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Cellular Response & Histopathological Studies of Murine Pyelonephritis Experimentally Induced by Aspergillus Flavus

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CELLULAR RESPONSE AND HISTOPATHOLOGICAL STUDIES OF MURINE PYELONEPHRITIS EXPERIMENTALLY INDUCED BY ASPERGILLUS FLAVUS

A Thesis

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the Faculty of the Department of Biology

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Anna M. F. Hansen

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CELLULAR RESPONSE AND HISTOPATHOLOGICAL STUDIES OF MURINE PYELONEPHRITIS EXPERIMENTALLY INDUCED BY ASPERGILLUS FLAVUS

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CELLULAR RESPONSE AND HISTOPATHOLOGICAL STUDIES OF MURINE PYELONEPHRITIS EXPERIMENTALLY INDUCED BY *ASPERGILLUS FLAVUS*

Anna M.F. Hansen August, 1980 pages

Directed by: S. Ford, D. Skean, and D. Bailey

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Five-week-old male BALB/c mice were anesthetized and incised mid-dorsally to expose the fascia over the right kidney. The kidney was then palpated up and an inoculum of 0.05 ml containing approximately 100,000 viable *Aspergillus flavus* spores in phosphate-buffered saline was injected into the cortex. A second group of mice underwent similar surgical procedures, but were injected with phosphate-buffered saline only into the right kidney. This group was designated the sham-operated control mice. All mice, including controls, were bled for differential blood counts 24 and 48 h after surgical procedures and subsequently on an every-other-day basis for the duration of the experiment. For Experiment 1, groups consisting of three infected mice each were killed at intervals of 1, 2, 4, 6, 8, 10, 13 and 16 d. For Experiment 2, three infected animals plus one sham-operated and a control mouse were killed at corresponding intervals as in Experiment

1 and also on day 21. Both kidneys were removed from all animals for weighing and measuring and then the right kidney was processed for histological study.

Differential blood counts showed an increase in polymorphonuclear neutrophils in both infected and sham-operated animals, suggesting that the primary change in white blood cell counts was the result of the surgical procedure rather than the infection. Histological studies demonstrated a chronological progression of the development of the fungus in the pelvic area of the kidney, its infiltration into the surrounding tissues, and its effect on tissues in the cortex region of the kidney. The histopathological symptoms of pyelonephritis were present from day four of the infection and continued to be enhanced through the duration of the experiment. Other cellular changes such as fibrotic tissue formations around abscesses, stromal fibrous replacement, and epithelial proliferation also occurred at various stages of the infection.

INTRODUCTION

Fungi usually exist in nature as saprophytes or obligatory parasites, but many normally saprophytic species can function as opportunists in certain animals. Fungi such as Cryptococcus neoformans, Rhizopus oryzae, Geotricum canididum, and members of the genus Aspergillus are becoming clinically more important with the increased usage of antibiotics and immunosuppressants. The aspergilli have, in recent years, become the focus of many studies due to the species involvement with organ transplant patients (Bach, et al., 1973) as well as in cancer patients. In fact, Aspergillus infections have become second only to candidiasis in mycotic complications in cancer victims (Young, et al., 1970).

Members of the genus Aspergillus, now known to number over 300, are very common in the environment and exist with few geographical restrictions (Freeman, 1979). The cosmopolitan distribution of this genus is enhanced by the abundant production of asexual spores throughout the life cycle. (Alexopoulos, 1962).

The spores of Aspergillus fumigatus, A. niger and A. flavus collectively cause a group of diseases with the common name aspergillosis. As the techniques used in medical mycology

have improved, more emphasis has been placed on A. flavus as the causative agent in more clinical cases and in more animal infections (Seabury and Samuels, 1963).

Aspergillosis in domestic animals is economically important. The fungus has been known to infect domesticated birds, chickens, and ducks via the ingestion of moldy grains or the inhalation of spores from moldy straw. The fungus has even been found within an incubating egg. Three types of infections commonly occur in birds, those of the air sacs and both the nodular and pneumonic forms of lung infections. Other domesticated animals, such as sheep, cattle and especially horses, have also been found to have contracted the fungal infection, but not to the same extent as observed in birds (Freeman, 1979). In pregnant cows, the fungus has been found to invade the uterus and infiltrate the placenta, causing abortions (Christenson, 1975).

In man, the question is not one of economics, but rather one of health or life itself. It is now known that subclinical cases of aspergilloses occur in the general population much more frequently than formerly realized. Lowering the resistance of such an individual with this infection may lead to a generalized systemic outbreak of the fungus (Freeman, 1979). Although currently a rare disease in adults and rarer still in children, disseminated aspergillosis occurs with the overwhelming use of antibiotics, antimetabolites, steroids, and radiotherapy (Khoo, et al., 1966). During the past two

decades, there has been a progressive increase in systemic infections due to these opportunistic fungi and the increased use of drug therapies (Merkow and Epstein, 1971).

The primary organ involved in human cases of the disease is the lung (Merkow and Epstein, 1971). This complication can involve itself in two forms: Aspergillus asthma, which is the result of sensitization by the inhalation of spores, and bronchopulmonary aspergillosis, in which the fungi produce mycelia within the lumen of the bronchioles. Both of these diseases are allergies and can be treated with steroids (Freeman, 1979). Colonizing aspergillosis, causing aspergilloma, is the next most common form of the infection. In this disease, the organism colonizes a preformed cavity, such as the pulmonary spaces, nasal sinuses, or old tubercular lesions. There is essentially no invasion of living tissues and the infection can usually be terminated with surgery (Freeman, 1979).

The rarest form of the fungal infection is the frequently fatal invasive aspergillosis. Mycelial elements invade the tissue and may rapidly progress to include systemic involvement (Freeman, 1979). The kidneys, brain, liver, and the intestines are the organs most favored by this form of fungal infection (Young, et al., 1970).

The incidence of Aspergillus infections has been found to surpass bacterial sepsis in renal transplant complications (Bach, et al., 1973) and it has also been reported that members of the genus Aspergillus are also responsible for large numbers of significant upper urinary tract diseases in

diabetics and immunosuppressed patients (Warshawsky, et al., 1975). This type of infection has also occurred in patients whose only abnormality was an obstruction of the ureteropelvic junction (Eisenberg et al., 1977).

At necropsy, patients infected with Aspergillus have been found to have well-demarcated lesions in the brain, trachea, larynx, lungs, myocardium, liver and the spleen (Grcevic and Matthews, 1959). Rare infiltrations of the thyroid have also been diagnosed in increasing numbers, both at necropsy and at biopsy, especially in patients who have undergone some type of immunosuppressive therapy (Wingelberg, et al., 1979).

Due to the growing importance of the aspergilli in the medical world, substantial research effort has been directed towards study of the host-parasite relationship between these fungi and experimental animal models. Animals such as mice, rats, hamsters, rabbits, and monkeys have been used to study the pathological and immunological response to the aspergilli under the influence of variables such as immunosuppressants, previous exposures, and age and sex differences.

Aspergillus flavus was studied by Ford and Friedman (1967) in a series of experiments designed to determine the pathogenicity of selected members of the genus Aspergillus. They found A. flavus to be the most pathogenic, even more so than A. fumigatus, and this finding was contrary to the most recent literature. They also found that animals which had been infected for long periods of time sometimes had a

kidney which appeared grossly abnormal or one of their kidneys would be missing. These mice had survived with only one functional kidney and, therefore, could serve as a potential model to study the host-parasite relationship between the murine system and A. flavus.

Thus, the objectives of this study were: (1) to establish and maintain an A. flavus infection in an organ of a population of mice, in this case a kidney, and (2) to determine the leukocytic response and pathological changes occurring in the murine kidney infected with viable spores.

MATERIALS AND METHODS

Experimental Design

For Experiment 1, 36 five-week-old male BALB/c mice, most weighing 17 to 20 g, bred at Western Kentucky University, were randomly distributed into groups of three and placed into 12 cages. All animals in cages 1 through 9 were infected with viable spores of A. flavus; mice in cage 10 underwent surgery, but were injected with only phosphate-buffered saline, and mice in cage 11 were controls. Animals in cage 12 were infected with A. flavus spores and were maintained to replace any other mice that did not survive the inoculation procedure.

For Experiment 2, 60 male BALB/c mice of the same age and stock as in Experiment 1, most weighing 17 to 20 g, were randomly distributed into groups of five among 12 cages. Cages 1 through 10 each contained 3 infected mice, 1 sham-operated mouse, and one control mouse. Cages 11 and 12 contained additional inoculated mice. More mice were used in Experiment 2 in order to give a more reliable blood record and ensure that an experiment would last 21 d. More blood counts from control and sham-operated animals were needed for daily averages to be more accurate. Additional mice were also inoculated to assure replacement of any animal that might not recover from surgery or might die from the infection.

Preparation of Conidial Suspensions

Five plates of Sabouraud Dextrose agar (Difco, Detroit, MI) were inoculated with A. flavus spores and incubated for 4 d at 37°C. After the first 4 d, the glass petri plate covers were replaced with porous asbestos tops and the cultures incubated for 2 d.

Dried spores were harvested by gently loosening the spores by raking the surface of the mycelium with a sterile microslide. The plates were then inverted into a sterile petri plate cover and tapped several times. All the spores from the five cultures were collected into one petri dish cover and then transferred to a sterile 120-ml bottle containing 40 ml of phosphate-buffered saline, pH 7.2, and enough 3-mm diameter glass beads to form a 2.54-cm layer on the bottom of the bottle.

Spores were then hydrated by shaking the saline spore suspension for approximately 30 min. After hydrating, the suspension was then filtered through several layers of sterile gauze. This procedure removed unwanted clumps of spores or pieces of hyphae. Filtered suspensions were labeled as stock and stored at 4°C until used. All suspensions were used within 48 h of preparation.

Viable spore counts of the stock suspension were made in duplicate sets immediately after collection of spores by plating on Littman's agar. Serial dilutions were made in sterile phosphate-buffered saline and pour plates were made in triplicate for each dilution. Plates were incubated at

37°C for 48 h before counting. An average count of 6 plates was obtained and then multiplied by the reciprocal of the appropriate dilution factor for the approximate spore count per ml. An inoculum was made by adjusting the stock solution to contain 10^6 spores per 0.5 ml. All inocula were prepared the day of the surgical procedures and stored at 4°C until used. When being used, the inoculum was kept iced in a beaker to inhibit spore germination.

At the beginning and at the end of each surgical procedure, viable spore counts were made in duplicate to determine if there was a significant decrease in spore viability over the duration of the surgeries.

Surgical Procedures

All infected and sham-operated mice were first given an interscapular, subcutaneous injection of Nembutal (Exp. 1) or Bio-Thol obtained from a local veterinarian (Exp. 2). Anesthetized mice were then placed in cages lined with paper rather than the normal bedding material in order to decrease the chance of respiratory complications.

Under aseptic conditions, an incision was made mid-dorsally over the area of the right kidney in such a way that only the skin was incised. The kidney was then palpated under the fascia and an inoculum of 0.05 ml, or approximately 100,000 viable spores, was administered as two injections to increase the dispersal of the spores. The incision was then closed with a 9-mm surgical staple. Sham-operated mice were surgically treated in the same manner, but their kidneys were injected with 0.05 ml of sterile phosphate-buffered saline,

pH 7.2. All mice, while anesthetized, were marked with an ear punch so that individuals could be easily identified.

Mice were weighed and killed by cervical dislocation on days 1, 2, 4, 6, 8, 10, 13, and 17 post-infection for Experiments 1 and 2 and also on day 21 for Experiment 2. The mice were incised from the region of the symphysis to the xiphoid process for removal of both kidneys. Both kidneys were then weighed and measured and the right kidney of each animal was placed into 37% formalin for fixation.

Tissue Preparation

After 24 h in formalin, tissues were dehydrated and embedded in Paraplast (melting point 54-56^{o}C). All blocks were serially sectioned at either 5 or 9 µm, depending on the friability of the tissue, using an American Optical Spencer rotary microtome. Serial sections were then cut to appropriate lengths and floated on a solution maintained at approximately 50^{o}C. The solution contained approximately 10 ml of 100% ethanol, approximately 0.5g of gelatin, and 500 ml of distilled water. Sections were mounted on slides that had been thoroughly cleaned with 100% ethanol and dried on slide warmers at 54^{o}C for at least 24 h. Slides were stained using Harris' hematoxylin and eosin, after which coverslips were applied using Permount. Finished slides were allowed to dry for at least 48 h before microscopic examination (See Appendix).

Differential Blood Counts

All mice were bled at 24 h and 48 h after inoculation

and then subsequently on an alternate-day basis for the duration of the experiment or until they were killed.

Blood was removed from tails that had been cleaned with ethanol and then wiped dry. Sterile scissors kept in a beaker of ethanol were used to remove between 1 and 2 mm of the distal portion of the tail to initiate blood flow. After duplicate slides were made and properly labeled, the mouse tails were disinfected with iodine. Subsequent bleedings involved only the removal of the scab from the end of the tail, but the ethanol-iodine regimen was followed at all times.

Blood slides were allowed to dry for 24 h and then were stained with xanthene and methylene-blue. Stained slides were air dried for 24 h before the differential counts were made as described by Brown (1976).

Photographic Procedures

Photographs of highly magnified representative sections of kidneys were taken with a Wild M-20 research microscope with an attached automatic camera during the progress of the Aspergillus infection. Photographs of whole kidney sections were taken with a Zeiss research dissecting scope with an attached camera. High magnification photographs were taken on Tri-Pan X film and other photographs were taken with Kodak Pan-X film.

RESULTS

Spore Viability

Plate counts demonstrated that the number of viable spores in the inocula did not decrease over the duration of the surgical procedures. This consistency was demonstrated by using the Student's t-test as seen in Table 1.

Anesthesia and Surgical Procedure

Most animals tolerated the surgical procedure without major complications. Adjustment of the anesthetic dose to the animal's body weight was the most critical step in this phase of the experiment. Early trials with an outdated supply of Nembutal bore out this particular fact in that calculated concentrations of the drug proved highly unreliable and mouse mortality was high with the use of this drug. Eight mice did not recover from the anesthetic in the first experiment.

Bio-Thol proved to be more reliable, but had unexpected side effects which may have affected the outcome of other phases of this experiment. All animals that survived revived within 30 to 60 min after the anesthetic was administered. No inflammation developed at the site of the incision and no animal died after recovering from the anesthetic.

Necropsy Findings

None of the organs observed, including the infected kidneys, appeared grossly abnormal until day 10 in Experiment

Table 1. Colony-Forming Units of Viable *Aspergillus* Spores[a]

Experiment	Plate	Before Surgery		After Surgery	
		Set 1	Set 2	Set 1	Set 2
1	1	89	87	89	90
	2	96	92	90	82
	3	95	90	93	88
	Mean[b]	91.5		89.1	
2	1	81	99	86	93
	2	81	96	89	90
	3	84	94	93	81
	Mean[b]	87.5		88.6	

[a]All counts were made from a 10^{-3} dilution of the stock suspension.

[b]By the Student's t-test, no significant ($p<0.01$) difference was noted between the means of spore CFU/ml before and after surgery for both experiments.

1 and day 13 in Experiment 2. At this stage of the infection the kidneys were decreased in size (Tables 2 and 3), the capsular epithelium was very friable, and purulent material could be forced out with gentle pressure. Random tissue samples of liver, spleen, lung, and uninfected kidneys were removed aseptically and ground for dilution and pour-plating on Littman's agar. No colonies developed on this selective growth medium after 96 h incubation.

Analysis of variance was performed on the weights of the normal and the infected kidney from each of the test animals used in Experiment 1. A significant difference between the weights of the infected and noninfected kidneys from the same animal was determined to have occurred from the very beginning of the experiment (Table 4).

The analysis for Experiment 2 compared: (1) the control animal kidneys with all others, (2) sham-operated mouse kidneys versus infected mouse kidneys, and (3) the infected versus the noninfected kidney from the same animal. No significant difference in kidney weights was found due to treatment although highly significant differences were found over time in all comparisons (Table 5).

