LOCALIZATION AND FATE OF ESCHERICHIA COLI IN HEMATOGENOUS PYELONEPHRITIS*, ‡

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PLATES 33 AND 34

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Elucidation of mechanisms responsible for the development of chronic pyelonephritis is necessary if its development is to be prevented or if therapy is to extend beyond the stage of empiricism. "Obstructive uropathy" is unquestionably the most common factor which enables Gram-negative bacilli initially to localize within the kidney and bring about acute pyelonephritis. Such obstruction may be either intrarenal or extrarenal (1, 2). Many of the factors which predispose to acute experimental pyelonephritis are potentially capable of producing intrarenal hydronephrosis. These factors include the induction of scars within the renal medulla by electrocautery (3), the production of scars following healing of staphylococcal renal infections (4), the production of interstitial edema and vascular engorgement by means of renal massage (5), or the causing of renal lesions either by means of specific toxins (6) or deficiency states such as potassium depletion (7).

Acute infection in the presence of continued significant obstructive uropathy results in chronic pyelonephritis with progressive destruction of renal parenchyma. But additional factors need to be considered in the pathogenesis of chronic pyelonephritis since obstruction is not always demonstrable. Local sequestration and proliferation of bacteria within the apex and fornix of the renal pelvic lumen following introduction of bacteria into the bladder has been reported by Andersen and Jackson to result in chronic non-obstructive pyelonephritis (8). We have demonstrated that chronicity in hematogenous pyelonephritis due to *Klebsiella pneumoniae* type C in rats relates to failure of animals to evoke an immune response against the capsular antigen (9).

The following experiments were designed to examine the possibility that

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chronic pyelonephritis also might be a response to degradation products of the bacteria initially responsible for the acute episode. The localization and fate of *Escherichia coli* and its antigenic residue following experimental hematogenous pyelonephritis was studied. Results of these studies demonstrated that acute hematogenous experimental pyelonephritis in rats due to *E. coli* O-111:B4 healed spontaneously, despite the observation that the sterile scars retained large amounts of amorphous bacterial antigens. Thus, chronic pyelonephritis did not result from the persistence of the antigenic residua of the initial infecting organism, but instead these antigens may constitute the major antigenic mass responsible for the development of the specific immunity observed in hematogenous experimental *E. coli* pyelonephritis.

Materials and Methods

Experimental hematogenous pyelonephritis was produced in male rats or the Sprague-Dawley strain obtained from Holtzman Farms, weighing 200 to 300 gm, by means of a modified technique originally described by Braude, Shapiro, and Siemienski (5). The modifications are described in detail elsewhere (9).

A serologically specific strain of *Escherichia coli* was used to enable localization by means of the fluorescent antibody technique of Coons and Kaplan (10). The strain of *E. coli* (O-111:B4) was obtained from Dr. Richard T. Smith (originally obtained from Dr. P. R. Edwards). Boiled, alcohol-acetone–extracted organisms were used for immunization. Suspensions of *E. coli* were prepared for use as antigens as follows: Flasks containing 20.5 liters of a buffered inorganic medium were inoculated with the *E. coli* O-111:B4 and incubated at 25°C with continuous agitation for 48 hours (11). The bacteria were then sedimented by centrifugation and suspended in an equal volume of 0.85 per cent saline solution and stored at 4°C for 48 hours. The saline suspensions were placed in a boiling water bath for 2 hours to inactivate both the H and L antigens. The boiled organisms were sedimented, the supernate was discarded, then 50 ml of 95 per cent ethyl alcohol was added, and the tubes were incubated at 37°C overnight. The organisms were then sedimented, and after decanting the supernatant, reincubated with 50 ml of 95 per cent alcohol for 4 hours. Following centrifugation and decantation of the alcohol, the organisms were then suspended in 50 ml of acetone, recentrifuged, washed once with acetone, and finally dried for storage.

