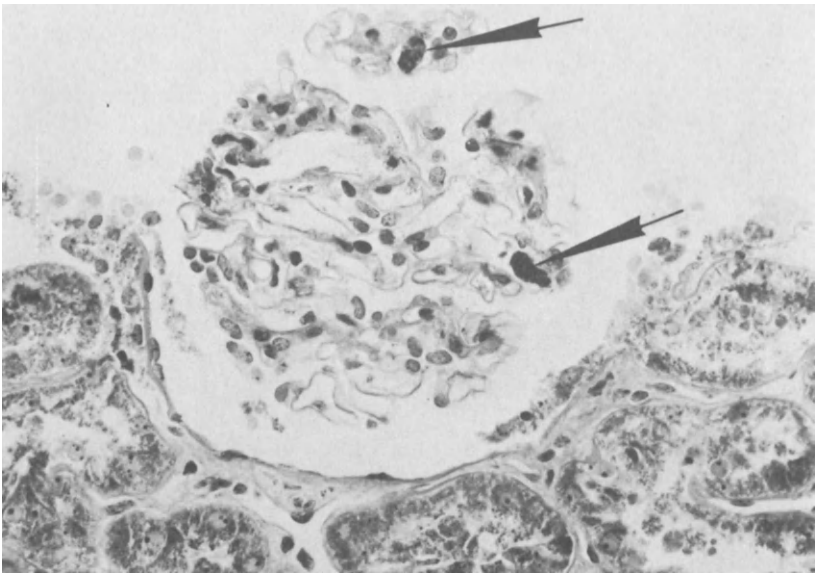


FIG. 6. Glomerular capillary thrombi (arrows) in a 1-hour posttransplant biopsy specimen from a renal allograft recipient pretreated with iv cyclosporine. PTAH, ×300.

cause of these changes. It is of interest that all 7 cases of glomerular capillary thrombosis in 1-hour posttransplant biopsies occurred in the winter months, and we have not observed this phenomenon before November or after mid-March. The seasonal predilection is like that seen in scleroderma renal failure (Cannon *et al.*, 1974), which is also characterized by intrarenal thrombosis.

Of the 7 patients in whom capillary glomerular thrombosis and/or endothelial injury were observed in the 1-hour posttransplant biopsy, one had primary nonfunction without signs of rejection and ultimately had the transplant removed; another had prolonged transplant dysfunction and never obtained a serum creatinine below 2.7 mg/dl, and the remainder had delayed function but ultimately lowered their serum creatinines below 2.7 mg/dl at times ranging from 14 to 150 days posttransplant (mean 74 days).

While intrarenal thrombosis has not been described by others as early as 1 hour posttransplant, there are numerous reports of thromboembolic complications occurring later in the posttransplant period in cyclosporine-treated renal allograft recipients (Vanrentergham *et al.*, 1985; Green *et al.*, 1985; Neild *et al.*, 1985). The first report of cyclosporine-induced vascu-

lopathy was that of Shulman *et al.* (1981) in bone marrow allograft recipients. These vascular changes may relate to the endothelial damaging and thrombogenic effects of cyclosporine observed in animal models.

Grace *et al.* (1987) have recently demonstrated increased platelet aggregation and thromboxane release in CsA-treated patients and normal volunteers. The calcium channel blocker nifedipine appeared to eliminate these effects.

D. Chronic Nephropathy with Interstitial Fibrosis

It is a common, widely accepted observation that renal allograft recipients on chronic cyclosporine therapy generally show some evidence of long-term nephrotoxicity with serum creatinine levels of 2.0 mg/dl or higher (Calne and Wood, 1985). Renal biopsy specimens from patients treated with cyclosporine for a year or more in which renal function impairment of this type is observed often show a striped form of interstitial fibrosis. However, as emphasized by Keown *et al.* (1986), it is often difficult to differentiate the changes of chronic cyclosporine toxicity from those of so-called chronic rejection in renal allografts. Chronic nephrotoxicity is somewhat easier to study in the cardiac allograft recipients who are transplanted at a time when they have healthy native kidneys (Myers *et al.*, 1984; Greenberg *et al.*, 1987). As is shown in Fig. 7, taken from Myers (1986), the prevailing level of serum creatinine among cyclosporine-treated 1-year survivors of heart transplantation at Stanford University is significantly higher than that in the historical control group treated with steroids and azathioprine. Serum creatinine concentration was below 1.4 mg/dl in most of the azathioprine-treated recipients, but exceeded 1.4 mg/dl in almost all of the cyclosporine patients. The mean serum creatinine concentration in the cyclosporine group of 2.1 mg/dl was significantly greater than the corresponding value of 1.2 in the azathioprine group ($p < 0.001$). About one-third of the patients in each group were selected at random for inulin clearance studies. As can be seen in the lower portion of Fig. 7, inulin clearance was in a normal range (>80 ml/min per 1.73 m^2) in most of the azathioprine-treated group, but was significantly reduced in all but one of the cyclosporine-treated patients. As with the serum creatinine levels, there was little overlap between the two groups. The mean GFR in the cyclosporine-treated group was decreased by almost 50% (53 ± 4 vs 91 ± 4 ml/min per 1.73 m^2, $p < 0.001$) (Myers, 1986; Myers *et al.*, 1984). Other features of chronic nephropathy in cyclosporine-treated heart transplant recipients included severe hypertension, mild proteinuria, and tubular dysfunction. A similar chronic cyclosporine toxicity has also been detected in liver transplant recipients (Datzman *et al.*, 1985). The markedly impaired

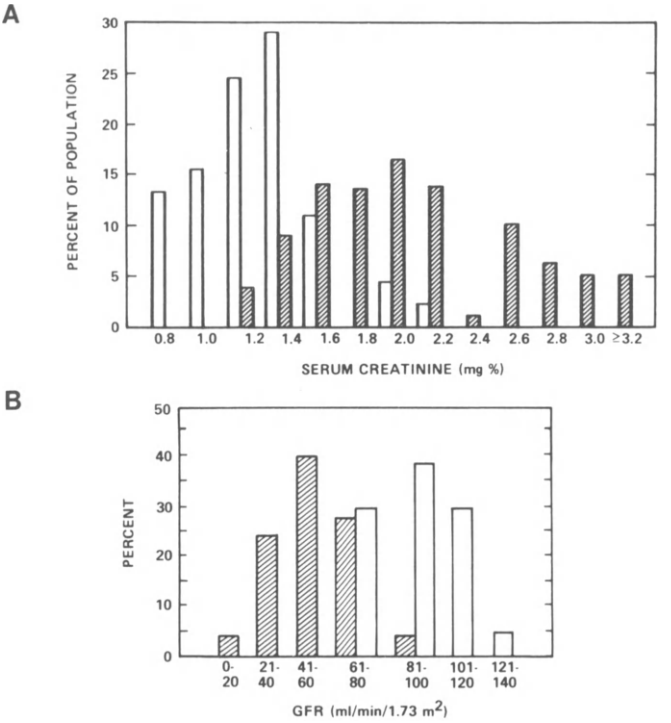


FIG. 7. (A) Histogram of serum creatinine levels 12 months after heart transplantation in all surviving Stanford University patients treated with prednisone and either cyclosporine (n = 79, hatched bars) or azathioprine (n = 56, open bars). (B) Histogram of distribution of GFR values (inulin clearance) in a representative sample of about one-third of each of the two treatment groups (cyclosporine, n = 25, hatched bars; azathioprine, n = 21, open bars) depicted in A. [From Myers (1986). Used by permission.]

GFR in stable long-surviving renal transplant patients receiving cyclosporine is probably a manifestation of the same type of chronic nephrotoxicity (Danovitch *et al.*, 1986). The renal failure brought about by chronic cyclosporine nephrotoxicity may not reverse when cyclosporine is discontinued (Rao *et al.*, 1985).

There is evidence that the structural basis of chronic cyclosporine nephrotoxicity is unique. In cardiac allograft recipients who developed end-stage renal disease on this basis, kidney size and weight were increased, not decreased as in most types of chronic renal failure (B. Myers, 1986, personal communication). Mesangial matrix was also increased (Myers *et al.*, 1988). Jackson and Humes (1986) have found evidence for proliferation of interstitial fibroblasts in rats treated with high-dose cyclosporine (100 mg/kg/day), and a similar phenomenon may occur in humans.

In studies of cardiac allograft recipients Greenberg *et al.* (1987) have

emphasized that although both acute reversible and chronic cyclosporine nephrotoxicity occur with high prevalence, the acute form does not appear to be a specific risk factor for the chronic form. In their study, two-thirds of cyclosporine-treated patients had renal dysfunction at 1 year in both the group which had experienced posttransplant ARF and the group which had maintained normal renal function in the perioperative period. In renal allograft recipients there is a relationship between cumulative cyclosporine dose and chronic nephrotoxicity, whereas acute nephrotoxicity is related to trough cyclosporine blood levels (Keown *et al.*, 1986; Kahan, 1986; Klintmalm *et al.*, 1984). Thus, one would not necessarily expect the same patients to be affected by both types of toxicity.

E. Summary of Studies of Clinical Cyclosporine Nephrotoxicity

As pointed out in Section I, there appear to be many differences between animal models of cyclosporine nephrotoxicity and clinical cyclosporine nephrotoxicity. Of the four types of nephrotoxicity seen in patients (acute reversible renal insufficiency, delayed renal allograft function, acute vasculopathy, and chronic nephropathy with interstitial fibrosis), only the first is easily reproducible in animals. The four types of nephrotoxicity are probably related, but it is not clear that episodes of acute reversible dysfunction increase the likelihood of chronic toxicity. Further experimental studies are clearly needed to improve our understanding of clinical nephrotoxicity and to devise ways of avoiding or lessening it. The acute vasculopathy may manifest itself within 1 hour after transplantation and appears frequently to be a self-limited process. Morphologically, cyclosporine nephrotoxicity appears fundamentally different from the nephrotoxicity of other drugs, but there remains a significant problem differentiating cyclosporine nephrotoxicity from acute rejection.

IV. Future Directions and Unanswered Questions

As we have seen, it is difficult if not impossible to separate cyclosporine's immunosuppressive effects from its nephrotoxic effects. This apparent linkage between immunosuppression and toxicity has led to a search for a unifying mechanistic hypothesis that would account for both effects. A number of authors have proposed that the immunosuppressive and nephrotoxic effects of cyclosporine may be occurring by similar mechanisms, which would explain the relative difficulty in separating these effects

clinically and experimentally. Neild *et al.* (1983b) and Kahan *et al.* (1985) have postulated that the plasma PSF, which has been shown to be deficient in cyclosporine-treated animals, may be a lymphokine produced by lymphocytes to regulate prostacyclin synthesis. In this construct, cyclosporine's inhibition of lymphocyte PSF release would be analogous to the drug's effects on generation of other lymphokines such as the interleukins. The possibility that interleukin 1 (IL-1) and PSF may be the same compound (Rossi *et al.*, 1985) suggests a very satisfying unitary hypothesis to account for both immunosuppression and nephrotoxicity as a consequence of the same general inhibitory effect on lymphokine release.

Clinically, there is a similarity between cyclosporine-induced renal failure and the renal dysfunction brought about by inhibitors of PG synthesis (Kahan, 1986). Coadministration of nonsteroidal antiinflammatory agents potentiates cyclosporine nephrotoxicity experimentally (Murray *et al.*, 1985). However, actual measurements of PG in renal transplant patients treated acutely with cyclosporine do not provide evidence for synthesis inhibition. Klassen *et al.* (1987) compared prostanoid levels in renal venous effluent of cyclosporine- or azathioprine-treated renal allograft recipients. The cyclosporine-treated group had significantly *higher* prostacyclin levels (measured as the metabolite 6-keto-$PGF_{1\alpha}$) than the azathioprine group at both 5 and 60 minutes after completion of the vascular anastamoses. There was no difference between groups in levels of Tx or PGE_2. Clearly, the role of prostacyclin alterations in the kidney's response to cyclosporine needs to be further evaluated.

Moss *et al.* (1985) have suggested that activation of the sympathetic nervous system by cyclosporine may have important effects on both renal function and immunity. As discussed in Section II,D, several investigators have found that denervation or α-adrenergic blockade prevents the decreases in RBF and GFR following acute or "chronic" cyclosporine treatment, and Moss *et al.* (1985) have directly demonstrated significant increases in renal and genitofemoral nerve activity in treated animals. Livnat *et al.* (1985) reviewed the involvement of peripheral and central catecholamine systems in neural–immune interactions. Direct sympathetic neural input has been demonstrated in lymphoid tissues in regions where lymphocytes (largely T cells) are found, and there is some evidence for receptors on cells of the immune system which are capable of receiving neural signals. The possibility that catecholamine systems may produce these two major actions of the drug is indeed intriguing, and deserves further investigation.

There is now considerable interest in the role of Ca^{2+} and Ca^{2+}-binding proteins in mediating effects of cyclosporine on the immune system. Colombani *et al.* (1985) and Hess *et al.* (1986) have demonstrated that CsA binds to five different cytoplasmic proteins, three of which will cross-react with a calmodulin antibody. CsA inhibits calmodulin activity, an inhibition

which is calcium dependent and in the micromolar ranges. Since T-cell activation, cell proliferation, and IL-2 production appear to be calcium-dependent phenomena (Nussenblatt *et al.*, 1983), the authors hypothesized that CsA interferes with calcium-dependent T-cell activation at the level of calmodulin and other calcium-dependent proteins, although there are other potential target proteins such as protein kinase C, phospholipase A_2, and phospholipase C which may be involved. These proteins are ubiquitous and critical for many cell functions, including synthetic activities and function of membrane pumps. It seems possible that inhibition of activity of these systems may play a role in the pathogenesis of CsA nephrotoxicity as well as CsA-induced immunosuppression, a possibility which should be investigated. General issues of binding kinetics and characteristics of CsA binding in the kidney also need to be further clarified.

Experimentally, the calcium channel blocker nifedipine reduces cyclosporine nephrotoxicity (Dieperink *et al.*, 1986b). In a retrospective analysis of the SEOPF data base, we (Solez *et al.*, 1988) found that patients receiving nifedipine and cyclosporine had better renal function and fewer treated rejection episodes than patients treated with other antihypertensives and cyclosporine. In this clinical context, nifedipine may be working through a combination of its intrinsic immunosuppressive and vasodilating properties, as well as through its inhibitory action on platelet aggregation and thromboxane release (Grace *et al.*, 1987; Mikhailidis *et al.*, 1987).

Prolactin modulates lymphocyte responsiveness, and there appears to be an important interaction of cyclosporine and prolactin in this regard. It has been suggested that the combination of cyclosporine and bromoceptine (which reduces endogenous prolactin production) may well result in improved immunosuppression with reduced nephrotoxicity (Hiestand *et al.*, 1986).

While *in vitro* effects of cyclosporine on cultured endothelial cells have been investigated in attempts to assess the propensity of cyclosporine to produce direct injury to endothelium (Zoja *et al.*, 1986; Brown and Neild, 1987), only two laboratories have reported studies with cultured renal tubular cells. One group (Regec *et al.*, 1985; Trifillis and Trump, 1985) reported increased numbers of Oil Red O-positive "vacuoles" in human proximal tubular cells exposed to CsA (0–10 μg/ml), which by EM appeared as "huge" non-membrane-bound cytoplasmic inclusions. However, as discussed above, the presence of these inclusions may not be an indication of true "toxicity." Assays of enzyme activities in these cells revealed a slight increase in activity of the brush border enzyme γ-glutamyl transpeptidase after 3 days of exposure to CsA (0–5 μg/ml), normalizing by 14 days, and increased lysosomal enzyme activities, followed by decreased activity after prolonged exposure. A second group (Hreniuk and Wilson,

1988) found increased lactate dehydrogenase release and nigrosine uptake following exposure of cultured human renal tubular cells to CsA. These effects were reduced in calcium-free media or in full media containing the calcium channel blocker verapermil (5×10^{-7} *M*) or the cysteine protease inhibitor E64 (50 μg/ml). Further studies in comparable systems using cells derived from humans or experimental animals could prove very useful in settling the issue of whether CsA has direct toxic effects on renal proximal tubular cells and in defining mechanisms of toxic effects, if they can be demonstrated.

There is suggestive experimental evidence that CsA may be altering glomerular ultrafiltration coefficient (K_f). Sullivan *et al.* (1985) noted a much greater decrease in inulin clearance (43%) than in RBF (15%) after administration of 10 mg/kg/day parenteral CsA for 7 days in their rat model. They suggested that a decrease in K_f could explain this discrepancy. Perico *et al.* (1986), in studies with a selective Tx synthetase inhibitor, found that treatment with the inhibitor UK-38,485 in CsA-treated rats (6 weeks of dosing at 40 mg/kg per 48 hours po) significantly reduced urinary TxB levels and increased GFR, but had no effects on RPF. Lack of correlation between levels of Tx synthesis and RPF in these studies suggested a reduction in K_f due to mesangial cell contraction, producing a reduced glomerular capillary filtration area. Myers also proposed a possible decrease in K_f to explain the chronic renal toxicity to cyclosporine (Moran *et al.*, 1985; Myers, 1986). However, alterations in glomerular K_f have been directly demonstrated experimentally in only one study (Barras *et al.*, 1987).

Chronic cyclosporine toxicity is an extremely fruitful area for future investigations. There are few published animal models for this type of cyclosporine toxicity. However, the recent work of H. Starklint *et al.* (1987) and Bertani *et al.* (1987) in rats appears quite promising. In the studies by H. Starklint *et al.*, the kidneys had interstitial fibrosis and yet were increased in size and weight, just as has been reported to be the case in human patients with end-stage chronic cyclosporine toxicity (B. D. Myers personal communication, 1986).

There are few animal studies thus far of cyclosporine toxicity in the transplanted kidney. Studies in our laboratory (L. C. Racusen and K. Solez, 1987, unpublished) show that cyclosporine toxicity is the major factor resulting in transplant renal dysfunction when it is given in a dose of 20 mg/kg/day. This same dose produces only mild renal dysfunction in the native kidneys of rats. Coffman *et al.* (1987a) have shown that chronic treatment with a Tx synthetase inhibitor improves renal allograft function in rats, and it would be of great interest to study the combined effects of such an inhibitor and cyclosporine on rat renal allograft function.

As was pointed out in Section I, much has been and will continue to be written about cyclosporine as more and more clinical indications for the drug are found (Tindall *et al.*, 1987). Since no viable successor compound has been identified, cyclosporine will remain an intensely interesting and widely used drug for some time, and its unique nephrotoxicity will remain an important and intriguing investigative issue. Much remains to be learned about cyclosporine's effects on the kidney. It is hoped that the reader will be stimulated by the information in this review to ask insightful new questions and perhaps further enhance our understanding of this important subject.

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Glomerular Antigens in Experimental Glomerulonephritis

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I. Models of Glomerulonephritis

Although diffuse glomerulonephritis satisfying all the criteria for the diagnosis of human disease has not yet been produced experimentally, evidence at hand points to the possibility that bacteria or the products of their growth and proteins may be responsible for the inception and development of glomerulonephritis. [Horn (1937a).]

More than a hundred years ago many investigators were concerned with the induction of glomerular diseases in animals to establish their etiology and study their natural history, pathogenesis, and response to treatment. The investigations were encouraged by Bright (1827), who established the association between "dropsy attended with coagulable urine" and structural alterations in the kidney. At first, the induction of experimental glomerulonephritis involved toxic action of different chemical compounds on the kidney (Horn, 1937a,b). Since Löhlein (1907) suggested that the renal changes observed by Bright were due in the majority of the cases to the action of streptococcal toxins, and Schick and von Pirquet (von Pirquet and Schick, 1905; Schick, 1907; von Pirquet, 1911) assumed that the lesions were caused by an allergic reaction to bacterial products, considerable effort was devoted to the induction of experimental glomerular lesions by the injection of bacteria or bacterial products (Faber and Murray, 1917; Duval and Hibbard, 1926, 1927; Wood and White, 1956), heterologous serum (Longcope, 1913; Lukens and Longcope, 1931; Klinge, 1930; Vauble, 1932), or purified serum proteins (Hawn and Janeway, 1947; Erich *et al.*, 1949; Wissler *et al.*, 1949; More and Waugh, 1949), or preformed immune complexes (Benacerraf *et al.*, 1960).

These experiments were based on the assumption that an immune reaction induced in the animals would lead to the formation of immune complexes which had been shown to possess phlogistic qualities (Arthus, 1903; Opie, 1924) and which could become deposited in the renal glomeruli and cause glomerulonephritis. Initially such induction of glomer-

ulonephritis was not very successful. Lindemann (1900) was the first to obtain results. In an attempt to define the toxicity of different serum components to the kidney, he raised a rabbit antiserum against rat kidney homogenate and injected it into rats. This resulted in a proliferative glomerulonephritis which was termed nephrotoxic serum nephritis (NTN). In this way he established a model of a human disease that was not to be discovered until 1967 (Lerner *et al.*, 1967). Even today, Lindemann's experiments represent progress in science that has given us an understanding of the pathogenetic mechanisms of at least one form of glomerulonephritis.

After the induction of NTN and a further characterization of this disease by Masugi (Masugi, 1933, 1934; Masugi and Sato, 1934), it was not until the early 1950s that further progress was made in the field of experimental glomerular diseases. The rapid developments in the fields of biology and immunology, the introduction of the renal biopsy technique by Iversen and Brun in 1951, and the use of new techniques in morphology such as electron microscopy (Farquhar *et al.*, 1951) and immunofluorescence microscopy (Coons and Kaplan, 1950), meant a giant leap forward for the study of glomerulonephritis and consequently for the understanding of its pathogenesis. Immunofluorescence methods in particular helped to establish firmly the involvement of immunological mechanisms in the pathogenesis of glomerulonephritis (Okuda *et al.*, 1965) as well as the participation of immune complexes in the majority of cases (Andres *et al.*, 1963). Understanding of "immune complex glomerulonephritis" was greatly advanced by the serum sickness model developed by Germuth and Dixon (Germuth, 1953; Germuth *et al.*, 1957; Dixon *et al.*, 1961).

The pathogenesis of glomerulonephritis now seemed to be based on one of two principally different mechanisms: glomerulonephritis caused by the binding of antikidney serum, as in NTN, or glomerulonephritis caused by the deposition of immune complexes.

With time, however, the relevance of some aspects of glomerulonephritis models has been questioned. The histopathological expression of several forms of glomerulonephritis showed characteristics which proved difficult to attribute to one of these two immunological mechanisms operating in experimental disease (Wilson and Dixon, 1981), and furthermore, glomerular lesions unrelated to immunological mechanisms were shown to express a similar variety of histopathological features. On these grounds, the importance of experimentally produced glomerular lesions should be regarded critically. Models of glomerulonephritis are usually based on immunization of animals with large nonphysiological doses of antigen, which is not likely to occur in humans. Furthermore, injection of heterologous nephritogenic antibodies into an animal almost always induces an

immune reaction to the foreign proteins injected. This reaction influences the originally induced glomerulonephritis considerably and complicates its pathogenesis.

There are also glomerulonephritis models based on an autoimmune reaction either restricted to the glomeruli or a systemic autoimmune reaction (Hoedemaeker *et al.*, 1984). In these cases the induced autoimmunity is usually the result of a temporary breakthrough of tolerance for renal antigens, which is not, however, chronic and is down-regulated with time (de Heer *et al.*, 1986). Although an autoimmune reaction of longer duration can be achieved by a graft-versus-host reaction (Fleuren *et al.*, 1982; Gleichmann *et al.*, 1984; Hoedemaeker *et al.*, 1984), a chronic autoimmunity resulting in glomerular lesions like those found in humans only occurs spontaneously in certain strains of mice (Wilson and Dixon, 1981).

Notwithstanding these restrictions, the models mentioned above have helped us to understand the pathogenetic mechanisms which are operating in human glomerulonephritis and may help us learn more about etiological factors (Hoedemaeker *et al.*, 1984). Within the limitations mentioned, the use of models of glomerulonephritis still can fill in gaps in our knowledge. It would therefore be more profitable, instead of looking for the best models of human glomerulonephritis (Albini *et al.*, 1985a,b), to use the available models to greater advantage in order to gain more insight into the complex but exciting events leading to glomerular disease.

II. Structure and Function of the Glomerulus

The filtering part of the nephron, the glomerulus, is composed of a coiled bundle of specialized capillaries which project into a capsular space bounded by Bowman's capsule. The centrilobular region of the glomerular tuft, which is called the mesangium, consists of mesangial cells embedded in an amorphous substance with a density similar to that of the lamina densa of the glomerular basement membrane (GBM) (Heptinstall 1983).

The structure of the glomerular capillary wall (GCW), across which the filtration occurs to produce the primary urine, is complex (Heptinstall, 1983). This complexity enables the GCW to retain large molecules selectively, while small ones have free access to Bowman's space. This filtration process is determined not only by properties of the GCW and by glomerular flows and pressures, but also by physicochemical determinants of the molecules to be filtered, such as size, shape, and charge (Brenner and Humes, 1977; Rennke and Venkatachalam, 1977; Brenner *et al.*, 1978; Venkatachalam and Rennke, 1980; Kanwar, 1984).

The GCW consists of three layers: (1) an inner layer of fenestrated

endothelium covered by the endothelial cell coat; (2) an outer layer of epithelial cells with interdigitating foot processes and also covered with a cell coat; and (3) between these layers, a basement membrane composed of an electron-dense layer, the lamina densa, and two peripheral radiolucent layers, the laminae rarae interna and externa. Although this model of the GCW has been generally accepted (Kanwar, 1984), freeze-fracture studies have never revealed a trilaminar structure (Simpson, 1986); in fact, very little is known about the structure of the GCW at the supramolecular level (Timpl, 1986).

The endothelial and epithelial cell surface coats are rich in polyanionic sialoproteins, histochemically defined as "polyanion" (Mohos and Skoza, 1969; Jones, 1969), which can be visualized in light and electron microscopy by cationic reagents such as colloidal iron, alcian blue, and polyethyleneimine (PEI) (Latta *et al.*, 1975; Caulfield and Farquhar, 1976; Schurer *et al.*, 1977) (Fig. 1). Both laminae rarae can also bind cationic reagents, because of the presence of anionic residues such as heparan sulfate and other glycosaminoglycans (Kanwar and Farquhar, 1979b; Stow *et al.*, 1985) (see also below). After fixation these anionic residues are arranged in discrete dots which have been designated anionic sites (Kanwar

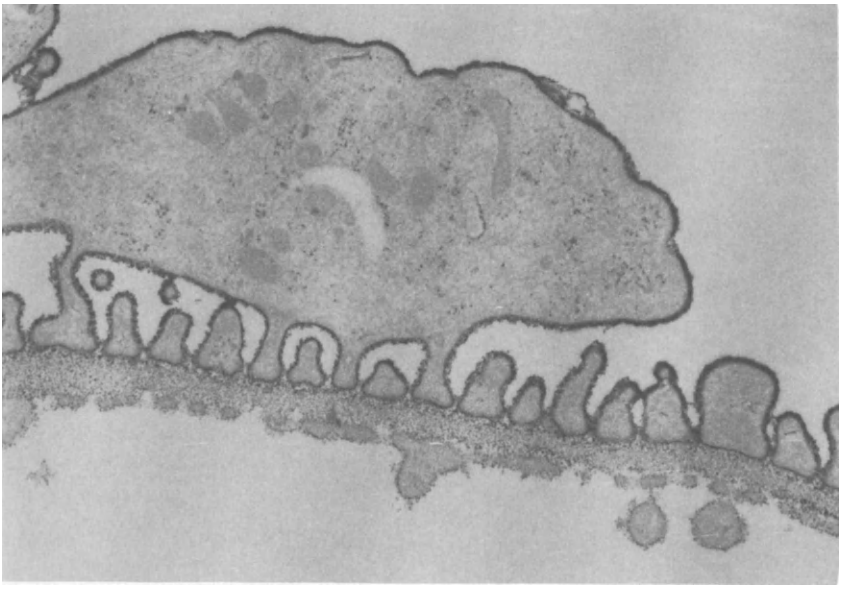

FIG. 1. PEI staining of polyanion of a rat glomerulus after a short fixation in paraformaldehyde. Reaction product is present along the epithelial cell membrane and to a lesser extent along the endothelial cell membrane and in the GBM. ×18,200.

and Farquhar, 1979a). These sites have an interspacing of 64 nm (Schurer *et al.*, 1980) (Fig. 2), and are arranged in a reticular pattern (Farquhar and Kanwar, 1982; Reale *et al.*, 1983) and embedded in filaments 100–160 nm long (Reale *et al.*, 1983).

Ultrastructural tracer studies with differently charged ferritins (Rennke *et al.*, 1975) and functional studies measuring the fractional clearances of differently charged polydisperse dextrans (Chang *et al.*, 1975) and protein tracers (Rennke *et al.*, 1978a) have shown that negatively charged macromolecules are restricted by the GCW more than neutral molecules are, whereas filtration of cationic macromolecules is facilitated. Filtration of neutral molecules depends solely on their molecular size and shape (Fig. 3). The main charge barrier is thought to reside in the more proximal constituents of the filter, that is, the endothelial cell coat and the inner lamina rara. The size-selective properties of the GCW probably depend on multiple structural components in the GCW, including filtration slits between the epithelial cell foot processes.

Mesangial Area

It is only since the early 1960s that the mesangial area has been recognized as a distinct area in the glomerulus (Vernier, 1961; Michielsen,

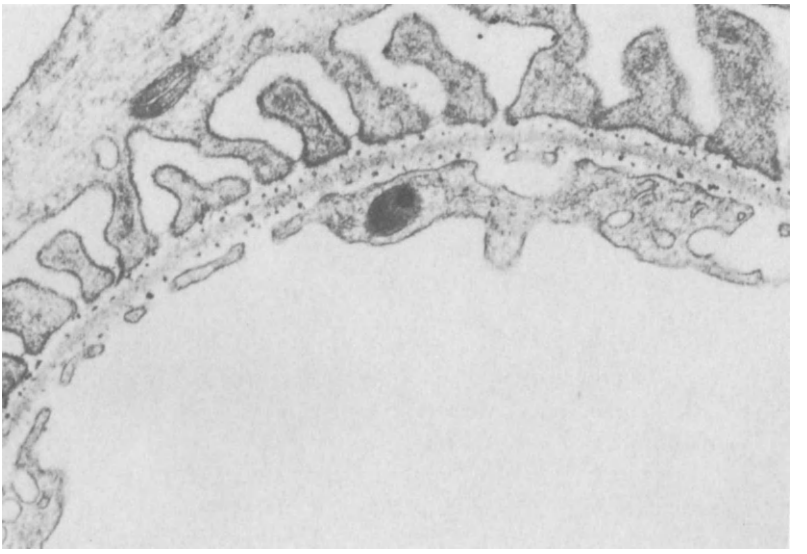

FIG. 2. Anionic sites in the glomerular basement membrane of a rat stained with PEI. Discrete particles are present in both laminae rarae. ×25,000. (Courtesy Dr. P. Hogedoorn.)

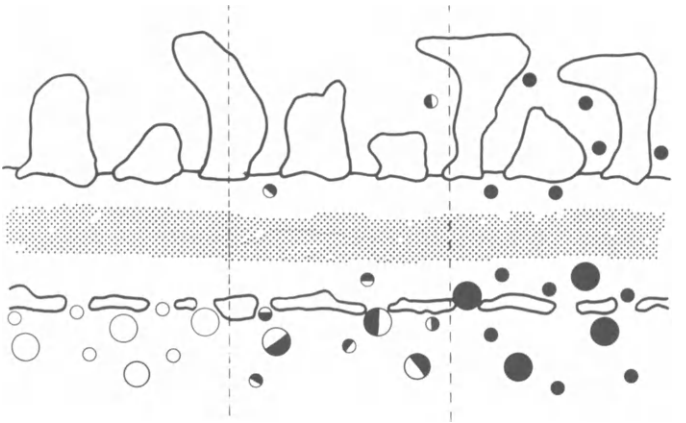

FIG. 3. Schematic representation of glomerular filtration. Filtration of neutral molecules (◑) depends mainly on molecular size and shape. The filtration of positively charged molecules (●) is facilitated, while negatively charged molecules (○) are not filtered at all.

1962). This area is situated in the hilar region of the glomerular capillaries and contains epithelioid cells separated by channels and embedded in an amorphous structure called the mesangial matrix (Latta and Maunsbach, 1960, 1962) (Fig. 4). The matrix has an electron density similar to that of the lamina densa of the GBM and contains many of the chemical components present in the GBM, including the anionic sites (Caulfield and Farquhar, 1976; Schurer *et al.*, 1977). The function of the mesangial area has not been elucidated completely. This area has been regarded as a physical support for glomerular capillaries (Farquhar and Palade, 1962) or as cleaning system for filtration residues (Elema *et al.*, 1976; Sterzel *et al.*, 1983a).

Recently, it was established that the mesangial cells originate from at least two different sources. The mesangial cell proper constitutes the majority and is of renal origin. It contains cytoplasmic contractile elements (Scheinmann *et al.*, 1974, 1976; Accinni *et al.*, 1975). Ausiello *et al.* (1980) demonstrated contractions of cultured mesangial cells after exposure to angiotensin II. On these grounds and because of its histochemical patterns, this cell may be considered a smooth muscle cell (Michielsen and Creemers, 1967; Yamamoto *et al.*, 1986). There is still debate as to whether mesangial contractile properties serve a functional purpose in glomerular hemodynamics (Michael *et al.*, 1980). The phagocytic properties originally thought to belong to all mesangial cells (Shinkai, 1982) are today attributed to the second cell population of this area (Haakenstad *et al.*, 1976; Striker *et al.*, 1979; Schreiner *et al.*, 1981; Seiler *et al.*, 1986).

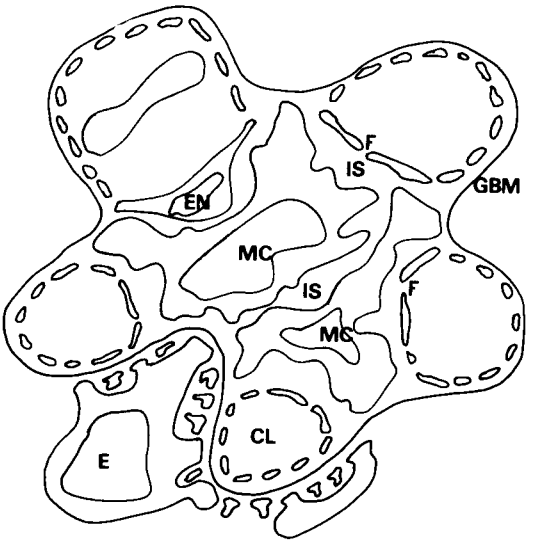

FIG. 4. Schematic representation of the mesangial area. CL, Capillary lumen; E, epithelium; EN, Endothelium; F, fenestration; GBM, glomerular basement membrane; IS, intercellular channel; MC, mesangial cell. [Modified from Latta and Maunsbach (1960); courtesy Dr. J. J. Grond.]

These cells form approximately 2% of the total glomerular cell population and are derived from the bone marrow (Striker *et al.*, 1979; Schreiner *et al.*, 1981; Schreiner and Unanue, 1984). Gurner *et al.* (1986) have showed that an influx of cells expressing the leukocyte common antigen (LCA) in irradiated Lewis kidneys transplanted in a normal recipient, originated from a circulating pool of mononuclear cells. In addition to the LCA, 50–60% of these cells bear major histocompatibility complex (MHC) class II antigen and are able to stimulate lymphocytes in an MHC-restricted manner (Schreiner and Unanue, 1984; Gurner *et al.*, 1986). These findings indicate that the monocyte population of the mesangium is involved in phagocytosis and may have a local functional activity in the afferent limb of the immune system.

The mesangial area is freely accessible to substances from the circulation. Since no basement membrane is present between the endothelial and mesangial cells in the hilus of glomerular capillaries (Latta and Maunsbach, 1962), the mesangial uptake of molecules is clearly influenced by hemodynamic changes (Keane and Raij, 1985) and depends on charge and/or size (Latta and Fliegel, 1985). The molecules taken up by the mesangium will remain for some time in the mesangial cells (Grond and Elema, 1981) (Fig. 5) or in the mesangial matrix (Seiler *et al.*, 1983), and some are taken

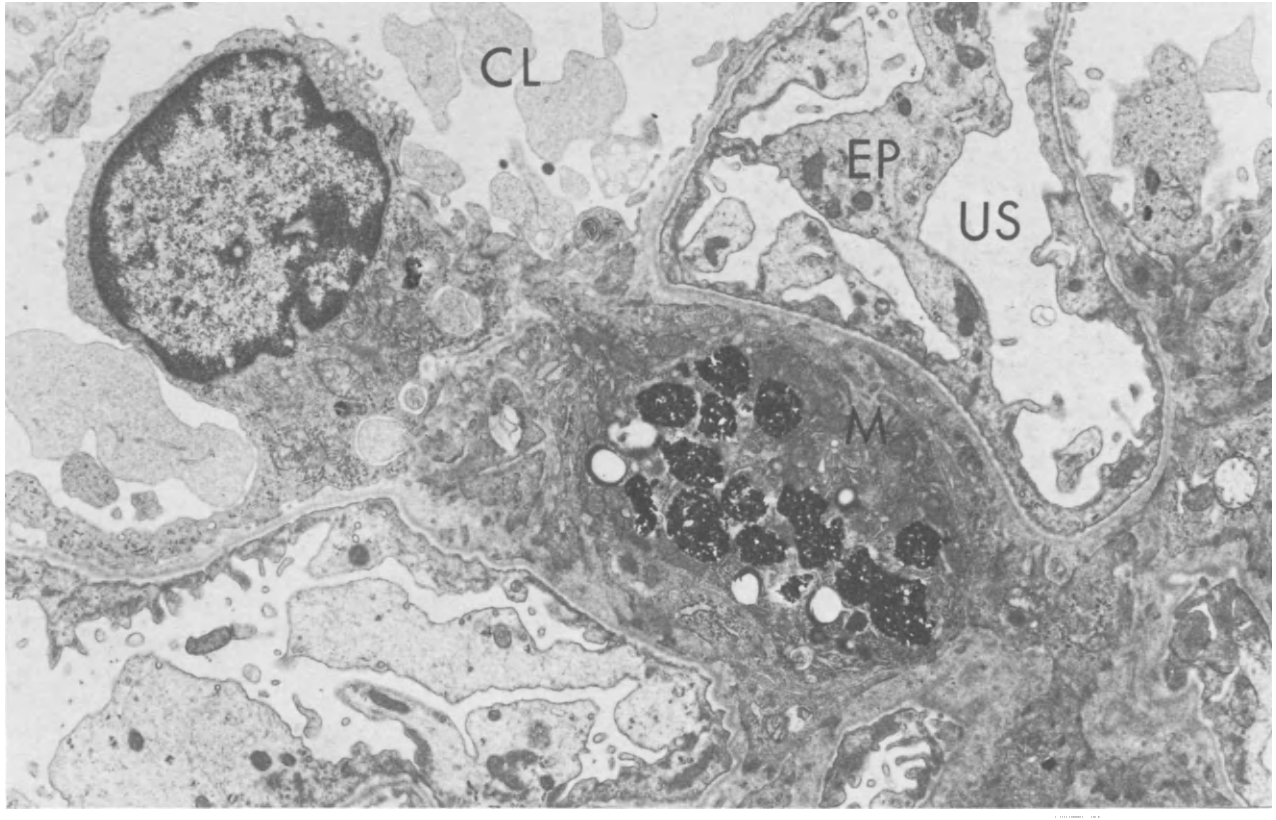

FIG. 5. Electron micrograph of the glomerular mesangium of a rat made nephrotic with aminonucleoside, 24 hours after an injection with colloidal carbon. Carbon particles are visible in the mesangial lysosomes but not in the mesangial matrix. CL, Capillary lumen; EP, epithelial cell; M, mesangial cell; US, urinary space. Uranyl acetate–lead citrate, ×9200. (Reproduced with permission.)

up by the mesangial monocytes (Sterzel *et al.*, 1983b). The molecules remaining in the matrix are transported to the hilar region of the glomerulus within a few days (Elema *et al.*, 1976). Whether the molecules are subsequently removed from this region by efferent lymph vessels is still uncertain. Although the structure of the mesangium and a few functional properties have been established, the exact role of this glomerular region in hemodynamics and filtration remains to be established.

III. Immune Mechanisms in the Pathogenesis of Glomerulonephritis

A. Mechanisms Mediated by Antibodies

The great majority of glomerular diseases are caused by immunological mechanisms (Wilson and Dixon, 1981), although nonimmunological factors have been shown to operate especially in chronic glomerular lesions such as focal segmental glomerulosclerosis. The latter will not be discussed in this article. In the pathogenesis of glomerular lesions, humoral immune reactions play the most important role. These immune reactions concern (1) antibodies reacting with the GCW, which leads to either a linear binding (Wilson, 1974) or a granular binding (van Damme *et al.*, 1978; Couser *et al.*, 1978; Hoedemaeker *et al.*, 1982a), and (2) deposition of immune complexes from the circulation into the glomerular filter or the mesangial area (Wilson, 1974). When an immune aggregate is present in the subendothelial or mesangial space of the glomerulus, polymorphonuclear leukocytes and macrophages are attracted by activated complement components. Subsequent phagocytosis will lead to the generation of toxic oxygen

TABLE I

Immune Mechanism in Glomerulonephritis

Antibody-mediated mechanisms
In situ formation of immune complexes
Renal antigens
Linear binding
Granular binding
Nonrenal antigens
Charge dependent
Charge independent
Deposition of immune complexes
Cellular immune mechanisms

products and halides (Johnson and Ward, 1982), with subsequent damage to the glomerular filter resulting in proteinuria, hematuria, and glomerulonephritis (Makino *et al.*, 1986). Localization of immune aggregates in the subepithelial space does not lead to phagocytosis. The mere presence of the aggregates and possibly the involvement of complement components may lead to filter damage and increased permeability (Salant *et al.*, 1980; Couser *et al.*, 1985; de Heer *et al.*, 1985b).

With respect to the binding of antibodies to glomeruli, a distinction must be made between a reaction of antibody with glomerular antigens and a reaction of antibody with antigens that are planted in the glomerulus from the circulation (Couser and Salant, 1980; Oite *et al.*, 1983; Kaysen *et al.*, 1986). These groups of antigens involved in the pathogenesis of glomerular lesions are listed in Table I and will be discussed in that order here.

1. *In Situ Formation of Immune Complexes*

a. Renal Antigens and Linear Binding (Fig. 6). The classical example of a glomerular lesion resulting from a reaction between antibody and GBM antigens, is the NTN, an entity introduced almost 90 years ago by Lindemann (1900), characterized by Masugi (1933, 1934), and studied further by many others (Kay, 1940, 1942; Krakower and Greenspon, 1951; Unanue and Dixon, 1964, 1965a,b). This form of glomerulonephritis is induced by an injection of heterologous antibody directed against noncollagenous antigens from the GBM, resulting in a linear binding in immunofluorescence (Hammer and Dixon, 1963) (Fig. 7). The injection leads to a proliferative glomerulonephritis characterized by an influx of polymorphonuclear leukocytes and monocytes and a variable degree of extra-

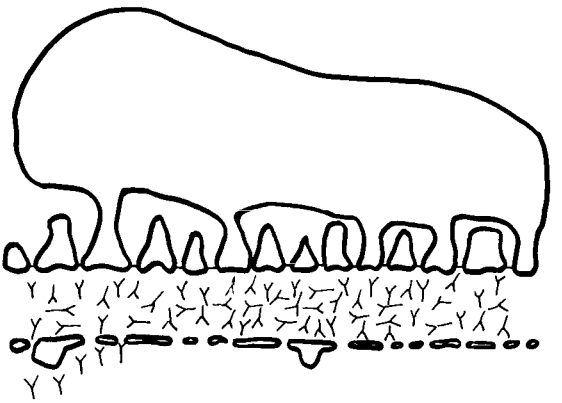

FIG. 6. Schematic representation of the glomerular capillary wall with binding of anti-GBM antibody throughout the basement membrane.

capillary proliferation. This extracapillary proliferation is associated with coagulation following separation of endothelium from the GBM (Silva *et al.*, 1984). Since a heterologous antibody is used, the injected animals develop an autologous phase during which antibodies directed against the injected IgG are also deposited along the GBM. At the ultrastructural level the bound antibody is present over the entire width of the filtering part of the GBM (Andres *et al.*, 1962; Arhelger *et al.*, 1963; Vogt *et al.*, 1966; Hoedemaeker *et al.*, 1972a) (Fig. 8). This disease can be induced in rats (Masugi, 1934; Kay, 1940, 1942), mice (Avasthi *et al.*, 1971), monkeys (Battifora and Markowitz, 1969), chickens (Bolton *et al.*, 1980), guinea pigs (Couser *et al.*, 1973), rabbits (Lerner and Dixon, 1968), and sheep (Unanue and Dixon, 1967).

Depending on the nature of the heterologous antibody used, the resulting proteinuria may either develop directly after the injection of anti-GBM antibodies or indirectly through the action of complement involving neutrophils and macrophages. The binding of anti-GBM antibodies facilitates the binding of nonimmune IgG to the GBM (Kawachi *et al.*, 1985).

In accelerated NTN (Naish *et al.*, 1975), rats are preimmunized with homologous IgG from the species used to prepare the anti-GBM antibody.

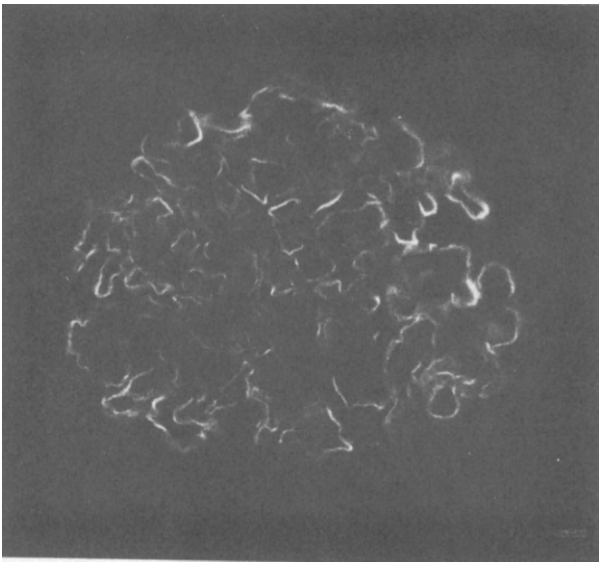

FIG. 7. Immunofluorescence of a glomerulus of a rat injected with rabbit anti-GBM antibody. A linear binding is observed throughout the GBM. ×250. (Courtesy Dr. J. J. Weening.)

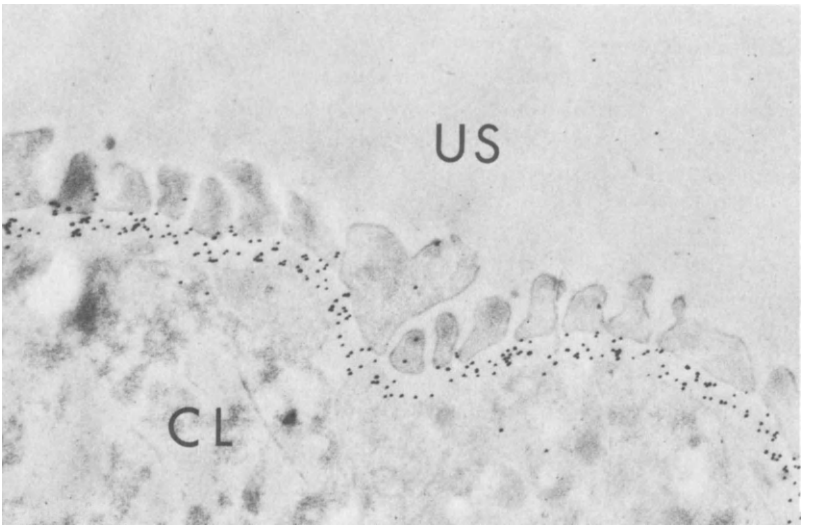

FIG. 8. Immunoelectron microscopy of a glomerular capillary wall of a rat with NTN, 24 hours after the injection of anti-GBM antibody. Gold particles indicating the bound antibody are observed throughout the GBM. Preembedding incubation with gold-labeled goat anti-rabbit IgG during 5 hours. CL, Capillary lumen; US, urinary space, ×11,000. (Reproduced with permission.)

A subsequent injection of this anti-GBM antibody induces a severe proliferative glomerulonephritis accompanied by an influx of mononuclear phagocytes (Schreiner *et al.*, 1978; Lowry *et al.*, 1984).

An autologous model of anti-GBM glomerulonephritis, known as experimental allergic glomerulonephritis (EAG), was introduced by Steblay in 1962 and is induced by immunizing animals with homologous or heterologous GBM. The resulting anti-GBM antibodies bind to the GBM in a linear fashion and induce a severe crescentic glomerulonephritis (Steblay and Rudofsky, 1968) which is sometimes associated with pulmonary hemorrhage resembling Goodpasture's syndrome in humans (Sado *et al.*, 1984). The disease can be transferred to naive animals by an injection of antibodies present in the circulation (Steblay and Rudofsky, 1969).

EAG can be induced in sheep (Steblay, 1962), rats (Heymann *et al.*, 1959), monkeys (Steblay, 1963), rabbits (Milgrom *et al.*, 1964; Unanue, 1966), goats (Williams and Steblay, 1965), and mice (Avasthi *et al.*, 1971). In the rat EAG usually runs a mild course (Fleuren *et al.*, 1982).

b. Renal Antigens and Granular Binding (Fig. 9). Recently it was established that binding of antibodies to glomerular antigens can also lead to local formation of glomerular immune aggregates (van Damme *et al.*, 1978;

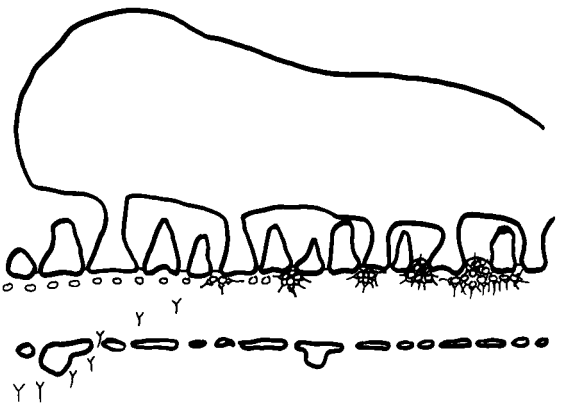

FIG. 9. Schematic representation of the glomerular capillary wall showing binding of antibody to antigens present in the GCW resulting in local formation of subepithelial immune aggregates.

Couser *et al.*, 1978). The best example of such a pathogenetic mechanism is the autologous immune complex glomerulonephritis (AICN) originally introduced by Heymann and Lund in 1951, and given its present name by Glassock *et al.* (1968). AICN can be induced in Sprague–Dawley, Lewis, Munich-Wistar, Wistar, Lewis-mai, Fisher, Long-Evans, Black and White hooded, PVG/c, and Buffalo rats (Hoedemaeker *et al.*, 1988), but not in mice (Ronco *et al.*, 1984b) or dogs (Behar *et al.*, 1986).

AICN is induced by immunizing animals with homologous kidney cortex homogenate (Heymann and Grupe, 1970), crude kidney fractions such as Fx1a (Edgington *et al.*, 1968), mitochondrial fraction (Barabas *et al.*, 1970), or antigens purified from the brush border of the proximal tubules (Kerjaschki and Farquhar, 1976), resulting in a granular immunofluorescence (Fig. 10). Van Damme *et al.* (1978) and Couser *et al.* (1978) found that in the absence of circulating immune complexes, perfusion of rat kidneys with antibodies directed to renal tubular antigens resulted in heterologous immune complex glomerulonephritis (HICN), also known as passive Heymann nephritis (Barabas *et al.*, 1969; Hoedemaeker, 1972; Feenstra *et al.*, 1975). Later on, this mechanism of *in situ* formation was recognized in AICN as well as many other forms of immune complex glomerulopathies (Couser and Salant, 1980; Hoedemaeker *et al.*, 1982a,b), thus providing another mechanism, besides the deposition of circulating immune complexes, by which immune complex glomerulopathies could be caused.

c. Nonrenal Antigens and Charge Dependence (Fig. 11). After it had been found that glomerular immune aggregates could be formed *in situ* by

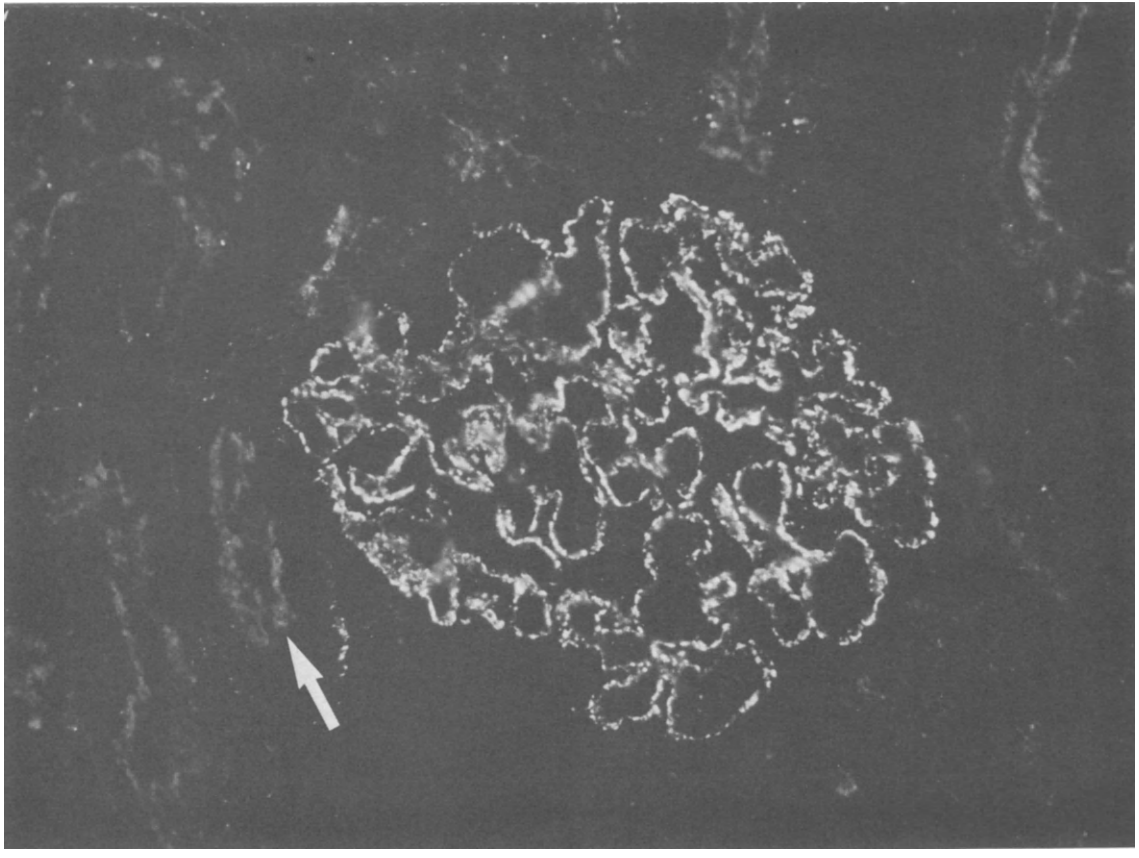

FIG. 10. Immunofluorescence micrograph of a glomerulus of a rat with AICN at week 15 showing the presence of rat IgG with a granular pattern along the GBM and occasionally along the tubular basement membrane. Note also the binding of rat IgG along the brush borders of the proximal tubules (arrow). ×400. (Reproduced with permission.)

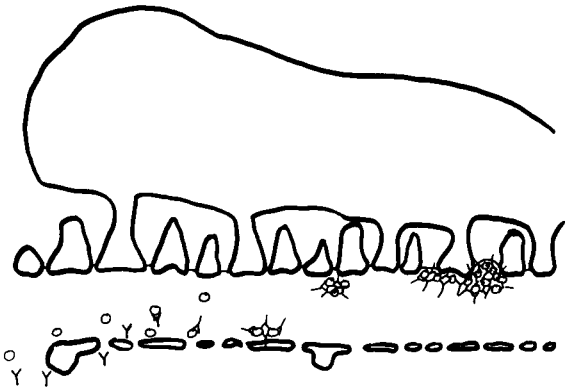

FIG. 11. Schematic representation of the glomerular capillary wall demonstrating local formation of immune aggregates with nonrenal antigens. Antigen and antibody travel separately into the GCW and form aggregates at both the endothelial and the epithelial sides. (Reproduced with permission.)

binding of antibodies to glomerular antigens, Fleuren *et al.* (1980b) showed that *in situ* formation could also occur when antigens not related to the glomerulus were first planted in the GCW and later reacted with an injected antibody. With an alternating perfusion system using bovine serum albumin (BSA) and anti-BSA antibodies, these authors demonstrated the formation of immune aggregates in the GCW of isolated rat kidneys. These investigations were extended by Border and co-workers (1981, 1982) and Oite *et al.* (1982, 1983), Vogt (1984), and Ward *et al.* (1984). Border and co-workers showed that in passive serum sickness glomerulopathy induced with cationized BSA, this antigen first bound to the negatively charged sites in the GBM and subsequently reacted with anti-BSA antibody, which led to the formation of immune aggregates. Injection of native BSA (pI 4.5) or anionic BSA in these cases did not result in the formation of immune complexes along the GBM, although medium-sized immune complexes were present in the circulation. Furthermore, cationic fractions of circulating anti-BSA can serve as a planted antigen by binding to anionic sites in the GBM and later reacting with the antigen, as has been demonstrated also for lysozyme (Fleuren *et al.*, 1980b) (Fig. 12). Other charged antigens or charged immune complexes used in the induction of serum sickness glomerulopathy are mentioned in Section IV,B,2 dealing with charged antigens.

Not only are positively charged antigens able to bind to the GCW, but also negatively charged antigens such as DNA have been shown to act as planted antigens, albeit through different mechanisms (Agodoa and Banis, 1985) (see also below).

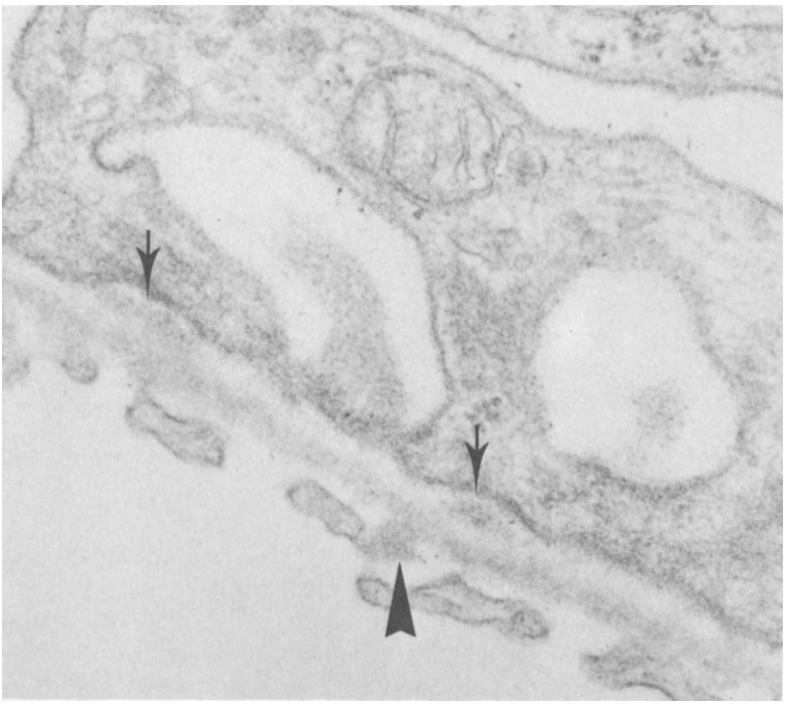

FIG. 12. Electron micrograph of a rat glomerulus following alternating perfusion with BSA and anti-BSA antibody. Electron-dense aggregates can be observed subendothelially (arrowhead) as well as subepithelially (arrows). Uranyl acetate–lead citrate, ×42,500.

d. Nonrenal Antigens and Charge Independence. In situ formation of glomerular immune aggregates leading to glomerulonephritis can also occur independent of the negative charges of the GCW. In 1979 Golbus and Wilson showed that animals injected with the lectin concanavalin A (Con A) developed an immune complex glomerulopathy. It is known that Con A has an affinity for the endothelial side of the GBM (Bretton and Bariéty, 1974). Golbus and Wilson demonstrated that Con A, once bound to the GBM, reacted with anti-Con A antibodies either administered systemically or actively formed by the recipient, resulting in immune aggregate formation and glomerulonephritis.

Immune complexes or protein aggregates present in the glomerulus may also serve as planted antigens and may react with circulating antibodies. Aggregated human IgG injected into rats by Mauer *et al.* (1973) became localized in the mesangium. A subsequent injection of anti-IgG antibody resulted in mesangial immune aggregate formation and glomerulonephritis.

