LEUKOPENIA WITH GRANULOCYTOPENIA IN EXPERIMENTAL MUCORMYCOSIS (RHIZOPUS ORYZAE INFECTION)*, ‡

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Plates 43 to 46

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Mucormycosis is a fungus infection which in most instances occurs as a complication in severely ill patients (1). Uncontrolled diabetes mellitus is often the underlying disease. The isolation of the phycomycete *Rhizopus oryzae* (2) and the reproduction of the disease in experimental animals have been previously reported (3). The behavior of this fungus is interesting since in rabbits with acute alloxan diabetes it produces an infection closely resembling the disease in man while normal animals show only rare minute fungus lesions at the site of inoculation. Fungus lesions of intermediate severity are encountered in rabbits with sustained infusion hyperglycemia without diabetes (4). In both experiments, the polymorphonuclear leukocytes uniformly reveal degenerative changes consisting of nuclear pyknosis and karyorrhexis which suggest impaired leukocytic function. In order to evaluate the role of the polymorphonuclear leukocyte in this infection we have studied the course of experimental mucormycosis in rabbits with sustained, severe leukopenia and granulocytopenia.

Methods

Nitrogen mustard (mustargen hydrochloride, Sharp and Dohme, Inc.) was injected in doses of 1 mg./Kilo into the tubing of an intravenous clysis of normal saline (0.9 per cent NaCl) running into the marginal ear vein of male rabbits weighing between 2000 and 3000 gm. One injection daily was given during the first 3 days. Thereafter, the administration of the drug was continued, the frequency of the injections being determined by daily hematologic studies, until the animals were sacrificed. Most animals required injections on alternate days.

Hematologic studies consisting of total white blood cell counts, differential counts, hemoglobin and hematocrit determinations were performed on all animals before and during the period of nitrogen mustard administration. The hemoglobin was determined with the Klett-Summerson photoelectric colorimeter and the hematocrit determination was measured by the microhematocrit technique of Guest and Siler (5). The differential white blood cell counts were recorded in the usual manner and also as the total percentage of granulocytes.

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Leukopenia with granulocytopenia was well established in all 37 rabbits of this experiment within 4 to 5 days after the first administration of nitrogen mustard. At this point, 25 rabbits were lightly anesthetized with intravenous nembutal and instilled intranasally with a standardized fungus spore suspension in sterile normal saline as previously described (3).[1] These rabbits were divided into 3 groups. Group I, comprising 13 animals, was sacrificed 2 days after fungus inoculation. Groups II and III, each consisting of 6 rabbits, were sacrificed 3 to 7 and 8 to 11 days after inoculation. A fourth group, consisting of 12 animals, was instilled intranasally with normal saline only. These rabbits served as controls and were sacrificed at time intervals corresponding to those of the other groups. At the end of the experiment, complete autopsies including bone marrow sections from the sternum and femur as well as fungus cultures of nose and lung were performed as previously described (3).

RESULTS

The hematologic data are summarized in Table I. The first line shows the range and/or median values of these determinations for all rabbits before the administration of nitrogen mustard. These data represent the baseline values. The results of the blood studies for each group of animals on the day of inoculation and the day of death are tabulated below.

The values shown in Table I refer only to the day of inoculation and the day of death but are representative of the severe leukopenia and granulocytopenia maintained throughout the entire experiment. All animals, including the controls, showed a slight transient rise in the percentage of neutrophiles on the 3rd or 4th day after inoculation, which was usually not associated with a corresponding rise in the total white blood cell count. Progressive anemia as indicated by a drop in hemoglobin and hematocrit occurred during the later phases of the experiment. Histologic study of the bone marrow in all rabbits showed hypocellularity with degenerating cells or aplasia (Fig. 1). The spleen and lymph nodes revealed marked lymphoid depletion and the testes showed arrest of spermatogenesis. Despite the severe hematologic abnormalities no hemorrhagic manifestations occurred. No animal developed diarrhea while receiving nitrogen mustard. The only instance of intercurrent bacterial infection consisted of a severe bronchopneumonia in one rabbit of group II which died on the 3rd day after fungus inoculation.

