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FACTORS INFLUENCING EXPERIMENTAL CANDIDIASIS IN MICE. I. ALLOXAN DIABETES.†

The frequent clinical observation of the association of diabetes mellitus and candidiasis[1-6] prompted an investigation of the effect of alloxan diabetes on experimental infections in mice caused by *Candida albicans, Candida tropicalis, Candida guilliermondii,* and *Candida parapsilosis.*

The results of this investigation reveal that alloxan diabetes enhances, in the mouse, experimental infections caused by various species of Candida and suggest that the metabolic alterations of diabetes mellitus produce alterations in resistance which are of signal importance in the pathogenesis of Candida infections. These experiments followed preliminary observations in this laboratory in 1959, which showed that candidiasis was enhanced in mice with alloxan diabetes.

MATERIALS AND METHODS

Mice. Female Swiss albino mice from the Animal Production Section of the National Institutes of Health were used in all experiments. Mice weighing 16 to 20 g. at the time of alloxanization and infection were housed in glass jars, five animals per jar, and were given commercial mouse food and water *ad libitum.* Although animals were not pair fed, the nutritional state of both alloxan-treated and control mice appeared good throughout the study period.

Organisms: Candida species. Four species of Candida were utilized in the lethality studies and one species in the tissue population study: 1) *C. albicans* (strain B-311), isolated from the urine of a patient who died from disseminated candidiasis, demonstrated considerable virulence for experimental animals; 2) *C. tropicalis* (strain 48), isolated from an abscess in a patient with carcinoma of the cervix uteri, also demonstrated considerable virulence for mice; 3) *C. guilliermondii* (strain 3163), from the collection of Dr. Margarite Silva; 4) *C. parapsilosis* (strain B-1234) was isolated from the blood of a patient who died with Candida endocarditis.

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Alloxan Administration

Alloxan monohydrate* was freshly prepared by weighing and then dissolving the powder in distilled water so as to attain a one per cent solution (W/V). This solution was sterilized by Seitz filtration and injected into the lateral tail vein of the mouse at a dose of 100 mg./kg. The volume of the injection was 0.2 ml. or less per mouse depending upon the exact weight of each animal. The successful production of experimental diabetes mellitus was confirmed by the persistence of glycosuria three plus or greater, and by weekly micro-determinations of blood sugar obtained eight hours after withholding food. Urine was obtained by holding the mouse and stroking the abdomen, and glycosuria was measured by catching "midstream" urine directly into Testape® and observing the color change. Micro blood sugar specimens were obtained by inserting a heparinized capillary pipette into the retro-orbital capillary plexus of the mouse's eye. Glucose was determined by the Nelson-Somogyi method.[7] The average blood sugar of normal mice was 80 $\pm$ 30 mg. per 100 ml. eight hours after last feeding. A level higher than 200 mg. per 100 ml. eight hours after the last feeding was considered an indication of diabetes. Urinary ketone determinations were not obtained.

Inoculation Schedules

Colonies were scraped from 2 per cent glucose, 1 per cent neopeptone agar slants, incubated at 30° C. for 24 to 48 hours and suspended in normal saline. The inoculum size was determined by direct hemocytometer count in a Spencer bright line Neubauer counting chamber. Serial dilutions were made so that 0.2 ml. of each inoculum contained the desired number of organisms. All inocula were injected intravenously into a lateral tail vein.

Since normal saline has some toxicity for Candida organisms, the interval between preparation and administration of each inoculum was brief (usually less than 90 minutes).

In addition, the number of viable organisms in each inoculum was rechecked by means of pour plate cultures of the inoculum obtained after the inoculation procedure had been completed. Diabetic animals were not infected until ten days after treatment with alloxan in an attempt to eliminate the hypoglycemic and nephrotoxic factors accompanying intravenous alloxan administration.

I. Lethality study. Paired groups of 10 alloxan-treated and 10 control mice received identical serial dilutions of yeast cell suspensions. For studies with *C. albicans* and *C. tropicalis* twelve groups, six paired groups of control and alloxan-treated mice, were infected with serial tenfold dilutions ranging from 10^7-10^2 yeast cells for each microorganism.

With *C. guilliermondii* and *C. parapsilosis* the same procedure was followed but the dilution schedule was 10^8-10^3 serial dilutions of yeast cell for the appropriate group of mice with each yeast species.

In addition, a group of 170 alloxan-treated, noninfected mice were observed to determine the per cent cumulative deaths due solely to alloxan administration.

II. Tissue population study. Three tissue population studies were carried out. Two groups, alloxan-treated and control mice, with 50 mice in each group were infected

* Eastman Organic-Chemicals.

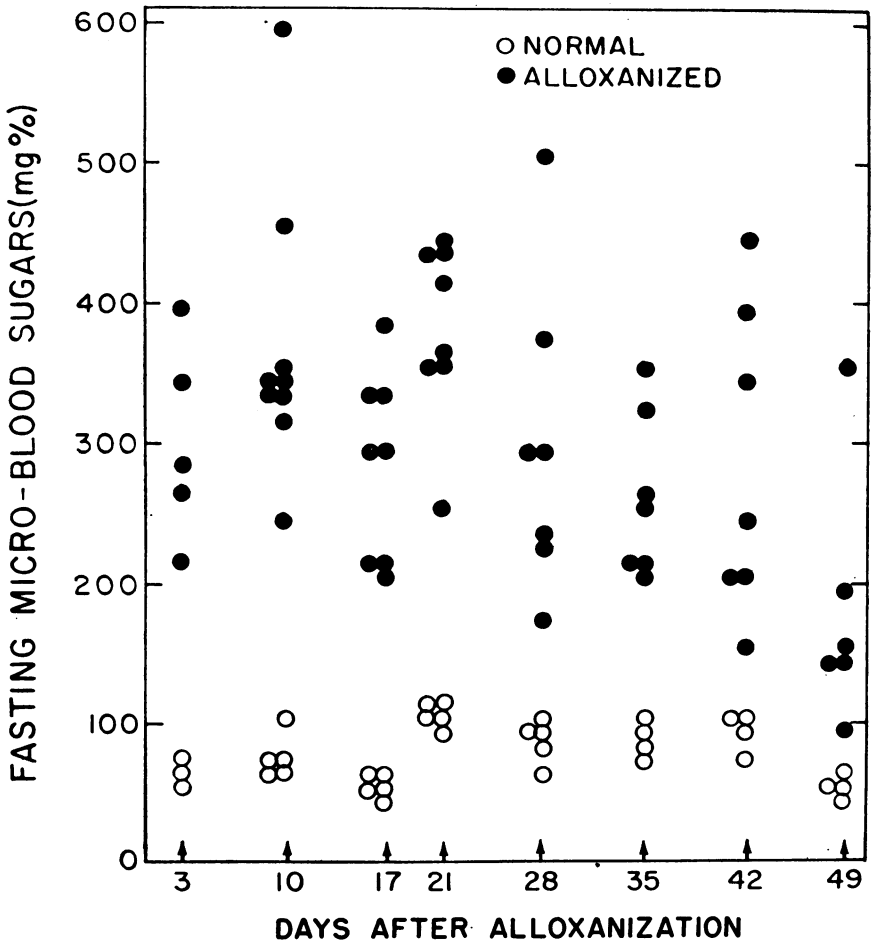

FIG. 1. Fasting blood sugars in normal and alloxanized mice.

with 10^8 *C. albicans* yeast cells. To confirm the number of viable fungi 0.1 ml. of the inoculum was plated, following serial dilution, in triplicate two per cent glucose trypticase soy agar pour plates. Tissue population studies were also obtained on a third group of 50 alloxan-treated noninfected mice.

