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THE EFFECTS OF METABOLIC ALTERATIONS ON EXPERIMENTAL RHIZOPUS ORYZAE (MUCORMYCOSIS) INFECTION§

Mucormycosis is a fungus infection which generally occurs in association with metabolic disorders, most frequently diabetes mellitus. The fungus has only recently been identified in culture when the phycomycetes Rhizopus oryzae and Rhizopus arrhizus were recovered from two diabetic patients with cerebral mucormycosis.[2,10] With these and other species of Mucorales, lesions closely resembling cerebral and pulmonary mucormycosis in man have been produced in rabbits with acute alloxan diabetes while non-diabetic animals showed only rare minute lesions at the site of inoculation.[3,9] Clinical, postmortem and experimental findings indicate, therefore, that severe metabolic alterations are essential in the pathogenesis of this fungus infection.[1,2]

To define the metabolic factors more accurately the following experiments were carried out. The effect of sustained hyperglycemia in rabbits without diabetes was studied since elevation of blood sugar is one of the principal metabolic abnormalities in diabetes. We had also noted previously that the fungus could be isolated in culture from non-diabetic rabbits without lesions as late as one week after inoculation. To test if experimentally induced metabolic alterations in a previously normal animal can change the fungus from a saprophyte to a pathogen, normal rabbits were inoculated with fungus at various times preceding the production of diabetes by alloxan administration.

FUNGUS INOCULATION PRECEDING ALLOXAN

METHODS

The details of the experimental procedures are the same as previously described.[8]

A standardized suspension of Rhizopus oryzae spores was instilled into one nostril of 19 male rabbits.¶ Acute diabetes was produced by alloxan injection which was given

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to five rabbits 24 hours and to nine rabbits 96 hours after fungus inoculation. Table 1 shows the blood sugar values at 48 hours after alloxan administration and on the day of death. Acetonuria is recorded qualitatively. Complete autopsies including fungus cultures of nose and lung were performed on all animals either when moribund or within two hours after death. Five inoculated rabbits received no alloxan and served as controls. They were sacrificed at intervals corresponding to the survival times of diabetic animals.

TABLE 1.

RHIZOPUS ORYZAE INOCULATION PRECEDING ALLOXAN BY ONE AND THREE DAYS

RABBIT NUMBER	BLOOD SUGAR	URINE ACETONE	SURVIVAL HOURS AFTER ALLOXAN	FUNGUS LESIONS			FUNGUS CULTURES	
				NOSE	LUNG	BRAIN	NOSE	LUNG
ONE DAY								
872	644	−	60	+	+	?+	+	+
873	532 868	+	124	+	−	+	+	+
889	924 924	+	102	+	+	?+	−	+
890	556 −	+	94	+	+	+	+	+
891	512 776	+	128	+	+	+	+	−
THREE DAYS								
827	366 552	+	120	+	+	−	+	+
828	398 560	+	120	−	+	−	−	+
830	360 426	+	96	−	+	−	+	+
831	318 −	Tr.	68	−	−	−	−	−
835	388 536	+	96	−	−	−	+	+
911	290 366	+	90	+	−	−	+	+
912	524 763	+	90	+	+	−	+	+
913	334 682	+	96	+	−	−	+	−
914	266 468	+	90	+	−	−	−	+

RESULTS

The data of this experiment are summarized in Table 1. All five rabbits which had been inoculated with fungus one day preceding alloxan administration showed nasal lesions. Pulmonary lesions were present in four of these animals. Definite meningoencephalitis occurred in three rabbits while slight meningeal inflammation without demonstrable mycelia was found in the two remaining animals. In the nine rabbits inoculated with fungus three days before alloxan injection, nasal lesions were found in five and pulmonary lesions in four. The latter occurred twice in association with nasal infection while in the other two animals the lesions were confined to the lung. In this group, no cerebral lesions were noted. In two rabbits, no lesions were found, although cultures from both nose and lung of one of