The findings in the infected animals are summarized in Table II. The median per group values of the total white blood cell counts, percentage of granulocytes, hemoglobin and hematocrit determination derived from the daily determinations are tabulated covering the entire period between inoculation and death.

All 13 rabbits of group I, 4 of group II, and 5 of group III showed fungus lesions in the nose. Of 4 animals with nasal lesions one rabbit each of groups I and III showed a minute area of bronchopneumonia and a small focus of meningitis occurred in one animal each of groups II and III. Cultures of nasal and lung tissue taken at autopsy yielded the fungus in 12 rabbits of group I, all of group II, and 5 of group III.

The nasal lesions of the rabbits in group I, sacrificed 2 days after inoculation, showed focal areas of mucosal necrosis (Fig. 2). Some lesions were small and superficial while others, formed by confluence of smaller foci, were larger and extended into the deeper

[1] We are indebted to Dr. L. Ajello, Chief, Mycology Section, Communicable Disease Center, Department of Health, Education, and Welfare, Chamblee, for the preparation of the fungus spore suspension.

structures of the nose, including bone. The lesions which were not circumscribed and were devoid of inflammatory cell response showed many, actively proliferating mycelia. There was no repair. A striking and constant finding was the invasion of mucosal capillaries and small veins by mycelia which produced many isolated fungus thrombi in the deeper tissues of the nose (Fig. 3). The thrombi consisted of masses of mycelia and fibrin with some red blood cells but without white blood cells (Fig. 4). These lesions frequently progressed by confluence and extended to adjacent structures including bone and, in rare instances, nerve trunks.

TABLE I

Leukopenia and Experimental Mucormycosis. Tabulation of Hematologic Data (by groups)

	Rabbits		Total WBC		Granulocytes, *per cent*				Hemoglobin	Hematocrit determination
	Group	No.	Range	Median	Neutro		Others			
					Range	Median	Range	Median	Median	
									gm. per cent	*mm.*
Before nitrogen mustard*			26,200–2,500	10,675	66–15	32	12–0	5.5	12.5	42
Day of inoculation	I	13	2,150–650	1,300	7–0	0	2–0	0	11.8	38
	II	6	2,000–1,350	1,475	4–0	0	3–0	0.5	13.2	42
	III	6	2,000–950	1,300	4–0	2.5	3–0	0	14.1	44
	IV	12	3,750–500	1,550	7–0	2	4–0	0.5	13.1	42
Day of death	I	13	1,950–150	1,200	10–0	2	18–0	2	10.5	33
	II	6	4,400–150	375	24–0	7	20–2	5	11.2	37
	III	6	950–350	575	31–2	5.5	11–2	5.5	8.3	26
	IV	12	1,850–250	925	5–0	0.5	33–0	1.5	10.8	35

* Baseline values representing all animals before nitrogen mustard.

The nasal lesions in the rabbits of group II, sacrificed from 3 to 7 days after inoculation, were characterized by early repair with some infiltration by large mononuclear cells and granulation tissue formation but contained no polymorphonuclear leukocytes. While some lesions were still diffuse, others tended to become circumscribed (Fig. 5). All lesions contained many actively proliferating mycelia. The invasion of small blood vessels by fungus still occurred but was rare.

The nasal lesions of group III rabbits, sacrificed 8 to 11 days after inoculation, were circumscribed and exhibited the characteristics of granulomata (Fig. 6). Repair with infiltration by large mononuclear cells, proliferation of fibroblasts and endothelial cells dominated the picture. Mycelia, although still readily demonstrable, were less numerous and appeared to be degenerating (Fig. 7). Some mycelia were partly surrounded by multinucleated giant cells of foreign body type. Occasional lesions contained some degenerating polymorphonuclear leukocytes. Vascular invasion was not demonstrable.

