EXPERIMENTAL BLASTOMYCOSIS IN MICE *

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In previous papers I have shown that the virulence, for mice, of the causative organisms from two cases of human blastomycosis was not enhanced by mouse passage,[1] and that the filamentous form was as infectious for mice as the yeast form.[2]

Since blastomycosis was being produced very consistently in this convenient laboratory animal, observations were made on the reactions of the tissues of the mouse to this large fungous organism. This study was augmented with an attempt to explain some of the tissue responses by noting the results of injections into mice of killed blastomycetes and of a phosphatide fraction of the fungus. Rabbits were utilized for an accompanying study of the effect of a polysaccharide fraction on the tissues. The chemical fractions were obtained from Peck and Hauser, whose published papers about the fractions may be consulted.[3,4]

LITERATURE

In all of these reports the injected organisms were obtained from proved human cases of blastomycosis as recently tabulated by Martin and Smith.[5]

Bowen and Wolbach [6] inoculated four mice intraperitoneally and obtained satisfactory abdominal and pulmonary lesions. They stated: "The type of lesion in the lung, the filling of the alveoli with large cells and organisms with little other reaction, is a peculiar one and deserves further study." Photographs show sections of the lesions of the lung of a mouse killed 40 days after intraperitoneal inoculation. The abdominal lesions had largely disappeared. DeMonbreun [7] found, from injection of yeastlike forms of the organisms, that "each of six mice died in from three to five weeks after inoculation, and numerous small abscesses containing the fungous cells were found in the lungs, liver, spleen and kidneys." In mice inoculated by Bergstrom, Nugent and Snider,[8] profuse lesions developed within 5 weeks, but the animals did not die until 10 weeks after inoculation. Davis [9] found that blastomycetes injected into the peritoneal cavity of guinea pigs were rapidly taken up by leukocytes, macrophages principally.

* Aided by the John and Mary R. Markle Foundation and the Duke University Research Fund.

Presented before the American Society for Experimental Pathology, Chicago, Ill., April 18, 1941.

Received for publication, September 4, 1941.

Materials and Methods

Two strains of *Blastomyces dermatitidis* were employed, the sources of which have been previously detailed.[1] Strain A was from a fatal case of systemic blastomycosis and strain B was from a cutaneous lesion which responded well to iodine therapy. Both strains were found to be capable of producing generalized blastomycosis in mice, and they are not differentiated in Table I.

The mice were inoculated intraperitoneally under rigid, aseptic technic with doses of pure cultures which varied from 0.5 cc. of a 1:200 suspension to 1 cc. of a 1:25 suspension, by volume. The yeast form of the organism was centrifuged in a calibrated tube at a standard speed and duration of time. Proper dilutions were made by adding saline solution. To obtain data to compare with the amounts of dead organisms and chemical fractions, the wet centrifuged blastomycetes were weighed, after the supernatant fluid was decanted. Another weighing after vacuum drying indicated that 1 cc. of the moist organism was equivalent to 52 mg. in the dry state. On this basis the dry weight injected varied from 0.1 to 2 mg. in different animals. The smaller dose was capable of producing generalized lesions, and no separation of the animals according to dosage has been made. Comment and data on the dosage factor are available in another report.[1]

Studies of the peritoneal fluid were made in mice sacrificed during the first 11 days following injection. The normal mouse has insufficient peritoneal fluid for cellular studies. Consequently, 0.5 cc. of physiologic saline solution was injected intraperitoneally just before the animal was killed. Total white counts were determined. The disposition of the blastomycetes and the comparative numbers of the cell types were determined by supravital study with neutral red and janus green, in a hot box, and by stained smears. Paraffin sections were found helpful in differentiating cell types, and gave topographic relations.

Thomas and Dessau [10] studied the cells of the peritoneal fluid in normal mice after rinsing the peritoneal cavity with 0.5 cc. of saline solution as just mentioned. The total cells averaged 5,900 per cmm. Undifferentiated cells made up 65.5 per cent in the differential counts. These "were small cells, about the size of the blood lymphocytes, with a small amount of cytoplasm and no constant cytoplasmic inclusions which stained with vital dyes." Monocytes numbered 32 per cent; polynuclear leukocytes, 1.5 per cent; basophilic leukocytes, 0.8 per cent, and clasmatocytes, 0.2 per cent. This classification has been followed in these experiments.