Rabbits weighing 3 kg received increasing quantities (0.5 to 10.0 mg) of boiled, alcohol-acetoned extracted antigen by intravenous injection over a 3 week period. The total quantity of antigen administered was 50 mg. Antibody titers were determined by agglutination procedures as follows: Twofold serial dilutions of serum were made in saline (0.5 ml volume). An equal volume of antigen solution containing 0.5 mg of antigen was added to each tube. Tubes were incubated at 56°C for 30 minutes and refrigerated for 48 hours at 4°C and the degree of macroscopic agglutination noted. Antisera obtained ranged in titer from 1:512 to 1:4096. The antisera did not cross-react with boiled, alcohol-acetone–extract *E. coli* O-55:B5. The antisera was preserved with 1.0 ml of 1/1000 merthiolate per 10 ml of serum and stored at −40°C.

The globulin fraction was obtained from this antiserum by ammonium sulfate fractionation and conjugated with fluorescein isothiocyanate according to the methods of Riggs and coworkers (12). Fluorescein isothiocyanate (BBL® Lot 11059) was used. The conjugated antisera was then filtered through a 0.45 micron Millipore filter and stored at −40°C until ready for use. Antisera were absorbed with rat kidney suspension prepared according to the technique of Kaplan (13). The absorbed fluorescein-antiserum conjugate was filtered through a 0.015 porosity Selas filter before use.

Experimental animals were sacrificed at intervals varying from 10 minutes to 6 months. Urine was obtained for culture at autopsy. The kidneys were removed aseptically and each was divided into three portions: One was ground up for quantitative cultures, the second was fixed in formalin for routine hematoxylin and eosin histologic sections, and the third was quickly frozen for preparation of immunofluorescent stained sections. The portion for fluorescent antibody staining was placed in a test tube, frozen in an alcohol-dry ice bath and stored at −40°C until sectioned. Sections were cut at 4 μ in a cryostatic cabinet at −15° to −20°C, then thawed on a slide, fixed in acetone for 10 minutes, and dried in a 37°C incubator for 30 minutes. Sections were stored over drierite at 4°C for periods of 1 week or less until stained. One drop of the absorbed conjugate was placed on the dried sections and allowed to react for 20 to 30 minutes within a moist chamber. The excess fluorescent antibody conjugate was washed off with a small amount of pH 7.0 buffered saline, then washed off with successive fresh portions of buffered saline for five 2-minute periods. The sections were drained and mounted in buffered glycerol.

The fluorescent antibody stained sections were examined by means of ultraviolet light provided by either a General Electric AH*6 1000 watt lamp or an Osram HBO 200 watt lamp. A 2 mm thick Corning 5840 glass filtered the light entering the substage condenser. Both Bausch & Lomb cardioid and Reichert darkfield condensers were used. A Wratten 2B ultraviolet filter was used in the ocular. Super Anscochrome daylight film was used for photographs.

Bacterial counts of urine were done by incorporating 0.1 to 1.0 ml of urine into trypticase soy agar "pour-plates." Bacterial counts of renal tissue were done by grinding one-third of a kidney in 1.0 ml of trypticase soy broth and sea sand with a mortar and pestle as described previously (9). Sections adjacent to those obtained for staining with the fluorescent antibody were streaked on eosin–methylene blue agar as another bacteriological control.