The pulmonary and meningeal fungus lesions consisted of an accumulation of large mononuclear cells surrounding a single mycelium. The meningeal lesions did not involve the underlying cerebral cortex. Invasion of the optic nerve by a single mycelium was seen in association with meningitis in one animal. The presence of fungus in the pulmonary and meningeal lesions could be ascertained only after prolonged search. No systemic dissemination of the fungus to other than these sites occurred.

Histologic study of the tissues from the control rabbits revealed no lesions except those attributable to the administration of nitrogen mustard. Fungus cultures, similar to those performed in the infected animals, yielded no growth.

TABLE II

Leukopenia and Experimental Mucormycosis. Hematologic, Morphologic, and Mycologic Findings at Autopsy

Rabbits		No. Days after inoculation	WBC per group median	Granulocytes, per cent Median/ Group		Per group		Fungus			
						Hemoglobin	Hematocrit determination	Lesions			Positive cultures nose and/or lung
Group	No.			Neutro	Others	Median		Nose	Lung	Brain	
						gm.	*mm.*				
I	13	2	1,200	2	2	10.5	33	13	1	0	12
II	6	3–7	375	7	5	11.2	37	4	0	1	6
III	6	8–11	575	5.5	5.5	8.3	26	5	1	1	5

Total No. of rabbits, 25.
With fungus lesions, 22.
With positive fungus cultures, 23.

DISCUSSION

The effect of host metabolism on infection can be studied advantageously in experimental mucormycosis because the agent, *Rhizopus oryzae*, produces no significant lesions in the metabolically normal rabbit while an aggressive spreading infection develops in metabolically abnormal animals. This fact has been established in previous experiments which attempted to define the nature of the metabolic alterations as well as their sites of action in the host (3, 4, 6). It was noted that one effect of altered host metabolism involved the polymorphonuclear leukocyte. Consequently an experiment was designed to study the effect of severe granulocytopenia on the course of mucormycosis in an otherwise relatively normal host.

In the present experiment, the polymorphonuclear leukocyte can be disregarded as a significant factor in the inflammatory response since the animals were maintained in a state of severe leukopenia and virtual agranulocytosis. A variable but transient rise in the percentage of granulocytes in the differential counts without concomitant increase in the white blood cell counts was observed in all rabbits between the 3rd and 5th day after instillation with either fungus or

saline. Since this rise also occurred in the control animals it could not be related to the presence of lesions. It appeared to be independent of the frequency of nitrogen mustard injections. This transient rise may be related to the presence of a few degenerating polymorphonuclear leukocytes in some of the lesions of group III. It is attributed to the release of previously sequestered leukocytes since the bone marrow of these animals showed no evidence of hematopoiesis and, in fact, was often aplastic.

Nitrogen mustard produces no known metabolic alterations aside from its effects on the hematopoietic, lymphoid tissues, and gonads (7). In our experiment, morphologic changes attributable to nitrogen mustard were found only in these tissues. No animal developed diarrhea indicative of intestinal injury. It seems reasonable to assume that the metabolic state of the animals in this experiment can be regarded as essentially intact except for the hematologic abnormalities.

Previous studies suggested that the polymorphonuclear leukocyte plays an important part in the pathogenesis of experimental mucormycosis. In the metabolically normal rabbit a pre-existing non-specific acute inflammatory process not only restrained the proliferation of *Rhizopus oryzae* but actually destroyed the inoculated spores (8). In the metabolically abnormal rabbit with either acute alloxan diabetes or infusion hyperglycemia without diabetes a vigorous response by polymorphonuclear leukocytes occurred in the fungus lesions but the leukocytes uniformly showed nuclear pyknosis and karyorrhexis suggesting altered leukocytic function (3, 4). There is considerable experimental evidence that metabolic alterations in the host impair leukocytic function. Cruickshank and Payne demonstrated that the bactericidal function of the leukocytes of alloxan diabetic rabbits is impaired (9). Martin and his associates have shown decreased lactic acid formation by the leukocytes of patients with diabetes mellitus (10). Diminished glycolytic activity manifested by a decrease in lactic acid production was also found in human leukocytes treated with adrenal steroids (11). Rutenburg and Fine have described morphologic changes as well as depression of the phagocytic index in rabbit leukocytes exposed to plasma of rabbits in hemorrhagic shock (12). The concept that changes in the biochemical environment of the leukocyte may affect its function has been emphasized by Dubos (13).