Period of Observation

Lethality studies. Alloxan-treated infected, normal infected, and alloxan-treated noninfected animals, were observed at 12-hour intervals for a period of 40 days and survival times were recorded for each group. The experiment was then terminated. The lethality of four species of Candida for normal and alloxan-treated mice was determined by plotting the per cent cumulative deaths observed in paired groups of normal and diabetic animals receiving identical intravenous inocula.

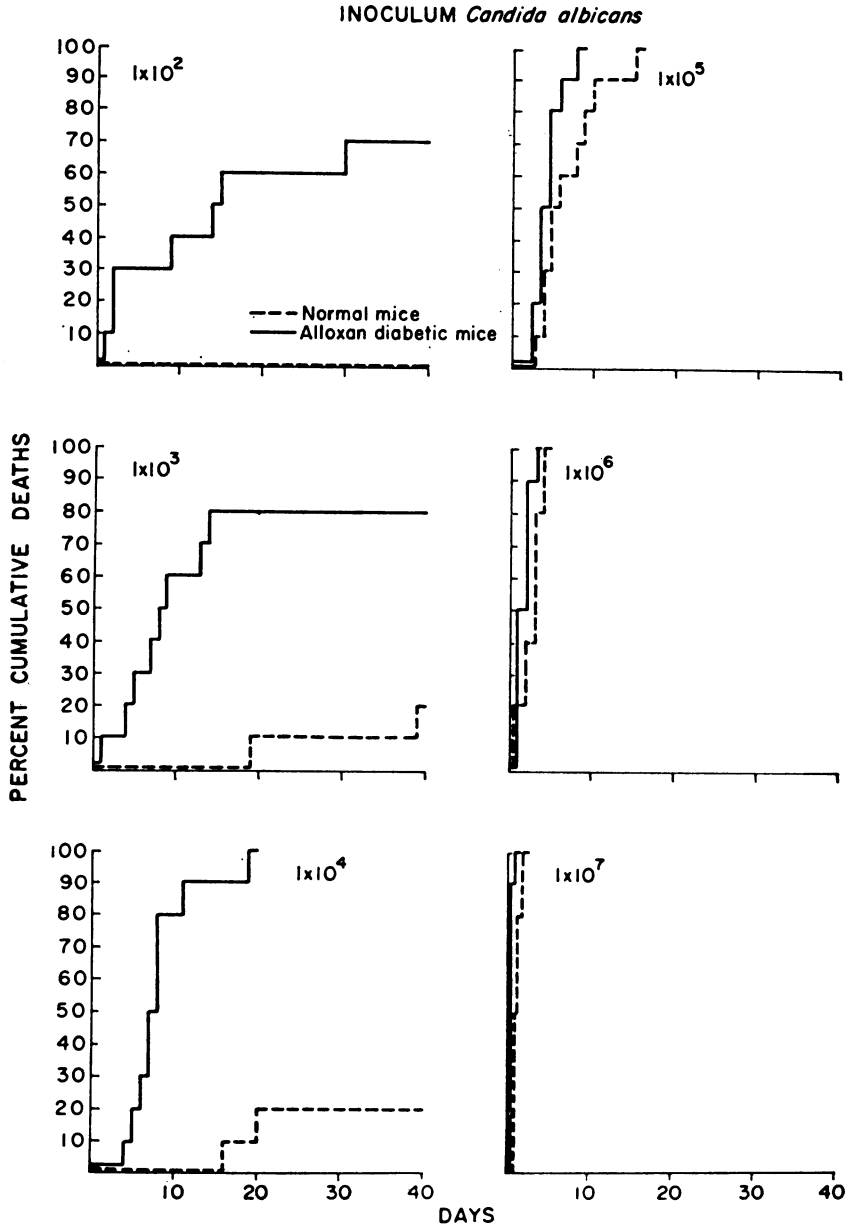

FIG. 2. Virulence of *C. albicans* in normal and alloxan diabetic mice. Ten mice per group.

At selected intervals (1, 2, 5, 7, 9, 14, 16, 21, 28 and 35 days after intravenous inoculation) four animals from each group (alloxanized infected, normal infected, and alloxanized noninfected) were killed. The organs of three animals were used to determine tissue populations, whereas those from the fourth were fixed in 10 per cent formalin; tissue sections were made and examined microscopically for histologic change.

Determination of Tissue Populations

Method of sacrifice: Animals were killed quickly and the blood allowed to run immediately into a sterile tube containing heparin. Spleen, liver, lungs, brain and kidneys were removed aseptically and placed immediately in separate sterile petri dishes. Similar organs from three animals of each group were pooled for culture in an attempt to eliminate animal variation in response to infection.

Method of culturing: The pooled organs were weighed, ground in a sterile mortar and pestle, and Ringer's solution was added to make a 1:10 suspension. Serial tenfold dilutions of this suspension were added to pour plates prepared with two per cent glucose trypticase soy agar. Each dilution was cultured in triplicate. Plates were incubated at 30° C. and colony counts were determined at 48 and 72 hours.

*Statistical Analysis**

I. Lethality studies. The effect of alloxan treatment was studied by applying the Kolmogorov-Smirnoff test[8] to the cumulative mortality curves of the alloxanized and control groups of animals injected with various species of Candida. A one-sided version of the test was used at the five per cent significance level.

II. Tissue population study. The effect of alloxan administration was studied by an analysis of the variance of transformed counts of colonies from mouse kidney tissue.

RESULTS

LETHALITY STUDIES

During the study period (35 days), the blood sugars of all but one alloxan-treated animal were 200 mg. per 100 ml. or greater (Fig. 1). No alloxan-treated animal with glycosuria three plus or greater had a blood sugar less than 200 mg. per 100 ml. In addition, a few alloxan-treated mice with one plus, two plus, or no detectable glycosuria, had blood sugars above 200 mg. per 100 ml.

