ACTIVATION OF QUIESCENT MUCORMYCOTIC GRANULOMATA IN RABBITS BY INDUCTION OF ACUTE ALLOXAN DIABETES* ‡

By WALTER H. SHELDON, M.D., AND HEINZ BAUER, M.D.

(From the Department of Pathology, Emory University School of Medicine, Emory University, Georgia)

PLATES 8 to 11

Previous experiments have demonstrated that spreading mucormycotic infection developed in rabbits with acute alloxan diabetes following inoculation with *Rhizopus oryzae* (1). Normal animals, however, showed only rare, minute, and self-limiting fungus lesions at the site of inoculation. In rabbits with severe sustained leukopenia and granulocytopenia which appeared otherwise metabolically normal, the fungus lesions showed an initial spread, but 3 days following inoculation they evolved into granulomata which tended to heal (2). Preliminary experiments included in the present studies showed that subcutaneous inoculation of *Rhizopus oryzae* spores into normal rabbits produced granulomatous lesions which did not spread, were well circumscribed after 7 days, yielded no growth of fungus after 70 days, and healed spontaneously. It seemed of interest to determine if the self-limiting character of well established fungus granulomata in normal rabbits could be affected by a superimposed severe alteration of host metabolism, namely acute alloxan diabetes.

Methods

Thirty-seven male rabbits weighing between 1600 and 2400 gm. were lightly anesthetized with intravenous nembutal and inoculated subcutaneously with 1 cc. of a standardized spore suspension of *Rhizopus oryzae* (1)[1]. The injections were placed in the shaved back at 4 sites which were in approximately the same location in each rabbit. These animals were divided into 3 groups; the pertinent data are summarized in Table I. Acute diabetes was produced by the injection of alloxan as previously described (1). The drug was administered to group I (5 rabbits) 8 days, to group II (22 rabbits) 10 days, and to group III (10 rabbits) 15 days after fungus inoculation. Blood sugar determinations using the micromethod of Nelson were

TABLE I

Activation of Mucormycotic Granulomata by Acute Alloxan Diabetes

Rabbits		Granuloma Age/Days			Acetonuria/Days			Blood sugar median	Granuloma activation grade
Group	No.	Biopsy	Alloxan injection	Death	Severe (3–4+)	Mild (Tr-2+)	Total		
I	10–60	7	8	11.5	1.5		1.5	567	1
	10–61	7	8	13	2.5		2.5	452	3
	10–63	7	8	13	2.5		2.5	452	3
	10–64	7	8	12	1.5		1.5	500	1
	10–65	7	8	13	2		2	495	2
II	12–69*, ‡		10	15	2.5		2.5	439	2
	12–71*, ‡		10	17	5		5	416	3
	12–72*, ‡		10	20	7	1	8	464	2
	13–01*		10	15	1.5		1.5	457	2
	13–07*, ‡		10	19	6	2	8	431	2
	11–21‡		10	17	2	5	7	420	3
	12–70*		10	13	2		2	508	2
	13–10*, ‡		10	14	3		3	428	2
	11–40*, ‡	9	10	26	6	6	12	475	2
	13–15*, ‡	9	10	16	4		4	439	2
	13–14*, ‡		10	13	1.5		1.5	566	2
	13–11*, ‡		10	16	3.5		3.5	371	2
	11–76*, ‡	9	10	15	3.5		3.5	674	2
	11–77	9	10	22	1	6	7	518	1
	12–11*, ‡		10	16	2.5		2.5	517	3
	12–12*, ‡		10	16	2.5		2.5	497	3
	13–23*, ‡	9	10	14	2		2	438	2
	13–25*	9	10	16	4		4	470	2
	13–51*		10	16	4		4	451	2
	13–53*, ‡	9	10	14	3		3	442	2
	11–75	9	10	22	1	6	7	518	1
	11–78	10	11	23	4	6	10	473	2
III	10–68	14	15	19	1.5		1.5	532	1
	10–69	14	15	19	1.5		1.5	655	1
	10–70	14	15	19	1.5		1.5	428	1
	14–23*		15	18	0.5		0.5	434	1
	14–25*		15	19	1		1	621	2
	14–26*		15	19	1.5		1.5	403	2
	14–27*		15	19	2		2	360	3
	14–28*		15	21	1	2	3	682	2
	14–29*		15	20	1		1	630	1
	14–30*		15	20	3.5		3.5	392	2