Microscopic Examination

All of the kidneys from Experiment 1 and randomly selected kidneys from Experiment 2 were serially sectioned and studied. All but 4 showed foci of infection and corresponding pathological change. Normal specimens of the

Table 2. Weights of Infected and Noninfected Kidneys From Sacrificed Animals in Experiment 1

Kill Day (PI)[a]	Animal No.	Kidney Weights(g) I[b]	N[c]
1	2-O	0.12	0.11
	4-R	0.16	0.13
	5-O	0.20	0.14
2	7-R	0.27	0.25
	12RR	0.23	0.18
	12-L	0.17	0.14
4	1-R	0.19	0.21
	1-O	0.22	0.22
	1-L	0.19	0.18
6	3-R	0.20	0.18
	3-O	0.21	0.20
	3-L	0.14	0.10
8	6-R	0.22	0.22
	6-O	0.15	0.16
	6-L	0.13	0.13
10	8-R[d]	0.24	0.24
	8-C[d]	0.18	0.17
	8-L[d]	0.16	0.20
13	9-R[d]	0.22	0.24
	9-C[d]	0.20	0.25
	9-L[d]	0.17	0.25
17	12-R[d]	0.19	0.26
	12-C[d]	0.26	0.27
Shams[e]	11-R	0.25	0.24
	11-C	0.23	0.23
	11-L	0.20	0.19
Controls[f]	10-R	0.18	0.18
	10-O	0.18	0.18
	10-L	0.19	0.19

Table 2. (continued)

---

[a]PI = Post-injection.

[b]I = Infected.

[c]N = Normal.

[d]Grossly abnormal.

[e]Received saline injection in lieu of spores.

[f]No surgical procedure performed.

Table 3. Weights of Infected and Noninfected Kidneys From Sacrificed Animals in Experiment 2.

Kill Day (PI)[a]	Animal No.	Kidney Weights(g) I[b]	N[c]
1	9-R	0.15	0.14
	9-O	0.18	0.15
	9-L	0.19	1.50
	9-S[d]	0.14	0.14
	9-C[e]	0.10	0.10
2	10-R	0.14	0.07
	10-O	0.09	0.06
	10-L	0.09	0.06
	10-S	0.06	0.06
	10-C	0.11	0.11
4	1-R	0.16	0.15
	1-O	0.14	0.13
	1-L	0.16	0.18
	1-S	0.17	0.17
	1-C	0.18	0.17
6	12RRLL	0.20	0.20
	6-O	0.22	0.23
	6-L	0.16	0.13
	6-S	0.16	0.12
	6-C	0.17	0.16
8	5-R	0.18	0.19
	5-O	0.20	0.18
	5-L	0.20	0.17
	5-S[f]	-	-
	5-C	0.19	0.20
10	3-R	0.16	0.16
	3-O	0.21	0.20
	3-L	0.18	0.20
	3-S	0.19	0.16
	3-C	0.25	0.25
13	2-R[g]	0.08	0.14
	2-O[g]	0.18	0.27
	2-L[g]	0.10	0.15
	2-S	0.17	0.18
	2-C	0.13	0.13

Table 3. (continued)

Kill Day (PI)[a]	Animal No.	Kidney Weights(g) I[b]	N[c]
17	4-R[g]	0.09	0.19
	4-O[g]	0.16	0.16
	4-L[g]	0.20	0.21
	4-S	0.20	0.21
	4-C	0.21	0.21
21	7-R[g]	0.22	0.18
	7-O	0.20	0.20
	7-L	0.19	0.20
	7-S	0.18	0.17
	7-C	0.20	0.20

[a]PI = Post-injection.

[b]I = Infected.

[c]N = Normal.

[d]S = Sham-operated (received saline injection in lieu of spores).

[e]C = Controls (no surgical procedures performed).

[f]Animal 5-S died after surgery.

[g]Grossly abnormal.

Table 4. Analysis of Variance of Kidney Weights for Experiment 1

	Df	ss	Ms	E
Total	45	0.0910		
Blocks	7	0.0170	2.428×10^{-3}	1.615
Kidneys	5	0.0259	5.180×10^{-3}	3.446*
Inf vs Non-Inf	1	0		
Error	33	0.0481	1.503×10^{-3}	

*$p<0.05$.

Table 5. Analysis of Variance of Kidney Weights for Experiment 2

	Df	ss	Ms	E
Total	89	0.20		
Blocks	8	0.08	0.01	7.14**
Treatments	3	0.01	0.003	2.14
Controls vs all	1	0.0		
Shams vs Infected	1	0.0		
Infected vs Noninfected	1	0.0		
Error	78	0.11	0.0014	

**$p<0.01$.

cortex (Figure 1), the medulla (Figure 2) and its compact duct system (Figure 4), and pelvic epithelium (Figure 3) were used for comparisons of pathological changes.

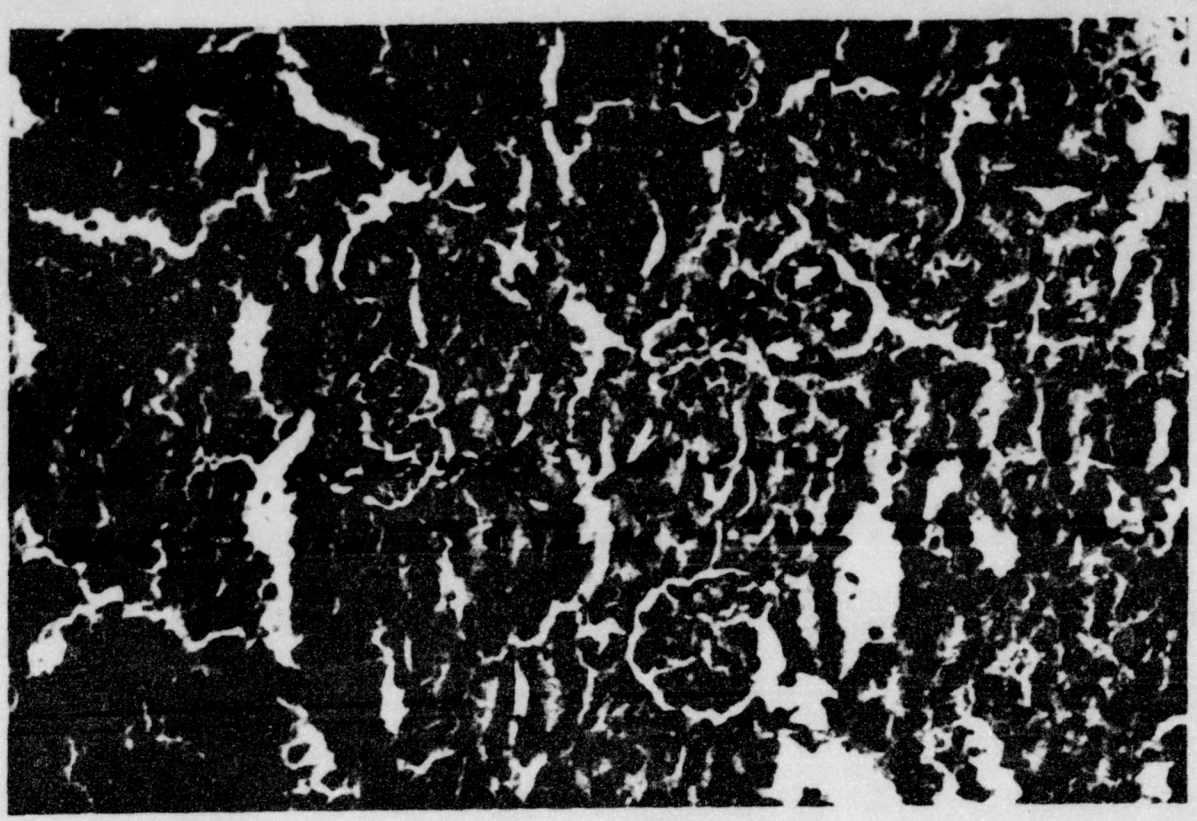
The microscopic examinations revealed the following general chronology of events. The kidney 24-h post-infection showed no fungal hyphae. Needle tracks were easily found and an infiltration of white blood cells was evident, but this infiltration was minimal and was expected as a result of the trauma to the organ (Figure 5). A small area of hyphal proliferation was observed in the medullary tubules of a kidney after 24 h (Figures 6a and b). This was an unexpected result. In experiments previously reported (Ford, et al., 1968) germination had only been observed after 48 h.

After 48 h, the infection had established itself in the pelvic areas of all kidneys observed from this time period (Figure 7). No direct infiltration of surrounding tissue was observed, but there was an increase in the numbers of white blood cells in the medullary region of the kidneys which could only be accounted for by the growth of the fungal ball. Most of these white blood cells were neutrophils (Figures 8 and 9).

After 4 d, the infection began to infiltrate the collecting tubules in the innermost layer of the medulla (Figures 10a, b, and c). The clogging of the tubules could be inferred by the beginning of tubular distension. Small spaces began to occur between the normally compact ducts and white blood

Figure 1. A section of normal kidney cortex taken from a control animal. X 120.

Figure 2. A section of normal medullary tissue taken from a control animal. X 120.



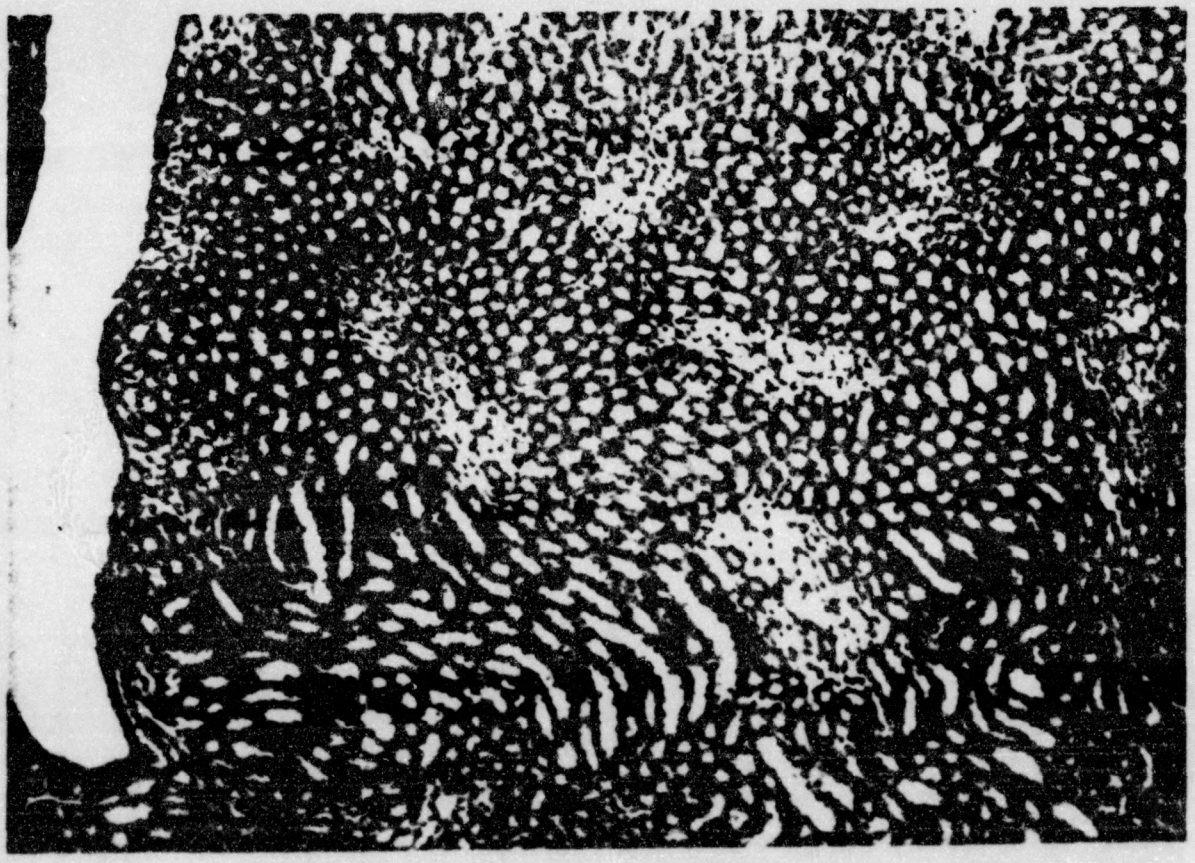


Figure 3. A section of normal pelvic epithelium taken from a control animal. X 800.

Figure 4. A section of normal condensed medullary tubules taken from a control animal. X 800.

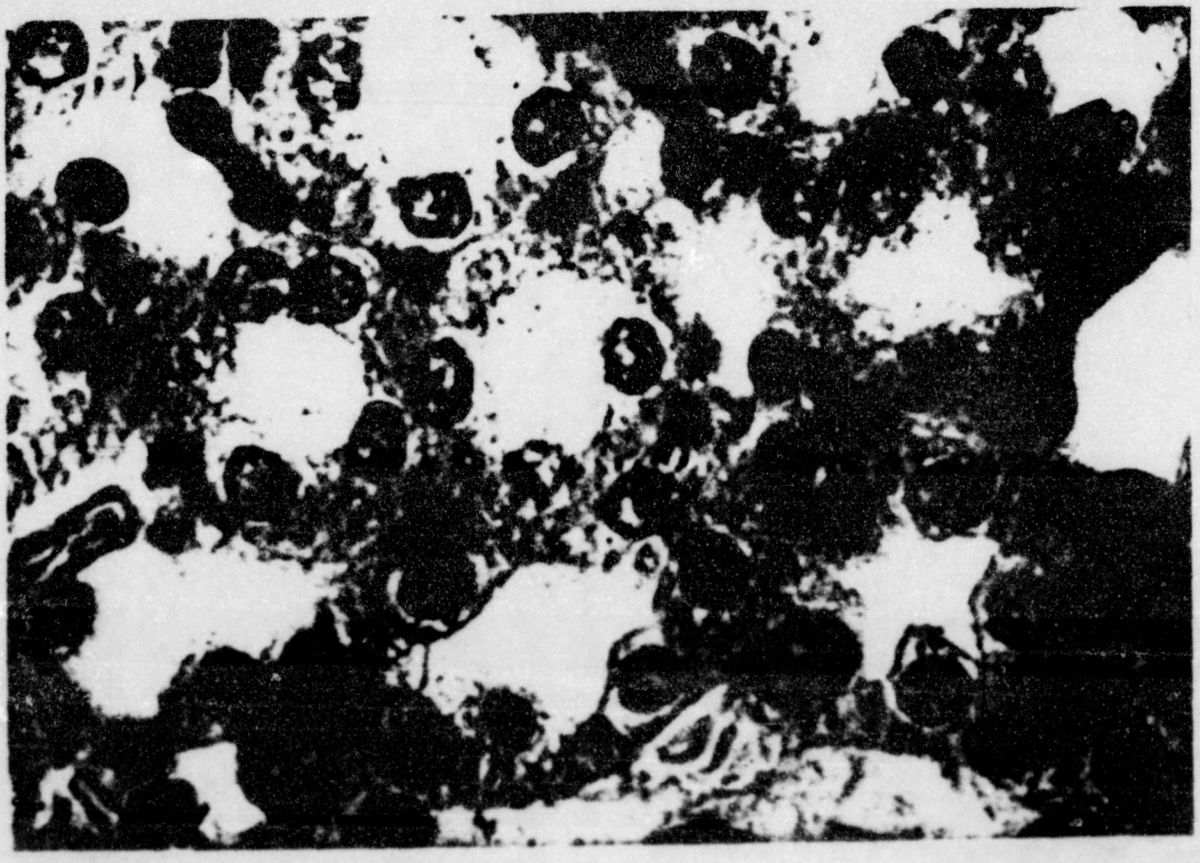


Figure 5. A section of cortex region taken from an animal killed 24 h after infection showing a needle track and a large number of white blood cells. X 120.