1. *Lethality of Candida albicans:* The results of infecting six paired groups of mice with *C. albicans* yeast cells (strain B-311) are shown in Figure 2. The differences in mortality observed in mice receiving 10^2, 10^3, and 10^4 yeast cells were statistically significant, whereas mice injected with 10^5, 10^6, and 10^7 doses did not show statistically significant differences. The survival times, however, of the alloxan-treated animals were slightly shorter than those of the control animals in the latter groups. This demonstrates, as

* We are indebted to Dr. David Alling for the statistical analysis of our data.

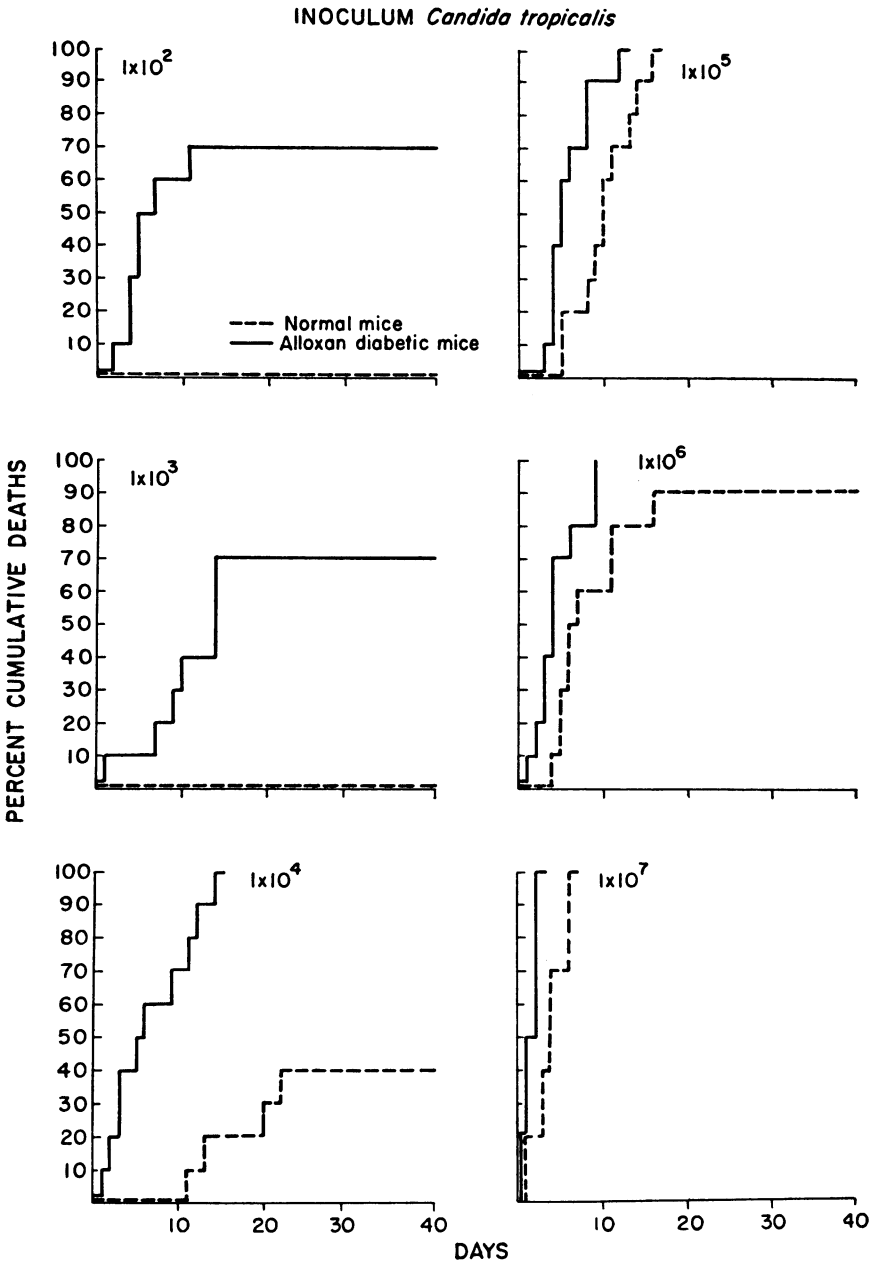

FIG. 3. Virulence of *C. tropicalis* in normal and alloxan diabetic mice. Ten mice per group.