* Received sodium bicarbonate.
‡ Received insulin.

performed prior to alloxan administration, and once daily throughout the experiment beginning on the 3rd day after alloxan (3). Urine sugar and acetone were also determined before and several times daily after alloxan, using clinitest and acetest tablets. In an attempt to prolong the survival of the rabbits in acidosis, 19 animals in group II and 7 in group III were given intraperitoneal injections of a 2 per cent solution of sodium bicarbonate in sterile normal saline (0.9 per cent NaCl) and/or insulin. Sodium bicarbonate was given in doses of 30 to 50 cc. one or more times daily. Regular insulin was injected intravenously in 1 or 2 daily doses of 2 units each, occasionally supplemented by 2 units of protamine zinc insulin intramuscularly. The administration of these substances was governed by the degree of acetonuria and the clinical state of the animals.

Some rabbits died in acidosis, while others were sacrificed moribund or when acetonuria decreased, indicating a transition from acute to chronic alloxan diabetes. Complete autopsies were performed, and the tissues fixed in Zenker's fluid with 5 per cent glacial acetic acid, 10 per cent formaldehyde and absolute alcohol. Histologic preparations were made from all tissues and stained with hematoxylin and phloxine and the Giemsa method where lesions were present. Each skin lesion was examined with multiple sections. Tissue fragments from the skin lesions and the lungs as well as cardiac blood samples were planted on Sabouraud-dextrose agar and incubated at room temperature.

Another 16 rabbits served as controls. They were inoculated with fungus in the same manner as the diabetic animals, but did not receive alloxan. Throughout the experiment they were tested repeatedly for the presence of hyperglycemia, glycosuria, and acetonuria. In 3 of these animals single skin lesions were excised 14, 28, and 42 days after fungus inoculation. At 70 days these rabbits were sacrificed and the last lesions excised. The remaining 13 animals were sacrificed 10 days (4 rabbits), 14 days (2 rabbits), 17 days (3 rabbits), 21 days (2 rabbits), 45 days (1 rabbit), and 52 days (1 rabbit) after fungus inoculation. Additional controls were obtained by excision of a single skin lesion 1 day prior to alloxan administration in all animals of group I, 9 of group II, and 3 of group III (Table I). Morphologic and mycologic studies identical with those of the diabetic animals were carried out in the controls.

RESULTS

On gross examination, when viewed *in situ* or on cut surface after excision, most of the skin lesions of all rabbits were of similar appearance. At 7 to 8 days after fungus inoculation they showed an inspissated, grayish yellow necrotic center completely enclosed by a well defined zone of fibrosis. With increasing age the central area of necrosis diminished in size, while the surrounding fibrosis became more pronounced. However, in 2 rabbits of group I and in 3 of group II delicate grayish streaks radiated from the skin lesions into the adjacent tissues, particularly the muscle. No significant changes were noted in the other organs.

Histologically, the skin lesions obtained 7 to 10 days after fungus inoculation from the non-diabetic rabbits and the control lesions excised before alloxan administration from the other animals consisted of a large area of central necrosis composed of polymorphonuclear leukocytes and many spores, singly or in small groups. Some of the polymorphonuclear leukocytes were degenerating, while others were morphologically normal. These spores stained poorly and appeared to be degenerating, and while a few looked viable, none showed evidence of proliferation and no hyphae were seen. The necrotic center

was completely surrounded by a wide zone of granulation tissue composed of vigorously proliferating fibroblasts, many large mononuclear cells, and a few polymorphonuclear leukocytes (Fig. 1). At the periphery many newly formed capillaries and some lymphocytes and plasma cells were present. At 14 to 15 days after inoculation the breakdown of polymorphonuclear leukocytes in the center had increased, while the number of visible spores had remained unchanged. Viable appearing spores were extremely rare. In the surrounding zone collagen deposition had increased, as had the number of large mononuclear and other chronic inflammatory cells. Multinucleated foreign body type giant cells were noted at the junction of necrotic and viable tissue. From then on until 70 days after inoculation the necrotic center decreased in size and was composed entirely of degenerated polymorphonuclear leukocytes and cellular debris. The number of visible spores diminished progressively. In the late stages spores disappeared completely, and the lesions became loculated by the formation of septa composed of fibrous tissue. The cellular infiltrate surrounding the necrosis consisted entirely of large mononuclear cells with many multinucleated giant cells. The thick walls of dense fibrous tissue still contained proliferating fibroblasts and a sprinkling of lymphocytes and plasma cells (Fig. 2). In the control animals the fungus lesions were always confined to the sites of inoculation and never involved the regional lymph nodes or other organs.