In our markedly leukopenic and granulocytopenic rabbits extensive mucormycotic lesions developed during the first 48 hours after instillation of the fungus. The lesions, however, were essentially confined to the site of inoculation in the nose. They consisted of mucosal ulcerations teeming with mycelia which commonly invaded capillaries and venules and thereby spread to the deeper tissues of the nose. At this stage, the lesions were not circumscribed and consisted of necrotic tissues without inflammatory cell infiltration or evidence of repair. After the 3rd or 4th day, the lesions began to be circumscribed by ingrowth of granulation tissue. Large mononuclear cells appeared, fungus pro-

liferation diminished, and vascular invasion by mycelia was rarely observed. Thereafter, until the termination of the experiment on the 11th day after inoculation, repair progressed and the lesions became distinctly granulomatous. Mycelia, although still readily demonstrable, were less numerous and appeared to be degenerating. Large mononuclear cells increased in number and some foreign body giant cells appeared. Vascular invasion no longer occurred. Occasional lesions now contained some degenerating polymorphonuclear leukocytes. Dissemination of the fungus did not occur except for rare minute foci of aspiration bronchopneumonia and meningitis without brain involvement.

Our findings indicate that experimental mucormycosis in granulocytopenic rabbits, after an initial phase of spread at the site of inoculation, is characterized by the predominance of a proliferative tissue response. The lesions become circumscribed and show a tendency to heal spontaneously. This course differs from mucormycotic infection in rabbits with acute alloxan diabetes when the tissue response is exudative and the disease progresses unchecked without evidence of healing. The fungus lesions are more extensive and destructive and show a vigorous inflammatory reaction by polymorphonuclear leukocytes. Massive mycelial invasion of blood vessels, particularly of arteries is common and produces infarction and fungus dissemination.

At the onset of the infection the leukopenic and granulocytopenic rabbit shows a greatly increased susceptibility to mucormycosis. Later, the lesions tend to heal and resemble in every respect those of the metabolically normal animal (14). This indicates that, despite the hematologic abnormalities, these rabbits respond like metabolically intact hosts. Decreased host resistance in the early phase of the infection is, therefore, attributed to the virtual absence of the polymorphonuclear leukocyte. The granulocytopenia, however, does not affect the later stages of the host response. Thus, the course of the infection in this experiment differs greatly from mucormycosis in the metabolically abnormal animal with acute alloxan diabetes when the unchecked progression of the infection indicates that metabolic alterations in the host affect all phases of host resistance and not only the response by polymorphonuclear leukocytes.[2]

SUMMARY

Mucormycosis was produced in rabbits with sustained, severe leukopenia and granulocytopenia induced by repeated injections of nitrogen mustard. Initially, these animals developed extensive fungus lesions at the site of inoculation which later became granulomatous and tended to heal. Only the early phases of host resistance appeared impaired by the virtual elimination of the polymorphonuclear leukocyte as a factor in the host response. Despite the persistent leukopenia and granulocytopenia, the later phases of host resistance

[2] The technical assistance of Miss Hillma Gheesling and Miss Elaine Schubert is gratefully acknowledged.

resembled those of the normal animal. Thus, the behavior of the infection in this experiment differs greatly from the unchecked progression of mucormycosis in the metabolically abnormal animal with acute alloxan diabetes. The differences in the course of the disease and in the morphologic appearance of the lesions indicate that metabolic alterations in the host affect all phases of host resistance and not only the polymorphonuclear leukocytic response.