TABLE I

The Cellular and Tissue Response to the Injection of Living Blastomyces dermatitidis into the Peritoneal Cavities of Mice*

Duration	No. mice	No. died	No. sacrificed	Peritoneal fluid						Gross
				White cells	Neutrophils	Undifferentiated cells	Monocytes	Eosinophils	Organisms	
				per cmm.	%	%	%	%		
½ to 3 hrs.	2	0	2	6,000	19	70	11		Free	Normal
4 hrs.	2	0	2	9,300	83	14	3		Surrounded by neutrophils	Normal
1 day	2	0	2	19,600	56	17	24	3	Surrounded by neutrophils	Normal
3, 4, 5 days	3	1	2	9,500	27	52	21		Surrounded by neutrophils and monocytes; fibroblasts present	Dull peritoneum
1 to 5 wks.	59	50	9	5,000†	54	16	29	1	Surrounded by neutrophils and monocytes; blastomycetes rare	Nodules on peritoneum and usually in lungs
5 to 8 wks.	4	2	2							Nodules on peritoneum and usually in lungs
Total	72	53	19							

* Dose varied from: 0.5 cc. of 1:200 to 1 cc. of 1:25 suspension by volume, or 0.1 to 2 mg. dry weight.
† Average of 3 mice: 8, 10, 11 days.

Experimental Data

Living blastomycetes were injected into the peritoneal cavities of 72 mice. The great majority of mice died between 1 and 5 weeks after inoculation (Table I) and presented peritoneal and pulmonary nodules, and usually microscopic lesions elsewhere. Most of the mice which died became less active and lost weight during the several days before death.

During the first 3 hours the injected blastomycetes lay free in the abdominal cavity and the white and differential counts were those of normal mice. After several hours (Fig. 1) the blastomycetes were found surrounded by polymorphonuclear neutrophils. The peritoneal fluid also showed an increase in the number of white cells and polymorphonuclear neutrophils predominated instead of undifferentiated cells.

At the end of 1 day the organisms were surrounded by cells which were difficult to identify. In the supravital preparations the cytoplasm contained numerous, neutral-red vacuoles, and nuclear staining sometimes occurred, which, in such preparations, indicates dead cells. The nucleus was swollen and usually was not polymorphous. In the stained films the cytoplasm was like that of neutrophils. Study of paraffin sections suggested that most of these surrounding cells were probably injured polymorphonuclear neutrophils with swollen nuclei. The same difficulty was experienced in interpreting the nature of the surrounding cells throughout the 11-day period during which observations of peritoneal fluid were made. No such difficulty was experienced in making differential counts of the peritoneal fluid. The total white count was definitely elevated and the polymorphonuclear cells predominated in the differential count. The coating on the peritoneum was found in paraffin sections to consist of blastomycetes, interspersed with polymorphonuclear neutrophils.

In the period from 3 to 5 days the blastomycetes were surrounded by cells which were judged to be polymorphonuclear neutrophils, but some stimulated monocytes and occasional fibroblasts were noted. The peritoneal surfaces were dull to the naked eye, and occasional unattached white nodules 1 mm. in diameter were seen. At 5 days (Fig. 3) the peritoneal coating of blastomycetes and interspersed polymorphonuclears had become necrotic centrally in the nodules, but peripherally the blastomycetes were proliferating and attracting polymorphonuclear neutrophils. Karyorrhexis among dead polymorphonuclear neutrophils was often noted. Monocytes were present but not prominent. Fibroblastic outgrowth from the serosa was well developed and vascularization had begun.

In the period from 1 to 5 weeks, free blastomycetes in the peritoneal fluid were less numerous. They occurred in a mass of cells which were

thought to be predominantly injured polymorphonuclear neutrophils, though some were monocytes. Fibroblasts extending from such a mass at the end of 8 days are shown in Figure 2.

At 1 week white nodules and plaques were visible over the peritoneal and omental surfaces, and these continued to increase in size.