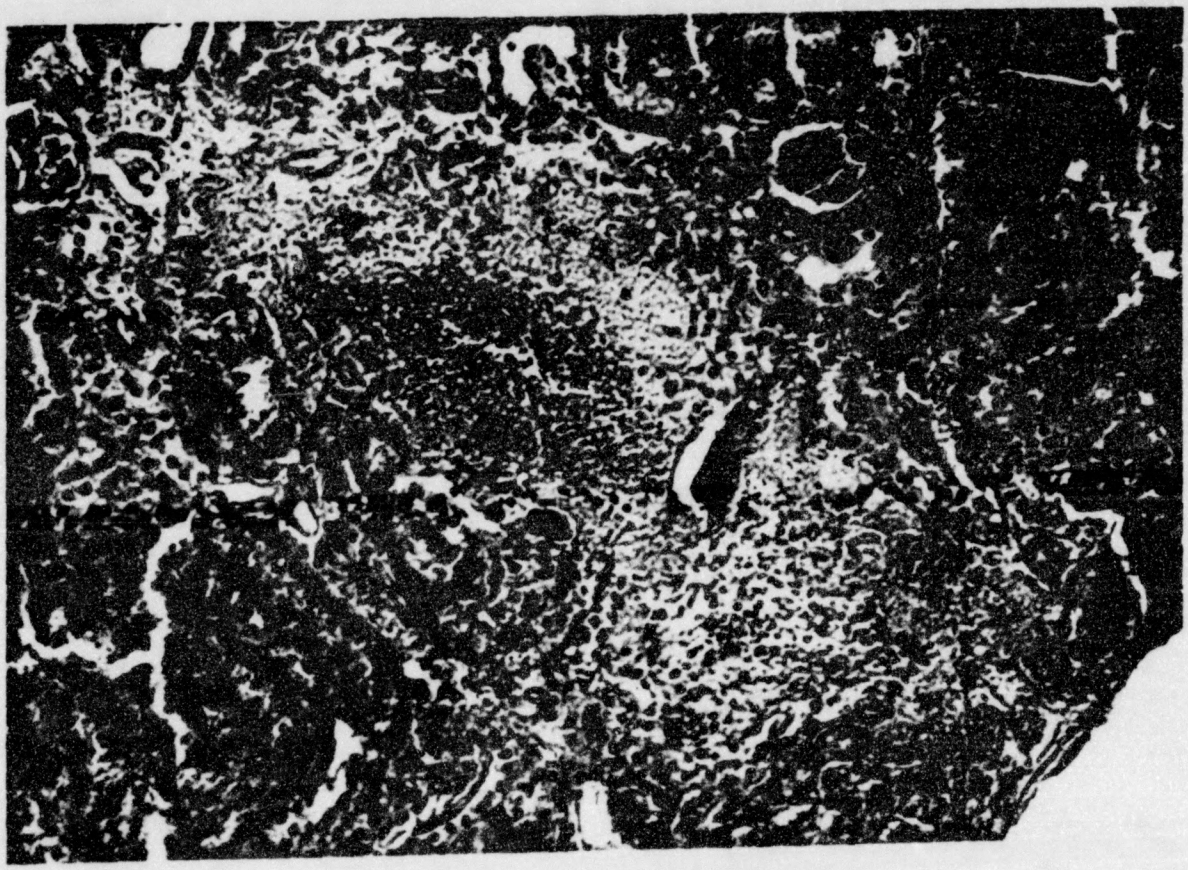
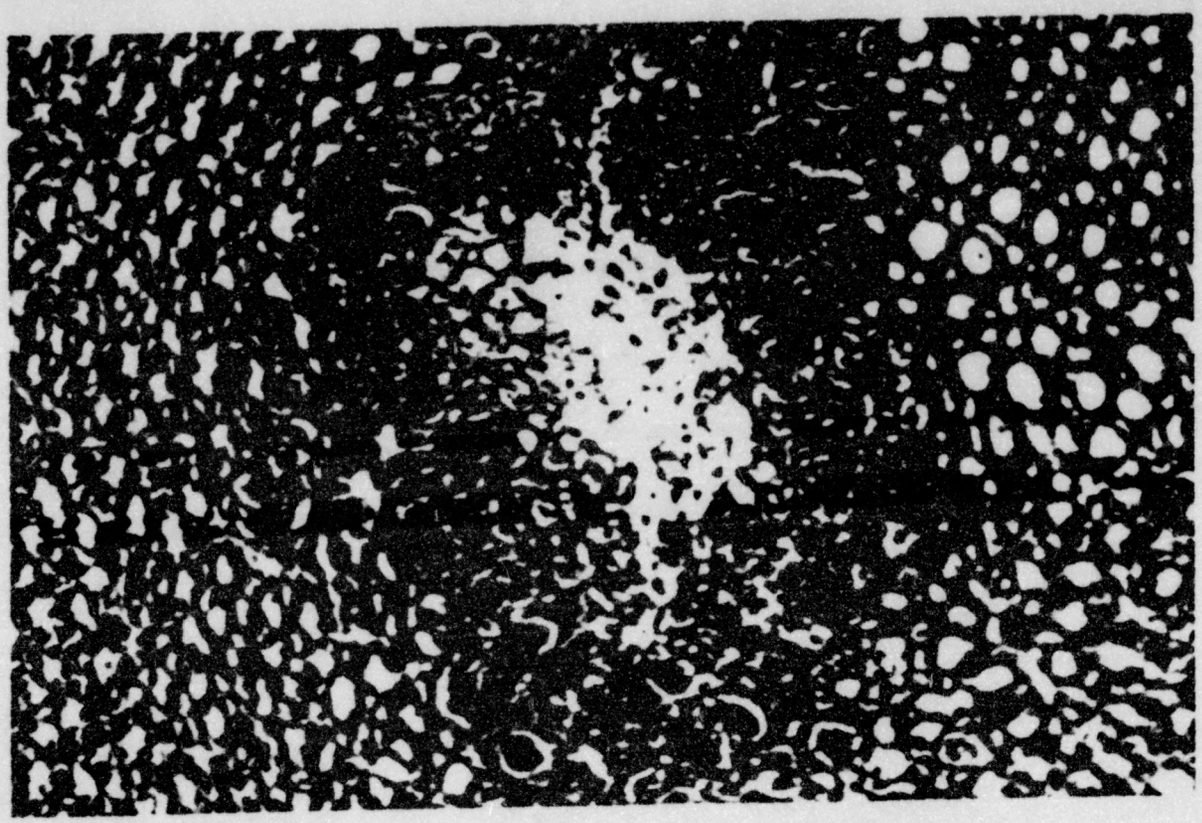


Figure 6a. A section of the medullary region from an animal killed 24 h after infection showing unusual hyphal proliferation and necrosis. X 120.

Figure 6b. Hyphal proliferation and necrosis in the medullary region of a kidney from an animal killed 24 h after injection. X 120.



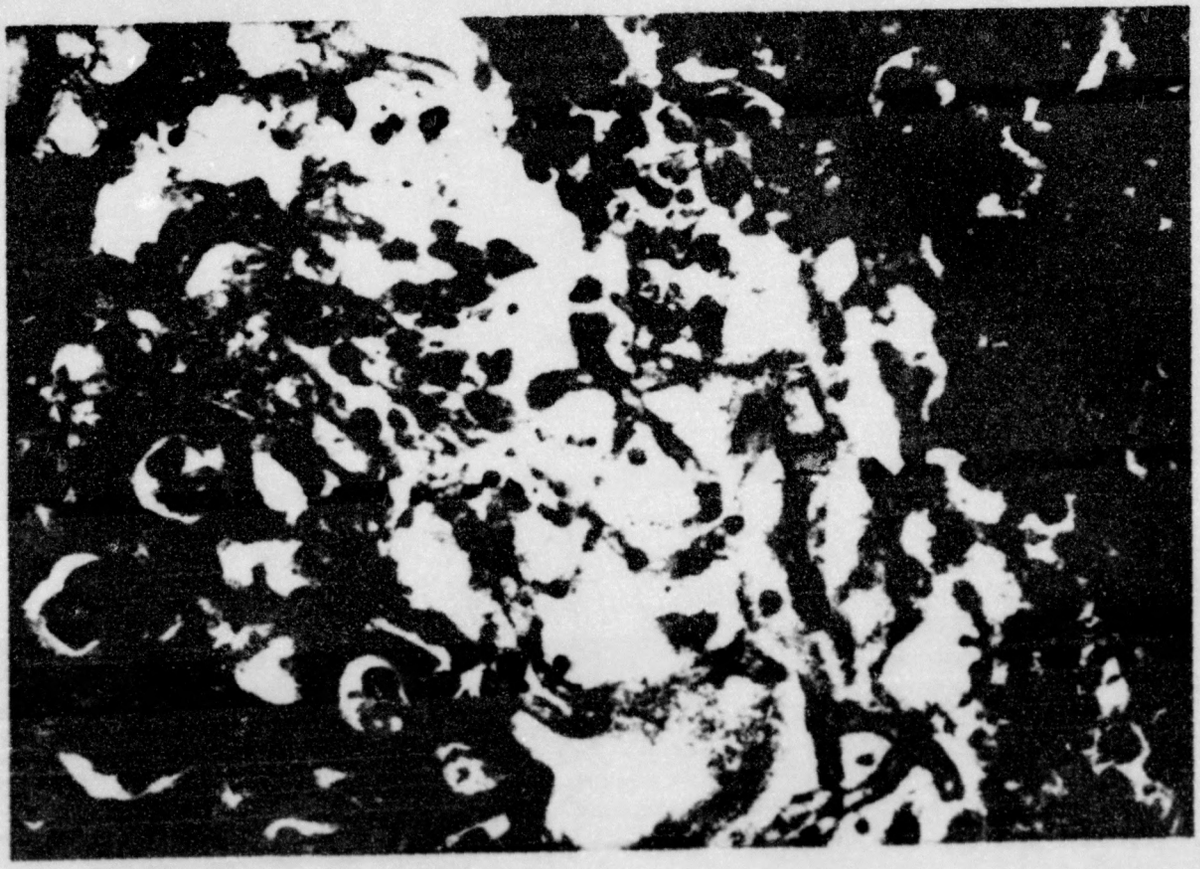


Figure 7. Section through the pelvic cavity of a kidney showing the proliferation of the hyphal ball and the increased number of white blood cells in the cavity and adhering to the hyphal walls. X 120.

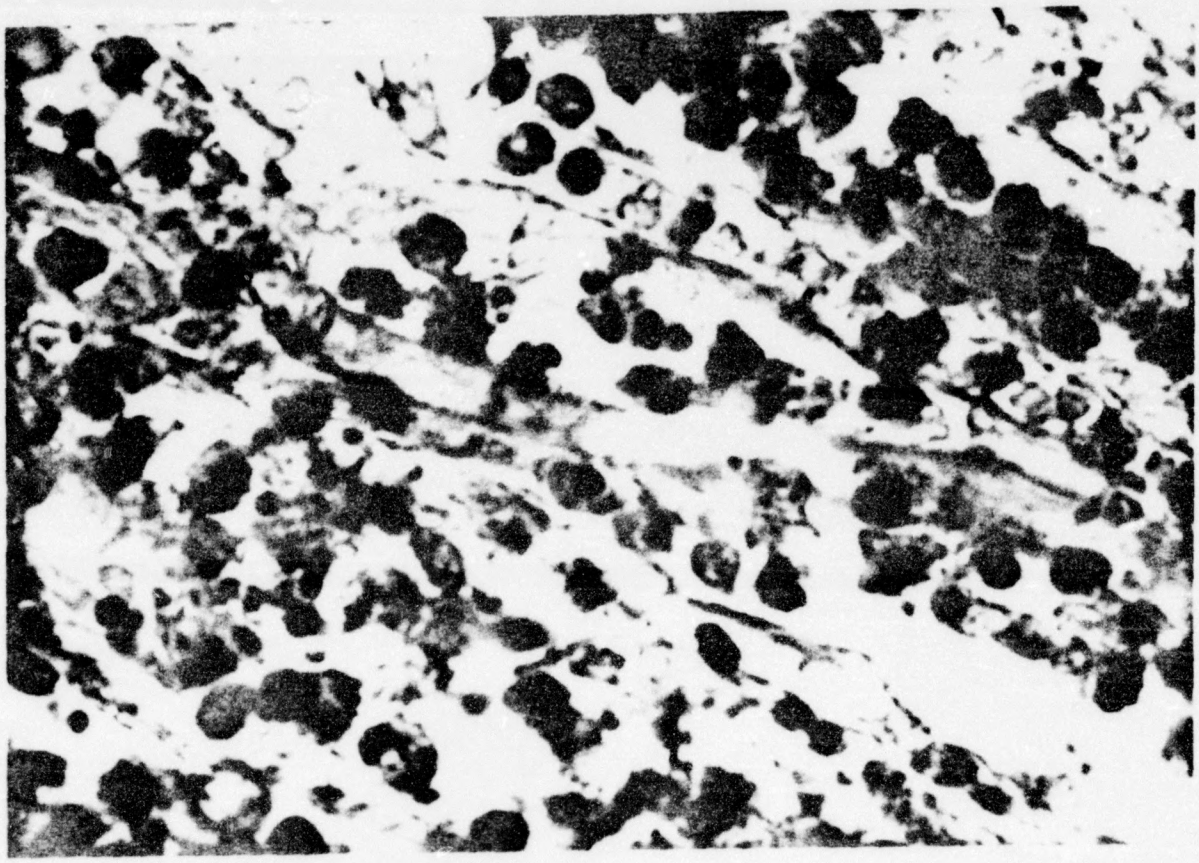


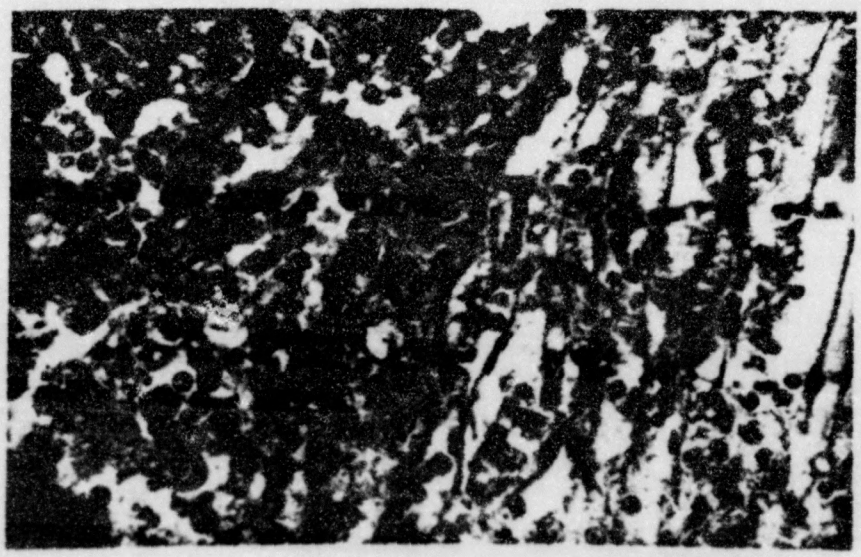
Figure 8. A section through the pelvic cavity of a kidney from an animal killed 48 h after infection showing large numbers of neutrophils. X 1500.

Figure 9. A section through the pelvic cavity of a kidney from an animal killed 48 h after infection showing large numbers of neutrophils. X 1500.

Figure 10a. A section through the pelvic cavity of a kidney from an animal killed 4 d after infection showing hyphal proliferation and invasion of medullary tubules. X 60.

Figure 10b. Hyphal proliferation and invasion of medullary tubules. X 100.

Figure 10c. Hyphal invasion of medullary tubules. X 200.



cells were seen entering the tissue area. The infiltration of white blood cells also had continued into the pelvis and resulted in their adherence to the outer surface of the hyphal ball (Figure 11).