Regardless of the age of the granulomata and the duration of acidosis, the skin lesions of all rabbits with acute alloxan diabetes differed from the controls by the presence of budding spores and the formation of mycelia, by fungus invasion of the fibrous wall and surrounding tissues, and by the appearance of an acute inflammatory cell reaction with focal breakdown of the granuloma wall. These changes did not occur simultaneously and to the same extent in all animals. In 2 rabbits of groups I and II each and in 5 of group III the changes consisted only of some budding of spores and rare mycelia formation in the necrotic center and an exudation by polymorphonuclear leukocytes into the surrounding granulomatous tissue (Fig. 3). This degree of change in the skin lesions was arbitrarily rated as grade 1. In 1 animal of group I, in 16 of group II, and in 4 of group III the wall showed early focal necrosis, definite invasion by fungus, and a more marked acute inflammatory cell infiltration (Fig. 4). In addition, budding spores and mycelia were more numerous in the necrotic center. These changes were classified as grade 2. The most extensive changes, designated as grade 3, were encountered in 2 rabbits of group I, 4 of group II, and in 1 of group III. These were characterized by a spread of the infection beyond the original lesion with frank invasion of the adjacent tissues, including blood vessels and muscle, by large numbers of mycelia (Figs. 5 to 8). Furthermore, the changes observed in the lesions graded as 1 and 2 were all present and markedly accentuated. Despite vascular invasion by mycelia, no fungus

lesions were found in the regional lymph nodes or other organs. The polymorphonuclear leukocytes in the rabbits with acute alloxan diabetes revealed the previously described nuclear changes, while degeneration of these cells in the control lesions was present only in the necrotic center (1, 4). The skin lesions of rabbits in groups I, II, and III uniformly yielded the fungus on culture. Cultures from the control lesions invariably yielded the agent up to 42 days, and except for one animal, up to 55 days after inoculation. At 70 days after inoculation the fungus could no longer be recovered from any of the lesions. Fungus cultures of lung tissue and heart blood of both diabetic and normal rabbits showed no growth.

The age of the fungus lesions at time of alloxan administration and at time of death, the degree and duration of acetonuria, the median level of hyperglycemia, and the grade of the skin lesions are shown for each rabbit of groups I, II, and III in Table I. Only rabbits with typical acute alloxan diabetes characterized by marked hyperglycemia and glycosuria, acetonuria, hyperlipemia, and dehydration were included in this experiment. Marked acetonuria was present in all of these animals within 48 to 72 hours after alloxan administration. In many rabbits of group II and some of group III survival in acidosis appeared to be prolonged by the injection of sodium bicarbonate and/or insulin. In 4 animals of group II and in 1 of group III the prolongation of acidosis was associated with a decline in the degree of acetonuria. Decreasing acetonuria, however, was also observed in some rabbits in which no attempt at treatment was made. None of the controls in group IV showed evidence of acetonuria, although occasionally a slight elevation of the blood sugar levels and glycosuria lasting for 12 to 48 hours were noted.

DISCUSSION

It is known that changes in the internal biochemical environment of the host may be a factor in altering the character of infectious lesions (5). The present experiments were designed to determine if alterations of host metabolism could induce activation and spread in a preexisting quiescent granulomatous lesion which in the metabolically normal animal remains confined to the site of inoculation and eventually heals. Experimental mucormycosis in the rabbit is particularly suited for this purpose, since it has been shown that derangements in host metabolism are essential in the pathogenesis of this infection (1, 4).