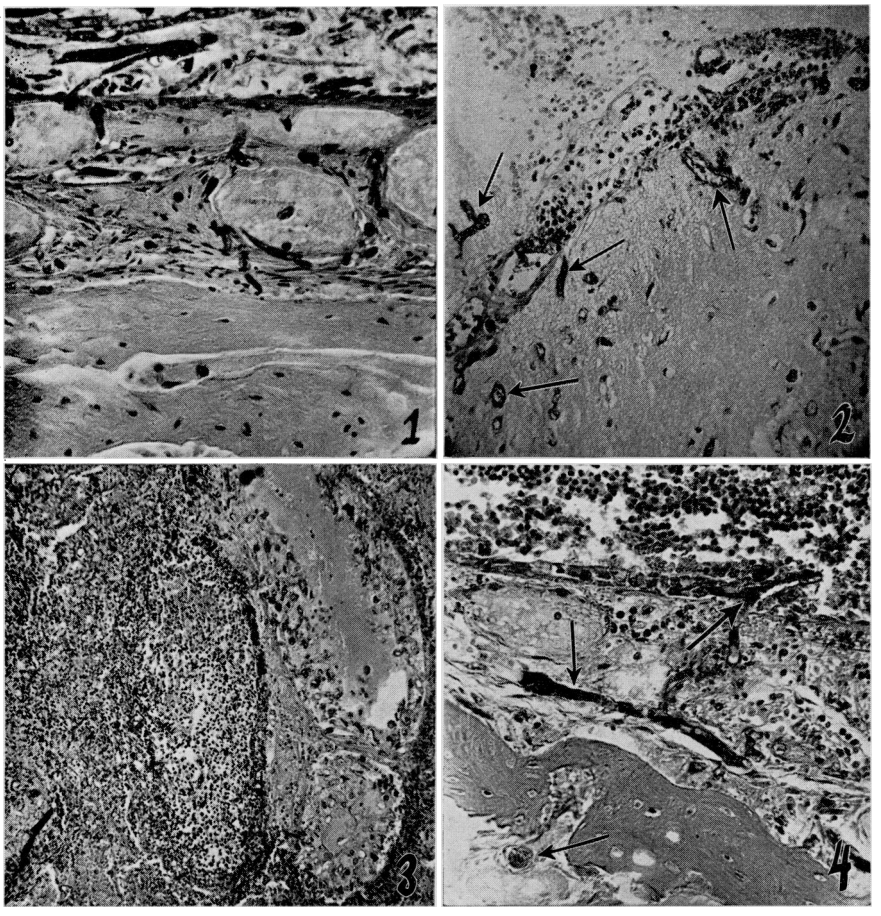


FIG. 1. Diabetic rabbit inoculated with fungus one day before alloxan administration. Extensive nasal lesion with mucosal ulceration, involvement of deep tissues, and many mycelia. Giemsa, x210.

FIG. 2. Same rabbit as Fig. 1. Early meningoencephalitis. Several mycelia are shown in the cortex (arrows). Giemsa, x180.

FIG. 3. Same rabbit as Fig. 1. Pulmonary lesion with involvement of bronchus and pulmonary artery. The vessel contains many mycelia. Giemsa, x115.

FIG. 4. Diabetic rabbit inoculated with fungus 3 days before alloxan administration. Extensive nasal lesion with bone necrosis. Note cross-section of mycelia in bone marrow (arrow) and other branching mycelia. Giemsa, x230.