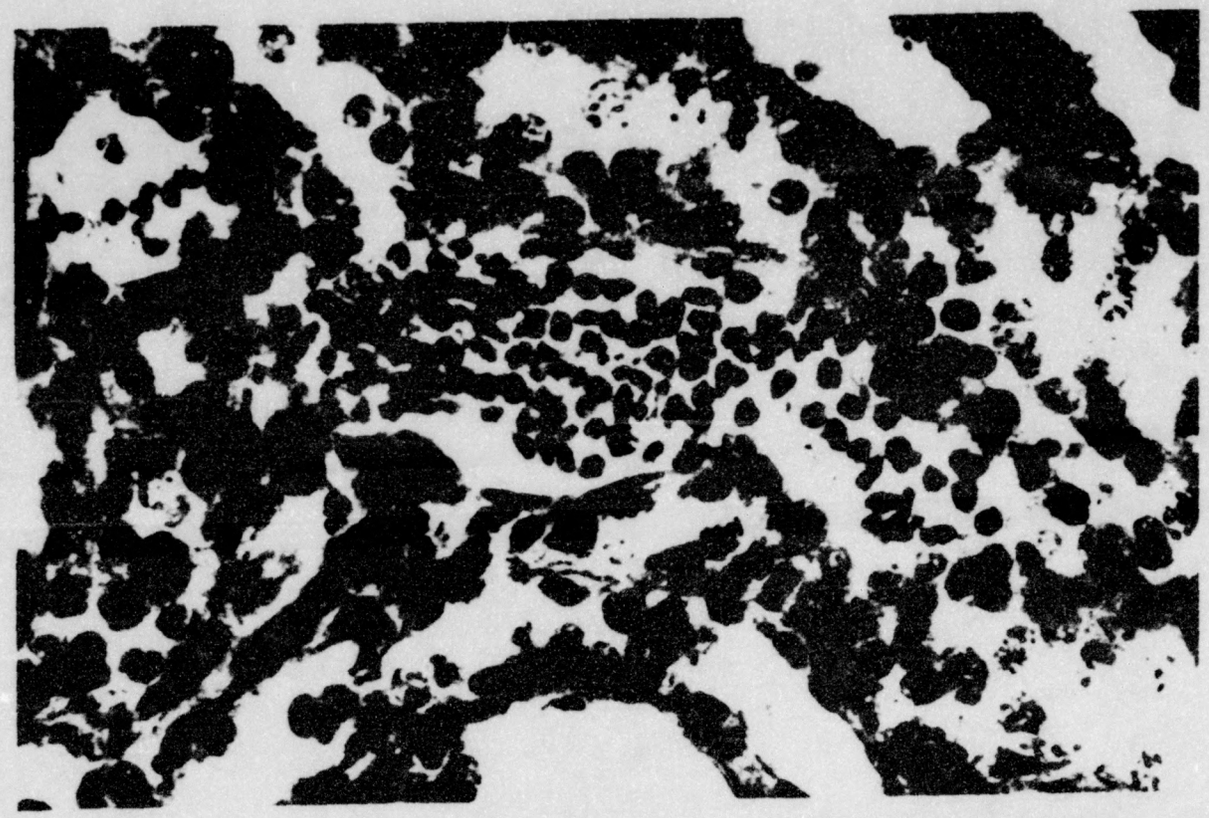
The kidney of an animal killed 6 d after infection was very congested with blood. Large numbers of neutrophils had become trapped in distended tubules causing lesion-like areas usually associated with chronic pyelonephritis (Figure 12). In some cases, the infiltration would cause even more distension of the tubules and the formation of abscesses (Figure 13). Neutrophils still could be observed adhering in large numbers to the outer surface of the fungal ball (Figure 14). Some macrophages were also seen, apparently engulfing necrotic material and other debris. Plasma cells also were seen, suggesting that the cellular response was also being assisted by the humoral portion of the immune system (Figures 15a and b).

In the cortex, tubules had become more distended (Figure 16) and large amounts of proteinaceous material were seen. The glomeruli were still normal in appearance, implying that the blood supply to the area was still adequate. A granuloma, filled with white blood cells and surrounded by fibroblasts, was seen in the lower margin of the cortex (Figure 17a, b and c). The epithelial lining of the cavity around the fungal ball was hyperplastic, implying that a reparative process was occurring.

Figure 11. A section through the pelvic area of a kidney from an animal killed 4 d after infection showing hyphal ball size increase and proliferation into medullary tubules. X 180.

Figure 12. A section through the medullary region of a kidney taken from an animal killed 6 d after infection showing tubules clogged by casts of proteinaceous material coated with white blood cells. X 400.

Figure 13. A section through the medullary region of a kidney taken from an animal 6 d after infection showing an abscessed tubule formed by the infiltration of large numbers of white blood cells. X 400.



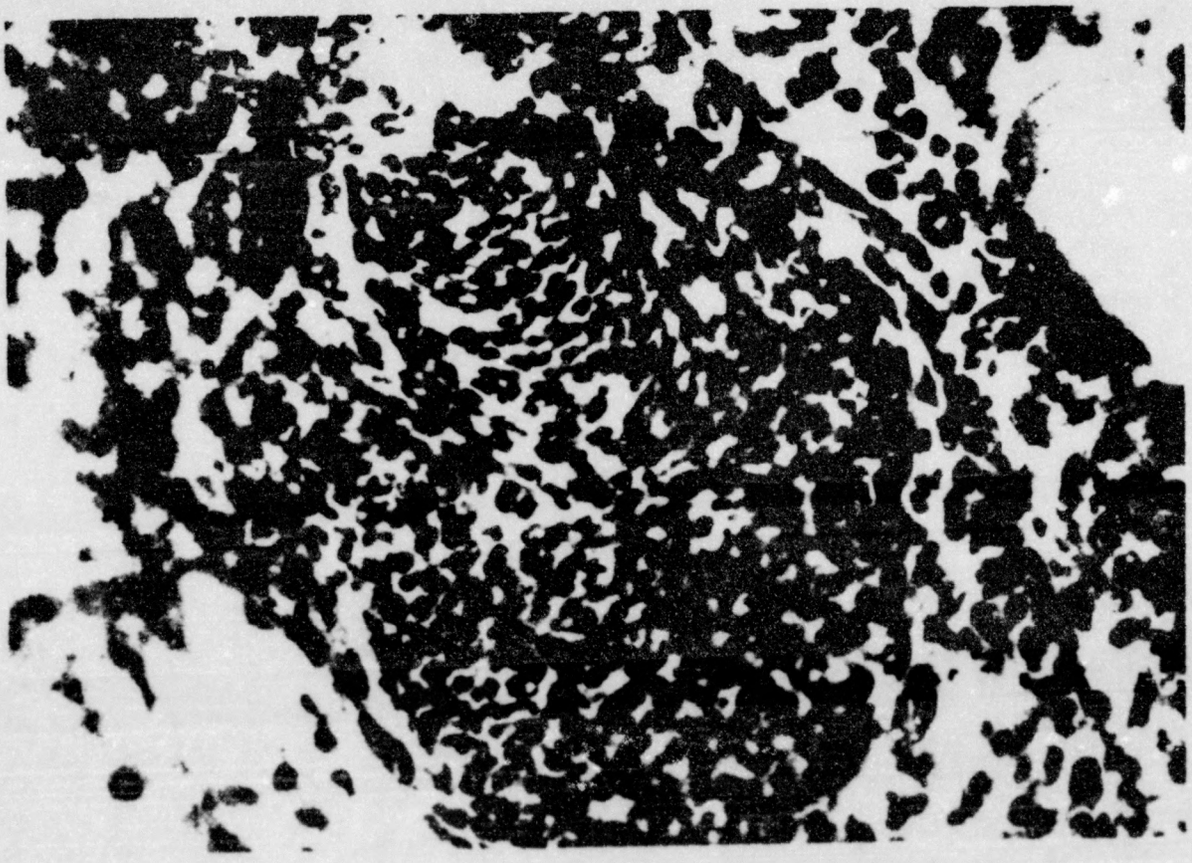
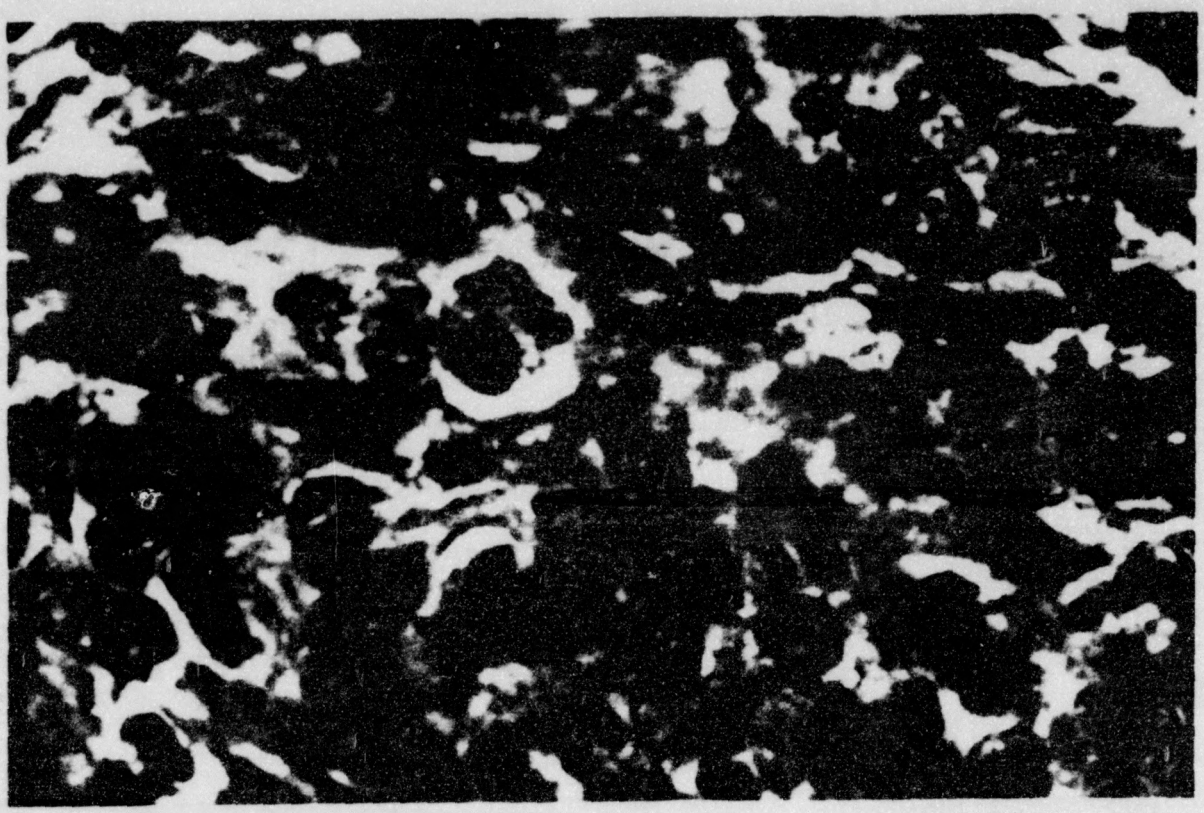


Figure 14. A section through the pelvic cavity of a kidney taken from an animal killed 6 d after infection showing fungal ball proliferation and the large numbers of white blood cells adhering to its outer surface. X 60.

Figure 15a. A section through the medullary region of a kidney taken from an animal killed 6 d after infection showing a plasma cell (center) infiltrating through tissues. X 600.

Figure 15b. Plasma cell infiltrating medullary tissue. X 1500.



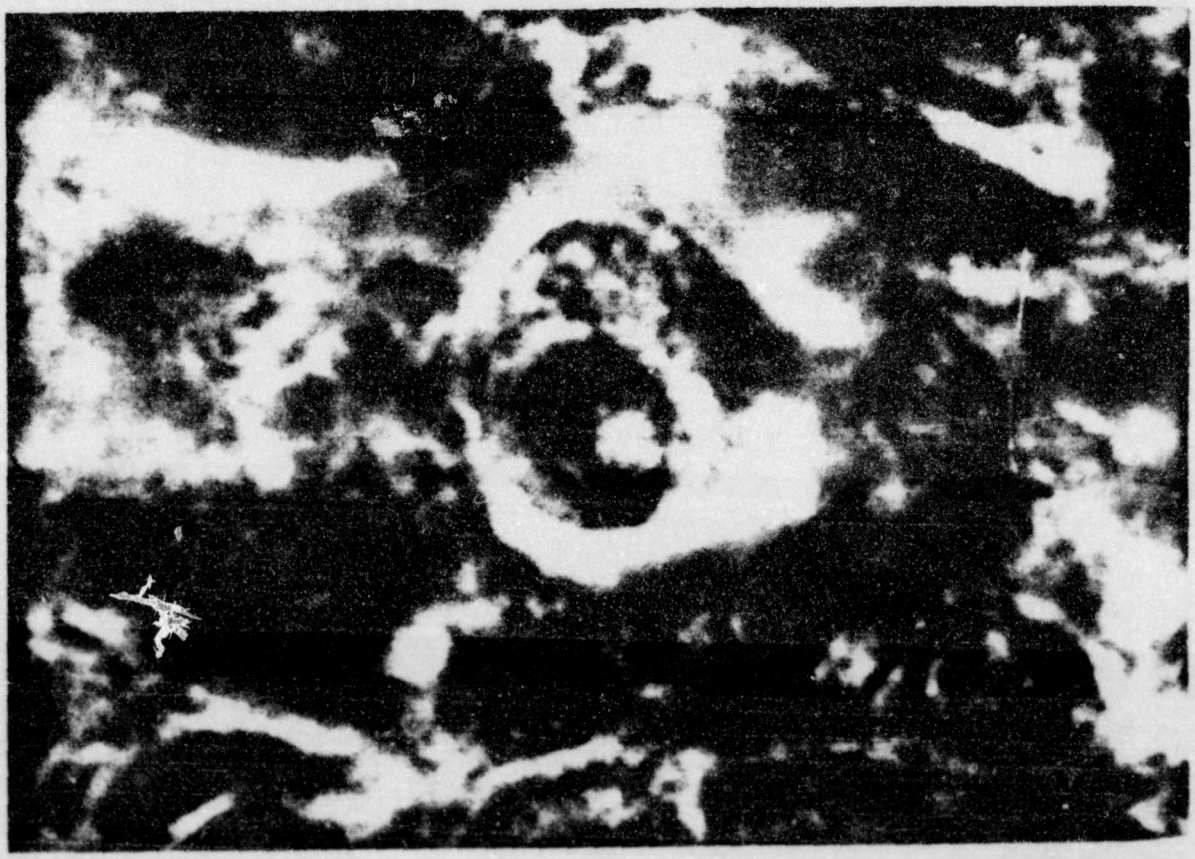


Figure 16. A section through the medullary region of a kidney taken from an animal killed 8 d after infection showing severe tubule distension due to clogging of collecting ducts by hyphae. X 60.