It was first established that subcutaneous inoculation of Rhizopus oryzae into normal rabbits produced localized granulomatous lesions which did not spread and healed within 3 to 4 months. The fungus could be recovered on culture from these lesions until 8 weeks after inoculation. Acute alloxan diabetes was then produced in rabbits with subcutaneous granulomatous lesions of 8, 10, and 15 days duration and, after varying periods of diabetic acidosis, autopsies were performed. The tissues were studied histologically and compared with

normal controls. The skin lesions of all animals with acute alloxan diabetes differed strikingly from those of the normal rabbits by an acute exacerbation and spread of the infection which could not be related to the age of the granulomata or the time of survival in acidosis. The preexisting granulomata showed a superimposed acute inflammation and active fungus proliferation with many mycelia which sometimes invaded the wall and surrounding tissues, including blood vessels. The wall of the granulomata usually showed areas of early focal necrosis. However, the regional lymph nodes were not involved by fungus and there was no systemic dissemination. As in previous experiments, the polymorphonuclear leukocytes of the animals with acute alloxan diabetes uniformly showed nuclear pyknosis and karyorrhexis, while the large mononuclear and other chronic inflammatory cells in the granulomatous lesions showed no morphologic changes. The skin lesions of all animals yielded the fungus on culture, while cultures of heart blood and lung tissue showed no growth.

Five rabbits inoculated with fungus and given alloxan 10 to 15 days later did not develop acute alloxan diabetes with acetonuria, but showed permanent hyperglycemia above 400 mg. per 100 cc. These animals were studied in the same manner as the others in order to observe the effects of chronic alloxan diabetes on the granulomatous lesions. Histologically, these skin lesions did not differ from those of the normal controls. It appears, therefore, that activation of the infection occurred only in the presence of acidosis. Acidosis as part of acute alloxan diabetes was present in all animals which showed activation of the granulomata. The acidosis as judged by the degree of acetonuria was initially severe in all rabbits. Although the duration of the acidosis and life of some animals may have been prolonged by the administration of sodium bicarbonate and/or insulin, no constant relationship between the total duration of acidosis and the degree of activation of the skin lesions could be established in this experiment.

Our findings show that acute alloxan diabetes profoundly alters the resistance of rabbits to preexisting, well established, and healing granulomatous lesions produced by the subcutaneous inoculation of *Rhizopus oryzae*. In the course of this drastic change in the metabolic state of the host, the granulomata which heal spontaneously in the metabolically normal animal change their character and become the site of acute mucormycosis. In this experiment the activation of a quiescent infection appears to be the direct result of a change in the metabolism of the host[2].

SUMMARY

In normal rabbits subcutaneous granulomata produced by the injection of a spore suspension of *Rhizopus oryzae* remained confined to the site of inocula-

tion, showed no fungus proliferation, no longer yielded the agent on culture 10 weeks after inoculation, and eventually healed. Similar well established granulomata in rabbits with acute alloxan diabetes induced 8, 10, and 15 days after injection of the fungus uniformly showed activation of the infection. This occurred only in animals showing acetonuria. In these animals the skin lesions showed proliferation of the fungus frequently associated with invasion and early necrosis of the granuloma wall. In some instances, spread of the infection to adjacent tissues with invasion of blood vessels had occurred. These experiments illustrate that changes in host metabolism can activate a preexisting quiescent infection.

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. R 14-02. Control skin lesion, 10 days after fungus inoculation, showing thick fibrous wall and portion of necrotic center. Giemsa. × 150.