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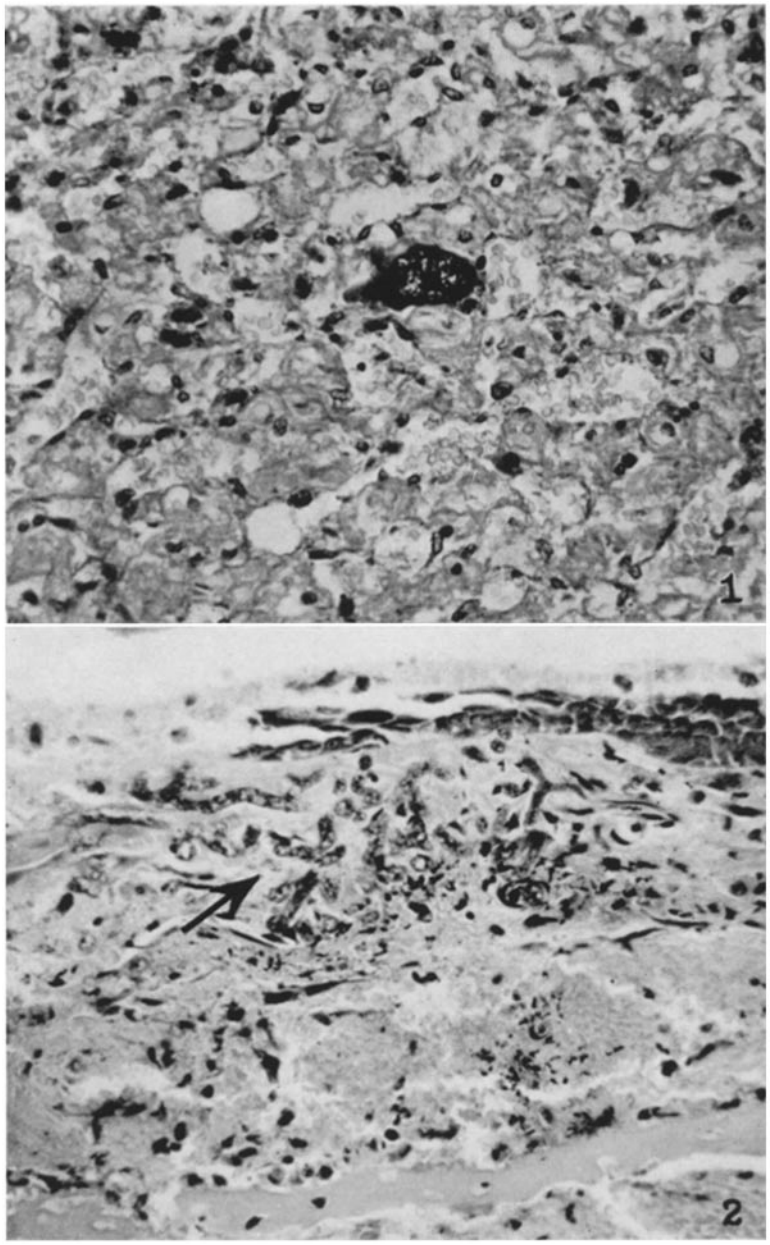
EXPLANATION OF PLATES

PLATE 43

FIG. 1. Aplastic bone marrow showing early fibrosis and a megakaryoblast in center. Hematoxylin-phloxine staining. × 290.

FIG. 2. Group I. Nasal lesion with extensive necrosis and many mycelia (arrow). Note absence of inflammatory cells. Giemsa stain. × 300.