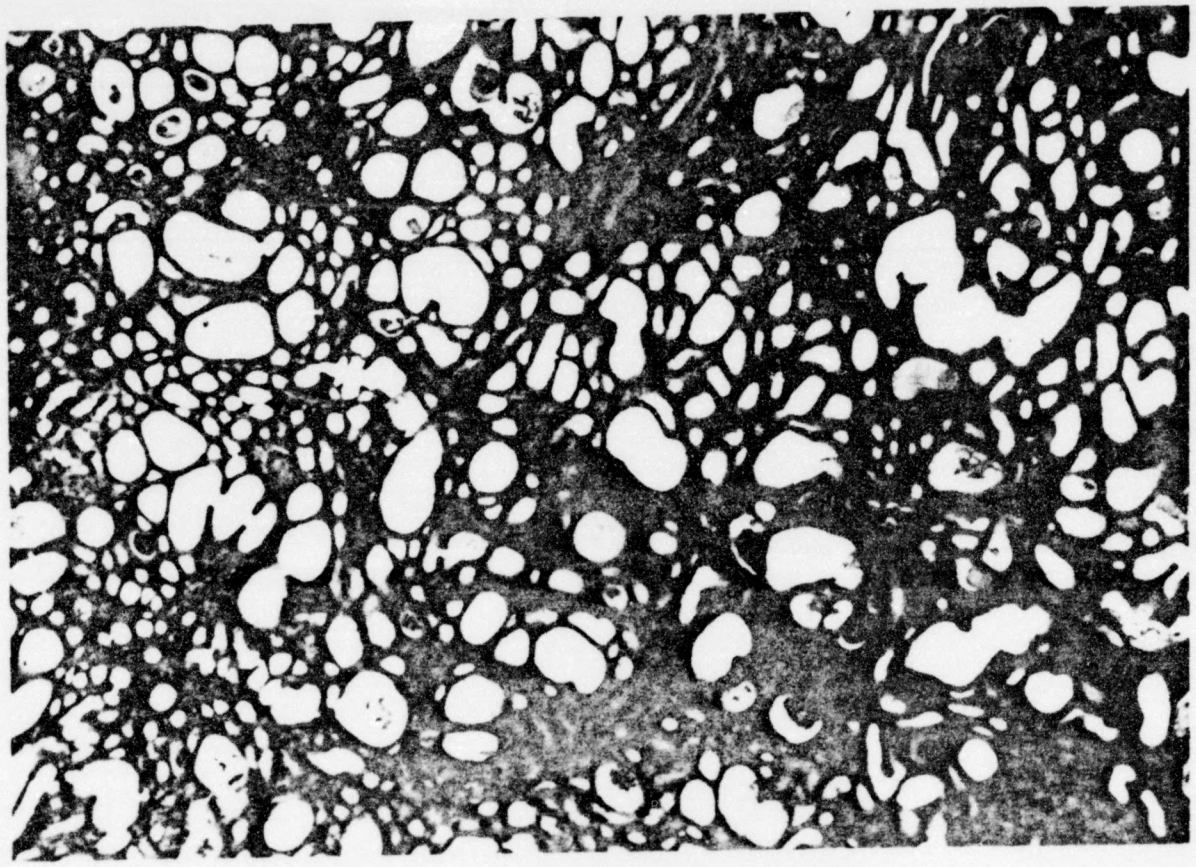
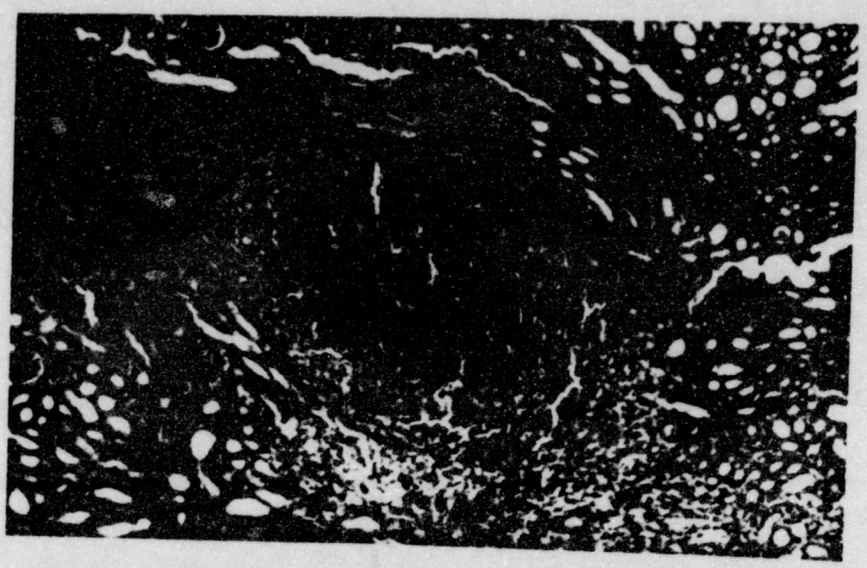
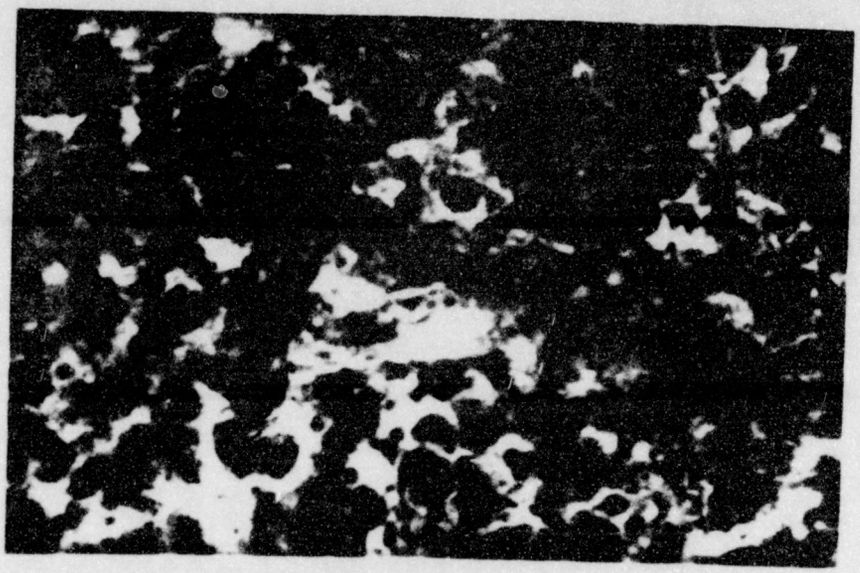


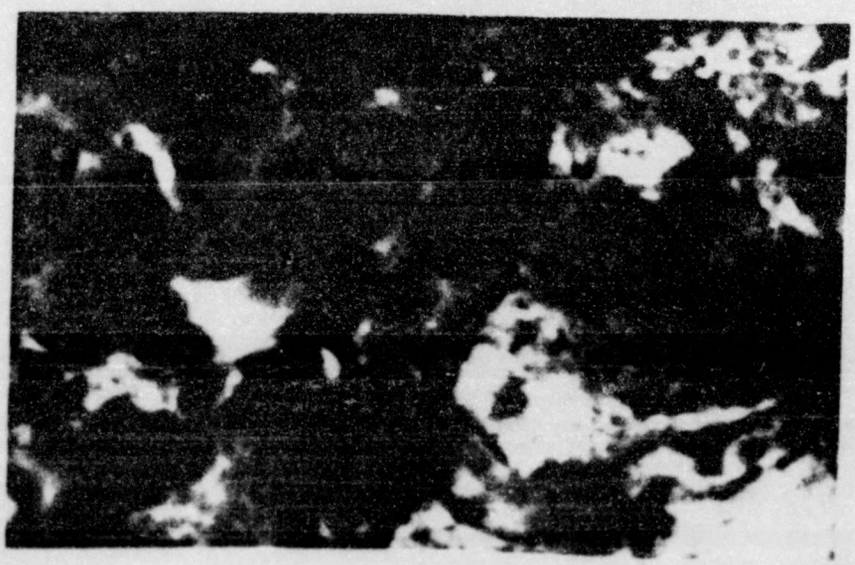
Figure 17a. Section through the cortex of a kidney taken from an animal killed 6 d after infection showing a granuloma with large number of white blood cells and a capsule of fibrous tissue. X 30.

Figure 17b. Center of granuloma showing the hyphal growth surrounded by white blood cells. X 100.

Figure 17c. Fungal hyphae at center of granuloma. X 1000.





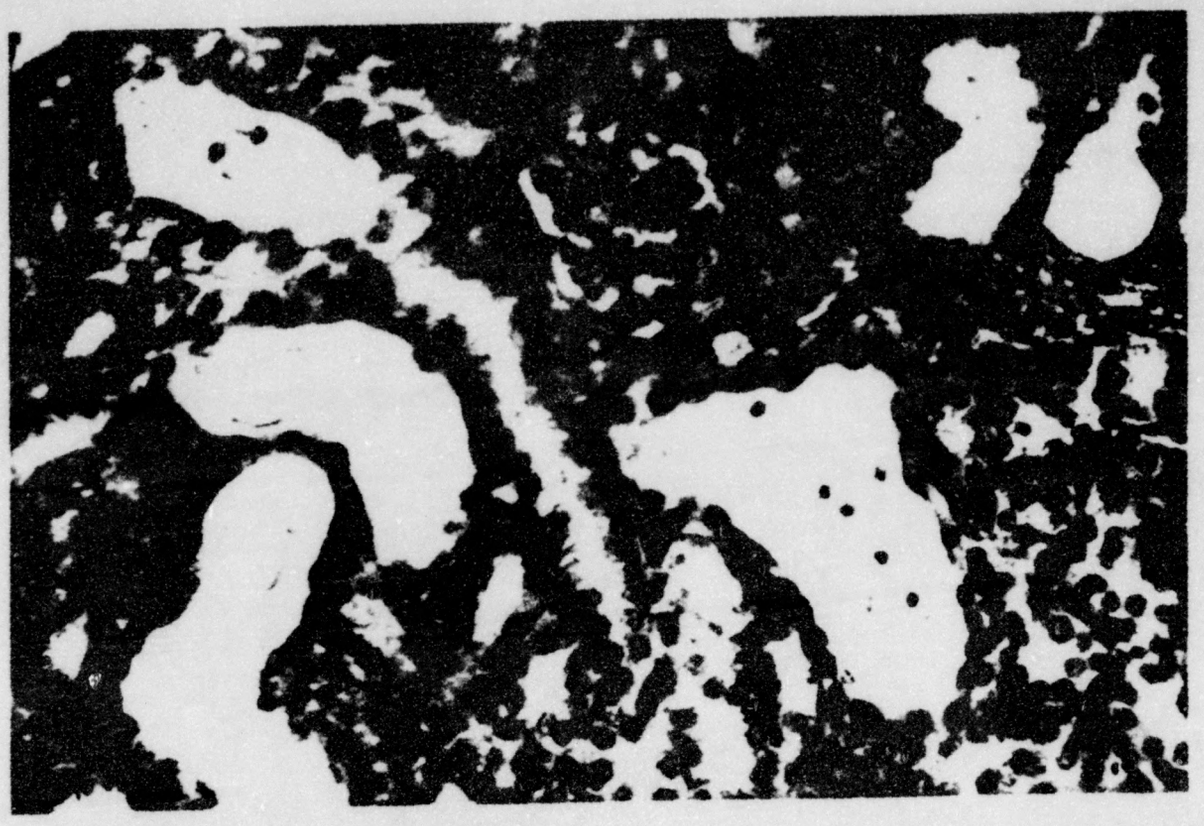


Kidneys taken from mice killed on the eighth and tenth day after infection showed tubule enlargement which had extended all the way through to the region of the cortex. So much enlargement had occurred that the tubules were distending the kidney capsule (Figures 18 and 19). Fibrotic tissue replacement had occurred in the remaining areas of the medulla (Figures 20a, b, and c) near the then-enlarged pelvic cavity (Figure 21). The few remaining medullary collecting tubules were extremely distended and fungal hyphae could be seen in the stroma surrounding them (Figures 22a, b and c). At this time, an epithelial layer completely lined the entire pelvic cavity. Large numbers of white blood cells still persisted in both the surrounding tissues and the pelvic cavity and were adhering to the surface of the fungal ball.

By days 17 and 21 post-injection only the cortex of the kidney remained (Figure 23 and 24). Under close microscopic examination the glomeruli appeared to be normal (Figure 25), but the remaining tubule systems were greatly enlarged due to clogging by proteinaceous material. Interstitial fibrosis had occurred in the remaining stroma and the concentration of white blood cells in this tissue had decreased. The fungal ball had become very diffuse and contained large areas of presumably dead hyphae (Figure 26a and b). The numbers of white blood cells in the cavity had also decreased substantially from previous levels. The squamous epithelial lining of the pelvic cavity persisted for the duration of the experiment.

Figure 18. A section of cortex from a kidney taken from an animal killed 8 d after infection showing distended tubules filled with proteinaceous material. X 200.

Figure 19. A section of cortex from a kidney taken from an animal killed 8 d after infection showing distended tubules pushing against capsule. X 200.



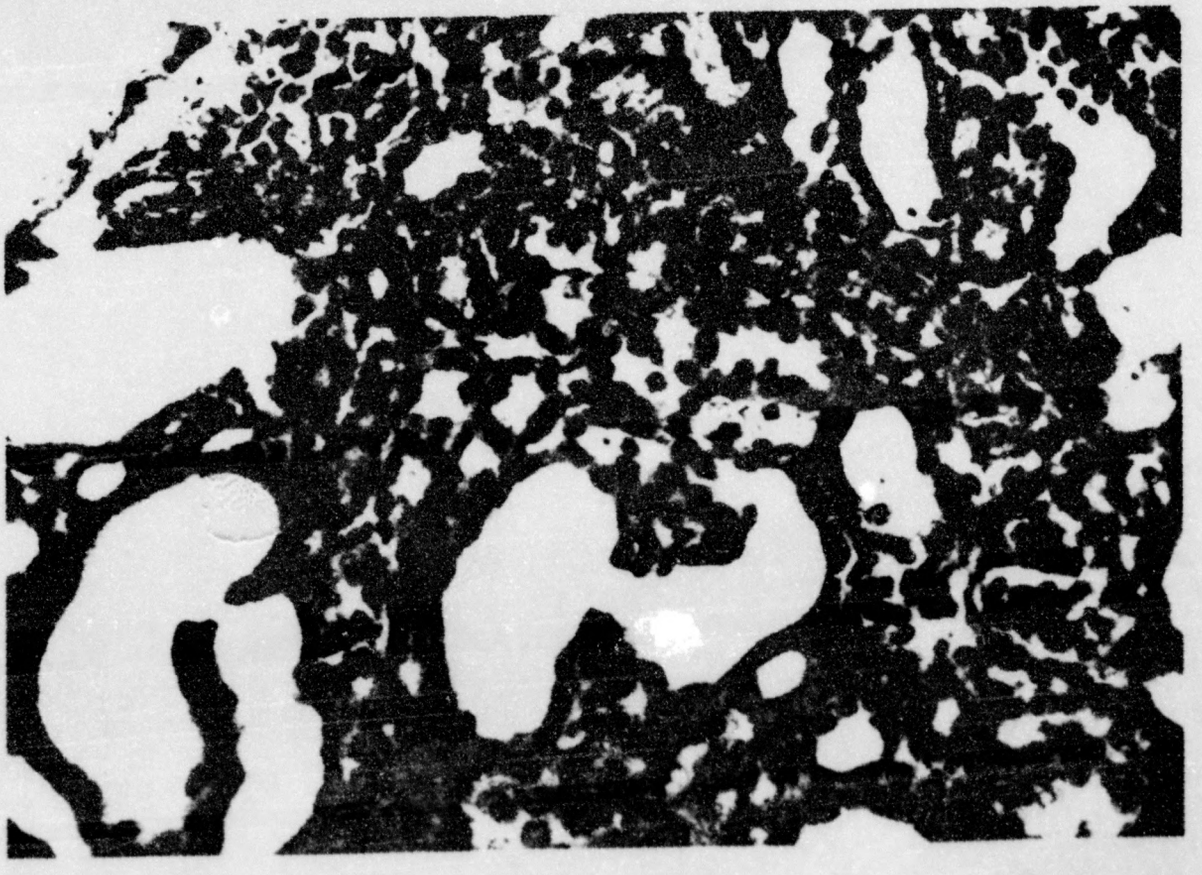
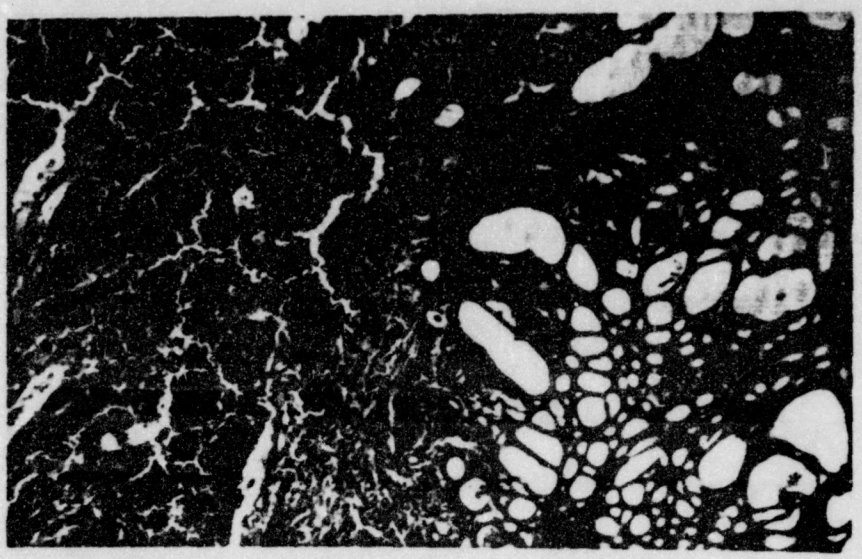
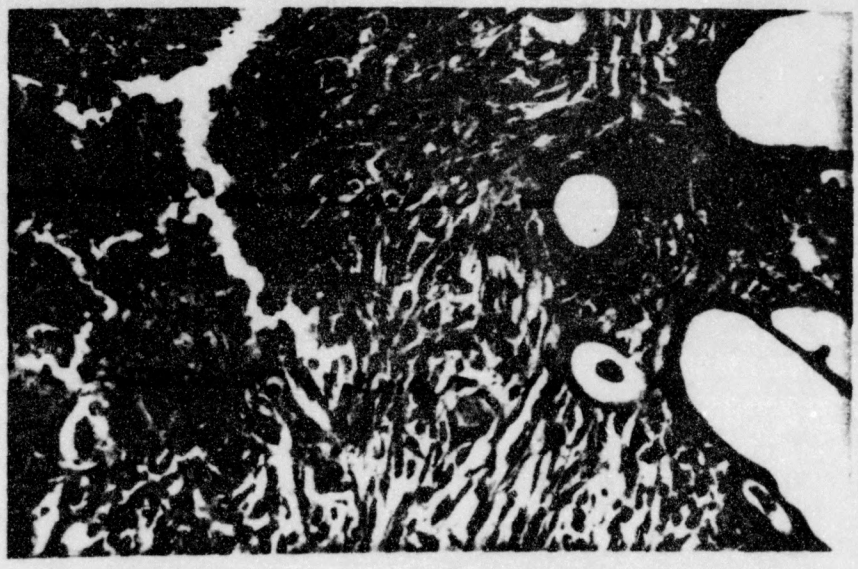


Figure 20a. A section of medullary tissue from a kidney taken from an animal killed 10 d after infection showing white blood cell invasion and interstitial fibrosis. X 30.