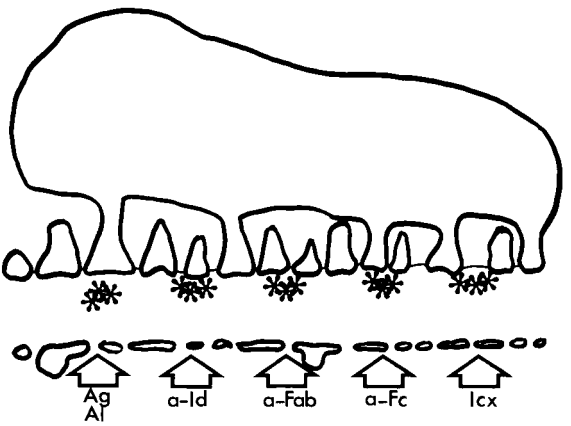

FIG. 13. Schematic representation of the glomerular capillary wall containing subepithelial immune aggregates. These aggregates can react with various substances derived from the circulation. AgAI, Antigen or antibody; a-Id, antiidiotypic antibody; a-Fab, anti-Fab antibody; a-Fc, anti-Fc antibody; Icx, immune complexes.

The same mechanism was found to operate in the autologous phase of HICN, in which rat antibodies reacted with the glomerular immune aggregates present in the GBM (van Es *et al.*, 1977; Adler *et al.*, 1983a).

Other reactants too have been shown to bind to immune complexes or immunoglobulin aggregates present in the GCW, for example rheumatoid factor (Ford and Kosatka, 1983), anti-Fab (Kano and Milgrom, 1980), and antiidiotypic antibodies (Zanetti and Wilson, 1983; Rose and Lambert, 1980) (Fig. 13).

2. *Deposition of Circulating Immune Complexes*

The serum sickness glomerulopathy introduced by Germuth and Dixon (Germuth, 1953; Germuth *et al.*, 1957; Dixon *et al.*, 1961; Wilson and Dixon, 1970) gave rise to the hypothesis that glomerular immune aggregates are formed by the deposition of circulating immune complexes (Okomura *et al.*, 1971; Haakenstad *et al.*, 1976).

Two forms of serum sickness glomerulopathy should be distinguished: an acute and a chronic form.

a. Acute Serum Sickness. This is the "one-shot" serum sickness which is induced in rabbits by one intravenous injection of a large dose of BSA (Dixon *et al.*, 1961; Germuth *et al.*, 1966). Nine days after the injection the disappearance rate of BSA from the serum is increased by immune elimination. During this period, symptoms of glomerulonephritis appear

due to the deposition in the glomeruli of immune complexes from the circulation (Dixon, 1962; Wilson and Dixon, 1970), which gives rise to inflammatory responses. Especially immune complexes formed in antigen excess which remain relatively small, can become deposited in the glomerular filter. Large immune complexes formed in equivalence or in antibody excess are deposited in the mesangial area (Germuth and Rodriguez, 1973; Fish *et al.*, 1966; Senterfit and Germuth, 1969).

The glomerular lesions consist of swelling and proliferation of endothelial cells (Feldman, 1958) and mesangial cells (Robertson and More, 1961), with an influx of polymorphonuclear leukocytes and macrophages (Shigematsu and Kobayashi, 1976). At the ultrastructural level, large electron-dense deposits representing immune aggregates are found mainly subepithelially (Feldman, 1958).

b. Chronic Serum Sickness Glomerulonephritis. This form can be induced in rabbits according to two protocols. The first of these involves daily injections of a fixed dose of BSA. The results are related to the level of the immune response of the injected animals (Germuth *et al.*, 1966). Rabbits with a good immune response develop a transient acute glomerulonephritis like that observed in acute serum sickness. Rabbits with no detectable antibody production develop no signs of glomerulonephritis at all. Rabbits with a moderate antibody response develop chronic immune complex glomerulonephritis, presumably resulting from immune complexes formed in slight antigen excess (Germuth and Rodriguez, 1973; Senterfit and Germuth, 1969).

The second protocol involves daily injections of variable amounts of BSA, adjusted to the antibody titer in the serum of the animal (Germuth and Rodriguez, 1973). In this way a continuous state of antigen excess can be created. This second protocol leads to a chronic immune complex glomerulonephritis (Dixon, 1962) in which the presence of antigen, antibody, and complement can be demonstrated by immunofluorescence, which shows a beadlike pattern along the GCW. Electron microscopy reveals aggregates at the epithelial side of the GBM (Fig. 14). Most rabbits with chronic serum sickness have a mixed membranous and proliferative form of glomerulonephritis; the endocapillary proliferation is probably related to subendothelial deposits.

Chronic serum sickness glomerulopathy can also be induced in rats (Fennell and Pardo, 1967; Yamamoto *et al.*, 1978; Arisz *et al.*, 1979l Miyazaki *et al.*, 1985; Noble *et al.*, 1986) and mice (Haakenstad *et al.*, 1983; Mannik and Striker, 1980; Noble *et al.*, 1980). From studies on passive serum sickness involving injections of preformed immune complexes, it has become clear that the site of localization of glomerular immune aggregates (i.e., whether mesangial, subendothelial, or subepithelial) depends on

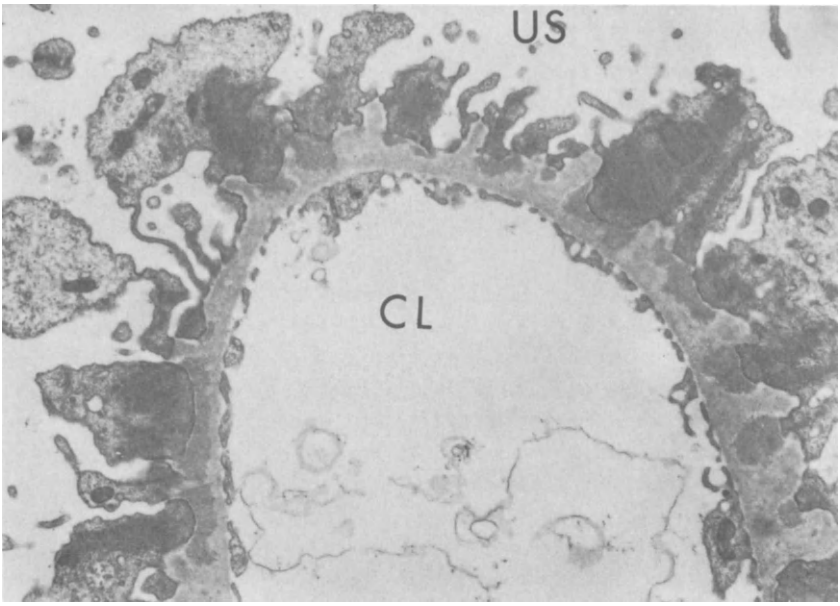

FIG. 14. Electron micrograph of a glomerular capillary of a rabbit with acute serum sickness. Electron-dense aggregates are present at the epithelial side of the GBM. CL, Capillary lumen; US, urinary space. Uranyl acetate–lead citrate, ×11,050. (Reproduced with permission.)

intrinsic properties of the immune complexes such as charge and avidity.

When cationized immune complexes are injected, a GCW localization may be obtained (Germuth *et al.*, 1979; Gallo *et al.*, 1981; Caulin-Glasser *et al.*, 1983) through an interaction of the immune complexes and the intrinsic negatively charged sites of the GBM (Koyama *et al.*, 1986). The nature of the antigen–antibody reactions also plays an important role. Koyama *et al.* (1986) showed that the size of immune complexes formed between cationized BSA and antibodies to native BSA was approximately 7 S, whereas that of immune complexes formed between anti-BSA antibodies and native or anionized BSA was approximately 19 S. Moreover, the 7 S immune complexes appeared to be of low avidity compared with the 19 S immune complexes. Iskander and Jenette (1983), Lew and Steward (1984), and Lew *et al.* (1984) showed that high-affinity immune complexes were deposited exclusively in the mesangial area, whereas low-affinity immune complexes were localized in the GCW. Under conditions of low affinity, the immune complexes might easily dissociate and re-form *in situ* in the GCW (Hoedemaeker *et al.*, 1982a,b; Vogt *et al.*, 1982; Vogt and Batsford, 1984) (see also below).

B. Cellular Immune Mechanisms

The role of cellular immunity in the induction of glomerular injury is not as well understood as that of antibody-mediated mechanisms (Bhan *et al.*, 1978, 1979; Wilson and Dixon, 1981; Neale and Wilson, 1982).

Although lymphocytes sensitized to a variety of glomerular antigens can be found in the majority of patients with various forms of glomerulonephritis, the pathogenetic significance of these lymphocytes is still obscure (Schmitt *et al.*, 1982).

In animals the role of cellular immunity has been studied in NTN, AICN, and EAG. Moorthy and Abreo (1983) found that rats receiving subnephritic doses of nephrotoxic serum showed increased proteinuria in the autologous phase when they were simultaneously given an injection of Freund's complete adjuvant. This amelioration proved to be by lymphocytes sensitized to the nephrotoxic IgG. Moreover, passive transfer of such lymphocytes caused albuminuria and glomerular histopathological alterations in animals treated with subnephritic doses of the antibody.

Schreiner *et al.* (1978) demonstrated that macrophages play an important role in the accelerated form of NTN. Total-body irradiation prevented glomerular infiltration of monocytes as well as the occurrence of proteinuria. The accumulation of macrophages is thought to occur through adherence to the Fc portion of glomerular-bound IgG (Holdsworth, 1983). Tipping *et al.* (1985) showed not only that in this model an accumulation of T-helper cells preceded the influx of macrophages, but also that treatment with cyclosporin A prevented the influx of both T-helper lymphocytes and monocytes. The same group (Boyce *et al.*, 1986) has demonstrated macrophage inhibition activity of these lymphocytes, indicating an active role of these cells in accelerated NTN. Bhan *et al.* (1979) showed that transfer of sensitized lymphocytes from rats with accelerated NTN to naive animals caused an influx of macrophages into the glomerulus. This possible role of cellular immunity expressed through macrophages is intriguing in view of the idea that Ia-positive resident macrophages in the glomerular mesangium may play a role in the afferent limb of the immune response (Schreiner and Unanue, 1984).

Hess *et al.* (1962) showed that AICN could be transferred to naive animals with sensitized lymphocytes. However, because of the long induction period needed, Glassock *et al.* (1968) supposed that the resulting glomerular disease was caused by a graft-versus-host reaction.

Studies on the role of cellular immunity in AICN also involved the induction of tolerance against Fx1A. In 1970, Heymann and Grupe described an induction of tolerance against Fx1A achieved by immunizing neonatal rats with kidney suspensions. These studies were extended by Harmon *et al.* (1980), who induced tolerance to Fx1A by immunizing rats

with Fx1A in incomplete Freund's adjuvant. Several investigators (Litwin *et al.*, 1979; Harmon *et al.*, 1980; Kherr, 1983; de Heer *et al.*, 1985a) concluded that suppressor cells are responsible for tolerance induced in this way. De Heer *et al.* (1985a) found that during the induction of AICN the natural tolerance for Fx1A is only abrogated locally, that is, in the lymph nodes draining the site of immunization. These authors showed that the immune response to Fx1A reached its maximum after 8 weeks and then declined, probably because of antigen-specific suppressor cells in the spleen and thymus.

In a study on the role of cellular immunity in HICN, Bakker *et al.* (1977) demonstrated the presence of T lymphocytes sensitized to Fx1A-containing immune complexes as well as their constituents.

A glomerulonephritis model in which the role of cellular immunity has been shown beyond doubt is EAG induced in chickens. Bolton *et al.* (1980) immunized chickens with bovine GBM in Freund's complete adjuvant, which resulted in a proliferative glomerulonephritis, occasionally with crescent formation. Proteinuria did not occur. In these animals bursectomy abrogated the antibody response completely, but did not influence the occurrence of EAG (Bolton *et al.*, 1984). Tucker *et al.* (1985) demonstrated that the disease could be transferred to naive animals with sensitized lymphocytes.

From the foregoing it may be concluded that cell-mediated immunity plays an important if not the sole role in EAG induced in chickens. An additional role of sensitized lymphocytes in other forms of experimental glomerulonephritis has yet to be established.

IV. Antigens in Experimental Glomerulonephritis

This section is concerned with the glomerular antigens involved in either the pathogenesis of glomerulonephritis or the genesis of functional damage to the glomerular filter. Antigens not known to be involved in these two situations will be mentioned briefly.

The glomerular antigens will be discussed before nonrenal antigens, which after binding to the glomerular capillary wall or mesangium may also play an important role in the pathogenesis of glomerular disease. A list of antigens is given in Table II.

A. Renal Antigens

1. *Epithelial Antigens*

Antigens present in the cell membrane of glomerular visceral epithelial cells are involved in the pathogenesis of at least three forms of experimental

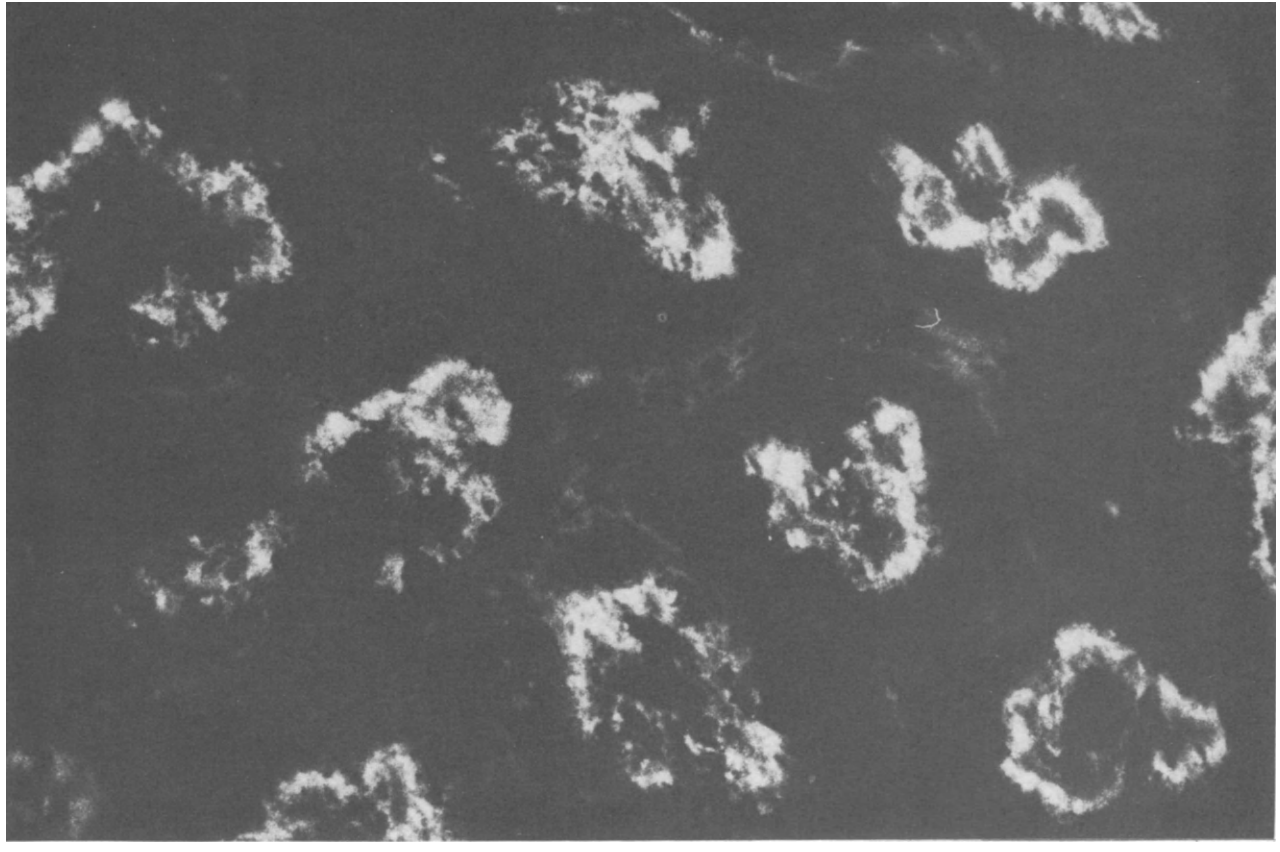

FIG. 15. Immunofluorescence of a rat kidney with FITC-labeled anti-Fx1A antibody. A bright fluorescence of the brush border of the proximal tubules can be observed. ×768.

glomerulonephritis: AICN, HICN, and a spontaneous glomerulonephritis occurring in New Zealand White rabbits.

It has long been known that antigens present in the brush border of proximal tubular epithelial cells of the rat kidney are essential for the induction of AICN (Edgington and Glassock, 1967; Edgington *et al.*, 1967, 1968) (Fig. 15). It was not until 1978, however, that van Damme *et al.* (1978) demonstrated that these antigens were also present in the glomerulus, presumably in the GCW. They and others made it likely that the immune aggregates containing these antigens were formed locally (van Damme *et al.*, 1978; Couser *et al.*, 1978; Fleuren *et al.*, 1980a). Kerjaschki and Farquhar (1982) purified the antigen in question and demonstrated that it was a glycoprotein with a molecular weight of 330,000 (gp 330). They showed, furthermore, that the antigen was present on the cell membrane of glomerular visceral epithelial cells, especially in coated pits (Kerjaschki *et al.*, 1983) (Fig. 16). In an elegant series of experiments, Camussi *et al.* (1985) demonstrated that *in vitro* the binding of this antigen with antibody caused clustering on the cell membrane with subsequent shedding of the immune aggregate.

These investigators extrapolated this finding to the *in vivo* situation and hypothesized that a similar mechanism might operate in AICN and HICN, and that immune complexes were shed in the space between glomerular

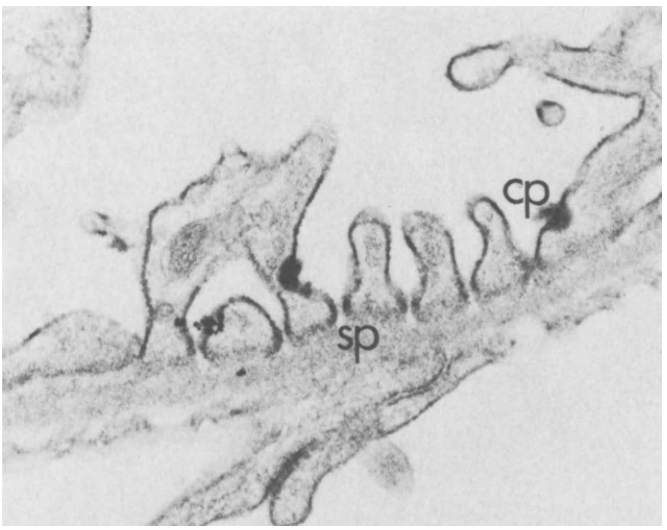

FIG. 16. Immunoelectron micrograph showing the binding of monoclonal anti-gp 330 antibody in the GCW of the rat glomerulus. Staining of coated pits (cp) and of the slitpores (sp) can be observed. ×32,000. (Courtesy Dr. E. de Heer.)

epithelial cells and the GBM, thus forming the immune aggregates observed in these glomerulopathies. The same group (Camussi *et al.*, 1986) has shown that pretreatment with chlorpromazine prevented clustering and shedding of the antigen, probably through its effect on the intracellular microtubules. Although a crude antigenic fraction known to contain the gp 330 antigen (Fx1A) is present on many epithelial cell membranes of organs associated with absorption and secretion (Miettinen and Linder, 1976), the presence of gp 330 is restricted to glomerular epithelial cells, cells of the epididymis and type II pneumocytes (Chatelet *et al.*, 1986b). gp 330 contains mannosyl groups (Miettinen *et al.*, 1980; Makker, 1980; Hoedemaeker *et al.*, 1982c) and differs from gp 300, which possesses maltase activity (Kerjaschki and Farquhar, 1985). The function of gp 330 is still unknown, but it may be related to a defined system of receptor-mediated endocytosis (Chatelet *et al.*, 1986a,b).

Although it is now generally accepted that in AICN immune aggregates are formed *in situ*, some investigators are convinced that at least some of the aggregates present in the glomerulus may be derived from the circulation. Their idea is supported by the results obtained by Naruse *et al.* (1976), who found minute amounts of Fx1A in the circulation of normal rats. Abrass *et al.* (1983), Abrass (1986), and Singh and Makker (1986) claimed that an antigen involved in the pathogenesis of AICN with a molecular weight of 70,000 is present in the circulation. Abrass (1986) found this antigen in the eluates of kidneys of rats in the early phase of AICN. However, these findings need further confirmation.

So far, the gp 330 antigen has only been found in rats. Rabbits and mice do not possess the antigen (Assmann *et al.*, 1985). Although it has been impossible to induce AICN in the dog, this is not due to the absence of gp 330 in its GCW (Behar *et al.*, 1986).

Controversy continues as to whether gp 330 is the only antigen involved in AICN. Up to the present, many investigators have confirmed the results of Kerjaschki and Farquhar (de Heer *et al.*, 1984; Ronco *et al.*, 1984a; Bhan *et al.*, 1985; Kimura *et al.*, 1986), but in addition some investigators claim to have isolated antigens characteristics different from those of gp 330, for which they claim involvement in the induction of AICN. The antigens which have molecular weights ranging from 70,000 to 1,000,000 are listed in Table II together with the literature referenes. Whether these antigens represent molecules possessing identical epitopes, or are dimeric forms of one antigen, or represent different molecules, is unknown at the moment. Because of the reported instability of the Heymann antigen (Kerjaschki and Farquhar, 1982), combined with its tendency to aggregate in highly concentrated forms (Edgington *et al.*, 1968), it is difficult to determine the molecular weight of the intact molecule.

TABLE II

ANTIGENS INVOLVED IN AICN

Antigen MW	References
1,000,000	Edgington *et al.* (1968)
600,000	Makker and Singh (1984)
330,000	Kerjaschki *et al.*(1983)
80,000–120,000	Miettinen *et al.* (1980)
108,000	Natori *et al.* (1986)
90,000	Ronco *et al.* (1984a); Bagchus *et al.* (1986a)
70,000	Abrass (1986)

Barabas *et al.* (1970) claimed to have isolated an antigen responsible for the induction of AICN, which he called F3, and which is derived from a mitochondrial fraction. Additional characteristics of this antigen are lacking.

Some of the antigens listed in Table II (MW 90,000 and 108,000) will be discussed in relation to HICN.

Several investigators have reported the induction of a passive Heymann nephritis with heterologous antibodies directed against a crude renal proximal tubular brush border preparation called Fx1A (Barabas *et al.*, 1969; Hoedemaeker, 1972; Feenstra *et al.*, 1975). In HICN, immune complexes are formed *in situ* immediately after the injection of anti-Fx1A antibodies (van Damme *et al.*, 1978; Couser *et al.*, 1978) (Fig. 17). Since the injection of eluates from rat kidneys with AICN (Sugisaki *et al.*, 1973; Fleuren, 1976) or polyclonal antibodies directed against gp 330 (Kerjaschki *et al.*, 1985) results in a pattern identical to that of HICN, it was concluded that identical antigens are involved in both forms of glomerulonephritis (Madaio *et al.*, 1983). However, it was suggested that in HICN, as in AICN, additional antigens could be involved in the pathogenesis, because other antigens besides gp 330 are shared between the brush border of the epithelial cells of the proximal tubules and the glomerular visceral epithelial cells (Ronco *et al.*, 1984a; Verroust *et al.*, 1986).

Bagchus *et al.* (1986c) showed that eluates from rat kidneys with HICN in addition to antibodies directed against gp 330, contained antibodies reacting with normal rat lymphocytes. The antigen involved was reported to be a glycoprotein with a molecular weight of 90,000 (Ronco *et al.*, 1984a; Kamata *et al.*, 1985). This finding was confirmed by Bagchus *et al.* (1986d). Chatelet and co-workers (1986b) demonstrated that this gp 90 antigen is present on the cell membranes of glomerular visceral epithelial and endothelial cells. Apart from this localization and the occurrence on lymphocytes gp 90 can be found on many structures such as gut brush border, the biliary pole of hepatocytes, and endothelial cells of the heart, lung, liver, and

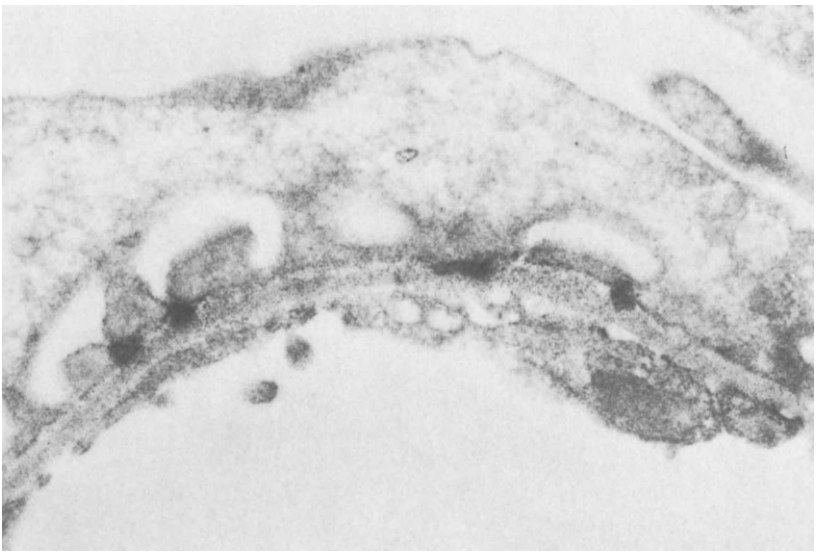

FIG. 17. Immunoelectron micrograph of a glomerular capillary of a rat with HICN at day 7. Peroxidase reaction product indicating the IgG in the immune aggregates is present in the lamina rara externa of the GBM. Unstained, ×22,950. (Reproduced with permission.)

spleen. It is associated with cell types having a high turnover of the cell membrane (Chatelet *et al.*, 1986b). Later, this antigen was characterized as a dipeptidyl-peptidase IV (Ronco *et al.*, 1986), which is also present on B and T lymphocytes (Leer *et al.*, 1986). Bagchus *et al.* (1986c) suggested that gp 90 plays a role in the pathogenesis of HICN. They showed that anti-Fx1A antisera from which the anti-gp 90 activity had been removed, did not become localized in rat glomeruli after perfusion. Ronco *et al.* (1984b) and Bagchus *et al.* (1984) demonstrated that anti-gp 90 antibodies bound transiently to the GBM following intravenous injection (Fig. 18). The conclusion drawn from these experiments was that anti-gp 90 might play a role in the cross-linking of epithelial cell membrane antigens and therefore in the formation of immune aggregates in HICN (Kamata *et al.*, 1985). Assmann *et al.* (1985) showed that in mice, gp 90 is the only antigen responsible for HICN. Whether this gp 90 is similar or identical to the gp 108 found by Natori *et al.* (1986) or gp 110 found by Bhan *et al.* (1985) is not known. Bhan *et al.* (1985) demonstrated the presence of gp 110 in immune aggregates of HICN. Natori *et al.* (1986) showed that injection of antibodies directed against gp 108 led to HICN. Furthermore, they found that eluates from AICN glomeruli reacted with gp 330 but not with gp 108.

FIG. 18. Indirect immunofluorescence showing the binding of monoclonal anti-gp 90 antibody to a normal rat glomerulus. A granular pattern is seen in the glomerulus (arrow). In addition, a binding is observed to the brush border of the proximal tubules. ×400.

On these grounds they concluded that gp 108 is the antigen responsible for HICN.

When all these results are taken together, there seem to be a number of epithelial antigens that could be responsible for the induction of AICN or HICN. On the other hand, it seems logical to consider still other antigens present at the junction of visceral epithelium and GBM as candidates for participation in the formation of immune aggregates once the proper antibody is available.

In 1978, Neale and Wilson reported on a glomerulonephritis occurring spontaneously in New Zealand White rabbits. This membranoproliferative glomerular lesion was not associated with the deposition of electron-dense material in the GBM. Neale *et al.* (1984) found that the disease was caused by antibodies to an intrinsic antigen present on the epithelial foot processes and giving rise to GBM damage, glomerulonephritis, and, in a small percentage of the cases, proteinuria.

The characteristics of this antigen are up till now unknown. In 1979, Thoenes *et al.* reported on a glomerulonephritis following transplantation. They transplanted kidneys from congenic Lew.1N rats which possess the MHC haplotype of BN rats combined with a Lewis background into normal BN recipients. This procedure led after 1–4 months to an immune complex glomerulopathy in the transplanted kidney but not in the kidneys of the recipient. The antigen involved in this glomerulopathy was found to have a different localization than gp 330. It was present in the cytoplasm of

epithelial cells of the midportion of the proximal tubules and presumably cross-reacted with a glomerular antigen. The characteristics of this epithelial antigen were not established.

Recently, Fleming *et al.* (1985) described an epithelial membrane antigen (EMA) occurring on distal tubular epithelium in humans. Howie (1986a) showed that this EMA could also be found in a ring situated at the glomerulotubular junction and might be involved in a glomerular lesion described by Howie and Brewer (1984) and by Howie (1986b) as a glomerular-tip lesion. As yet no experimental counterparts of this lesion are known.

Other epithelial antigens characterized by monoclonal antibodies (Mendrick *et al.*, 1983) are not known to be involved in the induction of glomerulonephritis.

2. *Endothelial Antigens*

Although endothelial cells produce and contain a variety of specific antigens such as the neutrophil adherence factor (Pohlman *et al.*, 1986), MHC class I and class II antigens (Gibbs *et al.*, 1985), prostacyclin (Bevilaqua *et al.*, 1985), interleukin I (Nawroth *et al.*, 1986), antihemophilic factor (Hoyer *et al.*, 1974), and several other anticoagulants, none of these antigens is known to participate in the induction of (experimental) glomerulonephritis. Antibodies to endothelial cells have been described in patients with systemic lupus erythematosus (SLE) (Cines *et al.*, 1982; Matsuo *et al.*, 1985). Binding of such antibodies to glomerular endothelial cells might result in granular deposits in the GCW by redistribution of the antigen, as was shown to occur in lung endothelial cells with antibodies to angiotensin-converting enzyme (Barba *et al.*, 1983). Whether such a mechanism plays a role in SLE glomerulonephritis is not known.

The only endothelial antigen which might participate in the induction of experimental glomerulonephritis is gp 90, as already discussed above in the section on the epithelial antigens. Since this antigen was found together with gp 330 in eluates of HICN (Ronco *et al.*, 1984a; Bagchus *et al.*, 1986c) and is known to bind transiently to the GCW (Bagchus *et al.*, 1984; Ronco *et al.*, 1984b), the finding of Jeraj *et al.* (1981) might be explained by the presence of this antigen. They demonstrated that early in the course of HICN, anti-Fx1A antibodies bound transiently at the endothelial side of the GBM and subsequently formed subepithelial deposits. However, this finding needs confirmation.

Huang and Langlois (1985) identified a cell surface protein they called podoendin. This protein of 62 kDa is present on the cell coats of glomerular endothelial and epithelial cells. An injection of a monoclonal antibody

against this protein resulted in a decoration of the endothelial cells associated with moderate proteinuria. There was no complement activation and no effacement of podocytes.

3. *Basement Membrane Antigens*

The GBM is composed of several specific proteins which are integrated into a highly ordered matrix via defined molecular interaction (Timpl *et al.*, 1984). The proteins of the GBM are a mixture of collagenous and noncollagenous proteins (Spiro, 1967, 1973; Kefalides, 1975, 1978). A list of the components of the GBM discussed in this section is given in Table III. Table IV lists the various antigens known to be involved in the production of experimental glomerulonephritis.

NTN is induced by an antiserum directed against the GBM. Such antisera usually contain antibodies to a variety of antigens. Depending on the antibody used and the species in which it is raised, administration of anti-GBM antibody can result either in immediate proteinuria (Wilson and Dixon, 1981) or delayed proteinuria, depending on the complement-binding properties of the antiserum, the inflammatory mediators involved (Neale and Wilson, 1982), and the occurrence of the autologous phase (Unanue and Dixon, 1967). In humans, glomerulonephritis caused by anti-GBM antibodies is known as Goodpasture's syndrome.

In 1967, Lerner *et al.* convincingly demonstrated the pathogenesis of Goodpasture's syndrome by showing that the disease could be transferred to subhuman primates with antibodies eluted from diseased human glomeruli. The Goodpasture antigen in humans has been characterized. Purified antibodies were found to react with the globular domain of type IV collagen of bovine GBM (Wieslander *et al.*, 1984), especially with antigens with molecular weights of 26,000 and 58,000, respectively (Pressey *et al.*, 1983).

TABLE III

COMPOSITION OF GLOMERULAR BASEMENT MEMBRANE

Category	Components
Collagenous protein	Type IV collagen
Noncollagenous protein (glycoproteins)	Laminin
	Fibronectin
	Entactin
	Nidogen
Proteoglycan	Heparan sulfate
	Chondroitin sulfate
	Dermatan sulfate
Disaccharide units	

TABLE IV

ANTIGENS IN EXPERIMENTAL GLOMERULONEPHRITIS

Antigen type	Examples
Renal	Epithelial antigens
	Endothelial antigens
	Basement membrane antigens
	Mesangial antigens
Nonrenal planted	Antigens involved in serum sickness
	Charged antigens
	Bacterial/protozoal antigens
	Viral antigens
	Nonrenal autoantigens
	Other antigens

Many attempts have been made to isolate the GBM antigen(s) responsible for the induction of NTN in rats, but the true nature of the antigen has not been elucidated yet and the results obtained so far remain controversial, although most investigators (Shibata, 1980; Neale and Wilson, 1982; Abrahamson, 1986) agree that the antigen resides in the noncollagenous protein fraction of the GBM (Thoenes and Hammer, 1967; Mendrick *et al.*, 1983; Cashman *et al.*, 1986).

Since species differences have been found in the composition of the GBM (Behar *et al.*, 1986), species-specific nephritogenic antigen(s) might not always be present in similar GBM components.

A GBM antigen related to the NTN-inducing antigen(s) and responsible for the induction of glomerulonephritis has been reported (Shibata *et al.*, 1978; Shibata and Miura, 1981). In a series of experiments these authors demonstrated that a noncollagenous glycopeptide, which they called nephritogenoside, could induce a chronic form of glomerulonephritis following immunization of normal rats (Shibata and Natori, 1984). Nephritogenoside is present in a linear distribution on the endothelial aspect of the GBM. The disease induced by immunization with this antigen is associated with proteinuria and resembles the sclerosing glomerulonephritis occurring in humans. Natori *et al.* (1986) have reported that a mixed membranous and proliferative glomerulonephritis was observed in rats immunized with impure nephritogenoside containing gp 108. It is of interest to know that in an effort to produce anti-GBM nephritis with antibodies directed to GBM antigens and purified according to Shibata *et al.* (1967), this procedure in the hands of Feenstra *et al.* (1975) led to an impurity similar to that reported by Natori *et al.* (1986) and thus to the discovery of an experimental membranous glomerulopathy that turned out to be HICN. In time, other

GBM components mentioned in Table III were identified, purified, and made available, and many investigators tried to induce glomerulonephritis or functional damage to the glomerular filter by injecting antibodies to the various purified components.

a. Collagen. One major component of the GBM is collagen. Both types IV and V have been found in the GBM (Daniels and Chu, 1975; Scheinman *et al.*, 1976; Tryggvason and Kivirrikko, 1978; Dixit, 1979; Roll *et al.*, 1980; Scheinman *et al.*, 1980a). Recent investigations have shown, however, that type V collagen is an interstitial fibrillar and not a basement membrane collagen (Schuppan *et al.*, 1986). Collagen type IV occurs mainly in the lamina densa, from which it extends in fine cords 2–8 nm long to form a network passing through the laminae rarae to the epithelial and endothelial cells (Inoue *et al.*, 1983; Mynderse *et al.*, 1983). This collagen network is covered with heparan sulfate, laminin, and fibronectin (Farquhar, 1983). The role of collagen protein in immunologically mediated glomerular diseases has not been completely defined (Scheinmann and Fish, 1978; Scheinmann and Michael, 1981). An injection of anticollagen antibodies gives rise to linear binding along the GBM (Rothbard and Watson, 1969), and only a mild and transient glomerulonephritis is observed in mice (Yaar *et al.*, 1982). When, however, anti-collagen IV antibodies are injected simultaneously with antilaminin antibodies, a segmental proliferative glomerulonephritis is induced accompanied by immune aggregates in both laminae rarae and a slight proteinuria in the autologous phase (Yaar *et al.*, 1982; Wick *et al.*, 1982). Once injected, an anticollagen antibody remains detectable linearly in the GBM for at least 1 year (Ph. J. Hoedemaeker, unpublished observations). In humans, the relative amount of collagen IV in the GBM tends to increase with age (Langeveld *et al.*, 1981; Karttunen *et al.*, 1986), but this phenomenon has not been studied in experimental animals.

b. Laminin. Laminin is a multidomain glycoprotein which promotes *in vitro* adhesion of epithelial, endothelial, and muscle cells to an underlying basement membrane (Abrahamson and Caulfield, 1982). With collagen, it constitutes the major component of the GBM and is present through its entire width (Madri *et al.*, 1980; Abrahamson and Caulfield, 1985) (Fig. 19) but more densely in both laminae rarae and the mesangial matrix (Scheinman *et al.*, 1980b; Foidart *et al.*, 1980; Martinez-Hernandez *et al.*, 1982; Mynderse *et al.*, 1983; Martinez-Hernandez and Amenta, 1983; Martinez-Hernandez and Chung, 1984). In aging human basement membranes the relative amount of laminin decreases with time (Karttunen *et al.*, 1986).

When antibodies to laminin are injected into rats, they bind linearly to the GBM as well as to the mesangium, without activation of complement

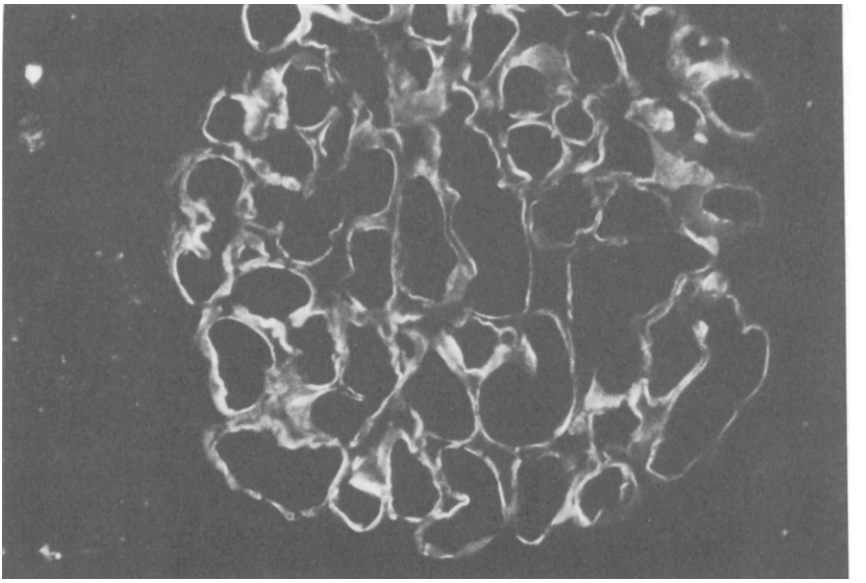

FIG. 19. Indirect immunofluorescence demonstrating the presence of laminin in the rat glomerulus. A binding of rabbit antilaminin antibody is observed along the GBM in a linear pattern and in the mesangium. ×500. (Courtesy Drs. Bruijn and Hogedoorn.)

(Abrahamson and Caulfield, 1982). This treatment is associated with the appearance of subendothelial deposits, an influx of macrophages and a dose-dependent albuminuria (Abrahamson and Caulfield, 1982). Transplantation of such kidneys into rats which have been passively immunized against the heterologous antibody injected, leads to an immediate detachment of endothelial and epithelial cells accompanied by severe proteinuria (Feintzeig *et al.*, 1986). This reaction was shown to be complement dependent (Feintzeig *et al.*, 1986).

Antilaminin antibodies injected into mice do not, however, induce glomerulonephritis (Yaar *et al.*, 1982). Mice immunized with laminin show linear binding of IgG along the GBM and in the mesangium and develop subendothelial electron-dense deposits associated with a splitting of the GBM (Murphy-Ullrich and Oberley 1984; Wick *et al.*, 1982). Abrahamson *et al.* (1983) reported aggravation of proteinuria after injection of antilaminin antibody with a nephrotic syndrome induced by aminonucleoside of puromycin.

An interesting finding was reported by Matsuo *et al.* (1986), who showed that the spikes formed in the GBM after the induction of AICN are composed solely of laminin.

The occurrence of antilaminin antibodies has been observed under several conditions unrelated to kidney disease. Bernard *et al.* (1984) observed the occurrence of antilaminin antibodies in rats after long-term exposure to cadmium. Foidart *et al.* (1986) found antilaminin antibodies in women with preeclampsia (Foidart *et al.*, 1981). Finally, Szarfman *et al.* (1982) observed antilaminin antibodies in patients with Chagas's disease.

c. Fibronectin. Fibronectin is a glycoprotein with a molecular weight between 200,000 and 500,000 that plays a role in cell–cell and cell–matrix adhesions (Mosher, 1980; Ruoslakti *et al.*, 1981; Hynes and Yamada, 1982). It is present in the matrix between endothelial and mesangial cells, and small quantities are found peripherally in the GCW, localized subendothelially and around the epithelial foot processes (Courtoy *et al.*, 1980; Oberley *et al.*, 1979). In spite of these findings it is considered doubtful that fibronectin is an intrinsic component of the GBM (Courtoy *et al.*, 1980, 1982). There is a tendency to explain its presence by deposition from the circulation (Boselli *et al.*, 1981; Amenta *et al.*, 1983; Courtoy and Boyles, 1983).

Immunization with fibronectin results in a mild proliferative glomerulonephritis associated with granular IgG deposits in the GCW. Ultrastructurally, electron-dense deposits are found within the GBM at the endothelial side (Murphy-Ullrich *et al.*, 1984).

d. Entactin. Entactin is a molecule of 158 kDa occurring along the cell membrane of glomerular epithelial and endothelial cells but not in the mesangium (Bender *et al.*, 1981; Martinez-Hernandez and Chung, 1984). Its function is unknown, but it may serve as an attachment protein (Carlin *et al.*, 1981). At present, no involvement in the induction of glomerulonephritis is known.

e. Nidogen. Nidogen is present in all basement membranes (Timpl *et al.*, 1983; Dziadek *et al.*, 1985). It is similar to laminin and fibronectin but its biological significance is not known (Hynes and Yamada, 1982). Its ability to bind to laminin as well as to collagen IV may give it a stabilizing function in the GBM (Abrahamson, 1986). It is not known to have a role in the induction of glomerulonephritis.

f. Proteoglycans. The proteoglycans, and especially heparan sulfate, constitute the anionic sites in the lamina rara interna and externa, and hence play an important role in the sieving properties of the GCW. Besides heparan sulfate, other proteoglycans such as chondroitin sulfate and dermatan sulfate are present in the GBM (Kanwar and Farquhar, 1979c; Kanwar *et al.*, 1981a), although heparan sulfate is the most important one (Brown *et al.*, 1981) (Fig. 20). Heparan sulfate occurs in the GBM in three forms, the largest of which binds to collagen IV (Inoue *et al.*, 1983). This sulfate is found mainly in the laminae rarae (Kanwar and Farquhar, 1979b).

However, Klein *et al.* (1983), who used a monospecific antiserum against bovine heparan sulfate proteoglycan, did not find binding in glomeruli of rats, mice, and humans.

The use of enzymatic methods to remove heparan sulfate and other sulfated proteoglycans from the GBM results in detachment of podocytes and a dramatic increase in the permeability of the glomerular filter for ferritin (Kanwar *et al.*, 1980) and albumin (Rosenzweig and Kanwar, 1982). When antibodies directed to heparan sulfate are injected into rats, binding is seen in both laminae rarae. This is associated with an activation of complement, an influx of leukocytes, and an associated dose-dependent proteinuria typical of anti-GBM nephritis (Miettinen *et al.*, 1986; Stow *et al.*, 1985). Some days after an injection of anti-heparan sulfate antibody, a subepithelial thickening of the GBM is observed (Wick *et al.*, 1982). It is not known whether this thickening, like the spikes in AICN, is produced mainly by the presence of laminin (Matsuo *et al.*, 1986).

Faaber *et al.* (1986) demonstrated a reactivity of polyclonal and monoclonal anti-DNA antibodies with heparan sulfate. This finding could be important for the explanation of the role of anti-DNA activity in human or murine SLE.

g. Disaccharide Units. The GBM contains two carbohydrates which account for approximately 10% of its total weight. One of these is a disaccharide consisting of glucose and galactose associated with collagenous proteins; the other is a heteropolysaccharide containing mannose, galactose, hexosamine, fucose, and sialic acid. The latter remains after digestion with collagenase and contains the major nephritogenic antigens responsible for the induction of NTN (Glassock *et al.*, 1968; Huang and Kalant, 1968; Misra, 1973; Holdsworth *et al.*, 1981; van Liew *et al.*, 1983; Pusey *et al.*, 1986).

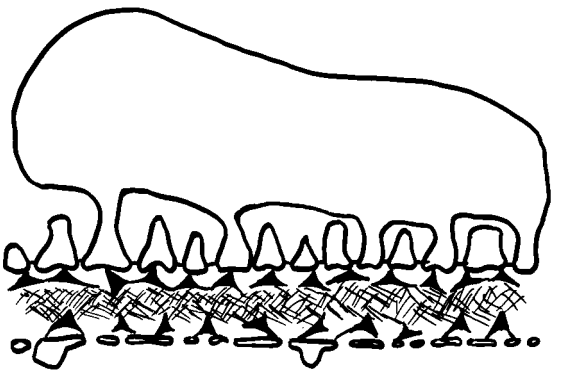

FIG. 20. Schematic representation of the GCW demonstrating the presence of proteoglycans (▲) in the laminae rarae of the GBM. (Modified after Farquhar and Kanwar, 1982.)

Other basement membrane components—such as amyloid P (Dyck *et al.*, 1980) or the antigen reactive with sera from patients with epidermolysis bullosa aquisita (Woodley *et al.*, 1984)—are not known to be involved in glomerulonephritis and will not be discussed in this article.

4. *Mesangial Antigens*

The Thy-1 antigen present on the cell membrane of mesangial cells (Figs. 21 and 22) is known to play a role in the induction of experimental glomerulonephritis. This glycoprotein, which is present on the cell membranes of thymocytes and brain cells (Kuchel *et al.*, 1978), also occurs on the cell membranes of mesangial cells of the kidneys of rats (Morris and Ritter, 1980; Barclay, 1981; Bukovsky *et al.*, 1981; Paul *et al.*, 1984; Bagchus *et al.*, 1986a; Yamamoto *et al.*, 1986), dogs (Dalchau and Fabre, 1979; McKenzie and Fabre, 1981), and humans (Dalchau anf Fabre, 1979). Thy-1 is a molecule of approximately 18 kDa; its function is not known (Kuchel *et al.*, 1978). Bagchus *et al.* (1986a,b) described a glomerulopathy resulting from an injection of a complement-activating monoclonal antibody directed against the Thy-1 antigen. This glomerulopathy is characterized by mesangiolysis (Figs. 23 and 24) and heavy proteinuria, and in later

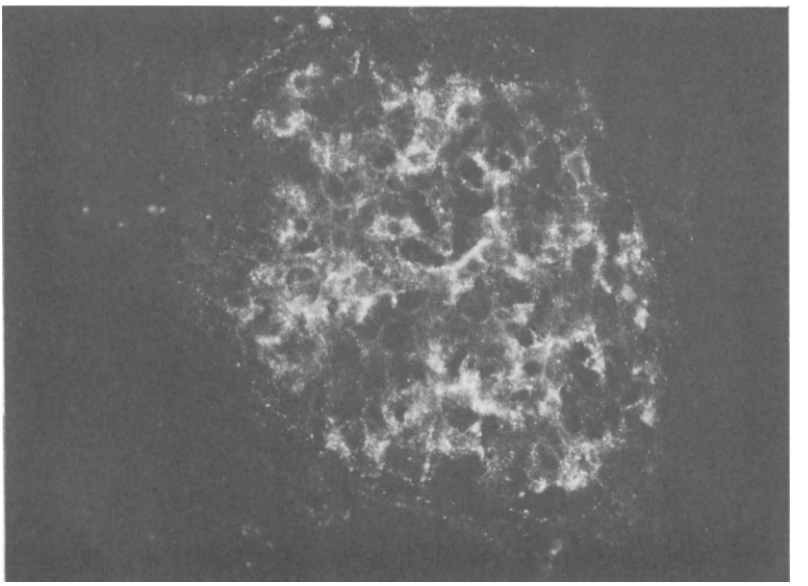

FIG. 21. Indirect immunofluorescence showing the binding of a monoclonal anti-Thy-1.1 antibody to a normal rat glomerulus. A fine granular staining is observed, mainly in the mesangium but occasionally along the GCW. ×400. (Courtesy Dr. W. M. Bagchus; reproduced with permission.)

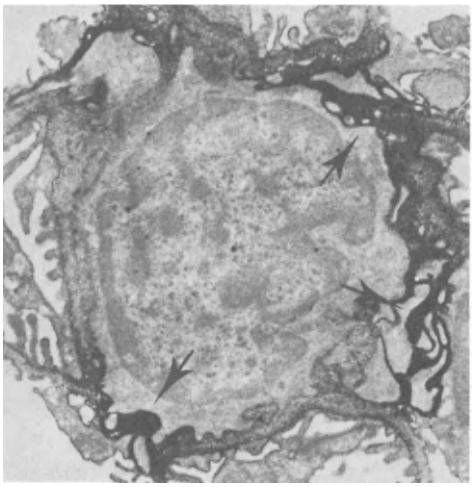

FIG. 22. Immunoelectron micrograph of a glomerulus from a rat kidney perfused with monoclonal anti-Thy-1.1 antibody. Peroxidase reaction product is observed in the mesangial matrix (arrows). ×10,000.

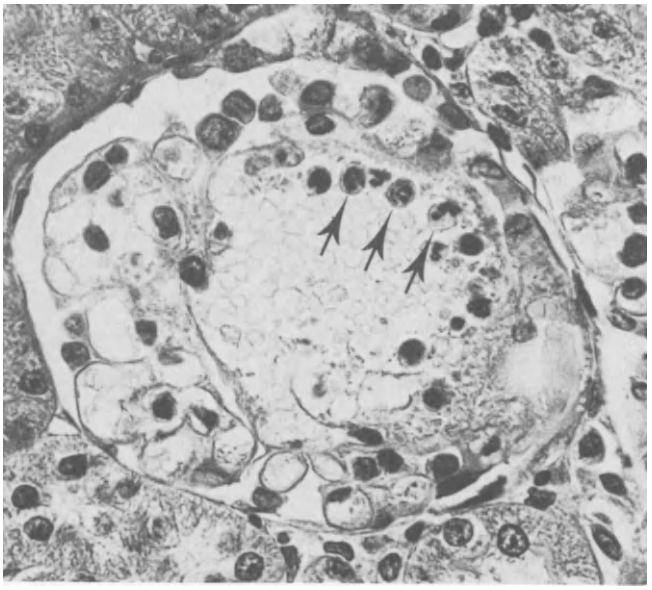

FIG. 23. Glomerulus from a rat 3 days after the injection of a monoclonal anti-Thy-1.1 antibody. Glomerular microaneurysms which are lined by neutrophils (arrows) can be observed. Hematoxylin–eosin, ×500.

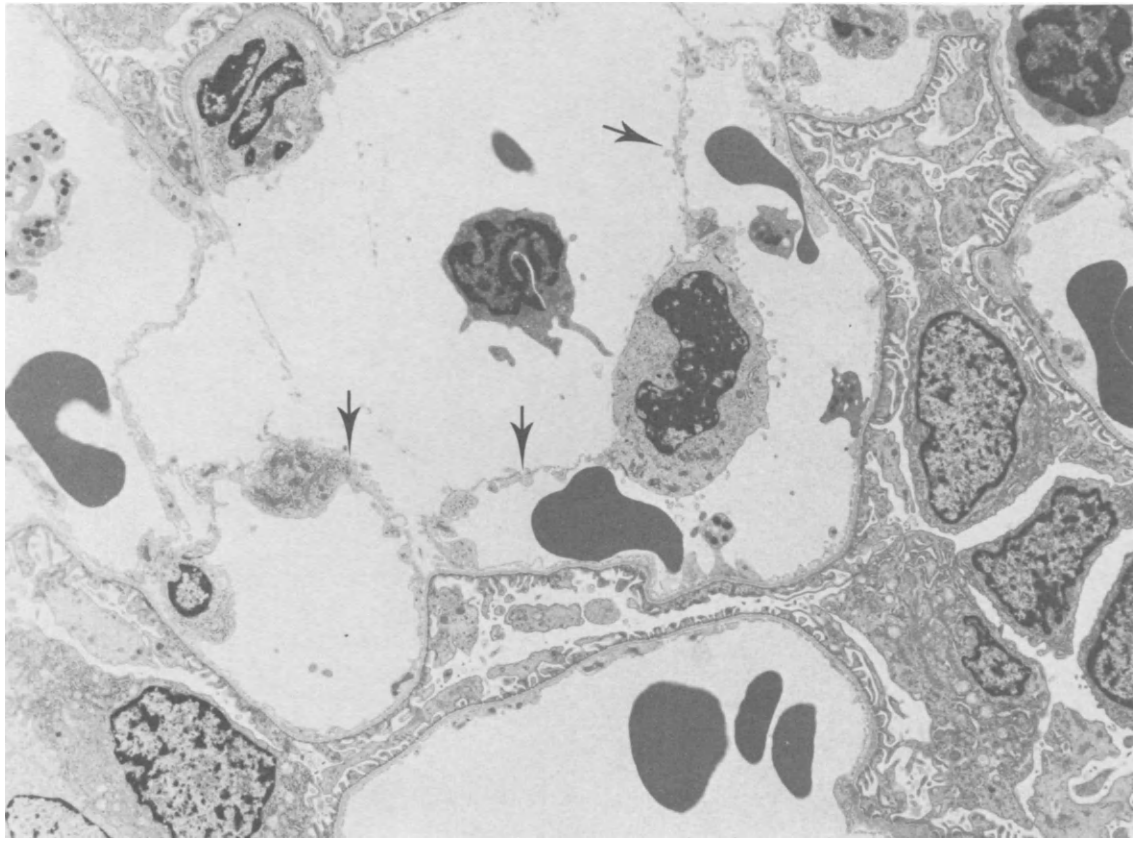

FIG. 24. Electron micrograph of a glomerulus of a rat injected 6 days previously with a monoclonal anti-Thy-1.1 antibody. Glomerular aneurysms containing thin strands of endothelial cells are seen (arrows). Uranyl acetate–lead citrate, ×2850.

stages by mesangial proliferation, sometimes associated with crescent formation. The observed mesangiolysis is complement dependent, since injection of non-complement-binding monoclonal anti-Thy-1 antibodies did not lead to a glomerular reaction. These findings were recently confirmed by Yamamoto and Wilson (1986). Some features of this experimental glomerulopathy caused by anti-Thy-1 antibodies resemble the mesangiolysis obtained after an injection of *Agistrodon* venom (Bradfield *et al.*, 1977; Cattell and Bradfield, 1977; Sakurai *et al.*, 1986). The extent to which cross-reactivity of monoclonal anti-Thy-1 antibodies with vimentin (Dulbecco *et al.*, 1981), actin (Dales *et al.*, 1983), or IgG subclasses (Bonewald *et al.*, 1984) plays a role, is not known.

Mendrick and Rennke (1986) described an intrinsic mesangial antigen which they characterized with a monoclonal antibody. Intravenous administration of this antibody led to immune aggregate formation in the mesangial area, similar to that in human IgA glomerulopathy, but neither proteinuria nor hematuria was observed.

Other mesangial antigens involved in the induction of a glomerular disease are not known at present.

B. Nonrenal (Planted) Antigens

1. *Serum Sickness*

Serum sickness glomerulopathy was first described by Germuth (1953) and by Dixon *et al.* (1961). In cases of acute serum sickness, a large amount of BSA is given to rabbits in one bolus injection. The resulting anti-BSA antibodies combine with the antigen in the circulation to form immune complexes, approximately between day 10 and day 12 (Fig. 25). In cases of chronic serum sickness, BSA injections are given daily in a dosage determined on the basis of the antibody titers such that a continuous antigen excess is obtained and immune complexes are formed. In both models the glomerulonephritis is thought to result from the glomerular deposition of soluble immune complexes present in the circulation. Depending on their size, the immune complexes are deposited either in the GCW or in the glomerular mesangium.

Physicochemical characteristics play an important role in the deposition of immune complexes. These characteristics determine the equilibrium between immune aggregates and immune complexes on the one hand and between immune complexes and their components on the other hand: $(AgAb)_n \leftrightarrows AgAb \leftrightarrows Ag + Ab$. If immune complexes are formed with antibodies of low avidity, the equilibrium shifts to the right, thus fulfilling a prerequisite for the *in situ* formation of immune complexes in tissues. This

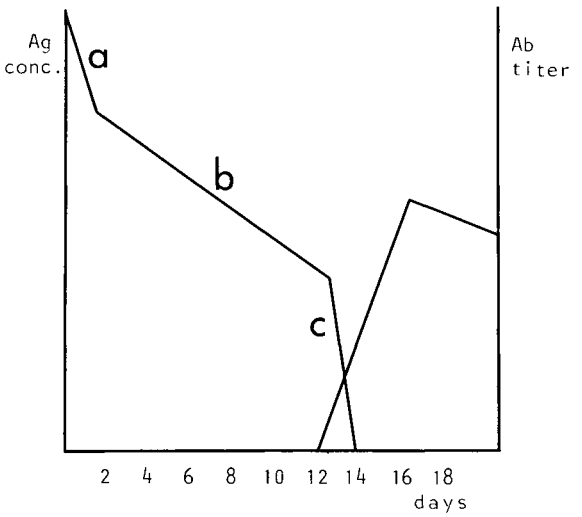

FIG. 25. Schematic representation of acute serum sickness. Serum concentration of the injected antigen decreases in three phases: (a) distribution among fluid compartments, (b) through catabolism, (c) through immune elimination. The maximum incidence of glomerulonephritis is found after 14 days. [Modified from Germuth (1953); reproduced with permission.]

hypothesis is consistent with results obtained by various investigators (Kuriyama, 1973; Steward, 1979; Lew *et al.*, 1984; Lew and Steward, 1984), who showed that in chronic serum sickness, membranous glomerulonephritis—in which glomerular immune aggregates are believed to be formed *in situ* (Couser and Salant, 1980)—develops only in animals producing low-affinity antibodies (Germuth *et al.*, 1979; Iskander *et al.*, 1986).

Once immune aggregates are present in glomerular structures, they can react specifically or nonspecifically with molecules and substances derived from the circulation. These include antibodies to IgG, especially in later stages of chronic serum sickness. Kano and Millgrom (1980) found IgM anti-Fab antibodies, and Penner *et al.* (1982) demonstrated the presence of rheumatoid factor in the deposits. Antiidiotypic antibodies too can be detected in glomerular immune aggregates (Rose and Lambert, 1980; Lambert *et al.*, 1982; Rose *et al.*, 1982) (see Fig. 13).

It is also known that antigens can react with glomerular immune aggregates. In cases of serum sickness (Valdes *et al.*, 1969), excess antigen can remove glomerular immune aggregates from both the GCW and the mesangial area (Mannik and Striker, 1980). The finding that it becomes progressively more difficult to remove glomerular immune aggregates by the use of an excess of antigens in the later stages of chronic serum sickness

(Haakenstad *et al.*, 1983) gives additional support to the idea that different antigen–antibody systems participate in glomerular aggregate formation in late stages of immune complex glomerulopathies.

Attention must also be given to the passive entrapment of substances in the mesangial area. Protein aggregates (Mauer *et al.*, 1973), immune complexes (Sterzel *et al.*, 1982; Mannik, 1980), or other substances such as polyvinyl alcohol (Sterzel *et al.*, 1983b), are taken up by the glomerular mesangium depending on their biological activity (Batsford *et al.*, 1985) or their size (Latta and Fliegel, 1985). This uptake is known to vary with the functional activity of the mesangium (Mauer *et al.*, 1972; Grond and Elema, 1981; Stein *et al.*, 1983; Keane and Raij, 1985).

Under three conditions substances deposited in the mesangium are known to be associated with glomerulonephritis. In 1973 Mauer *et al.* demonstrated that aggregated IgG taken up by the mesangium reacted with anti-IgG antibodies and led to acute glomerular damage accompanied by an influx of polymorphonuclear leukocytes. This glomerulonephritis model was the first model to establish the mechanism of *in situ* formation.