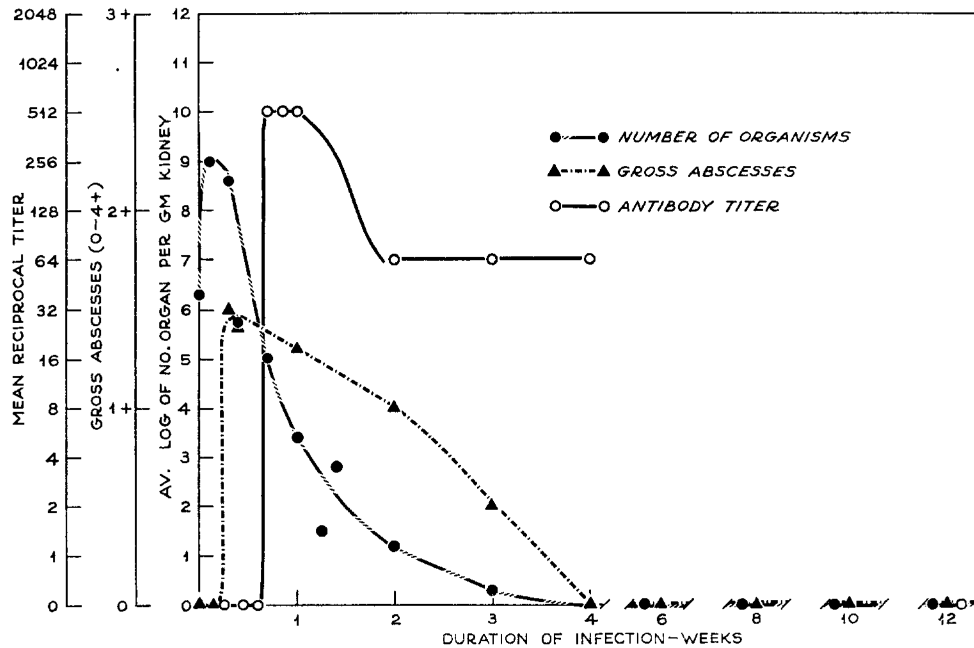
Experimental controls included rats which received (*a*) saline solution, (*b*) sterile trypticase soy broth, (*c*) the Seitz filtrate of the 18 hour cultures of *E. coli* O-111:B4, (*d*) boiled alcohol-acetone extracted *E. coli* O-111:B4 followed by renal massage and (*e*) viable *E. coli* O-111:B4 without renal massage. Controls for the fluorescent antibody procedure included (*a*) examination of tissue sections after treating with non-fluorescein labeled antiserum to detect autofluorescence and (*b*) elimination of fixation of the fluorescein-labeled antiserum by "pre-staining" with non-labeled antiserum to exclude non-specific precipitation of the labeled antiserum.

RESULTS

The kidneys of animals sacrificed during the interval from 10 to 60 minutes following completion of the period of renal massage contained relatively large numbers of *E. coli* (10^6 organisms per gm of kidney, wet weight) (Text-fig. 1). These organisms, which could be readily seen as short rods by the fluorescent antibody technique, were found individually within blood vessels in the glomeruli as well as within blood vessels in the medulla. Single organisms occurred in the interstitial tissue adjacent to blood vessels (Fig. 1). Organisms were not found in extravascular areas within glomeruli. Also, organisms were not present within the renal tubules, and urine cultures were uniformly sterile. By 60 minutes, bacterial multiplication within the interstitial tissue was detected with microcolonies consisting of 4 to 16 discrete short rods developing (Fig. 2). Leucocytic infiltration around colonies was not apparent during this interval. Bacterial multiplication also began within the glomeruli; however, the number of organisms usually was either 2 or 4.

Kidneys were obtained from animals sacrificed at 4, 6, 8, 12, 18, and 24 hours

after infection. The number of viable *E. coli* O-111:B4 increased during this interval to 10^9 organisms per gm of kidney. Likewise, during this period bladder urine cultures became positive. Microscopic examination of kidney sections obtained at these intervals showed progressive bacterial multiplication in the interstitial tissue with the development of microabscesses which contained several hundred discrete organisms (Fig. 3). Organisms which had begun to multiply within the glomeruli underwent degeneration with loss of structural



TEXT-FIG. 1. Acute hematogenous pyelonephritis in rats (*E. coli* O-111:B4).

details (Fig. 4). The glomeruli usually were entirely clear of material which localized the fluorescent anti-*E. coli* O-111:B4 antiserum by 24 hours.

During the period from 2 days to 14 days, gross abscesses were apparent. These lesions were typically conical shaped and extended down to the renal papillae. Intense polymorphonuclear leucocytic infiltration, tubular dilatation, and polymorphonuclear leucocytic casts had developed. The areas of active pyelonephritis demonstrated intense localization of the anti-*E. coli* O-111:B4 antiserum (Fig. 5). Renal tubules which drained from the infected areas were often filled with organisms, while adjacent tubules might be entirely clear (Figs. 6, 7). Toward the end of the 1st week, careful examination of the areas of acute infection revealed localized fluorescence which had an amorphous pattern

distinct from the morphologically discrete organisms. This material was immunologically specific, hence assumed to represent bacterial antigen (either the O or the B antigen) which persisted with the breakdown of organisms within the lesion.