Figure 20b. Areas of white blood cell invasion and interstitial fibrosis. X 100.

Figure 20c. Areas of interstitial fibrosis. X 200.





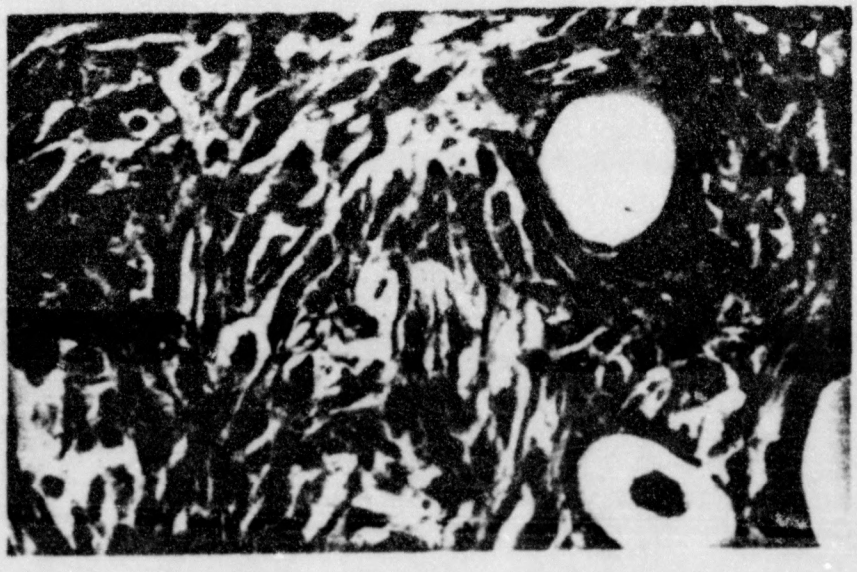


Figure 21. A section through the pelvic area of a kidney taken from an animal killed 10 d after infection showing extent of hyphal ball proliferation and distended tubules. X 38.

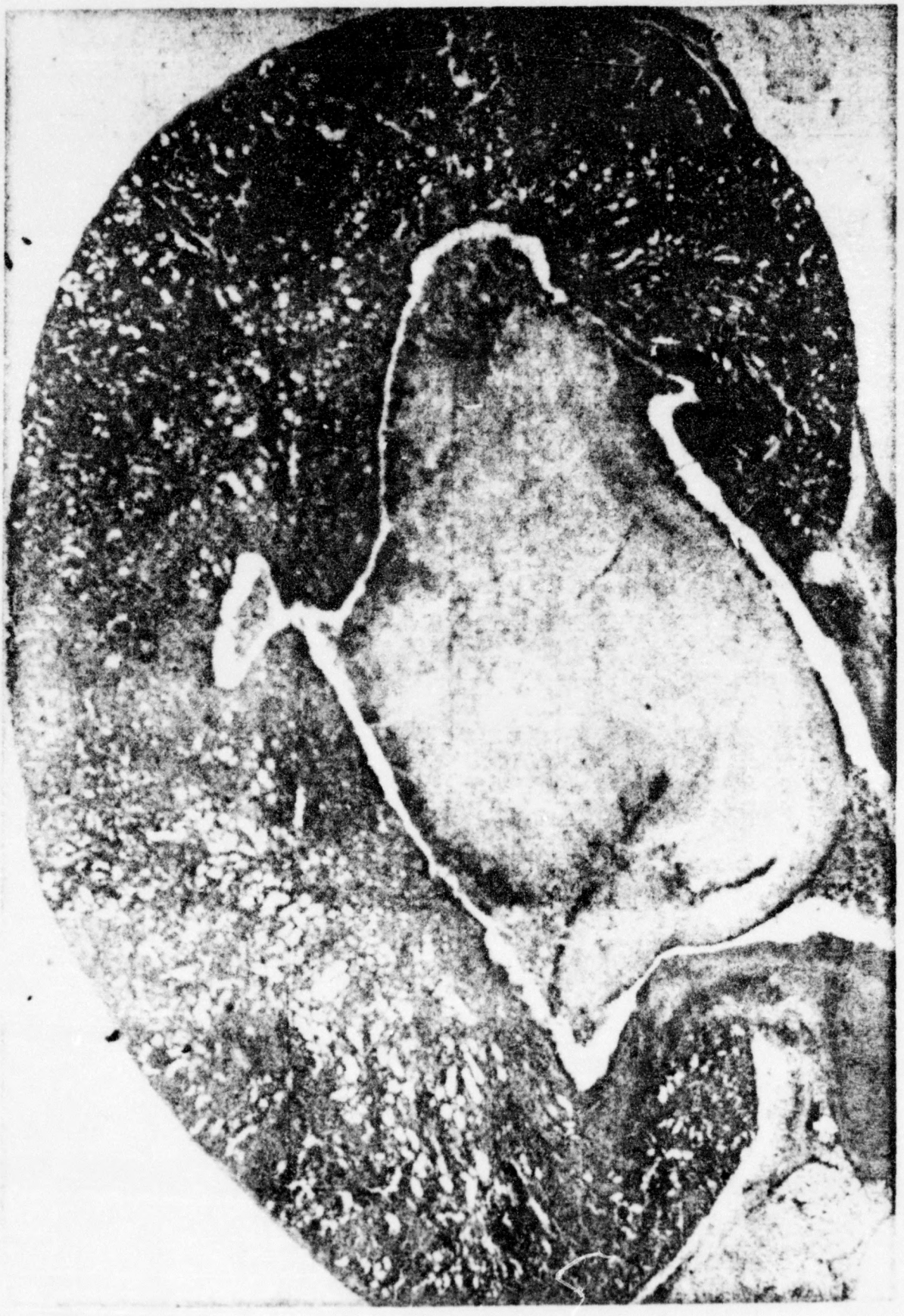


Figure 22a. A section through the pelvic area of a kidney taken from an animal killed 13 d after infection showing remaining tubule system completely surrounded by hyphae. X 30.

Figure 22b. Remaining tubules surrounded by hyphae and containing protein casts. X 100.

Figure 22c. Tubule containing protein cast coated with white blood cells. X 200.

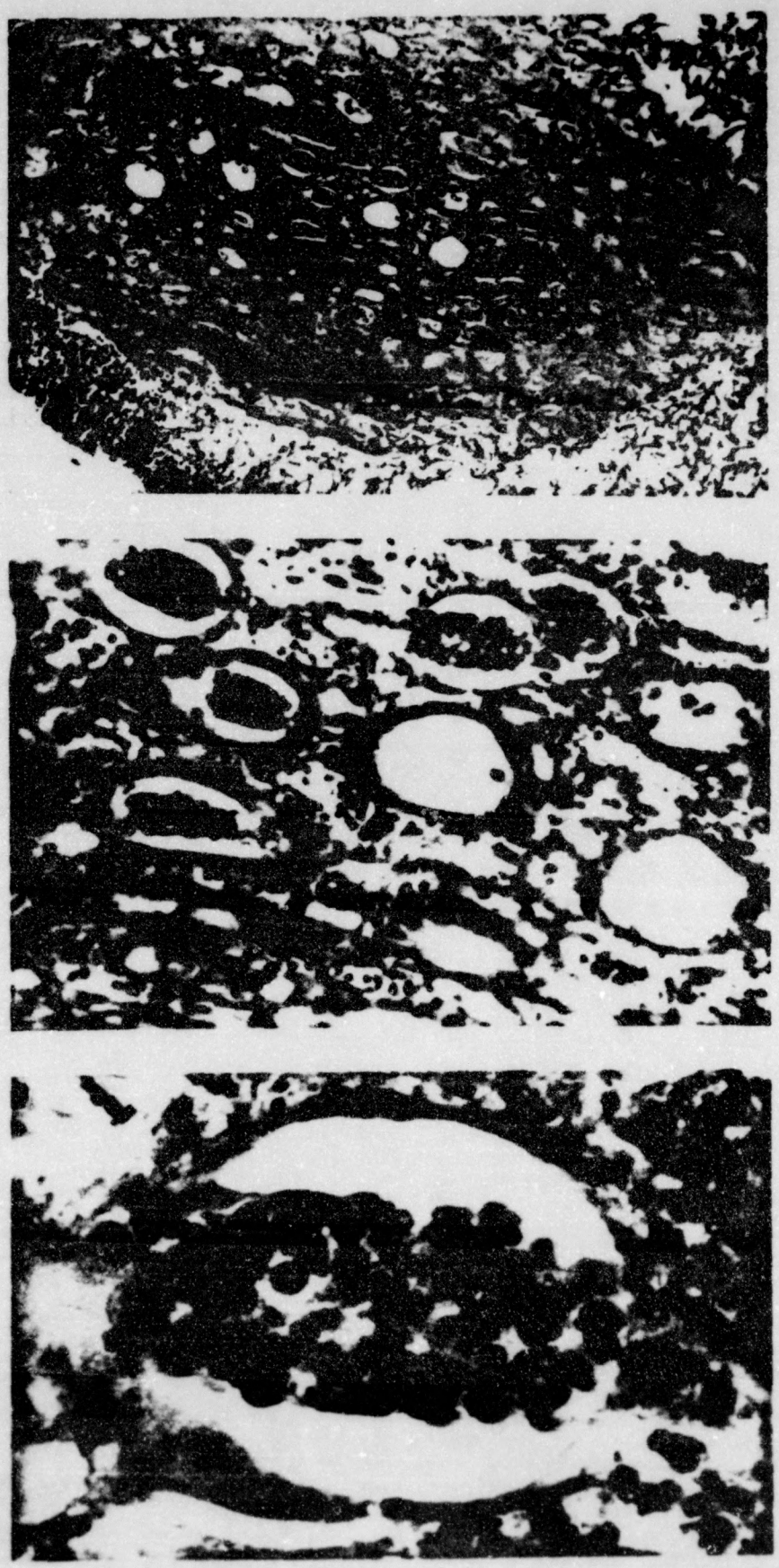
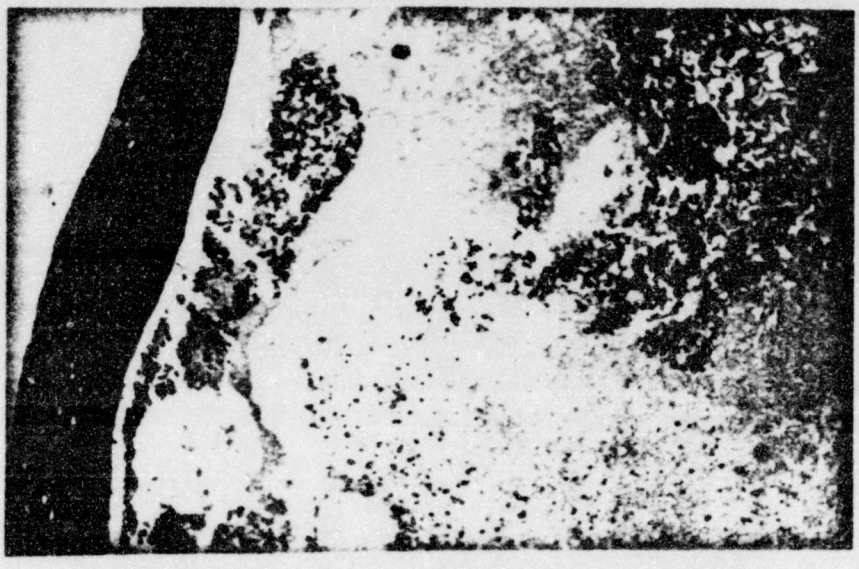


Figure 23. Section through the cortex area of a kidney taken from an animal killed 17 d after infection showing the remaining cortex and capsule. X 30.

Figure 24. Section through the pelvic area of a kidney taken from an animal killed 17 d after infection showing extensive hollowing of pelvic cavity, presumably dead hyphae, and remaining cortex. X 60.

Figure 25. Section through the cortex region of a kidney taken from an animal killed 17 d after infection showing remaining cortex with normal glomeruli. X 200.