Polyvinyl alcohol injected into rats is taken up by the mesangium (Sterzel *et al.*, 1983b). This mesangial uptake induces variable degrees of hypercellularity caused by an influx of monocytes into the mesangium (Seiler *et al.*, 1983, 1986).

The third example of passive entrapment of immune complexes in the mesangium involves models of human IgA glomerulopathy. Because many studies on human IgA nephropathy have shown an association of this disease with viruslike respiratory tract infections or gastrointestinal syndromes (Berger, 1969; McCoy *et al.*, 1974; Clarkson *et al.*, 1977; Woodroffe *et al.*, 1980), and since a significant elevation of IgA antibody levels in serum and exocrine fluids may occur in response to oral immunization (Craig and Cebra, 1975; Vaerman and Heremans, 1970; Crabbe *et al.*, 1979), Emancipator *et al.* (1983) tried to induce an experimental IgA glomerulopathy. Oral immunization of mice with ovalbumin gave rise to electron-dense IgA-containing mesangial deposits in 73% of the cases. In these cases complement activation induced hematuria but not an influx of leukocytes (Emancipator, 1986; Emancipator *et al.*, 1986).

Recently, Sato *et al.* (1986) succeeded in inducing an IgA-like glomerulonephritis in mice by the administration of oral lactalbumin combined with a reticuloendothelial system blockade with colloidal carbon. After 30 weeks, more than 90% of the animals had developed mesangial IgA-containing electron-dense deposits. Since polymeric IgA and immune complexes containing IgA are known to be transported through the hepatocyte by secretory component and excreted in the bile, Melvin *et al.* (1983) tried to induce an IgA glomerulopathy in rats by ligating bile ducts.

This ligation resulted in a selective increase of IgA, C3, and secretory component in the mesangial area, but these changes were not accompanied by signs of glomerulonephritis.

Whether *in situ* binding of IgA to mesangial matrix plays an additional role is unknown.

2. *Charged Antigens*

The elucidation of the glomerular filter as a charge- and size-selective barrier (Chang *et al.*, 1975; Venkatachalam and Rennke, 1978; Brenner *et al.*, 1978b) has had important implications for hypotheses on the pathogenesis of immunologically induced glomerular injury (Ward *et al.*, 1984). Whether immune deposits are formed *in situ* or by deposition of circulating immune complexes, the anionic charge of the GCW plays a significant role by providing either a charge barrier (Kanwar and Farquhar, 1979a) or a site for planted antigen, especially when the antigen posesses a positive charge (Vogt *et al.*, 1982). For this reason, the charged antigens have become important in the induction of experimental glomerulonephritis, and presumably also in the genesis of human glomerulonephritis. This group of antigens will therefore be discussed separately in this section.

The exclusion size for neutral molecules from the GCW is 4 nm (Vogt and Batsford, 1984), and cationized ferritin with an effective radius of 6.1 nm has free access to all layers of the GCW. Furthermore, Vogt and Batsford (1984) showed that molecular size is also important for fixation to the GCW. They found that molecules of sizes up to 40 kDa fix poorly to the glomerular filter but that larger molecules gave an increase in glomerular persistence. Given a right size for molecules to be filtered, an interaction of their cationic charges with fixed anionic sites in the GCW can induce a chain of events resulting in a loss of fixed negative charges of the GCW, epithelial cell detachment, and proteinuria (Batsford *et al.*, 1983). This was demonstrated by Seiler *et al.* (1975), who infused protamine sulfate into normal rat kidneys; this led to obliteration of epithelial foot processes, presumably due to the neutralization of anionic charges (Sonnenburg-Hatropoulos *et al.*, 1984). Hunsicker *et al.* (1981) showed that infusion of the polycation hexadimethrine in rats resulted in neutralization of the negative charges of the GCW associated with heavy proteinuria (Bertolatus *et al.*, 1984).

In 1982, Hoyer *et al.* demonstrated that alternating renal perfusion with heparin and protamine sulfate resulted in complexes with localizations in the GCW roughly indicating the negatively charged sites. The *in situ* formation of these spherical protamine–heparin complexes was shown to be unrelated to the observed proteinuria (Hoyer *et al.*, 1982; Weening *et al.*, 1983) (Fig. 26). In 1980, Fleuren *et al.* showed that alternating perfusion

with lysozyme and antilysozyme and with BSA and anti-BSA led to local formation of immune complexes in rat glomeruli (see Fig. 12). They interpreted this phenomenon as a reaction between a positively charged antialbumin antibody, which was first planted in the GCW by binding to the anionic sites, and its antigen. These results suggested that cationic proteins can be considered as candidates for nephritogenic antigens (Vogt *et al.*, 1980; Batsford *et al.*, 1980a,b; Oite *et al.*, 1982).

A series of investigations was undertaken in which a variety of cationic proteins were planted in the GBM and allowed to react with their respective antibodies to obtain immune aggregates in the GCW (Purtell *et al.*, 1979). Border *et al.* (1982) showed that immune aggregates were formed between cationized albumin molecules planted in the GCW and antialbumin antibodies, but this did not occur with either neutral or anionized albumin. Thaiss *et al.* (1986) were able to induce formation of immune aggregates in the GCW by injecting first cationized human IgG and then anti-IgG antibody. Oite *et al.* (1983) infused cationized human IgG into rats preimmunized for human IgG, which resulted in a severe crescentic glomerulonephritis. Similar results were obtained with injections of cationized ferritin into preimmunized animals (Oite *et al.*, 1985). With respect to this model the investigators reasoned that the immune complexes were initially formed subendothelially and were later dissociated and re-formed subepithelially (Vogt *et al.*, 1982; Vogt and Batsford, 1984). This re-formation of dissociated immune complexes probably also occurs in cases of passive serum sickness in which injection of preformed immune complexes is followed by *in situ* formation of immune complexes in the glomeruli (see preceding section).

This is not the only way in which circulating immune complexes can become localized in glomeruli, however. A finding that established the importance of anionic sites in the induction of experimental glomerulonephritis was made by Adler *et al.* (1983b). They found that an infusion of protamine sulfate could prevent the formation of immune aggregates between planted antigens and antibody in the GCW even to remove these aggregates by competitive binding to anionic sites.

Gallo *et al.* (1981) and Koyama *et al.* (1986) showed convincingly that immune complexes bearing a cationic charge could react with the anionic sites of the GCW. The more cationic these complexes are, the more they become localized in the subepithelial space and the more nephritogenic they are (Gallo *et al.*, 1983). Ford and Kosatka (1985) showed that the cationic moiety of IgM rheumatoid factor too can bind to the anionic sites of the GCW and act as a planted molecule that can bind circulating immune complexes or aggregated IgG.

Besides cationized proteins or immune complexes which bind to the

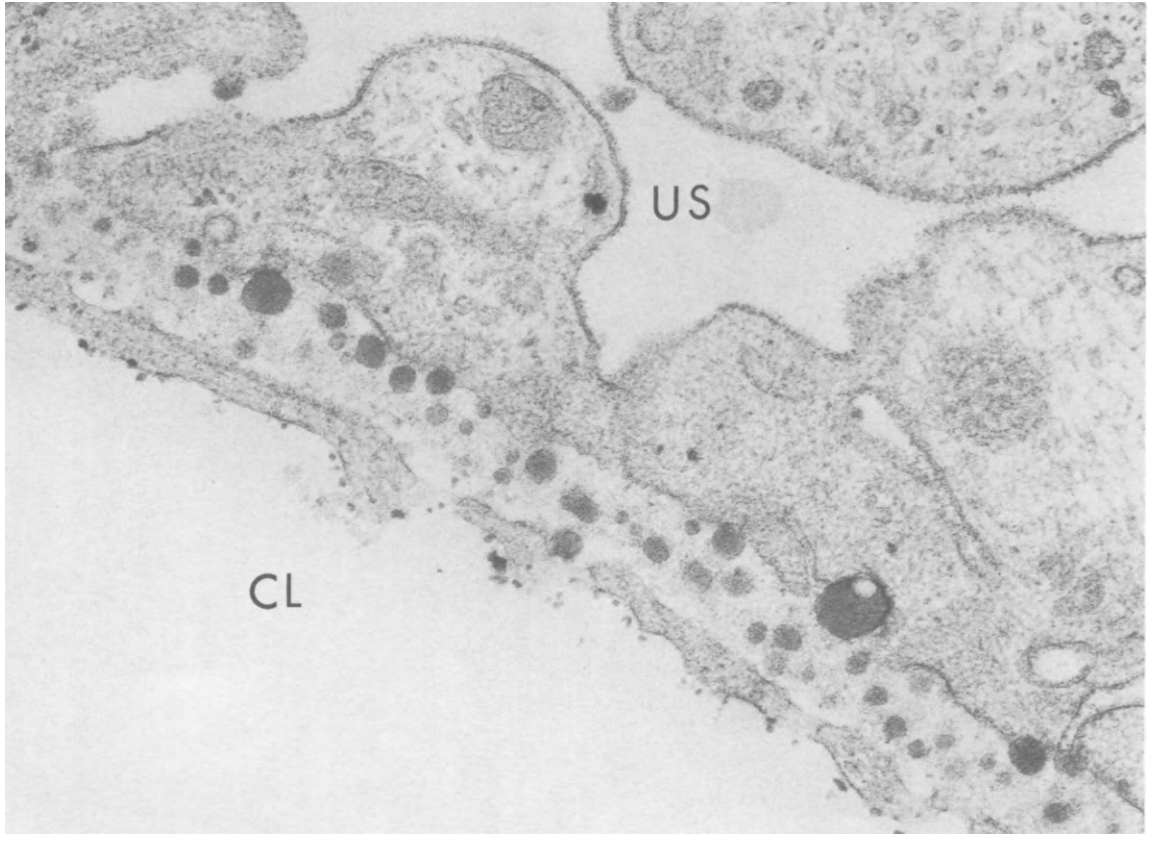

FIG. 26. Electron micrograph of a glomerulus from a rat made nephrotic with adriamycin and perfused alternate with protamine and heparin. Dense protamine–heparin aggregates can be seen in all layers of the GCW under fused epithelium. CL, Capillary lumen; US, urinary space. Uranyl acetate–lead citrate, ×61,600.

GCW, anionic substances have been shown to possess such an affinity on a different basis. Izui *et al.* (1976) showed that DNA can bind to collagen *in vitro*. Lake *et al.* (1985) postulated that fibronectin contains domains linking with both DNA and collagen, and which would mean that fibronectin could act as a mediator in the binding of DNA to the GCW. Furthermore, Melvin *et al.* (1984) demonstrated that negatively charged IgG and other anionic proteins can be electrostatically bound to normal GBM.

As in the studies done by Adler, mentioned above, binding of immune complexes to anionic sites could be prevented by neutralizing these charged sites in the GCW. Gauthier and Mannik (1986) showed that injection of protamine sulfate or cationized rabbit serum albumin prevented the deposition of cationized immune complexes in this region. These authors also showed that, once bound to the GCW, cationized immune complexes were readily replaced by these substances if the latter were injected not later than 1 minute after the administration of immune complexes. After 1 hour, however, this replacement appeared to be impossible, which implies that once the immune complexes in glomeruli condense to form larger deposits, other forces besides charge–charge interactions are responsible for their retention (Gauthier and Mannik, 1986).

The ability of antigen and antibody to interact also plays an important role in glomerular localization. Koyama *et al.* (1986) found that the size of immune complexes formed between cationized BSA and anti-native BSA is approximately 7 S, whereas that of those formed with native or anionized BSA is around 19 S. Moreover, anti-native BSA antibody possessed low affinity for cationized BSA and the resulting immune complexes were small and of low avidity.

Once immune aggregates are present in the GCW they can be modified by many reactants from the circulation. These reactants include albumin (Schneeberger *et al.*, 1979), nonspecific proteins (Bellon *et al.*, 1982; Kimura *et al.*, 1986), free antibodies (Wilson and Dixon, 1971), anti-IgG antibodies (Sindrey *et al.*, 1981), and antiidiotypic antibodies (Rose and Lambert, 1980; Lambert *et al.*, 1982; Rose *et al.*, 1982) (see Fig. 13). Feintzeig *et al.* (1985) showed that the GCW partially retards the passage of these reactants, and also that anionic antibodies are restricted in their passage across the GCW to bind to subepithelially planted antigens but that there is no such restriction where the antigen is planted subendothelially. Circulating immune complexes can be bound by the glomerular deposits, especially if they are of the same species and the planted immune complexes are formed in antibody excess (Ford and Kosatka, 1980), but also if they are of different species (Schneeberger *et al.*, 1974). Antigen can bind to glomerular aggregates as well. Binding of small amounts of antigen to immune complexes formed in antibody excess can result in lattice

formation, whereas binding of large amounts can lead to dissolution of the deposits (Valdes *et al.*, 1969; Wilson and Dixon, 1971; Mannik and Striker, 1980).

3. *Bacterial and Protozoal Antigens*

Since Longcope *et al.* (1927) reported the presence of an infection in 85% of the cases of acute diffuse glomerulonephritis, many investigators have tried to induce glomerulonephritis in animals by injecting bacteria or bacterial toxins (Horn *et al.*, 1937b). Antigens associated with streptococci (Treser *et al.*, 1969; Yoshizawa *et al.*, 1973; Seligson *et al.*, 1983) and staphylococci or pneumococci (Hyman *et al.*, 1975; Pertschuk *et al.*, 1976) have been found in the glomerulus at the onset of the infection, but their role in the induction of glomerulonephritis is not yet clearly understood. Although it was generally accepted that it was not the bacteria themselves but rather an immune reaction to bacterial products that played the most important role in the pathogenesis of glomerulonephritis, some of these studies were rather successful, and suggested that such an immune reaction indeed took place. In 1945, Cavelti and Cavelti tried to induce an autoimmune glomerulonephritis by immunizing rabbits with a mixture of β-hemolytic streptococci and a kidney homogenate. This led to a typical proliferative glomerulonephritis, as was later confirmed by Bykovskaya and Vihert (1965). In 1954, Reed and Matheson purified an antigen from the cell wall of nephritogenic streptococci. Rabbits injected with this antigen developed an acute glomerulonephritis which was associated with hypertension (Fardy *et al.*, 1969). Becker and Murphy (1968) injected rabbits with nephritogenic streptococci isolated from patients with glomerulonephritis. This, too, resulted in proliferative glomerulonephritis but here associated with proteinuria, hematuria, and in 20% of the cases with azotemia.

Lange *et al.* (1976) isolated an antigen from nephritogenic streptococci, which they called endostreptosin. They were able to show that in an early phase of the infection, this antigen was bound to the inner aspect of the GBM. After 7–10 days, when the patient developed an immune reaction against the streptococci and therefore to this antigen, binding of antibodies to this planted antigen led to immune aggregate formation and glomerulonephritis (Lange *et al.*, 1983). The same group (Lange *et al.*, 1986) demonstrated cross-reactivity between antigens from the cell membrane of streptococci and murine GBM. The significance of this finding for human or experimental glomerulonephritis is uncertain.

In 1979, Fiedel and Jackson obtained mesangial deposition of immunoglobulins after immunization of rats with a conjugate composed of BSA and

lipoteichoic acid isolated from group A streptococci; however, signs of glomerulonephritis did not develop. Bergholm and Holm (1983) described a proliferative glomerulonephritis in rabbits, resembling human poststreptococcal disease, following subcutaneous inoculation of the rabbits with group A streptococci. This glomerulonephritis was associated with granular electron-dense deposits of IgG along the GCW.

Finally, Albini *et al.* (1985a,b) induced a crescentic glomerulonephritis in rabbits after repeated intravenous injection of *Streptococcus mutans*. This glomerulonephritis was associated with linear IgG deposits along the GCW and humps in electron microscopy.

Although the rabbit seems to be a suitable animal for the induction of acute glomerulonephritis with streptococci, the antigenic part of the glomerular immune deposits was not identified and hence the relevance for human disease has yet to be established.

Apart from models based on *Staphylococcus aureus*-infected indwelling catheters in baboons, leading to a membranoproliferativelike glomerulonephritis (Leary *et al.*, 1981) and a mesangial proliferative glomerulonephritis induced in hamsters by an experimental infection with *Leishmania donovani* (Oliviera *et al.*, 1985), attempts to induce glomerulonephritis with many different bacteria have not been successful.

In 1983, Poels *et al.* reported on a glomerulonephritis in mice induced by a *Plasmodium berghei* infection. This glomerulonephritis was associated with immune aggregates along the capillary wall. Pakasa *et al.* (1985) found that the *Plasmodium* antigen was present in the mesangium and concluded that this might represent a basic mechanism for the disease. *Plasmodium*-induced glomerulonephritis was also studied by Geimann and Siddiqui (1969) and Hutt *et al.* (1975). Monkeys infected with *Plasmodium* developed a membranoproliferative glomerulonephritis associated with the nephrotic syndrome (Voller *et al.*, 1971, 1973).

Bacterial and protozoal antigens involved in glomerulonephritis induced by polyclonal stimulation will be discussed in the section on nonrenal autoantigens (Section IV,B,5).

4. *Viral Antigens*

A few forms of experimental glomerulonephritis have been described in which viral antigens play a role. One model of infection that has been studied extensively concerns the lymphocytic choriomeningitis virus in young mice. Inoculation during the neonatal period produces a carrier state which is associated with, among other things, an immune complex glomerulonephritis (Oldstone and Dixon, 1969). This glomerulonephritis is related to the amount of virus present, the antibody response of the animals, and

the interaction between virus and antibody, and is therefore thought to result from an immune reaction against viral antigens. Oldstone and Dixon (1968) showed that mouse strains vary in susceptibility to this kind of glomerulonephritis. They found that SWR/J mice harboring many viral antigens and having a high production of antibody, developed severe glomerulonephritis, whereas C3H mice had little virus, a low antibody production, and only occasionally developed glomerulonephritis.

In genetically susceptible Aleutian mink, a chronic persistent parvovirus infection has been described which is associated with various forms of glomerulonephritis, for example acute proliferative, membranoproliferative, and local proliferative glomerulonephritis (Müller-Peddinghaus and Trautwein, 1983). IgG antibodies eluted from the glomeruli of these animals proved to be directed against viral antigens (Müller-Peddinghaus and Trautwein, 1983).

In 1980, Smith and Wehner described a transient glomerulonephritis in HA/ICR mice associated with a cytomegalovirus infection. Cytomegalic changes were observed in the mesangial cells, which contained the viral antigen from Day 5 on. After that time the antigen was found in the hilar region and in association with hyalinization of the vascular pole.

In 1983, Wright and Cornwell showed that an adenovirus infection in dogs led to mesangial electron-dense deposits containing viral antigen. From the second day on, inclusion bodies were found in mesangial and endothelial cells.

5. *Nonrenal Autoantigens*

In recent years increasing attention has been given to glomerulonephritis associated with autoimmune diseases. The autoimmune response in the experimental animals discussed in this section usually originates from a polyclonal stimulation of B lymphocytes, which either occurs spontaneously as in SLE-like diseases or results from an immunoregulatory disturbance induced by external factors such as viral diseases, toxic agents, or a graft-versus-host reaction.

In this section the autoantigens involved in murine SLE will be dealt with first, after which polyclonal stimulation of B lymphocytes induced either by toxins or by graft-versus-host disease will be discussed as a cause of glomerulonephritis.

It has long been known that some mouse strains—NZB, NZW, BXB, MRL, and especially (NZB × NZW)F_1—spontaneously develop an SLE-like disease associated with an autoimmune response to DNA and an immune complex glomerulonephritis (Miyasato *et al.* 1967; Rosenberg *et al.*, 1984; Izui *et al.*, 1984a) (Fig. 27). After Koffler *et al.* (1971) reported

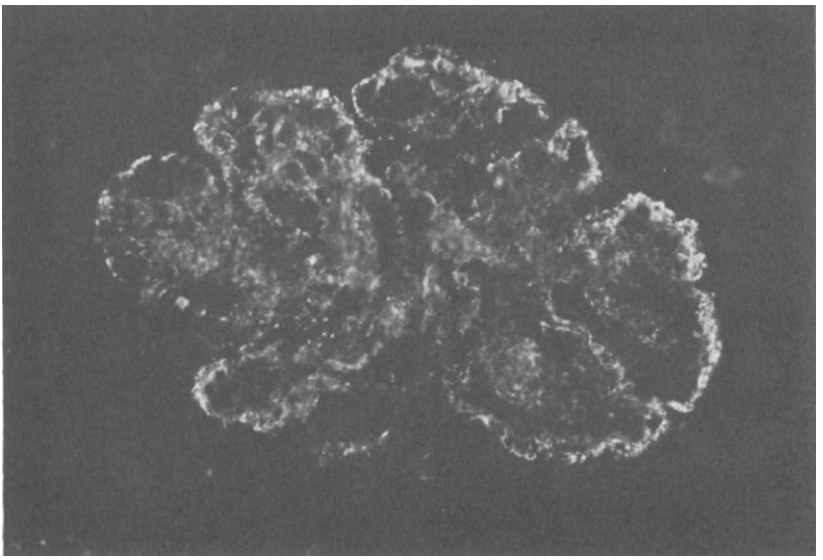

FIG. 27. Direct immunfluorescence demonstrating the presence of IgG in a glomerulus of a B/W mouse aged 6 months. Note the presence of IgG both along the GCW and in the mesangium. ×400.

that in human SLE the glomerular immune complexes were composed of DNA and anti-DNA, similar findings were reported in the above-mentioned mouse strains (Miyasato *et al.*, 1967; Seegal *et al.*, 1969; Chused *et al.*, 1972; Dang and Harbeck, 1982). Since 1976, when Izui *et al.* demonstrated an affinity of DNA molecules for the collagen moiety of the GBM *in vitro*, it has been thought that glomerular immune aggregates are formed *in situ* in the GCW in SLE glomerulonephritis. In this respect, a report by Faaber *et al.* (1986) is interesting. These authors found an affinity of anti-heparan sulfate antibodies for DNA *in vitro,* which might have implications for the pathogenesis of human or murine SLE.

O'Regan and Turgeon (1984) found that infusion of DNA into normal mice results in mesangial binding and not in a binding to the GCW. Furthermore, infusion of preformed DNA anti-DNA immune complexes into rabbits, led to a mesangial localization (Natali and Tan, 1972). Although Morimoto *et al.* (1982) reported that in SLE patients the disease activity was directly correlated with circulating DNA–anti-DNA immune complexes, Izui *et al.* (1977a) showed that only 6% of the circulating immune complexes in SLE patients had these constituents and that in at least 52% of the cases the immune complexes were different in composition. These findings raised doubts about the earlier conclusion that

DNA–anti-DNA immune complexes were of pathogenetic importance in (experimental) SLE glomerulonephritis.

Cavallo *et al.* (1983b) showed that the DNA–anti-DNA content was low in eluates from glomeruli of (NZB × NZW)F_1 (B/W) mice. Izui *et al.* (1981) found that the presence of serum antibodies to single- or double-stranded DNA was not significantly associated with glomerulonephritis. Granholm *et al.* (1985) even demonstrated that the glomerular eluates from B/W mice contained a factor that inhibited the interaction between DNA and anti-DNA. All of these studies led to the conclusion that other immune complex systems besides DNA–anti-DNA played a role in the pathogenesis of lupus nephritis.

Izui *et al.* (1981) found a correlation between the development of fatal glomerulonephritis in B/W mice, and the presence of gp 70-containing immune complexes (Izui *et al.*, 1984b). In 1984, the same investigators found that in BXSB mice glomerulonephritis was significantly correlated with abnormally elevated gp 70 serum levels and with the occurrence of gp 70–anti-gp 70 immune complexes. This was confirmed in B/W mice by Maruyama *et al.* (1983) and Magil *et al.* (1986).

gp 70 is a serum glycoprotein structurally related to the envelop glycoprotein of murine leukemia virus (Nagy *et al.*, 1979). Although this virus has a virtually ubiquitous occurrence in rodents, only lupus-prone animals develop autoantibodies against gp 70 spontaneously (Izui *et al.*, 1979). Izui *et al.* (1981a) concluded from these findings that gp 70–anti-gp 70 systems fulfill a pathogenic role in experimental lupus nephritis and that genes governing the production of autoantibodies against retroviral gp 70 may be the genes mainly responsible for the segregation of spontaneous renal disease in B/W mice (Izui *et al.*, 1981a). Andrews *et al.* (1986) questioned the essentiality of the anti-gp 70 immune response in murine SLE. Using a low-gp 70 MRL/l congenic line they could demonstrate that elmination of most of the serum gp 70 and virtually all of the immune complex gp 70 had no observable effect on the course or nature of the disease. In MRL Lpr/lpr mice, an unregulated production of interferon (IFN) seems to cause the development of autoimmunity. Rosenberg *et al.* (1984) showed that this strain lacks suppressor cells to regulate the production of IFN. Eisenberg and Cohen (1983) hypothesized that an immunoregulatory dysfunction caused by an altered recognition of class II MHC antigens by T cells formed the basis for the development of SLE, including glomerulonephritis. This potential mechanism was illustrated in an experimental graft-versus-host disease which in mice gave rise to a SLE-like disease (Rolink *et al.*, 1983). As already mentioned, many nonrenal autoantigens involved in the induction of experimental glomerulonephritis can arise from polyclonal stimulation of B lymphocytes induced

by a graft-versus-host reaction (Gleichmann *et al.*, 1984), bacterial products (Rodriguez-Iturbe, 1976; Fournie *et al.*, 1980; Bellon *et al.*, 1982b; Cavallo *et al.*, 1984a), or heavy metals (Druet *et al.*, 1982b).

Izui *et al.* (1977b) found that a single injection of endotoxin into mice resulted in hypergammaglobulinemia and circulating autoantibodies and immune complexes as well as a renal localization of immunoreactants. Chronic administration of lipopolysaccharides to mice induced a diffuse proliferative glomerulonephritis associated with the appearance of various autoantibodies (Cavallo *et al.*, 1984b) such as anti-DNA (Bellon *et al.*, 1982b). Bruijn *et al.* (1986) showed that experimental infection of BD IX rats with *Trypanosoma brucei* induced the formation of autoantibodies directed against laminin and collagen IV. These authors assumed that these autoimmune phenomena were caused by a polyclonal stimulation of B cells, which is a well-known feature of trypanosomiasis. Such polyclonal stimulation of B lymphocytes is also found after viral infections (Zinkernagel, 1977). A similar mechanism has been postulated to operate in the induction of glomerulonephritis in newborn mice following treatment with IFN (Morel-Maroger *et al.*, 1978; Gresser *et al.*, 1976). Polyclonal activation of B lymphocytes and autoimmune phenomena can also be induced by chemical reagents, e.g., drugs or heavy metals which cause an immune dysregulation and in the majority of cases a membranous glomerulopathy (Druet *et al.*, 1982b; Weening, 1983; Hoedemaeker, 1986). In 1971, Bariéty *et al.* reported that administration of mercuric chloride to Wistar rats induced a membranous glomerulonephritis. A similar lesion was induced in PVG/c rats by Weening *et al.* (1978) (Fig. 28), who found an association with the occurrence of circulating antinuclear antibodies (Weening *et al.*, 1978, 1980) due to a dysregulation of the immune system (Weening *et al.*, 1981). Sapin *et al.* (1977) showed that besides an immune complex glomerulonephritis like that in Wistar and PVG/c rats, BN rats treated with $HgCl_2$ developed a glomerulopathy mediated by an anti-GBM antibody (Fig. 29). In later phases of the disease glomerular immune aggregates along the GBM were also observed (Druet *et al.*, 1982a).

Prouvost-Danon *et al.* (1981) and Hirsch *et al.* (1982) showed that administration of $HgCl_2$ induced polyclonal stimulation of B lymphocytes which yielded various autoantibodies including anti-GBM antibodies. This effect is presumably attributable to a stimulatory effect of helper/inducer T lymphocytes exposed to mercury (Pelletier *et al.*, 1985). Lymberi *et al.* (1986) established polyclonal stimulation in $HgCl_2$-treated rats by fusing their spleen cells with rat myeloma cells and demonstrating autoantibodies against trinitrophenol and horseradish peroxidase, both of the D23 idiotype. Fleuren *et al.* (1985) injected BALB/c mice with $HgCl_2$ and observed an immune complex glomerulopathy (Fig. 30) which was associ-

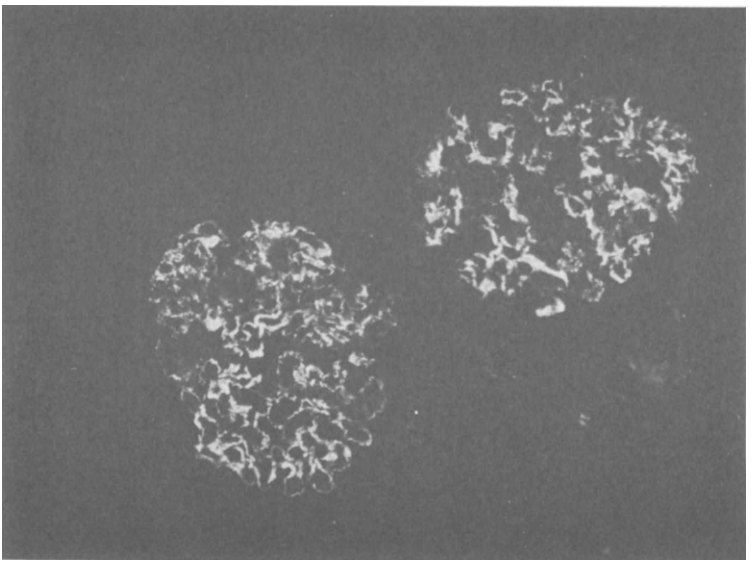

FIG. 28. Direct immunofluorescence of a kidney of a PVG/c rat treated with $HgCl_2$. Rat IgG is bound to the glomerular capillaries in a granular pattern. Mesangial fluorescence is also observed. ×150. (Courtesy Dr. J. J. Weening.)

ated with the development of antibodies directed against an epitope of gp 330 that does not itself occur in the mouse glomerulus.

Besides mercury, other heavy metals and such drugs as gold salts, penicillamine, and captopril have been shown to cause membranous glomerulonephritis in humans, possibly via polyclonal stimulation of B lymphocytes (Weening, 1983). Glomerulonephritis has also been induced with gold salts in rats and guinea pigs (Nagi *et al.*, 1971; Ueda *et al.*, 1985) as well as with cadmium (Joshi *et al.*, 1981) and penicillamine (Donker *et al.*, 1984), but autoantibodies were only found in animals given cadmium or gold (Donker *et al.*, 1982, 1984; Ueda *et al.*, 1985).

An exciting model for the induction of glomerulonephritis after polyclonal stimulation of B lymphocytes is provided by the experimental graft-versus-host disease. In 1968, Lewis *et al.* described an immune complex glomerulonephritis following the injection of BALB/c lymphocytes into (BALB/c × A/Jax)F_1 hybrid mice. Later, Gleichmann and Gleichmann (1976) and van der Veen *et al.* (1981) showed that this type of glomerulonephritis was associated with autoantibodies directed against DNA, erythrocytes, thymocytes, and the murine leukemia virus. In 1984, Gleichmann *et al.* and Tsuchimoto *et al.* found that polyclonal stimulation of B lymphocytes occurred in this model. Fleuren *et al.* (1982) and

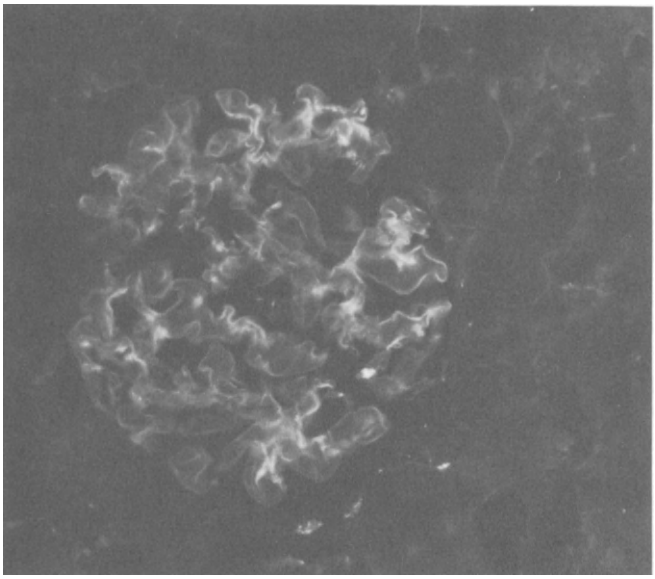

FIG. 29. Direct immunofluorescence of a kidney of a BN rat treated with $HgCl_2$. Rat IgG is bound to the GCW in a typical linear pattern. ×400. (Courtesy Dr. J. J. Weening.)

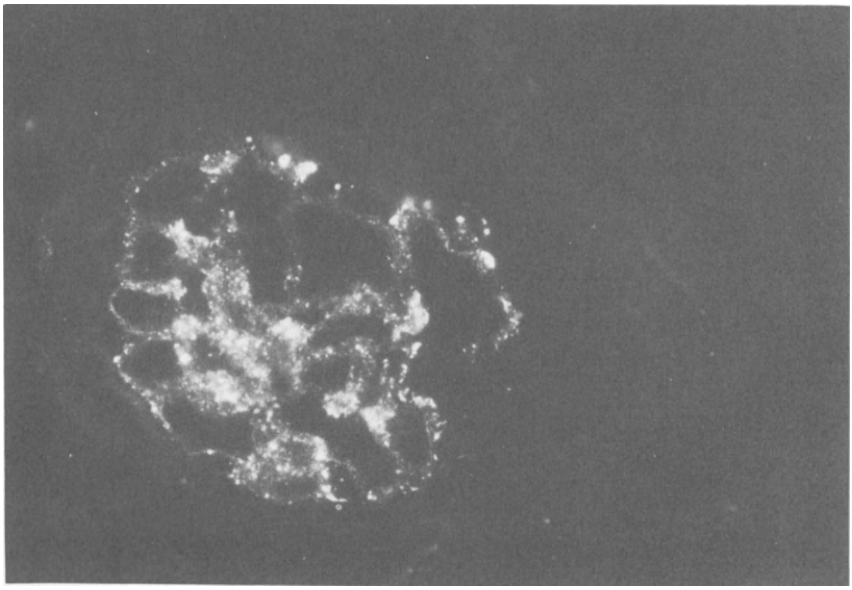

FIG. 30. Direct immunofluorescence of a glomerulus of a BALB/c mouse treated with $HgCl_2$. An intense binding of mouse IgG is present along the GCW and in the mesangium. ×400. Courtesy Dr. G. J. Fleuren.)

Hoedemaeker *et al.* (1984) reported that injection of low dosages of Lewis lymphocytes into (Lew × BN)F_1 hybrid rats led after 8 months to a membranous nephropathy associated with proteinuria, suggesting that this type of glomerulonephritis has an autoimmune character (Fig. 31). Rolink *et al.* (1983) had shown earlier that small differences between MHC class II antigens suffice to trigger the alloreactive donor T-helper cells to stimulate the autoreactive B cells, thus causing an SLE-like disease.

In sum, it may be said that in animals various experimental conditions can lead to polyclonal stimulation of B lymphocytes and thus to autoimmune phenomena. The resulting autoantibodies may react with renal autoantigens and give rise to either an immune complex glomerulopathy or an anti-GBM glomerulonephritis.

6. *Other Antigens*

In this section other substances or drugs possibly involved in the induction of glomerulonephritis will be discussed briefly. Usually for most cases the antigen involved is not known and the pathogenetic mechanism obscure. Drugs with a toxic influence on glomerular epithelial cells, such as aminonucleoside or puromycin (Frenk *et al.*, 1955) and adriamycin (Young, 1975), which induces the nephrotic syndrome without underlying glomerulonephritis, will not be dealt with here.

Many drugs are thought to be involved in the induction of glomerulonephritis (Hoedemaeker, 1986). These include volatile hydrocarbons and solvents (Glaser, 1982; Ravnskov *et al.*, 1983), ampicillin (Wright and Nash, 1984), and indomethacin (Sessa *et al.*, 1973). Treatment of rabbits with a high molecular weight polyfructosan was shown to ameliorate serum sickness glomerulopathy, presumably through complement activation (Stark *et al.*, 1985).

Huxtable and Dorling (1983) described a collagenizing glomerulopathy and the nephrotic syndrome in PVG/c rats following administration of an α-mannosidase inhibitor. Rehan *et al.* (1986) infused cobra venom into the renal artery of rats, which resulted in glomerular injury associated with an influx of neutrophils and proteinuria, probably due to the activation of complement. Finally, Kazama *et al.* (1985) showed that immunization of rats with the Engelbreth–Holm–Swarm (EHS) tumor led to a glomerulopathy similar to AICN. However, not only glomerular immune aggregates but also anti-tubular basement membrane antibodies were found. Moreover, antibodies eluted from the glomeruli did not react with antigens known to be involved in the pathogenesis of AICN.

Food antigens can also play a role in the pathogenesis of glomerulonephritis (van der Woude *et al.*, 1983), as was confirmed in a child with food

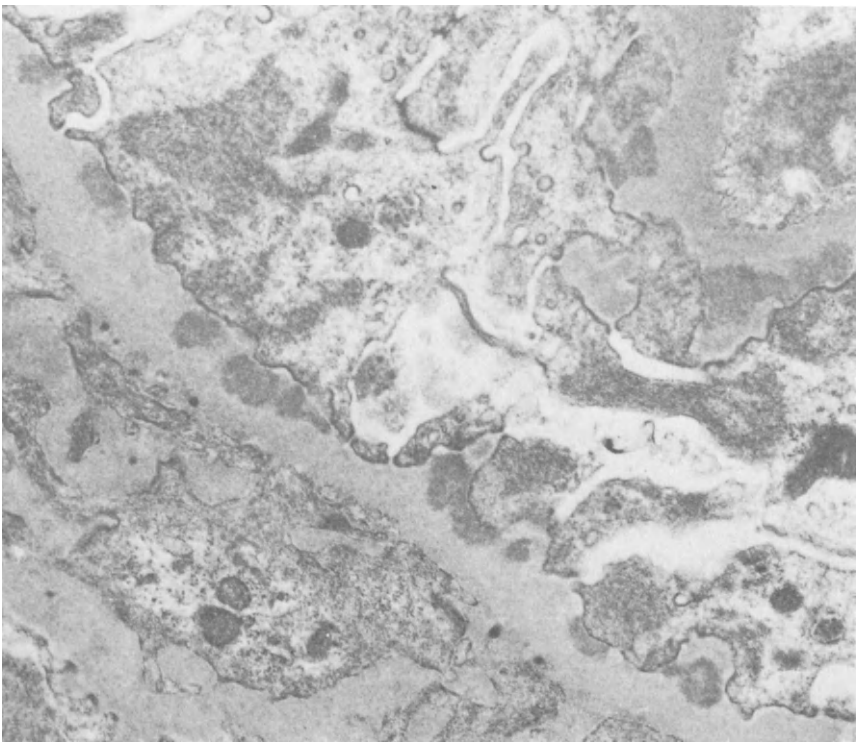

FIG. 31. Electron micrograph of a glomerulus of a rat with chronic allogeneic disease showing immune aggregates along the epithelial side of the GBM. Uranyl acetate–lead citrate, ×40,000. (Reproduced with permission.)

hypersensitivity (McCrozy *et al.*, 1986). No experimental models besides those mentioned in the section on serum sickness (Section IV,B,1) are available at present.

V. Genetic Influences

It has been known for some time that the MHC complex plays a role in the induction of several autoimmune diseases such as AICN (Stenglein *et al.*, 1978), $HgCl_2$-induced glomerulopathy (Druet *et al.*, 1977), and EAG (Stuffers-Heiman *et al.*, 1979; Sado *et al.*, 1986).

In 1968, Polak *et al.* reported that sensitization to heavy-metal compounds, including $HgCl_2$, is genetically controlled in guinea pigs. In 1977, Druet *et al.* extended these observations and demonstrated that the

TABLE V

GLOMERULAR BINDING OF AUTOANTIBODIES AND PROTEINURIA IN INBRED RAT STRAINS AND F_1 HYBRIDS TYPED FOR $RT1$ AND NON-$RT1$ ANTIGENS, AFTER TREATMENT WITH MERCURIC CHLORIDE[a]

Rat strain	RT1				Non-RT1	Autoantibodies at d15	Proteinuria
	A(I)	B(II)	D(II)	E(I)			
BN/Orl	n	n	n	n	BN	+	+
Lew/Pal	l	l	l	l	Lew	−	−
BN.1L/Han	l	l	l	l	BN	−	−
Lew.1N/Han	n	n	n	n	Lew	−	−
(BN/Orl×Lew/Pal)F_1	n/l	n/l	n/l	n/l	BN/Lew	+	+/−
(Lew.1N/Han×BN.1L/Han)F_1	n/l	n/l	n/l	n/l	Lew/BN	+	+/−
(Lew.1N/Han×BN/Orl)F_1	n	n	n	n	Lew/BN	+	+/−
(BN/Orl×BN.1L/Han)F_1	n/l	n/l	n/l	n/l	BN	+	+/−
(Lew.1N/Han×Lew/Pal)F_1	n/l	n/l	n/l	n/l	Lew	Not done	
(Lew/Pal×BN.1L/Han)F_1	l	l	l	l	Lew/BN	Not done	
MAXX/. . .	n	n	n	n	BN + Lew	+	−
AO/G	u	u	u	u	AO	+	+/−
(AO/G×BN/G)F_1	u/n	u/n	u/n	u/n	AO/BN	+	+
DZB/G	u	u	u	u	AO + BN	+	+
RP/G	u	l	l	u	RP	−	−

[a] Courtesy of Dr. J. Aten.

susceptibility for $HgCl_2$-induced glomerulopathy is also genetically restricted. They showed, furthermore, that rats bearing haplotype *RT1.n* develop anti-GBM antibodies and severe proteinuria after being injected with $HgCl_2$, whereas rats bearing haplotype *RT1.l* or *RT1.u* are resistant (Druet *et al.*, 1978). Other rat strains bearing various *RT1* haplotypes develop an immune complex glomerulopathy associated with antinuclear antibodies after treatment with $HgCl_2$ (Weening *et al.*, 1978; Druet *et al.*, 1978).

This susceptibility seems to be determined by both MHC- and non-MHC-linked genes, since the congenic Lew-1N rat bearing the *RT1.n* haplotype and a Lewis background is resistant to this type of glomerulopathy (Sapin *et al.*, 1981). Susceptibility for $HgCl_2$ of some inbred rat strains and F_1 hybrids as tested in our laboratory is summarized in Table V.

Stuffers-Heiman *et al.* (1979) showed a similar restriction in various rat strains immunized with bovine GBM. Rats bearing the haplotype *RT1.n* developed anti-GBM antibodies that bound to the GBM in a linear fashion, whereas Lew.1n rats did not respond, like other rat strains bearing various other haplotypes. These findings indicate a genetic predisposition for anti-GBM nephritis which is not, or not exclusively, controlled by genes linked to the *RT1* complex.

The susceptibility to AICN also differs between various rat strains. Stenglein *et al.* (1975) showed that rats bearing the haplotype *RT1.n* are relatively insensitive with respect to the induction of this type of nephritis, whereas rats bearing the haplotype *RT1.d* or *RT1.l* are more susceptible. On the other hand, hybrids of LEW.BN × BN cross showed a late onset of proteinuria after induction of AICN, which means that not only RT1 antigens but also non-RT1 antigens are involved in susceptibility to AICN. Finally, Sado *et al.* (1986) found a difference between various rat strains with respect to susceptibility to the induction of EAG associated with pulmonary hemorrhage.

VI. Conclusion

From the moment the hypothesis that immunological mechanisms played a role in the pathogenesis of glomerulonephritis was developed, many investigators tried to induce similar lesions in animals. Since a central role was attributed to immune complexes, presumably resulting from infections, investigators have tried to mimick such situations in animals by injecting various bacteria or bacterial products. Later also tissue fractions or purified proteins were injected to induce the disease. After the development of the experimental serum sickness in the 1950s and studies which led to the

finding that most of the glomerular-bound immune complexes were formed *in situ,* pathogenetic mechanisms for the development of glomerulonephritis seemed to be clear, such as deposition of preformed (circulating) immune complexes or the direct binding of antibodies to glomerular structures resulting either in a linear or granular binding. Whether cellular immune mechanisms play a role is still a matter of uncertainty.

The immune reactions involve two kinds of antigens: intrinsic antigens, which can be found on the cell membranes of the epithelial cells of glomeruli and tubules, on the mesangial cells, and in the structural components of the GBM; and extrinsic (nonrenal antigens), which consist of substances which either have chemical affinity to the GBM or bind to the charged sites of the GCW. The few forms of glomerulonephritis in humans that are known to be caused by extrinsic antigens (postinfectious glomerulonephritis) have virtually disappeared, which raises questions about the relevance of the models involving extrinsic antigens. Until today little evidence has been presented that the intrinsic antigens which are involved in various experimental glomerulonephritides play a role in glomerulonephritis in humans.

Recently several models of glomerulonephritis have been developed in which autoimmune phenomena play a role. This autoimmunity, which is caused by polyclonal activation of B lymphocytes, can be induced by a graft-versus-host disease or by toxins like mercuric chloride. Although most of the antigens involved in these models have not yet been characterized, it is tempting to believe that similar autoantigens and mechanisms are also involved in human pathology.

In spite of the fact that it is known that most antigens involved in models of glomerulonephritis are not playing a role in glomerulonephritis in humans, the value of glomerulonephritis models lies in the fact that these models helped us enormously to gain insight in the mechanisms of disease, a knowledge that eventually might lead to the prevention of glomerulonephritis.

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INTERNATIONAL REVIEW OF EXPERIMENTAL PATHOLOGY, VOL. 30

Spontaneous Renal Disease in Laboratory Animals

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I. Introduction

Spontaneously occurring renal diseases are common in laboratory animals. However, the diseases vary with each species examined and, as is the case with the pathological evaluation of any organ, one must recognize the normal background of spontaneous lesions for that organ and be able to distinguish them from experimentally induced lesions. The importance of knowing the background disease patterns in laboratory animals has been emphasized (Burek, 1978; Zurcher *et al.*, 1982; Dunn, 1954). Knowing the background diseases in a species or strain of animal will aid an investigator in selecting or avoiding a given species for experiments. Such knowledge is also important to our understanding of the similarities or differences between animal and human renal diseases. In some cases the renal diseases in animals are identical to a human disease, in other cases the diseases in animals have no human counterpart.

An example of a disease occurring in animals but not in humans is the chronic renal disease of rats. All rat strains develop chronic renal disease. The cause is unknown, but it is rat-specific with no counterpart in humans. The disease in Sprague–Dawley rats affects virtually 100% of males older than 1 year and can be the leading cause of death. Female Sprague–Dawley rats and rats of some other strains (i.e., F344) also have the disease, but the incidence and severity are less. Few of these rats die from renal failure. Some mouse strains (i.e., MRL, NZB, and RFM) also develop an immuno-

logically mediated chronic renal disease which may be the leading cause of death in those animals. The lesions produced and the pathogenesis of the disease in these mice are different than in the rat, but are similar to some diseases in humans. Other strains of mice (e.g., CD-1) and Syrian golden hamsters develop generalized amyloidosis, with the kidney being one of the organs most commonly affected. In these animals, amyloidosis is the most common cause of death. Rabbits, on the other hand, often have a low-grade, persistent parasitic infection (Encephalitozoonosis) in their kidneys (an incidence of 20–50% is not uncommon). The disease is not life-threatening, has not been reported in humans, but can compromise the value of rabbits for use in research on renal disease.

The renal anatomy, histology, and physiology of laboratory animals and humans are similar but not identical. Like humans, all of the animals discussed in this chapter are similar in that grossly their kidneys have a uniform tan to brown color and a smooth glistening capsular surface.

The functional unit of the kidney is the nephron and there are, for example, approximately 30,000–40,000 nephrons in an adult rat kidney.

The distal tubule is connected to a system of collecting ducts. The nephrons and collecting ducts arise from different embryological primordia.

The glomerulus consists of a tuft of capillaries enclosed within Bowman's capsule. The capillaries are lined by thin endothelium and a thin visceral layer of epithelium.

Proximal tubules are divided into a convoluted portion (pars convoluta) and a straight portion (pars recta). The pars convoluta can further be divided into S_1 (P_1) and S_2 (P_2), which are usually located in the cortex. The pars recta (S_3 or P_3) is usually located in the outer stripe of the medulla. Based on morphology, the different segments of the proximal tubules can be characterized as summarized in Table I.

Laboratory animals have a single renal papilla, in contrast to humans, where there are several conical regions called pyramids. There are other anatomical, histological, and physiological differences investigators should recognize. For example, the male mouse has cuboidal epithelial cells lining Bowman's membrane while the hamster has a long renal papilla. Also, the product of the kidneys can be very different. Humans and most other species produce clear, amber-colored urine. Rabbits, on the other hand, produce a cloudy urine filled with large amounts of sediment, a feature making it very difficult to do a proper urinalysis. The urinary pH differs among the species. The urine of humans and dogs normally has an acid pH ($\pm$4 to 6) while that of rats and mice tends to be basic (pH $\pm$6 to 8). These differences can lead to differences in experimental results. Some drugs or their metabolites may be excreted easily and without toxicity at one pH, but may precipitate out, may cause local irritation, or may produce toxicity in a species that has a different pH.

TABLE I

COMPARISON OF STRUCTURAL FEATURES OF VARIOUS SEGMENTS OF RAT PROXIMAL TUBULE

Feature[a]	S_1 (P_1)	S_2 (P_2)	S_3 (P_3)
Shape	Tall/low columnar	Shorter than S_1	Cuboidal
Brush border width	2–3 μm	2μm	2.5–4 μm
Vesicles	Numerous	Numerous	Fewer than S_1 and S_2
Vacuoles	Large, numerous	Fewer, smaller than S_1	Fewer than S_2
Golgi complex	Small, well developed	More developed than S_1	Better developed than S_1 and S_2
RER	Present	Not as prominent as in S_1 or S_3	Present
SER	Present	Not as prominent as in S_1	More prominent than S_1 and S_2
Lysosomes	Numerous, small and large, pale	Fewer than S_1	Fewer than S_1, dark, small
Mitochondria	Numerous, elongated	Fewer than S_1, shorter	Fewer than S_1, shorter than S_1 and S_2
Peroxisomes	Few	More numerous than S_1	Larger, more numerous than S_1 and S_2
Interdigitations	Numerous	Fewer and more shallow than S_1	Very few or none

[a] RER, Rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

A classic example of species differences in toxicity is that caused by chloroform in male mice (Eschenbrenner and Miller, 1945). Male mice of some strains (BALB/c and C3H) are extremely sensitive to exposures of chloroform with resulting renal tubular necrosis and death. Females of the same mouse strain, as well as males and females of different mouse and rat strains, can be unaffected at the same exposure concentrations. Not only do different strains differ in their susceptibility to chloroform, but the pattern of renal damage can vary. C3H male mice may survive the acute toxicity but necrotic tubules calcify. Other strains may develop tubular necrosis but calcification does not occur.

Several excellent textbooks and monographs are available that deal with renal disease in animals. A recent text by the International Life Sciences Institute (Jones *et al.*, 1986) provides a review of many common diseases found in the urinary system of laboratory rodents. *The Pathology of*

Laboratory Animals (Benirschke *et al.*, 1978) also provides a review of common renal diseases in laboratory animals. Robinson and Dennis (1980) and Osborne *et al.* (1977), have written excellent overviews on animal models of human renal disease. Additional reviews dealing with individual species are available for the mouse (Foster *et al.*, 1981, 1982a,b, 1983), rat (Baker *et al.*, 1979, 1980; Burek, 1978), and hamster (Schmidt *et al.*, 1983).

In this article we try to examine renal diseases in laboratory animals based on our experience using such animals in toxicity and carcinogenicity studies. In these studies, differences in spontaneous and induced renal disease in a variety of species given an even wider variety of treatments can be seen. The spontaneous diseases observed, rather than providing models of human disease, provide diagnostic difficulties in distinguishing the normal variability of background disease from variations induced by drugs, chemicals, or experimental manipulative procedures. On the surface such differences should be easy to assess, but in practice they can result in one of the more difficult challenges a pathologist must deal with in this field of research. To differentiate normal from induced changes often requires an extensive historical data base so one can evaluate the normal variation in incidence and severity of the lesions in question. Even something as straightforward as comparing differences in organ weights between treated and control animals can be difficult without an overall perspective of what is expected in the species and organ being evaluated. Therefore, the review provided in this article is intended as a general overview of the spectrum of spontaneous lesions that is likely to be encountered in the kidney of the commonly used animals. These lesions may complicate the conduct and interpretation of results of studies on renal disease in biomedical research. Emphasis is placed on those species, strains, or stocks of animals with which we have experience and a reasonable historical data base. The species include the rat, mouse, hamster, rabbit, guinea pig, dog, and monkey. Obviously there are many strains and stocks of rodents, many sources of dogs, and numerous species of primates. All are not reviewed. The goal of this article is to provide a broad overview of the commonly occurring spontaneous renal diseases in laboratory animals; the literature is reviewed and emphasis has been placed on our experience with these species.

II. Rat

A. Normal Anatomy, Histology, and Organ Weights

Development of the kidney in most animal species goes through three successive stages: pronephros, mesonephros, and metanephros. The first two stages are primitive and act as inducers of the third, and permanent,

metanephros. In the rat the development of the kidney extends through the third week of postnatal life. The kidneys of newborn rats consist of cortex and medulla. The cortex appears mature at postnatal day 12. The medulla is ontogenetically younger than the cortex, maturing around postnatal day 21.

The medulla ends in a single papilla. It is further divided into the inner and outer medulla. The latter can be further divided into the inner and outer stripes. An excellent and detailed review of the normal histology and ultrastructure of the rat kidney is available by Bachman *et al.* (1986).

In the rat there are sex differences in the morphology of various proximal tubular segments. These are shown in Table II.

The role of sex hormones in establishing and maintaining morphological characteristics of proximal tubular cells has been reported (Schiebler and Danner, 1978; Daigeler, 1981).

There are also histochemical differences among various segments of the proximal tubule in the rat. The brush border can be stained with the PAS technique and certain plant-derived lectins due to the presence of sugar residues (glycocalyx) on the plasma membrane. Other plasma membrane markers include alkaline phosphatase, γ-glutamyl transpeptidase and Na^+,K^+-ATPase. Various lysosomal, peroxisomal, and mitochondrial markers have been described in humans, mice, and rats (Zabel and Schiebler, 1980). These markers show segmental differences between S_1, S_2, and S_3 segments. In addition, significant histochemical differences have been noted between male and female pars convoluta and pars recta, and their differences are dependent on sex hormones.

TABLE II

SEX DIFFERENCES IN MORPHOLOGY OF VARIOUS PROXIMAL TUBULAR SEGMENTS OF RATS

Segment[a]	Male	Female
S_1		
Apical vacuoles	Larger, more numerous than in female	—
Lysosomes	—	More giant lysosomes
S_2		
Apical vacuoles	Larger, more numerous than in female	—
Lysosomes	Larger, more numerous than in female	—
S_3		
SER	—	More abundant than in male
Lysosomes	—	Fewer, but larger than in male
Peroxisomes	Larger than in female	—

[a] SER, Smooth endoplasmic reticulum.

In the rat the weight of the kidney varies with sex and body weight. The median weights of two kidneys of male and female Sprague–Dawley rats of different body weights from our laboratory are shown in Tables III and IV.

B. Congenital Diseases

In the rat, the most common congenital malformations are renal agenesis, hypoplasia, and hydronephrosis.

1. *Renal Agenesis and Hypoplasia*

Agenesis and hypoplasia have been observed in many strains including the Wistar rat, a cross between a Wistar and a hooded variety (Hain and Robertson, 1936), ACI rats (Solleveld and Boorman, 1986), and Sprague–Dawley rats. In some strains it is usually the left kidney that is affected, but in the Sprague–Dawley it is usually the right. In ACI rats the incidence in males and females was 12 and 14%, respectively. Among 8629 Sprague–Dawley fetuses examined at our laboratory there were two fetuses with renal agenesis and seven fetuses with renal hypoplasia. The hypoplastic kidney was usually cystic.

TABLE III

Weights of Kidneys from Untreated Male Crl:CD(SD)BR Rats

		Absolute weight (g)		Relative weight (% body weight)	
Weight range (g)	Number of animals	Median value	Range of actual values	Median value	Range of actual values
100–150	194	1.43	1.05–2.12	1.08	0.88–1.46
151–200	196	1.70	1.35–2.23	0.98	0.75–1.21
201–250	388	1.98	1.03–3.63	0.86	0.45–1.68
251–300	461	2.26	1.69–3.10	0.83	0.64–1.14
301–350	183	2.47	1.94–4.01	0.78	0.57–1.19
351–400	271	2.59	2.06–4.20	0.68	0.53–1.13
401–450	734	2.80	2.16–7.31	0.66	0.52–1.68
451–500	831	3.06	2.35–7.73	0.65	0.50–1.56
501–550	561	3.26	2.48–7.18	0.62	0.48–1.35
551–600	364	3.42	2.50–7.27	0.60	0.42–1.26
601–650	262	3.62	0.76–12.37	0.58	0.12–1.92
651–700	186	3.90	2.92–8.89	0.58	0.42–1.32
701–750	125	4.04	3.04–7.46	0.56	0.43–1.00
751–850	154	4.36	3.34–8.37	0.55	0.41–1.04
851–999	47	4.55	3.15–10.06	0.51	0.35–1.08

2. *Hydronephrosis*

Hydronephrosis is an abnormal collection of urine in the renal pelvis that results in distention and subsequent atrophy of the surrounding tissue. It may occur as a congenital or an acquired disease (Section II,D,9) and has been described in many strains of rats (Wistar, Slonaker–Addis, Long Evans, Sprague–Dawley, and Brown Norway) (Cohen, *et al.*, 1970). In the Slonaker–Addis strain the incidence is approximately 50% (Sellers *et al.*, 1960). Higher incidences have been reported in ACI and M520 rat strains (Solleveld and Boorman, 1986).

C. Infectious and Parasitic Diseases

Estimates of the incidence of infectious diseases of rat kidney are not available in the literature. It is rare on the basis of our laboratory experience. Diseases caused by bacteria (*Escherichia coli* and *Corynebacterium* sp.) are the most common infectious diseases in the rat kidney. Parasitic diseases are now very rare due to improved laboratory care.

TABLE IV

Weights of Kidneys from Untreated Female Crl:CD(SD)BR Rats

		Absolute weight (g)		Relative weight (% body weight)	
Weight range (g)	Number of animals	Median value	Range of actual values	Median value	Range of actual values
100–150	469	1.29	1.01–1.92	1.03	0.70–1.70
151–200	789	1.47	1.10–2.06	0.85	0.60–1.16
201–250	1095	1.63	1.19–3.42	0.71	0.51–1.51
251–300	1252	1.83	1.31–6.10	0.67	0.50–2.27
301–350	536	2.04	1.52–12.06	0.64	0.47–3.49
351–400	340	2.34	1.66–4.80	0.62	0.46–1.22
401–450	198	2.56	1.73–3.89	0.61	0.39–0.89
451–500	131	2.75	1.96–4.48	0.58	0.42–0.95
501–550	63	2.79	2.04–4.64	0.54	0.37–0.88
551–600	34	3.02	2.27–4.33	0.52	0.40–0.74
600–650	22	3.15	2.66–4.56	0.51	0.44–0.71
651–700	8	3.56	2.98–4.31	0.52	0.44–0.65
701–750	6	3.31	2.20–3.71	0.46	0.31–0.50
751–850	3	3.04	2.92–3.86	0.37	0.36–0.51
851–999	2	4.50	3.80–5.21	0.52	0.44–0.59

1. *Bacterial Diseases*

Bacterial infections most often manifest themselves in the form of pyelonephritis or suppurative nephritis.

Pyelonephritis is inflammation of the renal pelvis and parenchyma and is usually caused by bacteria from the lower urinary tract. The types of bacteria involved are usually *E. coli* and *Corynebacterium* sp., although others such as *Streptococcus*sp., *Enterococcus* sp., *Proteus* sp., *Pseudomonas* sp., and *Klebsiella* sp. are also reported (Duprat and Burek, 1986). Spontaneous pyelonephritis is not very common in most rat strains. Besides lower urinary tract infection secondary to obstruction, vesicoureteral reflux is the most common contributing factor. Suppurative nephritis occurs as a primary lesion in the parenchyma by embolism of bacteria. This lesion can ultimately extend to the pelvis. In acute cases, the kidney has pockets of purulent exudate, and red to white streaks radiate from the cortex to the pelvis. Microscopically, the acute lesions are characterized by severe inflammation and destruction of tissue including necrosis of renal papillae. The tubular lumen may contain masses of bacteria. In chronic cases, the surface of the kidneys show scarred areas that extend deep into the cortex. Cysts containing brownish viscous fluid are often present in the cortex and medulla. Histologically, chronic lesions show a continuous spectrum from fibrosis to areas of suppuration. The papillae are frequently distorted by fibrous tissue. The glomeruli may or may not be sclerotic, and periglomerular fibrosis is characteristic.