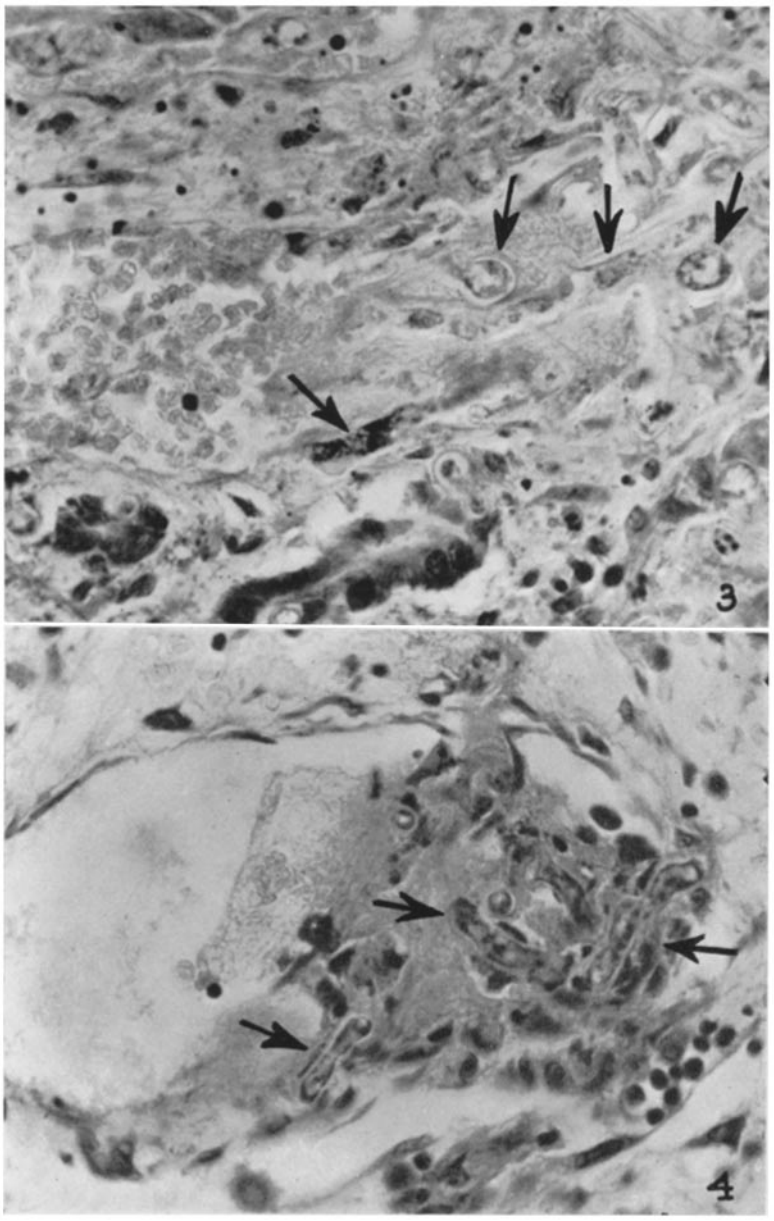


(Bauer and Sheldon: Leukopenia with granulocytopenia in mucormycosis)

PLATE 44

FIG. 3. Group I. Venule at edge of superficial nasal lesion, partly thrombosed and invaded by mycelia (arrows). Giemsa. × 620.

FIG. 4. Group I. Deep nasal venule with thrombus composed of fibrin and mycelia (arrows). Giemsa. × 590.