The gross appearance typical of the period between 1 and 5 weeks has been shown in previous papers.[1,2] Often a single, large, caseous nodule occurred between stomach and spleen, and smaller nodules elsewhere. At 1 week blastomycetes had been first noted in the retrosternal lymph nodes and in the lungs. From then on involvement of the lung was usually prominent and was noted in 90 per cent of the animals which died in this period (Fig. 5; also Fig. 4 in previous paper [2]). The route from the peritoneal cavity to the lungs was never by direct extension through the diaphragm because the lungs were free in the pleural cavities. The uniform distribution of nodules in the lungs indicated an hematogenous spread, either via the retrosternal lymphatics and nodes to the venous system, or directly from the peritoneal cavity into the vascular system. Hematogenous spread to cardiac muscle (Fig. 5 in previous paper [2]), brain, spleen and liver was demonstrated microscopically in a number of mice, and probably would have been demonstrated much more frequently if all of these organs had been systematically studied microscopically. An interesting involvement of the liver consisted of growth of organisms into portal veins with the production of large areas of infarction.

Microscopically, this period was characterized by the great proliferation of blastomycetes, whether in the abdominal cavity, lungs (Fig. 6) or elsewhere. There was little reaction of any kind in some masses but usually the blastomycetes were interspersed with polymorphonuclear cells (Fig. 6), fragments of nuclei, or less frequently with monocytes, and very rarely with the undifferentiated cells. Necrosis of the centers of blastomycetic masses was the rule; and fibroblastic growth developed at the periphery after a time, but generally the cellular reaction was less conspicuous than the blastomycetic proliferation.

Microscopic abscesses like those of human cutaneous blastomycosis rarely occurred. This may have been due to the fact that the blastomycetic growth was so massive and diffuse that microscopic collections of a few blastomycetes with surrounding polymorphonuclear cells were not frequent. Figure 4 shows a microscopic "abscess" adjacent to the pancreas in a mouse injected 11 days before.

Phagocytosis of blastomycetes by macrophages and giant cells was never a frequent appearance but it did occur, especially in the animals which had been injected for the longer intervals. Several giant cells

containing blastomycetes were noted in a mouse injected 34 days before.

In the few mice examined in the interval between 5 and 8 weeks, the central areas of blastomycotic masses were not only necrotic, but the outlines of the blastomycetic shells had disappeared, so that the appearance of caseation was produced. Dense fibrosis occurred about such areas.

"Toxicity" of Process

Some of the sacrificed animals were active and appeared healthy, but showed extensive abdominal and pulmonary lesions. It is not clear, therefore, that blastomycetic infection in itself is especially productive of a toxic state. The same may be said for human cases of blastomycosis, except in the terminal stages of the process. As possible causes for death in the mouse, aside from a toxic effect of the organism or of the products of disintegration of the lesions, are the presence of blastomycosis of the peritoneum which may interfere with peristalsis of the intestines, or blastomycosis of the liver which may interfere with the function of that organ. The arguments in favor of the toxicity of dead organisms will become apparent in the following section on killed blastomycetes.

Summary of Changes

The intraperitoneal inoculation of mice with *B. dermatitidis* led to a massive growth of organisms in the peritoneum with dissemination to the lungs and elsewhere. The blastomycetes attracted cells, chiefly polymorphonuclear neutrophils, and when the organisms occurred in masses they tended to develop necrotic centers. The early, peripheral, fibroblastic growth developed into an encapsulating, fibrous layer. The monocyte and giant cell appeared to be less important in the mouse than the polymorphonuclear neutrophil and the fibroblast.

Injection of Killed Blastomycetes

Killed blastomycetes were injected into the peritoneal cavities of 14 mice to determine, first, how closely the tissue response to the living organisms could be duplicated, and second, how toxic the suspension was.

A single small dose of killed organisms produced no apparent effect. Mice which had received 0.1 mg. were sacrificed after 8 weeks. No gross or microscopic abnormality could be demonstrated. It has already been shown that this amount of living fungus produced progressive blastomycosis and death.

To duplicate the effects of the proliferating, living yeast cells, repeated large doses of killed organisms were used. Ten mg. were given

every other day. (Dosage is in terms of equivalent dried weight of suspension.) Killing was effected by heating at 60° C. for 2 hours. After each aspiration of suspension from the rubber-stoppered vaccine bottle, the heating was repeated to insure sterility.

The mice tolerated the large doses poorly, and appeared ill after injections. Most of them died in 5 to 11 days, apparently of the toxic effects of the suspension.