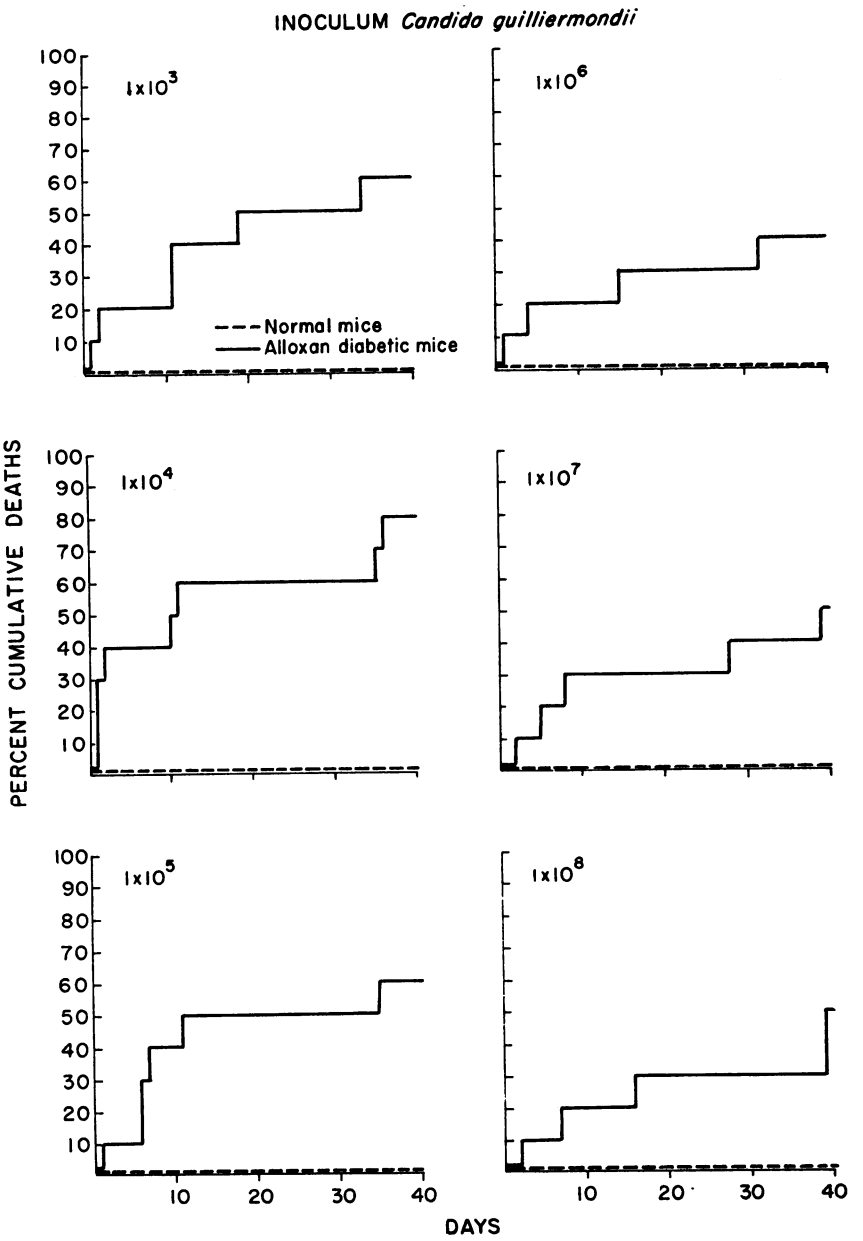

FIG. 4. Virulence of *C. guilliermondii* in normal and alloxan diabetic mice. Ten mice per group.

expected, progressively shorter survival times in each group challenged with greater inoculum.

2. *Lethality of Candida tropicalis:* The results of infecting six paired groups of mice with *C. tropicalis* yeast cells (strain 48) are shown in Figure 3. A statistically significant difference between the per cent cumulative deaths of the control and alloxan-treated mice is observed with the six inocula.

3. *Lethality of Candida guilliermondii:* The results of infecting six paired groups of mice with *C. guilliermondii* yeast cells (strain 3163), are shown in Figure 4. Alloxan-treated mice receiving 10^3, 10^4, and 10^5 cells are significantly more susceptible than controls, whereas those receiving 10^6, 10^7, and 10^8 cells are not. This strain appears to be avirulent for normal control mice since none of these animals died after receiving doses containing as many as 10^8 yeast cells. In addition, the larger inocula (10^6, 10^7, and 10^8) appear to be less lethal for alloxan-treated mice than inocula containing 10^3, 10^4, and 10^5 organisms. This apparent difference, however, is not statistically significant and is within the expected experimental variation.

4. *Lethality of Candida parapsilosis:* The results obtained following the injection of the six paired groups of mice with *C. parapsilosis* yeast cells (strain B-1234) are shown in Figure 5. The groups of animals receiving inocula of 10^5, 10^6, and 10^7 yeast cells show significant differences, whereas the difference in cumulative deaths between the normal and alloxan-treated mice infected with inocula of 10^3 and 10^4 cells fails to attain statistical significance. A challenge of 10^8 cells was equally lethal for both groups and may be related to the endotoxic effect of a large inoculum.

5. *Lethality of alloxan administration:* The per cent cumulative deaths caused by alloxan alone are shown in Figure 6. Nine deaths (5.3%) in 170 alloxan-treated noninfected mice occurred during the 40-day study period, and did not significantly alter the observed differences in the infected groups.

TISSUE POPULATION STUDY

In three separate experiments, normal and alloxan-treated mice were injected intravenously with 10^3 *C. albicans* cells. At specified intervals colony counts of fungi were made of a variety of tissues. This inoculum was chosen because it was the median statistically significant infective dose observed during the earlier lethality studies (Fig. 2). Colony counts of *C. albicans* were negligible in the blood, liver, spleen, lung and brain of both control and alloxan-treated animals. The kidneys, however, appeared to be the target organs and the tissue populations obtained at one, two, five, seven,

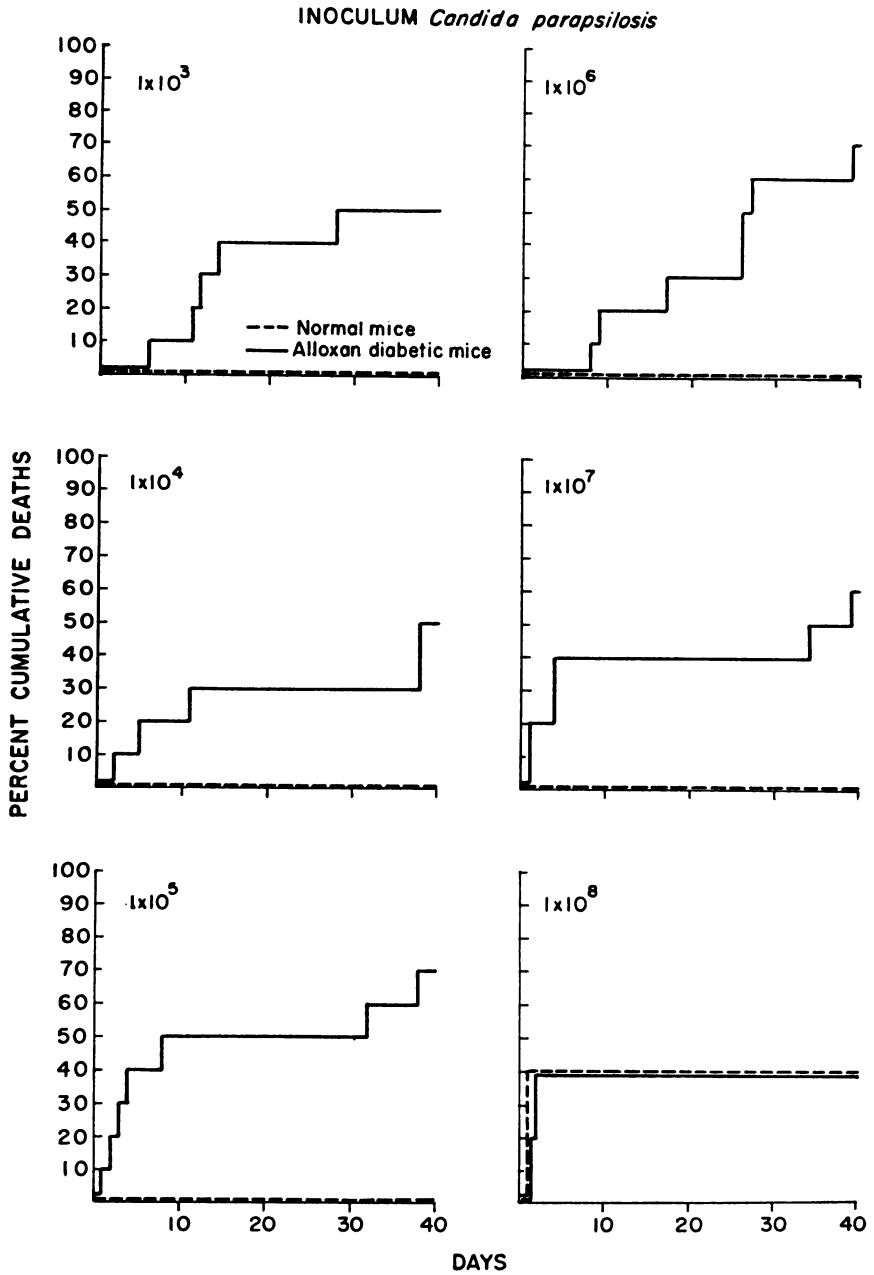

FIG. 5. Virulence of *C. parapsilosis* in normal and alloxan diabetic mice. Ten mice per group.