FIG. 2. R 10-87. Control skin lesion, 10 weeks after fungus inoculation, with fibrous wall, large mononuclear and giant cells, and residual necrotic center. Giemsa. × 130.
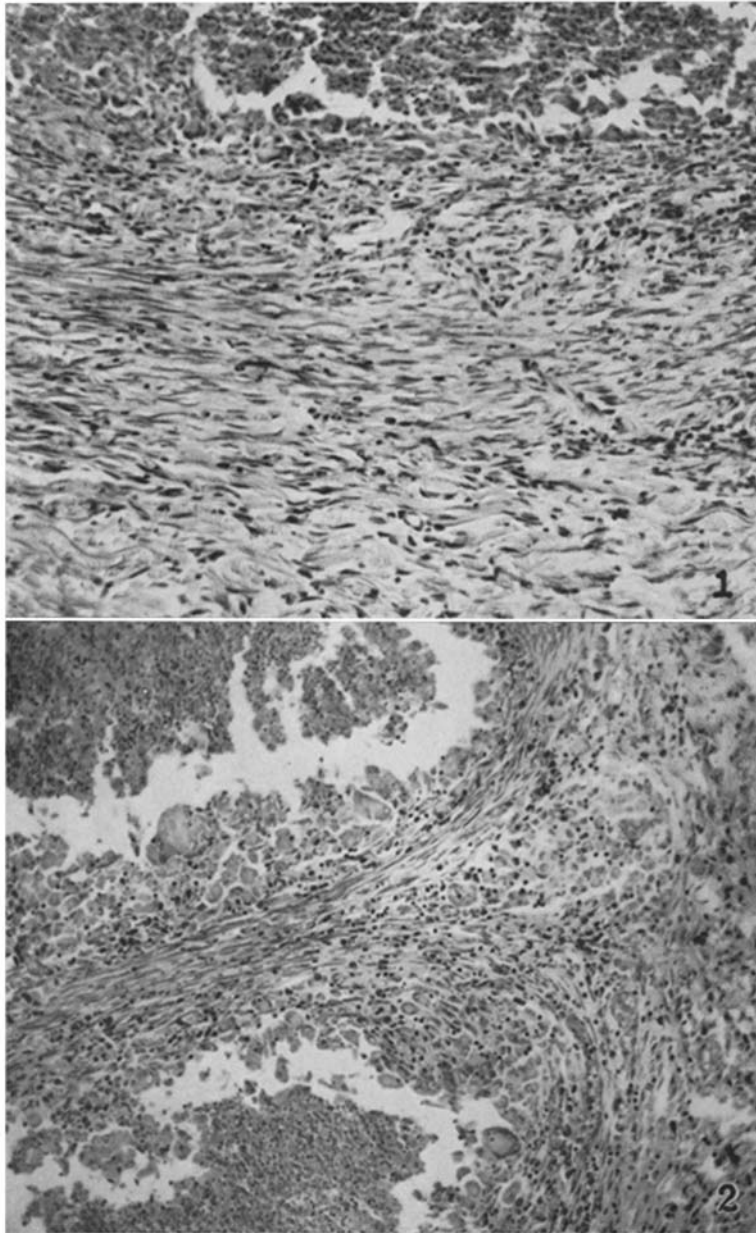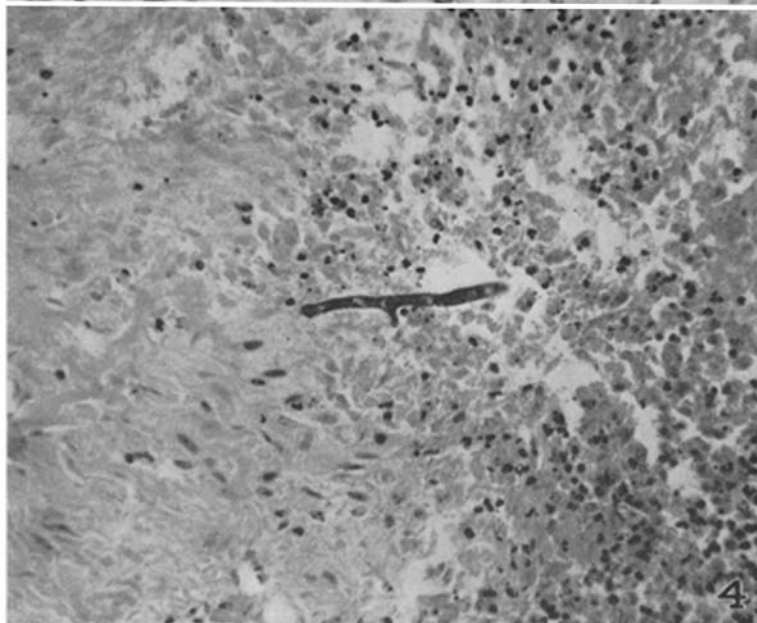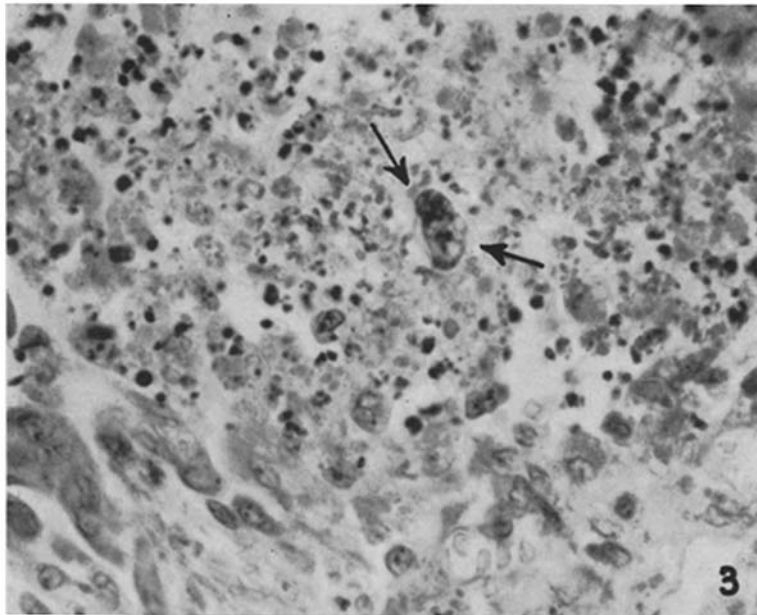
(Sheldon and Bauer: Mucormycotic granulomata activation)