In paraffin sections the fact that the fungus was dead was clearly evident in the failure of the blastomycetes, especially the internal substance, to stain with hematoxylin. Uniform staining with eosin occurred.

The peritoneum was lined with a friable, yellow layer. This was composed largely of the deposit of killed blastomycetes (Fig. 7). Between them, as between the living organisms in the first experiments, polymorphonuclear cells and a few mononuclear cells occurred. There was the same necrosis in the centers of large blastomycotic masses as was noted with the living organisms; but with the killed blastomycetes the necrosis applied only to the infiltrating cells, since the blastomycetes themselves were already dead. Eosinophils, and macrophages and giant cells containing blastomycetes, were more abundant than in the mice which received the living organisms. In the mouse examined 11 days after injection the outlines of the blastomycetes had largely disappeared, leaving "caseous" areas.

Pulmonary lesions were not produced. The retrosternal lymph nodes contained very large, finely granular reticulo-endothelial cells, but no blastomycetes.

In comparison with the living organism it was concluded that the initial cellular response, the central necrotizing process and the peripheral, fibroblastic response were similar. The early toxicity of the large doses of killed organisms was correlated with the toxicity of the living organisms after several weeks. By this time the formerly viable masses had become necrotic centrally and had probably liberated some toxic substance in the nature of an autolysate. Enzymes from the dead polymorphonuclear neutrophils may have produced the necrosis.

Blastomycetic Phosphatide in Mice

For the method of extraction and an account of the properties of this phosphatide the paper by Peck and Hauser [3] may be consulted.

Thomas and Dessau,[10] who studied the effects of chemical fractions of the tubercle bacillus in mice, stated: "It is of interest that while in guinea pigs and in rabbits the reaction to tuberculo-phosphatide resembles very closely the lesions produced by the living organisms, this mimicry is best accomplished in the mouse by the injection of the waxy

fractions." They found that the phosphatides of the tubercle bacillus stimulated the multiplication and partial maturation of monocytes. The formation of true epithelioid cells was not the rule.

The blastomycetic phosphatide had been passed through a Berkefeld filter for sterilization when it was in the stage of ether extraction. For injection of mice, 10 mg. were suspended in 1 cc. of distilled water. Microscopically the suspension showed droplets of which none was as large as a human red blood cell. After the suspension stood for a day, a small amount of material settled out.

Seven mice received the phosphatide intraperitoneally, in amounts varying from a single dose of 10 mg. to daily doses of 30 mg. for 24 days, with sacrifice 3 days later. The higher dosage resulted in prominent, white, peritoneal plaques.

A peritoneal reaction, predominantly of the monocytic series of cells, was seen in sections. Most of these cells were of moderate size. A few had fine vacuoles, some of which, in frozen sections, absorbed a little scharlach R. In addition to the predominant "large mononuclear cell," polymorphonuclear neutrophils and eosinophils, fibroblasts and reticulum were occasionally noted, the latter in mice allowed to survive for the longer periods.

Apparently, then, the phosphatide of the blastomycete acts on the mouse in much the same manner as does the phosphatide of the tubercle bacillus.

The significant point here is that the large majority of reacting cells were of the monocytic series (Fig. 8) rather than of the polymorphonuclear series. Moreover, a layer of the same thickness caused by the living or killed blastomycetes would show necrosis centrally, as was demonstrated by direct comparisons. Hence the phosphatide is not the chemical fraction responsible for the polymorphonuclear reaction and the necrosis, the two most striking features in connection with the living or killed organisms.

Further studies with chemical fractions have not been pursued in the mouse.

Blastomycetic Polysaccharide in Rabbits

A polysaccharide from *B. dermatitidis* was injected intraperitoneally into rabbits, because repeated samples of blood are easier to obtain in rabbits than in mice, and because a report on the effects of polysaccharides of certain bacteria on rabbits was already available for comparison.[11] Preparation 1 as described by Peck, Martin and Hauser[4] was used.

During the first few hours after the injection of 10 mg. in distilled

water there were produced sterile peritonitis, retrosternal lymphadenitis and the impressive changes in cells of the peripheral blood stream shown in Chart 1. These changes in the white blood cells consisted of (1) almost immediate leukopenia, (2) lymphopenia reaching the lowest point in 5 or 6 hours and (3) a great increase in immature polymorphonuclears (amphophils) which reached the maximum in 5 or 6 hours. The curve of these immature, non-filamented polymorphonuclears in Chart 1 ascends and crosses the curve of the more mature, filamented polymorphonuclears. This is thought to indicate that the bone marrow was stimulated to send these immature forms into the blood stream.