nine, 14, 16, 21, 28 and 35 days after infection during three experimental periods are shown in Figure 7. Twenty-four hours after infection, the colony counts were similar for both groups in the three experiments. From two to 14 days after infection, however, there was a two to tenfold difference between kidney populations in the normal and diabetic animals. Only a few

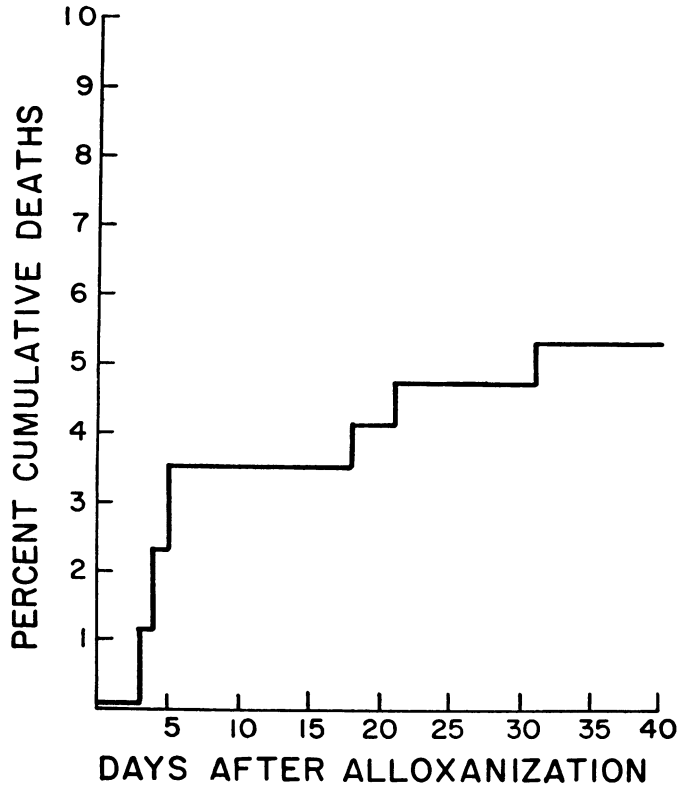

FIG. 6. Per cent cumulative deaths in alloxanized noninfected mice.

viable yeast cells could be cultured from either group at 16 days and colony counts were negligible during the last three weeks of the study period. Greater yeast cell populations in the kidney were observed in normal infected than in diabetic infected mice on two occasions (Exp. 2 on day seven and Exp. 1 on day 14). These inverted results do not alter statistical significance and fall within the expected experimental variation. No colonies of *C. albicans* were recovered from platings of similar organs from diabetic noninfected mice obtained at the same intervals.

Differences between colony counts of fungi from mouse kidney tissue were statistically significant as shown in Table 1.

HISTOLOGICAL STUDIES OF THE KIDNEYS OF DIABETIC AND NONDIABETIC MICE

All tissue sections were stained with hematoxylin-eosin and by the Bauer method as modified by Lillie.

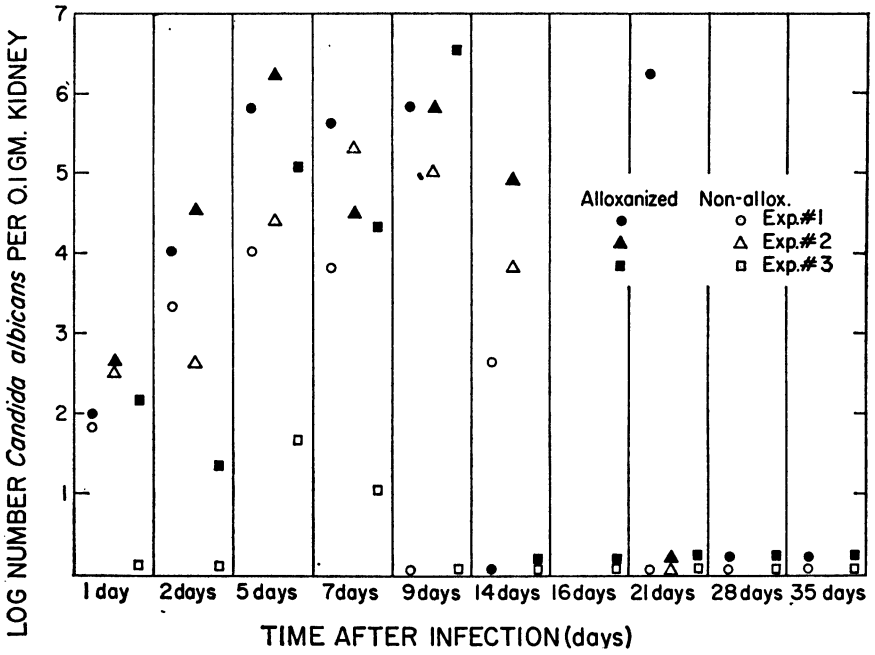

FIG. 7. Candida populations in the kidneys of diabetic and nondiabetic mice following intravenous challenge with 10^8 yeast cells of strain B-311.

Cortex. Twenty-four hours after infection a focal distribution of both yeasts and hyphal forms accompanied by an intense cellular response with formation of micro-abscesses was observed in interstitial tissue adjacent to glomeruli in both diabetic and nondiabetic animals (Fig. 8). The interstitial lesions progressed, involving larger areas of the cortex. Long hyphal forms associated with intense cortical abscesses were observed within five days and were more severe in diabetic animals. Proliferating fibroblasts and mononuclear cells were present in the cortical lesions by nine days. Yeast and hyphal forms were decreased in number and were not observed after 14 days

in nondiabetic and 21 days in diabetic animals. Cellular scars had replaced the cortical lesions by 21 days in nondiabetic and 28 days in diabetic animals.