FIG. 3. R 10-60. Skin lesion, grade 1, with early mycelia formation (arrows) in necrotic center. Giemsa. × 345.

FIG. 4. R 10-65. Skin lesion, grade 2, with branching hypha penetrating into wall of granuloma showing early necrosis. Giemsa. × 320.
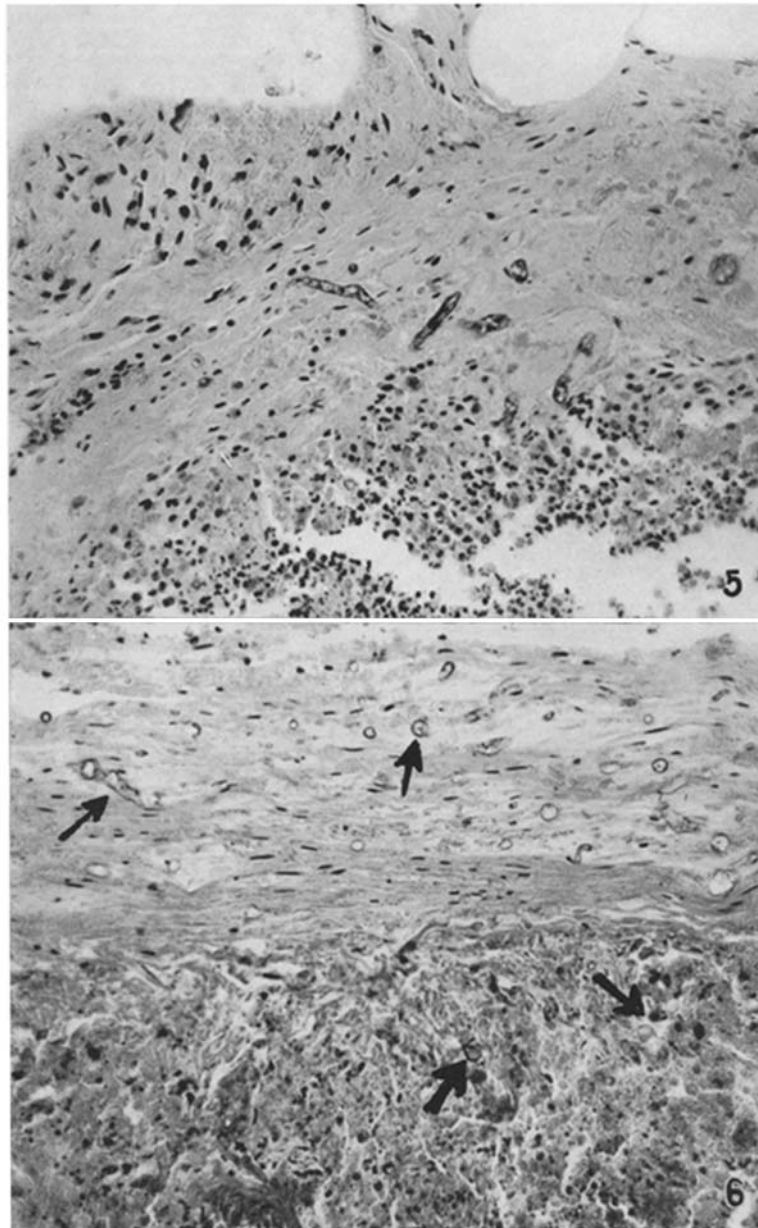
(Sheldon and Bauer: Mucormycotic granulomata activation)

PLATE 10

FIG. 5. R 12-71. Skin lesion, grade 3, with several hyphae invading granuloma wall. Giemsa. × 260.