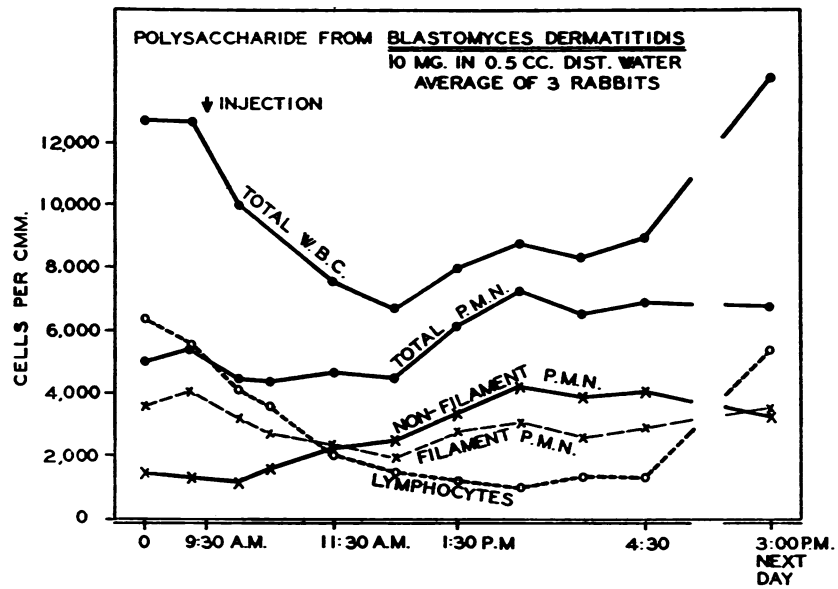

Chart 1. Changes in the number of white cells in the blood streams of three rabbits which received a polysaccharide of *Blastomyces dermatitidis* intraperitoneally.

The transient nature of the changes in the levels of the blood cells is indicated in Chart 1. They tended to return to the starting points on the second day when the rabbits were killed. Each of the individual graphs from which this average graph was constructed showed the same trend in lymphocytes and in non-filamented (immature) amphophils. The curve of the non-filamented forms of each rabbit crossed and went above the curve of the filamented forms.

Control studies in 13 rabbits injected intraperitoneally with distilled water, dextrose, saline solution, barium sulfate and lamp black, indicated that the described reactions in the blood stream were not specific for the blastomycetic polysaccharide, but could be elicited by other substances. However, the degree of change was greater with the blastomycetic polysaccharide.

Comment could be made on the microscopic character of the peritoneal exudates, the retrosternal nodes and the spleens. However, nothing additional to what has already been reported [11] concerning phagocytosis of polymorphonuclear neutrophils and peritoneal reaction was learned, and details are omitted.

It is apparent that these experimental results are very similar to those obtained from the injection of the tuberculo-polysaccharide as reported by Sabin, Joyner and Smithburn.[11] The increase in the immature polymorphonuclear cells and the decrease in the lymphocytes of the blood stream occur as strikingly after the injection of blastomycetic polysaccharide as they do after the injection of the polysaccharides which these authors employed.

Without the employment of numerous control polysaccharides derived from many sources it would be difficult to maintain that the polysaccharides so far employed have any highly specific effect. The control substances have been dissimilar with respect to molecular weight and solubility.

Of greatest general interest is the information, not new, but confirmed, that exceedingly minute quantities of various substances injected intraperitoneally produce not only the mild, acute peritonitis which might be expected but also significant changes elsewhere in the body, as in the retrosternal nodes, blood stream and spleen (and bone marrow also, according to Sabin, Joyner and Smithburn [11]).

Summary and Conclusions

A study was made of the effects on mice of intraperitoneal injections of living *Blastomyces dermatitidis*, killed suspensions of the same organism and a phosphatide fraction of this fungus; also a polysaccharide fraction was studied in rabbits. The following results and conclusions are recorded:

1. The mouse was preëminently suited to the experimental production of blastomycosis. The experimental disease was characterized by the continued response of polymorphonuclear neutrophils to the luxuriant growth of the fungus throughout the infected animal and by the necrosis of the blastomycotic masses. The lesions in mice consisted mainly of organisms, whereas the lesions in most infectious diseases consist mainly of the reacting cells of the hosts.