Medulla. Focal interstitial inflammatory lesions were observed at 48 hours. These lesions progressed to micro-abscesses and at five days were more florid and more numerous in the diabetic animals (Fig. 9). Micro-abscess formation in the medullae of nondiabetic animals at nine days (Fig. 10) was comparable to lesions observed at five days in diabetic animals. Intense and destructive abscesses associated with many yeast and large hyphal forms were scattered throughout the entire medulla and concentrated

TABLE 1. ANALYSIS OF VARIANCE OF TRANSFORMED COLONY COUNTS* MADE ON MOUSE KIDNEY TISSUE

Source of variation	*d.f.*	*SS*	*MS*	*F*
Among days	9	135.63	15.07	6.16†
Diabetic vs. nondiabetic	1	22.46	22.46	9.18†
Among experiments	2	35.75	17.87	7.13†
First vs. second	1	3.42	3.42	1.40
First, second vs. third	1	32.33	32.33	13.22†
Residual	39	95.41	2.446	

* For cultures showing growth of Candida the logarithm of the number of colonies was taken. Cultures showing no growth were coded as zero. The observations for the 16th day, first experiment, and for the 16th, 28th, and 35th days, second experiment, were estimated by the usual procedure.

† Significant at 5 per cent level.

in the papilla of diabetic animals at nine days (Fig. 11). By 14 days gradual resolution of the medullary lesions was observed in the nondiabetic animals, whereas large abscess formation involving the entire medulla was observed in the diabetic animals (Fig. 12). There appeared to be complete healing of the lesions and replacement of destroyed renal parenchyma by scar tissue in mice surviving 28 and 35 days after infection.

Our observations suggested that the kidney is more susceptible to infection with *C. albicans* in diabetic mice than in corresponding control animals. Although alloxan characteristically causes damage to renal tubules, renal lesions are most conspicuous during the first four days after the administration of alloxan, are dose related, and are reversible if death from uremia does not occur.[9] The intravenous inoculation of Candida was delayed, therefore, until 10 days after the administration of alloxan in an effort to minimize nephrotoxicity. The kidneys of noninfected mice studied 10 days

after alloxan administration and at further intervals, were normal histologically. It is recognized, of course, that this does not rule out some subtle physiological disturbance.

DISCUSSION

These studies suggest that:

1) Alloxan diabetes alters the relationship of host metabolism to infection by providing more favorable growth conditions for the pathogen, or altering host resistance. There is an increased mortality in mice infected with these strains of *C. albicans* and *C. tropicalis*. Death is produced readily in alloxan diabetic mice infected with normally nonlethal strains of *C. guilliermondii* and *C. parapsilosis*. Tissue susceptibility to infection with this strain of *C. albicans* is increased in the diabetic mouse.

2) The intravenous injection of small inocula of highly virulent fungi (10^3 and 10^4 *C. albicans* cells and 10^4 *C. tropicalis* cells) are lethal (20%, 20% and 40% respectively) for normal mice. Hasenclever and Mitchell[10] have reported similar observations using the same strains of *C. albicans* and *C. tropicalis*. This finding is in contrast to the observations of Adriano and Schwarz in which a small inoculum of their strain of *C. albicans* produced lesions of a mild nature, but proved to be incapable of producing deaths in mice.[11]

3) These strains of *C. albicans* and *C. tropicalis* are highly pathogenic for normal mice, killing 90-100 per cent of animals receiving intravenous inocula of 10^5, 10^6 and 10^7 yeast cells. This observation is in agreement with recent studies by several investigators.[10, 12, 13, 14]

4) The kidney was the organ most susceptible to infection with *C. albicans*. Although cortical and medullary lesions were observed, lesions in the medulla were more numerous, persisted longer, and were more destructive than corresponding cortical lesions in both nondiabetic and diabetic animals. Similar lesions in the kidney of normal animals infected with *large* inocula of *C. albicans* have been observed by other investigators.[11, 14, 15, 16]

There is both clinical and experimental[17] evidence that certain bacterial organisms find the kidney a more favorable environment than other tissues of the body. Studies by Guze[18] and Freedman and Beeson[19] have demonstrated that the medulla is more susceptible to bacterial infections than is the renal cortex of experimental animals. Histologically, one sees the development of micro-abscesses which progress to larger lesions and either kill the animal or eventually regress and heal completely. Our histological observations suggest that chronic experimental *C. albicans* infections produce a "mycotic pyelonephritis" in both normal and diabetic mice.