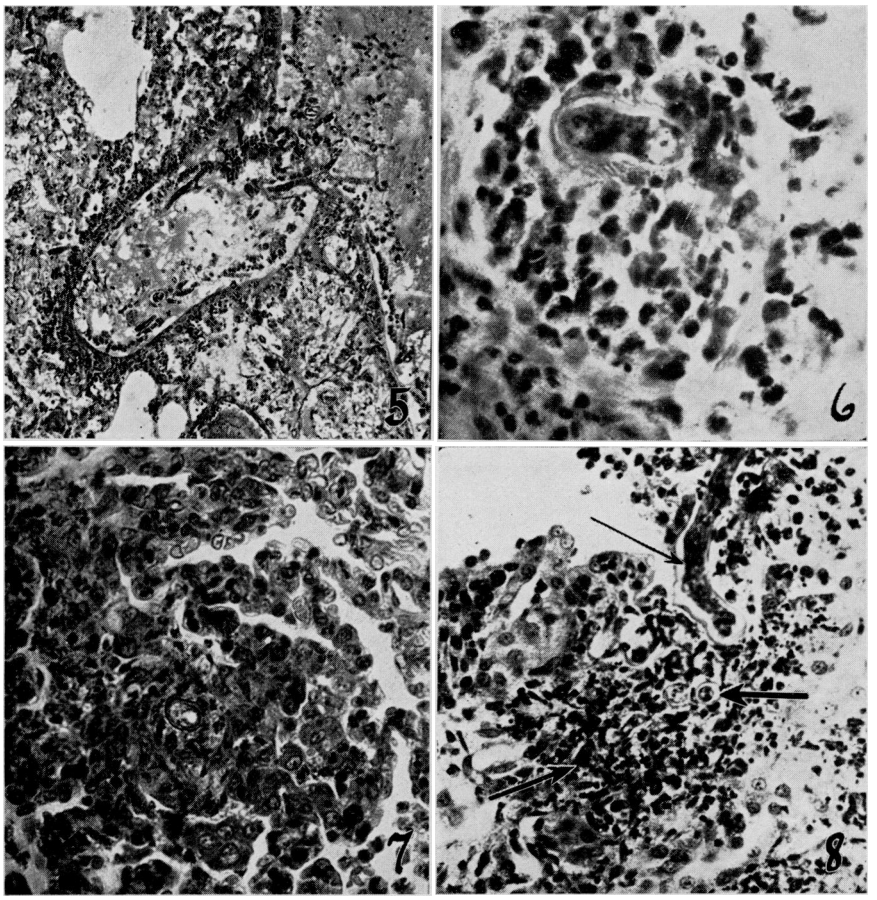
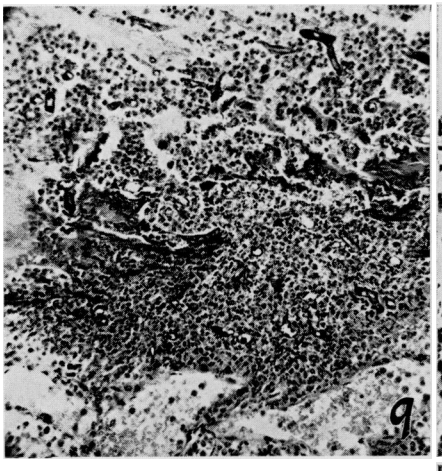


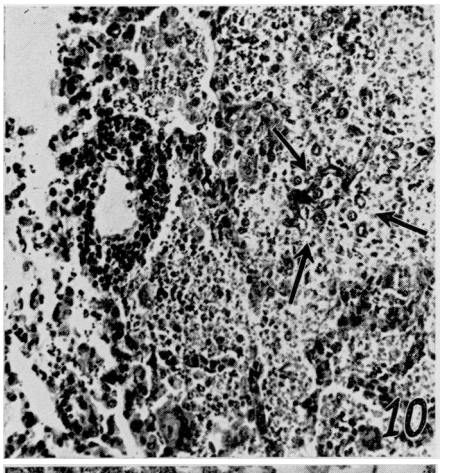
FIG. 5. Same rabbit as Figure 4. Extensive pulmonary lesion with many mycelia involving bronchus. Giemsa, x230.

FIG. 6. Rabbit with infusion hyperglycemia. Small nasal lesion (graded +) showing early hypha surrounded by inflammatory cells. Giemsa, x1000.

FIG. 7. Rabbit with infusion hyperglycemia. Pulmonary lesion (graded +) with degenerating spore surrounded by inflammatory cells. Giemsa, x440.

FIG. 8. Infusion hyperglycemia. Nasal lesion (graded ++) showing mucosal ulceration with several mycelia, some in cross-section (arrows). Giemsa, x400.





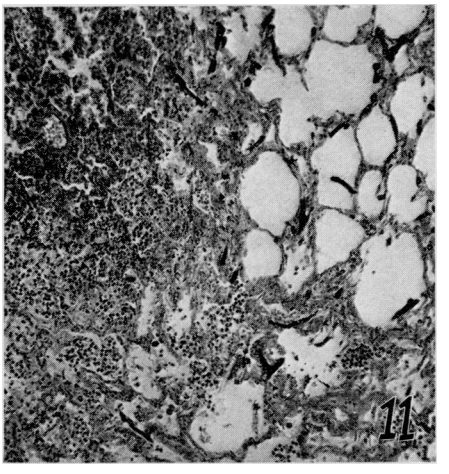


FIG. 9. Infusion hyperglycemia. Extensive nasal lesion (graded +++) involving deep structures and showing massive mycelial growth. Giemsa, x175.