Between 2 and 4 weeks following infection, bladder urine cultures became sterile though *E. coli* O-111:B4 could be isolated from the kidneys. In the gross the kidneys appeared to be healing with the development of pitted areas on the cortical surfaces.

Four weeks following the acute *E. coli* O-111:B4 infection, the kidneys uniformly contained numerous pitted and scarred areas without gross evidence to suggest active infection. Cultures of these kidneys were uniformly sterile and remained so in animals sacrificed at monthly intervals up to 6 months. Examination of hematoxylin and eosin sections revealed scarring with crowding of glomeruli, loss of tubules, and moderate to minimal mononuclear and plasma cell infiltration. These areas still contained large amounts of amorphous material which stained specifically with the anti-*E. coli* O-111:B4 antiserum (Fig. 8). No morphologically discrete organisms could be seen in these areas and the consecutive serial sections on both sides of such an area were culturally sterile. Specific immunofluorescence could also be observed in occasional tubules arising from these areas. Thus, bacterial antigens persisted in areas of healing acute infection which had become culturally sterile.

The specific antigens could not be detected in scars at approximately the 10th week following acute infection; at this time an autofluorescence appeared in these areas which rendered specific localization of the amorphous antigen equivocal.

DISCUSSION

The localization and fate of *E. coli* O-111:B4 during the course of acute hematogenous pyelonephritis has been defined by means of fluorescent antibody staining which enables visualization both of intact organisms and nonviable bacterial antigens. Bacterial multiplication within the glomeruli was abortive during the first few hours after infection, and a glomerular inflammatory response was not manifest. After the first 24 hours organisms were not again seen within the glomerulus. Likewise, multiplication was not observed within renal tubular cells. Active bacterial multiplication was apparent only after organisms had moved through vascular walls into the interstitial tissue. The mechanism whereby renal massage predisposed to bacterial localization has not been defined; however, a secondary alteration in peritubular blood flow with relative stasis might favor movement of organisms into the interstitium. Once organisms were present in the interstitium, multiplication was as rapid as noted *in vitro* with doubling times of approximately 20 minutes (14). Organisms did not appear within the lumina of tubules until microabscesses

were well developed. Thus, bacteriuria in hematogenous experimental pyelonephritis appeared relatively late (4 to 24 hours) and represented secondary invasion from outside the tubule rather than either multiplication within the tubular cells or glomerular filtration (Table I). The failure to observe filtration of bacteria confirmed the observation of Monma (15).

The kidneys spontaneously became sterile when studied by the usual bacteriological techniques. But, while sterile, the scars contained large amounts of bacterial antigens which were specifically stained with the antiserum prepared against the O and B antigens of *E. coli* O-111:B4. The O antigens characteristically have "endotoxic" properties and are often described as "endotoxins." Endotoxins have many physiological and immunological activities including the capacity for eliciting both immediate and delayed hypersensitivity and the enhancement of immune responses to a variety of antigens (16). Kováts has reported that typhoid endotoxin can modify homologous proteins to become antigenic and thus to produce a hypersensitive state resembling an autoimmune process (17). Kalmanson and Guze failed to demonstrate antikidney antibodies in rats with chronic *Streptococcus faecalis* pyelonephritis (18). These observations do not necessarily millitate against the role of autoimmunity in chronic pyelonephritis since *Streptococcus faecalis* differs from the Gram-negative bacteria capable of producing pyelonephritis in that it contains little if any endotoxin. However, if autoimmunity is mediated by the persistence of endotoxins in the kidney, the present studies constitute evidence against this hypothesis since acute *E. coli* pyelonephritis healed completely despite the

TABLE I

Genesis of Experimental Chronic E. coli Pyelonephritis

Duration	Histologic features		Cultural Features	
	Glomeruli	Interstitium	Urine	Kidney
10 to 60 min.	Intravascular bacteria degenerate	Invasion and multiplication	0	+
4 to 24 hrs.	? Degenerate bacteria	Microabscesses Discrete bacteria	+	+
1 to 2 wks.	Clear	Macroabscesses Discrete bacteria Endotoxin	+	+
4 to 10 wks.	Clear	Macroabscesses Endotoxin	0	0

persistence of abundant endotoxin in the kidney. In contrast, such animals were resistant to reinfections by homotypic organisms (9).