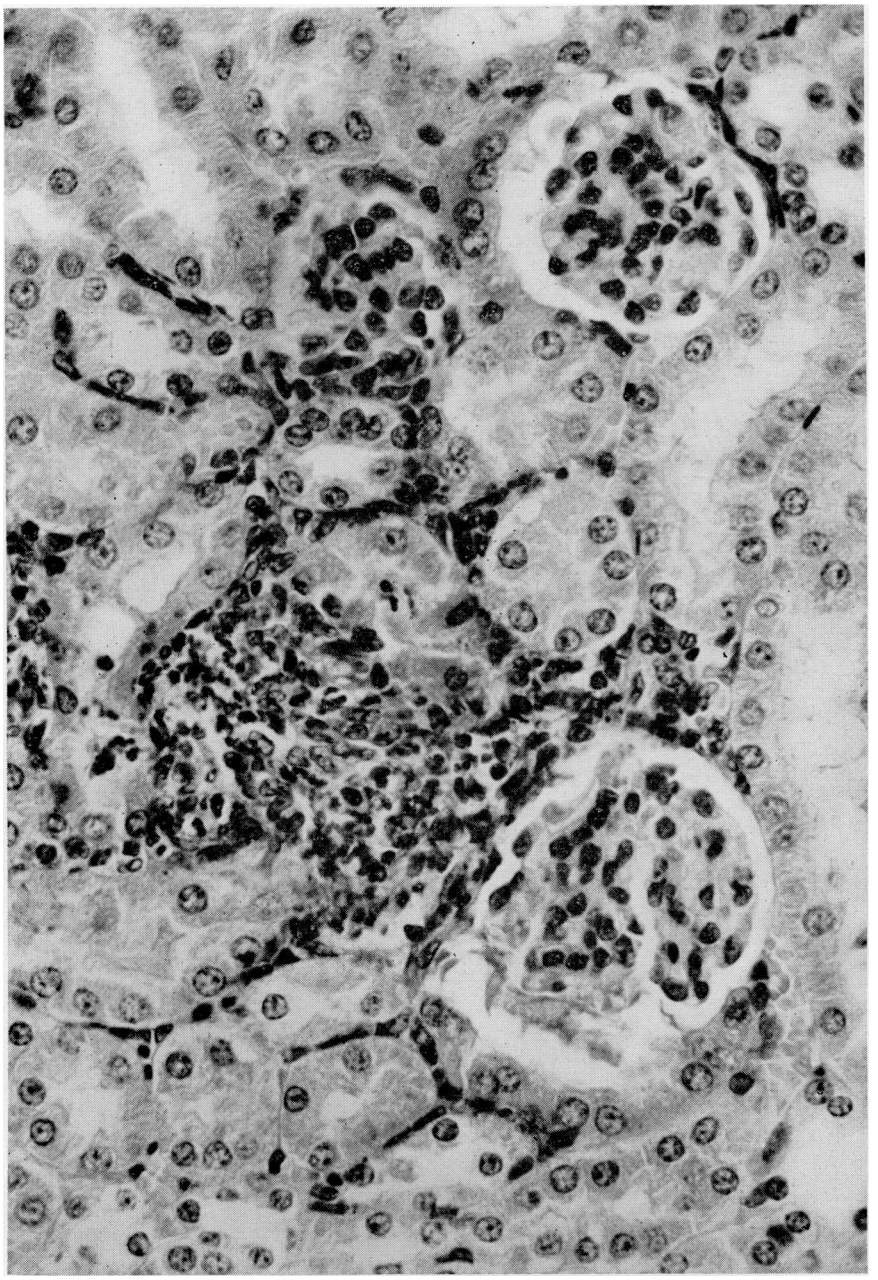

FIG. 8. Microscopic section of kidney of alloxan diabetic mouse showing micro-abscess formation in interstitial tissue adjacent to glomerulus. Hematoxylin and eosin. x375.

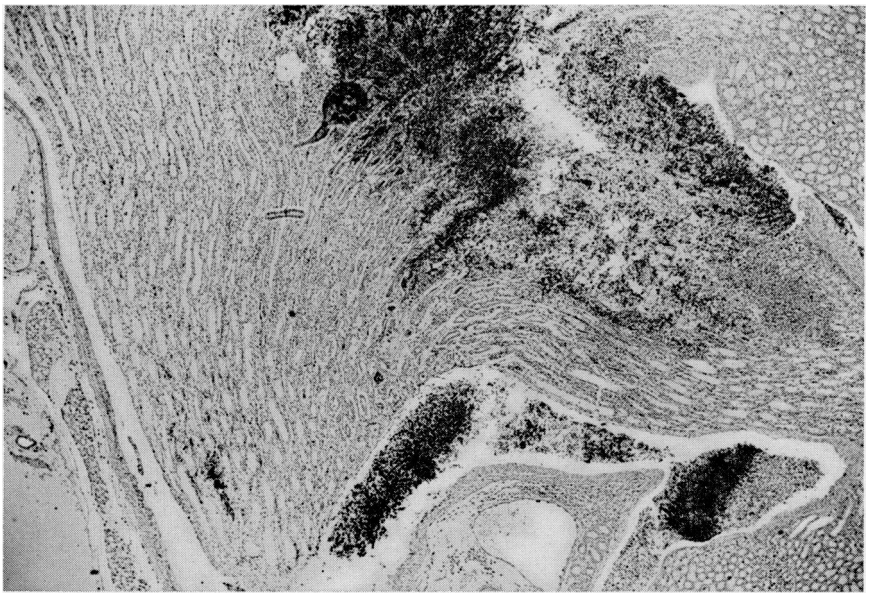

FIG. 9. Microscopic section of kidney of alloxan diabetic mouse five days after infection with 10^3 *C. albicans* cells showing abscess formation in the medulla. Bauer. x39.

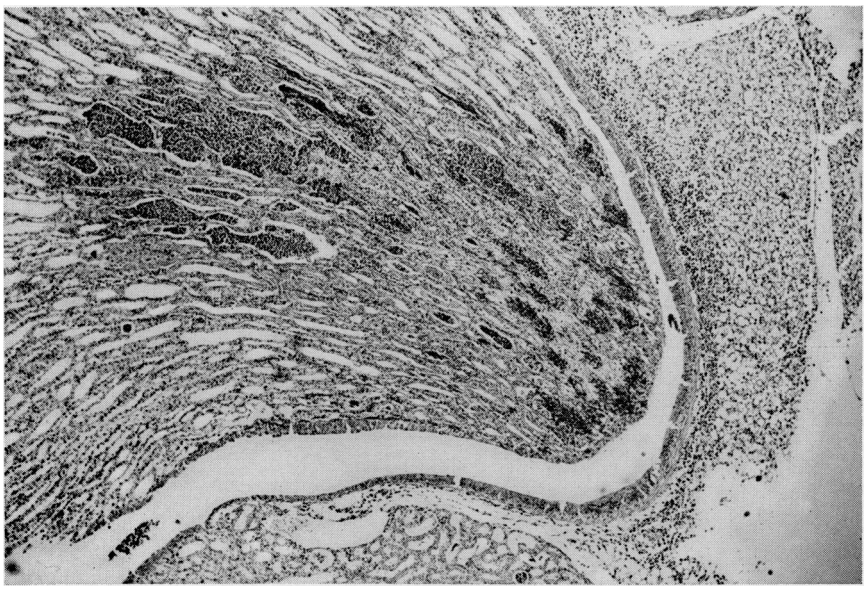

FIG. 10. Microscopic section of kidney of nondiabetic mouse nine days after infection with 10^3 *C. albicans* cells showing microabscess formation in the medulla. Bauer. x39.

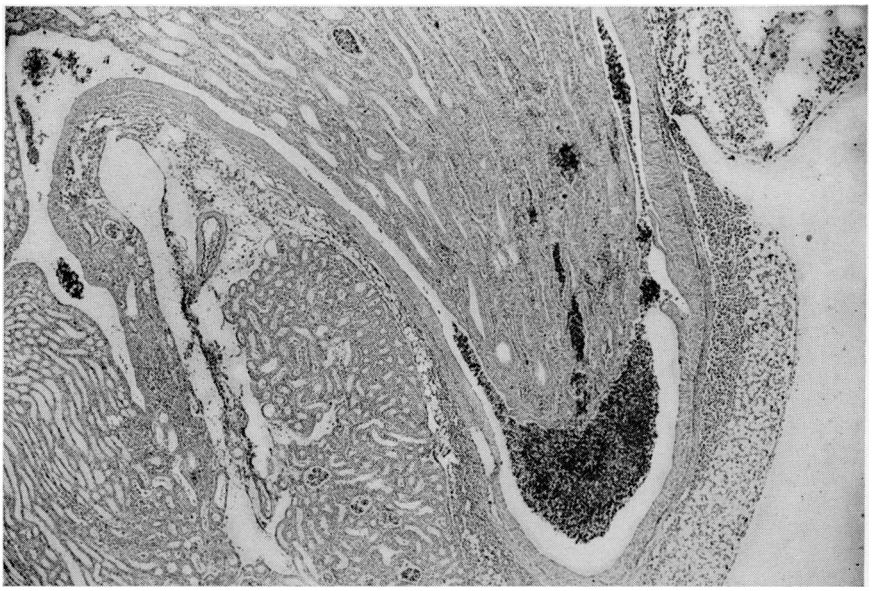

FIG. 11. Microscopic section of kidney of alloxan diabetic mouse nine days after infection with 10^3 *C. albicans* cells, showing involvement of papilla. Bauer. x39.