FIG. 10. Bronchopneumonic focus of infusion hyperglycemia (graded ++) with cluster of mycelia (arrows). Giemsa, x220.

FIG. 11. Pulmonary lesion of infusion hyperglycemia with infarction of tissues and massive mycelial growth. Giemsa, x814.

these animals yielded fungus growth. Fungus cultures from the other rabbit without lesions showed no growth. Fungus cultures of nose and/or lung were positive in all other animals including the controls.

The fungus lesions in the diabetic animals resembled in every respect those reported previously.[8] The lesions were extensive and were characterized by marked mycelial proliferation and invasion of tissues, particularly blood vessels, which frequently produced infarction and led to dissemina-

TABLE 2.

INFUSION HYPERGLYCEMIA AND RHIZOPUS ORYZAE INOCULATION

RABBIT NUMBER	BLOOD SUGAR			SURVIVAL HOURS AFTER INOCULATION	FUNGUS LESIONS		FUNGUS CULTURES	
	MIN.	MAX.	MEAN		NOSE	LUNG	NOSE	LUNG
803	224	674	433	48	−	−	+	−
804	360	903	624	49	+	+	+	+
805	320	674	625	46	+	−	+	+
806	230	796	647	23	+ +	−	+	−
814	440	970	722	48	+++	+ +	+	+
815	290	636	461	49	+	−	+	+
816	340	670	565	49	+ +	+	+	−
817	414	690	536	49	+	−	+	+
866	378	520	466	45	+ +	−	+	−
867	422	616	510	48	+ +	−	+	+
868	436	623	467	49	+	−	+	+
869	218	404	342	50	+	−	+	−

tion. Degenerative changes in the polymorphonuclear leukocytes of the inflammatory reaction were another striking and constant finding. In the nose, there was infarction of tissue with bone necrosis and invasion of blood vessels and nerve trunks by numerous mycelia (Figs. 1, 4). The cerebral lesions in this series were quite early and consisted of invasion of leptomeninges and their vessels by fungus with beginning extension into the cerebral cortex which showed early infarction (Fig. 2). The pulmonary lesions consisted either of bronchopneumonic foci or of infarcted areas (Figs. 3, 5). In the latter, vessel invasion and occlusion by mycelial clusters dominated the picture.

The lesions in the non-diabetic controls were few and of minute size. They consisted of circumscribed foci of superficial ulceration of the nasal mucosa with many normal appearing polymorphonuclear leukocytes surrounding rare degenerating spores.

INFUSION HYPERGLYCEMIA

METHODS

The blood sugar determinations and inoculation procedures were the same as described previously.[8] The data of this experiment are summarized in Table 2.

Twelve male rabbits weighing between 2000-2500 gm. were given a constant intravenous infusion of 10% glucose in 0.45 saline with 20 mEq/L of potassium. The animals were immobilized on a stand with the neck secured in a yoke. They withstood the infusion well and showed no evidence of abnormal fluid retention except for mild dependent edema of the hindlegs. This usually appeared during the second day of the experiment. The infusion was given into the marginal ear vein through a 20-gauge hypodermic needle. The rate of infusion was adjusted on the basis of blood sugar determinations performed two to four times daily. Blood for biochemical determinations was obtained from the ear which was not the site of infusion. The minimum, maximum, and mean blood sugar levels are shown in Table 2. After blood sugar levels of more than 400 mgm% were reached, which occurred usually within two to four hours after the beginning of the infusion, the animals were lightly anesthetized with intravenous Nembutal and a standardized suspension of Rhizopus oryzae spores was instilled into one nostril. Hyperglycemia was maintained for 45-50 hours except in one animal which died 23 hours after inoculation because of technical difficulties. The other 11 rabbits were sacrificed by air injected into the infusion tubing after a final blood sample had been obtained. Complete autopsies including fungus cultures from the nose and lung were performed. The extent of the morphologic findings is shown graded from one to three plus in Table 2.

Plasma sodium, potassium, chloride, and CO_2 combining power determinations were performed daily during the experiment on rabbits Nos. 866-869 after base line values had been obtained for each animal. Plasma sodium and potassium were determined on the Baird flame photometer.* The method of Schales and Schales was used for the chloride determinations.[13] The microgasometric method of Scholander and Roughton was employed for estimation of the plasma CO_2 combining power.[14] All determinations were performed in duplicate. The values obtained in this manner checked closely and the mean values were recorded.