2. *Parasitic Diseases*

There are two parasites—one a coccidia, the other a nematode—which may affect the rat kidney. Neither is common, and both should be controlled by proper animal husbandry practices in today's breeding and experimental colonies.

Klossiellosis has been reported in Sprague–Dawley and Wistar strains of rats (Hortig and Hebold, 1970). The organism is a coccidian protozoan that is seen in the tubular epithelial cells and occasionally in tubular lumens. The organisms are oval to crescent shaped and 2–4 μm long during proliferative stages. Generally the parasite causes little or minimal damage to the surrounding tissue. They are generally an incidental finding in kidney sections.

The normal habitat of *Trichosomoides crassicauda* is the mucosa of the urinary bladder, but it is occasionally found in the ureter and renal pelvis (Bone and Harr, 1967). This nematode parasite is approximately 1 cm in length and 200 μm in diameter and is usually embedded in the mucosa.

TABLE V

INCIDENCE OF COMMONLY OCCURRING DEGENERATIVE AND INFLAMMATORY CHANGES IN THE KIDNEY OF UNTREATED MALE Crl:CD(SD)BR RATS

Type of change	Incidence by age in weeks (%)							
	0–20 (n = 1905)	21–34 (n = 463)	35–48 (n = 57)	49–62 (n = 310)	63–76 (n = 82)	77–90 (n = 199)	>90 (n = 781)	Total (n = 3797)
Tubular basophilia	8	9	12	19	4	1	1	7
Cellular infiltration	6	10	12	7	6	0	1	6
Chronic progressive nephrosis	5	9	33	55	65	75	100	34
Pyelonephritis	<1	1	0	1	4	3	2	1

D. Nonspecific Inflammatory, Degenerative, and Proliferative Diseases

Degenerative diseases constitute the most common group of spontaneous diseases in the rat kidney. A few of the most commonly seen degenerative diseases are briefly described below, and the incidence in untreated control male and female Sprague–Dawley rats in our laboratory is shown in Tables V and VI, respectively.

1. *Chronic Progressive Nephrosis*

This renal disease in rats is known in the literature by a variety of names; nephropathy, nephrosis, glomerular nephrosis, chronic nephritis, glomerular nephritis, chronic renal disease, and chronic progressive nephrosis (Fig. 1). The term chronic progressive nephrosis is most fitting, as the process is degenerative and not inflammatory. It is progressive and of a chronic duration. This is the single most common spontaneous renal disease in adult and aged rats (Gray, 1963, 1986; Blatherwick and Medlar, 1937; Armstrong and Horsley, 1966). The onset is earlier and the severity greater in males of most rat strains. It is well accepted that the earliest change is protein leakage which precedes the earliest microscopic changes, namely, cast formation and thickening of the glomerular basement membrane. The tubular epithelium undergoes progressive degeneration and later atrophy. The tubular basement membrane becomes increasingly thickened and the tubules become isolated. The inflammatory cell infiltrate is generally considered secondary.

Few gross changes are seen in the early stages. As the lesions progress, the kidneys become paler than normal and later become light tan; the surface becomes granular and pitted, and the kidneys gradually increase in size. The cut surface shows mottling, radial striations, and cysts. There is gradual narrowing of the medullary zone with enlargement of the cortex.

The earliest microscopic changes are thickening of the tubular basement membranes and cast formation. As the disease progresses, there is degeneration of tubular epithelium and subsequent atrophy. The interstitium shows an increase in fibrous connective tissue as well as lymphocytic infiltration. In advanced cases, the cast formation is widespread and is the most conspicuous aspect of the disease. The epithelium of the affected tubules becomes flattened, giving the kidney section the appearance of a thyroid gland. Occasionally, hyperplasia of the lining cells is seen. The glomerular changes tend to be quite variable. The glomerular capsule may be dilated and often contains proteinaceous material. In some animals the glomerular tuft

TABLE VI

INCIDENCE OF COMMONLY OCCURRING DEGENERATIVE AND INFLAMMATORY CHANGES IN THE KIDNEY OF UNTREATED FEMALE Crl: CD(SD)BR RATS

	Incidence by age in weeks (%)							
Type of change	0–20 (n = 1877)	21–34 (n = 448)	35–48 (n = 38)	49–62 (n = 328)	63–76 (n = 125)	77–90 (n = 201)	>90 (n = 740)	Total (n = 3757)
Tubular Basophilia	6	5	0	4	5	4	2	4
Cellular infiltration	4	6	16	2	5	1	1	4
Chronic progressive nephrosis	4	2	11	19	18	38	44	15
Pyelonephritis	<1	<1	8	1	2	2	1	1

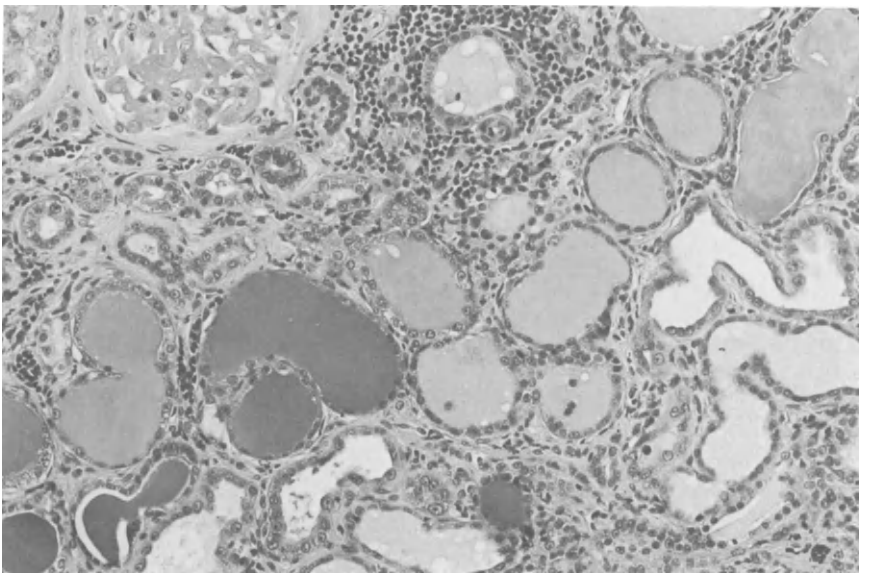

FIG. 1. Chronic progressive nephrosis in a rat. Note the tubular dilation, protein casts, aggregates of interstitial mononuclear cells, fibrosis, and thickened glomeruli. Hematoxylin–eosin, ×250.

appears swollen and some may be atrophied. Adhesions and proliferation of parietal cells of Bowman's capsule may be seen.

2. *Nephrocalcinosis*

Focal mineralization of the renal tubules, especially at the corticomedullary junction, is a frequent finding in the rat. The incidence is age related and higher in females than in males. The incidence in females Sprague–Dawley rats ranges from 7% (1–26 weeks of age) to 54% (105–130 weeks of age). In male rats the incidence range is 1–7%. This higher incidence in females is thought to be related to estrogen levels. Experimental studies have shown that the development of the lesion can be prevented by ovariectomy and that calcium deposition could be induced in castrated male and female rats by administering estrogen (Armstrong and Horsley, 1966).

3. *Tubular Epithelial Hyaline Droplets*

Large numbers of naturally occurring intracellular hyaline droplets have been seen in male rats (Logothetoporlos and Weinbren 1955; Peter *et al.*,

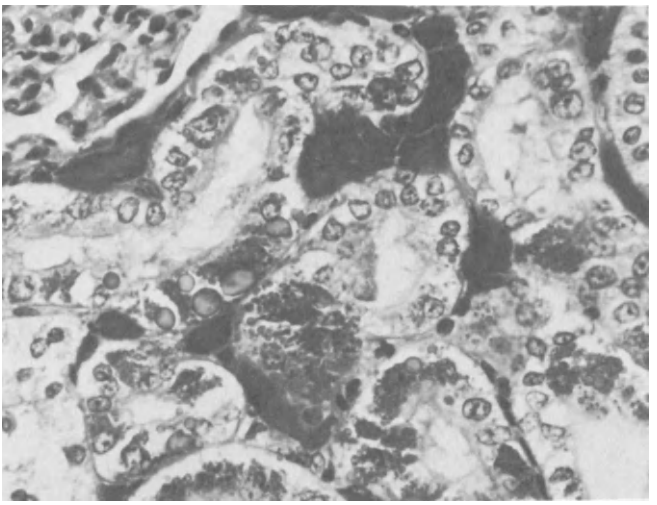

FIG. 2. Rat renal tubular epithelial cells containing hyaline droplets. Hematoxylin–eosin, ×500.

1986). All strains appear to have the droplets; however, they are more prominent in some strains (e.g., F344) than others (e.g., Sprague–Dawley). These droplets appear in the upper two-thirds of the proximal tubules from approximately 60 days of age, and the number of droplets increases with age. Two types of hyaline droplets are seen. One type is characterized as lightly eosinophilic, irregular, and usually single. The other type is deeply eosinophilic, refractile, round, and multiple (Fig. 2). The hyaline droplet formation is directly correlated with the degree of proteinuria. This proteinuria is dependent on testosterone levels. This is supported by a decrease in proteinuria in castrated males and an increase in proteinuria in testosterone-treated castrated males. There is some evidence that this protein is a low molecular weight α_2-microglobulin (Alden, 1986). It has been proposed that chemicals that bind to this protein can accumulate in the proximal tubule cells and thereby induce nephrotoxicity (Gray *et al.*, 1982).

4. *Tubular Basophilia*

Renal cortical epithelial basophilia (tubular basophilia), independent of any other renal change, is a relatively frequent spontaneous finding ($\leq$80%) in young rats of various strains (Peter *et al.*, 1986). The incidence is higher in males than in females. The lesion is characterized by widely scattered individual tubules or small clusters of tubules lined by slightly basophilic cells with the vesicular nuclei and marginated chromatin (Fig. 3). There is

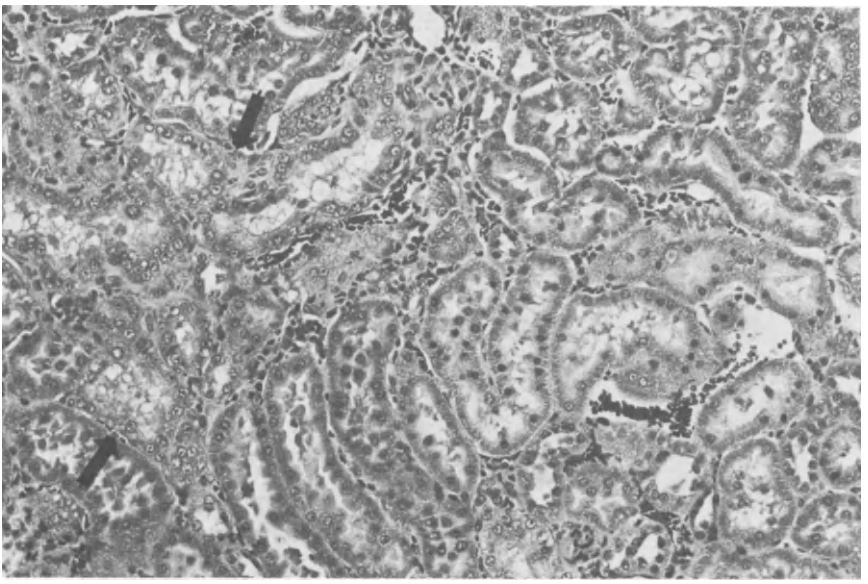

FIG. 3. Tubular basophilia present in the tubules between the arrows in a rat kidney. Although the color is not obvious in this photograph, the difference in morphology of these cells and their nuclei can be seen compared to the normal nonbasophilic cells. Hematoxylin–eosin, ×250.

usually thickening of the basement membrane. The altered tubules are generally located in the midcortical region. The pathogenesis is not known; however, it could be initially regenerative due to basophilic (RNA-rich) cytoplasm and later degenerative and atrophic as the basement membrane becomes altered. It is possible that the basophilic change is an early change of chronic progressive nephrosis, since this lesion is seen most frequently in strains with a high incidence of chronic progressive nephrosis later on in life (e.g., Sprague–Dawley rats).

5. *Renal Tubular Hypertrophy*

This lesion is characterized by the presence of large tubules lined by a single layer of large eosinophilic cuboidal to columnar cells with the luminal portion wider than the basal portion (Fig. 4). This is in contrast to renal tubular hyperplasia that occurs spontaneously associated with the chronic progressive nephrosis. In renal tubular hyperplasia, the cells are generally smaller than normal, basophilic, and are arranged in several layers, sometimes causing obliteration of the tubular lumen. Renal tubular hypertrophy has been reported in various strains of rats with or without accompanying

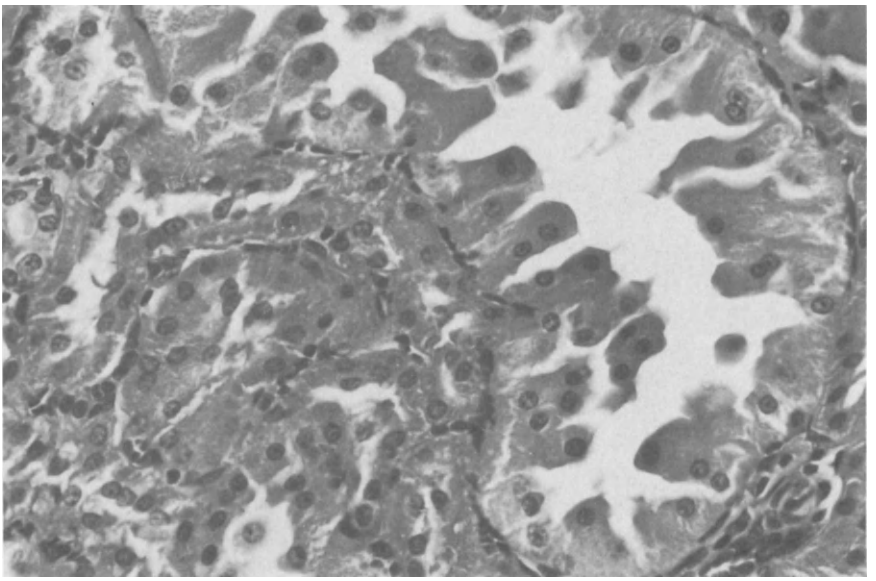

FIG. 4. Hypertrophy of rat renal tubular epithelial cells. Hematoxylin–eosin, ×500.

chronic progressive nephrosis (Gray *et al.*, 1982). This change may be present in up to 100% of full-life-span animals of some strains. The incidence is age and sex dependent and increases rapidly during the second half of the life-span in most strains, and the incidence is higher in males than in females (13% vs 7%) of some Sprague–Dawley rats. Others (Bannasch *et al.*, 1986) have referred to this lesion as an oncocytoma. However, in our experience, there is no evidence that this progresses to tumor formation. It is age associated and can have an incidence of 100% in some strains (Peter *et al.*, 1986), but it is not preneoplastic or neoplastic in its biological behavior.

6. *Renal Papillary Necrosis*

Renal papillary necrosis has been reviewed by Elliott (1986). It has been reported to occur spontaneously in adult JJ Gunn rats, a mutant strain of albino rat with a genetic predisposition (deficiency in glucuronyl transferase) for hyperbilirubinemia. Renal papillary necrosis in this strain is associated with deposits of bilirubinlike material (Baum *et al.*, 1969). Renal papillary necrosis in rats is most often seen with pyelonephritis or associated with nonsteroidal antiinflammatory and analgesic toxicity. Microscopically, the lesion is characterized by coagulative necrosis of the tip or entire papilla without inflammatory cell infiltration. At the junction of the

necrotic area and viable tissue, hyperemia and inflammatory cell infiltration are generally seen.

7. *Urolithiasis and Renal Papillary Epithelial Hyperplasia*

The incidence of spontaneous urolithiasis varies considerably. In some strains (Wistar) this is very rare, and in others (Sprague–Dawley), renal calculi are frequently observed (Berg, 1967). Chemically, most spontaneous renal calculi in rats are either phosphates of calcium and magnesium or ammonium magnesium phosphate (Magnusson and Ramsey, 1971). However, stones found in NMRI rats fed low-protein and/or high-mineral diets, consist almost entirely of calcium citrate (Van Reen *et al.*, 1959). Uroliths in Sprague–Dawley rats are most often renal and vary in form from irregular to spherical or disklike and in size up to 6 mm in diameter. These stones may be either free in the renal pelvis or adherent to the surface of the renal papilla. The smaller calculi usually have a concentric pattern and the larger ones are usually structureless. Urolithiasis usually begins as mineralization of the degenerating epithelial cells, and the mineralized nodules can break off and act as foci for further stone formation. Even microscopic renal calculi are usually associated with epithelial hyperplasia on the surface of the papilla (Fig. 5).

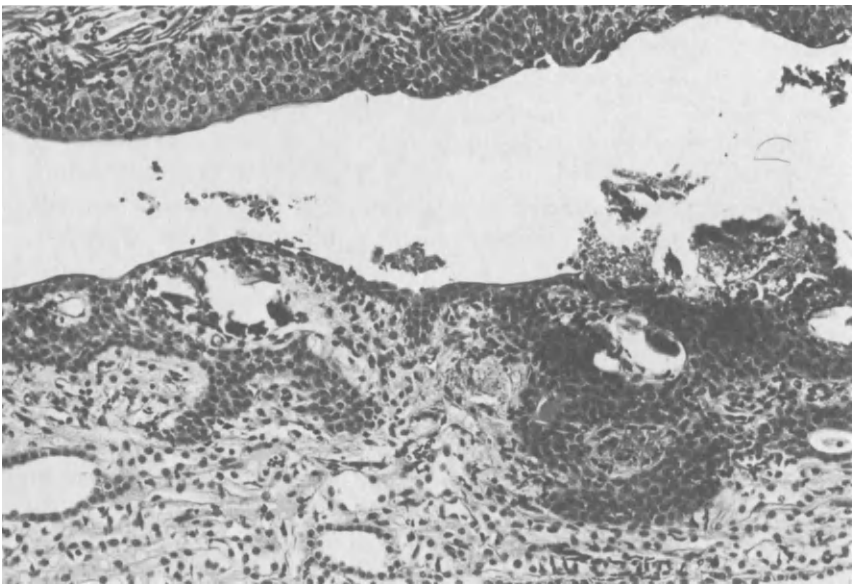

FIG. 5. Mineral and associated focal transitional cell hyperplasia of the renal pelvic epithelium in a rat. Hematoxylin–eosin, ×250.

8. *Interstitial Cellular Infiltration*

Cellular infiltration, primarily lymphocytes and plasma cells, in the interstitium independent of other concurrent renal disease, is a common spontaneous change in the rat kidney, especially among young rats. The incidence is higher in males than in females. Cellular infiltration in the interstitium may be seen with renal tubular basophilia which may be an early change of chronic progressive nephrosis. In older animals, cellular infiltration is often seen associated with chronic progressive nephrosis.

9. *Hydronephrosis*

Rats have a variable incidence of hydronephrosis; however, it is often difficult to determine if the disease in these rats is congential or acquired. In our Sprague–Dawley rats, there is a near 0% background of congenital hydronephrosis (see Section II,B,2), but there is a 2% background in 3757 female and 3797 male adult aging rats in our laboratory. This condition is more common in males than females. The cause is not always apparent, but some have suggested it results from partial obstruction of the ureter by compression between the ovarian or spermatic artery and ileolumbar artery (Maronpot, 1986).

10. *Immunologically Related Disease*

Unlike mice, renal disease induced through immunological mechanisms does not appear to be common in rats. There have been numerous studies to define an immune mechanism for chronic progressive nephrosis in rats, but a clear relationship has not been recognized. Nevertheless, immune complexes that have been associated with glomerular and tubulointerstitial lesions in rats have been reported. These data were summarized by Hoedemacker *et al.* (1986).

E. Neoplastic Diseases

Primary renal tumors are rare in most strains and are most often seen in older rats (Snell, 1967). The incidences of neoplastic diseases in control male and female Sprague–Dawley rats from 2-year carcinogenicity studies performed in our laboratory are summarized in Tables VII and VIII. Tumors of cortical epithelial cell origin (adenoma and adenocarcinoma), lipomatous tumors (lipoma and liposarcoma), and embryonal nephromas (nephroblastomas) are the most common renal tumors in rats.

Renal cortical epithelial tumors tend to be circumscribed grossly and range in size from microscopic lesions to neoplasms several centimeters in

TABLE VII

INCIDENCE OF COMMONLY OCCURRING PROLIFERATIVE CHANGES IN THE KIDNEY OF UNTREATED MALE Crl:CD(SD)BR RATS

Type of change	Incidence by age in weeks (%)							
	0–20 (n = 1930)	21–34 (n = 483)	35–48 (n = 65)	49–62 (n = 370)	63–76 (n = 133)	77–90 (n = 279)	>90 (n = 1401)	Total (n = 4661)
Epithelial hyperplasia, renal papilla	0.3	0.6	0	1	3	4	6	2
Tubular adenoma	0	0	0	0	0	0.7	0.8	0.3
Tubular adenocarcinoma	0	0	0	0	0	0	0.07	0.02
Lipoma	0	0	0	0	0	0	0.07	0.02
Liposarcoma	0	0	0	0	0	0	0.2	0.06
Embryonal Nephroma	0.05	0.2	0	0	0	0	0.07	0.02
Transitional cell carcinoma	0	0	0	0	0.8	0	0	0.02

TABLE VIII

INCIDENCE OF COMMONLY OCCURRING PROLIFERATIVE CHANGES IN THE KIDNEY OF FEMALE Crl:CD(SD)BR RATS

Type of change	Incidence by age in weeks (%)							
	0–20 (n = 1903)	21–34 (n = 466)	35–48 (n = 43)	49–62 (n = 385)	63–76 (n = 175)	77–90 (n = 311)	>90 (n = 1330)	Total (n = 4613)
Epithelial hyperplasia, renal papilla	0.3	1	5	4	3	7	11	4
Tubular adenoma	0	0	0	0	0	0	0.4	0.1
Tubular adenocarcinoma	0	0	0	0	0	0	0.08	0.02
Lipoma	0	0	0	0	0	0	0.08	0.02
Liposarcoma	0	0	0	0	0	0	0.08	0.02
Embryonal nephroma	0.05	0	0	0	0	0	0	0
Transitional cell carcinoma	0	0	0	0	0	0	0.15	0.04

diameter. Microscopically, the tumor cells may have a granular (acidophilic or basophilic) or clear cytoplasm. Individual tumors may have both granular and clear cells. Arrangement of the cells further characterizes the tumor morphologically as tubular, lobular, solid, papillary, or cystic. These various morphological patterns may occur in different parts of the same tumor. The tumors in which the cells are highly pleomorphic and disorganized and which display invasive properties are considered carcinomas or adenocarcinomas. Epithelial tumors often stimulate a mild fibrous reaction which condenses around the periphery and forms a pseudocapsule. These differences are illustrated in Figs. 6 and 7, which show a renal cortical cell adenoma and carcinoma, respectively.

Lipomatous tumors are occasionally seen, especially in aged rats. These are controversial lesions and have been variously classified in the literature as lipomas, liposarcomas, and lipomatous hamartomas (Hard, 1976; Gordon, 1986). The lipomas range from microscopic aggregations of mature fat cells in the outer medulla which cause very little distortion of the renal architecture to a large collection of fat cells that widely separate the tubules and glomeruli (Fig. 8). In some lipomas there may be lipoblasts which have large central nuclei and small vacuoles in their cytoplasm. Areas of hemorrhage and necrosis may be seen. Liposarcomas often cause marked distortion of renal architecture and extend beyond the limits of the capsule to the pelvis. They have a more heterogeneous population of cells than

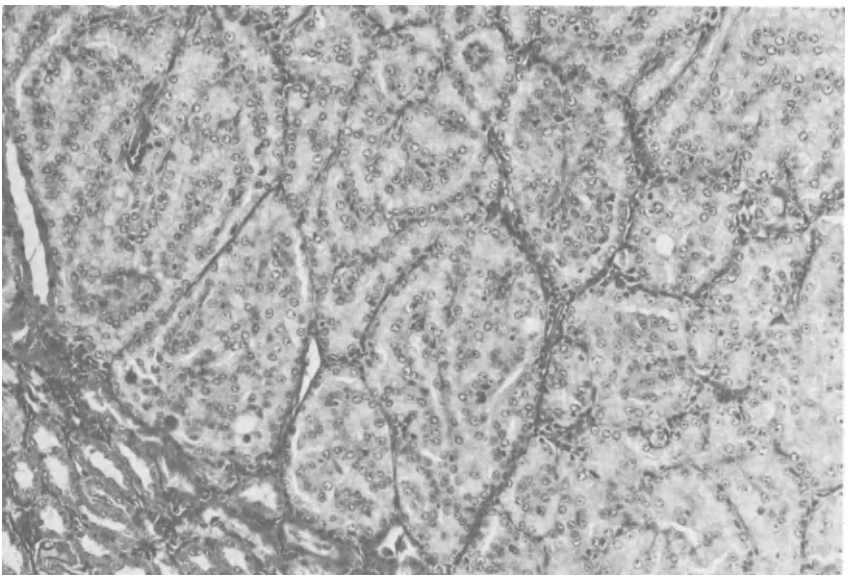

FIG. 6. Renal cortical cell adenoma in a rat. Hematoxylin–eosin, ×250.

lipomas, namely, lipocytes, lipoblasts, and mesenchymal cells. Hemorrhagic and necrotic foci are more prominent in liposarcomas; distant metastases are rare.

Nephroblastoma is the most commonly diagnosed renal tumor in rats (Snell, 1967). This is a neoplasm of the young rat (5–12 months of age). The histogenesis of the tumor is not well established, and for this reason, the lesion is usually considered separately from epithelial tumors, although its consistent structure suggests that it should be included among epithelial tumors. Details of the histology of nephroblastomas have been presented by Hottendorf and Ingraham (1968) and are illustrated in Fig. 9.

Small nephroblastomas are usually well circumscribed and well demarcated from the normal renal parenchyma. Large tumors can occupy the whole organ as a multilobulated mass and may occupy much of the abdominal cavity. Nephroblastomas consist of islands of densely packed basophilic spherical cells with scant cytoplasm. The nuclei are generally hypochromatic and often large, oval to round, and vesicular with prominent nucleoli. These nests of neoplastic cells are scattered throughout a relatively scant connective tissue stroma. Most tumors contain tubular structures conforming to "focal blastemal" type in the center of the nests. Cell nests are also associated with larger ducts and on occasion the proliferating cells seem to form indentations into the larger ducts. The tumor growth is usually associated with a chronic inflammatory reaction in the surrounding

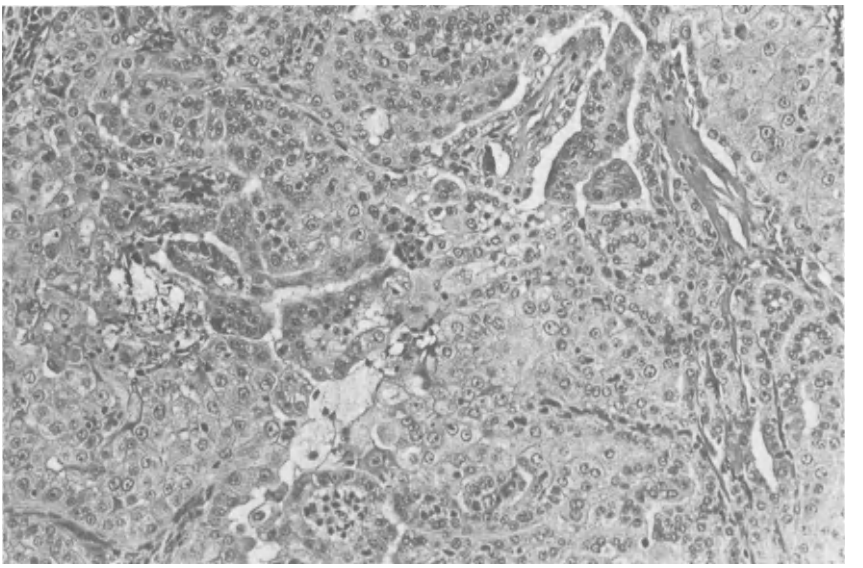

FIG. 7. Renal adenocarcinoma in a rat. Hematoxylin–eosin, ×250.

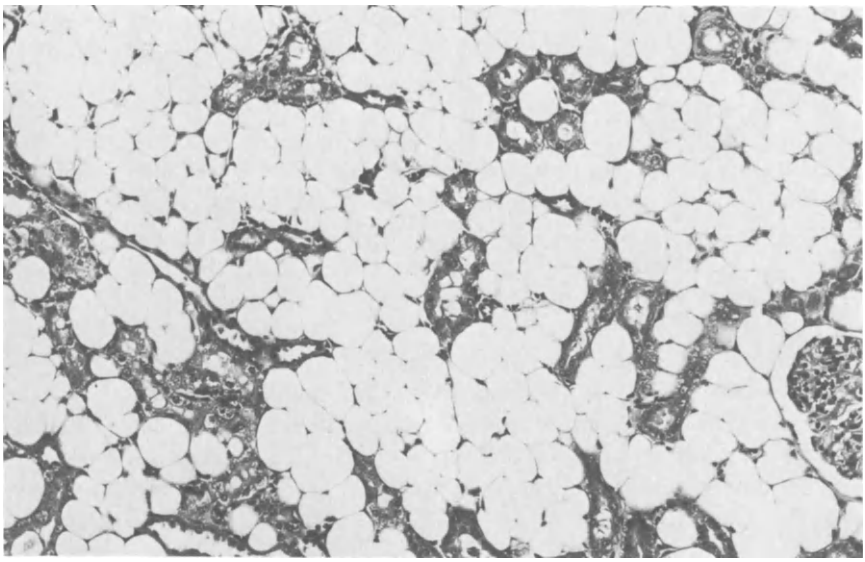

FIG. 8. Lipoma in renal cortex of a rat. Hematoxylin–eosin, ×250.

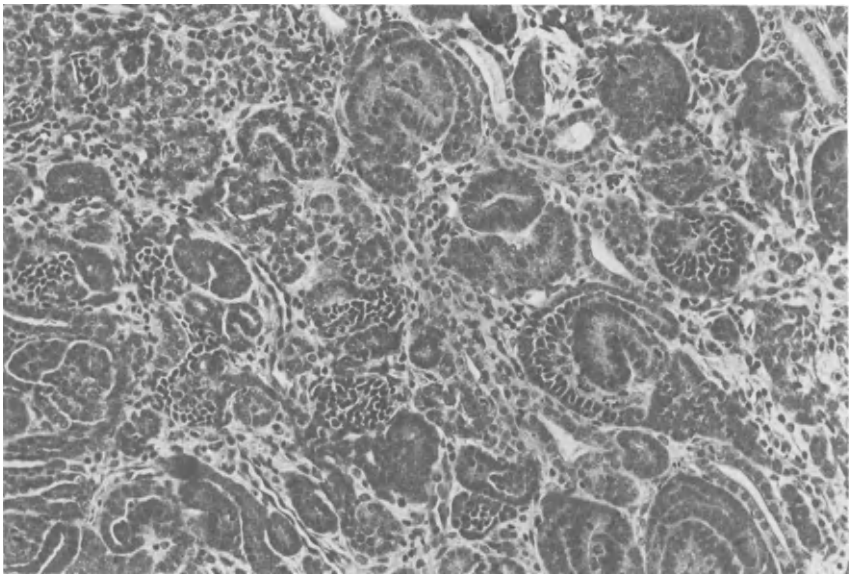

FIG. 9. Embryonal nephroma (nephroblastoma) in a rat. Hematoxylin–eosin, ×250.

normal parenchyma. The tumor usually grows by expansion, thus causing compression of the surrounding tissue and formation of a pseudocapsule. Metastases have been reported, although they are rare.

Tumors arising from the renal pelvis may be transitional cell carcinomas or squamous cell carcinomas. Transitional cell carcinomas (Fig. 10) are usually papillary and are covered by multilayered transitional epithelium. They invade the parenchyma in strands, columns, or irregular masses. Squamous cell carcinomas are usually solid tumors and consist predominantly of sheets of poorly differentiated polygonal cells with areas of keratinization and pearl formation. Renal pelvic carcinomas are often associated with an extensive inflammatory reaction and necrosis.

III. Mouse

A. Normal Anatomy, Histology, and Organ Weights

Liebelt (1986) has reviewed the anatomy, histology, and ultrastructure of the mouse kidney. As in the rat (Section II,A), the sexes differ in the amounts and distribution of certain enzymes and proteins in renal tubules,

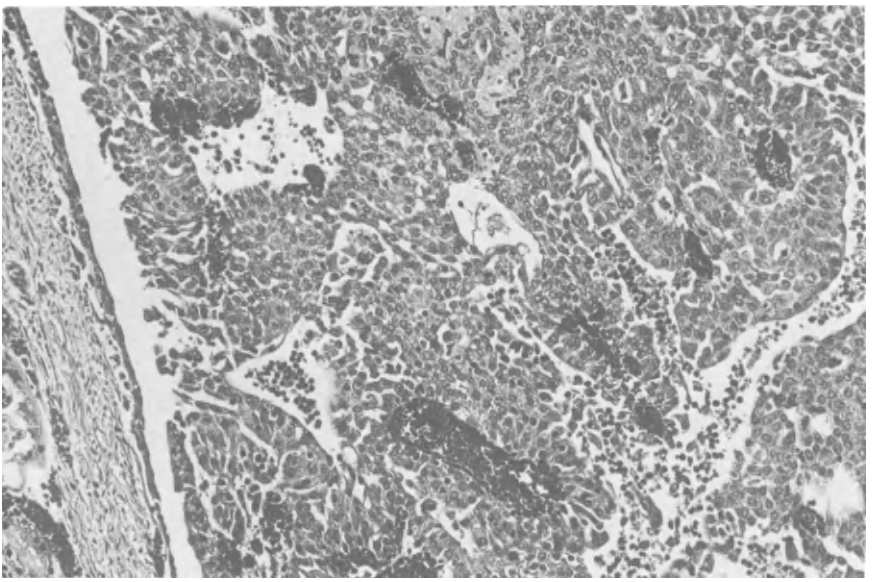

FIG. 10. Transitional cell carcinoma arising from the renal pelvic epithelium in a rat. Hematoxylin–eosin, ×250.

as well as morphology. However, the mouse kidney is anatomically unique due to the presence of cuboidal, parietal epithelial cells lining Bowman's capsule in adult males (Fig. 11). Crabtree (1941) found cuboidal epithelium lining Bowman's capsule by 11 weeks of age in nearly 100% of all glomeruli in two inbred stocks of male mice. At this same age, the capsule of females was lined by a single layer of squamous epithelium. Males and females were similar until approximately 3 weeks of age. Cuboidal epithelium lining Bowman's capsule can occasionally be observed in some female mice (Selye, 1939), but it is much less common than in males and the number of glomeruli affected is much less. These cells are histologically and ultrastructurally similar to proximal tubular cells (Butterfield, 1972). Little is known about the function of these cells, although they are hormone dependent as evidenced by their sexual dimorphism, absence in immature males, absence in castrated males, and presence in androgen-treated females. The kidney weights of mice, like rats, vary with sex and body weight as summarized in Table IX for control CD-1 mice from our laboratory and as summarized by Casey *et al.* (1978).

B. Congenital Diseases

Mice are susceptible to all varieties of congenital kidney alterations. Reviews of this subject have been published (Perraud, 1976). We use Crl:CF-1 BR mice for our teratology studies, and Table X summarizes our experience from examining over 1000 litters and more than 5500 mice from our control population. Congenital malformation of the kidney has a low incidence. Other strains of mice may have a higher spontaneous background rate. C58 black mice have a 10% incidence of renal aplasia while other strains, such as C57BL/6 (Mandell *et al.*, 1983) and CRWwd (Werder *et al.*, 1984), are reported to have a high background of congenital polycystic renal disease which resembles the human disease. Congenital or hereditary hydronephrosis is rare in our Crl:CF-1 mice, but is more frequent in other strains (Collins *et al.*, 1972; Warner, 1971), as reviewed by Hsu (1986). Hydronephrosis in rodents and humans has been reviewed by Wallace and Spickett (1967), and this disease in adult mice is discussed elsewhere (Section III,D,5) in this article.

C. Infectious and Parasitic Diseases

Mice may be infected by a large variety of infectious agents (Squire, 1971). Most cause systemic disease and only a few infect the kidney as the principal target organ. Examples of a few agents that are more likely to

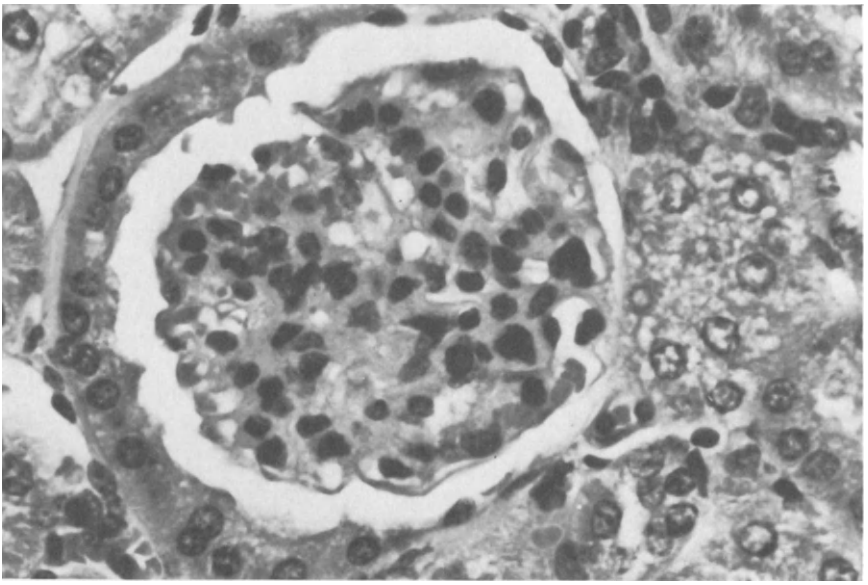

FIG. 11. Glomerulus from a mature male mouse illustrating cuboidal epithelial cells lining the inner surface of Bowman's capsule. Hematoxylin–eosin, ×500.

infect the mouse kidney are discussed below. If proper husbandry is followed, infectious diseases can be controlled and infections of the kidney should be rare events.

1. *Viral Diseases*

Most viral diseases in mice produce systemic disease with the kidneys sporadically involved and often affected to a minimal degree. Such diseases are summarized in more detail elsewhere (Foster *et al.*, 1982a). As an example, mouse adenovirus can affect suckling mice causing unthriftiness, stunting, and death. The disease may have a predilection for the intestinal tract (Otten and Tennant, 1982) or the heart, with necrosis and inflammation. A characteristic feature of the disease is the presence of intranuclear viral inclusion bodies in cells of numerous organs including the kidneys. Mousepox (ectromelia) is another example of a systemic infection which may secondarily involve the kidney (Fenner, 1982). Skin lesions, sudden death, hepatic necrosis, splenomegaly, and lymphoid necrosis are some of the features of this infection. Intracytoplasmic viral inclusion bodies may be found in any organ, including the kidney.

Lymphocytic choriomeningitis (LCM) virus is a murine virus that can infect the central nervous system of several species, resulting in a nonsup-

TABLE IX

WEIGHTS OF KIDNEYS FROM UNTREATED Crl:CD-1(ICR)BR MICE

Sex	Weight range (g)	Number of animals	Absolute weight (g)		Relative weight (% body weight)	
			Median value	Range of actual values	Median value	Range of actual values
Female	20–24	53	0.30	0.22–0.43	1.35	0.96–2.00
	25–29	130	0.36	0.24–0.55	1.31	0.96–1.92
	30–34	86	0.42	0.31–0.60	1.32	0.96–1.90
	35–39	42	0.44	0.33–0.68	1.18	0.86–1.88
	40–100	15	0.48	0.36–0.58	1.12	0.82–1.32
Male	20–24	5	0.36	0.34–0.04	1.56	1.46–1.74
	25–29	46	0.45	0.31–0.56	1.65	1.19–2.20
	30–34	94	0.54	0.42–0.77	1.66	1.31–2.21
	35–39	114	0.61	0.43–1.05	1.66	1.23–2.97
	40–100	31	0.68	0.52–0.97	1.59	1.27–2.19

purative meningitis or meningoencephalitis (Lehmann-Grube, 1982). If neonatal or suckling mice are infected, a persistent viremia may develop and this can lead to the formation of immune complex deposits in the glomeruli (see Section III,D,1). The renal disease produced is a result of the development of immune complex glomerulonephritis (GN), a disease which can progress to end-stage kidney disease and death.

2. *Bacterial Diseases*

Bacterial infections in the urinary system are sporadic and usually of minimal consequence. However, from time to time these infections do occur, resulting in nephritis or pyelonephritis. Most of the time, cultures are not taken and the causative agent is not identified. As a result, pyelonephritis and suppurative nephritis may be occasionally observed histologically. Most are probably bacterial in origin, but the etiology is never determined. Also, under experimental conditions additional stress may be imposed upon the animals from certain experimental procedures such as surgical manipulation, treatment with immunosuppressive drugs, or exposure to irradiation. Such procedures may lead to a depressed immune system and secondary infections.

Corynebacterium kutcherii is a gram-positive rod-shaped bacterium which reaches the kidney via the hematogenous route resulting in focal or multifocal liquefactive necrosis, suppurative inflammation, and abscess formation. The kidney is a frequent site of lesion development, but other

TABLE X

INCIDENCE OF RENAL CONGENITAL ANOMALIES IN CONTROL Crl:CF-1 BR MOUSE FETUSES AND LITTERS

Anomaly	Overall incidence		Highest incidence in a single study	
	Number of fetuses with change (%)	Number of litters with change (%)	Number of fetuses with change (%)	Number of litters with change (%)
Agenesis	1/5544 (0.018%)	1/1439 (0.069%)	1/129 (0.775%)	1/35 (2.857%)
Hypoplasia	1/5544 (0.018%)	1/1439 (0.069%)	1/62 (1.613%)	1/22 (4.545%)
Malformation	1/5544 (0.018%)	1/1439 (0.069%)	1/141 (0.709%)	1/35 (2.857%)
Fusion	0/5544 (0.000%)	0/1439 (0.000%)	—	—
Displacement	0/5544 (0.000%)	0/1439 (0.000%)	—	—
Hydronephrosis	0/5544 (0.000%)	0/1439 (0.000%)	—	—

organs will usually be affected as well. Bacteria may be seen in glomeruli and in areas of necrosis or inflammation.

Proteus sp. and *Pseudomonas* sp. have been known to infect the urinary system of mice. *Proteus mirabilis* can produce mortality, necrotizing pyelonephritis, and suppurative nephritis as reported by Jones *et al.* (1972) and Maronpot and Peterson (1981). In the former report only two mice were reported with the infection, while in the latter 16% of 2836 mice had *Proteus*-induced nephritis and this agent was an important cause of mortality in the male mice of their strain. Likewise, *Pseudomonas aeruginosa* (Tuffery, 1966) has been isolated from cases of pyelonephritis in mice. *Pseudomonas* sp. can result in significant mortality in young mice, but in those outbreaks contaminated water is often the source and the disease is an acute systemic infection as opposed to a primary urinary tract or kidney disease.

Other organisms such as *Pasteurella* sp. *Streptococcus* sp., and *Staphylococcus* sp. may be isolated from cases of urinary tract infections in mice, but none are frequent and all should be controlled using proper animal husbandry procedures.

3. *Parasitic Diseases*

Klossiella muris is a variety of coccidia that parasitizes several tissues in mice with the convoluted tubules of the kidney most commonly affected. Like all coccidia, the life cycle is complex with formation of oocytes, sporocytes, schizonts, mirazoites, macrogametes, and microgametes (Hsu, 1982). When present, the disease is usually minimal with focal or multifocal clusters of parasites present in the tubular epithelium. Severe cases can have over half of the tubules affected.

Grossly, the kidneys may have pinpoint pale, depressed foci scattered over the cortical surface (Otto, 1957; Yang and Grice, 1964). Histopathologically, there is damage to the renal tubular epithelial cells. Inflammation is usually minimal in slight cases, but can be more pronounced in advanced cases. Areas of tubular vacuolation, degeneration, necrosis, and mononuclear inflammatory cell infiltration may be present. Characteristic of the disease is the presence of the organism in the tubular epithelial cells.

Klossiella can be common in wild-caught mice and in the past was seen in colony-bred mice. Today this disease has virtually been eliminated from commercial colonies. Proper hygiene in both the breeding colony and the experimental rooms can control and eliminate this infection. Its presence suggests a need to examine carefully a facility's rodent hygiene practices.

D. Nonspecific Inflammatory, Degenerative, and Proliferative Diseases

There are two common diseases in this category—namely, glomerulonephritis and renal amyloidosis—which are associated with specific strains of mice. Also, there are a number of other diseases and incidental lesions which can be seen mostly in aging mice, but the incidence is variable depending on the strain. The most common lesions, based on our experience with control CD-1 mice from several recently completed carcinogenicity studies are summarized in Tables XI and XII.

1. *Glomerulonephritis*

Glomerulonephritis (GN) in humans is a well-characterized disease entity (Mostofi *et al.*, 1971) with an immunological basis leading to a glomerular inflammatory response associated with the deposition of antigen, antibody, and complement. It is recognized in diseases such as systemic lupus erythematosus, poststreptococcal GN, and membranous GN. Similar types of GN do occur in mice as reviewed in detail by Sass (1986b) and others (Theofilopoulos and Dixon, 1985; Osborne *et al.*, 1977; Robinson and Dennis, 1980), although the etiology is not known in all cases.

LCM virus can lead to the formation of GN (Buchmeier and Oldstone, 1978; Oldstone and Dixon, 1969, 1970). The virus normally produces choriomeningitis. Under certain conditions, such as when mice are congenitally infected or infected at birth (Kajima and Pollard, 1970), hypergammaglobulinemia develops because of persistent viral infection. The viral antigen has been demonstrated in glomerular epithelial cells which presumably provides antigen for circulating antibody to form the deposits of immune complexes and resulting GN. The lesions observed are typical of the disease process, with antigen, antibody, and complement deposition in the glomeruli. Fibrinoid material is deposited along a thickened mesangium and in subendothelial spaces. The mesangial cells proliferate and the basement membrane is thickened by fibrinoid material and intramembranous dense bodies. There is evidence of fusion of epithelial foot processes.

Other viruses have also been associated with secondary immune complex GN in mice including the lactic dehydrogenase virus (Oldstone and Dixon, 1971; Porter and Porter, 1971), murine leukemia viruses (Oldstone *et al.*, 1972), mouse mammary tumor virus (Pascal *et al.*, 1975), and encephalomyocarditis virus (Burch *et al.*, 1972). Although the evidence suggests a strong link with these viruses and the formation of GN, the evidence is strongest and best documented with the LCM virus.

TABLE XI

INCIDENCE OF COMMONLY OCCURRING DEGENERATIVE AND INFLAMMATORY CHANGES IN THE KIDNEY OF UNTREATED MALE CONTROL Crl:CD-1(ICR)BR MICE

Type of change	Incidence by age in weeks (%)									
	1–13 (n = 72)	14–26 (n = 66)	27–39 (n = 20)	40–52 (n = 17)	53–65 (n = 61)	66–78 (n = 203)	79–91 (n = 332)	92–104 (n = 566)	>104 (n = 23)	Total (n = 1360)
Amyloidosis	0	0	0	12	39	77	79	55	100	57
Cellular infiltration	0	15	15	0	23	32	31	44	44	33
Nephrosis	0	0	0	0	7	14	18	11	74	13
Cyst	0	0	0	0	3	5	7	22	26	12
Nephritis	1	0	0	0	7	3	7	14	0	9
Cast	0	0	0	0	3	8	5	11	0	7
Mineralization	0	2	0	0	2	4	5	13	0	7
Necrosis	0	2	0	0	7	8	7	3	9	5
Tubular basophilia	0	0	0	0	0	<1	0	15	0	6
Hydronephrosis	0	0	0	0	2	<1	2	1	0	1
Pyelonephritis	0	3	5	6	2	0	1	<1	0	1
Glomerulonephritis	0	0	0	6	2	0	0	0	0	<1
Glomerulosclerosis	0	0	5	0	0	0	0	0	0	<1

TABLE XII

INCIDENCE OF COMMONLY OCCURRING DEGENERATIVE AND INFLAMMATORY CHANGES IN THE KIDNEY OF UNTREATED FEMALE CONTROL Crl:CD-1(ICR)BR MICE

Type of change	Incidence by age in weeks (%)									
	1–13 (n = 95)	14–26 (n = 51)	27–39 (n = 23)	40–52 (n = 26)	53–65 (n = 34)	66–78 (n = 117)	79–91 (n = 227)	92–104 (n = 693)	>104 (n = 98)	Total (n = 1366)
Amyloidosis	0	2	0	8	32	58	70	54	93	52
Cellular infiltration	1	4	13	19	27	29	25	42	12	31
Nephrosis	0	0	4	4	3	9	13	9	29	10
Cyst	0	2	0	8	3	5	13	7	0	5
Nephritis	0	4	0	4	3	3	6	9	0	6
Cast	0	0	0	0	3	9	9	11	0	8
Mineralization	1	0	9	0	0	2	4	5	0	4
Necrosis	0	0	0	0	9	9	5	3	1	4
Tubular basophilia	0	0	0	0	3	1	0	5	0	3
Hydronephrosis	0	0	0	0	0	2	1	<1	0	<1
Pyelonephritis	0	2	0	0	3	1	<1	<1	0	<1
Glomerulonephritis	0	0	0	0	0	1	1	<1	0	<1
Glomerulosclerosis	0	0	4	4	0	0	0	0	0	<1

Spontaneous autoimmune disease also results in GN in certain inbred mouse strains, and these have been well characterized. For example, NZB mice (Talal and Steinberg, 1974; Kelley and Winkelstein, 1980), C57BL/6 mice (Linder *et al.*, 1972), RF mice (Gude and Upton, 1962), and MRL mice (Kolaja and Fast, 1982) develop a naturally occurring GN which leads to a shortened survival of these strains. Death in some of these strains can result from renal failure in as short a time as 4–6 months of age. The NZW, NZB, and NZB/NZW hybrid mice have been studied extensively, as reviewed by Robinson and Dennis (1980) and Osborne *et al.* (1977). A lupus erythematosus-like syndrome develops in the NZB and NZB/NZW mice characterized by decreased weight, anemia, enlarged liver, enlarged spleen, circulating autoantibodies, hemolytic anemia, and membranous GN. Antinuclear antibodies are demonstrated on the glomerular basement membrane, and subendothelial and subepithelial basement membrane dense bodies occur that increase in size as the disease progresses.

Another strain, the MRL mouse, has been developed as an animal model of autoimmune disease, since it too develops immune compolex GN (Kolaja and Fast, 1982; Accinni and Dixon, 1979). In the MRL strain, animals that are about 2 months of age have minimal or no lesions, whereas by 5 months of age immunofluorescence reveals deposits of IgG and C3. Morphological changes can be detected in the glomerular mesangial cells, with electron-dense material noted by electron microscopy. This disease has many of the features in common with immune complex GN observed in the NZB mouse, as well as in humans.

It was found that ddy mice, derived from noninbred dd stock mice, spontaneously developed an IgA-dominant deposition in the glomerular mesangium. This disease also appears to occur spontaneously with no or minimal deposition at a younger age, but by 40 weeks of age mice are affected by proliferative GN, with the IgA component being prominent (Imai *et al.*, 1985).

Markham *et al.* (1973) evaluated 12 strains of normal and 2 strains of germ-free mice to determine the extent of immune complex deposition in their glomeruli. They concluded that immune complex localization was ubiquitous in all mice, including the germ-free groups.

In summary, immune complex-induced glomerular lesions and GN are common in mice. The cause is not always apparent and may be either secondary to a viral infection or spontaneous. The incidence and severity of GN usually increases with age, but there is considerable strain and sex variation. Zurcher *et al.* (1982) examined four strains of aging mice and found the following incidences: CBA males 84% and females 66%, RFM males 100% and females 100%, C57BL males 100% and females 100%, and NZB males 80% and females 96%. However, only 4–13% of the RFM

and NZB mice developed a severe form of the disease. Ward *et al.* (1979) found a low background of GN in $B63CF_1$ mice with 1.2% and 2.4% of 2543 male and 2522 females, respectively. Cosgrove *et al.* (1978) reported an incidence of 98% in their RFM and 74% in their BALB/c mice.

Our experience with CD-1 mice indicates GN is not an important disease in our strain. Of nearly 3000 control mice examined, the incidence of GN is less than 1%, regardless of age. Similarly, other CD-1 mice also appear to have a very low background of GN (Homburger *et al.*, 1975).

2. *Amyloidosis*

Amyloidosis in mice, like hamsters, is strain, sex, and age dependent. Some sources of mice have relatively little, while it is extremely common in others and may approach 100% in older animals (West and Murphy, 1965; Heston and Deringer, 1948; Dunn, 1967). In some studies nearly all LLC mice (Chai, 1978), KK (Soret *et al.*, 1977), and SJL/J (Scheinberg *et al.*, 1976) mice over 1 year of age had the disease. The incidences of the disease in various strains as well as a literature review were presented by Conner *et al.* (1983). They also reported their experience with the disease in CD-1 mice where the incidence was 100% in mice surviving beyond 14 months.

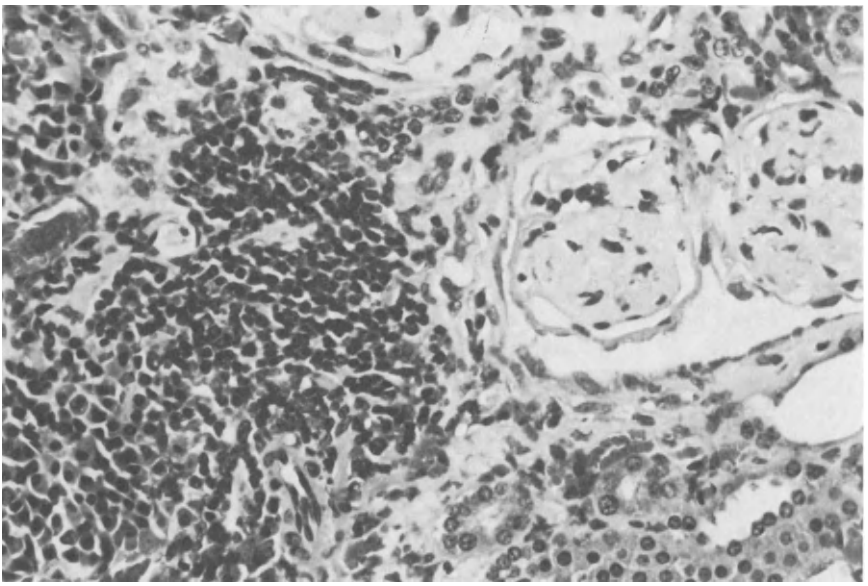

FIG. 12. Mouse kidney illustrating two common lesions. One is amyloidosis of the glomerulus and interstitial tissue and the other is focal aggregation of mononuclear cells. Hematoxylin–eosin, ×500.

In our experience with CD-1 mice, the overall incidence of amyloidosis is approximately 57% in males and 52% in females (Tables XI and XII). It is the most common disease process affecting the kidney and is illustrated in Fig. 12. It is the most common disease process resulting in the death of older mice. It is rare in animals less than 6 months of age and increases dramatically in incidence and severity beyond 1 year of age. The overall incidence of amyloidosis in our male and female CD-1 mice is high, but there can be considerable variation from study to study. This is illustrated in Fig. 13 for females and Fig. 14 for males, which shows the incidence of renal amyloid in control mice from eight recent carcinogenicity studies done in our laboratory. We normally have two control groups of 50 mice per sex for each study. Therefore, of 800 control mice per sex, one can see the overall incidence and variability per study.

3. *Hypertensive Kidney Disease in Mice*

As reported by Rosenberg *et al.* (1982), kidneys of genetically hypertensive mice tend to be larger and possess fewer glomeruli per kidney than do

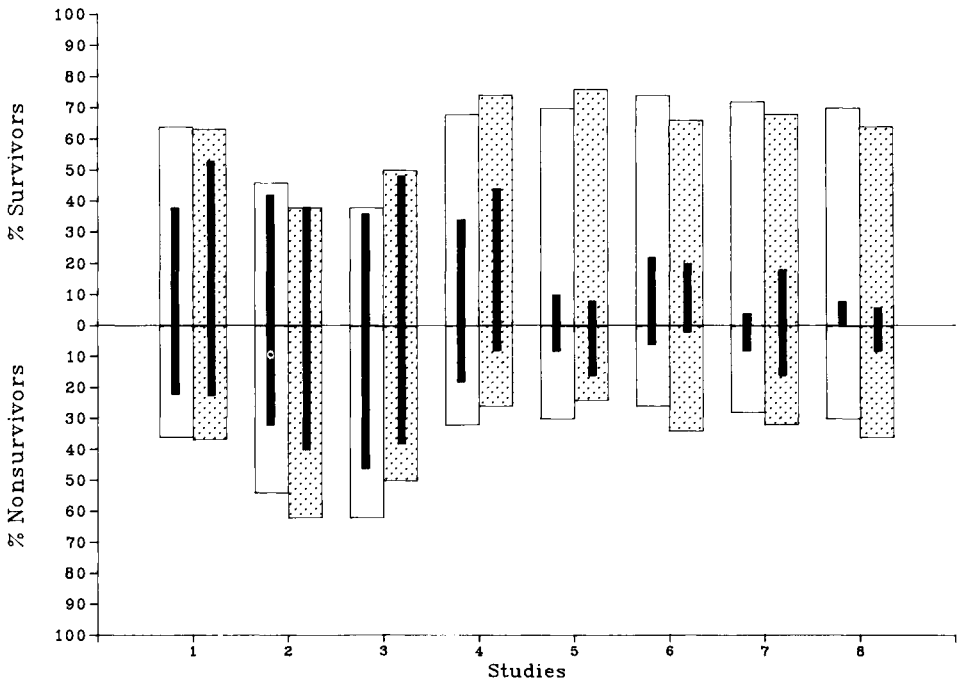

FIG. 13. Percentages of survivors, nonsurvivors, and renal amyloidosis in control female CrL:CD-1(ICR)BR mice from eight recent carcinogenicity long-term studies. Control I, open bars; control II, shaded bars; renal amyloidosis, solid bars.

normotensive mice. Also, their superficial cortical glomeruli tend to be smaller in volume with smaller juxtaglomerular zones. Simpson (1980) describes in more detail a mouse model in the BW mouse of spontaneously occurring renal hypertension and examines parameters such as blood pressure, heart weight, kidney weight, and proteinuria in these mice. The disease is characterized by increased kidney and heart weight (which increases with age), and their conclusion was that this was a good model for spontaneous renal hypertension. Histologically, chronically hypertensive animals showed alterations in the glomerular basement membrane with numerous subepithelial focal thickenings which were largely absent in normotensive controls. Except for the changes in the glomerular capillary loops, the hypertensive animals appeared relatively unremarkable with no evidence of other renal parenchymal changes (Rosenberg *et al.*, 1979).

NZB/NZW and MRL mice have a high incidence of hypertension associated with severe nephritis. The NZB/NZW mouse frequently develops low-renin hypertension during the last phase of the renal disease, whereas the MRL mouse dies from renal disease before blood pressure

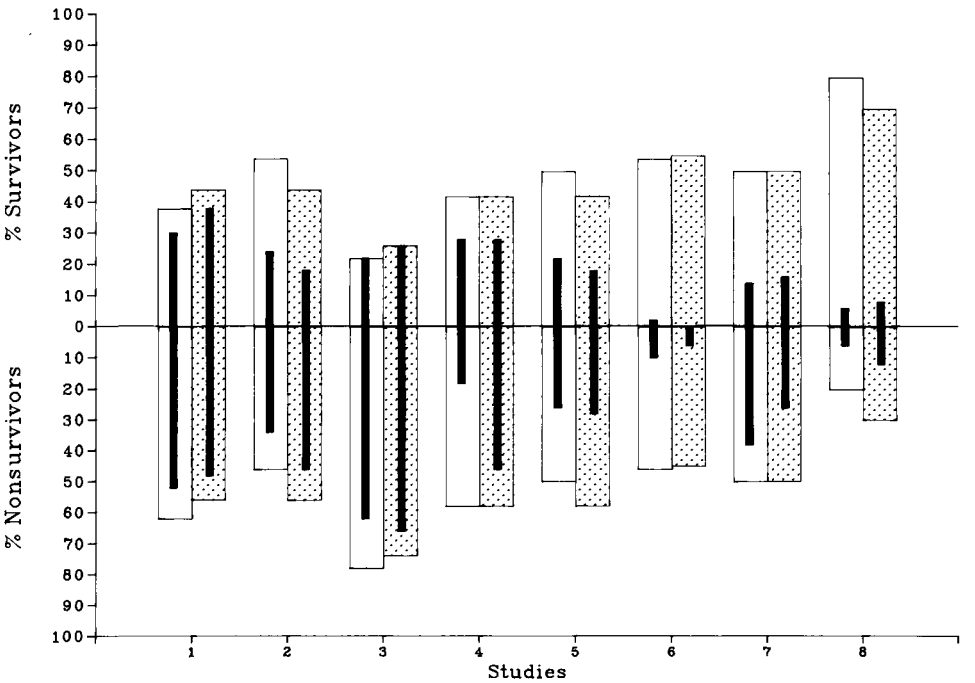

FIG. 14. Percentages of survivors, nonsurvivors, and renal amyloidosis in control male CrL:CD-1(ICR)BR mice from eight recent carcinogenicity long-term studies. Bar shading as in Fig. 13.

elevations can be detected (Rudofsky *et al.*, 1984). Therefore, there appear to be reasonable murine models for hypertensive renal disease. However, the natural occurrence of this disease is relatively uncommon and the diagnosis of renal-induced hypertension in aging mouse colonies is not likely to be made without more sophisticated studies of the colonies in question.