The enhanced resistance to reinfection in experimental pyelonephritis is associated with specific antibodies against the O antigen of *E. coli* O-111:B4. The persistence of *E. coli* O-111:B4 antigen, as demonstrated in these observations, may constitute an important factor in the development and duration of such type-specific immunity. Cotran and Kass have recently reported the persistence of specific antigenic material of *Bacillus proteus* in macrophages within renal interstitium up to 13 months following experimental ascending *B. proteus* pyelonephritis in rats (19). Since the persistence of bacterial antigens may constitute a continued antigenic stimulus responsible for active immunity, their observations of persistence of *B. proteus* antigen support our studies demonstrating that specific immunity is also an important factor in the acquisition of resistance to ascending pyelonephritis produced by a strain of *Proteus mirabilis* (20).

Many of the reactions involving endotoxins are not solely the result of the development of specific antibodies. Such reactions would include tolerance to the pyrogenic effects of endotoxin and the Shwartzman phenomenon. Braude and Siemienski demonstrated an accelerated clearance of homologous but not heterologous endotoxin from the plasma of pyelonephritic as compared with with normal rats (21), accelerated clearance being a factor in tolerance (22). Along similar lines, McCabe demonstrated heterologous tolerance to a pyrogenic stimulus both in rabbits and in patients with pyelonephritis as compared with normals (23, 24). Pears and Houghton have demonstrated an increase both in pyuria and in bacteriuria following the administration of purified *Salmonella abortus equi* pyrogen to patients with pyelonephritis as compared with subjects without pyelonephritis (25).

From these studies it is clear that endotoxin persists in the kidneys after acute *E. coli* pyelonephritis has healed completely. Endotoxin therefore cannot be implicated as a causal factor in the pathogenesis of chronic experimental *E. coli* pyelonephritis either by a direct toxic action on the kidney or by eliciting an autoimmune response. Quite to the contrary, the evidence seems strong to suggest that the persistence of endotoxin is a major factor both in protecting against reinfection since its presence may facilitate brisk antibody responses and in being responsible for a variety of non-specific protective reactions.

SUMMARY

1. Hematogenous *E. coli* pyelonephritis was produced in rats. The localization of the organisms and the persistence of bacterial antigens was followed by fluorescent antibody techniques as well as by standard histological and bacteriological methods.

2. Salient sequential features were as follows: Single organisms passed through vessel walls into the renal interstitium and began multiplication and subsequently evoked a leucocytic response. Bacterial multiplication did not occur in glomeruli or renal tubular cells. Bacteria did not appear within renal tubular lumina until microabscesses were well developed in the renal interstitium. Bacteriuria appeared late and represented secondary invasion rather than filtration of organisms. The infection healed spontaneously but, while sterile, the parenchymal scars contained large amounts of residual bacterial antigen. The persistence of bacterial antigen did not result in continuing inflammatory changes or progressive scarring.

3. The persistence of bacterial antigens is postulated to constitute a major antigenic stimulus responsible for active immunity in experimental hematogenous pyelonephritis.