2. Repeated intraperitoneal injections of heat-killed *B. dermatitidis* were toxic for mice, and often lethal. This is thought to explain the final lethal effect in the experimental disease, in which masses of organisms and intermingled reacting cells became necrotic, and probably permitted the absorption of substances like those associated with the suspensions of the heat-killed organisms.

Heat-killed and living blastomycetes provoked similar cellular responses in the peritoneal cavities of mice.

3. Blastomycetic phosphatide repeatedly injected intraperitoneally into mice caused cells of the monocytic series to respond. This fraction is therefore not responsible for the polymorphonuclear response and the necrotizing effect related to the living organisms.

4. In rabbits, single intraperitoneal injections of blastomycetic polysaccharide produced, in the first few hours, sterile peritonitis, retrosternal lymphadenitis and remarkable changes in the blood stream consisting of leukopenia, lymphopenia and increase in the numbers of immature amphophils. These changes are similar to those which have been produced by polysaccharides from tubercle bacilli and pneumococci, as described by Sabin, Joyner and Smithburn.

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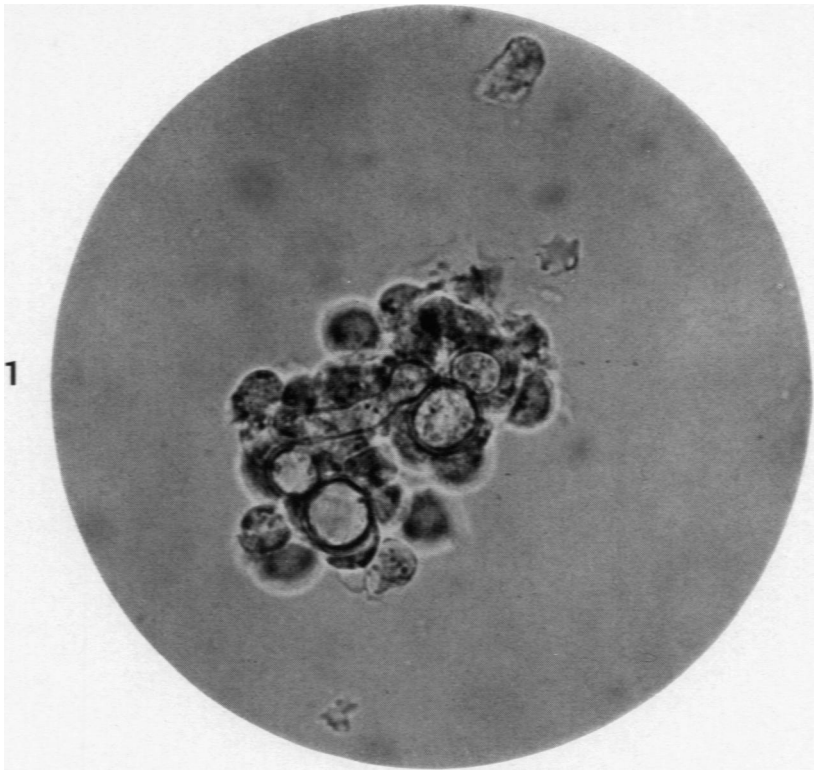
DESCRIPTION OF PLATES

PLATE 76

FIG. 1. Cellular reaction about living blastomycetes several hours after intraperitoneal injection. Peritoneal fluid of mouse. Neutral red, supravital preparation. × 715.

FIG. 2. Similar preparation, 8 days after injection. Two blastomycetes below in mass of reacting cells; fibroblasts above and to the right. × 715.