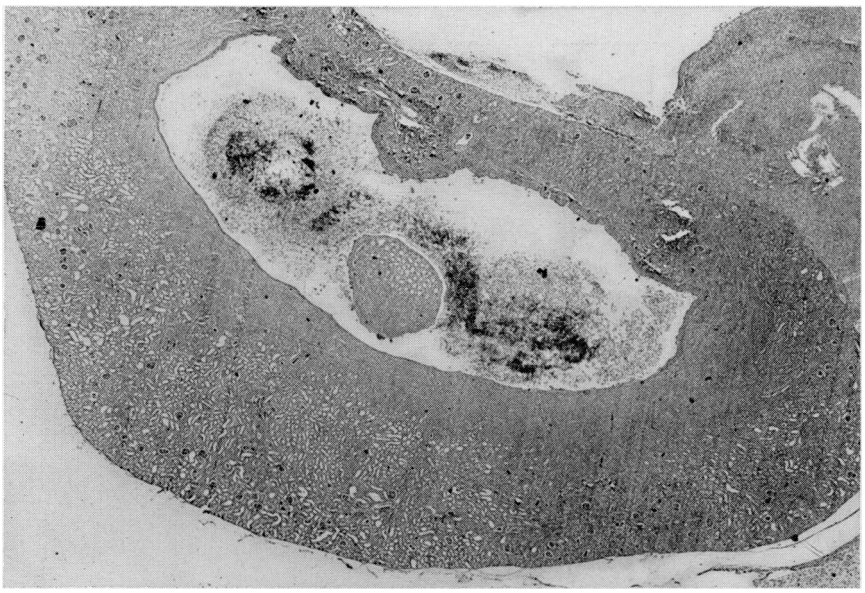

FIG. 12. Microscopic section of kidney of alloxan diabetic mouse 14 days after infection with 10^3 *C. albicans* cells showing large abscess formation in the medulla. Bauer. x15.

Although Ashford, in 1916,[20] noted that rabbits in the course of generalized candidiasis developed large infarct-like lesions in the renal cortex, and Redaelli, in 1924,[21] first described the kidney as the site of maximum multiplication in experimental infections with *C. albicans,* the mechanisms underlying renal susceptibility to infection in normal animals have not been delineated as yet. A number of theories, however, have been proposed. Redaelli[21] believed that the renal lesions were caused by vascular occlusions from embolization of fungi. The studies of Stovall and Pessin,[22, 23] in contrast, suggested that embolism was not the pathogenic mechanism of systemic Candida infections. Fuentes, Schwarz, and Aboulafia[24] suggested local toxic damage to vascular endothelium as a mechanism for the pathogenicity of *C. albicans.* Salvin[25] demonstrated that cells of *C. albicans* contain a soluble endotoxin and that filtrates from suspensions of these organisms produced the same lesions as live organisms. Mourad and Friedman[26] demonstrated that soluble substances, obtained by sonic destruction of *C. albicans* yeast cells, were lethal for mice when inoculated intravenously. Gresham and Whittle[27] called attention to the possibility of a capsular aggressin of pathogenic *C. albicans* which is probably cytotoxic for vascular endothelium. Louria, Fallon and Browne[14] suggested that the renal localization of Candida infections was related to the protection offered the fungus by intratubular residence.

Some other hypotheses must be considered in an attempt to explain the susceptibility of the kidney to infection with *C. albicans* during the physiologically altered conditions of diabetes mellitus.

1) The studies of Sheldon and Bauer[28] have shown that the onset of the response by polymorphonuclear leukocytes to experimental cutaneous mucormycosis was delayed by several hours, reduced in intensity and apparently less effective in acute alloxan diabetic rabbits than in control animals. This alteration in polymorphonuclear response appears to provide protection for *C. albicans,*[29] in addition to the protection offered by the concept of intratubular residence.[14]

2) Since Candida grows more readily in carbohydrate enriched media, their growth may be directly enhanced by the production of hyperglycemia and glycosuria. Rabbits with infusion hyperglycemia inoculated with *Rhizopus oryzae* develop fungus lesions in the nose and lung.[30]

3) A reaction which requires the cooperation of all four components of complement, such as bactericidal and quite possibly fungicidal action, cannot occur if one component is missing, even though the other three components are present in abundance. The evidence presently available suggests that there is something in kidney tissue which interferes with the bactericidal

action of normal serum against gram negative bacilli. Recent studies by Beeson and Rowley[31] suggest that this mechanism of interference appears to be an anticomplementary action, involving inactivation of the fourth component of complement by ammonia. The ammonia formed in the kidney is largely derived from glutamine and this process is activated in the presence of acidosis. The mouse has very little of the fourth component of complement, at least, by serum assay,[32,33] suggesting greater ease in the interference of the action of this component by the production of diabetes and acidosis. In addition, the complement activity of normal serum is destroyed by zymosan, an insoluble carbohydrate derived from yeast. The substance inactivated by yeast is the third component or C'3 of complement which seems to behave like a catalyst during those reactions involving complement. Young and Lemanas[34] demonstrated that both the zymosan fraction and whole *C. albicans* cells inactivated C'3 component of complement.

The mechanism of the susceptibility of the kidney to infection with *C. albicans* in both normal and alloxan diabetic mice was not defined in this study. Under the conditions of these experiments, however, Candida infections were clearly enhanced to a far greater degree in the alloxan diabetic mouse kidney than in the kidney of the normal mouse. The observations made during the course of this investigation elucidate some of the factors concerning the host-parasite relationships that exist during experimental Candida infections.

SUMMARY

Alloxan diabetes decreased the survival times of mice with experimental Candida infections to a significant degree when compared to the survival times of nondiabetic mice.

The kidney was demonstrated to be the only tissue in which progressive infection occurred following the intravenous injection of a small inoculum of *C. albicans* in both normal and alloxan diabetic mice.

Tissue populations of *C. albicans* were significantly higher in the kidneys of alloxan diabetic mice than in normal mice.

Histological studies suggested that the lesions in the kidneys of alloxan diabetic mice developed earlier, were more severe, and persisted longer than lesions in the kidneys of normal mice.

The metabolic alterations associated with experimental alloxan diabetes mellitus appear to influence essential factors in the pathogenesis of Candida infections.

ACKNOWLEDGMENT

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