4. *Diabetic Nephropathy in Mice*

Diabetic nephropathy is a rare event in most mouse strains except for those mice that have been developed as animal models for diabetes. These mice are useful animal models of nephrogenic diabetes. Mice with diabetes may have a mild form of the disease or a more severe form with an inability to concentrate urine and the development of a severe form of diabetes. Shortening of the loop of Henle in the kidney may be observed. Histologically there is a decreased number of glomeruli per section and an overall decrease in renal weight with formation of microangiopathic lesions in the glomeruli typical of the diabetes syndrome.

5. *Hydronephrosis in Aging Mice*

Hydronephrosis in mice is strain and sex dependent and can be congenital or acquired. Several reviews on this subject are available (Wallace and Spickett, 1967; Taylor and Fraser, 1973). In aging mice both conditions may occur and may be unilateral or bilateral. Nakajima *et al.* (1983) reported an incidence of 100% in male and 38% in female adult ddd mice. Zurcher *et al.* (1982) found hydronephrosis in 2%, 0%, 6%, and 4% of male CBA, RFM, C57BL, and NZB mice, respectively, and in 0%, 0%, 9%, and 14% of females of the same strains. Ward *et al.* (1979) found less than 1% incidence in $B6C3F_1$ mice.

In our experience with aging CD-1 mice, we also have a low incidence of hydronephrosis that is less than 1%.

6. *Nephritis, Pyelitis, and Pyelonephritis*

As reported earlier (Section II,C,1), bacterial infections of the mouse kidney do occur but are poorly documented and unless there is a colony-wide problem, cultures are seldom taken. Also, even if ascending infections were detected based on routine histopathology, such data would not be reported in the literature, since such findings are of little interest without cultures and identification of causative agents. Montgomery (1986a) has reviewed the literature on this subject.

In our mouse colony, about 10% have an inflammatory pelvic and/or

cortical disease. Therefore, about 10% of our aging mice develop what appears to be an infection of the kidney; most appear to be ascending infections. It is important to remember that amyloid-associated renal disease, lymphoreticular tumors, and diseases of other organs are present in these mice leading to stress and debilitation. Therefore, most of the cases of inflammatory kidney disease are likely secondary to some other primary disease.

7. *Tubular Basophilia*

Tubular basophilia is a term used in our laboratory to describe foci of tubules with more basophilic epithelial cell cytoplasm and is discussed elsewhere in this article (Section II,D,4). These cells are often slightly larger than normal and may or may not be associated with minimal mononuclear interstitial cell infiltration. It occurs in about 3% of our female and 6% of our male CD-1 mice. It may be seen at any age and probably represents a focal degenerative or regenerative process in the renal tubules.

8. *Mineralization*

Mineral deposits are frequent in C3H and DBA mice but are less common in CD-1 and BALB/c mice (Morrissey, 1986). They may appear as small, focal, or multifocal basophilic deposits often at the corticomedullary junction and medullary region. Such deposits are usually minimal and incidental findings in routine studies.

9. *Pigments*

Pigments are common in renal tubular epithelial cells of older mice (Brown, 1986), but are usually present in very minute amounts. The usual pigments may be hemosiderin- and lipofuscinlike deposits.

10. *Tubulointerstitial Disease*

CBA/J mice have been reported to develop renal tubulointerstitial disease by 2 months of age (Rudofsky, 1978). The disease was characterized by mononuclear cell infiltration in the interstitium with multinucleated cells and destruction of tubules. Glomeruli remained normal. This disease was age associated, with all mice affected by 12 months. The cause of the lesions could not be determined, but anti-tubular basement membrane autoantibodies and immune complexes were ruled out as a cause. Also, C57BL/6J mice did not develop the disease even when housed with affected CBA/J mice.

Neilson *et al.* (1984) reported a similar disease in AB mice (kdkd strain). The disease was progressive, and antibodies to the tubulointerstitial tissue could not be demonstrated. However, the cellular immune system was thought to play a role in the pathogenesis of the disease.

Our CD-1 mice have a high incidence (~30%) of mononuclear cell (predominantly lymphocytes and plasma cells) interstitial infiltration (Fig. 12). Such infiltrations are common in other mouse strains, like the $B6C3F_1$ (Montgomery, 1986a). It is usually an incidental observation generally not associated with tubular degenerative changes, and its cause is unknown.

11. *Papillary Necrosis*

Papillary necrosis (Fig. 15) in mice is a rare primary lesion, but can be seen more frequently associated with renal amyloidosis.

12. *Vacuolation of Renal Tubular Epithelial Cells*

Vacuolation is usually the result of lipid accumulation in tubular epithelial cells. Some lipid can almost always be detected. Occasionally the amount is increased (Fig. 16). The cause and significance of these lipid accumulations are unknown.

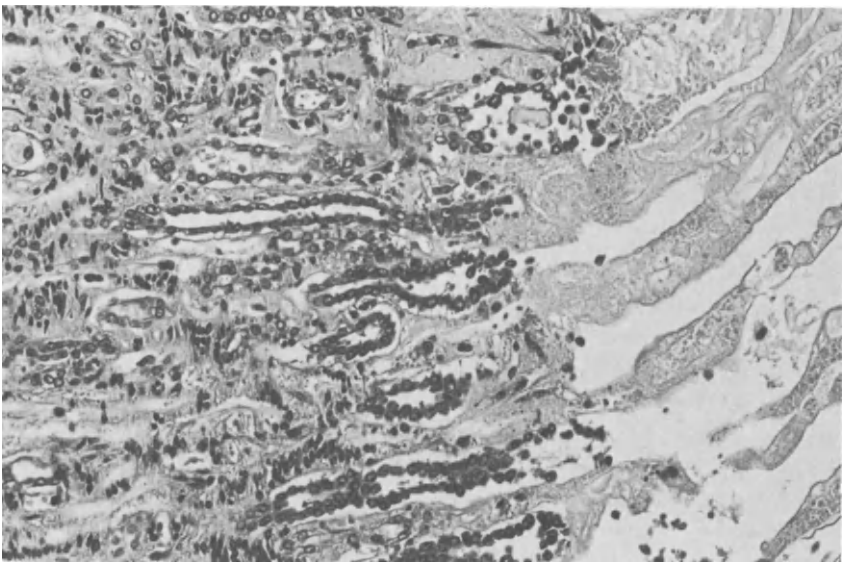

FIG. 15. Extensive renal papillary necrosis in a mouse. Hematoxylin–eosin, ×250.

E. Neoplastic Diseases

The morphological classification of renal tumors in mice has been reviewed (Sass, 1986a; Terracini and Campobasso, 1979). Primary renal tumors include adenomas and adenocarcinomas of tubular epithelial cells, transitional cell papillomas, and carcinoma of the pelvic epithelium and sarcomas. Unfortunately, criteria for the classification of mouse kidney tumors has been based on experience with induced tumors, because spontaneously occurring tumors of the mouse kidney have a very low incidence in most mouse strains and stocks. One notable exception is the Claude BALB/c. Claude (1958) originally reported a 60–70% incidence of renal tumors. Later, Sass *et al.* (1976) evaluated two substrains of the BALB/cfCd mice and, although lower than Claude's first observation, still reported renal tumors with an incidence in the range of 25–48%. The tumors in both studies were adenomas, papillary cystadenomas, and adenocarcinomas of renal tubular epithelial cells.

Sass (1986a) reviewed spontaneous and induced renal epithelial tumors in mice. To illustrate the low frequency of spontaneous renal tumors in most other mice, a few specific references are cited here. Sheldon and Greenman (1979) summarized the most frequently observed neoplasms in

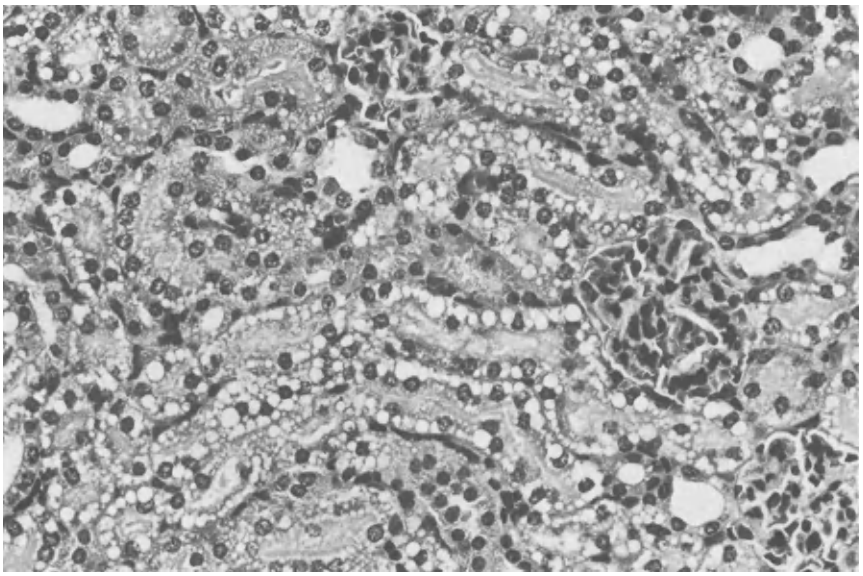

FIG. 16. Prominent vacuolation of renal tubular epithelial cells in a mouse. Hematoxylin–eosin, ×250.

2376 $B6C3F_1$ mice from a large study at NCTR and did not report any renal tumors. However, Shinohara and Frith (1980) selected and evaluated 13 renal cell tumors (11 adenomas and 2 carcinomas) among 3495 animals involved in that same study, suggesting the overall incidence of renal tumors in their $B6C3F_1$ mice was less than 0.5%. Nakanishi *et al.* (1982) reported only 1 pelvic tumor in 96 control $B6C3F_1$ mice, even though phenacetin-treated males developed treatment-related renal cell tumors. Ward *et al.* (1979) provided more data supporting the low frequency of renal tumors in $B6C3F_1$ mice with 2 adenomas, 3 adenocarcinomas, and 1 renal pelvic transitional cell papilloma in 5065 mice (a combined incidence of 0.12%).

Lombard *et al.* (1974) found renal tumors in 2% of control C3H × C57BL hybrid mice. In another review (Sher *et al.*, 1982) of CD-1 and $B6C3F_1$ mice, renal tumors were not reported. Our computer files at MSDRL indicate that in 5056 control CD-1 mice, only eight (0.16%) primary epithelial kidney tumors were observed (five adenomas and three adenocarcinomas). There were no nephroblastomas or transitional cell pelvic tumors. However, in addition to the epithelial tumors, six sarcomas (one leiomyosarcoma, one fibrosarcoma, and four unspecified sarcomas) were recognized.

Primary renal tumors are infrequent, but secondary tumors are not as unusual. For example, of 5056 control CD-1 mice from our laboratory, lymphoreticular tumors (leukemia, lymphoma, lymphosarcoma, reticulum cell sarcoma, histocytic sarcoma, and plasma cell tumors) involved the kidney in 301 (6%) of the cases.

IV. Hamster

A. Normal Anatomy, Histology, and Organ Weights

The anatomy of the hamster kidney differs from other rodents in that the renal papilla tends to be much longer and extends into the ureter, a feature which has led to its extensive use in renal physiology studies.

Hamster body weights, absolute and relative (expressed as a percentage of body weight) kidney weights have been summarized by Frank (1976) for 17-week-old animals and by Thomas *et al.* (1979) for hamsters ranging from 30 to approximately 500 days of age. Their data indicates that kidneys in adult males weigh between 250 to 530 mg, and the kidney weight expressed as a percentage of body weight ranges from 0.5 to 0.4 between 30 and 500 days old, respectively. The same data for females ranged from 280 to 800 mg for kidney weights and 0.7 to 0.8 for percentage of body weight. We have had experience with hamsters from Sasco and Charles River

Laboratories, and our renal weight data are summarized in Tables XIII and XIV for these animals, respectively.

B. Congenital Diseases

Malformations in hamsters have been reviewed by Shenefelt (1978). Spontaneous malformations in hamsters occur at a low rate (usually 0–3%). Malformations of the urinary system are rarer still in most hamster colonies. An exception is the piebald hamster, which may have a 20% incidence of urogenital anomalies, especially in females (Orsini, 1952; Bock, 1953; Foote, 1955), consisting of renal aplasia and hypoplasia. Hamsters are being used with increasing frequency in experimental teratology, so additional data should be available which should confirm the low spontaneous malformation rate for this species.

C. Infectious and Parasitic Diseases

Hamsters are susceptible to a number of viral, bacterial, fungal, and parasitic diseases. However, most either result in systemic infection or

TABLE XIII

Weights of Kidneys from Untreated SAS;E(SYR) Hamsters

Sex	Weight range (g)	Number of animals	Absolute weight (g)		Relative weight (% body weight)	
			Median value	Range of actual values	Median value	Range of actual values
Female	100–109	2	1.35	1.35–1.35	1.24	1.24–1.25
	110–119	5	1.32	1.19–1.46	1.18	1.08–1.27
	120–129	13	1.34	1.21–1.45	1.10	0.98–1.15
	130–139	10	1.44	1.29–1.65	1.05	0.98–1.22
	140–149	6	1.46	1.27–1.64	1.00	0.86–1.12
	150–159	6	1.55	1.33–1.66	1.00	0.86–1.08
	160–169	2	1.68	1.63–1.72	1.01	0.98–1.04
	200–209	1	1.49	1.49–1.49	0.74	0.74–0.74
Male	90–99	1	1.07	1.07–1.07	1.09	1.09–1.09
	100–109	5	1.09	1.00–1.18	1.04	0.92–1.13
	110–119	3	1.09	1.07–1.13	0.96	0.96–0.97
	120–129	14	1.22	1.11–1.27	0.96	0.86–1.06
	130–139	5	1.22	1.14–1.30	0.90	0.86–1.00
	140–149	5	1.25	1.22–1.46	0.87	0.82–1.02
	150–159	6	1.24	1.14–1.30	0.81	0.73–0.84
	160–169	5	1.37	1.27–1.48	0.84	0.78–0.90

TABLE XIV

WEIGHTS OF KIDNEYS FROM UNTREATED LAKEVIEW LVG HAMSTERS

Sex	Weight range (g)	Number of animals	Absolute Weight (g)		Relative weight (% body weight)	
			Median value	Range of actual values	Median value	Range of actual values
Female	80–89	8	1.00	0.91–1.06	1.15	1.11–1.30
	90–99	3	1.02	0.98–1.18	1.11	1.06–1.23
	100–109	9	1.12	1.05–1.17	1.07	1.05–1.10
	110–119	9	1.18	1.13–1.38	1.04	0.98–1.16
	120–129	11	1.24	1.12–1.37	0.99	0.93–1.10
	130–139	6	1.29	1.15–1.39	0.97	0.87–1.00
	170–179	1	1.65	1.65–1.65	0.95	0.95–0.95
Male	80–89	7	0.93	0.90–1.01	1.11	1.06–1.20
	90–99	6	0.94	0.90–1.19	1.00	1.00–1.20
	100–109	7	0.98	0.91–1.19	0.92	0.89–1.10
	110–119	5	1.10	1.07–1.10	0.94	0.92–1.00
	120–129	10	1.10	1.01–1.13	0.87	0.81–0.92
	130–139	10	1.12	0.99–1.23	0.82	0.76–0.94
	140–149	1	1.15	1.15–1.15	0.79	0.79–0.79
	150–159	1	1.20	1.20–1.20	0.76	0.76–0.76
	160–169	1	1.27	1.27–1.27	0.76	0.76–0.76

affect organs other than the kidney. Only a few agents affect the kidney or urogenital system as the primary site. Those most likely to produce lesions in the kidney include viral agents such as LCM virus, producing a similar disease to that seen in mice (Section III,D). Likewise, bacterial infections may occur either via the hematogenous route or as ascending urinary tract infections. However, as in mice, infections are sporadic and seldom complicate studies.

D. NONSPECIFIC INFLAMMATORY AND DEGENERATIVE DISEASES

Syrian hamsters have a spectrum of spontaneous nonneoplastic renal lesions. Most do not or should not affect studies using younger animals (<14 weeks of age). However, hamsters older than 6 months and especially those older than 1 year are likely to have significant renal damage. For simplicity, hamster renal diseases can be divided into two major categories: amyloid and nonamyloid. The former has been well characterized, while the latter has not been studied in detail and likely represents a spectrum of unrelated diseases. Regardless, of the cause, the incidence of deaths related

to end-stage renal disease can be very high in colonies of aging Syrian hamsters; in some colonies renal amyloidosis is the major cause of death, while in others it is nonamyloid-related end-stage renal disease; more often both causes can be observed.

1. *Amyloidosis*

The most common disease affecting Syrian hamsters is amyloidosis. It is a systemic disease affecting many organs including the kidney. Amyloidosis in hamsters is well characterized (Lewis and Mezza, 1986; Renshaw *et al.*, 1975; Casey *et al.*, 1978; Gleiser *et al.*, 1971). The disease is age associated. It is unusual in animals younger than 6 months, but progresses with age so that older hamsters (18–24 months) have the greatest incidence and severity of the disease. The incidence varies with the strain, source, and age of hamsters, and the incidence ranges from 30% to over 80% (Gleiser *et al.*, 1971; Burek *et al.*, 1984; Schmidt *et al.*, 1983). The incidence may actually be 100% in hamsters that reach an age of 24 months.

Grossly, the kidneys are enlarged, pale, and have a rough or irregular cortical surface. The histopathological lesion in the kidney usually begins in glomeruli but can be interstitial, or both. Histologically, the early changes begin as hyaline, eosinophilic, homogeneous, multifocal deposits. Progression continues until the disease becomes diffuse and severe. The severity of the disease can progress until it leads to end-stage kidney disease with renal failure and death (Murphy *et al.*, 1984). Amyloid has a characteristic staining affinity with several stains including green birefringence with Congo red, metachromasia with crystal violet, and secondary fluorescence when stained with thioflavin-S or thioflavin-T. Also, amyloid has a characteristic electron microscopic appearance characterized by fibrils that have a diameter of approximately 70–100 Å. The earliest lesions may be Congo red negative, but as the disease progresses, the characteristic appearance with special stains can be seen.

2. *Nonamyloid Renal Disease(s)*

In addition to amyloidosis, a spectrum of chronic degenerative renal diseases also occur in Syrian hamsters. This spectrum has been poorly documented. The etiology, pathogenesis, and strain predilection are unknown. Also, the following lesions may represent several independent pathological processes or some could be stages of the same syndrome.

Young hamsters (1–6 months old) may have multifocal nodular thickening of the glomerular basement membrane as noted by both light and electron microscopy (van Marck *et al.*, 1978; Klei *et al.*, 1974a). This early change may occur in the absence of glomerulosclerosis or alterations in the

mesangial matrix, and without IgG or amyloid deposition, but slight proteinuria may result. Its etiology, incidence, and potential for progression are unknown.

An age-associated reduction in the size of glomerular tufts was reported by Maguire *et al.* (1974). Glomerular tufts were small, the urinary space filled with amorphous pink fluid, and glomeruli at all cortical levels were affected. The change was occasionally associated with thickening of Bowman's capsule. This observation occurred in the absence of amyloid or interstitial infiltration. Tubular casts were seen in some animals. The authors concluded it was a spontaneous age-related degenerative change.

A progressive age-associated degenerative atrophy of tubules was reported by Schmidt *et al.* (1983). The early lesions were minimal, radial areas of atrophic tubules with increased interstitial connective tissue. Severe cases had numerous linear or wedge-shaped areas with collapsed tubules. The most severe cases resulted in end-stage kidney disease and death. The size and number of areas varied, and irregular cortical surface resulted. These authors also noted some atrophic glomeruli, but in most cases the glomeruli were normal. Many tubular lumens were dilated but empty, while others contained protein casts. Although features of the lesions suggested a vascular origin, no vascular lesions were observed to account for the changes.

Calcinosis affecting the arteries of most organs in outbred Syrian hamsters of the Eppley colony has been reported (Pour and Birt, 1979). The disease caused progressive calcification and degeneration of arteries. Renal artery involvement lead to altered renal blood flow and degeneration of the kidneys even to the point of chronic renal failure, secondary hyperthroidism, and renal fibrous osteodystrophy. The authors indicated that alterations in diet could decrease or eliminate this condition.

Polycystic disease in hamsters has been recognized as an age-associated disease consisting of cyst formation, with the kidney being one of numerous organs affected (Gleiser *et al.*, 1970; Renshaw *et al.*, 1975; Fox, 1979). The cysts are single or multiple, thin-walled, and filled with clear to amber-colored fluid.

Tables XV and XVI summarize nontumor data from 505 control LVG hamsters from our MSDRL files. The most common disease was amyloidosis, which affected 32% of males and 49% of females. The second most common disease was a nonamyloid, nonspecific, degenerative renal tubular lesion (nephrosis). It was present in 25% of the females and 20% of the males. The third most common diagnosis was cellular (mononuclear) infiltration followed by mineralization. These changes were similar to those seen in mice and appeared as very slight to slight lesions. Other lesions were

TABLE XV

INCIDENCE OF COMMONLY OCCURRING DEGENERATIVE AND INFLAMMATORY CHANGES IN THE KIDNEY OF UNTREATED MALE LAKEVIEW LVG HAMSTERS

Type of change	Incidence by age in weeks (%)								
	1–13 (n = 21)	14–26 (n = 42)	27–39 (n = 13)	40–52 (n = 16)	53–65 (n = 20)	66–78 (n = 16)	79–91 (n = 21)	92–104 (n = 103)	Total (n = 252)
Amyloidosis	0	0	15	25	20	25	38	56	32
Cellular infiltration	0	0	0	0	0	6	0	7	3
Nephrosis	0	0	8	31	35	50	29	23	20
Nephritis	0	0	8	0	0	0	0	1	1
Cast	0	0	0	0	20	19	5	6	6
Mineralization	0	5	15	19	15	19	0	7	8
Tubular basophilia	0	12	0	0	0	0	0	0	2
Tubular dilatation	0	24	0	0	0	0	0	0	4

TABLE XVI

INCIDENCE OF COMMONLY OCCURRING DEGENERATIVE AND INFLAMMATORY CHANGES IN THE KIDNEY OF UNTREATED FEMALE LAKEVIEW LVG HAMSTERS

Type of change	Incidence by age in weeks (%)								
	1–13 (n = 21)	14–26 (n = 42)	27–39 (n = 14)	40–52 (n = 17)	53–65 (n = 20)	66–78 (n = 36)	79–91 (n = 103)	92–104 (—)	Total (n = 253)
Amyloidosis	0	0	21	29	65	78	72	—	49
Cellular infiltration	0	0	0	6	10	28	22	—	14
Nephrosis	0	0	7	24	35	36	36	—	25
Nephritis	0	14	0	12	0	6	6	—	6
Cast	0	0	0	0	0	3	0	—	<1
Mineralization	0	0	21	12	5	25	6	—	8
Tubular basophilia	0	2	0	0	0	0	0	—	<1
Tubular dilatation	5	12	0	0	0	0	0	—	2

much less frequent, and included tubular basophilia (similar to that seen in rats and mice), casts, tubular dilatation (Fig. 17), and nephritis.

E. Neoplastic Diseases

The classification of hamster epithelial kidney tumors has been reviewed by Pour *et al.* (1976a,b).

Naturally occurring tumors are uncommon in the kidneys of Syrian hamsters. Detailed surveys of a few colonies have been done confirming this fact. Pour *et al.* (1976a,b) examined two hamster colonies. Kidney tumors were found in 0% of male and 0.7% of female Eppley colony hamsters and 0% of males and females from the Hannover colony hamsters. The one tumor in the 516 hamsters examined was a cortical cell adenoma. In another study Pour *et al.* (1979) examined tumors in three inbred hamster colonies (CH, WH, and AH lines) from the Eppley colony. Renal tumors were not observed in 71 female or 54 male CH hamsters or in 71 female or 65 male WH hamsters. The AH line had multiple adenomas in 3 of 68 (4.4%) females and 1 of 81 (1.2%) males. Also, two (2.9%) females had nephroblastomas. Schmidt *et al.* (1983) found only one renal adenoma among 750 control male Syrian hamsters. Others (Toth and Nagel, 1977) found no renal tumors in 100 male and 100 female hamsters used in a

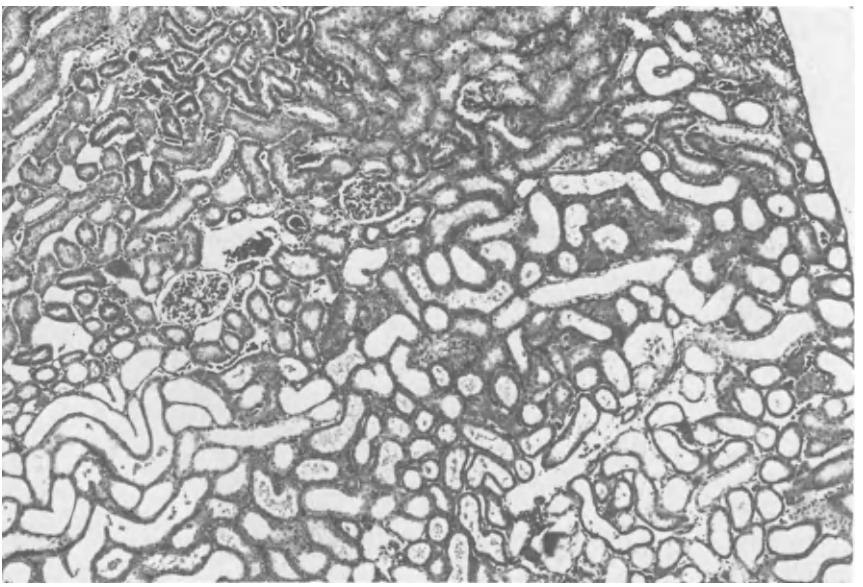

FIG. 17. Hamster kidney with extensive renal tubular dilatation. Hematoxylin–eosin, ×100.

lifetime study. McMartin (1979) also evaluated an aging Syrian hamster colony and found no renal tumors in 72 males and 52 females.

Homburger's group has reported tumor data on a new hybrid BIO FID Alexander Syrian hamster from their laboratory (Homburger *et al.*, 1983; Bernfeld *et al.*, 1986). In their first article, two adenomas were observed in 177 males and no renal tumors were observed in 177 females. In their most recent article, they did not report any renal tumors in approximately 400 hamsters examined.

Our experience with hamsters has been with LVG Charles River colony animals, and we have observed two adenomas and five carcinomas in the 820 control hamsters we have examined in our carcinogenicity studies.

Therefore, the incidence of primary epithelial renal tumors in Syrian golden hamsters is low (generally $<1\%$) in both sexes. It is interesting that despite the low spontaneous renal tumor rate, hormones (estrogens) have a unique and well-defined action on the hamster kidney leading to adenomas and adenocarcinomas (Liehr, 1983; Liehr *et al.*, 1983; Lin *et al.*, 1982; Llombart-Bosch and Peydro-Olaya, 1986).

Lymphoreticular tumors affect a variety of tissues in addition to the lymphoid system. They may infiltrate a wide range of tissues, and the kidney is a frequent site of such involvement. Of the 820 hamsters we have examined, lymphoreticular tumors affected the kidney of 5 (0.6%).

V. Rabbit

A. Normal Anatomy, Histology, and Organ Weights

The kidneys of the rabbit are smooth, tan, bean-shaped organs, firmly fixed to the dorsal body wall. The right kidney is more craniad than the left. The ventral surface of each kidney is covered by peritoneum and frequently by an accumulation of fat in the subperitoneal connective tissue forming an indefinite adipose capsule (Craigie, 1951). The renal capsule is a thin, transparent, and tough membrane.

On the cut surface of a bisected kidney, a single renal papilla and the undivided renal pelvis are evident (Craigie, 1948).

The weights of kidneys from untreated young adult, male and female New Zealand White rabbits, the breed most commonly used in the laboratory, are given in Table XVII. Limited kidney weight data from Dutch Belted rabbits have been reported (Kozma *et al.*, 1974).

The histological appearance of the rabbit kidney does not present any remarkable species-specific features, and will not be discussed further.

B. Congenital Diseases

A variety of congenital anomalies involving the kidneys has been reported in the rabbit, most of these being derived from observations on fetuses as part of teratological studies. In New Zealand White rabbit fetuses, unilateral renal agenesis occurs with a frequency ranging from 1/6000 to 2/3185 (0.017–0.063%); misshapen and fused (horseshoe) kidneys, 1/4300 (0.023%); ectopic kidney, 1/8500 (0.012%); and hydronephrosis, 1/4300 (0.023%)(Palmer, 1968; Stadler *et al.*, 1983). Malformed or misshapen kidney was the most commonly observed renal anomaly in 11,831 control New Zealand White rabbit fetuses examined in our laboratory in the course of teratological studies. As is shown in Table XVIII, this change occurred with an overall frequency of 0.085%, although the highest incidence within a single study was 5.71% of fetuses and 20.0% of litters.

Renal cortical cysts, occurring either singly or in small to large numbers (50–100 or more), occur in almost all breeds of rabbits to some extent, although the incidence is generally low (e.g., 2/3185 New Zealand White rabbit fetuses) (Stadler *et al.*, 1983). In some strains of rabbits, the incidence of cortical cysts is considerably higher, such as in several strains of JAX rabbits: 456/2544 (18%) in strain III/J, 706/5370 (13%) in strain IIIVO/J, and 110/750 (15%) in strain IIIEP/J (Crary and Fox, 1980). Renal cortical cysts often do not appear until 3 or 4 weeks of age, and usually are of no clinical or pathological importance. Polycystic kidneys have been shown to

TABLE XVII

Weights of Kidneys from Untreated Young Adult New Zealand White Rabbits

Sex	Weight range (kg)	Number of animals	Absolute weight (g)		Relative weight (% body weight)	
			Median value	Range of actual values	Median value	Range of actual values
Female	1.0–1.9	1	18.67	18.67–18.67	1.01	1.01–1.01
	2.0–2.9	13	17.30	11.80–28.18	0.61	0.51–1.13
	3.0–3.9	57	18.66	12.72–34.71	0.54	0.38–1.04
	4.0–4.9	26	19.66	15.07–33.40	0.45	0.37–0.81
	5.0–5.9	4	23.50	21.92–24.26	0.45	0.41–0.47
Male	2.0–2.9	8	15.82	12.31–17.43	0.62	0.52–0.66
	3.0–3.9	70	20.38	14.50–33.79	0.58	0.45–0.87
	4.0–4.9	28	22.68	17.46–26.04	0.49	0.42–0.65
	5.0–5.9	1	25.02	25.02–25.02	0.49	0.49–0.49

TABLE XVIII

INCIDENCE OF RENAL CONGENITAL ANOMALIES IN CONTROL NEW ZEALAND WHITE RABBIT FETUSES AND LITTERS

Anomaly	Overall incidence		Highest incidence in a single study	
	Number of fetuses with change (%)	Number of litters with change (%)	Number of fetuses with change (%)	Number of litters with change (%)
Agenesis	4/11,831 (0.034%)	2/1,536 (0.130%)	3/131 ((2.29%)	1/13 (7.69%)
Hypoplasia	2/11,831 (0.017%)	2/1,536 (0.130%)	1/244 (0.41%)	1/30 (3.33%)
Malformation	10/11,831 (0.085%)	8/1,536 (0.521%)	2/35 (5.71%)	1/5 (20.0%)
Fusion	2/11,831 (0.017%)	2/1,536 (0.130%)	1/35 (2.86%)	1/5 (20.0%)
Displacement	3/11,831 (0.025%)	3/1,536 (0.195%)	2/466 (0.43%)	2/55 (3.64)

have a genetic basis, resulting from a single autosomal recessive gene, in at least one strain of JAX rabbit (IIIVO/J) (Lindsey and Fox, 1974).

C. Infectious and Parasitic Diseases

1. *Bacterial Diseases*

The bacterial diseases are among the most common of the spontaneous diseases of rabbits, and are a significant cause of morbidity and mortality in this species. There are many bacterial diseases which may either directly or indirectly involve the kidney during their clinical course. It is not within the scope of this article to discuss these at length; detailed information on many bacterial diseases of rabbits has been published (Flatt, 1974; Carlton and Hunt, 1978). In this section, a brief review will be given of several multisystemic bacterial diseases which may, at times, include the kidney as one of the affected organs.

a. Pasteurellosis. This disease is caused by *Pasteurella multocida,* a short gram-negative bipolar rod. A variety of clinical expressions of the disease occurs, including rhinitis and sinusitis ("snuffles"), enzootic pneumonia, otitis media, conjunctivitis, pyometra, orchitis, generalized septicemia, and abscesses. The last-mentioned clinical form, abscessation, may involve the kidney, as well as virtually any organ or tissue in the body. Such a presentation is usually the result of chronic pasteurellosis, which develops in animals that survive the acute disease, especially the upper respiratory or septicemic forms. Grossly, renal abscesses caused by *P. multocida* contain thick, tan, creamy exudate and they may have a well-formed capsule. Bacterial culture of the exudate often yields a pure isolate of the organism.

b. Staphylococcosis. The disease caused by *Staphylococcus aureus* is relatively common in rabbits. The disease may be present as an acute fatal septicemia with few or no clinical signs, a subacute form with swelling, reddening, and induration of the skin and mammary glands, or a chronic form with abscessation in several different organs, including the kidneys. The abscesses that form in chronic staphylococcosis are thick-walled and contain thick, white exudate. Clusters of gram-positive cocci are usually easily demonstrable in tissue sections, and the causative organism can be readily isolated from affected tissues.

c. Yersiniosis (Pseudotuberculosis). This disease is caused by *Yersinia pseudotuberculosis,* a pleomorphic, gram-negative coccobacillus. Typically, the disease involves the gastrointestinal tract, liver, spleen, and mesenteric lymph nodes. At times the kidney, as well as other organs, may be involved. Grossly, prominent white or yellow, slightly raised nodules are seen in affected organs. Histologically, such lesions are granulomas, with central

caseous necrosis surrounded by a zone of macrophages, lymphocytes, and occasional giant cells.

Yersiniosis must be distinguished from tuberculosis, caused by *Mycobacterium tuberculosis,* var. *bovis,* which very rarely occurs spontaneously in rabbits. The lesions of tuberculosis have a similar organ distribution, and grossly and microscopically closely resemble those of yersiniosis. The presence of acid-fast bacilli in the granulomas of tuberculosis is the most important distinguishing feature.

d. Necrobacillosis (Schmorl's Disease). This is a sporadic disease of rabbits caused by *Fusobacterium necrophorum,* which is a pleomorphic, filamentous to coccoid, gram-negative, non-spore-forming rod. The usual clinical signs include abscessation and ulceration of the lips, skin of the face, head, neck, and feet. Bacteremia may result, giving rise to necrotic lesions in the parenchymatous organs, including the kidneys. The lesions in these organs are nonspecific, and bacterial isolation often is necessary to make a definitive diagnosis.

2. *Parasitic Diseases*

The most important parasitic disease involving the rabbit kidney is encephalitozoonosis, caused by the microsporidian *Encephalitozoon cuniculi.* The organism usually causes an inapparent infection, but acute disease with neurological signs may occur, reflecting involvement of the central nervous system (granulomatous encephalitis). The disease is common in many conventional rabbit colonies (Flynn, 1973), and it has also been described in gnotobiotic rabbits, suggesting vertical transmission (Hunt *et al.*, 1972).

Affected kidneys grossly show multiple white-gray pinpoint foci scattered over the cortical surface. Often, discrete depressed areas, several millimeters in diameter, may be seen on the surface. Typically, the lesions are characterized histologically by granulomatous inflammation or focal chronic interstitial nephritis, with lymphocytic and plasma-cellular infiltration, fibrosis, tubular degeneration, and dilatation (Pakes, 1974). Sometimes more acute lesions are seen, which are characterized by multifocal necrosis and which are often associated with a large number of organisms, both in tubular epithelial cells, and free within tubular lumens (Fig. 18).

The organisms are sometimes difficult to identify in routine hematoxylin- and eosin-stained sections, although large clusters of organisms within cystlike spaces in tubular epithelial cells are readily visible. The organism is gram-positive, oval, approximately 1.5×2.5 μm, and has an affinity for silver stains (Shadduck and Pakes, 1978). A definitive diagnosis can be made based on the identification of the organisms in typical lesions in the

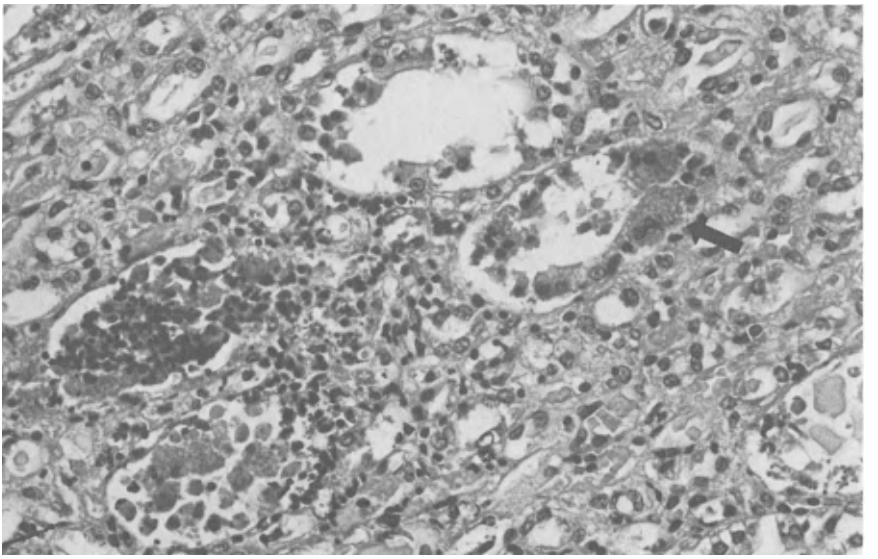

FIG. 18. Rabbit kidney with localized inflammation and tubular degeneration caused by *Encephalitozoon cuniculi*. Organisms appear in epithelial cells as finely granular material (arrow). Hematoxylin–eosin, ×500.

kidneys and brain, as well as by detecting antibodies in affected animals using an immunofluorescence test (Shadduck and Pakes, 1978).

D. NONSPECIFIC INFLAMMATORY, DEGENERATIVE, AND PROLIFERATIVE DISEASES

As in the other laboratory animal species, a variety of nonspecific inflammatory and degenerative lesions can occur spontaneously in the rabbit kidney. Often, the etiology of such lesions cannot be determined based on the histological examination alone. In the case of certain inflammatory lesions, special stains may at times be useful in arriving at a presumptive diagnosis.

Focal interstitial nephritis occurs spontaneously in many laboratory animals, including rabbits. The lesion is usually characterized by focal accumulation of mononuclear inflammatory cells, primarily lymphocytes, although plasma cells, macrophages, and occasional neutrophils may also be present. This lesion is often diagnosed simply as focal (mononuclear) cellular infiltration, and may be solitary or multifocal, and can be found anywhere in the organ. In the rabbit, it is considered that most such lesions

probably result from infection with *Encephalitozoon cuniculi* (see above), although often the organisms cannot be demonstrated, and a specific diagnosis cannot be made. It is likely that a variety of infectious agents, bacterial as well as parasitic, can produce such lesions, and immunological mechanisms also may play a role.

Suppurative nephritis, including pyelonephritis, is generally the result of a bacterial infection of the kidney. Several different bacterial agents which can cause such lesions in the rabbit have already been discussed. In many instances, however, bacterial cultures will not be available, and special stains will fail to demonstrate the presence of organisms in such lesions. In these cases, only a presumptive diagnosis of bacterial nephritis can be made on the basis of the histological features of the lesion.

Based on the lack of reports in the literature, spontaneous renal amyloidosis can be regarded as a rare lesion in rabbits, in contrast with other laboratory animal species, especially mice and hamsters. Amyloidosis has been experimentally induced in rabbits, mostly by the administration of casein (Cohen *et al.*, 1959).

Pregnancy toxemia is a disease of rabbits and guinea pigs that can affect the kidneys. This metabolic disease is not common, and may affect pregnant as well as postparturient and pseudopregnant does. The cause of the disease is still largely unknown, although many factors appear to influence its development, including breed, age, sex, amount of body fat, number of previous pregnancies, and undefined hormonal factors (Flatt *et al.*, 1974).

Clinically, affected rabbits may show depression, dyspnea (with acetone odor on the breath), oliguria, and death. Death may be rapid and sudden, or may be preceded by incoordination, convulsions, and coma. At necropsy, affected animals often are obese and have lactating mammary glands, large corpora lutea, and an enlarged pituitary. The kidneys, as well as the liver, heart, and other organs, are swollen, pale, and slightly yellow. Histologically, diffuse tubular fatty change is seen in the kidneys, and severe fatty change often with degeneration and necrosis characterizes the other affected organs, particularly the liver (Flatt *et al.*, 1974; McClure *et al.*, 1978).

Vacuolation not associated with stainable lipids is occasionally seen in renal tubules. The cause and significance of this change is unknown. A variety of other changes in renal tubules may be found during the routine histological examination of rabbit kidneys, including focal mineralization, pigmentation, basophilia, dilatation, and cast formation. Such changes resemble those seen in other laboratory animal species and are described elsewhere in this article. The frequency with which these lesions as well as other nonspecific lesions affecting the kidney have been diagnosed in 344

untreated young adult (≤1 year of age) New Zealand White rabbits in our laboratory is given in Table XIX.

E. Neoplastic Lesions

1. *Embryonal Nephroma (Nephroblastoma)*

According to Weisbroth (1974), this neoplasm is relatively common in rabbits and he cites 20 cases in the literature. The frequency of occurrence of this neoplasm in rabbits is second only to that of uterine carcinomas. The exact incidence of embryonal nephromas in rabbits is now known; in our laboratory, one case (6.5-month-old female) has been identified among a total of 344 controls.

Embryonal nephromas are usually incidental findings at necropsy, and have been found in both young and old animals. All reported cases have been benign, and the somewhat larger size of these tumors in older animals as compared with those in younger animals suggests that they are slowly growing neoplasms (Weisbroth, 1974). Grossly, the neoplasms appear as gray-white sharply demarcated nodules within, or projecting above, the renal parenchyma. Histologically, embryonic mesenchymal cells, immature tubules lined by columnar epithelial cells and pseudoglomeruli are seen.

TABLE XIX

Incidence of Commonly Occurring Degenerative and Inflammatory Changes in Untreated New Zealand White Rabbits ≤1 Year of Age

Type of change	Incidence (%)	
	Females (n = 220)	Males (n = 124)
Nephritis, focal/multifocal, chronic interstitial	20	20
Mineralization, focal	10	2
Tubular basophilia	5	4
Tubular dilatation	4	3
Tubular degeneration, focal	2	1
Tubular cast formation	2	1
Tubular vacuolation	1	2
Pyelonephritis/pyelitis	1	2
Cyst	1	—

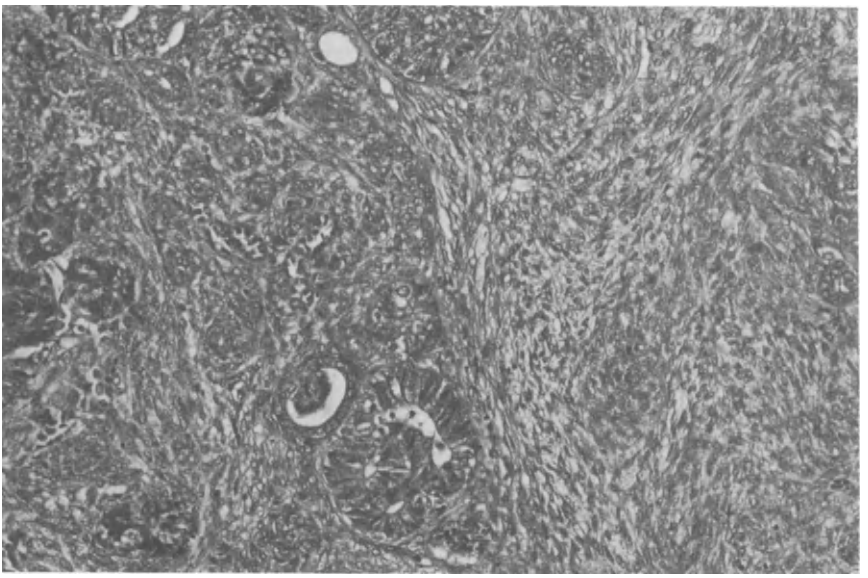

FIG. 19. Embryonal nephroma (nephroblastoma) in a rabbit. Hematoxylin–eosin, ×250.

The neoplasm is usually separated from the normal parenchyma by fibrous connective tissue (Fig. 19).

Embryonal nephromas appear not to interfere with renal function in the rabbit. Recently, an association between secondary polycythemia and embryonal nephroma in rabbits has been reported (Wardrop *et al.*, 1982; Lipman *et al.*, 1985). In one case, removal of the neoplasm resulted in a return to normal of the hematocrit (Wardrop *et al.*, 1982). Markedly elevated serum erythropoietin levels could not be demonstrated in that animal, although it could not be ruled out whether low levels of erythropoietin may have been produced by the neoplasm. The authors found evidence of slight vascular compression at the margin of the neoplasm, and they postulated that, alternatively, the diseased kidney may have produced a slightly increased amount of erythropoietin in response to decreased renal perfusion. Polycythemia as a result of erythropoietin-secreting Wilm's tumors has been described in human patients (Shalet *et al.*, 1967; Thurman *et al.*, 1966).

2. *Renal Carcinoma*

A single case of renal carcinoma has been reported (Kaufman and Quist, 1970) in a New Zealand White rabbit. The neoplasm appeared as a smooth-surfaced mass arising in the cortex, and was composed of solid

sheets and nests of cuboidal to spindle-shaped epithelial cells, with scattered irregular tubular structures. The histological appearance was quite distinct from that seen in embryonal nephromas.

VI. Guinea Pig

A. Normal Anatomy, Histology, and Organ Weights

The kidneys of the guinea pig are smooth, somewhat bean shaped, and pale tan. The dorsal surface is slightly flattened, and the ventral is strongly convex. The right kidney is more craniad than the left, with the caudal pole of the right kidney being at the same transverse level as the cranial extremity of the left. The renal capsule is a thin, transparent membrane. The renal pelvis is characterized by a single renal papilla with lateral calyces. Only limited renal weight data have been reported (Breazile and Brown, 1976; Casey *et al.*, 1978).

Histologically, the guinea pig kidney has no unique species-specific characteristics, and its appearance conforms to the general description given in Section I.

B. Congenital Diseases

Although a variety of spontaneous as well as induced congenital malformations has been reported in guinea pigs, the kidney is apparently rarely involved (Wilson *et al.*, 1978). Unilateral agenesis of the left kidney and ureter was reported in an 8-week-old random-bred Rockefeller albino Moen–Chase guinea pig (Scher and Weisbroth, 1974).

C. Infectious and Parasitic Diseases

1. *Bacterial Diseases*

Guinea pigs are susceptible to a variety of bacterial diseases, which at times can cause high mortality in a colony. Some of the more common bacteria causing spontaneous disease in guinea pigs include *Salmonella* sp., *Bordetella bronchiseptica,* and *Yersinia pseudotuberculosis,* although renal involvement in these diseases is uncommon. Streptococcosis, a common disease of guinea pigs, may involve the kidneys, and will be described briefly below.

Streptococcosis. This disease, caused by β-hemolytic, Lancefield Group C

organism *Streptococcus zooepidemicus,* occurs worldwide, and affects guinea pigs of all ages and both sexes. The organism may be spread by the oral or respiratory routes, and typically induces cervical lymphadenitis, or "lumps," although a septicemic form of the disease can also occur. Affected lymph nodes become firm and up to several centimeters in diameter, and they may rupture and heal spontaneously. In chronic cases, the animals become emaciated and, at necropsy, widespread abscessation and purulent inflammation may be evident in a variety of organs, including the kidneys (Ganaway, 1976).

2. *Viral Diseases*

Cytomegalovirus. The causative agent of cytomegalic inclusion disease is a DNA virus of the herpesvirus group termed *Cytomegalovirus.* The infection in guinea pigs is considered to be widespread, although the disease is typically subclinical and produces no gross lesions (Van Hoosier and Robinette, 1976). Microscopically, eosinophilic or amphophilic intranuclear inclusion bodies are seen in the ductal epithelium of the salivary glands, and epithelium of the proximal and distal convoluted tubules of the kidney. The inclusion bodies are about 10 μm in diameter and are surrounded by a clear halo with margination of the chromatin along the nuclear membrane. Inclusion body-containing renal tubular epithelial cells are not greatly enlarged as compared with affected ductal cells in the salivary glands, which may be 40 μm or more in diameter (Hunt *et al.,* 1978).

Inclusion body-containing cells, without associated tissue damage, are usually the only evidence of infection by this virus, although occasionally mononuclear cell infiltrates may be found in affected areas. Generalized fatal disease can be induced by immunosuppression of latently infected animals, or by enhancing the virulence of the virus by multiple serial passages (Hunt *et al.,* 1978).

3. *Parasitic Diseases*

a. Klossiellosis. This is a sporadic disease of guinea pigs caused by *Klossiella cobayae,* a coccidian parasite. Although the disease is widespread in its distribution (Vetterling, 1976), there are few data regarding its prevalence in guinea pig colonies.

Klossiella infection usually does not result in clinical disease, and the diagnosis is made during routine light microscopic examination of the kidneys. Taylor *et al.* (1979) cite a report which indicated that a massive infection by this parasite can result in renal dysfunction and death.

Grossly affected kidneys may show no lesions or a small number of pinpoint, slightly depressed white foci scattered over the cortical surface.

Light microscopically, focal or multifocal chronic nephritis may be evident, characterized by focal mononuclear cell infiltration and mild fibrosis. In such foci, the organisms are readily seen within distended tubular epithelial cells as eosinophilic spherical structures surrounded by a clear space (Fig. 20). The schizonts are usually found in epithelial cells of the proximal convoluted tubule, which ultimately rupture, releasing gametocytes, which in turn invade the epithelial cells of Henle's loop. The life cycle has been reviewed in detail by Taylor *et al.* (1979).

b. Encephalitozoonosis. Infections caused by *Encephalitozoon cuniculi* have also been reported in guinea pigs, although in this species they are usually asymptomatic (Vetterling, 1976) and occur less frequently than in the rabbit. The lesions in kidneys of affected guinea pigs are similar to those described previously for rabbits (see Section V,C,2), and excellent reviews dealing with this organism have been published (Pakes, 1974; Shadduck and Pakes, 1978).

c. Toxoplasmosis. Sporadic spontaneous infections with *Toxoplasma gondii* have been reported in guinea pigs (Vetterling, 1976). Nearly all infections are asymptomatic, and the diagnosis is made during routine examination of histological sections. *Toxoplasma* cysts, as a result of chronic infections, are usually found in the brain or myocardium, although they may occur in other organs, including the kidney.

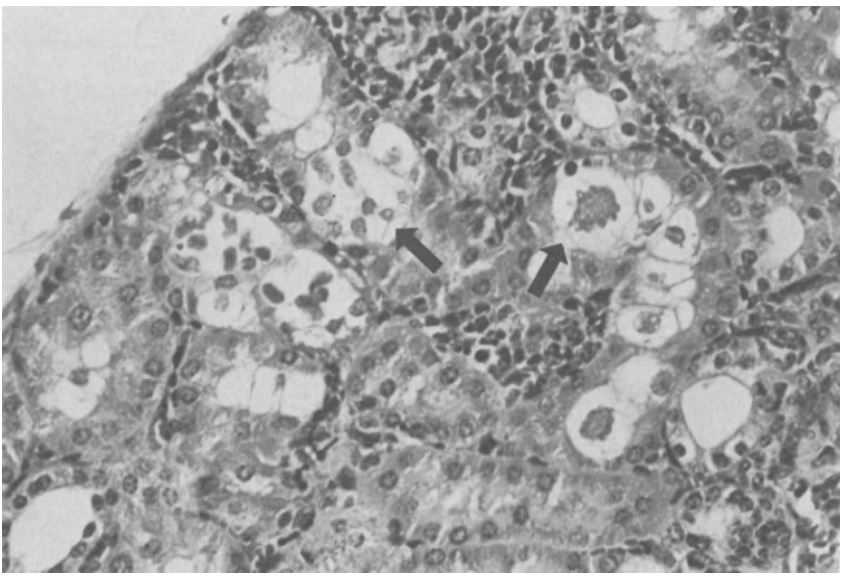

FIG. 20. Kidney of a guinea pig infected with *Klossiella cobayae* (arrows). Hematoxylin–eosin, ×500.

D. Nonspecific Inflammatory, Degenerative, and Proliferative Diseases

A number of species of laboratory animals have been investigated extensively to gain a broader understanding of the immunologically mediated diseases of the kidney. The guinea pig has played a prominent role in these investigations; especially in those dealing with tubulointerstitial nephritis (Brentjens *et al.*, 1982; McCluskey, 1983). In view of the widespread use of the guinea pig in this area of research, it is surprising that relatively few descriptions of spontaneous renal disease in this species have been published.

Spontaneous renal lesions which resembled human nephrosclerosis have been reported in inbred Abyssinian and Hartley strain guinea pigs (Takeda and Grollman, 1970). The lesions were first recognized grossly at 2 months of age, and were characterized by slight pitting of the surface. These changes progressed, until at 15–17 months of age the surface of the kidney was pale, granular, and had irregular areas of infarction and cortical narrowing.

Histologically, the earliest lesions appeared as thickening and narrowing of renal arterioles as a result of smooth muscle and endothelial cell proliferation. The more advanced changes were characterized by arteriolar hyalinization. Evidence of interstitial or pelvic inflammation was lacking, but interstitial fibrosis with tubular atrophy and focal glomerulosclerosis accompanied the vascular changes.

The authors were unable to demonstrate the presence of γ-globulin in these kidneys by immunofluorescence methods. They did show, however, that a gradual increase in blood pressure occurred with age, suggesting that the renal lesions were causally related to mild hypertension in this species.

Another study describing spontaneously occurring renal lesions in Albany and Hartley strain, strain 2 and 13, and English short-hair guinea pigs (Steblay and Rudofsky, 1971), presents some interesting contrasts to the findings reviewed above.

Grossly, pitting and increased granularity of the cortical surface was evident, which progressed in severity from the age of 6 months to the oldest (24 months) animals examined. Light microscopically, these authors found a variety of renal lesions, including obsolescent, atrophic, and cystic glomeruli, focal degenerative changes in tubules, vacuolization of tubular epithelium, and foci of mononuclear cellular infiltration, tubular atrophy, and interstitial fibrosis. These changes were found to some degree in most animals, increasing in severity with age.

By immunofluorescence, deposits of IgG and C3 were seen along most of the mesangial basement membranes and the basement membrane of many

of the peripheral capillary loops. These deposits were discrete and granular in young animals, and became heavy and confluent with age.

No correlation could be found between proteinuria or BUN level with age, immune complex deposition, or degree of glomerular cellularity. The pathogenesis and the nature of the immune complexes were not further defined. Also, the relationship of these deposits to the observed renal lesions, and the absence of renal impairment, remained unclear. Others have commented on a causal relationship between chronic renal disease (chronic interstitial nephritis) and a wasting syndrome followed by death in aged pet guinea pigs (Wagner, 1976).

Renal lesions have been described in guinea pigs with spontaneous diabetes mellitus presumably of viral origin (Lang *et al.*, 1977). The lesions were most pronounced in animals which had been diabetic for longer than 6 months, and consisted of diffuse thickening of the glomerular basal lamina and focal or nodular mesangial expansion. Occasionally, focal deposits of PAS-positive material in Bowman's capsule were seen, sometimes accompanied by prominent fibrosis and scarring. In further investigations with this animal model (Langner *et al.*, 1981), deposits of IgG were seen in glomeruli of diabetic guinea pigs. The authors speculated that the age-related glomerulopathy seen in nondiabetic animals can be enhanced by hyperglycemic conditions, such as diabetes mellitus.

As was described in the section dealing with rabbits, pregnancy ketosis—or toxemia of pregnancy—also occurs in guinea pigs and may affect the kidneys. It is a disease of adult female guinea pigs which occurs sporadically late in pregnancy, and probably has a multifactorial, although unknown, etiology. Obesity and stress, especially fasting, in late pregnancy are capable of inducing the syndrome experimentally.

The lesions of pregnancy ketosis primarily are severe fatty change in the liver; hemorrhage, necrosis, and depletion of lipids in the adrenal zona fasciculata; and fatty change of variable severity in kidneys as well as other organs (Navia and Hunt, 1976).

Amyloidosis in a variety of organs has been induced in guinea pigs by several methods, including casein administration. Amyloidosis also occurs spontaneously in this species, often affecting the kidney as well as liver, spleen, and adrenals. Such generalized amyloidosis has been found in animals with chronic pododermatitis and osteoarthritis caused by *Staphylococcus aureus* (Wagner, 1976).

Spontaneously occurring nephrocalcinosis has been described in a colony of guinea pigs with metastatic calcification (Sparschu and Christie, 1968). Marked nephrocalcinosis, accompanied by increased serum creatinine and inorganic phosphorus levels, was present in animals with severe metastatic calcification. The exact etiology of the condition was not determined,

although an imbalance of dietary phosphorus and magnesium was implicated.

E. Neoplastic Diseases

Renal neoplasms in guinea pigs are extremely uncommon and no reports of epithelial renal neoplasms were found. The only primary renal tumors described include an osteosarcoma and a round cell sarcoma (Blumenthal and Rogers, 1965).

VII. Dog

A. Normal Anatomy, Histology, and Organ Weights

The kidneys of dogs are located in a similar position to those of other quadripeds, and their anatomical relationships differ from other species because of interspecies variations in position and anatomy of other abdominal viscera. The single papilla or renal crest is elongated craniocaudally. The pelvis is of the highly folded type (Pfeiffer, 1968), and the architecture of the pelvis is compatible with that of an animal with high capacity for urinary concentration (Lacy, 1980). Beeuwkes and Bonventre (1975) have described the tubular organization and vasculotubular relations. Although the

TABLE XX

Weights of Kidneys from Untreated Male Beagle Dogs

		Absolute weight (g)		Relative weight (% body weight)	
Weight range (kg)	Number of animals	Median value	Range of actual values	Median value	Range of actual values
3.1– 5.0	6	30.65	27.60–33.80	0.67	0.61–0.78
5.1– 7.0	12	33.97	30.18–46.28	0.61	0.47–0.80
7.1– 8.0	12	39.50	34.04–51.28	0.50	0.43–0.67
8.1– 9.0	60	43.48	34.00–59.23	0.51	0.38–0.71
9.1–10.0	99	48.00	32.90–74.00	0.51	0.36–0.79
10.1–11.0	152	51.02	38.99–66.93	0.48	0.37–0.63
11.1–12.0	169	54.86	40.40–79.31	0.47	0.34–0.67
12.1–13.0	100	58.13	42.97–86.09	0.47	0.34–0.70
13.1–14.0	61	57.49	39.44–80.00	0.43	0.28–0.58
14.1–19.0	37	66.00	51.55–76.71	0.44	0.32–0.53

nephrons are similar to those of other species in our laboratory, a variation in the pars recta of proximal tubules occurs. In some dogs the epithelial cells and the tubules appear hypertrophic. Vacuolation and lipid accumulations in the medullary rays is a normal feature and is more common in females (72%) than in males (40%). After birth, superficial nephrons continue to be formed. The median absolute and relative (to body weight) kidney weights in mature beagle dogs are shown in Tables XX and XXI.

Two textbooks, one on canine nephrology (Bovee, 1984) and another on veterinary nephrology (Hall, 1983), review canine renal diseases. With the exception of a few 7-year toxicity studies performed with beagle dogs, the most commonly used laboratory-raised and maintained dogs are less than 2 years of age at necropsy. As a result, age-related changes are not important in laboratory-maintained beagle dogs. Also, compared with pound dogs, the health status, vaccination history, parasite burden, and age of laboratory-reared dogs are controlled.