For controls, four rabbits were subjected to the same experimental procedures including electrolyte studies and determination of plasma CO_2 combining power but were not inoculated with fungus and received only 1.5 cc. of saline intranasally. These animals were sacrificed at the end of 48-50 hours when the infusion was terminated. Six other rabbits were given glucose infusion for 48-50 hours under the same experimental conditions but were not inoculated with fungus or saline. They were permitted to survive from 10-16 days after termination of the infusion at which time they were sacrificed and complete autopsies were performed. Blood sugar levels were determined before the infusion was discontinued, again 8-12 hours later and several times thereafter. Two additional rabbits were immobilized for 50 hours in the same manner as the other experimental animals but were neither infused nor inoculated. They were fed and watered without being removed from the stand. Plasma CO_2 combining power, Na, Cl, and K determinations were performed at 24 and 48 hours after base line values had been obtained. These animals were not sacrificed.

* We are indebted to Dr. Frank W. Fales, Department of Biochemistry, Emory University, for the plasma sodium and potassium determinations.

RESULTS

The data of this experiment are summarized in Table 2.

Nasal lesions were present in 11 of the 12 rabbits and pulmonary lesions were found in three of these. In six of the 11 animals, the nasal lesions (listed as one plus in Table 2) were few in number and consisted of minute superficial mucosal ulcerations. These showed viable-appearing, sometimes budding spores and hyphae (Fig. 6), and a vigorous inflammatory response by polymorphonuclear leukocytes which revealed marked nuclear pyknosis and sometimes karyorrhexis. In two of these six rabbits, rare minute foci of leukocytic exudate surrounding an occasional degenerating spore were found in the lung (Fig. 7), chiefly in alveolar ducts. The polymorphonuclear leukocytes of these lesions revealed the same nuclear changes as described above. In four other rabbits, the nasal lesions (graded as two plus in Table 2) were more numerous and extensive (Fig. 8). They consisted of circumscribed, but frequently confluent, areas of ulceration which extended beyond the mucosa into the deeper tissues. There was a vigorous response of polymorphonuclear leukocytes with purulent exudate in the nasal cavity. The leukocytes revealed pyknosis of nuclei and frequent karyorrhexis. Considerable mycelial proliferation was present in the purulent exudate of the nasal cavity and in the tissues with occasional mycelial invasion of capillaries, nerve trunks, and bone. There was no infarction of tissue and no pulmonary lesions were encountered. In another animal, widespread and marked nasal lesions (graded as three plus in Table 2) were seen to have formed by confluence of many small ulcerations. The deeper tissues, including bone and nerve trunks, were invaded by many mycelia (Fig. 9). Some capillaries and veins were invaded by fungus but neither involvement of arteries nor infarction of tissue was found. The inflammatory response was marked in the tissues and in the nasal cavity and consisted of polymorphonuclear leukocytes with large masses of mycelia. The nuclear alterations which have been described were present in the polymorphonuclear leukocytes of all lung lesions. On histological examination the purulent exudate of all nose lesions was found to contain some scattered bacteria. One animal showed no lesions. No cerebral involvement occurred in any of the rabbits. Cultures from the nose and/or lung yielded the fungus in all animals.

In one of the four control rabbits receiving intravenous glucose infusion and inoculated intranasally with saline, the nasal cavity showed on histological study a considerable amount of purulent exudate with large masses of bacteria, chiefly cocci and some bacilli. The mucosa revealed some small erosions confined to the uppermost layer with an infiltration by polymorphonuclear leukocytes. These cells showed the same regressive changes as seen

in the other hyperglycemic animals. The remaining three control animals showed no lesions.

All animals receiving glucose infusion revealed marked glycogen infiltration of the liver and of the renal tubular epithelium. The pancreatic islets appeared normal on routine histological study.

In the six control rabbits, with infusion hyperglycemia but without fungus inoculation, the blood sugar had returned to normal levels 8-12 hours after the infusion was discontinued and remained normal. The animals ate and acted normally, and autopsies performed 10-16 days after termination of the infusion revealed no lesions.