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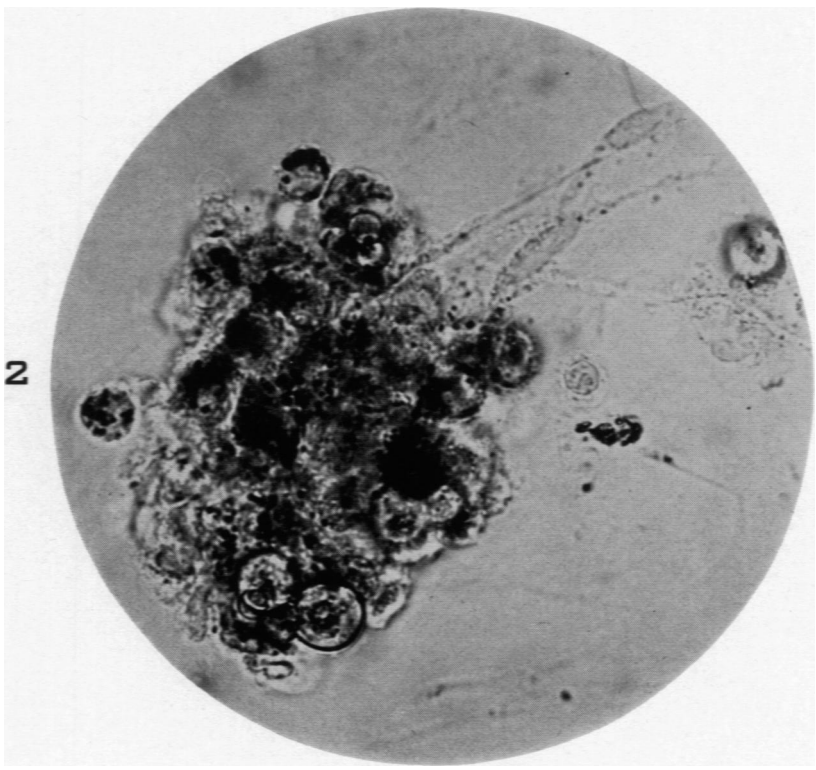

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PLATE 77

FIG. 3. Necrosis in center of peritoneal blastomycotic mass. Experimental blastomycosis in a mouse, 5 days after intraperitoneal injection. × 174.

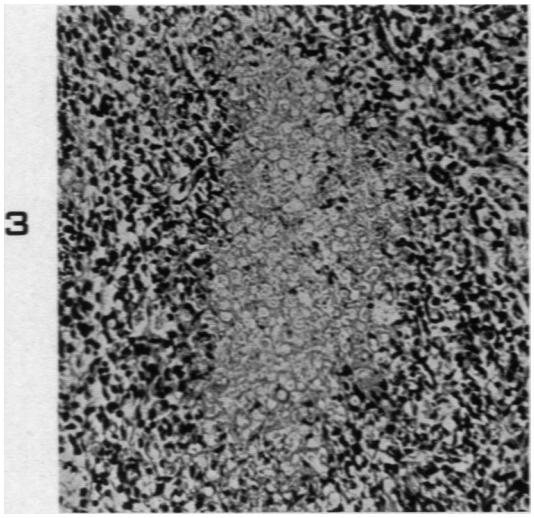
FIG. 4. Focal lesion in peritoneum; single blastomycete surrounded by polymorphonuclear neutrophils. Mouse, 11 days after injection. × 760.

FIG. 5. Large pulmonary nodules in a mouse, 20 days after intraperitoneal injection. The pale, central areas are largely necrotic. × 14.

FIG. 6. Higher magnification of area in the preceding figure. This field is from the periphery of a pulmonary nodule where the blastomycetes are viable, as indicated by the staining of the internal substance of the blastomycete. The interstices between blastomycetes are filled with cells, chiefly polymorphonuclear neutrophils, and nuclear fragments. × 760.

FIG. 7. Cellular response in peritoneum to heat-killed blastomycetes, 5 days after initial intraperitoneal injection. There is a similarity to Figure 6, except for the lack of staining of the central portions of the blastomycetes, indicating that they are dead. × 760.