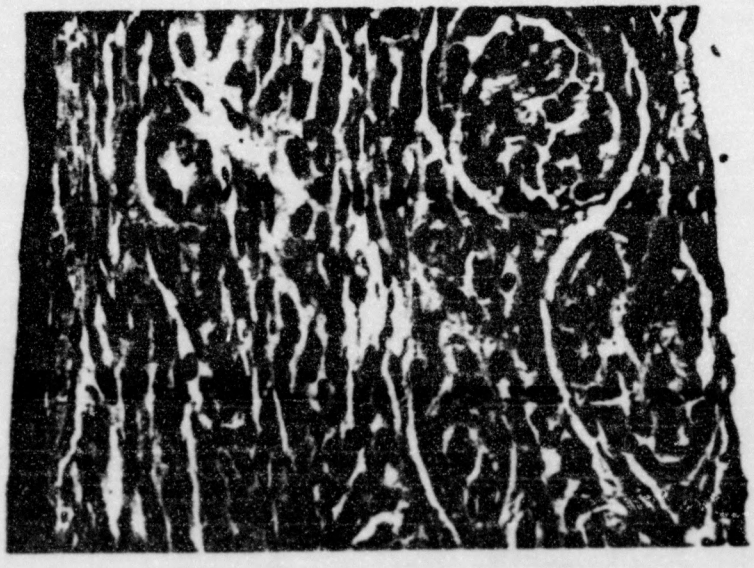
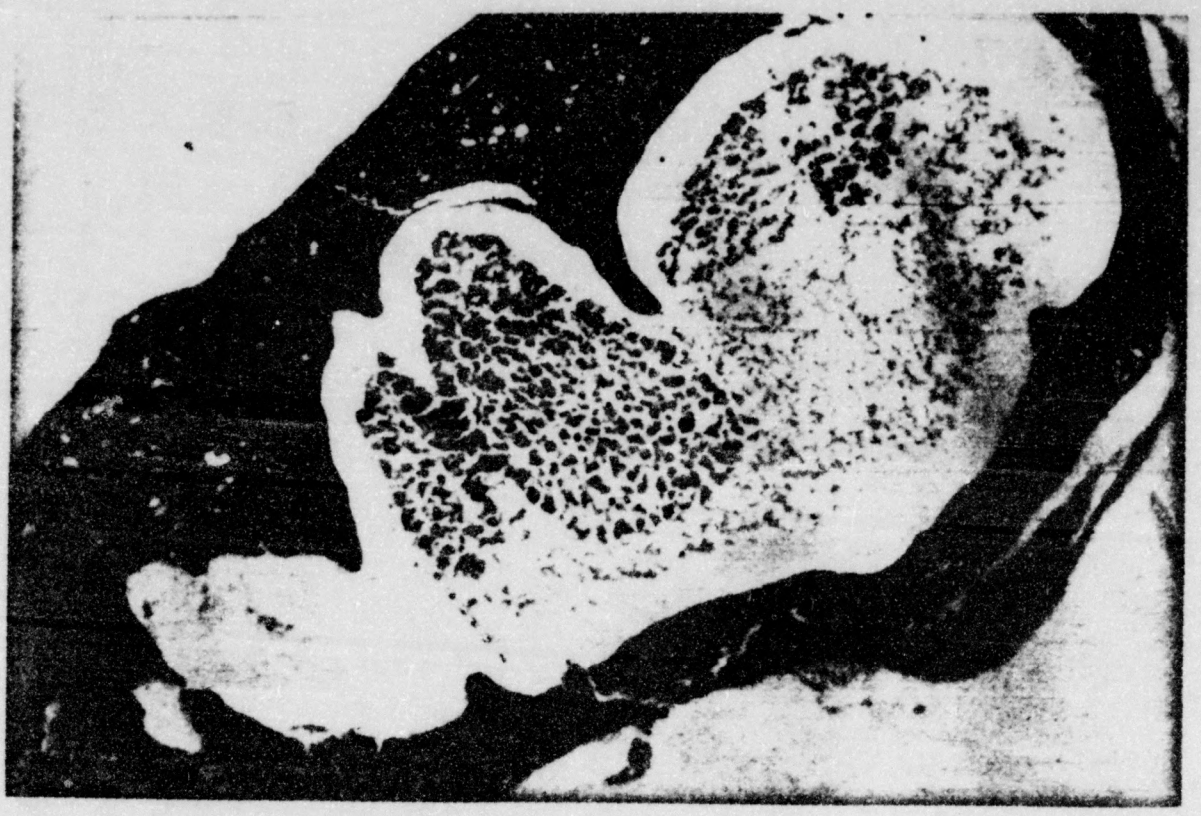
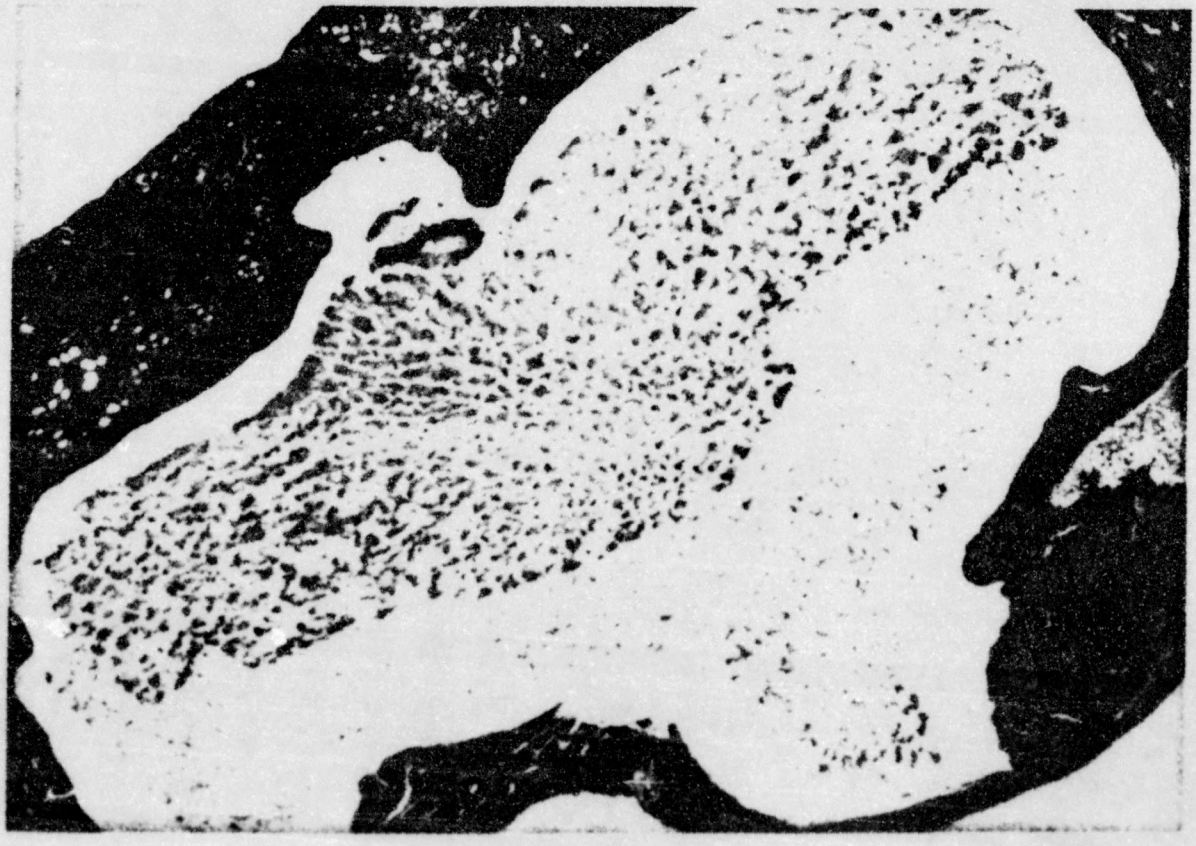


Figure 26a. A section through the pelvic area of a kidney taken from an animal killed 21 d after infection showing the enlarged cavity and the remaining cortex. X 55.

Figure 26b. A cross section through a kidney taken from an animal killed 21 d after infection showing the greatly enlarged pelvic cavity, profuse hyphae, and the remaining rim of cortex. X 55.





Differential Blood Counts

In mice the highest percentage of white blood cells are the agranulocytes: monocytes and lymphocytes. Neutrophils, eosinophils, and basophils make up the remaining proportion of the total white blood cell population. An experimental treatment may change the normal percentage of certain white blood cells depending on the type of response mounted by the animal's immune system.

This hypothesis was given credence by the results of the blood counts from both experiments. An increase of neutrophils occurred 24 h post-injection and peaked after approximately 4 d in infected mice. Subsequently neutrophil counts dropped to near normal by about 4 d after this initial peak. Counts of neutrophils from sham-operated mice were high at 24 h, but then decreased steadily over the duration of the experiment. The controls showed a somewhat irregular differential blood count, but this could be attributed to stress or any other variable which could not be accounted for or controlled (Figure 27).

Monocytes increased dramatically in both the sham-operated and the infected animals 6 d after the start of the experiment, but controls also showed a slight increase at about the same time.

The results from Experiment 2 (Figure 28) did not show the same peak of neutrophils in the infected animals, but rather both the sham-operated and infected mice had high neutrophil counts at 24 h post-surgery and these high counts were maintained in both groups for approximately 16 d. On

Figure 27. The results of differential blood counts from animals in Experiment 1 comparing: a) neutrophil counts, b) lymphocyte counts, and c) monocyte counts of control, sham-operated, and infected animals.

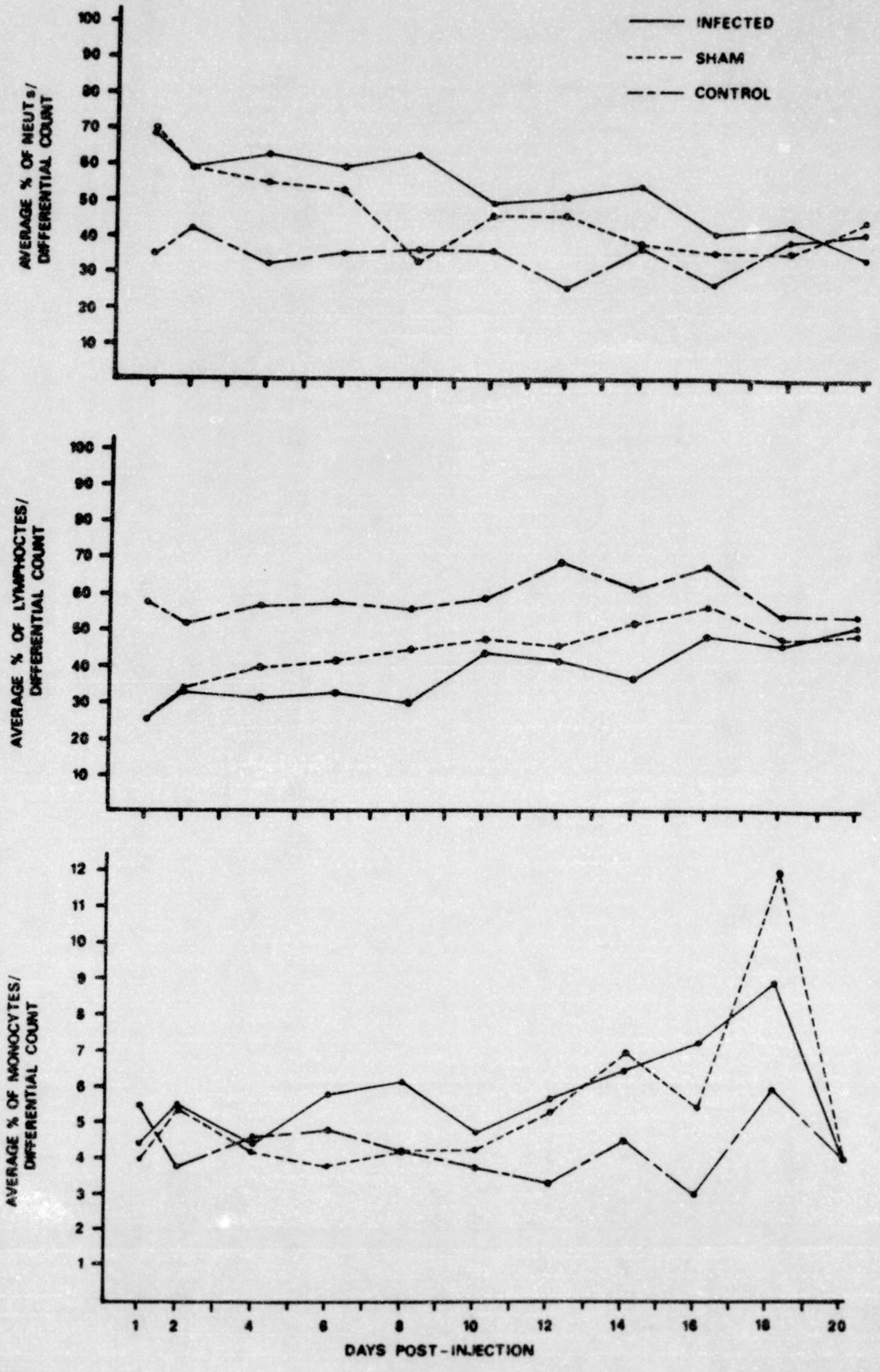
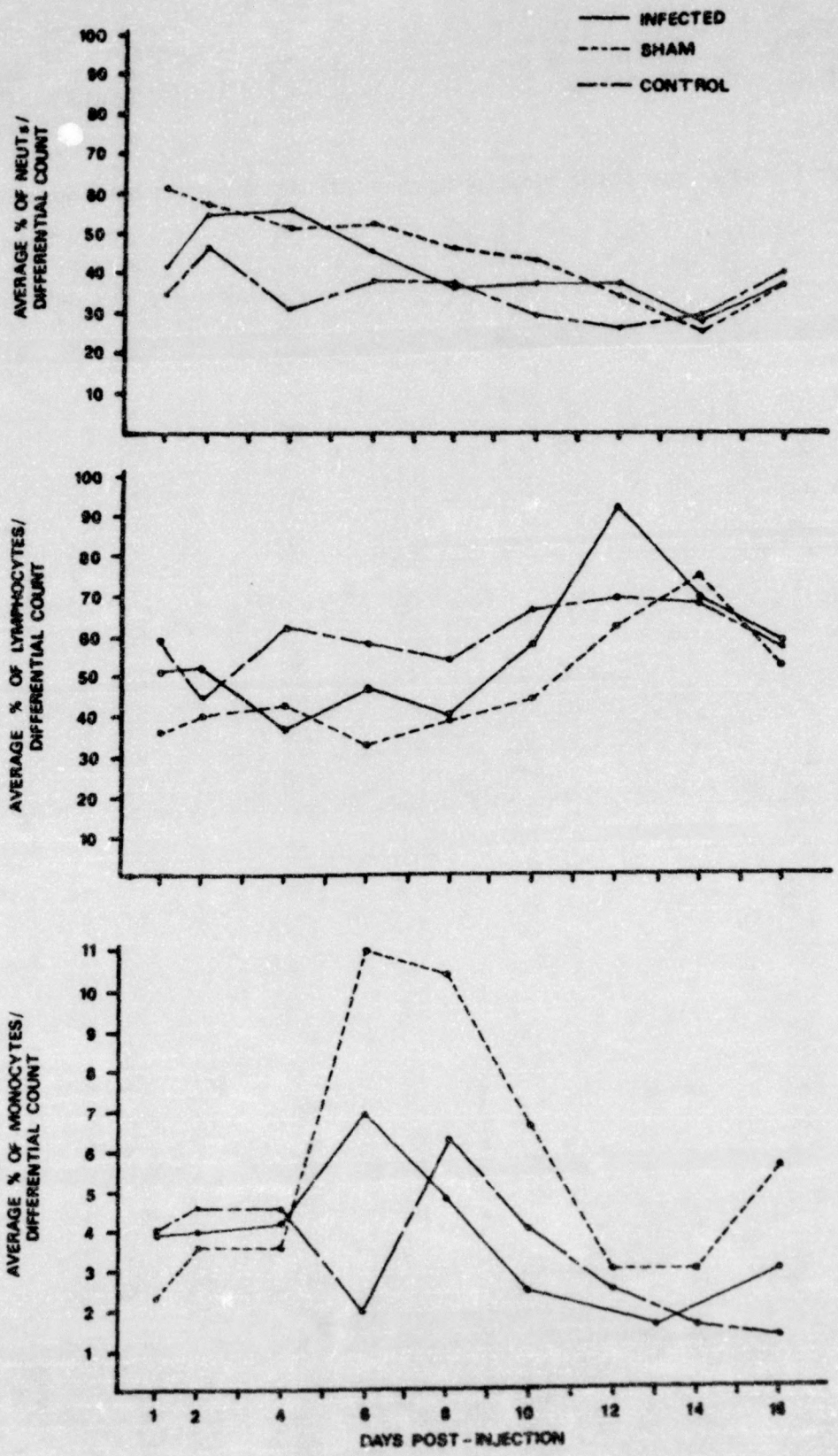

INFECTED
SHAM
CONTROL
AVERAGE % OF NEUTS/ DIFFERENTIAL COUNT
AVERAGE % OF LYMPHOCTES/ DIFFERENTIAL COUNT
AVERAGE % OF MONOCYTES/ DIFFERENTIAL COUNT
DAYS POST-INJECTION

Figure 28. The results of differential blood counts from animals in Experiment 2 comparing: a) neutrophil counts, b) lymphocyte counts, and c) monocyte counts of control, sham-operated, and infected animals.


INFECTED
SHAM
CONTROL
AVERAGE % OF NEUTs/ DIFFERENTIAL COUNT
AVERAGE % OF LYMPHOCYTES/ DIFFERENTIAL COUNT
AVERAGE % OF MONOCYTES/ DIFFERENTIAL COUNT
DAYS POST-INJECTION

day 18, monocytes peaked in counts of both the sham-operated and infected individuals much later in comparison to the monocyte peak in Experiment 1 which occurred after 6 d. Controls in this experiment showed a consistent neutrophil and monocyte number which made comparisons more outstanding.

DISCUSSION

The experiments reported here differed from other experiments in that the method of inoculation was directly into the target organ, the kidney. Most of the previous methods involved the introduction of spores through the intravenous route using the tail vein (Lehman and White, 1976; Rippon and Anderson, 1978), or the direct inhalation of an aerosol with a controlled number of viable spores (Merkow, et al., 1971). One group of researchers also studied the effects of subcutaneous injections of the spores (Turner, et al., 1975).

The method of direct inoculation was successful in that it assured a high infection rate. Out of all the tissues examined, only 4 kidneys showed no sign of infection. None of the animals infected by this method died. Thus, the infection could be localized and then could be studied over a long period of time.

The kidneys taken from animals killed 24 h after injection showed no hyphal growth, with one exception. Therefore, locating needle tracks left in the kidney was the only way to determine if these kidneys indeed had been inoculated. Needle tracks were observed in kidneys from animals sacrificed at 24, 48, and 96 h after inoculation. After that time, tracks were no longer visible.