The electrolyte determinations in infected and control rabbits with hyperglycemia revealed a slight drop in plasma sodium within the first 24 hours. The values returned to base line levels at 48 hours. Plasma potassium determinations showed no significant changes. Plasma CO_2 combining power determinations at 24 hours showed a uniform fall of 20-25 vol.% from average base line values of 40-50 vol.%. The values at the termination of the experiment either remained the same or rose to 30-35 vol.%. After 24 and 48 hours, plasma chloride values were elevated about 10-15 mEq/L above average base line values of 95-105 mEq/L.

The two rabbits which served as controls of the effects of prolonged immobilization alone showed at 24 hours a drop in plasma CO_2 combining power of 8-12 vol.% with a rise towards normal values at 48 hours. Plasma Na, Cl, and K determinations revealed no significant changes.

DISCUSSION

Our findings re-emphasize the importance of metabolic alterations in the pathogenesis of Rhizopus oryzae infection.

Normal rabbits inoculated with fungus show only rare minute lesions at the site of inoculation, but fungus cultures from the tissues at autopsy are positive for as long as 13 days after inoculation. It appears, therefore, that the fungus persists in the tissues of the metabolically normal animal and that the host cannot readily destroy the agent. An aggressive fungus infection is, however, precipitated when acute alloxan diabetes is induced in rabbits which have been inoculated with fungus either one or three days before alloxan injection. In this experiment, all but one rabbit developed extensive lesions in one or several sites (Table 1).

The greater frequency and more extensive dissemination of fungus lesions among the rabbits inoculated one day prior to alloxan administration may result from the persistence of a larger number of viable spores as compared to the animals inoculated three days before alloxan injection. Rabbit No.

831 was injured while struggling during alloxan administration and died before diabetes with acidosis developed, which may account for the absence of lesions.

During this and previous experiments it had been noted by us and others that mucormycosis can be produced in rabbits with acute alloxan diabetes.[3, 9] Animals with chronic alloxan diabetes survive. They do not develop acidosis and are not susceptible to mucormycosis. Acute alloxan diabetes in rabbits is clinically quite similar to diabetes mellitus with acidosis and coma in man. This similarity includes the chemical changes which consist of glycosuria, hyperglycemia with ketosis, hyperlipemia, decreased plasma CO_2 combining power, hyponatremia, and dehydration.[8]

The second group of experiments shows that hyperglycemia produced by glucose infusion and not associated with diabetes induces changes in the host which render the fungus pathogenic. The lesions differ, however, in number, size, and appearance from those of the diabetic as well as the metabolically normal rabbits.

In six of 11 rabbits, the nasal lesions resembled those of the metabolically normal control animals in size and superficial location. They differed from the findings in the controls by an increase in frequency and the presence of viable, sometimes budding spores and hyphae. In two of the six animals, rare minute foci of bronchopneumonia with a few degenerating spores were seen, while with intranasal inoculation pulmonary lesions had previously been encountered only in diabetic rabbits. In another four of the 11 rabbits, the nasal lesions were still more numerous. They were also more extensive having formed by confluence of small mucosal ulcerations. The lesions showed mycelial proliferation and involvement of deeper tissues including nerve trunks and bone. In this respect, the findings resembled the lesions of diabetic rabbits from which they differed, however, by their more circumscribed appearance and by the absence of mycelial invasion of blood vessels with infarction of tissues. Occasional mycelia, however, were present in a few capillaries. In the 11th rabbit, the nasal lesions were still more widespread, approaching but not quite reproducing those of the diabetic animals. The ulcerations extended into the bone, and there was massive invasion by mycelia with involvement of nerve trunks, veins, and capillaries. The arteries, however, were spared, and there was no infarction. In the same animal, lesions were present in the lung which consisted of one area of infarction and many foci of bronchopneumonia. The infarcted area closely resembled similar pulmonary lesions in the diabetic animals. The bronchopneumonic foci differed from their counterpart in the diabetic rabbits in their smaller size, lesser number of mycelia, and the absence of vascular and bronchial wall invasion by fungus.