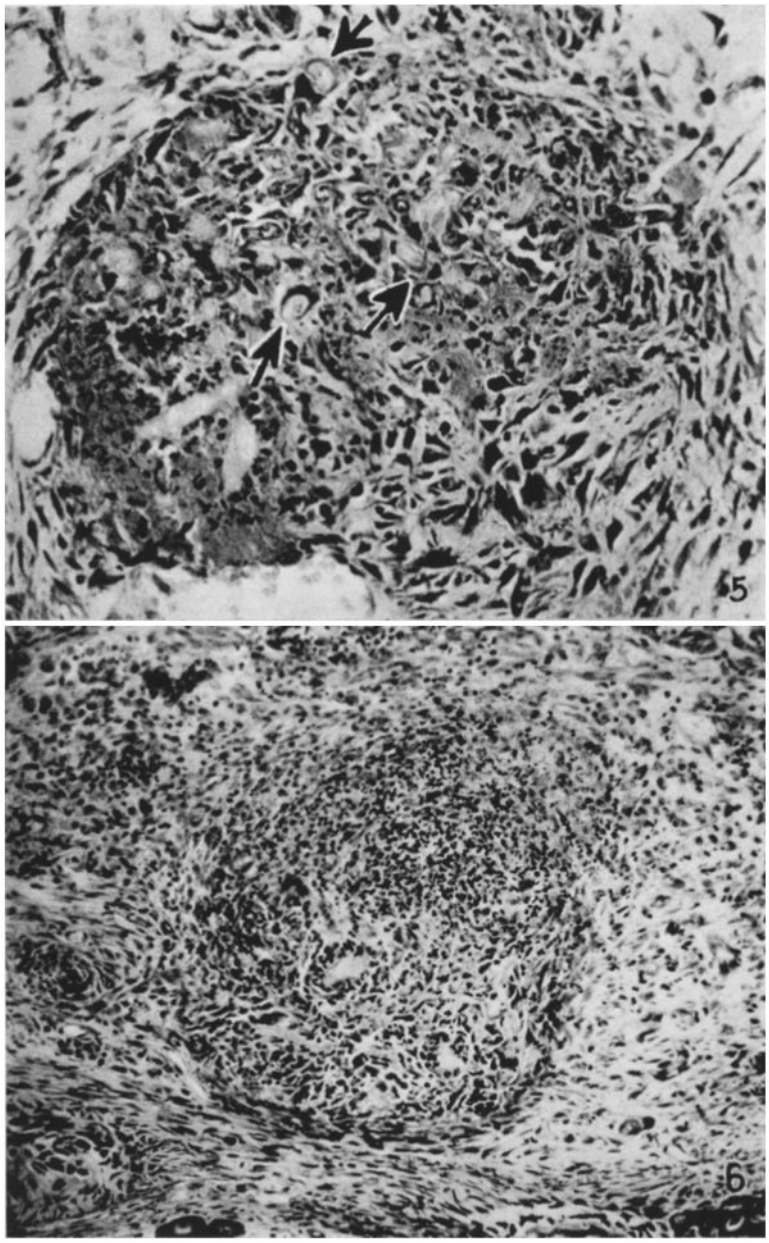


(Bauer and Sheldon: Leukopenia with granulocytopenia in mucormycosis)

PLATE 45

FIG. 5. Group II. Circumscribed nasal lesion with beginning peripheral repair. Note mycelia (arrows). Giemsa. × 280.

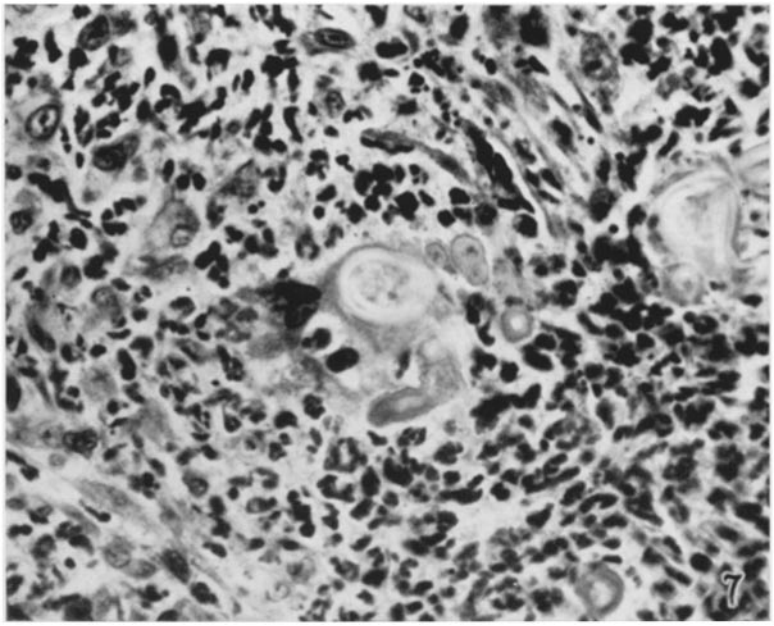
FIG. 6. Group III. A granulomatous lesion. Giemsa. × 155.



(Bauer and Sheldon: Leukopenia with granulocytopenia in mucormycosis)

PLATE 46

FIG. 7. Group III. Center of granulomatous lesion. Note degenerating mycelia and polymorphonuclear leukocytes. Giemsa. × 665.



(Bauer and Sheldon: Leukopenia with granulocytopenia in mucormycosis)