B. Congenital Diseases

Although congenital diseases are rare in beagle dogs (Worden, 1972), renal agenesis (Robbins, 1965), renal aplasia (Vymetal, 1965), and polycystic kidneys have been described (Fox, 1964). Young dogs of several breeds show dysplastic renal lesions of three types (Lucke *et al.*, 1980): predominantly cystic and connective tissue changes; atypical connective tissue

TABLE XXI

Weights of Kidneys from Untreated Female Beagle Dogs

Weight range (kg)	Number of animals	Absolute weight (g)		Relative weight (% body weight)	
		Median value	Range of actual values	Median value	Range of actual values
3.1–5.0	11	29.97	28.04–34.63	0.66	0.60–0.74
5.1–7.0	64	34.74	24.97–40.90	0.53	0.39–0.66
7.1–8.0	102	36.00	26.00–54.84	0.47	0.35–0.76
8.1–9.0	166	38.96	29.77–55.12	0.46	0.36–0.63
9.1–10.0	154	40.88	28.93–56.20	0.43	0.32–0.58
10.1–11.0	100	44.20	33.00–58.33	0.42	0.31–0.56
11.1–12.0	47	43.84	34.07–54.00	0.39	0.29–0.48
12.1–13.0	35	46.19	36.83–66.49	0.38	0.30–0.54
13.1–14.0	11	47.23	39.14–53.00	0.36	0.29–0.39
14.1–19.0	2	48.33	47.83–48.83	0.32	0.32–0.33

changes with segmental bands of fibrous tissue containing primitive glomerular and tubular structures; and glomerular and connective tissue changes characterized by degrees of glomerulosclerosis and widespread calcification of glomeruli, tubules, and blood vessels.

The more important hereditary and congenital renal diseases, including cystinuria and Fanconi syndrome, which are similar to conditions in humans, are shown in Table XXII. They are included here because of their potential usefulness as experimental models for familial diseases in humans.

C. Infectious and Parasitic Diseases

Beagle dogs, bred and reared for laboratory use, are maintained under tightly controlled conditions. They are vaccinated against canine distemper, infectious canine hepatitis, and leptospirosis, and are treated for parasites. Generally, before commencing studies, the dogs are allowed to acclimatize to their environment and physical examinations, serum biochemistry, hematology, and urinalyses tests are performed. Unhealthy animals are

TABLE XXII

Hereditary and Congenital Renal Diseases in Dogs

Disease	Breed	References
Cystinuria	Irish terrier	Brand and Cahill (1936); Brand *et al.* (1940)
Fanconi syndrome	Norwegian elkhound Shetland sheepdog Schnauzer	Bovee *et al.* (1979)
Renal glycosuria	Basenji Scottish terrier Norwegian elkhound Mongrels	Easley and Breitschwerdt (1976); Bovee (1984)
Familial renal disease with dysplasia	Norwegian elkhound Cocker spaniel Samoyed	Finco *et al.* (1977) Johnson *et al.* (1972) Bernard and Valli (1977)
Hypoplasia and renal failure	Keeshund Bedlington terrier Lhasa apso Shi tzu	Klopper *et al.* (1975) Oskanen and Sittnikow (1972) Osborne *et al.* (1972) Bovee (1984)
Familial glomerulonephritis	Doberman pinscher	Wilcock and Patterson (1979)
Multifocal renal cystadenocarcinoma and dermatofibrosis	German shepherd	Lium and Moe (1985)

excluded so serious diseases are effectively excluded from the laboratory. A brief review of the more important aspects of some diseases is included here for completeness.

1. *Bacterial Diseases*

Before routine vaccination, acute interstitial nephritis due to *Leptospira canicola* or *L. icterohaemorrhagiae* was common (MacIntyre and Montgomery, 1952), and the pathological features and stages well defined (Bloom, 1941; MacIntyre and Montgomery, 1952; Taylor *et al.*, 1970). Although subclinical and mild forms of the disease are common, hyperacute interstitial nephritis with death may occur. Under these circumstances, grossly the kidneys are swollen, turgid, and pale with petechial hemorrhages in the cortex. Histologically, tubular epithelial cells may show degeneration, necrosis, and regeneration. The most prominent feature is a diffuse cortical peritubular infiltration with mainly lymphocytes, plasma cells, macrophages, and some polymorphonuclear leukocytes. At this time, organisms may be demonstrated in some tubules with Levaditi's silver stain. Subacute forms of the disease may show the most striking changes at the corticomedullary junction with extensions into the cortex. Histologically, convoluted tubules undergoing degenerative changes are surrounded or replaced by dense masses of cells, including lymphocytes, plasma cells, macrophages and occasional neutrophils (Jones and Hunt, 1983). Because of these acute and subacute forms of interstitial nephritis, for many years it was considered that the commonly diagnosed chronic interstitial nephritis was the sequela. This is now known not to be the case (Robertson, 1984).

2. *Viral Diseases*

Canine herpesvirus and canine adenovirus-1 infection cause nephritis in dogs. Canine herpesvirus produces focal necrosis and hemorrhage as part of fatal systemic disease of the newborn (Cheville, 1983). Of more interest is the natural and experimental infection of dogs with canine adenovirus-1. A proliferative glomerulonephritis develops, accompanied by hematuria and proteinuria (Wright *et al.*, 1974; Morrison *et al.*, 1975; Morrison and Wright, 1976). The glomerular lesions are characterized by mesangial expansion, hypercellularity, and variable infiltrations of polymorphonuclear leukocytes into the capillary loops. The localization of immune complexes containing viral antigen and IgG and C3 in the mesangium has been demonstrated.

This acute response is similar to serum sickness GN, and normally resolves when high titers of antiviral antibody are present in the serum. Later, following infection of renal tubular epithelium, focal acute interstitial

nephritis may develop (Wright *et al.*, 1971). This change is characterized by necrosis and sloughing of the viral-infected tubular cells and interstitial cellular infiltrates composed of polymorphonuclear leukocytes, macrophages, plasma cells, lymphocytes, and occasional syncytial giant cells. Viral particles are present in large numbers in infected tubules. These focal lesions heal by fibrosis with minimal scarring.

3. *Parasitic Diseases*

The parasite burden of laboratory-maintained dogs is low. However, parasitic granulomas (Fig. 21) due to visceral larvae migrans of *Toxocara canis* are occasionally seen in the kidneys. On one occasion in our laboratory the giant kidney worm *Dioctephyme renale* was found in a beagle dog. Microfilaria of *Dirofilaria immitis* are reported to cause renal lesions including GN (Osborne *et al.*, 1972; Klei *et al.*, 1974b; Casey and Splitter, 1975), but we have not found these in dogs at our laboratory.

D. Nonspecific Inflammatory, Degenerative, and Proliferative Diseases

The most important canine glomerular diseases are glomerulonephritis and amyloidosis. However, they are virtually absent in laboratory-maintained beagle dogs. Glomerular lipoidosis, which is due to eosinophilic foamy lipoid material mainly in mesangial cells (Zayed *et al.*, 1976), is occasionally seen in a few glomeruli in some of our dogs. Glomerulosclerosis is also seen focally at a low frequency.

Although GN is not seen commonly in laboratory dogs, when it does occur, there are many similarities with human GN. Although, according to Robertson (1986), "an appreciation of the scope of canine glomerular disease is decades behind that which is known in man," the situation is changing. In the last 15 years, a number of case reports and series have emphasized the importance of canine GN (Halliwell and Blakemore, 1972; Kurtz *et al.*, 1972; Murray and Wright, 1974; Rouse and Lewis, 1975; Muller-Peddinghaus and Trautwein, 1977a,b). The WHO classification scheme for human GN (Churg and Sobin, 1982) has been used by some workers. However, Winter and Majid (1984) have proposed a morphological classification of GN for domestic animals: membranous, mesangial proliferative, mesangial sclerotic, membranoproliferative and membranosclerotic, intra- and extracapillary proliferative, exudative, and chronic. A prospective survey has shown that GN is a common cause of canine chronic renal disease (MacDougall *et al.*, 1986). In this survey, all classes of GN found in humans were seen in dogs, with the exception of mesangial IgA

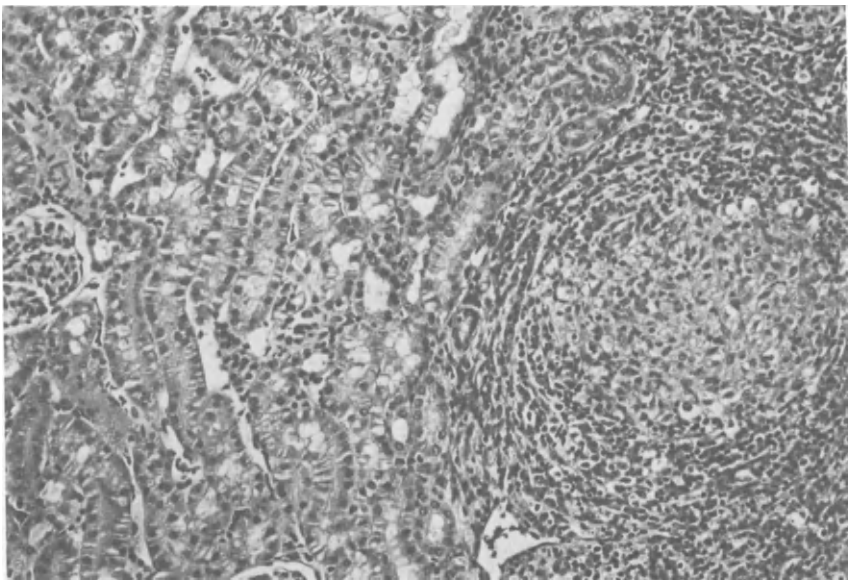

FIG. 21. Focal granuloma in the kidney of a dog. Such lesions are usually the result of larval parasite migration. Hematoxylin–eosin, ×250.

disease and membranous GN. Other workers have described membranous GN in dogs (Murray and Wright, 1974; Wright *et al.*, 1981).

Electron microscopy, immunofluorescence, and elution studies have shown that GN is of the immune complex type; anti-basement membrane disease has not been recorded in the dog (Lewis and Center, 1984; Robertson, 1984, 1986). The cause is usually not identified, but GN has been recorded after acute adenoviral infection (Wright *et al.*, 1973, 1974, 1976), dirofilariasis (Klei *et al.*, 1974b; Casey and Splitter, 1975), *Escherichia coli* (Asheim, 1964), malignant neoplasms, chronic pancreatitis (Murray and Wright, 1974), and systemic lupus erythematosus (Osborne *et al.*, 1973).

Spontaneous tubular lesions are seen with varying frequency in dogs. The most frequent diagnoses made in our laboratory are shown in Table XXIII. The histopathological diagnosis of these changes is self-explanatory and some, such as mineralization (Fig. 22), have previously been recorded as a common occurrence in control beagle dogs (Hottendorf and Hirth, 1974). Of course, other lesions are observed with much less frequency, such as focal or segmental degenerative glomerular (Fig. 23) or tubular changes.

Damaged renal proximal tubular epithelial cells can regenerate (Slauson and Cooper, 1972). The regenerating tubules show increased mitotic

TABLE XXIII

INCIDENCE OF COMMONLY OCCURRING DEGENERATIVE AND INFLAMMATORY CHANGES IN UNTREATED BEAGLE DOGS ≤3 YEARS OF AGE

Type of change	Incidence (%)	
	Females (n = 470)	Males (n = 473)
Tubular basophilia	1	1
Casts	2	3
Cellular infiltration	1	3
Cyst	2	1
Mineralization	7	8
Nephritis	<1	1

activity and the cells are initially cuboidal with basophilic cytoplasm and lack the brush border of normal proximal tubules. The problem occurs in a study when at high dosage levels clear-cut tubular necrosis and regeneration is seen and the changes occurring at low dosage levels need to be assessed to establish a no-effect level, since tubular basophilia, which is usually interpreted as a regenerative phenomenon, also occurs at a low frequency in control dogs.

Renal lesions involving both tubules and interstitial tissue are common incidental findings in the kidneys of laboratory-maintained beagle dogs. Typically the mildest changes are focal and confined to the cortex or corticomedullary region and consist either of focal cellular infiltration or small groups of abnormal tubules with basophilic cytoplasm, thickened basement membrane, and interstitial mononuclear cell infiltration or fibrosis. The cause is unknown.

Pyelitis and pyelonephritis occur at a low incidence in young dogs maintained in laboratories. The term pyelonephritis is used to describe inflammatory disease of the renal pelvis and parenchyma, and is usually of ascending origin (Kelly, 1984). The most common bacteria are *Escherichia coli* and *Proteus* sp.; *Staphylococci* and *Streptococci* are less commonly encountered (Osborne *et al.*, 1972). Classically and experimentally, the disease is usually associated with persistent lower urinary tract infections, such as cystitis and prostatitis, and obstruction is also of importance for establishing disease. If cystitis and prostatitis previously described in beagle dogs by Hottendorf and Hirth (1974) are the source of infection, then vesicoureteral reflux may account for infection of the kidneys. Normal young dogs show transient primary vesicoureteral reflux, the incidence of which

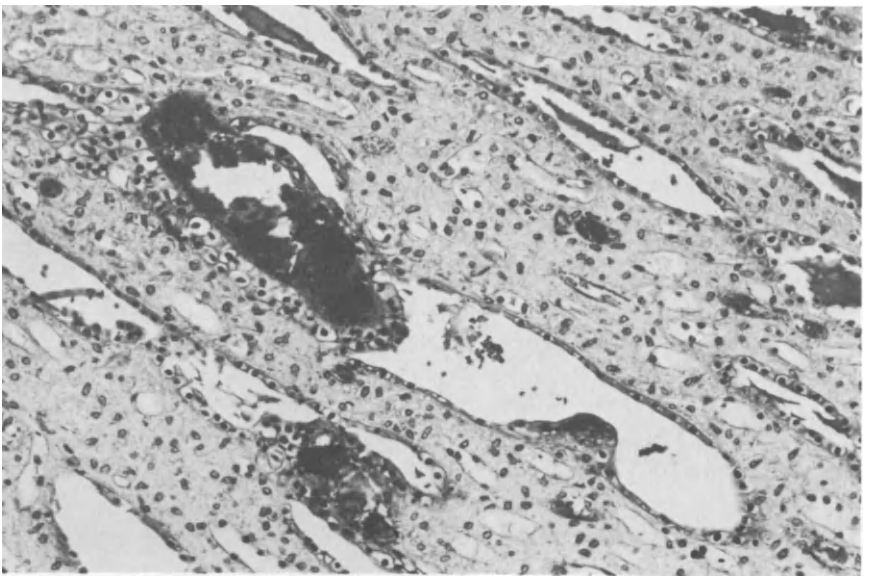

FIG. 22. Multifocal mineral deposits in the renal medulla of a dog. Hematoxylin–eosin, ×250.

declines with age from 79% to 10% between 3 months and 10 years of age (Christie, 1971a,b, 1973). It seems that this is a transient phenomenon related to growth and maturation of the vesicoureteral junction. Thus, the combination of infectious cystitis and prostatitis with reflux of organism from the bladder to the renal pelvis are believed to cause pyelitis and pyelonephritis. Typically, pyelitis is seen as a dense subepithelial lymphocytic infiltration. Although the overlying epithelium is intact, it may be thinner.

Kelly (1984) classified the types of pyelonephritis occurring in dogs as acute, active progressive, chronic, and focal renal scarring. In our laboratory, we see acute pyelonephritis, often focal in distribution, and accompanied by a papillitis. Advanced lesions have not been encountered. Occasionally nonspecific focal renal scarring is also seen, and Kelly (1984) has emphasized that radial interstitial fibrosis, together with tubular atrophy with lymphocytic and plasma cellular infiltration that constitutes this lesion is more likely to be due to focal pyelonephritis and hardly ever due to renal infarcts.

Spontaneous end-stage renal disease is very rare in laboratory-maintained beagles, but a brief review of this topic is important. Often, dogs with scarred, shrunken, pitted end-stage kidneys and chronic renal failure have been diagnosed as having chronic interstitial nephritis. In fact, many of

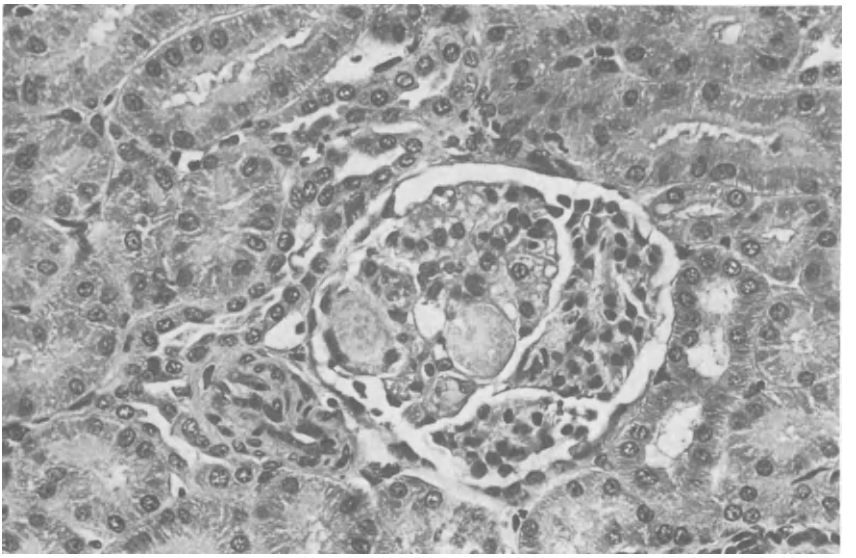

FIG. 23. Segmental glomerular degeneration in a dog. Hematoxylin–eosin, ×500.

these probably had either chronic GN or chronic pyelonephritis (Robertson, 1984). Although clinically and biochemically, chronic GN and chronic interstitial nephritis are indistinguishable, the two diseases may be distinguished on the pattern and degree of fibroplasia, fibrin deposition in the glomeruli, and immunofluorescence findings (Wright *et al.*, 1976). In cases of chronic interstitial nephritis, the fibrosis tends to be more severe, concentrated at the corticomedullary junction; glomerular fibrin deposits are much less important than in GN; and glomerular IgG and C3 are absent. Chronic pyelonephritis is recognized because of parenchymal and pelvic inflammation, with wedge-shaped scars extending from the renal pelvis to the capsular cortex, and tends to be more severe in one kidney than in the other (Robertson, 1984). In general, fibrosis is mildest and more uniform in chronic GN, the two kidneys are affected equally, and the cortex is of a normal thickness.

E. NEOPLASTIC DISEASES

Renal adenoma, adenocarcinoma, tumors of the renal pelvis, nephroblastoma, mesenchymal tumors, and metastatic renal neoplasms are found in dogs (Moulton, 1978; Goldschmidt, 1984). With the exception of one renal adenocarcinoma described in a young laboratory-maintained beagle dog by

Hottendorf and Hirth (1974), these tumors are extremely rare in laboratory beagles because the dogs are young.

VIII. Monkey

A. Normal Anatomy, Histology, and Organ Weights

The kidneys are intraabdominally located in contact with liver and adrenals. Usually, the left kidney is opposite of the right, except in *Papio,* where it is more caudal (Swindler and Wood, 1982). The hilus is the concave medial region of each kidney where vessels, nerves, and ureters enter or leave. A longitudinal section of the nonhuman primate kidney shows a single papilla, as opposed to the human multipapillary kidney. Anatomically and physiologically, the kidneys of nonhuman primates are similar to those of other laboratory animals. The microscopic morphology of nonhuman primate kidneys has been described by Bulger (1985) in relation with segmental function. Body weights and absolute and relative kidney weights for rhesus monkeys used in our laboratory at MSDRL are summarized in Tables XXIV and XXV.

B. Congenital Diseases

Naturally occurring renal malformations in nonhuman primates were sufficiently rare to warrant case-by-case descriptions (Hill, 1964; Kim and

TABLE XXIV

Weights of Kidneys from Untreated Male Rhesus Monkeys

Weight range (kg)	Number of animals	Absolute weight (g)		Relative weight (% body weight)	
		Median value	Range of actual values	Median value	Range of actual values
1.5–1.9	2	8.88	7.97–9.78	0.48	0.44–0.51
2.0–2.4	22	10.54	9.26–13.61	0.46	0.39–0.58
2.5–2.9	42	11.88	9.00–15.68	0.44	0.33–0.59
3.0–3.4	70	13.00	9.74–17.42	0.40	0.32–0.56
3.5–3.9	73	15.00	11.00–20.05	0.40	0.30–0.56
4.0–4.4	51	15.90	12.79–21.58	0.38	0.31–0.53
4.5–4.9	34	16.90	12.00–21.29	0.36	0.26–0.45
5.0–5.4	10	17.77	14.00–25.52	0.35	0.26–0.47
5.5–6.0	5	18.20	15.00–19.21	0.33	0.26–0.34

TABLE XXV

WEIGHTS OF KIDNEYS FROM UNTREATED FEMALE RHESUS MONKEYS

Weight range (kg)	Number of animals	Absolute weight (g)		Relative weight (% body weight)	
		Median value	Range of actual values	Median value	Range of actual values
1.5–1.9	6	9.34	8.70–11.41	0.50	0.48–0.60
2.0–2.4	21	10.75	8.11–13.57	0.46	0.39–0.59
2.5–2.9	45	12.00	9.00–17.55	0.44	0.35–0.60
3.0–3.4	65	13.48	10.42–17.59	0.42	0.32–0.52
3.5–3.9	72	14.16	10.00–19.13	0.38	0.27–0.52
4.0–4.4	58	15.93	12.00–20.25	0.38	0.28–0.49
4.5–4.9	19	17.50	12.00–21.84	0.38	0.25–0.48
5.0–5.4	3	20.00	18.30–21.01	0.37	0.34–0.41
6.1–7.0	1	21.97	21.97–21.97	0.36	0.36–0.36

Kalter, 1972; Jerome *et al.*, 1985). These case reports were either necropsy findings in primates from zoos, those used in medical research, or observations of stillbirths in breeding colonies. Now, with the increased use of nonhuman primates in teratological evaluation of new drugs, more animals are being examined and a more accurate record of congenital renal anomalies can be expected in the near future.

Renal anomalies are recognized either grossly at necropsy or only upon microscopic examination. In the first category fall unilateral renal aplasia and accessory kidney reported in several species (Hill, 1964; Kim and Kalter, 1972; Stills and Bullock, 1981). The second group includes mainly ectopic adrenal and tubular cysts. In our experience, ectopic adrenal was more commonly found in rhesus monkeys (6 cases among 108) than in the cynomolgus (none of 104). Various degrees of tubular cysts are encountered in nonhuman primate kidneys. A detailed examination of these cysts made with scanning and transmission electron microscopy by Miyoshi *et al.* (1984), derived from dilaceration of both layers of the Bowman's membrane of the glomeruli.

C. INFECTIOUS AND PARASITIC DISEASES

1. *Bacterial Diseases*

Human strains of tuberculosis produce a systemic disease in Old World primates, with the lung or intestinal tract the primary site of infection, but spread to numerous organs is common. Grossly and microscopically, renal

tuberculosis is not different from that in other animals. The lesions are multifocal granulomas often with giant cell formation. Although common in the past, due to systematic detection by skin tests of monkeys newly arrived and maintained in quarantine, this disease has become extremely rare.

Other mycobacterial infections, granulomatous lesions which contained acid-fast microorganisms have been seen in monkey kidneys (Fleischman *et al.*, 1982). Cultures revealed *Mycobacterium intracellulare* (serotype 10), but again as previously stated for tuberculosis, the kidney was not primarily involved and identical lesions were found in several organs.

2. *Viral Diseases*

Simian hemorrhagic fever is a systemic viral disease which occurs naturally in macaques. Other species have been either naturally exposed or experimentally infected but do not contract the disease or present overt clinical signs (Simpson, 1972). Severe hemorrhagic diathesis results in severe hemorrhages in most organs and occasionally under the renal capsule. It is not clear, however, if renal hemorrhage is a primary event or secondary to animal handling (Simpson, 1972). Upon microscopic examination, hemorrhages can be seen together with venous stasis, vascular dilatation, and thrombosis (Simpson, 1972). Degenerative tubular changes secondary to hypoxia resulting from blood stasis can also be encountered (Simpson, 1972).

Cytomegalic inclusion disease due to host-specific herpesvirus is known to affect several varieties of nonhuman primates (Hunt *et al.*, 1978). Histologically, renal lesions consist of megalocytes. These are epithelial cells with typical cellular changes characterized by hypertrophied epithelial cells containing large, usually eosinophilic inclusion bodies (Hunt *et al.*, 1978). These megalocytes are seen in the renal tubular epithelium, but also in salivary glands. There is neither inflammation nor necrosis accompanying this cellular enlargement.

Measles is a highly infectious viral disease of children and also for several species of nonhuman primates, especially rhesus monkeys, when brought into captivity (Meyer *et al.*, 1962). The disease produces the formation of multinucleated syncytial giant cells, often with eosinophilic intracytoplasmic viral inclusion bodies, in numerous organs including the lung, skin, gastrointestinal tract, uterus, urinary bladder, and kidneys.

During outbreaks of simian acquired immunodeficiency syndrome (SAIDS) in the past 20 years at the California Primate Research Center, immunological and pathological examinations were conducted on 27 rhesus monkeys. Besides direct and indirect evidence of immunodeficiency, there were, respectively, 12 (48%) and 5 (19%) cases on interstitial nephritis and

glomerulonephritis (Henrickson *et al.*, 1984). Although these nephropathies are not pathognomonic of the disease, these observations suggested an opportunistic increase of the incidence of these renal changes.

3. *Parasitic Diseases*

Parasitic nephritides are not common lesions encountered in nonhuman primates (Casey *et al.*, 1978), in the sense that there are no parasites specifically noted in kidneys. However, parasitic granulomas (at various stages from the acute suppurative to the terminal fibrotic process undergoing secondary mineralization) are occasionally found. *Oesophagostomum* sp. larvae from the colon may migrate into the kidney; filarial parasites (e.g., *Dipetalonema* sp.) can be seen in renal blood vessels, and the kidneys remained unaffected.

In squirrel monkeys, *Encephalitozoon cuniculi* (see Section V,C,2) induced renal inflammatory lesions are frequent. For example, pseudocysts have been reported in 17 of 22 animals examined by Zeman and Baskin (1985), with the kidneys being the second most commonly affected organ. The renal lesions consisted of areas of interstitial nephritis but in more advanced cases, tubular atrophy, interstitial fibrosis, and sclerotic glomeruli. The authors stressed that vascular (encompassing various types of vasculitis) and perivascular changes were not uncommon.

Spontaneous systemic infections due to a variety of fungi (principally nocardiosis, coccidioidomycosis, histoplasmosis) have been reported to occur uncommonly in nonhuman primates (Al-Doory, 1972), and the literature is composed of case reports and overviews summarizing these cases. Disseminated suppurative inflammation, abscesses, and pyogranulomas at various stages of evolution are lesions in which branching hyphae can be detected. They are usually seen in lungs and draining lymph nodes considered as primary affected organs. Renal lesions are occasionally seen and are probably dependent on the dissemination of the fungal disease (Al-Doory, 1972; Liebenberg and Giddens, 1985).

D. Nonspecific Inflammatory, Degenerative, and Proliferative Diseases

Particularly of note is that in approximately 75% of wild-caught cynomolgus (and to a lesser extent in rhesus) monkeys, multinucleate epithelial cells are present in the collecting tubules from the corticomedullary junction to the tip of the papilla (Seeman, 1986), sometimes leading to stratified layering of these ducts. In addition, upon routine examination of

the kidney in toxicological studies, minute perivascular lymphoid infiltrates, generally without tubular involvements, are commonly seen (Brack, 1981).

Polycystic disease is associated with multiple cysts present in several organs including the kidney. Renal changes are characterized grossly by enlarged and spongy organs and microscopically by dilated tubules in fibrotic tissue interspersed among normal renal parenchyma (Baskin *et al.*, 1981).

Suppurative glomerulonephritis, generally bilateral and due to bacterial embolism, is not a primary event in nonhuman primates. It represents a multifocal renal location of a more advanced suppurative lesion as in other animals and reported in monkeys (Giddens *et al.*, 1981). Generally embolic suppurative nephritis is not severe, is clinically inapparent, and may be found only at necropsy (Migaki *et al.*, 1979). It has also been reported in 4 of 60 catheterized baboons (Heidel *et al.*, 1981), together with renal infarcts from thromboemboli originating from the catheter. Grossly renal suppurative inflammation or abscesses can be seen. Microscopically the description of the suppurative embolic GN does not differ from that of other laboratory animals.

Immune-mediated GN in nonhuman primates has been infrequently reported (Casey *et al.*, 1978) but appeared extensively studied probably because of its mechanism and the search of an animal model for the human disease. Several monkey species are affected. Microscopically the lesion does differ from that reported in other laboratory animals and included proliferation of mesangial and epithelial cells, increase in mesangial matrix, thickening of basement membranes, hyalinization, and presence of immunofluorescent deposits together with eosinophilic tubular casts and interstitial edema. It occurs either spontaneously in some species, such as owl (Hunt *et al.*, 1976; King *et al.*, 1976) and pigtailed (Boyce *et al.*, 1981) monkeys. It is seen in primates used experimentally, such as baboons after parasitic infection (Houba *et al.*, 1977), after catheter implantation (Leary *et al.*, 1981), or spontaneously in galagos (Burkholder, 1981) and baboons (Brack, 1981) maintained in captivity. Occasionally, mesangial thickening is observed in the glomeruli of primates (Fig. 24), but the cause is seldom explored and the incidence is low.

Renal amyloidosis is a rare observation in nonhuman primates. It represents either a generalized primary disease (Casey *et al.*, 1972) or the location of a secondary event, especially after chronic indwelling venous catheters (Doepel *et al.*, 1984) or chronic diseases (Benditt and Eriksen, 1972) in rhesus monkeys. Grossly and microscopically, amyloid deposits in nonhuman primates have no special feature which distinguishes them from amyloidosis in other laboratory animals.

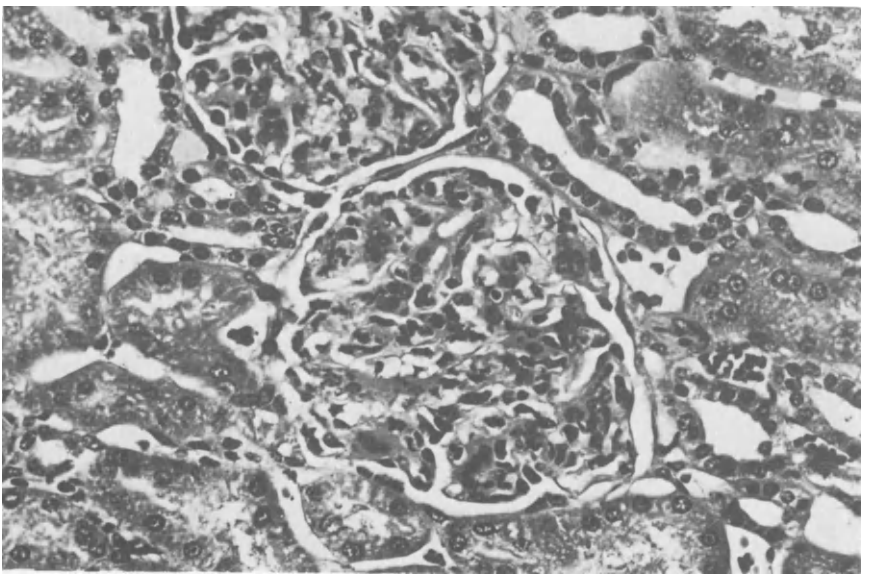

FIG. 24. Segmental mesangial thickening in the glomerulus of a rhesus monkey. Hematoxylin–eosin, ×500.

Cases of idiopathic muscle necrosis with myoglobinuria, similar to Meyer–Betz disease in humans, are rarely reported (Seibold *et al.*, 1971; Brack, 1981). Microscopically, Bowman's capsules were dilated and glomerular tufts compressed; distal convoluted and collecting tubules contained casts. Renal failure was the cause of death.

A retrospective study conducted by Giddens *et al.* (1981) reported tubular necrosis in approximately 20% of adult pigtailed monkeys, two-thirds being previously treated with known nephrotoxic antibiotics principally for diarrhea. Epithelial cell degenerative (loss of brush border, flattened cells, necrosis) and regenerative changes did not differ from those induced by these drugs in other species.

Mineralization in kidneys of nonhuman primates has seldom been described except for squirrel monkeys where 85% of necropsied monkeys "had some degree of mineralization in the kidney" (Stills and Bullock, 1981). The intensity ranged from minute foci to pelvic stones, In addition, approximately 1% of adult squirrel monkeys had renal urolithiasis. The reported cases of nephrolithiasis in other monkey species (Stephens *et al.*, 1979) generally included pelvic stones either alone or with urinary bladder and/or ureter blockage by calculi.

Most macaque monkeys have very slight to slight amounts of fat in the

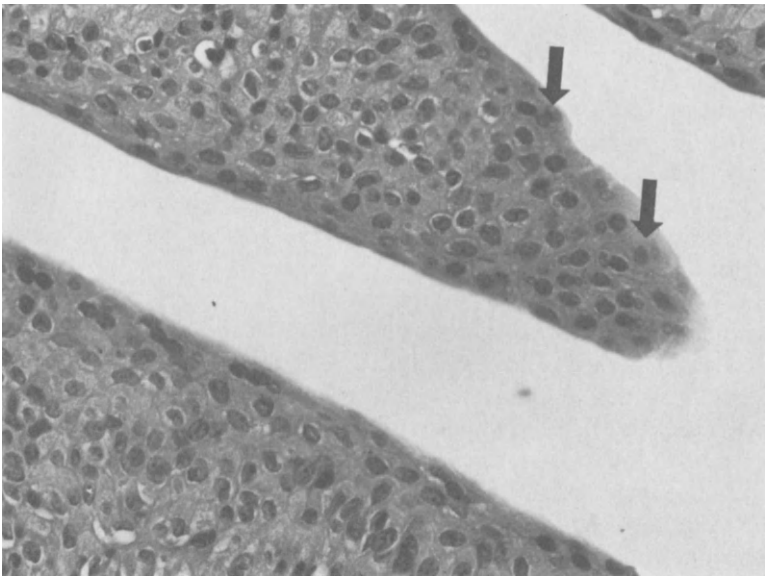

FIG. 25. Transitional epithelial cells in a rhesus monkey containing eosinophilic intracytoplasmic inclusion bodies (arrows). Hematoxylin–eosin, ×500.

renal tubules. This is characterized by very small intracytoplasmic droplets of neutral fat (oil red O-positive) in the straight segment of proximal convoluted tubules. In addition, examples of fat accumulation in nonhuman primate kidneys were reported by Bronson *et al.* (1982), either in obese macaques together with fatty livers and occasionally other changes, or at necropsy without known clinical signs (Giddens *et al.*, 1981). Grossly the kidney appeared enlarged, and microscopically various degrees of vacuolation were seen in the epithelial cells of the proximal convoluted tubules associated with scanty cellular necrosis. Neutral lipids were confirmed in a few cases only. The mechanism of the change is not known, although two possibilities—shock or antimicrobial agents—have been proposed.

A variety of pigments (seen in routine hematoxylin- and eosin-stained sections or demonstrated using special staining reactions) may be encountered in the cytoplasm of tubular epithelial cells. In nonhuman primates, as well as in other laboratory animal species, they usually do not represent a primary event but are indicative of a concurrent disease in other organs. Hemosiderin is seen concomitantly with hemolysis, and biliary pigments in liver disease; lipofuscin is the sole pigment which can be seen spontaneously without major disease.

Eosinophilic cytoplasmic inclusions occur in urinary bladder and renal

pelvic transitional epithelial cells of rhesus (Burek *et al.*, 1972; Lucas *et al.*, 1972) and stumptail (Bischoff *et al.*, 1968) monkeys (Fig. 25). They may be present in as many as 5–10% of the monkeys. They are incidental findings, but could be confused with some viral inclusion bodies.

Interstitial nephritis (Fig. 26), characterized by various degrees of primary inflammation of the renal interstitium (together with limited tubular involvements), seemed the most commonly encountered changes in nonhuman primate kidneys, although causes were not known (Casey *et al.*, 1978; Brack, 1981).

Focal interstitial nephritis consisted of small foci of predominantly mononuclear cell infiltration distorting the adjacent tubules. They usually resolve spontaneously and scarring is occasional.

Diffuse interstitial nephritis due to *Leptospira* sp. was reported in monkeys (Fear *et al.*, 1968; Shive *et al.*, 1969). Description of gross (swollen hyperemic kidneys) and microscopic changes (interstitial inflammation, tubular degeneration, and necrosis), did not differ from diffuse interstitial nephritis in dogs. As in the canine disease, tubular changes resolve and the interstitium undergoes fibroplasia followed by fibrosis.

As in other laboratory animals, chronic interstitial nephritis should be differentiated from chronic pyelonephritis.

Pyelonephritis has been encountered in clinically healthy nonhuman

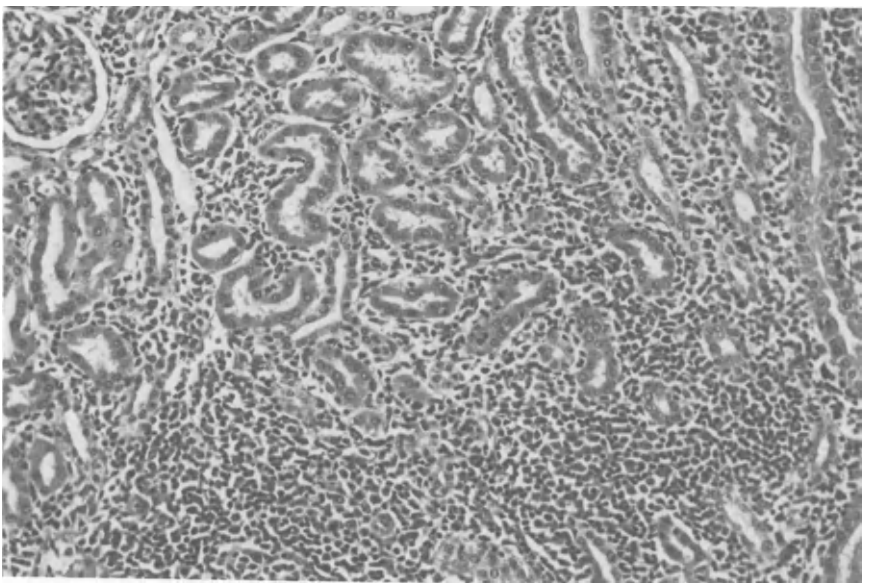

FIG. 26. Pyelonephritis in a monkey. Hematoxylin–eosin, ×250.

TABLE XXVI

INCIDENCE OF COMMONLY OCCURRING DEGENERATIVE AND INFLAMMATORY CHANGES IN UNTREATED RHESUS MONKEYS

Type of change	Incidence (%)	
	Females (n =227)	Males (n = 237)
Cellular infiltration	19	17
Nephritis, glomerulonephritis, papillitis, inflammation	17	17
Giant cell formation	1	1
Granuloma	2	1
Mineralization	<1	1
Pyelonephritis, urolithiasis	0	<1
Tuberculosis	1	0

primates. In most cases it is only seen upon microscopic examination of kidneys. Pyelonephritis is usually a low-grade renal and non-life-threatening disease; it is seldom acute (O'Neill *et al.*, 1978) and apparently never progresses to massive fibrosis as seen in rats. Grossly and microscopically, pyelonephritis in monkeys is identical to that previously described for other laboratory animals.

End-stage kidney disease has been reported in owl monkeys (Chapman *et al.*, 1973) and squirrel monkeys (Stills and Bullock, 1981). Kidneys were firm, mottled, with a granular and/or pitted capsule. Microscopically, hyalinized glomeruli and considerable fibrosis of cortex and medulla replacing atrophic tubules and interspersed with mononuclear cells are typical features of this change.

The wasting syndrome affecting specifically marmosets is believed to be related to protein deficiency. Animals become cachectic and die. At necropsy, besides hepatic and pancreatic changes, kidneys appear pale and granular (Brack and Rothe, 1981). Microscopically, peritubular lymphocytic infiltration and massive interstitial fibrosis are the predominant changes. Initially, unaltered glomeruli show dilatation of Bowman's spaces (secondary to tubular compression); later, while disease progresses, they tend to disappear in the generalized interstitial fibrosis and the entire kidneys become fibrotic.

The major reports of cause of death and associated lesions in owl monkeys (Chapman *et al.*, 1973; Hunt *et al.*, 1976; Chalifoux *et al.*, 1981) include hemolytic anemia together with GN. Grossly, the kidneys varied from normal (early lesion) to larger and granular (severe cases) due to pale foci in cortices; the medullas and pelvises remain unaffected. Cortical

streaks (possible infarcts) and a brownish color in case of anemia are occasionally seen. In addition, with lesions typical of anemia (centrilobular necrosis, extramedullary hematopoiesis, iron pigment, and lipofuscin in liver; presence of actively phagocytizing cells in lymph nodes; ecchymoses in brain and spinal cord), GN was concomitantly found with a good correlation. The changes included inflammation and fibrosis and progressed from minute inflammatory cell collections around Bowman's capsule to chronic interstitial inflammation and fibrosis involving glomeruli. The changes were classified into six types of lesions by Chalifoux *et al.* (1981) by analogy with humans, with the most commonly encountered glomerular lesions consisting of mesangial cell and matrix proliferations.

Table XXVI provides a summary of the more common renal lesions observed in control rhesus monkeys used in our laboratory.

E. Neoplastic Diseases

The overall known incidence of spontaneous neoplasms in nonhuman primates is low, and primary renal tumors are very rare. In safety studies, nonhuman primates are sacrificed at an early age (careful initial animal selection and studies of relatively short duration when compared to possible life-span), and this explains the paucity of data. The list of tumors (Squire *et al.*, 1978; Jones and Casey, 1981) is thus updated by newly reported cases (Bennett *et al.*, 1982; Chrisp *et al.*, 1985).

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INTERNATIONAL REVIEW OF EXPERIMENTAL PATHOLOGY, VOL. 30

The Use of Video Imaging Techniques to Study Ultrafiltration and Blood Flow in the Normal and Diseased Kidney

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I. Background

A. Evaluation of Renal Function Using Clearance Techniques and Micropuncture

The study of renal function has traditionally depended on clearance techniques that involve the measurement of the appearance of substances in the urine. Deductions about the mechanisms by which the substance makes its way into the urine through the use of various experimental manipulations that alter the renal clearance must then be made. Clearance is a complex function dependent on the delivery of the test substance to the kidney via perfusion and the subsequent processing of the substance through filtration, active and passive reabsorption, and secretion. The importance of perfusion rate and pressure has long been recognized, and in recent years the role of perfusion in determining filtration rate has been elucidated in a series of exacting and elegant micropuncture experiments (Tucker and Blantz, 1977; Ott *et al.*, 1976; Navar *et al.*, 1977; Brenner *et al.*, 1972). Data from these studies show that single-nephron glomerular filtration rate (SNGFR) is dependent on both glomerular perfusion rate and intracapillary pressure. These techniques do not allow direct evaluation of vascular function at the level of the single vessel. In this review I will summarize recent studies in which video microscopy has been used to define the reactivity of the renal microvasculature and the hydraulic permeability of the glomerular capillaries.

B. Direct Observation of Vascular Function Using Video Microscopy

Optical observations and analyses of video recordings permit precise and quantitative measurement of the vascular response of the vascular bed either *in situ* or in isolated segments *in vitro*. Video observation permits the use of low light levels that limit possible injury to tissues, time averaging, background subtraction, and other forms of image enhancement to visualize otherwise inapparent details. Since the quality of the image may be monitored during the experiment, the adequacy of the data is immediately evident and the experiment may be repeated if necessary. Stop-action playback permits the analysis of events that occur in very rapid time frames (50–240 Hz). Quantitative objective measurements may be made using the digitized output from the video signal, and replicate analyses may be made on the same or different areas of the recorded field. Replicate studies on a specific vessel may also be performed following experimental manipulations. Variations between populations of vessels (e.g., in deep vs superficial cortex) may also be evaluated. Disadvantages of video-microscopic techniques include the time limitations for replicate images, the limitations of resolution by the number of rastar lines and pixels of the recording, carry-over of images from one image to the next, and nonlinearity of the signal–light intensity relationship.

II. Documentation of Filtration by Isolated Glomeruli

A. Glomerular Isolation and Estimation of Ultrafiltration Coefficient and Hydraulic Conductivity

The advantages of video recording of events within the microvasculature of the kidney are illustrated by the studies of filtration by individual isolated glomeruli (Savin and Terreros, 1981). Glomerular capillaries are quite long and tortuous and relatively impermeant to protein. Because of these properties, their cellular and macromolecular contents are conserved in glomeruli isolated in medium with oncotic concentration similar to that of plasma. In contrast, water and small molecules are readily exchanged, resulting in equilibration of the activities of these molecules across the capillary wall. Replacement of isooncotic medium by hypooncotic medium results in a net oncotic force that favors net movement of fluid across the capillary wall from bath to lumen, luminal expansion, and ejection of capillary contents including both plasma and erythrocytes (RBC).

Normal glomeruli are adherent to Bowman's capsule and the early proximal tubule only at the vascular pole and may be easily isolated from surrounding tissue. The medium bathing the capillary–epithelial complex may then be rapidly changed. Glomeruli were isolated in isotonic medium containing an oncotic agent, usually bovine serum albumin (BSA), by established sieving techniques or by microdissection. Isolation by sequential sieving was carried out in the following manner. The kidneys were removed from the perirenal tissue; the capsule was discarded and the renal cortex separated from the medulla and papillary tissue. The renal cortex was then minced into fragments of 1–2 mm dimensions with iris scissors. Medium was added during this process to cover the fragments and prevent dehydration of the tissue. Fragments were transferred to a stainless-steel screen (80 or 100 mesh according to the size of the glomeruli), and tissue was pressed through this screen with a stainless-steel spoon spatula. The number of glomeruli retrieved could be increased by more extensive maceration of tissue through the screen, but the resulting suspension usually also contained a larger number of torn glomeruli that were not usable in filtration studies and more tubule fragments. Many glomeruli were adherent to the bottom of the initial screen. A disproportionate number of these are contained within Bowman's capsule or retain adherent tubule or arteriolar fragments. The suspension that resulted from passage through the initial screen was subsequently enriched for suitable glomeruli by passing it through smaller screens (100 or 120 mesh) that retained most encapulated glomeruli and larger tubule fragments, and finally over a fine screen (150–200 mesh) that retained glomeruli but permitted blood cells and small tissue fragments to pass and be discarded. Isolation was monitored by light microscopic examination of the suspension at each step and the sequence of sieving modified appropriately. This was necessary because glomeruli vary widely in size with both age and species studied, and also because the physical characteristics of the other tissues of the cortex determine the extent of contamination. Isolation was performed at room temperature because glomeruli become rigid and excessively subject to fragmentation at low temperatures. At room temperatures filtration characteristics remain unchanged for at least 60 minutes after isolation. When longer intervals between nephrectomy and study were required, glomerular suspensions were chilled after isolation and rewarmed 20–30 minutes prior to study.

When only a small specimen of renal cortex is available, as in studies of biopsy samples of renal cortex, glomeruli were isolated by microdissection. In this case the tissue was placed in a small Petri dish in isooncotic medium and observed with a dissecting microscope. Tissue was teased apart with dissecting needles or forceps, and glomeruli fell away from the surrounding tissue. Individual glomeruli were picked up in a Pasteur pipette and

transferred to the previously prepared observation chamber for filtration studies. In studies of human renal biopsy samples, 4–10 glomeruli could be obtained from samples of 2–3 mm^3.

After isolation, a sample of glomeruli was transferred to a chamber with a coverslip bottom. A single glomerulus that was free of Bowman's capsule and appeared to be intact was selected and held by gentle suction on a glass micropipette 10–20 μm in diameter. If the pipette tip was too small in relation to the glomerulus it did not hold well enough to permit vigorous washing; if it was too large part of the capillary tuft was drawn into the pipette lumen and removed from the experimental medium exchange. In general, we have studied glomeruli that have no apparent afferent or efferent arteriolar fragments. In a small series of experiments we have selected glomeruli that have retained both arterioles and have crimped these in the holding pipette so that no fluid could be ejected.

During isolation and incubation, the water and electrolyte activities of the fluid within the capillary equilibrated with those of the bathing medium, and ionic or oncotic gradients across the capillary wall were dissipated. Filtration was then produced by abruptly washing the glomerulus with medium with a lower oncotic concentration. The change in medium created an oncotic gradient across the capillary and caused influx of fluid into the capillary. The capillary distended to its maximum within about 0.2 second; further fluid influx results in ejection of plasma and RBC from the arteriolar fragments. The configuration of glomerulus, holding pipette, and washing pipette is shown in Fig. 1. The washing pipette was located about 100 μm from the glomerulus. As fresh medium was introduced, the glomerulus was enveloped in a stream of medium providing for turbulent flow and minimizing the effect of unstirred layers. Flow through the wash pipette was rapid, but no attempt was made to exchange the medium within the chamber completely. Rather, all measurements were made during the

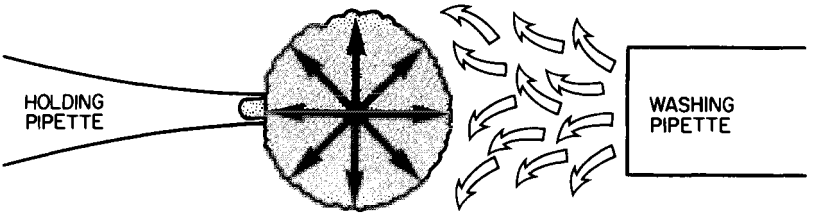

FIG. 1. Diagrammatic representation of an isolated glomerulus during exchange of external medium. A single glomerulus was selected and held on a micropipette while new medium was delivered by the washing pipette. Four diameters were measured from the video image and averaged for each 0.2-second time interval. [Savin and Terreros (1981), reproduced with permission.]

period when the glomerulus was being bathed by incoming medium. The difference in media protein concentration resulted in different retractive indexes and visible Schlerin effect at interfaces between media. This was used as a marker for the beginning of measurement.

After washing the glomerulus for about 10 seconds, RBC ejection slowed and washing was stopped. Events that occurred during filtration were analyzed using stop-action that displays a single video field. Each field represented the configuration of the glomerulus during a single 1/60-second interval but contained only one-half the rastar lines of a complete frame (1/30 second). Glomerular diameter was measured from the video monitor using a clear plastic ruler. Magnification of 20–40× objectives produced a final magnification in the range of 100–200×. Four diameters were measured at 45° intervals and the glomerular volume calculated using the geometric mean of these diameters and the formula for the volume of a sphere: $V = 4/3\pi r^3$. The rate of filtration was estimated from the maximum rate of change in glomerular volume during a single interval within the first 0.1 second following the initial application of the oncotic gradient. Ultrafiltration coefficient was then calculated as the ratio of the maximum filtration rate ($\Delta v/\Delta t$) and the calculated or measured oncotic difference between isolation–incubation medium and washing medium ($\Delta\pi$).

$$K_f = \Delta v/\Delta t \cdot \Delta\pi$$

An example of the measured change in glomerular volume and calculation of K_f is shown in Fig. 2.

B. Studies of Glomeruli from Normal Mammalian Kidneys

The ability to study isolated glomeruli permitted the investigation of the intrinsic filtration properties of the glomerular capillary in isolation from the influence of perfusion rate and pressure as well as the study of glomeruli that are inaccessible to micropuncture such as deep cortical glomeruli, glomeruli of species that do not have surface glomeruli, human glomeruli from both normal kidneys and from pathological specimens. To date, studies to define the filtration characteristics of normal glomeruli of several species have been performed. These studies have included the measurement of K_f, estimation or morphometric measurement of capillary area and calculation of capillary hydraulic conductivity, definition of the relationship between oncotic gradient and glomerular volume, and definition of the pressure required for ejection of RBC from the capillaries. The response of glomerular capillaries to intravascular volume depletion and expansion has also been documented. Finally, experimentally induced renal failure in

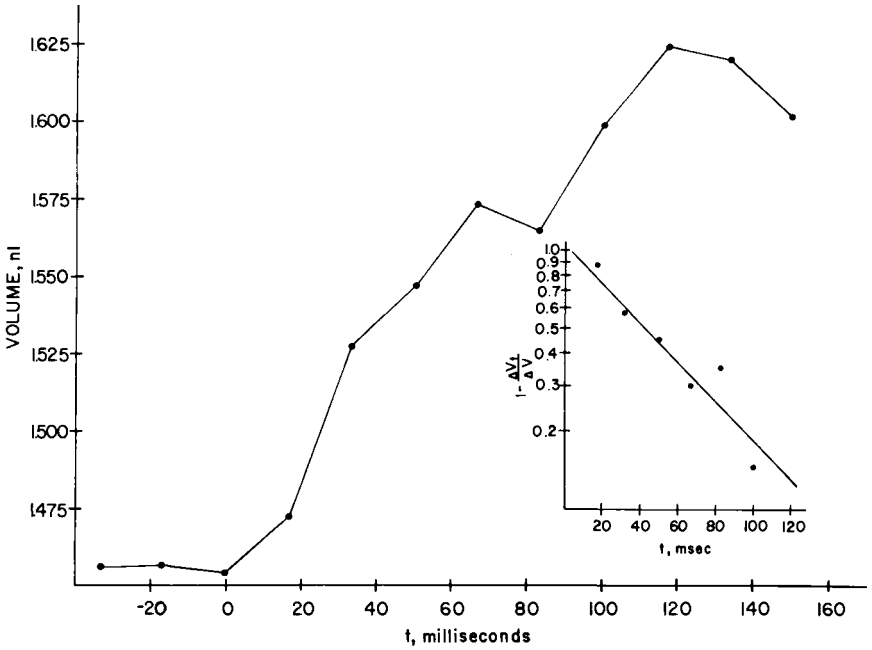

FIG. 2. Example of K_f calculation in a single glomerulus. The increase in volume of a rat glomerulus during change of medium from 12 to 4 g/dl (~65 mm Hg) is shown on the arithmetic coordinates. K_f calculated using the maximum v/t was 2.8 nl/min/mm Hg. The inset shows the transposition of the data to a logarithmic form in which $v;inf/t/v$ is the fractional decrease in volume. The line was calculated using the least-squares method. For this glomerulus, $t_{1/2}$ was 42.2 seconds and the rate constant (k) was 0.016. K_f determined in this way was 2.0 nl/min/mm Hg. [Savin and Terreros (1981), reproduced with permission.]

animals and naturally occurring renal disease in humans have been studied to determine whether altered capillary function contributes to the renal failure.

Several basic concepts emerged from these studies. First, in normal mammals, area (A) was the strongest determinant of K_f. This is true whether glomeruli from superficial and deep cortex of a single animal are compared, whether glomeruli from animals of the same species but of different sizes or ages are compared, or whether glomeruli from several species are compared (Fig. 3). The ultrafiltration coefficient is, by definition, equal to the capillary hydraulic conductivity (Lp) times the filtering area, A. Thus Lp may be estimated from the slope of the line that provides the best fit between K_f and A for many glomeruli or many individuals, or Lp may be calculated according to formula $\text{Lp} = K_f/A$.

The capillary area of individual glomeruli was initially estimated using the formula of Renkin and Gilmore: $A = 3\pi d^2$ (Renkin and Gilmore, 1973).

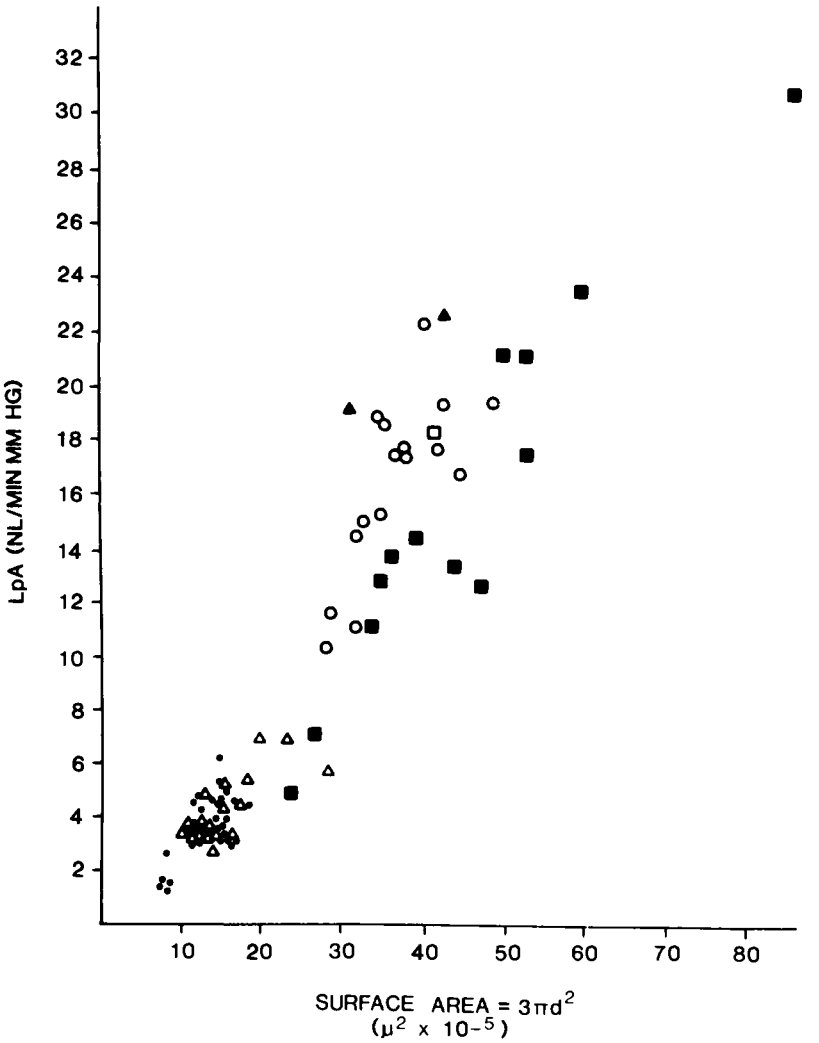

FIG. 3. Relationship between hydraulic conductance (LpA) and surface area (A) for glomeruli from several mammalian species: rats (●), rabbits (△), dogs (○), sheep (▲), cows (□), and humans (■). LpA was determined for isolated glomeruli using the maximum v/t and averaged for three to six glomeruli from each individual. Area for each glomerulus was calculated using the empiric formula $A = 3\pi d^2$. [Data from Savin and Patak (1981).]

This empirical approach may be applied to every glomerulus without requiring extensive morphometric studies. Area derived in this way varied directly with measured capillary area of glomeruli sampled from a single relatively hemogeneous population, such as superficial cortical glomeruli of

Munich–Wistar rats of 150–200 g body weight. However, when it was used to estimate area in samples of glomeruli from several populations such as superficial and deep cortical glomeruli of the same rats, the relationship between $3\pi d^2$ and measured capillary area was not linear. In this circumstance morphometric measurements showed that capillary surface density was the same in the two populations; that is, area varied with d^3 rather than d^2 (Pinnick and Savin, 1986). Thus, while the empiric estimate may permit qualitative estimates of Lp, more precise calculations depend on measurement of capillary areas of the particular glomeruli under study. In some subsequent investigations (Pinnick and Savin, 1986; Savin, 1983; Savin *et al.*, 1985a) the area of capillary basement membrane and of the peripheral capillary were calculated using morphometric measurements of surface density derived from transmission electron micrographs.

We have designated Lp derived as $K_f/3\pi d^2$ as Lp^D, Lp derived using morphometric estimates of total basement membrane area as Lp^S, and Lp derived using only peripheral capillary area as Lp^P. Small variations in both Lp^S and Lp^P are apparent during development of the young rat (Savin *et al.*, 1985a). In this case, Lp is greater in the early postnatal period prior to 14 days of age and diminishes somewhat thereafter. Lp^S and Lp^P are stable through the period of sexual maturity. The mechanisms responsible for this change in Lp during maturation are under investigation.

C. Glomerular Response to Depletion and Expansion of Intravascular Volume

The second principle that has emerged from studies of filtration by isolated glomeruli is that K_f and Lp are modulated during experimentally induced states of intravascular volume depletion and expansion. Several series of experiments regarding K_f of glomeruli isolated from rat kidney following experimental manipulation of intravascular volume were carried out. In initial experiments, glomeruli from rats on standard laboratory chow were compared to those from rats on sodium-restricted chow, and to those from rats that had been volume depleted by furosemide administration or by hemorrhage (Ridge *et al.*, 1984). In these experiments the entire cortex was used in glomerular isolation, and glomeruli studied were selected without systematic regard to their size or shape. Because midcortical glomeruli are most numerous and because the diameter of these glomeruli fell between that of superficial and deep glomeruli from rats of similar body weight, the glomeruli in this study probably represented primarily midcortical glomeruli. K_f and Lp^D varied directly with the degree of intravascular volume depletion as measured by hemoconcentration following sodium

restriction or furosemide administration or decrease in hematocrit following hemorrhage. K_f and Lp^D were independent of changes in plasma protein concentration or hematocrit. Both K_f and Lp returned to normal values when rats were allowed to drink normal saline *ad lib* overnight.