FIG. 8. Reaction to repeated injections of blastomycetic phosphatide. Cells of the monocytic series on the parietal peritoneum; fibroblasts are also present. This mouse received intraperitoneally 30 mg. of phosphatide in distilled water daily for 24 days, and was sacrificed 3 days after the last injection. × 760.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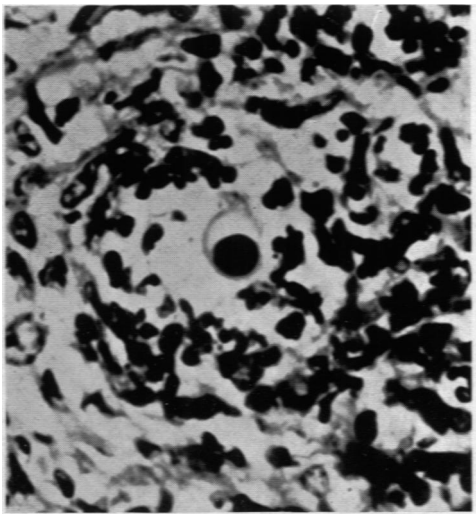
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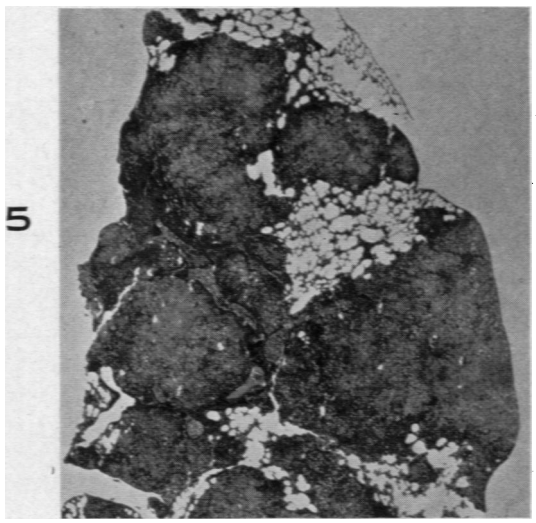
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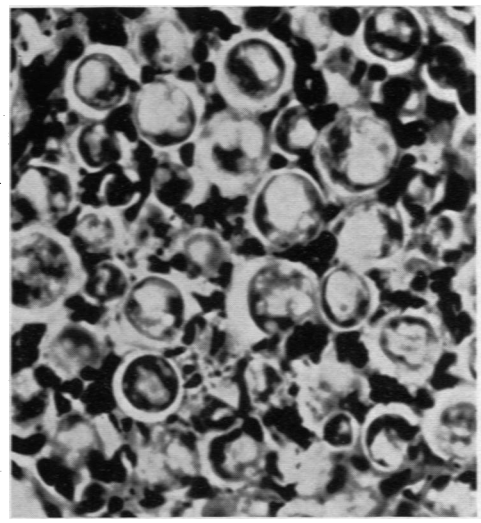
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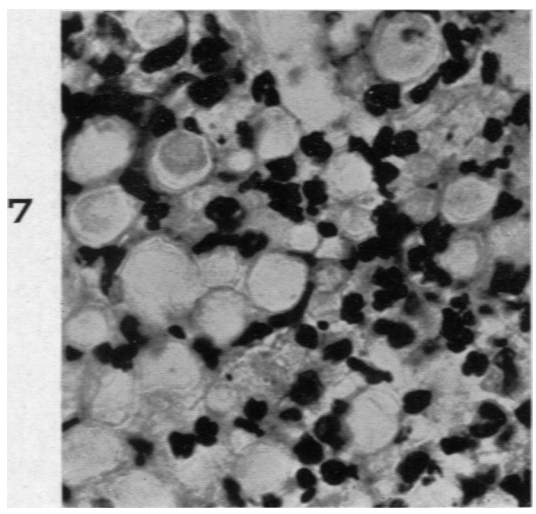
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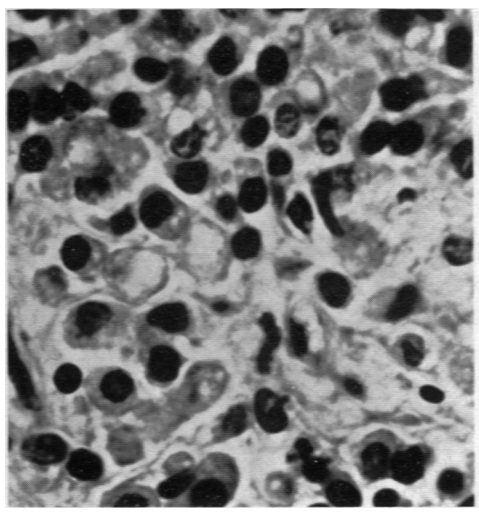
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