The inflammatory response in all lesions consisted chiefly of polymorphonuclear leukocytes which were numerous and uniformly showed marked nuclear changes of pyknosis and karyorrhexis. The degree of leukocytic infiltration and the nuclear alterations closely resembled those observed in the lesions of rabbits with acute alloxan diabetes. The lesions associated with infusion hyperglycemia may be regarded as representing an intermediate stage in the susceptibility of the host to Rhizopus oryzae infection. They are distinctly more frequent and active than the rare minute ulcerations of the metabolically normal animal but lack the aggressiveness, particularly the characteristic mycelial invasion of arteries with subsequent infarction of tissue which is seen in diabetic animals.

The electrolyte changes associated with infusion hyperglycemia consisted of hyperchloremia, decreased plasma CO_2 combining power and slight hyponatremia. These changes may be the result of an excess intake and excretion of chloride ions combined with a decrease in respiratory CO_2 excretion resulting from prolonged immobilization. In diabetes mellitus and probably also in acute alloxan diabetes, the acidosis largely results from a greatly increased utilization of fat and subsequent ketonemia which may be an important factor in the biochemistry of infection.[8]

The blood sugar levels produced by glucose infusion were generally the same as those of rabbits with acute alloxan diabetes and can, therefore, not account for the differences of the lesions in the two experiments. A significant effect of an increased glucose content of body fluids and tissues on the fungus is unlikely since similar fungus lesions can be produced in normoglycemic rabbits rendered agranulocytic with nitrogen mustard.[5] The metabolic changes leading to the formation of fungus lesions, therefore, appear to affect mainly the host. We have shown that despite certain similarities the lesions induced by infusion hyperglycemia differ from those produced in animals with acute alloxan diabetes. In both states of altered metabolism, the polymorphonuclear leukocytes in the lesions display similar degenerative changes which suggest impaired leukocytic function. Other investigators have shown that the function of the leukocyte is affected by metabolic disturbances.[12] Martin and associates have demonstrated *in vitro* that the polymorphonuclear leukocytes of diabetic patients have impaired glycolytic power and form less lactic acid.[11] This may reduce the bactericidal capacity of the leukocyte and can be reversed by the addition of insulin. The findings of Cruickshank and Payne indicate that the bactericidal power of the leukocyte in the alloxan diabetic rabbit is impaired.[7] In another study, Cruickshank attributes the increased susceptibility to infection of the diabetic animal to peripheral circulatory failure which inhibits the migration of leukocytes into the infected tissues.[6] In our experiments, we have found no

difference in the degree of leukocytic reaction in the fungus lesions associated with either infusion hyperglycemia or acute alloxan diabetes. Cruickshank's observations, however, refer to bacterial skin lesions which may account for some of the differences between his and our results.

Our findings suggest that the metabolic similarities and differences of infusion hyperglycemia and acute alloxan diabetes are related to the morphological resemblances and dissimilarities of the fungus lesions. Hyperglycemia and decreased plasma CO_2 combining power together with mycelial proliferation in the tissues and degenerative nuclear changes in the leukocytes are encountered in infusion hyperglycemia as well as in acute alloxan diabetes. Ketonuria, hyponatremia, hyperlipemia, dehydration, and massive fungus lesions with extensive blood vessel invasion and infarction of tissues occur only in acute alloxan diabetes. The changes in the polymorphonuclear leukocytes appear to be a common denominator in the lesions of both metabolic disturbances and suggest that altered leukocytic function is an important factor in the pathogenesis of Rhizopus oryzae infection. Distinct differences in the appearance of the lesions and in the behavior of the infection exist between infusion hyperglycemia and acute alloxan diabetes which, however, cannot be entirely explained by the changes in the leukocytes and indicate that other, as yet unrecognized, factors are present. These include the biochemical alterations of acute alloxan diabetes other than hyperglycemia and the mechanism of their effect on host resistance and will be the subject of further studies.

SUMMARY

Rabbits inoculated with Rhizopus oryzae will develop the lesions of nasal, pulmonary, and cerebral mucormycosis even when the fungus inoculation precedes acute alloxan diabetes by several days. Rabbits with infusion hyperglycemia inoculated with Rhizopus oryzae develop fungus lesions in the nose and lung. These lesions are more frequent and active than those of metabolically normal rabbits but lack the aggressiveness found in the lesions of rabbits with acute alloxan diabetes and suggest an intermediate stage of host susceptibility. In both acute alloxan diabetes and infusion hyperglycemia the polymorphonuclear leukocytes show degenerative changes which suggest that altered leukocytic function is an important factor in the pathogenesis of this infection.

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