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EXPLANATION OF PLATES

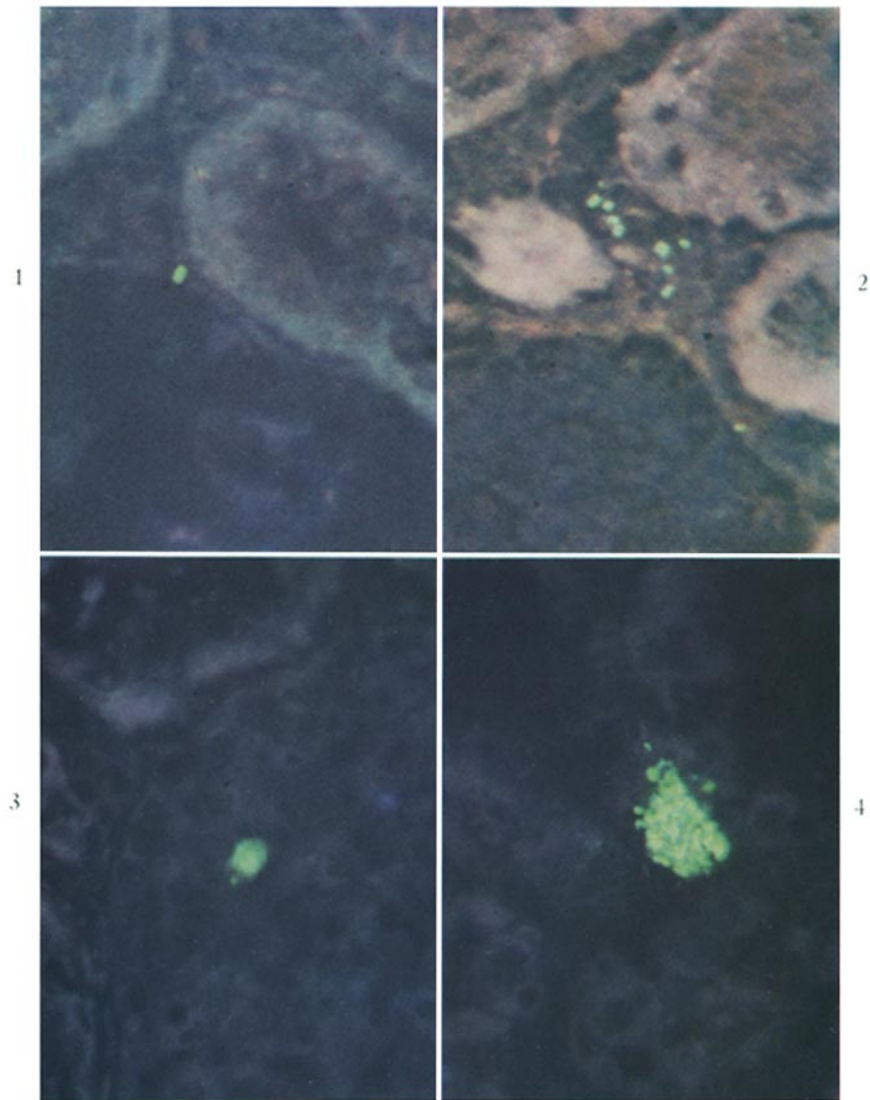
PLATE 33

FIG. 1. High power view of rat kidney obtained 10 minutes following intravascular injection of *Escherichia coli* O-111:B4 followed by renal massage. Section stained with fluorescent rabbit anti-*E. coli* O-111:B4 antiserum. Note single short rod in interstitium adjacent to blood vessel. × 430.

FIG. 2. Rat kidney obtained 60 minutes following *E. coli* O-111:B4 and renal massage. Section stained with fluorescent rabbit anti-*E. coli* O-111:B4 antiserum. Note microcolony consisting of approximately 10 short rods. × 430.

FIG. 3. Rat kidney obtained 4 hours following *E. coli* O-111:B4 and renal massage. Section stained with fluorescent rabbit anti-*E. coli* O-111:B4 antiserum. Note microabscess containing several hundred discrete organisms. × 430.

FIG. 4. Rat kidney obtained 24 hours following *E. coli* O-111:B4 and renal massage. Section stained with fluorescent rabbit anti-*E. coli* O-111:B4 antiserum. Note glomerulus containing an amorphous deposit of antigen. × 430.



(Sanford *et al.*: *Escherichia coli* in hematogenous pyelonephritis)