Further studies of volume depletion and expansion were performed in which superficial cortical (S) glomeruli were isolated from the outermost 2–3 mm of cortex and deep (D) glomeruli from the area to the corticomedullary junction (Pinnick and Savin, 1986). Area was measured from replicate samples of isolated glomeruli. In these experiments average K_f of control S glomeruli was less than that of larger D glomeruli. Lp^S and Lp^P did not differ in the two populations. After mild volume depletion (~10%) K_f and Lp of S glomeruli decreased by about 50%. K_f and Lp of D glomeruli were not altered. Experiments were also conducted to determine whether K_f and Lp could be increased by intravascular volume expansion (Duncan *et al.*, 1986). When intravenous saline was administered at a rate sufficient to suppress plasma renin activity and increase fractional excretion of sodium, there was no appreciable change in intravascular volume as estimated by hematocrit or plasma protein concentration and there was no change in K_f of either S or D glomeruli. When hyperoncotic albumin was infused, intravascular volume was increased by about 40% and K_f and Lp of S glomeruli increased by about 25%, while K_f of D glomeruli again remained unaffected. The results of experiments during volume depletion and expansion are summarized in Fig. 4.

The modulation of K_f and Lp was in proportion to the alteration in intravascular volume both in direction and magnitude but was limited to the superficial and midcortical glomeruli; K_f and Lp of inner cortical glomeruli remained remarkably constant despite marked perturbations of vascular volume. K_f and Lp returned to normal within a few hours of reexpansion of vascular volume. These findings suggested a sensitive physiological system that permitted assessment of intravascular volume, and included receptors or effectors that are present in the superficial but not the deep nephrons. The set point for Lp in rats fed standard chow appeared to be near its maximum value. The slope of the relationship between intravascular volume and Lp was greater during volume depletion than during volume expansion.

D. Relationship between Glomerular Contraction and K_f

The third principle that has emerged from studies of *in vitro* glomeruli was that filtration by nonperfused glomeruli is independent of glomerular contraction or relaxation induced by vasoactive substances. In an attempt to discern the factors that might be operative during the glomerular response

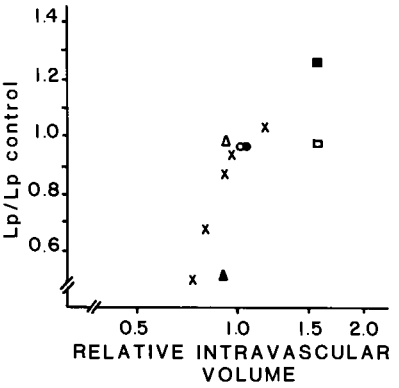

FIG. 4. Relationship between relative change in intravascular volume and Lp during volume depletion and expansion of the rat. Volume depletion was accomplished using furosemide and dietary sodium restriction or by hemorrhage. Volume expansion was induced by infusion of hyperoncotic albumin solution. Relative intravascular volume was calculated from the change in hematocrit, assuming a constant RBC mass. Data displayed are those presented in Ridge *et al.* (1984), Pinnick *et al.* (1986), and Duncan *et al.* (1986).

to volume depletion and expansion, glomeruli were incubated in media containing one of several vasoactive substances prior to induction of filtration. Glomerular diameter decreased during exposure to angiotensin II (A II) (Fig. 5), norepinephrine (NE), or vasopressin, and increased during incubation with medium containing acetylcholine, bradykinin, or prostaglandin E_2 (PGE_2). However, despite alterations in glomerular diameter, K_f and Lp were not altered by incubation with A II in concentrations up to 5.3×10^{-6} *M* (Fig. 6) (Savin, 1986). In these experiments the dissociation between the vasoconstrictor or vasodilator actions of the substances tested and the stability of K_f was striking. It seems most likely that the effects of these substances on K_f *in vivo* are mediated, at least in part, by diverting perfusion away from part of the glomerular capillary. *In vitro* filtration by nonperfused glomeruli was unaffected by local constriction of capillaries caused by mesangial cell contraction, and stable K_f reflects the stability of both Lp and *A* in this circumstance.

The response to *in vitro* incubation with A II stands in direct contrast to the persistent diminution of K_f and Lp following volume depletion. Additional studies have been performed to clarify the mechanisms responsible for this discrepancy. In these studies A II was infused systemically into anesthetized rats or into isolated perfused rat kidneys. In both these circumstances K_f was significantly lower in A II-treated than in control kidneys (Savin *et al.*, 1986; Savin and Beason-Griffin, 1987). It seems likely

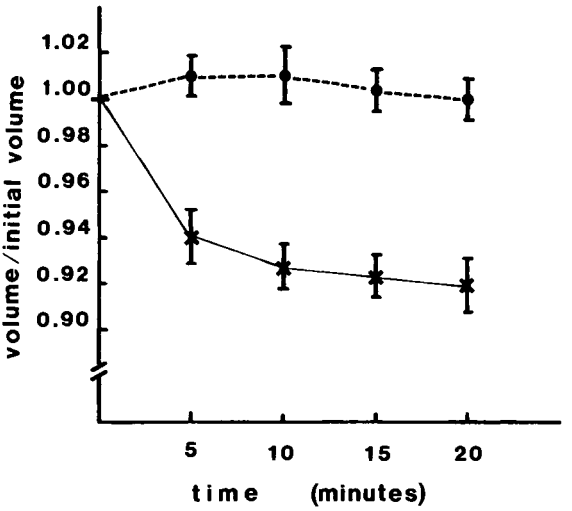

FIG. 5. Contraction by individual rat glomeruli with occluded arterioles during incubation with control medium (●, $n = 5$) or A II (X, 5.3×10^{-6} M, $n = 7$). [Savin, 1986.]

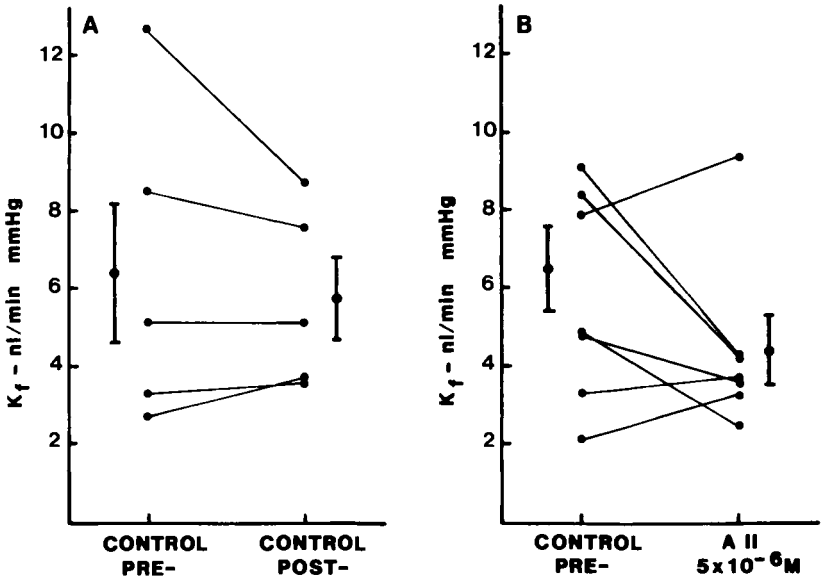

FIG. 6. Replicate studies of filtration before and after incubation for 20 minutes at 37°C in control medium (A) or medium containing A II (5.3×10^{-6} M) (B). Glomerular ultrafiltration (K_f) was not significantly altered by incubation. [Savin, 1986.]

that although A II causes mesangial cell contraction, a secondary response within the kidney is required for the alteration in Lp. Whether this effect requires humoral intermediates such as PG, kinins, or other substances produced within the kidney but not by isolated glomeruli or whether physical forces such as increased intraglomerular pressure or increased filtration rate with introduction of protein into the capillary wall are required is not known.

E. Studies of Ultrafiltration Coefficient in Experimental and Human Renal Disease

Filtration by isolated nonperfused glomeruli have also been performed in animals with several forms of experimentally induced renal disease and in glomeruli from human renal biopsy specimens. The results of these studies document that although altered glomerular function often occurs in acute renal failure (ARF), it is neither necessary nor sufficient in degree to account for diminished glomerular filtration rate (GFR). Rather, the degree of renal insufficiency is more closely correlated with impaired renal perfusion, with the degree of tubular dysfunction, or, in chronic renal insufficiency, with the number nephrons that are globally obsolescent.

Four models of ARF have been studied. Renal ischemic injury was induced in canine kidneys by 90 minutes of complete pedicle clamping (Savin *et al.*, 1983a). The contralateral kidney was removed prior to ischemia and glomeruli from this kidney served as controls. Samples of glomeruli taken immediately following reperfusion of the ischemic kidney and 1 hour later had K_f values that did not differ from control. At these periods GFR was markedly diminished despite normal total renal blood flow. Filtration failure must at that time be attributed to extraglomerular factors. In contrast, K_f of glomeruli isolated and studied 24 or 48 hours after the initial insult averaged about 50% of their control values (Fig. 7). An increase in the oncotic gradient required to cause vigorous RBC ejection was also noted in glomeruli studied at 24–48 hours. While the degree of impairment in GFR still exceeded the relative decrease in K_f, it seems possible that glomerular permeability and impaired perfusion may contribute to the persistent renal insufficiency in established postischemic ARF.

In established ARF following administration of uranyl nitrate (5 or 15 mg/kg), K_f was also decreased. However, in ARF after mercuric chloride (4 mg/kg), K_f was maintained at normal levels (Cachia *et al.*, 1981). Only when the dose of mercuric cloride was increased to 10 mg/kg, a dose that caused death within 48 hours, was K_f diminished. These studies again suggested that altered glomerular hydraulic permeability is not a universal

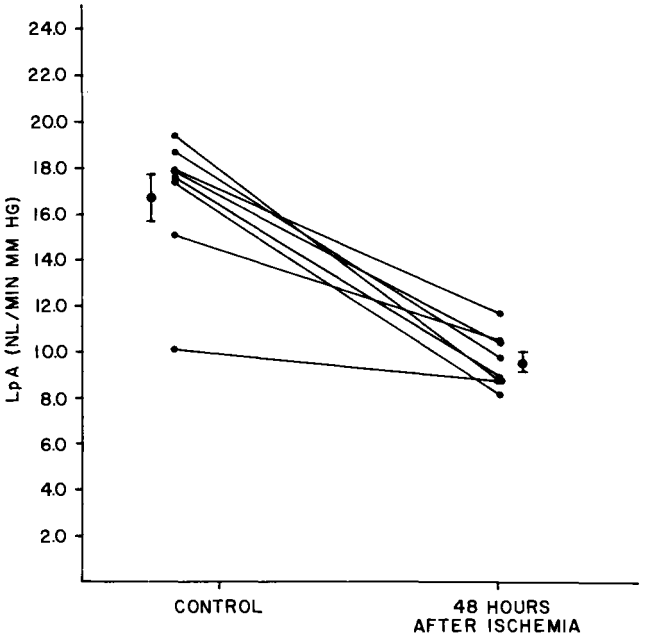

FIG. 7. Ultrafiltration coefficient (LpA) of isolated canine glomeruli before ischemia (control) and 48 hours following 90 minutes of renal pedicle clamping. Symbols represent average of four to six glomeruli for each dog. LpA or K_f was diminished in every case. [Data from Savin *et al.* (1983a).]

concomitant of ARF. More extensive correlations between glomerular and tubular functional abnormalities were possible in studies of gentamicin toxicity in rabbits (Savin *et al.*, 1985b). Gentamicin (15 mg/kg SC bid) was given to both sexually immature (1.4–1.8 kg) and sexually mature rabbits (3.8–4.5 kg). Gentamicin had little effect on creatinine clearance of young rabbits while older rabbits exhibited renal insufficiency within 5–10 days. K_f was measured in isolated glomeruli, and tubule function was assessed by the *p*-aminohippuric acid (PAH) uptake by isolated proximal tubules. Glomeruli from both young and older rabbits had decreased K_f and Lp^D. Tubule uptake of PAH was mildly impaired in young and mature nonazotemic rabbits. PAH uptake was more severely impaired in azotemic mature rabbits. The degree of renal insufficiency was correlated with tubular dysfunction but not with K_f or Lp.

Studies have also been carried out to determine the function of glomeruli in a variety of immunological and other renal diseases. To date only one study of experimental nephritis has been reported. K_f was not altered during renal insufficiency associated with immune complex glomerulone-

phritis in rats infected with *Trypanosoma rhodesensiae* (Savin *et al.*, 1982). In these glomeruli there was mesangial hypercellularity associated with increased numbers of mesangial macrophages as well as mesangial deposition of IgG and complement. Narrowing of capillaries was evident on histological examination of glomeruli, and increased intracapillary resistance to perfusion was evidenced by the fact that an oncotic gradient averaging 40–60 mm Hg was required to produce ejection of RBC from the glomerular capillaries.

Study of glomeruli from biopsied human kidneys has permitted the survey of a wide variety of renal diseases including such diverse conditions as ARF secondary to rhabdomyolysis, acute and chronic glomerulonephritis, and transplant rejection (Richardson *et al.*, 1987). The general principle of direct correlation between glomerular size and K_f is confirmed in this study. The correlation coefficient for the relationship between glomerular diameter and K_f was 0.72 ($p < 0.001$). In most cases the relationship between diameter and K_f fell within the normal range defined by the 95% confidence intervals from studies of glomeruli from normal humans. Notable exceptions were patients with ARF, malignant hypertension, or severe sclerosing processes; in these cases K_f was lower than that predicted for glomeruli of the same size. Glomeruli from one patient with diabetes and glomerulonephritis and nephrotic syndrome exhibited higher than predicted K_f. Multiple regression analysis showed that K_f was also correlated with mesangial proliferation and with the severity of capillary injury as judged from light microscopy and with the thickness of the capillary basement membrane measured from transmission electron micrographs. Together, diameter and three other variables accounted for 66% of the observed variation in K_f. Renal function as measured by the index, 1/serum creatinine, was correlated with capillary injury, with percentage obsolescent glomeruli, and with diastolic blood pressure. These variables plus the body surface area of the patient accounted for 76% of the variation in 1/serum creatinine. We conclude that capillary Lp may remain within the normal range despite acute inflammation or glomerular hypertrophy. In contrast, Lp is diminished in acute tubular injury and severe hypertension. Diminished K_f in glomeruli with sclerosis is most likely secondary to loss of actual capillary area.

F. Glomerular Function following Subtotal Nephrectomy

Finally, a series of experiments regarding the alterations in glomerular function in progressive renal disease following subtotal nephrectomy is in

progress. In an initial series of experiments, K_f was increased concurrently with glomerular hypertrophy in the first 2 months following subtotal nephrectomy (Savin *et al.*, 1983b). By 6 months, K_f remained stable despite further hypertrophy and increased filtration area, indicating that Lp had diminished. Protein restriction prevented both glomerular hypertrophy and Lp decrease (V. J. Savin, unpublished data). The relationships between glomerular hypertrophy, diminished Lp, and eventual glomerular sclerosis remain to be determined. Further studies are being conducted to determine whether other interventions that permit hypertrophy but may slow the progression of glomerular sclerosis may also prevent the late decrease in Lp.

III. Studies of Isolated Renal Microvessels

A. Isolation of Measurements of Vascular Responses

Video microscopy has been used to study the segmental effects of vasoactive substances on isolated renal microvessels *in vitro*. Using techniques analogous to those established for the dissection of isolated tubules of the rabbit kidney, Edwards has been able to dissect the renal vascular tree (Edwards, 1983). Kidneys were removed from adult female New Zealand White Rabbits (1.5–2.5 kg), thin slices were cut along the corticomedullary axis and placed in chilled artificial medium. A slice in which an arcuate artery was visible was chosen, and dissection was performed with sharpened forceps at magnifications up to ×9. An interlobular artery was identified at its origin from the arcuate artery and gently pulled from the slice. Adherent tubule fragments were stripped off to reveal a vascular tree which included the interlobular artery, its branches, afferent arterioles, glomeruli, and efferent arterioles. Vessel segments for study were then chosen and removed. Segments of at least 150–175 μm were required for study. Unbranched segments of interlobular arteries of up to 500 μm were cut away at each end using iridectomy scissors. Afferent and efferent vessels were studied with their glomeruli attached. Afferent arterioles were torn from the interlobular artery at their origins. Efferent arterioles were already severed from the distal postglomerular circulation and were separated from the more proximal vascular tree at the junction of afferent arteriole and glomerulus. Only superficial cortical vessels have been studied because efferent arterioles of midcortical nephrons branch almost immediately. Isolated vessel segments were transferred to a temperature-controlled chamber (37°C), cannulated, and perfused for 30 minutes using a modified Krebs–Ringer bicarbonate buffer solution (pH 7.4) containing 3.0 m*M*

$CaCl_2$ and 6 g/dl BSA. Bath medium consisted of the same solution but with only 2.0 m*M* $CaCl_2$ and 2 g/dl albumin. Solutions were equilibrated with 80% O_2–20% CO_2 prior to and during the experiments to maintain oxygenation and pH. Examples of isolated microvessels are shown in Fig. 8.

Evaluation of vascular reactivity was made using occluded vessels. Intralobular arteries were occluded by crimping them into a pipette; afferent and efferent arterioles were occluded as close to the glomerulus as possible. Intraluminal pressure was then set to predetermined levels. The relationship between intraluminal pressure and lumen diameter was defined for each type of vessel. In subsequent experiments constant pressure was employed and the change in lumen diameter during exposure to vasoactive substances was studied. Pressure used for interlobular arteries was 100 mm Hg and for afferent and efferent arterioles 90 and 20 mm Hg, respectively.

Measurements of the vessel diameter in initial experiments were made using direct visualization and an image-splitting eyepiece (Vickers Instruments, Woburn, Massachusetts). This technique permitted only a single measurement at a given time point and required that the operator remain attentive to his task at all times. Experiments since then have been conducted using video recording and subsequent measurements of vascular diameter. Measurements were made directly from the video monitor screen and quantified using calibration made using the image of a micrometer. Video recording has permitted measurement of multiple diameters at any time point; these diameters may then be averaged or, in the case of efferent arterioles that do not contract uniformly along their length, the contractile response may be followed at several points. The luminal diameter of interlobular arteries averaged about 45 μm, that of afferent arterioles about 18 μm under basal conditions (100 and 90 mm Hg, respectively). Diameters of these vessels were relatively uniform along the extent of the segments studied. The lumens of efferent arterioles tended to narrow adjacent to the glomerulus; average lumen diameter was about 11 μm (20 mm Hg).

B. Responses of Isolated Microvessels to Vasoconstrictors and Vasodilators

The diameter of preglomerular vessels (interlobular arteries and afferent arterioles) increased with increasing pressure up to pressures of about 70 mm Hg but decreased or remained constant as pressure was further increased from 70 to 180 mm Hg (Fig. 9). Lumen diameters of efferent arterioles increased over the entire range of intraluminal pressures studied.

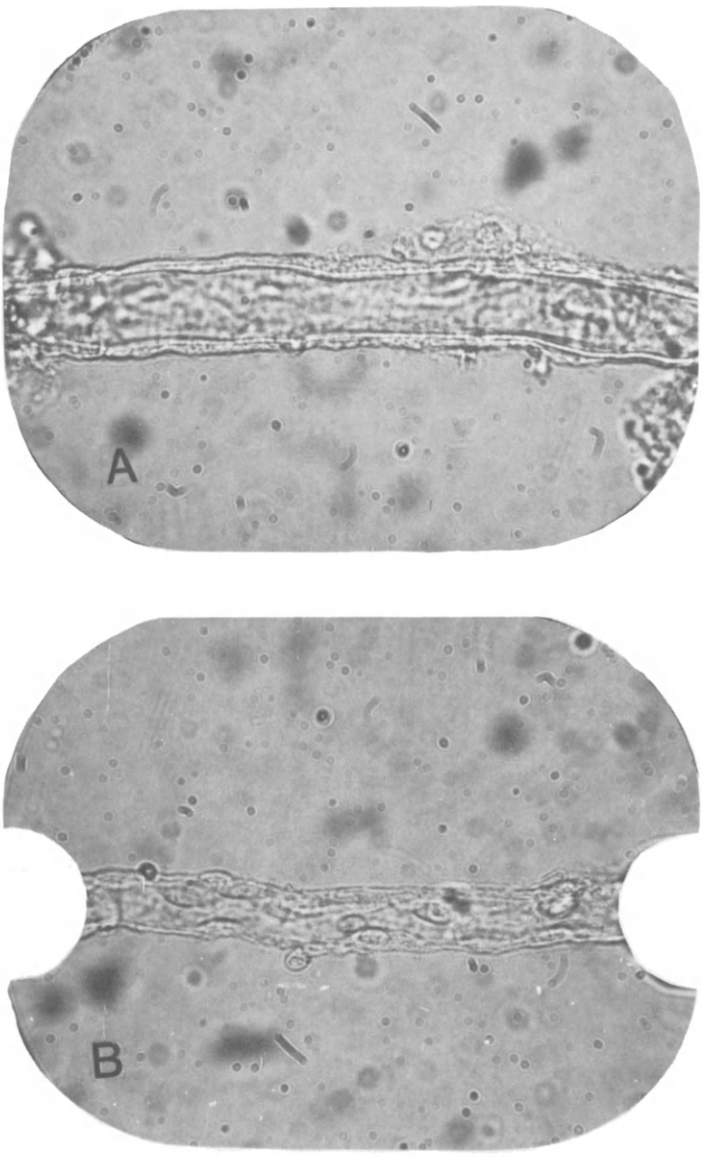

FIG. 8. Isolated rabbit renal microvessels used to study vascular reactivity. (A) Afferent arteriole, (B) efferent arteriole. (Courtesy of Dr. Richard Edwards.)

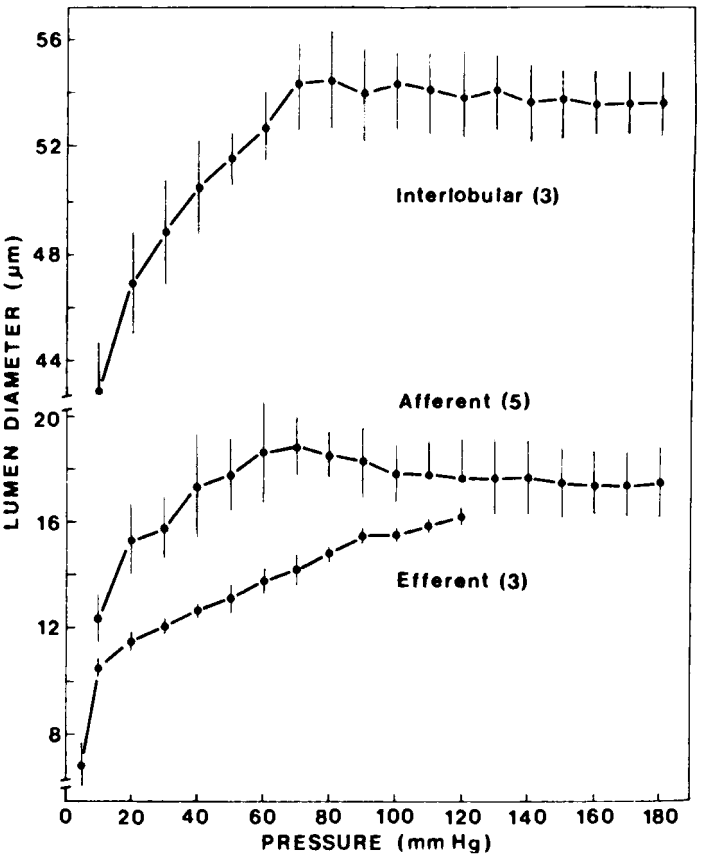

FIG. 9. Pressure–diameter characteristics of rabbit renal microvessels. Intraluminal pressure was increased in increments of 10 mm Hg, and lumen diameter was measured at each pressure. Each point represents mean ±SE. Numbers in parentheses indicate number of vessels studied. [Edwards (1983), reproduced with permission.]

When intraluminal pressure was held constant, NE caused contraction of both pre- and postglomerular vessels; sensitivity, as indicated by the dose required to produce a half-maximal response, was greater in efferent than afferent arterioles or intralobular arteries. In contrast, A II caused contraction only of the efferent arteriole and contraction was limited to the 50–75 μm immediately distal to the glomerulus. Vasodilators acetylcholine, dopamine, and bradykinin also affect renal microvessels in a heterogeneous fashion (Edwards, 1985a). Vessels were isolated, cannulated and occluded as before, and treated with NE to induce partial constriction (tone).

Vasodilators were then assessed according to the relaxation produced in these partially constricted vessels. Acetylcholine caused similar relaxation in all three types of microvessels. Dopamine caused relaxation in afferent and efferent arterioles but was less effective on interlobular arterioles. Bradykinin was only effective in relaxing efferent arterioles and had no effect on the preglomerular circulation. Further experiments concerning the mechanism of action of PG on partially constricted microvessels (Edwards, 1985b) indicated heterogeneity in response to these substances as well. Incubation in medium containing arachidonic acid caused relaxation of each microvascular segment. PGE_2 and PGI_2 caused relaxation of interlobular artery; PGD_2 also caused relaxation but a higher dose was required, while $PFG_{2\alpha}$ was ineffective in this segment. Similar effects were observed in afferent arterioles. Efferent arterioles, in contrast, responded only to PGI_2.

The effects of selective dopamine DA_1 receptor agonists Fenoldopam and SK&F R-87516, and DA_1 receptor antagonist SK&F R-83566 have also been studied using isolated microvessels (Edwards, 1986). Vessels were first treated with NE (3×10^{-7} *M*) or A II (10^{-10} *M*) to induce stable contraction. Fresh medium containing NE or A II and the agonist to be tested were introduced and lumen diameter measured after it had stabilized. Results were expressed as percentage relaxation. Potencies of both dopamine agonists were similar to that of dopamine itself. The DA_1 receptor antagonist produced a right shift in the concentration–response curve to Fenoldopam without itself altering the baseline response to NE. This study provided direct evidence that dopamine-induced relaxation of renal arterioles is due to activation of DA_1 receptors.

Most recently Edwards has reported the insensitivity of rabbit glomerular arterioles to the effects of atriopeptin II (AP II) (Edwards, 1987). AP II had no effect on the diameter of isolated microvessels, nor did it alter the resopnse of vessels to NE or A II. AP II concentrations employed ranged from 10^{-12} to 10^{-7} M; AP II was ineffective in changing luminal diameter whether it was delivered in the bathing medium or into the vessel lumen. Edwards suggests that renal vascular effects of AP II observed *in vivo* are due to an indirect action of the peptide rather than direct effects on the arterioles of superficial glomeruli.

These experiments demonstrate the unique potential from examining the differential effects of vasoactive substances on specific segments of the microvasculature using optical observation. Video monitoring and recording lends an added dimension by permitting data analysis using several points within the vessels, measurements made within a brief time period, and reanalysis of the same experiment if it is required.

IV. Observations of Glomeruli during *In Situ* Perfusion

A. Methods for Visualizing Glomerular Capillaries and Estimation of Perfusion Rate and Hydraulic Conductivity

Steinhausen and co-workers have employed video microscopy and epiillumination using fluorescently tagged RBC, latex particles, or dextran to define the glomerular capillaries and microcirculation. Video microscopy replaces high-speed photomicrography used in earlier studies. In these studies, fluorescently labeled latex particles (1.8 μm in diameter) remained in the circulation for a few cycles after injection and their velocity could be calculated using their position in consecutive video frames. The capillary diameter was defined by fluorescent dextrose and measurements used to calculate cross-sectional area and flow rates. The initial report using this technique (Steinhausen *et al.*, 1981) documented simultaneous observations of capillary diameter, velocity of microspheres, and measured intracapillary and intratubular pressures of capillaries of glomeruli that were visible on the surface of kidneys of anesthetized Wistar–Firth rats (200–250 g). The kidney was exposed by a flank incision and immobilized as for standard micropuncture experiments. Observations were made using incident light (Leitz Ultropac or Fluopac) and a water immersion objective (Leitz U055, NA 0.80). A xenon light source with heat filter, blue-green filter, and interference filter (490 nm) was used for fluorescence observations; emitted light was filtered at 530 nm. Latex particles were suspended in physiological saline and injected in a bolus of 0.05–0.10 ml via the jugular vein. Capillary diameter was measured following a bolus injection of 0.1–0.3 ml of a 4% solution of dextran (MW 150,000) labeled with fluorescein isothiocarbamyl (FITC). Fluorescent particles and dextran could be visualized simultaneously. The velocity of a particle (V) was calculated from the traversed path during 20 milliseconds and capillary diameter (d) was measured as the distance between boundary lines produced by the stained rims of the capillary contents (Fig. 10). Flow rate was calculated as the product of V and $(d/2)^2$. Capillary pressure was measured in loops adjacent to those in which flow and diameter were being assessed during puncture of individual capillary loops using a servo-nulling device filled with FITC in saline. Filtration by individual capillaries was estimated as the difference in flow rates of particles in adjacent segments of the same capillary and expressed in units of nl/min μm^{-2}. Perfusion casts of the glomeruli studies were made by injection of epoxy resin following the studies so that the capillaries observed *in vivo* could be studied in detail.

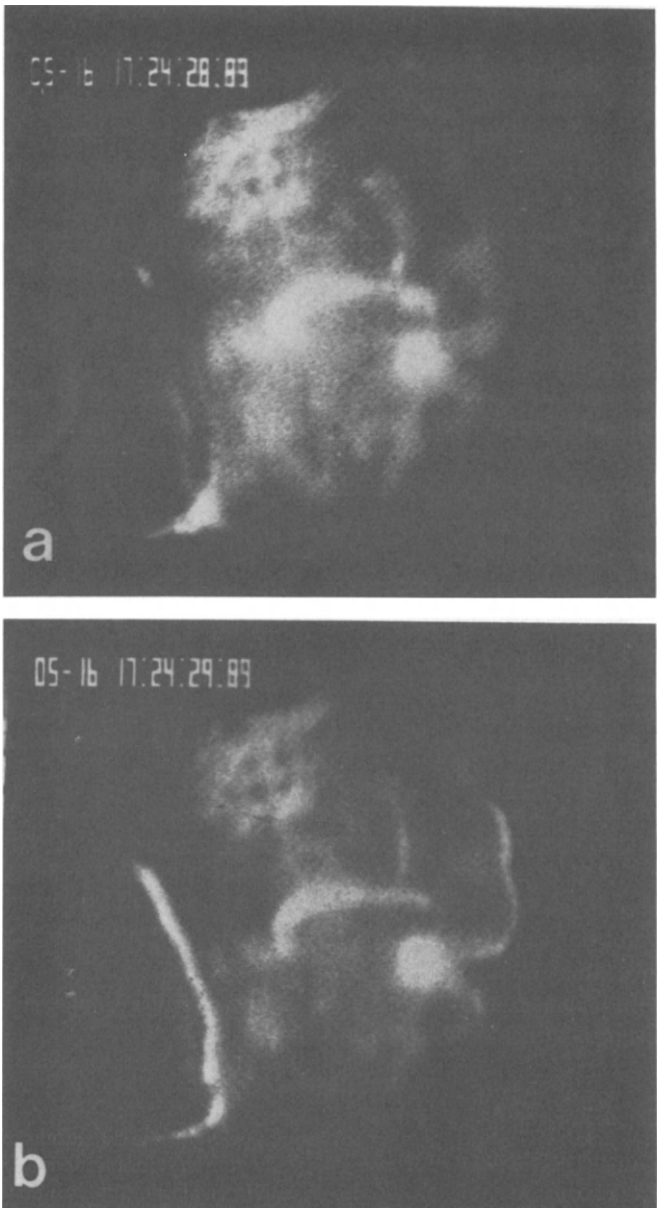

FIG. 10. (a) Photograph of the television picture of a glomerulus at the surface of the kidney of a living rat. The capillaries fluoresce because of the FITC-labeled plasma flowing through them. At the left is an insert of blood pressure registration. (b) Photograph of the same glomerulus 1 second later. The winding white lines represent the bright fluorescence given off by the latex particles as they swiftly flow through the capillaries. 1 cm $\triangleq$ 40 μm. [Steinhausen *et al.* (1981).]

Reported velocity of particles varied widely (100–1300 μm/sec) and averaged 781 ± 271 μm/sec in 25 rats. Capillary diameter ranged from 7.5 to 10.9 μm, averaging 8.4 ± 1.4 μm. Flow rate within capillaries averaged 2.6 ± 1.2 nl/min. Filtration rate was estimated in 15 capillaries of 12 rats and averaged 6.0 ± 3.5 × 10^4 nl/min μm^2. Both particle velocity and loop flow rate were positively correlated with filtration rate. Capillary pressure of adjacent capillaries was also correlated with both velocity and flow rate. When tubules were occluded by injection of a wax block, neither flow velocity nor flow rate were significantly altered, but loop filtration rate decreased in every case. The authors estimated mean ultrafiltration pressure in the loops studied from the measured intracapillary and intratubular pressures and the plasma oncotic pressure af 15 mm Hg and further estimated the capillary hydraulic conductivity of 2.7 × 10^{-5} ml/min/μm^2/mm Hg. This value is approximately the same as that derived from micropuncture studies.

In subsequent studies from the same investigators (Zimmerhackl *et al.*, 1983), video observations of glomerular perfusion and filtration rates were refined by the introduction of FITC-tagged RBC to replace latex beads. RBC exhibited normal morphology and survival in the circulation and could be visualized in the glomerular circulation. Calculation of RBC velocity was automated by the use of a dual-slit photometric circuit similar to that of Wayland and Johnson (1967) and Intaglietta *et al.* (1975). Studies were carried out using concentrations of tagged RBC of about 200 cells/nl packed RBC. RBC velocity was determined from the taped video signal using a dual photometric analyzer system (Instrumentation for Physiology and Medicine, Inc., San Diego, California). For these measurements, photometric windows were positioned at two different sites over a capillary. Fluorescent cells appeared as bright images and produced an increased voltage output in the corresponding channel. The voltage signals were digitized and processed using a microprocessor. The digital output of the first channel was monitored at a rate of 1000 per second. After a labeled cell was detected in the first photometric window, the output of the second channel was sampled to determine whether the labeled cell appeared in the second window within a predetermined time (40–200 milliseconds). The latency between the appearance of the cell in the first and second windows was used to calculate the transit time. Individual transit times could be determined only to a resolution of 20 milliseconds, since the video images were produced at 50 Hz. Thus, only a mean transit time could be determined using this method. A schematic drawing of the circuit used for this analysis is shown in Fig. 11. Results of measurements of seven capillaries of surface glomeruli of three Wistar rats indicated a mean diameter of 9.4 ± 4 μm, RBC velocity of 837 ± 145 μm/sec, RBC flux of 1.32 ± 0.17 nl/min. Microhematocrit within the capillary deviated from the

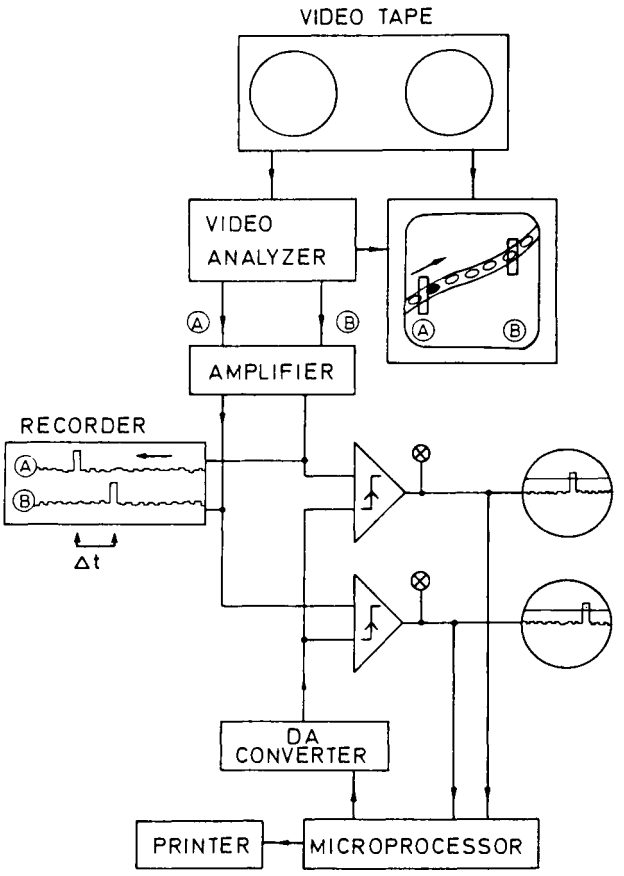

FIG. 11. Schematic drawing of the modified dual-slit technique for measuring RBC velocity and RBC flux in glomerular capillaries [Zimmerhackl *et al.* (1983), reproduced with permission.]

systemic hematocrit by −51 to +28%. There was considerable variation of RBC velocity within a single capillary as well as a 3-fold variation in average velocities between several capillaries. The use of these techniques to assess the flow and filtration characteristics of the glomerular capillaries is documented in additional reports.

B. GLOMERULAR RESPONSE TO ANGIOTENSIN

The same system has been used to study the effects of systemic infusion of A II on the glomerular circulation (Zimmelhackl *et al.*, 1985a). Kidneys

were exposed as before and the time delay between the appearance of an RBC at each of the photometric slits was used to calculate RBC velocity. Each capillary was observed for 3 minutes and the data analyzed as six consecutive subperiods of 30 seconds. RBC velocity and capillary diameter were measured prior to and during the systemic infusion of A II at 0.4 μg/min/kg. RBC velocity and capillary diameter could not be measured simultaneously because the presence of FITC-dextran interfered with the contrast necessary for the analysis of RBC velocity. Because of this difficulty, velocity before and during A II infusion and diameter during control and A II infusion were studied sequentially in a group of 10 rats. Two additional groups of rats were used to document the reversibility of the observed responses. A II infusion increased systemic mean arterial pressure from control of 109 mm Hg to 120 mm Hg and decreased total renal blood blow (measured with an electromagnetic flow meter) by 42 ± 5%. RBC velocity and flux during the initial control period averaged 880 ± 77 μm/sec and 1.32 ± 0.17 nl/min, respectively, and did not differ significantly from these values during later control periods. During A II infusion mean velocity of RBC did not change significantly, but paired analysis showed a decrease in velocity of 22 ± 7% ($p < 0.01$). RBC flux decreased by about 25% ($p < 0.01$). Volume flow, calculated from the RBC velocity and the cross-sectional area of the capillaries, decreased from 3.2 ± 0.4 to 2.4 ± 0.4 nl/min and averaged 23% in paired measurements. There was no change in the calculated microhematocrit during control and A II infusion periods. The mean diameter of capillaries also did not differ during control and A II periods; average relative change in the diameter was 2.9 ± 2%.

More recently, experimental hydronephrosis in rats has been used to visualize and document flow through the vascular structures of the kidney (Steinhausen *et al.*, 1983). In these experiments, unilateral hydronephrosis was induced by complete ureteral ligation and 60 minutes of renal artery occlusion. This resulted in progressive tubular atrophy with good preservation of vascular structures and glomeruli. Nine weeks after the induction of hydronephrosis the kidney was exposed by a flank incision and immobilized in a kidney spoon. The thin shell of renal cortex was split along the greater curvature and one-half of the kidney sutured to a wire form and observed with transmitted light microscopy. Fluorescence microscopic equipment was similar to that used in previous experiments. Optical sectioning was performed using a 75× lens with NA of 0.90 and a depth of field less than the diameter of the glomerular capillaries. Fluorescently labeled RBC were used and the glomerular architecture defined through the use of optical sectioning techniques. About half of the glomerulus could be visualized using optical sections of 5 μm. Inner and, in some cases, outer

capillary diameters were measured using a shearing monitor and the direction of flow determined in the several segments of capillary that could be visualized. RBC velocity and volume flow were measured under control conditions and the infusion of A II at 0.2 or 0.4 μg/min/kg or following the intravenous bolus infusion of A II (0.06 μg). Blood velocity in afferent and efferent arterioles was studied using the dual-slit method in a separate series of experiments. Arteriolar internal diameter was measured electronically and arteriolar volume flow calculated. Under control conditions the inner and outer diameter of the vas afferens at a point 50 μm from the vascular pole averaged 7.9 $\pm$ 0.5 and 10.9 $\pm$ 0.5 μm, respectively, while the inner and outer diameters of the vas efferens averaged 7.7 $\pm$ 0.5 and 11.1 $\pm$ 1.1 μm. There were, in both segments, narrowed portions of the vessel near the glomerular capsule. The diameter at these locations was termed "minimal diameter." The minimal inner and outer diameters of the vas afferens averaged 4.5 $\pm$ 0.5 and 6.6 $\pm$ 0.8 μm and those of the vas efferens 4.3 $\pm$ 5.0 and 6.0 $\pm$ 0.8 μm. After bolus injection of pharmacological doses of A II the glomerular circulation ceased altogether because of complete constriction and occlusion of the lumen of the efferent arteriole. This constriction was reversible and directly correlated with increase in mean arterial pressure. The afferent arteriole also constricted following bolus A II infusion, but the lumen was not completely occluded. Continuous infusion of A II in pressor doses had a variable effect on the minimal diameter of the afferent arteriole. At low doses (0.2 μg/min/kg) the lumen was dilated; at higher doses (0.4 μg/min/kg) the lumen constricted. In contrast, the minimal diameter of the efferent arteriole was diminished during A II infusion in every case and became dilated after the infusion was discontinued. In an additional series of studies, A II infusion caused constriction of both afferent and efferent vessels and decrease in volume flow; flow velocity was increased in some cases and decreased in others. A II infusion failed to alter either glomerular diameter or the diameter of single capillaries in this preparation. Since there was no filtration by these glomeruli, no comment could be made regarding the effect of A II on hydraulic conductivity. Additional studies in intact kidneys have documented similar decreases in both RBC velocity and RBC flux without alterations in capillary diameter or local hematocrit during A II infusion. These data fail to support the notion that a decrease in the area of glomerular capillaries can account for observed alterations in the ultrafiltration coefficient as measured in micropuncture studies and may be used to implicate either a decrease in hydraulic conductivity or regional alterations in flow rate that lead to local filtration equilibrium as the mechanisms by which K_f is altered during A II infusion.

C. Microvascular and Glomerular Response to Dopamine

The split hydronephrotic rat kidney has also been used to study the effects of locally applied dopamine on the renal microvessels (Steinhausen *et al.*, 1986). Vessel diameters measured were those of the arcuate artery proximally, near the interlobar artery and distally, near the interlobular artery; the interlobular artery proximally and distally, near the branching point of the terminal afferent arteriole; the afferent arteriole proximally and at its narrowest point near the glomerulus; and the efferent arteriole within 50 μm of the glomerulus and at the welling point. Three series of investigations addressing the dose–response curve of dopamine of the several segments of the renal vasculature, the role of specific dopamine receptors in mediating dopamine effects and the stability of the preparation under control conditions were reported. Dopamine effects were examined at bath concentrations of 10^{-6}–10^{-3} M; vessel diameters were measured after 15 minutes of incubation in experimental medium. Inhibition of dopamine effect by inhibition of dopamine receptors was assessed by adding haloperidol (10^{-4} M) to the bath and inhibition by blocking of α- and β-adrenergic receptors by the addition of phentolamine (10^{-4} M) or propranolol HCl (10^{-6} M), respectively. Vessel diameter under control conditions diminished from about 40 μm for the proximal arcuate artery to about 8 μm for the afferent arteriole near the glomerulus. Minimal diameter of the efferent arteriole was even less (~6 μm), and the efferent arteriolar diameter increased to about 15 μm at the welling point. Dopamine in concentrations of 10^{-6} to 3×10^{-5} M produced a concentration-dependent increase in the diameter of the preglomerular vessels and of the efferent arteriole near the glomerulus. High concentrations of dopamine (3×10^{-4} to 10^{-3} M) tended to reduce the diameter of preglomerular sites except for the afferent arteriole near the glomerulus. Pretreatment with haloperidol did not alter vessel diameters in the absence of dopamine but did attenuate the dilator effect of low-dose dopamine and the constrictor effect of high-dose dopamine at all sites except the welling point. Pretreatment with phentolamine enhanced vasodilatation by low-dose dopamine and attenuated the constriction produced by high-dose dopamine. Propranolol enhanced the dilatory effect of low-dose dopamine except at the afferent arteriole closest to the glomerulus and inhibited the vasoconstrictive effect of high-dose dopamine leading to vasodilatation at some sites even with dopamine concentrations of 10^{-3} M. The unique ability to study the vascular diameters of the renal microcirculation at many sites simultaneously and to perform replicate studies of the same vessels under a variety of conditions is demonstrated by these studies.

V. Studies of Blood-Perfused Juxtamedullary Nephrons

A. Preparation of Isolated Perfused Juxtamedullary Cortex

Neither studies of isolated vascular segments *in vitro* nor those of *in situ*-perfused surface glomeruli give definitive information about the vascular responses of juxtamedullary regions. A technique has been developed that permits the visualization of both pre- and postglomerular vascular structures of the juxtamedullary cortex of the rat (Casellas and Navar, 1984). Kidneys of male Sprague–Dawley rats (350–450 g) pretreated with the converting-enzyme inhibitor captopril were exposed, decapsulated, and removed to Petri dishes. The cortex from one side of the kidney was removed so that the intact papilla was visible. Cortical tissue was resected, the papilla reflected, and pelvic mucosa removed to reveal branches of the renal vein. Venous and adipose tissues were removed to expose the inside cortical tissues without manipulating tubules, superficial arteries, or glomeruli. The perfusion cannula placed in the arcuate artery after the portion of the arcuate artery that supplied the superficial glomeruli was tied off. The pressure responses and filtration rate were documented in initial studies using this model (Casellas and Navar, 1984; Casellas *et al.*, 1985). Glomerular pressure averaged 49 mm Hg at perfusion pressure of 100 mm Hg, and SNGFR averaged 34 nl/min when perfusate had no albumin and 23.3 nl/min when colloid osmotic pressure was maintained with albumin.

B. Microvascular Responses to Vasoactive Substances

This preparation has been used to study arteriolar and glomerular capillary pressures following bolus injection of A II or epinephrine (Casellas *et al.*, 1985). A II caused a dose-dependent and reversible decrease in glomerular capillary pressure. Epinephrine also decreased glomerular capillary pressure. Afferent arteriolar pressure increased after A II infusion, but decreased following epinephrine infusion. The investigators suggested that this constellation of findings indicated that epinephrine acted in a generalized fashion in the preglomerular circulation, while A II acted selectively in the late afferent arteriole and possibly within the glomerulus itself. The effects of A II on vessel diameter have been documented recently using the same preparation perfused with blood containing FITC-labeled BSA and observed with fluorescence video microscopy. (Carmines *et al.*, 1986). Video techniques permitted visualization of arcuate and

interlobular arteries, afferent and efferent arterioles. Vessel diameter was studied before, during, and after topical application of A II, NE, or sodium nitroprusside. Rats were treated with captopril, the left kidney was perfused with Krebs–Ringer bicarbonate solution containing 6 g/dl BSA and amino acids. The kidney was removed and dissection performed to expose the inner cortical surface. Blood perfusate with hematocrit 30% and colloid osmotic pressure 18 mm Hg was prepared and perfusion and pressure measurements carried out using a double-barreled cannula. Perfusion pressure was maintained at 110 mm Hg. Tissue was maintained at 37°C during the observation period. Microvessel diameters were made using epifluorescence (Leitz LABORLUX 12-FS microscope, PLOEMOPAK epifluorescence, 20, 32, or 40× objectives). A xenon light source with a 450–490 nm bandpass filter and a 515-nm long-pass emission filter were used. Images from a Newvicon camera was enhanced and vessel diameter was measured using a video caliper (304r, Vista Electronics, Ramona, California). Diameter was measured at the midplane of the vessel, defined as the widest possible image; the caliper output was recorded on a polygraph and the operator was not permitted to see this output during measurement. Vessels were exposed to superfusate concentrations of A II of 0.01, 0.1, or 1 n*M*. Measurements of the same vessel during control, experimental, and recovery periods were compared. Control diameters of interlobular arteries, mid, and late afferent arterioles, and efferent arterioles avergaed 64, 24, 21, and 19 μm, respectively. Vessels responded to sodium nitroprusside (380 n*M*) by increasing their diameters by an average of 10–30% and to NE (700 n*M*) by constriction of 15–20%. A II caused constriction of every segment studied. This response was dose related in 62% of the preglomerular vessels studied. A minority of preglomerular vessels showed greater sensitivity to 0.001 μM A II than to higher concentrations. A II responses were inhibited but not entirely blocked by the concurrent exposure to saralasin (10 μM). In a parallel series of studies A II (0.01 n*M*) decreased SNGFR by 28%.

Vascular diameters in this study were greater than those reported for the split hydronephrotic kidney. Vasodilatory response to nitroprusside documents that vessels are not maximally vasodilated. NE-induced constriction of both pre- and postglomerular vessels is in agreement with other observations both from micropuncture and *in situ* perfusion studies and studies of isolated microvessels *in vitro*. The finding of A II responsiveness of preglomerular vessels at even the lowest dose tested is unique and in direct contrast to observations in isolated rabbit microvessels. The heterogeneity of the dose–response curve in these vessels remains to be explained.

VI. Documentation of Medullary Blood Flow

In addition to documenting perfusion and filtration patterns in the glomerular circulation, video techniques have been used to determine blood flow rates and patterns in the renal medulla and to make hypotheses concerning the vascular requirements for the making of a concentrated urine. Jamison and co-workers (Holliger *et al.*, 1983; Jamison *et al.*, 1985) have reported a number of studies in which the video tracking or dual-slit techniques have been used to determine the RBC velocity in vasa recta capillaries. Papillary perfusion was studied in kidneys of anesthetized Munich–Wistar rats in which the papilla had been exposed by excising the ureter. Reflected rather than transmitted light was used and images were recorded using a Leitz intravital microscope and a SIT camera. The video signal was digitized and the passage of RBC monitored by voltage changes in two windows positioned over the image of a capillary. RBC velocity was computed using the distance between the two windows and the time delay that resulted in maximum cross-correlation. Images were made at the rate of 120–240/second by using an external synchronizing generator. In the first of these studies, they found that RBC velocity was increased following saline infusion in male but not female rats (Gussis *et al.*, 1979). Antidiuresis produced by intravenous antidiuretic hormone infusion did not alter RBC velocity (Zimmerhackl *et al.*, 1985b).

In the system as described, video recording provides several advantages including permitting repeated measurements of the same or different vessels, electronic computation of velocities, immediate visualization and evaluation of the data, and the potential for signal analysis for analysis of frequency components. Limitations stem from the inability to resolve velocity at rates that exceed 2.4 mm/sec, the limitation of resolution of capillary diameter and hematocrit resulting from limitations in optical resolution. RBC velocity was used as an index of vasa recta blood flow rate, but the validity of extrapolation was not assessed directly since neither the number of patent capillaries nor the diameter of capillaries nor the microhematocrit within the capillaries studied was measured. In subsequent studies, capillary diameter was measured directly from the video monitor following intravenous injection of fluoresceinated bovine γ-globulin and illumination with a mercury lamp (Zimmerhackl *et al.*, 1985c). Renal papillae were exposed as before and RBC velocity determined using a modification of the original technique. The relationship between RBC velocity and blood flow rate was calculated using F of Fahraeus factor derived from empirical observations of RBC flowing through small-bore quartz capillaries at a known rate of perfusion. This factor is required, since

RBC are excluded from the peripheral region of vessels with diameter of less than 200 μm, but their velocity may exceed that of plasma adjacent to the vessel wall. Total papillary blood flow was calculated from single-vessel blood flow using estimates of the number of descending and ascending vasa recta (DVR and AVR) derived from transmission electron micrographs of slices of the renal papilla. During hydropenia (mean urine osmolality 1250), RBC velocity averaged 1.0 ± 0.1 mm/sec DVR and 0.38 mm/sec in AVR. Diameter of DVR and AVR averaged 15.6 ± 0.5 and 20.0 ± 0.4 μm, respectively. The *F* factor averaged 1.4 ± 0.3 and was independent of hemotocrit or capillary diameter in the range studied. Using the measured RBC velocities, capillary diameters, and *F* factor, DVR blood flow averaged 8.8 ± 1.0 nl/min and was almost twice that in AVR (4.8 ± 0.3 nl/min). The blood flow rate at the papillary tip averaged 5.2 μl/min for DVR and 11.3 μl/min for AVR. The total perfusion rate derived using this method was greater than those previously reported using other methods. The increment in blood flow between the DVR and AVR was sufficient to account for the removal of water reabsorbed from the papillary collecting duct. Additional studies (Zimmerhackl *et al.*, 1985d) using fluorescent-tagged RBC have demonstrated that the dynamic hematocrit in the vasa recta averages about 58% of the systemic hematocrit. Discrepancies between the results of micropuncture studies and video measurements combined with morphometric studies have been assessed in concurrent studies in the same animals (Zimmerhackl *et al.*, 1985c). Functional ratios of DVR to AVR differed from morphometric estimates and permitted the data from the two sorts of studies to be reconciled. Studies in which vascular diameter as well as RBC velocity were measured demonstrated that AVP has a direct vasoconstrictive effect on the medullary microcirculation but that its effect on urinary concentration is not mediated by this effect alone (Zimmerhackl *et al.*, 1985b).

Roman and Smits have developed a surgical technique for exposing the papilla of adult rats for video microscopic studies of the vasa recta circulation, and they have evaluated the use of a laser Doppler flow meter for continuous measurement of cortical and papillary blood flow (Roman and Smits, 1986; Smits *et al.*, 1986). Their results indicate that papillary blood flow may be twice as high in adult rats (>300 g) than in younger animals (100–150 g) which have typically been used in papillary micropuncture studies. They have also studied autoregulation of cortical and papillary blood flow using the Doppler flow meter. Their studies suggest that papillary blood flow may not be autoregulated as efficiently as cortical and whole-kidney blood flow over the range of pressures from 100 to 150 mm Hg (Roman, 1986; Takezawa *et al.*, 1987). Figure 12 depicts the appearance of the papilla of a rat as renal perfusion pressure increased the

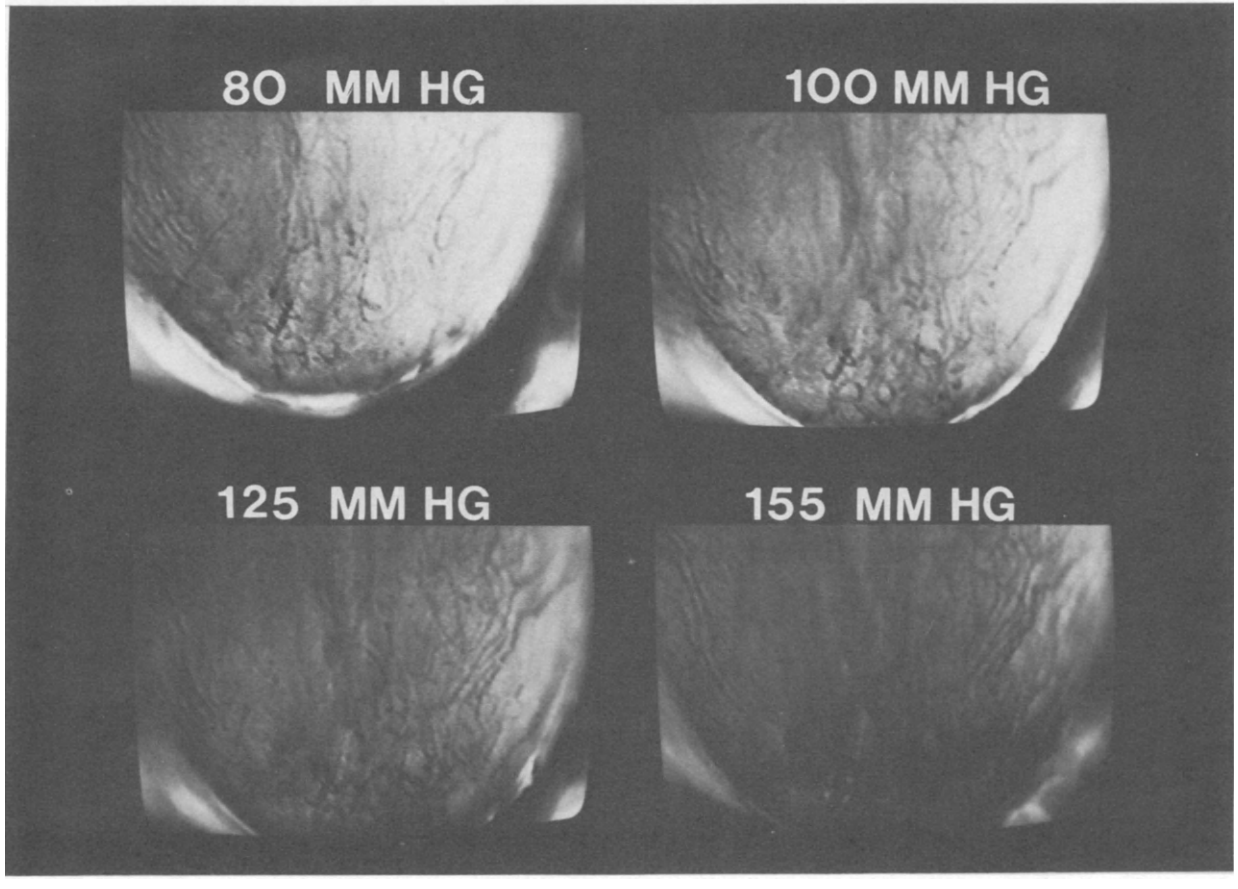

FIG. 12. Video microscopic appearance of the papilla of an adult Sprague-Dawley rat perfused at different levels of pressure. Approximately ×35. (Courtesy of Dr. Richard Roman.)

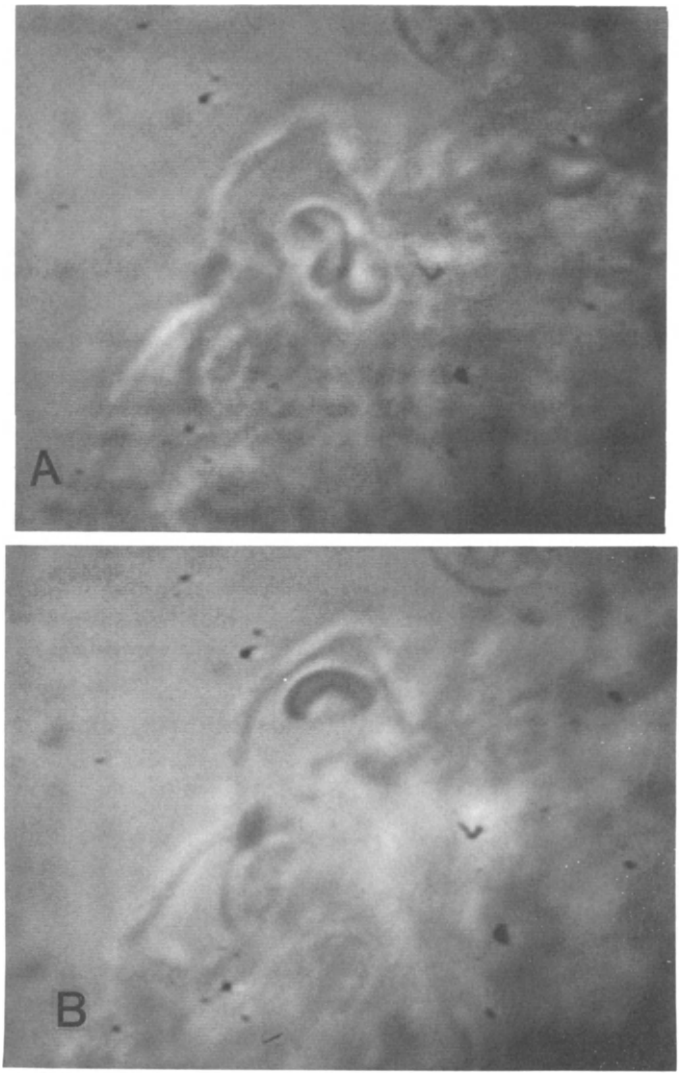

FIG. 13. Glomerular capillary viewed using differential interference contrast (DIC) video microscopy with image enhancement. (A) Glomerulus in 4 g/dl BSA medium, (B) glomerulus following incubation with AII 10^{-6} *M*. Capillary has the same configuration as prior to AII but is drawn toward the central part of the glomerulus by mesangial cell contraction.

number of perfused vasa recta capillaries. This finding suggests that changes in capillary recruitment, as well as an increase in blood flow in individual vasa recta capillaries, may contribute to the rise in papillary blood flow at elevated renal perfusion pressure. It remains to be determined whether

changes in the number of perfused vasa recta are also associated with the changes in papillary blood flow produced by various drugs and hormones.