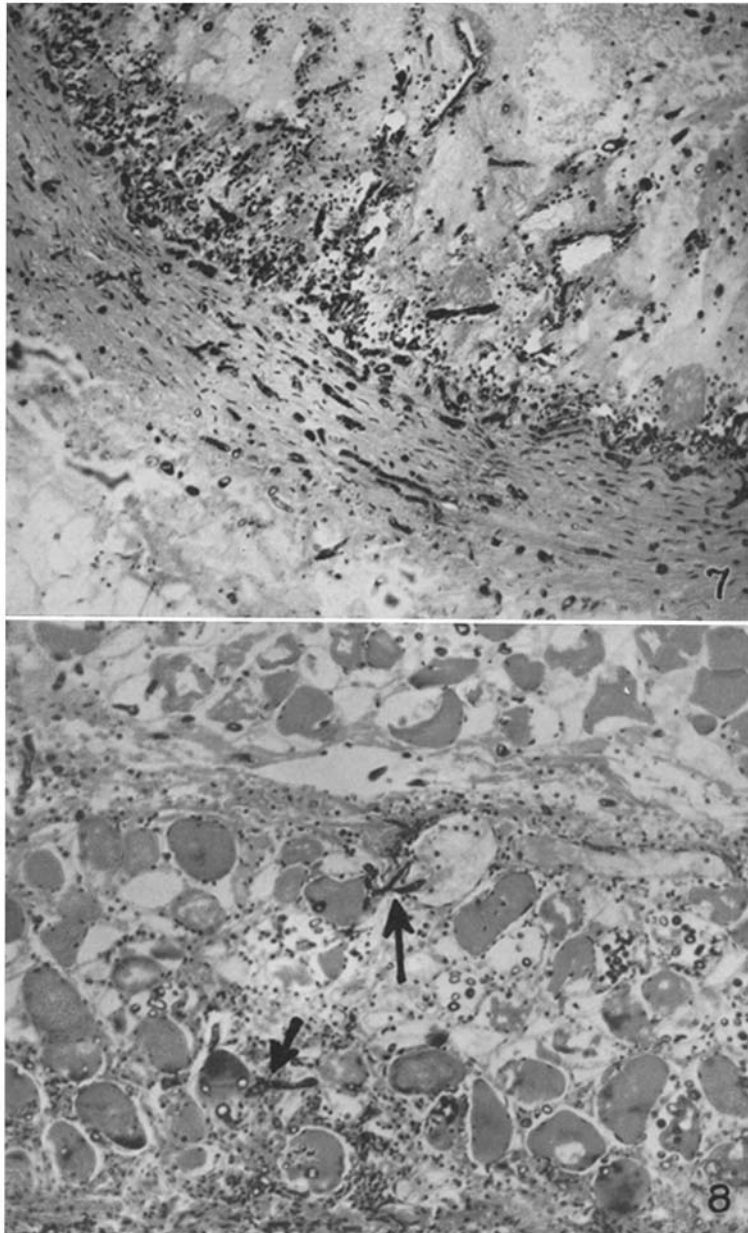FIG. 6. R 14-27. Skin lesion, grade 3, with many hyphae (arrows) in granuloma wall and among necrotic center. Giemsa. × 265.

(Sheldon and Bauer: Mucormycotic granulomata activation)

FIG. 7. R 11-21. Large artery deep to skin lesion, grade 3, with many mycelia in lumen, wall, and adventitia. Giemsa. × 115.

FIG. 8. R 11-21. Muscle adjacent to skin lesion, grade 3, and small vein invaded by numerous mycelia (arrows). Giemsa. × 130.

(Sheldon and Bauer: Mucormycotic granulomata activation)