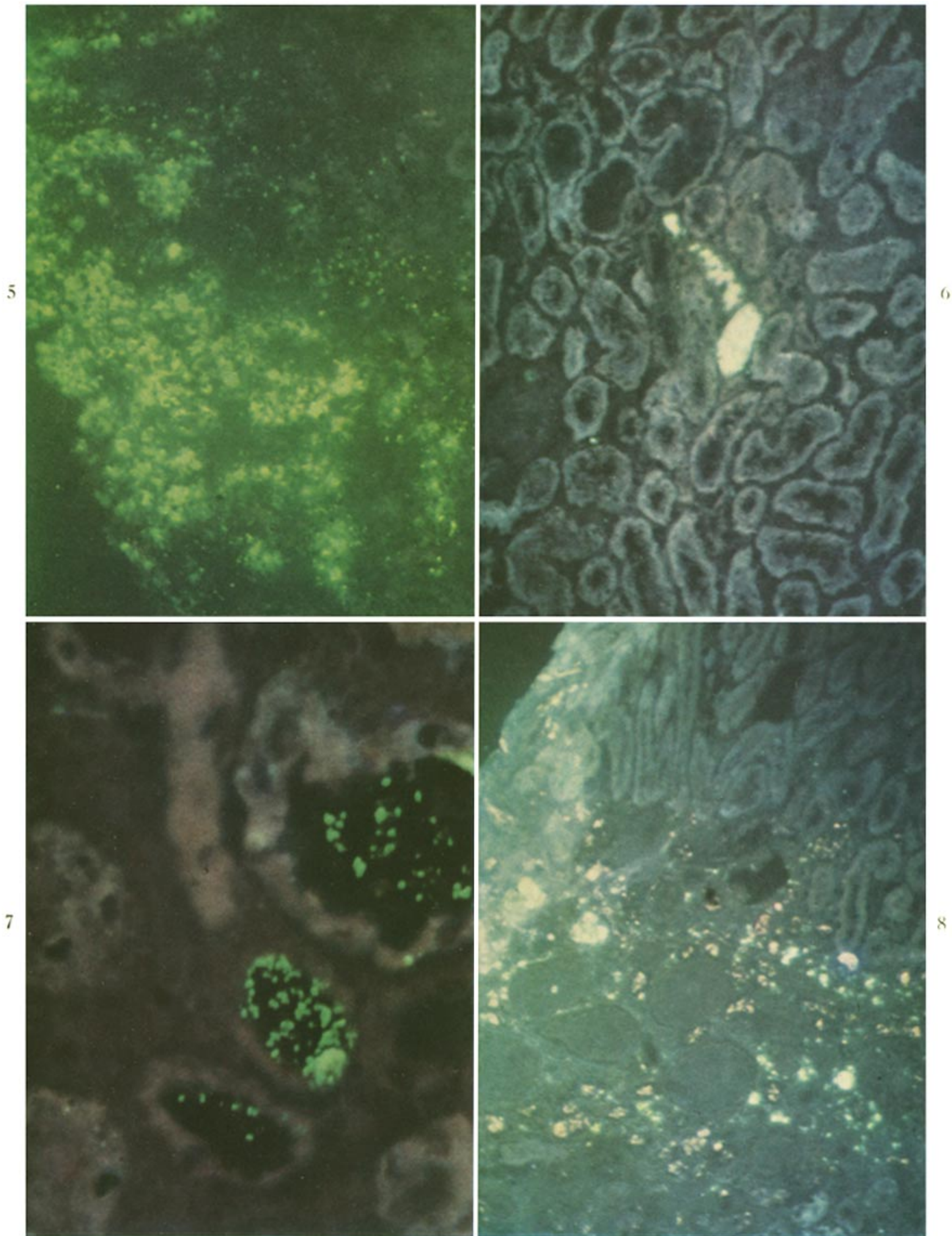
PLATE 34

FIG. 5. Low power view of rat kidney obtained 7 days following *E. coli* O-111:B4 and renal massage. Section stained with fluorescent rabbit anti-*E. coli* O-111:B4 antiserum. Note large abscess containing discrete organisms as well as amorphous antigen. × 100.

FIG. 6. Rat kidney obtained 24 hours following *E. coli* O-111:B4 and renal massage. Section stained with fluorescent rabbit anti-*E. coli* O-111:B4 antiserum. Note tubule solidly filled with antigen. × 100.

FIG. 7. Rat kidney obtained 24 hours following *E. coli* O-111:B4 and renal massage. Section stained with fluorescent rabbit anti-*E. coli* O-111:B4 antiserum. Note three tubules containing discrete organisms. × 430.

FIG. 8. Low power view of rat kidney obtained 4 weeks following *E. coli* O-111:B4 and renal massage. Section stained with fluorescent rabbit anti-*E. coli* O-111:B4 antiserum. Note scarring with crowding of glomeruli and amorphous antigen. Adjacent serial sections were sterile on culture. × 100.



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