VII. Prospects for the Future

Reports to date have focused on establishing and validating methods for making and analyzing data derived from optical observations of the renal vasculature *in situ* or in isolated segments of arterioles or glomerular capillaries. It now remains for these techniques to be widely applied to physiological and pathological circumstances. The microvasculature of the kidney appears to have altered responsiveness in several types of renal disease. In diabetes mellitus, for instance, there appears to be vasodilatation that permits hyperperfusion even in the very early stages of the disease. Consequently, hyperfiltration precedes any demonstrable structural abnormality and may in itself contribute to the later changes of mesangial and peripheral extracellular matrix deposition and eventual global sclerosis. It is attractive to hypothesize that documented abnormalities in receptor number may lead to impaired response to vasoconstrictor agents, but no direct evidence bearing on this issue has been produced. The action of many pharmacological agents on renal vasculature remains to be defined. The responsiveness of vessels following acute tubular injury is most likely a strong determinant of decreased filtration rate; this too has not yet been documented. The function of the glomerular capillary may be further defined using images of individual capillaries rather than the entire glomerular tuft. It will be especially important to determine whether vasoactive agents actually alter glomerular capillary diameter or whether the mesangium contracts and serves to draw the capillaries closer together and perhaps to affect regional perfusion patterns without altering total capillary diameter, volume, or length. Observations of medullary blood flow during several states of antidiuresis, solute and water diuresis may be used to determine the effects of altered perfusion rates and pressures on net function. Finally, high-resolution video micrography may permit the visualization of the structure of the endothelial and smooth muscle cell layers as well as the glomerular epithelial cells during function and modification by pharmacological agents (Fig. 13). Such concurrent observations of function and microstructure may permit a better understanding of the cellular processes involved in the work of the kidney.

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Chemically Induced Renal Epithelial Neoplasia in Experimental Animals

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I. Introduction

The kidneys of experimental animals have been the target for the neoplastic action of over 100 different chemicals (Hamilton, 1975; Hard, 1976). A number of compounds from diverse chemical classes have induced renal neoplasms in different species of experimental animals. The types of chemicals capable of inducing renal carcinoma include indirect-acting chemical carcinogens which require metabolism to exert their carcinogenic potential, direct-acting alkylating agents, metals, and a number of compounds with poorly understood mechanisms of action. While the kidneys of experimental animals can respond to carcinogenic stimuli with the development of a number of different morphological types of neoplasms, renal epithelial neoplasms constitute the vast majority (Hard, 1976). Renal neoplasms of mesenchymal cell origin, and to a lesser extent, nephroblastomas have also been induced by carcinogenic agents. The pattern of neoplasm induction is similar to the pattern in humans, where over 90% of the diagnosed renal neoplasms are of epithelial origin (Bennington and Kradjain, 1967).

The classifications of renal epithelial neoplasms can generally be divided into two categories according to the cell of origin: renal tubule cell neoplasia, which is postulated to be derived from epithelial cells lining the proximal tubule, and transitional cell neoplasia derived from the transitional epithelial cells lining the renal pelvis.

In this article, we will discuss chemically induced renal tubular epithelial cell neoplasia in experimental animals. The discussion will focus on the types of etiological agents, their mechanisms of action, and the pathogenesis of renal epithelial neoplasia.

II. Renal Tubule Epithelial Cell Neoplasia

A. Etiological Agents

Since a large number of diverse chemicals have been shown to induce renal neoplasia, we will not discuss them all individually. Rather, we will consider general chemical classes, including nitroso compounds, halogenated hydrocarbons, polycyclic aromatic hydrocarbons, aromatic amines and amides, metal compounds, aliphatic compounds, hormones, volatile hydrocarbons, naturally occurring chemical agents, and other miscellaneous agents such as nitrilotriacetic acid (NTA).[1]

1. *Nitroso Compounds*

Nitrosamines and other nitroso derivatives have been shown to be potent renal carcinogens in experimental animals (Zak *et al.*, 1960; Magee and Barnes, 1959; Ito *et al.*, 1966; Druckery *et al.*, 1969; Hard and Butler, 1971a,b; Schmahl *et al.*, 1978). Initial investigations of dimethylnitrosamine (DMN) demonstrated its hepatocarcinogenicity (Magee and Barnes, 1959). When Magee and Barnes (1959, 1962) varied the feeding schedule of DMN to Porton rats, they noted a propensity for the development of renal neoplasia including renal adenomas and carcinomas. Short exposure to high doses of DMN (100 and 200 ppm) resulted in acute liver necrosis which was followed by repair but no hepatocellular neoplasia. This type of exposure regimen produced a high incidence of renal neoplasms in male and female rats killed 26–48 weeks after exposure (Magee and Barnes, 1962). They also speculated on the pathogenesis of these neoplasms, indicating their probable development was from large tubules with irregular epithelium which progressively increased in size to small adenomas and finally large carcinomas. They noted a similarity in histological appearance between renal adenocarcinomas induced by DMN in rats and renal adenocarcinomas seen in humans. Subsequent to this study, a number of investigators reported detailed observations on DMN-induced renal carcinogenesis (Ito *et al.*, 1966; Jasmin and Cha, 1969; Hard and Butler, 1970, 1971a,b). A single dose of DMN was found to be sufficient to induce renal carcinogenesis in rats fed protein-deficient diets (Terracini and Magee, 1964; Murphy *et al.*, 1966; McLean and Magee, 1970; Hard and Butler,

[1] Abbreviations: DENA, diethylnitrosamine; DMN, dimethylnitrosamine; DNA, deoxyribonucleic acid; EHEN, *N*-ethyl-*N*-hydroxyethylnitrosamine; FBPA, *N*-(4′-fluoro-4-biphenyl)acetamide; FNT, formic acid, 2-[4-(5-nitro-2-furyl)-2-thiazolyl]-hydrazide; NTA, Nitrilotriacetic acid; PAS, periodic acid–Schiff's reagent; TMP, 2,2,4-trimethylpentane.

1970; Hard, 1979). This regimen induces a high incidence of both mesenchymal neoplasms and epithelial neoplasms. The distribution of the type of neoplasm was dependent on the age of the animal at the time of dosing (Hard, 1979). Other nitroso compounds have also been shown to have been effective inducers of renal carcinoma (Druckery *et al.*, 1969; Lombard and Vesselinovitch, 1971; Lombard *et al.*, 1974; Fox *et al.*, 1975; Rosenberg *et al.*, 1985).

2. *Arylamines and Amides*

These compounds induce a variety of neoplasms in different organs of experimental animals (Kreik and Helgeveld, 1977, 1978). Their carcinogenicity is dependent on metabolic conversion to reactive metabolites via N-hydroxy derivatives (Weisburger and Weisburger, 1973; Kreik and Helgeveld, 1978). With respect to renal carcinogenesis, one of the most extensively studied compounds in this class is *N*-(4′-fluoro-4-biphenyl)acetamide (FBPA). Morris *et al.* (1957) first reported the renal carcinogenicity of FBPA. They developed a series of transplantable neoplasms with well-differentiated phenotypes for study (Morris *et al.*, 1970). Further morphological and histogenetic analysis of FBPA-induced renal neoplasia was reported by Reuber (1975) using Buffalo rats. The ultrastructural characteristics of FBPA-induced renal adenocarcinoma were reported by Dees *et al.* (1976). The neoplasms were characterized by abnormal brush border on all aspects of the cells and appeared to reflect a proximal tubule cell origin for the neoplasms. Subsequent studies using FBPA in a Fischer-344 rat model, detailed the enzyme histochemical patterns and histogenesis of the renal neoplasms (Heatfield *et al.*, 1976; Dees *et al.*, 1980; Hinton *et al.*, 1980). The neoplasms were proceeded in development by areas of abnormal hyperplasia and carcinoma *in situ* (microscopic neoplasms) and were further characterized as arising from proximal tubule epithelium. Other amines including acetylaminofluorene (Shinohara and Frith, 1980) and 4′-fluoro-4-aminodiphenyl (Matthews and Walpole, 1958) also induced renal neoplasia in animals.

3. *Nitrofurans*

Some nitrofurans have been shown to induce renal neoplasia in experimental animals (Cohen *et al.*, 1970; Erturk *et al.*, 1970). The neoplasms and their histogenesis have been studied and seem identical to that seen with nitrosamine and arylamide-induced renal neoplasia. The nitrofuran studied most extensively, formic acid, 2-[4-(5-nitro-2-furyl)-2-thiazolyl]-hydrazide (FNT), induced mostly renal epithelial cell neoplasia and few mesenchymal neoplasms.

4. *Metal Compounds*

A number of metal compounds have been shown to induce renal neoplasia in experimental animals (Sunderman, 1971). Lead acetate has been shown to induce renal tubular hyperplasia, renal adenomas, and adenocarcinomas in rats (Boyland *et al.*, 1962; Van Esch *et al.*, 1962; Mao and Molnar, 1967). Zollinger (1953) also demonstrated the induction of renal neoplasia in rats exposed to lead phosphates. The mechanism of lead acetate-induced renal neoplasia is unknown. Lead is not mutagenic in most test systems (Rossman, 1981). It is, however, a potent mitogen in the rat and mouse kidney (Choie and Richter, 1974), and lead decreased the fidelity of deoxyribonucleic acid (DNA) repair polymerases (Gerber *et al.*, 1980). Lead acetate can also act as a cocarcinogen to enhance the incidence of renal neoplasia and decrease the latency period for neoplasm development (Hinton *et al.*, 1979; Tanner and Lipsky, 1984).

Nickel subsulfide has also been shown to induce renal neoplasia (Jasmin and Riopelle, 1976), as has nickel carbonyl (Law *et al.*, 1972). A single dose of nickel subsulfide induced nucleolar and mitochondrial alterations in renal tubular cells which were followed, after longer time intervals, by renal adenocarcinomas.

5. *Mycotoxins*

Certain mycotoxins are potent carcinogens A great deal of study has focused on understanding aflatoxin-induced carcinogenesis. Aflatoxins, the products of the fungus *Aspergillus flavus,* are carcinogenic to a variety of organs, including kidney. Initial studies reported rare incidences of renal neoplasia in experimental animals exposed to aflatoxin B_1 (Newberne *et al.*, 1964; Madhavan and Gopalan, 1968). A subsequent study (Epstein *et al.*, 1969) reported a high incidence of renal epithelial neoplasms in male Wistar rats fed aflatoxin B_1. The neoplasms were preceded by karyomegaly and tubular hyperplasia.

Ochratoxin A, a mycotoxin produced by fungal strains within the genera *Aspergillus* and *Penicillium* (Krough, 1974), has been shown to produce renal neoplasia in male mice fed the toxin at 40 ppm (Bendele *et al.*, 1985). Ochratoxin was shown to concentrate in the liver and proximal convoluted tubule cells of the mouse kidney (Lee *et al.*, 1984). However, it is not known if metabolism occurred in the kidney cells, although this is one potential mechanism for its carcinogenicity (Bendele *et al.*, 1985).

6. *Aliphatic Compounds and Antibiotics*

The antibiotics streptozotocin and daunomycin can induce renal epithelial neoplasia in experimental animals. Streptozotocin, an antibiotic isolated

from *Streptomyces achromogenes,* is an antineoplastic agent (Evans *et al.*, 1965) which produces renal neoplasm when administered to rats (Arison and Feudale, 1965; Rakieten *et al.*, 1968; Kazumi *et al.*, 1978). Daunomycin is another antibiotic and antineoplastic agent isolated from a type of *Streptomyces*. It induces renal neoplasia in rats (Sternberg *et al.*, 1972). Both agents contain an aglycone similar to the carcinogen methylazoxymethanol (Bennington and Beckwith, 1975).

The antischistosomal drug Niridazole induces renal neoplasia in rats as well as nephrosclerotic lesions, karyomegaly, and tubular hyperplasia (Bulay *et al.*, 1979). Other aliphatic compounds such as ethyl carbonate (Urethan) (Vesselinovitch and Mihailovich, 1968), ethyl- and methylethanesulfonate (Swann and Magee, 1969), and cycasin (Spatz, 1969; Fukunishi *et al.*, 1985) produce renal neoplasia in experimental animals.

7. *Hormones*

Estrogens have been used to induce renal neoplasia in Syrian golden hamsters (Kirkman, 1959). This model has been used to study the role of hormones in the neoplastic process (Li *et al.*, 1983). It was demonstrated that estrogen-induced neoplasia in hamsters could be blocked by administration of antiestrogens which inhibit estrogen receptor binding (Li *et al.*, 1980). Evidence has also been presented for a role for estrogen metabolites (Li and Li, 1984; Liehr, 1984) and mixed-function oxidases (Li and Li, 1984) in the mechanism of estrogen-induced renal neoplasia development.

8. *Volatile Hydrocarbons*

A number of diverse volatile hydrocarbon compounds and mixtures have been shown to induce renal carcinomas in male rats (Brunner, 1984; McFarland, 1984; Craig, 1986; Eustis, 1986). Examples of carcinogenic volatile hydrocarbons are unleaded gasoline (McFarland, 1984), the synthetic propellents RJ-5 and JP-10 (Brunner, 1984, 1986), and the "pure" hydrocarbon 1,4-dichlorobenzene (Chem. Regul. Report, 1986). Neither female rats nor males and females of other species have developed renal neoplasms after exposure to volatile hydrocarbons. The induction of renal neoplasia is preceded by a characteristic nephropathy with the most consistent component being accumulation of hyaline droplets containing the male rat urinary protein, α_{2u}-globulin (Alden *et al.*, 1984; Busey and Cockrell, 1984; Phillips, 1984; Stonard *et al.*, 1985). The relationship between the acute nephropathy and the later appearance of renal adenocarcinomas is unclear. The hydrocarbons tested to date have not been genotoxic (Stott *et al.*, 1981; Loury and Butterworth, 1986; Loury *et al.*, 1986). The mechanism(s) by which they exert their carcinogenic potentials is not understood.

9. *Other Agents*

A wide variety of different chemicals can also induce renal neoplasia in experimental animals, including the halogenated hydrocarbons chloroform, hexachloro (1 : 3) butadiene, and hexachloroethane (Weisberger, 1977; IARC, 1979; Jorgenson *et al.*, 1985); the polycyclic aromatic hydrocarbon 7,12-dimethylbenz[*a*]anthracene (Huggins and Grand, 1966); the flame retardant tris(2,3-dibromopropyl)phosphate (Reznik *et al.*, 1979, 1981); the cationic chelator NTA (Alden and Kanerva, 1982a,b; NCI, 1977); and dichloroacetylene (Reichert *et al.*, 1984).

B. Gross and Morphological Characteristics of Renal Tubule Cell Adenocarcinoma

1. *Gross Features*

Renal neoplasms induced by chemical carcinogens in experimental animals can range from the limit of gross detection (<0.5 mm^2) to over 10 cm in diameter. They can occur as single or multiple masses and are often bilateral in distribution. Renal neoplasms have been described as appearing gray to white to yellow-tan in color and as involving up to two-thirds of the renal mass. The consistency varied from smooth and rubbery to rough and friable, with the cut surfaces often showing hemorrhage, necrosis, and/or cystic areas. The neoplasms are generally well circumscribed but can show areas of direct, local invasion into surrounding parenchyma. Metastasis of chemically induced renal carcinomas in experimental animals is rare.

2. *Histological Features*

The histological features of chemically induced renal adenocarcinoma in experimental animals vary, both in the appearance of the constituent cells and in their architectural organization (Bennington and Beckwith, 1975). In general, this variety is limited to granular or clear constituent cells and either a solid, papillary, or cystic organization. There does not appear to be any relationship between the morphological appearance of the adenocarcinoma and the inducing carcinogen. Individual neoplasms, however, generally demonstrate a single morphological appearance in terms of both the cell type and organizational pattern.

The predominant morphological appearance of renal adenocarcinoma cells in experimental animals is that of granular, basophilic cells. These cells are well differentiated when compared to the normal tubular epithelium (Magee and Barnes, 1962; Hard and Butler, 1971a,b; Butler, 1975; Dees *et al.*, 1976). These cells were cuboidal to columnar in shape with basophilic-

staining cytoplasm (Fig. 1). The nuclei were usually round and regular in shape with prominent nucleoli. Occasional large, atypical nuclei can be observed (Magee and Barnes, 1962). In periodic acid–Schiff's reagent (PAS)-stained sections, basement membrane material can usually be seen between and among neoplastic cells.

Some of the chemically induced neoplasms were composed of large, clear-staining cells with small hyperchromatic nuclei (Fig. 2). These cells were usually arranged in a solid pattern and are reminiscent of clear-cell adenocarcinomas prominent in humans. The clear areas of these cells are thought to be composed of abundant glycogen and lipids with scant organelles (McGiven and Ireton, 1972; Bennasch, 1974; Ericsson *et al.*, 1976; Dees *et al.*, 1980).

3. *Enzyme Histochemical Features*

The enzyme histochemical pattern observed in renal adenocarcinoma cells revealed some marked differences between those cells and normal proximal tubule epithelial cells. Adenocarcinomas induced by different carcinogens contained similar histochemical patterns for specific enzymes. Glucose-6-phosphatase usually displayed little to no reactivity in neoplastic cells (Jasmin and Riopelle, 1968; Ito, 1973; Heatfield *et al.*, 1976). Weak to absent staining activity was also noted for the mitochondrial enzyme succinic dehydrogenase (Jasmin and Riopelle, 1968; Ito, 1973; Heatfield *et*

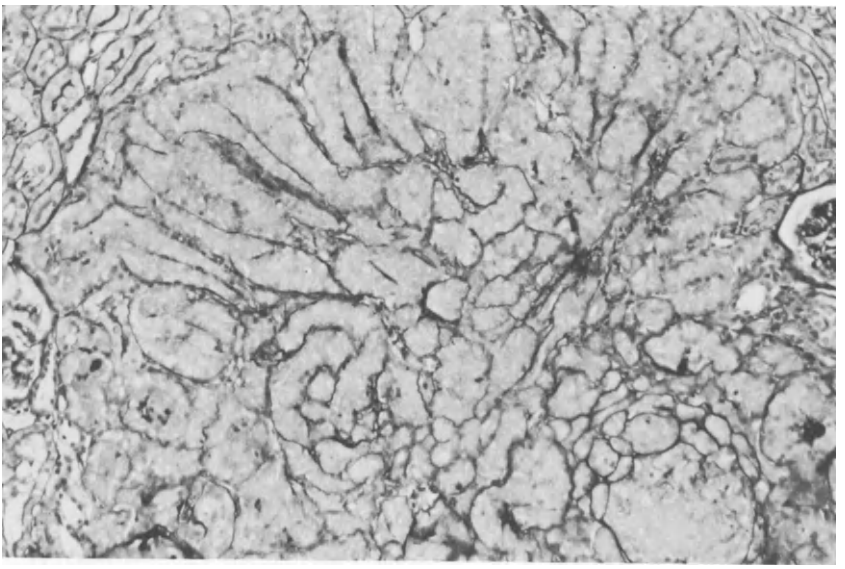

FIG. 1. Renal cell carcinoma in a Fischer-344 male rat fed FBPA. PAS, ×125.

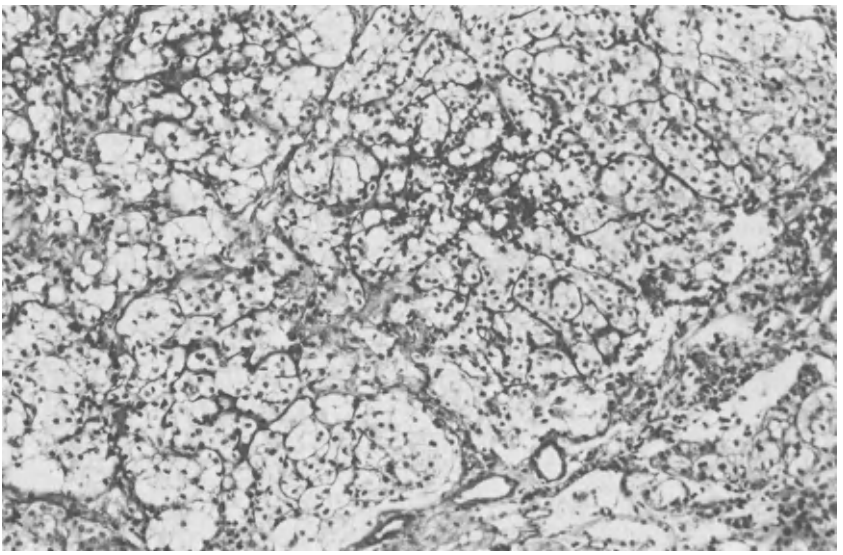

FIG. 2. Clear-cell carcinoma of the kidney induced by FBPA in a male Fischer-344 rat. PAS, ×130.

al., 1976), and the cell membrane markers alkaline phosphatase and 5′-nucleotidase (Ito *et al.*, 1966, 1974; Mao and Molner, 1967; Jasmin and Riopelle, 1968; Heatfield *et al.*, 1976). The cytoplasmic enzymes glucose-6-phosphate dehydrogenase and lactic dehydrogenase displayed moderate to intense staining activity (Jasmin and Riopelle, 1968; Heatfield *et al.*, 1976). In general, the enzyme histochemical staining parallels both functional and ultrastructural characteristics of neoplastic cells. The decreased glucose-6-phosphatase and succinic dehydrogenase activities and relatively high lactic dehydrogenase activity is indicative of a shift toward the glycolytic production of ATP in the neoplastic cells (Heatfield *et al.*, 1976). In addition, it was noted that higher levels of lactic dehydrogenase activity were seen in cells adjacent to areas of cell necrosis and may be related to a metabolic adaptation to a hypoxic environment (Heatfield *et al.*, 1976; Rosada *et al.*, 1969). The consistently high levels of glucose-6-phosphate dehydrogenase activity may indicate increased pentose shunt activity, important in the formation of NADPH (Heatfield *et al.*, 1976). The simplification of the plasma membrane surface specializations of the neoplastic cells correlates, in general, with the low levels of 5′-nucleotidase, ATPase, and alkaline phosphatase. Decreased alkaline phosphatase activity could be related to reduced transport activity in neoplastic cells (Mao and Molnar, 1967; Heatfield *et al.*, 1976).

4. *Electron Microscopic Observations*

The granular, basophilic cells comprising the majority of chemically induced renal adenocarcinomas had a rather characteristic appearance. The most constant feature observed in renal adenocarcinoma cells, from well- to poorly differentiated neoplasms, was the presence of brush border-like aggregations of microvilli (Hendry *et al.*, 1955; Oberling *et al.*, 1960; Mao and Molner, 1967; Seljelid and Ericsson, 1965; Hard and Butler, 1971a,b; Hruban *et al.*, 1973; Merkow *et al.*, 1973; Butler, 1975; Dees *et al.*, 1976, 1980). This could be noted on any aspect of the cell surface, not just surfaces bordering lumens or cystic spaces. In adjacent cells the microvilli formed complex interdigitations and/or canalicularlike structures. These microvilli often extended into invaginations of adjacent cells and appeared to be associated with pinocytotic vesicles (Morris *et al.*, 1970; Hard and Butler, 1971; Hruban *et al.*, 1973; Dees *et al.*, 1976).

Another ultrastructural feature of renal adenocarcinoma cells that appears consistent among the different reports is the presence of prominent or hypertrophied Golgi (Seljelid and Ericson, 1965; Hard and Butler, 1971; Hruban *et al.*, 1973; Dees *et al.*, 1976, 1980). Golgi cisternae were numerous and highly organized, with numerous vesicles present at the periphery of the saccules.

The mitochondria of renal adenocarcinoma cells varied in amount and organization that was related to the degree of cellular differentiation. In well-differentiated cells the mitochondria were small, round, and numerous, while in less well-differentiated cells they were elongated and convoluted (Dees *et al.*, 1976, 1980).

Nuclei were generally round and regular in shape in well-differentiated neoplastic cells and contained prominent, dense nucleoli (Riopelle and Jasmin, 1969; Hard and Butler, 1971; McGiven and Ireton, 1972; Dees *et al.*, 1976). Although the nuclei tended to remain regular in size and shape, atypia characterized by indentations, pseudoinclusions, and irregular borders were noted in less well-differentiated neoplastic cells.

Descriptions of the ultrastructural appearance of other organelles varied among studies, but was generally unremarkable, with no consistent alterations being noted.

C. Species

1. *Rats*

The rat has been the most extensively used species for the induction and study of renal carcinoma. The rat kidney is responsive to the carcinogenic

action of a wide array of chemicals, as discussed above. The incidence of spontaneous renal tubule epithelial neoplasms in most strains of rats used in toxicologic research is low (<1%) (Tarone *et al.*, 1981; Owen and Haywood, 1986; Solleveld and Boorman, 1986). This rarity of spontaneous, primary renal epithelial neoplasia is important for evaluating the neoplastic potential of chemical agents (Solleveld and Boorman, 1986). The greatest confounding issue in evaluating renal toxicity and carcinogenicity is the role of the aging rat nephropathy in the induction of neoplastic lesions. The aging rat nephropathy will be discussed in greater detail in a later section of this review (Section II,E,5), and in the review by Burek *et al.* in this volume. In short, the aging rat nephropathy destroys renal parenchyma and alters renal function (Owen and Haywood, 1986). Lesions characteristic of this condition may be hyperplastic and serve as progenitors of neoplastic cells. It is also possible that the entire condition may exert physiological stimuli conducive to promoting or enhancing chemically induced renal carcinogenesis (Trump *et al.*, 1984). The presence of the aging nephropathy makes the rat less than ideal for evaluating potential renal carcinogens and necessitates that neoplastic development by evaluated in light of this progressive disorder.

2. *Mice*

The mouse has been used less extensively than the rat for the study of renal carcinogenesis. In fact, renal tubular epithelial neoplasia is relatively rare in mice, either untreated or treated with chemical carcinogens (Stevenson and Van Haan, 1962; Guerin *et al.*, 1969; Prejean *et al.*, 1973). Renal epithelial neoplasms have been induced in mice with ethylnitrosourea (Lombard and Vesselinovitch, 1971; Lombard *et al.*, 1974), DMN (Takayama and Oota, 1963; Toth *et al.*, 1964; Terracini *et al.*, 1966), urethane (Toth *et al.*, 1961), procarbazine (Kelly *et al.*, 1969), lead acetate (Van Esch *et al.*, 1962; Van Esch and Kroes, 1969), tris(2,3-dibromopropyl)phosphate (Reznik *et al.*, 1979), 2-acetylaminofluorene (Shinohara and Frith, 1980), methylmercury chloride (Mitsumori *et al.*, 1981), and 3-methylcholanthrene (Stevenson and Van Haan, 1962). Renal neoplasms have also been reported as minor constituents of total neoplasm incidences in mice treated with some other compounds (Guerin *et al.*, 1969). The morphological appearance and biological behavior of renal epithelial neoplasms in mice is similar if not identical to that reported for renal neoplasia in rats. The incidence of spontaneous renal epithelial neoplasia in mice is rare (Lombard *et al.*, 1974; Tarone *et al.*, 1981), and mice do not appear to develop a chronic renal nephrosis as is seen in the rat.

3. *Hamsters*

Hamsters have been primarily used to study estrogen-induced renal tubule epithelial neoplasia (Kirkman, 1957, 1959) The spontaneous evidence of renal neoplasia in hamsters is rare, as is the occurrence of a chronic renal nephrosis.

D. HISTOGENESIS

Most chemical carcinogens which induce renal adenocarcinoma induce a variety of other renal tubular cell lesions, many of which have been implicated in the genesis of adenocarcinoma. Renal tubular cell lesions which have been consistently associated with chemical carcinogen exposure include karyomegaly, renal tubular cell hyperplasia, and renal adenoma (carcinoma *in situ*). A number of studies have concluded that the proximal tubule cell is the site of origin for these epithelial lesions and adenocarcinoma (Riopelle and Jasmin, 1969; Erturk *et al.*, 1970; Hard and Butler, 1971a; McGiven *et al.*, 1972; Merkow *et al.*, 1973; Butler, 1975; Stula *et al.*, 1978; Dees *et al.*, 1976; Reznik *et al.*, 1981). This localization has been based on the morphological similarity of cells in renal lesions to normal tubule epithelial cells. More detailed analyses have further refined the cell of origin of renal neoplasia with mixed results. Some investigations implicated cells in the S_1 or S_2 segment of the proximal tubule as the likely cell of origin of DMN-induced renal adenocarcinoma (Hard and Butler, 1970, 1971a). Dees *et al.* (1976) and Hinton *et al.* (1980), however, postulated that the S_3 segment of the proximal tubule was the site of origin for neoplasms induced by FBPA and lead acetate.

1. *Karyomegaly*

One of the lesions commonly associated with carcinogen treatment has been karyomegaly. These cells have been noted in the proximal tubule after exposure to a large number of carcinogens including lead acetate (Boyland *et al.*, 1962), DMN (Hard and Butler, 1971a), daunomycin (Sternberg *et al.*, 1972), 4,4′-methylene bis(2-carbomethoxyaniline) (Stula *et al.*, 1978, tris(2,3-dibromopropyl)phosphate (Reznik *et al.*, 1981), and FBPA (Dees *et al.*, 1976, 1980). The cells are usually enlarged and protruding into the tubular lumen with nuclei that may be up to three or four times as large as normal epithelial cell nuclei (Zak *et al.*, 1960; Magee and Barnes, 1962). These nuclei are often hyperchromatic with irregular borders and may contain multiple nucleoli. At times the axis of the nuclei appears to be

rotated with respect to the basal lamina. For the few chemical compounds where the histogenesis of renal adenocarcinoma has been studied in detail, karyomegaly is often seen as one of the earliest cellular alterations which can be related to carcinogen exposure. However, when exposure to the carcinogen is continuous, karyomegaly may be seen at any time that the animals are sacrificed. It is not uncommon to see karyomegaly in tubules of a kidney containing a renal adenocarcinoma. The significance of this particular lesion is unclear at the present time. It has been suggested that the appearance of karyomegaly in chemically exposed animals may be an indication that the chemical has the potential for renal carcinogenesis. It should be noted that the karyomegaly encountered in the carcinogen models is easily distinguished from the enlarged nuclei commonly seen in aged animals. Additionally, in studies where the lesions were localized with some degree of reliability, karyomegaly seems to occur in the S_3 segment of the pars recta and sometimes at the end of the proximal convoluted tubule near the S_2–S_3 junction (Dees *et al.*, 1980). Some studies have attempted to correlate karyomegaly quantitatively with the late appearance of renal adenocarcinomas in animals treated with different carcinogenic regimens (Tanner and Lipsky, 1984). Karyomegaly was seen between 4 and 8 weeks after treatment with either FBPA, lead acetate, or a combination of the two. The data from these studies failed to demonstrate a quantitative correlation between the number of karyomegalic cells and the degree of carcinogenicity of the inducing regimen (Tanner and Lipsky, 1984). For example, the animals with the largest number of renal adenocarcinomas were those receiving the combination diet; however, these animals had the least number of karyomegalic cells. It should be noted that, although there was no correlation between cell number and degree of carcinogenicity, karyomegaly was seen in all animals receiving a carcinogenic diet. No karyomegalic cells were seen in the control animals.

2. *Hyperplasia of Proximal Tubule Cells*

Proliferative lesions of tubular epithelial cell origin have been observed in experimental animals exposed to most renal carcinogens. Some of the chemicals which induced tubular hyperplasia include nitrosamines (Magee and Barnes, 1962; Hard and Butler, 1971a), arylamides (Dees *et al.*, 1976, 1980), aflatoxin B_1 (Epstein, 1969), and lead acetate (Boyland *et al.*, 1962). Tubule cell hyperplasia can occur as solid clusters of hyperplastic cells (Fig. 3) and as cells proliferating within cystic or dilated tubule profiles (Fig. 4) (Hinton *et al.*, 1980).

The cells comprising these lesions can vary in morphological appearance

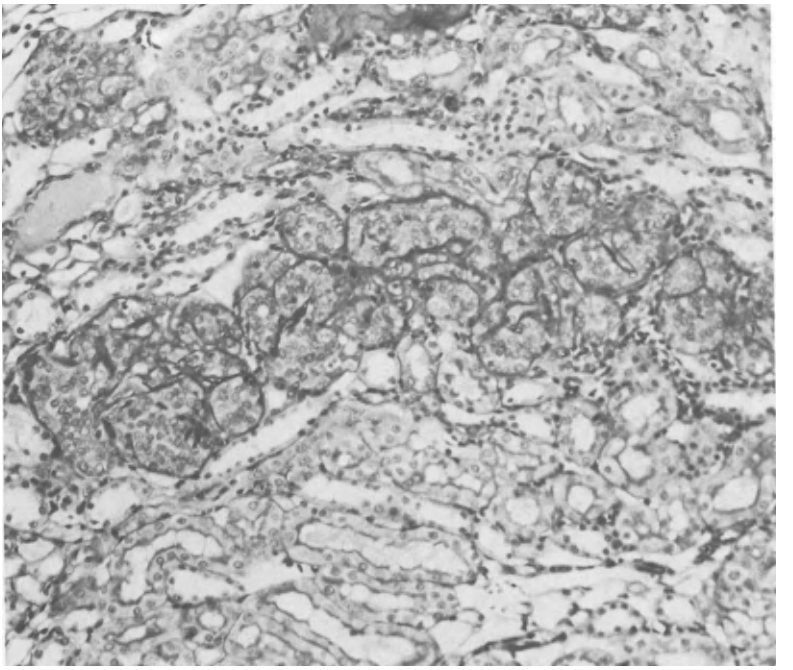

FIG. 3. Area of tubule cell hyperplasia in a male Fischer-344 rat exposed to FBPA. PAS, ×160.

but generally fall into one of two categories, based upon characteristics observed in hematoxylin- and eosin-stained sections, that is, small basophilic cells or clear cells (Fig. 5) (Riopelle and Jasmin, 1969; Erturk *et al.*, 1970; Hard and Butler, 1971b; Bulay *et al.*, 1979; Dees *et al.*, 1980). These cells generally had an increased nuclear–cytoplasmic ratio with hyperchromatic nuclear staining. Mitoses were occasionally noted in these tubule profiles. The ultrastructural characteristics of the basophilic, proliferative cells have been described in some detail (McGiven and Ireton, 1972; Dees *et al.*, 1980). One prominent characteristic of the cells was the formation of brush border on the luminal surface of hyperplastic cells and on nonluminal surfaces of cells arranged in solid patterns. The brush border was often atypical in shape. There was also a general simplification of the basal infoldings accompanied by a thickening of the basal lamina (Dees *et al.*, 1980). The nuclei were irregular in contour with invaginations and prominent nucleoli. The prominence of brush border in these cells, as well as their general ultrastructural appearance, indicates that they originated in

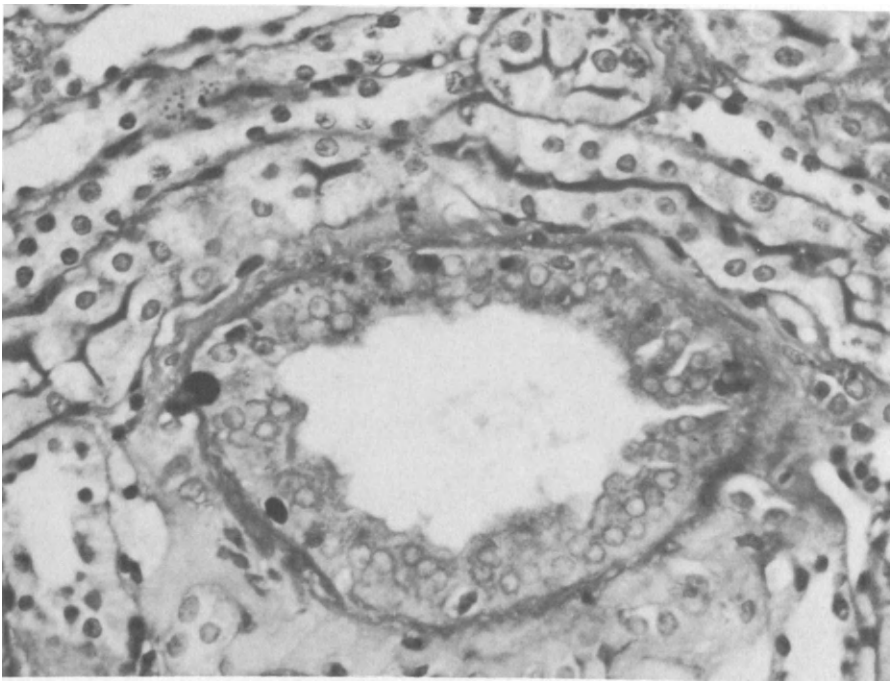

FIG. 4. Hyperplastic cells in a dilated tubule of a Fischer-344 male rat exposed to FBPA. PAS, ×160.

proximal tubules. However, as discussed above, the precise origin in terms of proximal tubular segments is debated and may vary between experimental models.

3. *Renal Tubular Adenoma (Microcarcinoma)*

Throughout the literature on renal carcinoma development, there are descriptions of small, often microscopic, nodular lesions composed of cells which appear similar, if not identical, to cells within renal carcinomas. These small nodules appear to arise from overgrowth and/or coalescence of tubular cell hyperplasias (Fig. 6). Some of these lesions appear to be multilobular with basement membrane-like material present between lobules. The nodules can also appear in a papillary pattern, often within cystic or dilated tubules. The cells comprising these small nodules are generally basophilic, with increased nuclear–cytoplasmic ratios and atypical nuclei with prominent nucleoli (Magee and Barnes, 1962; Hard and Butler, 1971b; Butler, 1975; Dees *et al.*, 1980). Clear-cell variants of this lesion also occur. At the ultrastructural level, the cells were characterized by

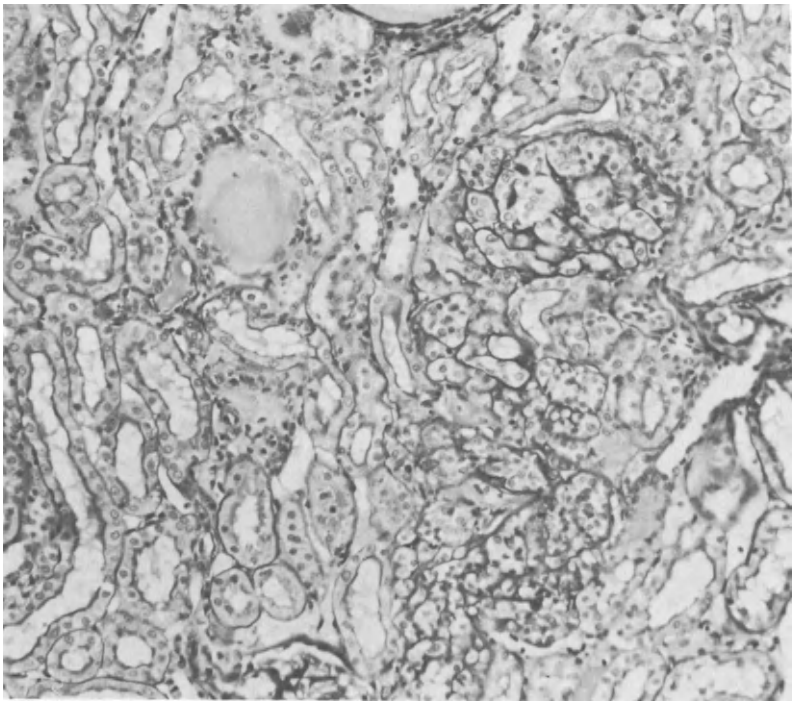

FIG. 5. Clear-cell hyperplasia in a Fischer-344 male rat exposed to FBPA. PAS, ×160.

abnormal brush border development (Dees *et al.*, 1980; Reznik *et al.*, 1981). Brush border-like processes could occur on any aspect of the neoplastic cell surface. Frequently, the structures appeared to invaginate in vacuoles or infoldings of the plasma membrane of adjacent cells. This characteristic suggested that the probable cell of origin of the neoplastic cells is the proximal tubule epithelial cell. Other ultrastructural features were less consistent in the neoplastic cells. In general, the Golgi were well developed and relatively prominent. Junctional complexes were evident between cells and included tight and intermediate junctions, and desmosomes. Microbodies, rough endoplasmic reticulum, and lysosomes were evident and unremarkable. Mitochondrial morphology varied from small, round forms to elongated, bizarre configurations. The basal infoldings of cell membranes seen in normal proximal tubule cells were often lacking or greatly simplified in cells which abutted areas of basal lamina deposition.

It has been suggested by different investigators that lesions classified as renal adenomas in experimental animals are more accurately described as carcinomas *in situ* or microcarcinomas (Bennington and Kradjian, 1967;

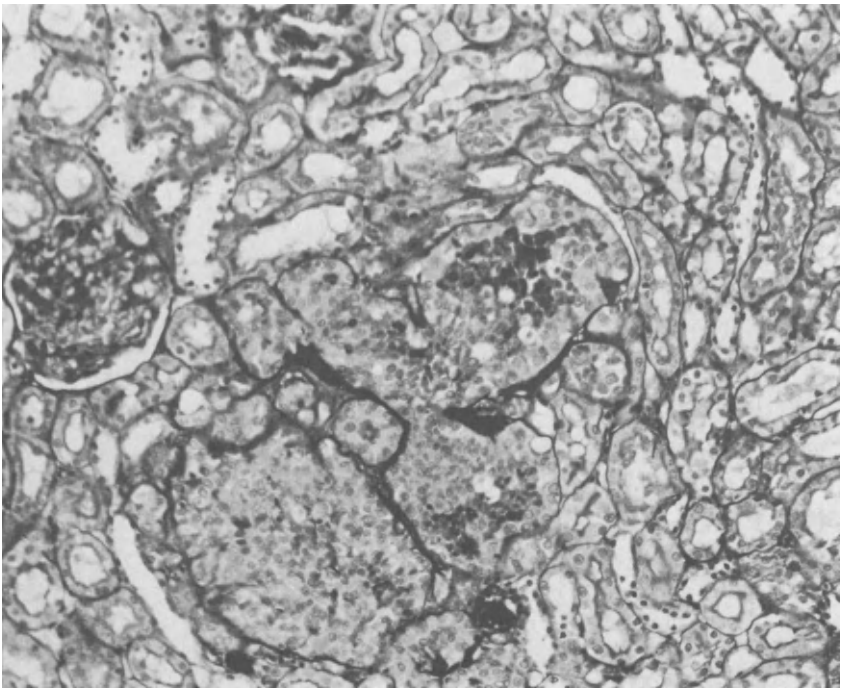

FIG. 6. Small basophilic adenoma in a Fischer-344 male rat exposed to FBPA. PAS, ×160.

Riopelle and Jasmin, 1969; Erturk *et al.*, 1970; Butler, 1975; Dees *et al.*, 1980; Hiasa *et al.*, 1983). Evidence for this classification is based on morphological and behavioral observations. The cells comprising the small nodules were strikingly similar if not identical in morphology to cells comprising adenocarcinomas. This was true at the light and electron microscopic levels. Nuclear pleomorphism, enlarged nucleoli, and the presence of mitosis are a few of the cellular characteristics shared by the adenomas and adenocarcinomas. In addition, it is not uncommon to observe areas of focal necrosis and/or calcification in the small nodules (Fig. 7). Enzyme histochemical patterns and ultrastructural features for adenomas and adenocarcinomas are also strikingly similar (Heatfield *et al.*, 1976). There are also data to suggest that the small nodules are readily transplantable and develop in the recipient animal into nodules with a morphological appearance of well-differentiated adenocarcinoma (Heatfield *et al.*, 1976; Dees *et al.*, 1980).

4. *Role of Tubular Epithelial Lesions in Adenocarcinoma Development*

Examination of many different models for renal adenocarcinoma development, utilizing a wide variety of chemical carcinogens in different

species, reveals similarity in the types of epithelial lesions induced. These lesions generally fall into the categories discussed above. In experimental protocols designed to analyze sequentially renal adenocarcinoma development, a consistent pattern emerges. Karyomegaly is often seen relatively early after exposure to carcinogenic agents. This lesion, however, does not appear to play a direct role in the carcinogenic process (Tanner and Lipsky, 1984). There is no evidence that karyomegalic cells can divide or give rise to any other renal epithelial lesions. This lesion arises in the proximal tubule, probably in all three segments. Tubular cell hyperplasia also arises within proximal tubule profiles and has been reported, with different chemicals, to originate in either the S_1, S_2, or the S_3 segment. The distinction between larger, advanced hyperplasia and small adenoma is difficult, if not impossible, to make at the light and electron microscopic levels. This morphologic similarity is true for solid, granular-cell hyperplasias and adenomas and for clear-cell hyperplasias and adenomas. Little is known about the relationship of clear-cell lesions to the more granular-cell lesions. In the rat and mouse, the granular-cell lesions are the predominant type induced by chemical carcinogen treatment. However, clear-cell lesions fall into the same general categories of hyperplasias and adenomas as the granular-cell lesions. There do not appear to be intermediate forms, indicating a basic difference in phenotypic expression between clear and granular cells. The relationship between these two distinct morphological entities awaits discovery.

The overwhelming evidence from morphological studies indicated that chemically induced renal carcinoma originates from proximal tubule epithelial cells. The initiated cells in this tubular segment proliferate into hyperplastic lesions, at least some of which can serve as the developmental bed for further growth to microcarcinomas (adenomas). The eventual development of metastatic carcinoma would seem to involve the further growth and neoplastic progression of microcarcinomas.

E. Mechanisms of Chemically Induced Renal Neoplasia in Experimental Animals

1. *Genetic Mechanisms*

A majority of renal carcinogens studied appear to act via a genotoxic/mutagenic mechanism; that is, the chemicals or their metabolites interact with DNA, resulting in a heritable alteration in the target cells (genotoxic mechanism). A variety of thorough reviews in the mechanisms of chemical carcinogenesis are available (Miller, 1978; Miller and Miller, 1981; Weisburger, 1981). In general, a chemical carcinogen and/or its metabolite covalently binds to one of the bases in the DNA of a target cell. The

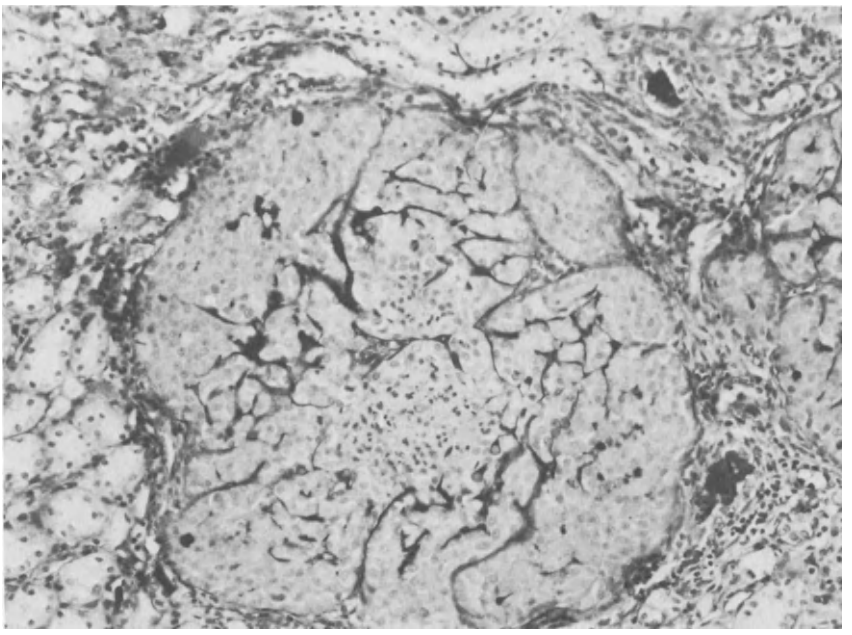

FIG. 7. Renal adenoma with a central area of necrosis in a Fischer-344 male rat exposed to FBPA. PAS, ×160.

chemical–DNA adduct can then be translated into a heritable alteration, commonly believed to be a mutation, after DNA replication and a round of cell division are completed. This process results in an initiated "altered cell" believed to be a precursor for carcinoma development (Farber, 1980, 1984). A number of renal carcinogens including most nitrosamines, arylamines and amides, and polycyclic hydrocarbons are in this category. For individual chemicals a variety of specific enzymatic interactions may occur enabling the formation of a metabolite–DNA adduct. These reactions are beyond the scope of this review; however, the literature in this area is voluminous and readily available to the interested reader.

2. *Epigenetic Mechanisms*

Epigenetic mechanisms of chemical carcinogen action are far less common and less understood. Epigenetic hypotheses for carcinogen action are generally invoked when carcinogenesis results from exposure of experimental animals to chemicals which have not been shown to be mutagenic. One good example is the chemical NTA. NTA is not metabolized or biotransformed and is nonmutagenic in experimental animals (Michaels and

Wakim, 1971). When fed at relatively high concentrations, NTA induces renal neoplasia in rats and mice (NCI, 1977; Alden and Kanerva, 1982a). The neoplasia is preceded by the development of alterations in urinary cation balance and renal tubular hyperplasia (Anderson *et al.*, 1982; Alden and Kanerva, 1982b). It is this alteration in cation balance and/or the presence of chronic injury and regeneration in the proximal tubule that are hypothesized as contributing to renal carcinoma development. This is in the absence of any detectable genotoxicity.

3. *Enhancement and Promotion*

That chemical carcinogenesis could be divided into distinct stages was first described by Friedewald and Rous (1944) for mouse skin. Subsequent investigations have repeatedly demonstrated the multistage nature of chemical carcinogenesis in a number of organ systems including skin (Berenblum and Shubik, 19479), liver (Peraino *et al.*, 1973; Pitot and Sirica, 1980), and others. In general, multistage carcinogenesis can be separated into two distinct phases: initiation and promotion. Promoting agents are not carcinogenic and can only induce neoplasia if they are administered subsequently to administration of a chemical carcinogen.

A number of chemical compounds have been examined for the ability to act as promoters of renal carcinoma development. The design of most of the studies of these compounds precludes the classification of their action as promoters; however, neoplastic enhancement did occur. One study (Rosenberg *et al.*, 1985) has produced results consistent with the classical definition of neoplastic promotion utilizing exposure to nicotinamide as the promoting stimulus. In this study design, male Fischer-344 rats were given a single intraperitoneal injection of diethylnitrosamine (DENA) (25 mg/kg body weight) 24 hours after a 70% partial hepatectomy. Two weeks after the DENA treatment, the rats were given nicotinamide (6.7 or 30 m*M*) in the drinking water for 18 months. Appropriate control groups were used to assess the effect of nicotinamide in the absence of DENA treatment and the effect of DENA alone. One renal neoplasm was found among the 20 rats comprising the two control groups. There was a statistically significant increase in renal neoplasms observed in the two experimental groups: a 59% incidence when nicotinamide was given at 30 m*M* and a 28% incidence when nicotinamide was given at 6.7 m*M*. The results of the study are consistent with the role of nicotinamide as a promoter of DENA-initiated renal neoplasia. Nicotinamide, by itself, was not carcinogenic for rat kidneys and appeared to be nontoxic (Rosenberg *et al.*, 1985). The mechanism of action, however, remains unclear. It should also be noted that other studies showed conflicting results of nicotinamide on renal carcino-

genesis. It decreased the incidence of kidney neoplasms induced by streptozotocin (Rakieten *et al.*, 1971) but increased the incidence of renal neoplasms induced by prenatal DENA treatment (Schoental, 1977).

A number of other compounds have been evaluated for their effects on renal carcinogenesis including lead acetate (Hiasa *et al.*, 1983), β-cyclodextrin (Hiasa *et al.*, 1982), and NTA (Hiasa *et al.*, 1984a). These studies utilized 2-week dietary administration of *N*-ethyl-*N*-hydroxyethylnitrosamine (EHEN) as the initiating protocol followed by continuous administration of one of the three aforementioned compounds (for 20–30 weeks) as the enhancing–promoting regimen. Lead acetate, β-cyclodextrin, and NTA all enhanced the incidence and number of renal neoplasms induced by EHEN exposure. The data from these studies are summarized in Table I. While these protocols clearly lead to enhanced renal tumorigenicity, the study designs and results do not allow for the designation of any of the three compounds as promoters of renal carcinogenesis, at least according to the classical definition of a promoter. In all three studies, EHEN treatment alone resulted in a significant incidence of renal neoplasia (33–50%) (Hiasa *et al.*, 1982, 1983, 1984a). Therefore, the three compounds being evaluated as "promoters" were more properly enhancing

TABLE I

INCIDENCE OF RENAL NEOPLASMS IN EHEN-INDUCED RATS

Study	Number of renal neoplasms	Rats with neoplasms (%)
Hiasa *et al.* (1982)		
EHEN (1,000 ppm)	12	50
EHEN (1,000 ppm) + β-cyclodextrin	32	100
EHEN (500 ppm)	0	0
EHEN (500 ppm) +β-cyclodextrin	8	30
β-cyclodextrin	0	0
Hiasa *et al.* (1983)		
EHEN (1,000 ppm)	12	50
EHEN (1,000 ppm) + lead acetate (1,000 ppm)	51	100
EHEN (500 ppm)	0	0
EHEN (500 ppm) + lead acetate (1,000 ppm)	16	45
Lead acetate	0	0
Hiasa *et al.* (1984a)		
EHEN (1,000 ppm)	27	33
EHEN (1,000 ppm) + NTA (10,000 ppm)	304	100
EHEN (1,000 ppm) + NTA (500 ppm)	31	39
NTA (10,000 ppm)	0	0
NTA (500 ppm)	0	0

the existing carcinogenic development induced by EHEN. Regardless of the terminology used, lead acetate, β-cyclodextrin, and NTA increased the incidence and number of renal neoplasms occurring during EHEN carcinogenesis. Lead acetate has also been shown to increase the number of preneoplastic renal lesions induced by FBPA (Tanner and Lipsky, 1984). Several other compounds have also been shown to enhance renal neoplasia. These chemicals include ethylmethanesulfonate (Montesano *et al.*, 1974), *N*-(3,5-dichlorophenyl)succinimide (Ito *et al.*, 1974; Shinohara *et al.*, 1977; Shirai *et al.*, 1984), potassium bromate (Kurokawa *et al.*, 1985), citrinin (Shinohara *et al.*, 1976), sodium arsenate (Shirachi *et al.*, 1983), and DL-serine (Hiasa *et al.*, 1984b).

4. *Toxicity and Renal Neoplasia Development*

At the present time, it is difficult to estabish a definitive causal link between chemically induced renal toxicity (acute or chronic) and renal neoplasia. Most renal carcinogens induce toxicity in proximal tubule epithelial cells. In studies where the carcinogen is administered continuously, the toxic lesions precede and occur along with the development of neoplasia. The toxicity and carcinogenicity of DMN, however, could be dissociated in models using a single intraperitoneal injection of DMN to induce neoplasia (Swann and McLean, 1968; Hard and Butler, 1970; Evarts *et al.*, 1982). The case for a central role of renal toxicity in the carcinogenic process is more compelling for the neoplastic potential of nongenotoxic agents such as NTA and volatile hydrocarbons.

For example, NTA produces distinctive proximal tubule cell toxicity after acute and chronic exposure in rats. The lesions include vacuolated epithelium, occasional cell necrosis, and tubular cell hyperplasia (Nixon *et al.*, 1972; Alden *et al.*, 1981; Alden and Kanerva, 1982b). The acute, toxic lesions were dose dependent and not observed in rats given NTA at levels up to 0.1 (3.7 mmol/kg) to 0.5% (Mahaffey and Goyer, 1972; Merski, 1981). In addition, these toxic effects (in a chronic protocol) were reversible after 18 months of treatment and resulted in decreased neoplasm incidence (Alden and Kanerva, 1982b). The toxicity, and presumably the carcinogenicity, of NTA has been hypothesized to act via alterations in divalent cation balance (Anderson and Kanerva, 1978; Anderson *et al.*, 1982). This results in increased levels of Zn^{2+} and Ca^{2+}. Such a persistent increase in divalent cations, such as Ca^{2+}, could provide a constant stimulus for cell proliferation in the proximal tubule, potentially leading to neoplasia development. As noted above, however, the data to support such a hypothesis are incomplete. This could be an interesting and relevant area for further research on the relationship between renal toxicity and carcino-

genicity. It should also be noted that the vacuolated lesions characteristic of NTA toxicity primarily involve fluid accumulation, presumably in the lysosomal compartment. The relationship between lysosomal distention and neoplasia development needs further investigation.

The volatile hydrocarbons also produce acute and chronic toxicity involving the accumulation of protein within the lysosomal compartment. The acute toxicity induced by volatile hydrocarbons includes lysosomal distention and cell degeneration (Alden *et al.*, 1984; Busey and Cockrell, 1984; Short *et al.*, 1986). When the hydrocarbon 2,2,4-trimethylpentane (TMP) was administered to male rats, the acute toxicity was also accompanied by an increase in DNA synthesis in cells lining the S_2 and S_3 segments of the proximal tubules (Short *et al.*, 1986). The mechanism of hydrocarbon toxicity is not understood, but appears to be related to the accumulation of the male rat urinary protein α_{2u}-globulin in lysosomes of proximal tubule epithelial cells. Studies using an *in vitro* model system demonstrated that α_{2u}-globulin was toxic to proximal tubule cells isolated from male rats (Lipsky *et al.*, 1987). This toxicity was only seen at high concentrations of α_{2u}-globulin (3 mg/ml). This would appear to correlate with the cell degeneration, necrosis, and compensatory increases in DNA synthesis seen in proximal tubules of rats exposed to TMP (Short *et al.*, 1986). A chronic cycle of cell degeneration and regeneration could be related to the mechanisms of weak carcinogenic activity of volatile hydrocarbons (Short *et al.*, 1986).

5. *Old Rat Nephropathy and Renal Neoplasia*

It is well known that the aging male rat exhibits a morphologically striking lesion, often termed glomerular nephrosis or old rat nephropathy (Andrew and Pruett, 1957; Bras, 1969; Hirokawa, 1975; Bolton *et al.*, 1976; Haley and Bulger, 1983). It is also clear that, regardless of the strain, the male is more severely affected than the female, although old rat nephropathy is by no means absent in the female.

Grossly, the lesion is characterized by large, pale kidneys with a knobby surface. These multiple surface elevations correspond to hypertrophied tubules, and the intervening depressions to tubular atrophy. By light microscopy, the lesion is characterized by variable amounts of hyalinization of the renal corpuscles, with abundant PAS-positive deposits. In some studies, immunoglobulin deposition has been demonstrated by immunofluorescence. The proximal tubule shows a variety of changes including severe atrophy, apparently beginning in the S_1 segment, and areas with increased cell division. In the atrophic areas the cells are low-cuboidal in shape and have a basophilic hue, probably due to the high nuclear–cytoplasmic ratio. The basement membranes in these regions are very thick

and often serrated. The interstitium contains abundant connective tissue with increased amounts of collagen and a variety of chronic inflammatory cells including both lymphocytes and plasma cells. The distal portions of the nephron, especially in the cortex and outer stripe, contain abundant colloid casts that give a thyroidlike appearance to the kidney. The lesion is characterized by alternating involved and noninvolved regions, creating a lobulated appearance. Although in the present observations the medium-sized and small arteries appeared thickened, the literature in general agrees that vascular changes are not a prominent feature.

Studies of Haley and Bulger (1983) are compatible with earlier observations which indicate that the earliest changes occur in the renal corpuscle. Changes in glomerular blood flow and subsequently in peritubular capillary flow could well account for the segmental distribution of the subsequent lesions. Other mechanisms proposed to account for the old rat nephropathy include renal ischemia due to vascular damage, immunopathological mechanisms, and chronic pyelonephritis. With regard to chronic pyelonephritis, it is interesting that rats maintained in a germ-free environment have a markedly reduced incidence of old rat nephropathy.

The significance of old rat nephropathy in carcinogenesis in the male rat kidney remains a mystery. Based on reports of greatly increased adenocarcinoma incidence in human dialysis patients (Hughson *et al.*, 1980; Ishikawa *et al.*, 1980), one could argue that the presence of old rat nephropathy could well foster or promote the development of renal adenocarcinomas. At the same time, the lesion can be morphologically distinguished from both preneoplastic and neoplastic lesions induced in the male rat by several model carcinogens. However, the increased mitotic rates and chronic inflammation seen with the old rat lesion, along with current knowledge concerning initiation–promotion, suggests that this lesion might well act as a promotional and/or cocarcinogenic stimulus.

The similarity of old rat nephropathy to human arterial and/or arteriolar nephrosclerosis and/or chronic pyelonephritis is at least superficially striking. As in the rat, with age the human kidney exhibits a decreasing glomerular filtration rate, a diminished capacity to conserve sodium, and a significantly diminished ability to concentrate urine (Epstein, 1979). As in the rat, the glomeruli in human kidney show sclerotic changes with variable amounts of tubular atrophy and interstitial inflammation. But perhaps different from the rat, the principal lesions appear to involve the blood vessels.

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