The insult to the kidney initiated an inflammatory response which was observable at the site of the needle track after 24 h. This trauma was expected to affect the rate at which white blood cells would respond to the presence of the A. flavus spores. According to Ford, Baker, and Friedman (1968)., hyphae were observed in the tissue five days after inoculation with no obvious cellular response having occurred. In this experiment, white blood cells were observed "sticking" to hyphae after only 48 h.

In most experiments where a kidney infection due to A. flavus spores were observed, the primary focus of the infection was the pelvis. This location was consistent with observations made in previous research (Turner, et al., 1975; Ford, et al., 1969).

One possible explanation for the rather consistent localization of infection is that the renal pelvis fulfilled one of the favored conditions for growth, a preformed cavity. It is possible that the initial fungal growth was saphrophytic in nature and the normal cellular response was not initiated until tissue infiltration occurred.

The renal tubules of the medulla were the first site of tissue invasion, this was a similar finding of other investigators (Pore and Larsh, 1968). However in this case, even with the presence of large numbers of white cells, mostly neutrophils, at 48 h, the spread of the hyphae into the tissues did not appear to be hindered. This observation suggested a second possible reason for the rapid growth of the fungus in the kidney, the decreased degree of phagocytic activity.

The ability of phagocytes to ingest particles that they recognize as foreign depends on interactions occurring upon their external membranes and responses that occur in their peripheral cytoplasm (Stossel, 1977).

In the kidney, this recognition step necessary for ingestion is hindered due to an unusual degree of anti-complimentary activity found in this organ. Extensive comparisons of these activities of several tissues from mice, rats, and rabbits have been done. In more limited experimentation, the kidneys of pigs, calves, oxen, and man were also studied in comparison to other tissues. The results from all the experiments showed that the kidney had the highest degree of anticomplimentary activity (Beeson and Rowley, 1960).

Pieces of cortical and medullary tissues were separated and tested to see if this anticomplimentary effect differed from area to area of the kidney. It was found that both areas had the same degree of the activity and therefore, the component was associated with the renal tubules and not localized in one zone of the kidney (Beeson and Rowley, 1960).

The only chemicals known to inactivate selectively the third component of the complement system are ammonia and primary amines. In the kidney, the main source of ammonia is the tubular epithelial cells. Immunological processes in the kidney encounter wide variations in solute concentrations and osmotic pressures that exist in no other extracellular fluids. Phagocytosis has been found to be greatly inhibited by urines with high urea concentrations. It has also shown that high osmotic pressure is also an important factor responsible

for antiphagocytic effects. In some *in vitro* experiments, a direct correlation was made between the antiphagocytic properties of solutions and their osmotic pressures. The appearance of lymphoctes in the area depended on the environmental tonicity (Chernew and Braude, 1962).

In *in vitro* experiments, increasing concentrations of sodium chloride, urea, or hydrogen ions were found to depress phagocytosis until it was virtually abolished at a point well below the highest concentrations of these ions anticipated in the area of the renal pelvis. The inhibitory effect of the hydrogen ion occurred rather abruptly between pH 6.2 and 5.5. Because the pH of urine in distal tubules may fall well below this level, the acidity becomes an even more important factor in the inhibition of phagocytosis. In addition, complement has been found to be rapidly destroyed at pH 5.5 or lower. Many studies have indicated that high concentrations of urea found in the medullary region of the kidney depressed phagocytosis and thus favored infections by agents not normally pathogenic in other organs (Chernew and Braude, 1962).

It would seem that in these experiments that even though a large population of white blood cells was present in the organ due to the inoculation technique, these cells were unable to respond to the dormant spores, germinating spores, or to the original small hyphal balls. The initial inability to respond may have been due to the antiphagocytic climate of the kidney, but eventually, after approximately 2 d, the hyphal mass was already too large for its growth to be inhibited. In previous experiments, the hyphal ball was

already of a sufficiently large size to resist inhibition of its growth before a cellular response was even observed (Ford, et al., 1969).

One researcher, when studying the aspergilli in the lung, found, at necropsy, a single kidney with lesions containing hyphae while the other kidney remained normal (White, 1977). This finding stimulated more research and the results formed a hypothesis for a third reason for the seemingly high affinity of certain Aspergillus species for the kidney (Lehman and White, 1978).

In the latter experiments, extracts of various organs were used in a hyphal extension assay. It was clear from the results that both the kidney and the spleen were stimulatory to hyphal growth, and the degree of enhancement of hyphal extension was linearly related to the $\log_{10}$ dilution of the tissue extract. This effect was postulated to have allowed a more rapid conidiospore germination in this organ (Lehman and White, 1978).

These researchers hypothesized that a water-soluble factor in kidney extracts stimulated germination in vitro. This factor could also have been present in vivo and been the determinant of the kidney's susceptibility to infection. Rapid germination would allow for rapid mycelial growth which could easily overcome host defenses. Examples where specific chemicals were responsible for the localization of the growth of pathogens in particular organs or host tissues exist, but more work should be done in this area (Lehman and White, 1978).

Spores germinated in the renal pelvis and the hyphal balls were localized in this area until approximately 4 d after inoculation. After this time, the hyphae began to invade the nearby medullary papillae consisting of the proximal and distal convoluted tubules and Henle's loop of the nephron. The same loci of primary tissue invasion were also observed by others (Ford, et al., 1968; Pore and Larsh, 1968). The question of what facilitated such an invasion has been studied by several investigators (Turner, et al., 1975; Sidransky, et al., 1972).

The latter group suggested that the organisms may liberate digestive or proteolytic enzymes which facilitate penetration into the tissue. Meanwhile, Turner and co-workers stated that hyphae were only found after necrosis was prominant in abscesses. This fact led these researchers to suspect that tissue death preceded the fungal invasive phase of the organism which then utilized the dead tissue as an effective substrate. In experiments reported here, invasion of the tissue by hyphae occurred in normal tubules without the aid of necrosis.

A personal communication from Schwab to Turner (1975) and his group pointed out that a virus particle had been found in the hyphae of A. fumigatus and this observation raised the possibility that tissue necrosis, where found, may have been the result of the rapid multiplication in the tissues by the virus. It was unknown at that time if these parasites of fungi were pathogenic to the fungal host.

As the invasion of the hyphae continued, the size of the hyphal ball within the pelvic area also continued to fill the available space and even began to extend into the ureter. With this blockage, many histological changes began to occur.

Urine retention due to the ureter blockage resulted in enlarged tubule lumen and pelvic sinuses. Both of these changes were indicative of hydronephrosis. These results corresponded to those found in previous work (Williams, 1973). In this disorder, atrophy of the renal papillae was evident, and the lobes of the right kidney begin to hollow out leaving only a rim of tissue under the thickened capsule. Thus, as the hollowing continued, more space became available for the fungal ball to proliferate and the dead tissue may have served as substrate. It is also possible that bacterial sepsis occurred within this blocked area and resulted in tissue necrosis.

A corresponding pyelonephritis was also observed from 4 d post-infection throughout the duration of the experiment. This disorder was chronic and certain pathological changes corresponded to this type of infection. In most chronic cases, as found here, the kidney decreased markedly in size, and its shape was sometimes distorted by the fibrous replacement of lost or damaged nephrons. Surviving tubules usually compensated by enlargement and were dilated and contained protein casts. Sometimes, casts were totally "coated" with white blood cells. White blood cells had infiltrated enlarged tubules and, due to this infiltration, tubules became more

distended and abscesses were formed. No hyphae were ever observed within these abscesses. White blood cells had also infiltrated surrounding tissues in large numbers, a typical manifestation of pyelonephritis.

Throughout both experiments the cortex was minimally involved. Glomeruli remained intact and appeared to function normally. However, by day 21 post-infection, interstitial fibrous replacement had begun. This finding corresponded with that of another research group (Pore and Larsh, 1968).

In an organ removed 6 d after inoculation, a granuloma was found in the upper medullary, lower cortex region. This particular observation posed a theoretical problem in that it is difficult to determine why the hyphal growth could be completely surrounded by white blood cells, mostly neutrophils, and then the entire "abscess" surrounded by fibrous tissue to form a thickened capsule. In this case, the system had completely delimited this outbreak of the fungal infection. Perhaps this particular response was possible due to the high numbers of white blood cells already present in the area which could have surrounded the germinating spore and effectively inhibited its development. Perhaps this area of the kidney supported phagocytic activity better than was postulated.

At the same time that the aforementioned granuloma was observed, plasma cells were seen to have infiltrated the area of the remaining medulla. This would imply that some sort of humoral response was being mounted, but it was impossible to state emperically that their presence was due

to the fungal infection alone. Bacterial sepsis may have occurred and the response could have been for some unknown pathogen. By day 6, it appeared that the function of the infected kidney had ceased.

From day 6 until the termination of the experiment, the medulla was gradually being destroyed. The necrotic tissue was probably used as substrate for the ever-increasing fungal ball and bits of the dead tissue were also seen within the cytoplasm of wandering macrophages. At any stage after day 6, the pelvic cavity was always lined by a highly proliferated, flattened, cuboidal epithelium; this included the stage when only the cortex of the infected kidney remained. This may have been an attempt of the organ to limit the infection or it may simply have been a response typical of any organ to replace dead or dying tissue in order to maintain its integrity. Within the remaining cortex, functional glomeruli were observed, but so was interstitial fibrosis. Fibrosis could also be termed a response of delimitation by the organ, but more likely this was the result of replacement of dead tissue or a scar-like reaction due to the combined effects of hydronephrosis and pyelonephritis. Other researchers noted similar findings in that the entire kidney would disappear after several months of the infection (Ford and Friedman, 1967).

Even though no aflatoxin studies were done in this work, the question of aflatoxins must always be addressed when most species of the Aspergillus are used in experimentation.

One of the most outstanding effects of the toxin was demonstrated at the DNA level. In *in vitro* studies using green monkey kidney cells, aflatoxin B_1 was transformed by the system into active metabolites which bound covalently to DNA, RNA, and protein (Swensen, *et al.*, 1974). It was also shown *in vivo* that this action led mainly to the inhibition of RNA and protein synthesis (Sarasin and Moule, 1975). Even unactivated aflatoxin B_1 was found to bind to double- and single-stranded DNA through weak interactions and thus, even without activation, one could expect an effect on processes at the genetic level. Most authors agree that all inhibition began at the step of DNA synthesis when the aflatoxin functioned at this level (Mereghini and Schumacher, 1977). However, these results were found only when purified aflatoxins were added in high concentrations to the experimental system. The question remains whether the colonies in the *in vivo* system were producing enough aflatoxin to cause cell death and large areas of necrosis.

It was reported that the most important factor influencing metabolic activities of fungi was temperature (Purchase, 1974). The optimum temperature for growth of *A. flavus* was reported as 36-38°C with a range of 8-48°C, while the optimum temperature for aflatoxin B_1 production was shown to be 25°C (Purchase, 1974). There was an absence of any toxin production at 41°C and no aflatoxin B_1 was produced at 36°C (Schindler, *et al.*, 1967). It was hypothesized then that B_1 at least, one of the most potent aflatoxins, was not the

cause of the tissue necrosis. Some attempts were made to determine if aflatoxins were being made *in situ* within experimental systems, but the results of analysis were uniformly negative (Ford and Friedman, 1967).

The analysis of variance of the kidney weights seemed to suggest conflicting conclusions as to what was happening as the result of the chronic *A. flavus* infection. In Experiment 1, the statistics show that a difference in weights existed in the infected kidney and the noninfected kidney from the same animal at the very beginning of the experiment. Because comparisons with controls or sham-operated mice could not be done due to inappropriate kill times, all sham-operated and control animals were killed at the end of the experiment, the reason for the weight difference could not really be determined.

There are several possibilities for the differences that can neither be substantiated nor disregarded. The differences in weights could have been due to surgical procedure alone. The act of puncturing the kidney may have increased the blood flow to the organ, thus engorging the kidney and causing an increase in the weight. As the infection progressed, changes in weight between the two kidneys may have occurred due to the fungal ball proliferation and the combined pathological effects of hydronephrosis and the pyelonephritis. Another reason could simply be the normal asymmetry that exists in the body. It is not uncommon to find paired organs or appendages to differ in size or weight.

In Experiment 2, a significant difference in blocks (over time) ($p<0.01$) could be found, but not in treatments. Comparisons were made between controls, sham-operated, and infected and noninfected kidneys from the same animal. It is obvious from Table 5, that significant differences occurred over certain time periods, but it appears that the differences in weights of these kidneys at different time periods seem to cancel each other. At certain times, usually at the beginning of an experiment, the kidneys were increased in size, while at the later stages of the experiment, the kidney weights were decreased. Adding across treatments caused an "averaging out" of the weights. Several other more sensitive techniques were tried in order to get a better analysis, but the same type of problem occurred. One of the major problems in trying to draw conclusions from these data was the fact that, over time, the animal was also maturing and thus an increase in the weight of normal kidneys was expected. It was almost impossible to tell whether the change was due to normal changes within the system, or the chronic infection, or to the surgical procedure alone.

The blood analysis work proved equally difficult and inconclusive. In Experiment 1, the blood level of neutrophils in infected animals peaked 4 d after the surgical procedure and then declined to the level of the normals used in the system. This trend was very similar to results obtained from previous published experiments performed at Western Kentucky University by Peter Didier. Unlike this original

experiment, blood counts from sham-operated mice were also recorded and the white blood cell population of neutrophils increased at the same rate as those of the infected animals. Also an increase in the monocyte population was found at 6 d after the injection in both the infected and sham-operated animals. From these results, one can hypothesize that the change of the white blood cell populations in the peripheral blood was due to the surgical techniques rather than the A. flavus infection. Vascillations in the blood cell counts of normal, sham-operated, and infected animals could be expected also, due to the stress of the experiment and bleeding schedule.

Unfortunately, the blood counts from Experiment 2 did not duplicate the data from Experiment 1. Neutrophil counts from both the sham-operated and infected animals started much higher than the normals and these elevated levels were maintained for an unusually long period of time. The monocyte peak, which occurred at 6 d in Experiment 1, was not evident until approximately day 18 in Experiment 2.

The probable cause for these conflicting results may have been the use of a different, more potent anesthetic. Bio-Thol, in high enough concentrations, can cause deterioration of a dog's vein. It was assumed that the dilution of the anesthetic and the low inoculation dosage would lower the chance of any reaction by the animals. After several days, it was obvious that there was a tissue reaction in the larger animals which had been given a larger dose of the anesthetic.

Scabs were felt between the scapulae, and at autopsy prominent reddened areas were easily seen on the under surface of the skin. It is possible that this reaction to the anesthetic was responsible for the prolonged neutrophil count which was maintained for the major part of the experiment.

SUMMARY

1. An *Aspergillus flavus* infection can be localized and effectively maintained in the murine kidney for an extended period of time whithout causing death to the animal.
2. A chronological study of the infection gave a clear and reproducible histopathological response.
3. Analysis of blood counts gave inconclusive results in that the changes in the percentages of the white blood cells were apparently due to the surgical procedures and any response to the infection may have been sufficiently masked.

APPENDIX

Methods for Preparation of Tissues for Histological Study

Embedding

After fixation, the tissues were dehydrated in ethanol and embedded in Histowax (melting point 52-54°C) using the following schedule:

70% ethanol	1 h
95% ethanol	1 h
100% ethanol	1 h
100% ethanol	1 h
xylene	1/2 h
xylene	1/2 h
50% xylene-50% Histowax	1 h
50% xylene-50% Histowax	1 h
melted Histowax	1 h
embed	

Staining

Permanent, stained slides of the specimens were prepared for histological study using the following steps:

xylene	1/2-1 h
100% ethanol	15 min
95% ethanol	15 min
70% ethanol	15 min
distilled water	15 min
Harris' hematoxylin	10 min
running tap water	rinse
1% HCl in 70% ethanol	until destained
70% ethanol	rinse
0.5% $NaHCO_3$ in 70% ethanol	10-15 min
70% ethanol	rinse
95% ethanol	10 min
eosin	10 min
95% ethanol	rinse
95% ethanol	rinse
100% ethanol	15 min
100% ethanol	15 min
xylene	15 min
xylene	15 min

Upon completion of the staining and dehydration procedures, the specimen sections were mounted with Kleermount and covered with coverslips.

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