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Opposite Effects of Human Monocytes, Macrophages, and Polymorphonuclear Neutrophils on Replication of *Blastomyces dermatitidis* In Vitro

ELMER BRUMMER* AND DAVID A. STEVENS

Division of Infectious Diseases, Department of Medicine, Santa Clara Valley Medical Center, and Institute for Medical Research, San Jose, California 95128, and Stanford University, Stanford, California 94305

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The purpose of this study was to determine the effects of human monocytes, macrophages, and polymorphonuclear neutrophils (PMN) on the fungal pathogen *Blastomyces dermatitidis* in vitro. Peripheral blood monocyte monolayers significantly inhibited the replication of a virulent strain (V) and an avirulent strain (AV) of *B. dermatitidis* by 35 and 38%, respectively. Macrophage monolayers, derived from monocytes by in vitro culturing for 9 days, also inhibited the replication of V and AV in 24-h cocultures; in 72-h cocultures, the inhibition was increased (85 and 88%, respectively). By contrast, PMN stimulated the replication of V and AV in 24-h cocultures (i.e., V, 45%; AV, 18%) and in 72-h cocultures (V, 68%; AV, 65%). No effect was observed in 2-h cocultures of PMN and *B. dermatitidis*, even though *Candida albicans* was killed by PMN in concurrent experiments. PMN stimulated replication of V in a dose-dependent manner, and viability of PMN was not a requirement for the achievement of this effect. These results indicate that monocytes and macrophages significantly inhibited the replication of *B. dermatitidis*, whereas PMN had an opposite effect. Our findings raise the possibility that these phagocytic cells may have similar opposing effects on the replication of *B. dermatitidis* in vivo.

The analysis by Baker (1) of tissue reactions in human blastomycosis indicates that abscess formation was a consistent feature of the 13 cutaneous and 10 generalized or thoracic cases that he studied. Although some lesions in some systemic cases resembled lesions seen in cases of turberculosis, human blastomycosis was interpreted as being primarily pyogenic, with abscesses containing mainly polymorphonuclear neutrophils (PMN) along with giant cells. The prominence of PMN in these lesions could, to some extent, be accounted for by the reported production of chemotaxins for PMN by *B. dermatitidis* (9). Despite their prominence in lesions, PMN do not appear to be particularly effective in eradicating *B. dermatitidis*; however, they have been reported to have some fungicidal activity against *B. dermatitidis* in vitro (9). The role and importance of other phagocytic cell types found in these lesions in the resolution of *B. dermatitidis* infection has not been defined. To investigate this, we studied the interactions of human monocytes and monocyte-derived macrophages with *B. dermatitidis* in vitro. In concurrent experiments, these interactions were compared with those between PMN and *B. dermatitidis*.

(Part of this report was presented at the 1981 Annual Meeting of the American Society for Microbiology.)

MATERIALS AND METHODS

Isolation of PMN and mononuclear leukocytes. Heparinized blood (10 U of heparin per ml) from healthy donors (without histories of blastomycosis) was mixed with 6% Dextran 70 (McGraw Laboratories, Glendale, Calif.) in a ratio of 5:1 and sedimented at 37°C for 45 min. The leukocyte-enriched plasma was removed and diluted 1:1 with minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.); 8 ml was then layered over 3 ml of Ficoll-Hypaque (d = 1.09) in 15-ml plastic conical centrifuge tubes (no. 2905; Falcon Plastics, Oxnard, Calif.). Density gradient centrifugation was done at 400 × *g* for 40 min. Mononuclear leukocytes were harvested from the interface layer, washed twice in minimal essential medium, counted, and suspended to 10 × 10^6 cells per ml of complete tissue culture medium (CTCM). CTCM consisted of RPMI 1640 (GIBCO), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fresh autologous serum. The pellet consisting primarily of PMN was resuspended in minimal essential medium, washed twice, counted, and resuspended to 5 × 10^6 cells per ml of CTCM. PMN were 98% viable, as assessed by the trypan blue dye exclusion test, and 99% of the Giemsa-stained cytocentrifuge preparations consisted of PMN.

Selection of monocytes. Mononuclear leukocytes (10 × 10^6/ml) were incubated (0.2 ml per well) in flat-bottom microtest plate wells (no. 3040; Falcon Plas-

tics) for 2 h at 37°C in 5% CO_2–air. Nonadherent cells were removed by aspiration, and each well was washed with 0.2 ml of RPMI 1640 medium. The number of adherent cells per well was calculated by subtracting the mean number of nonadherent cells per well from the original number of cells per well. In four experiments, the mean number of adherent cells per well was $0.94 \times 10^6 \pm$ a standard deviation (SD) of 0.13×10^6. When mononuclear leukocytes were allowed to adhere to glass cover slips under the same conditions, 51% of the cells adhered, and 90% of the adherent cells were esterase positive (4).

Monocyte-derived macrophages. Monocytes selected as described above were cultured in CTCM. Every 3 days, the medium was removed and fresh medium was added. Most of these cells underwent pronounced morphological changes which correlated with time in culture, comparable to those reported by Musson et al. (8) and discussed below.

B. dermatitidis. Three strains of *B. dermatitidis*, ATCC 26199 (virulent [V]), an attenuated mutant of ATCC 26199 (A), and ATCC 26197 (GA-1; avirulent [AV]) (5), were used in these studies. The virulence designations were derived from studies of murine pulmonary blastomycosis (5). Stock cultures were grown at 37°C on brain heart infusion agar slants and stored at 4°C. Liquid yeast phase cultures were prepared by seeding 3 ml of defined medium (5) with growth from brain heart infusion agar slants. Log-phase growth was harvested from 48- to 72-h liquid cultures grown on a rotary shaker (200 rpm) at 37°C. Yeast cells were washed twice in saline, resuspended in CTCM, and counted with a hemacytometer, and appropriate dilutions were plated in triplicate on blood agar plates to determine the number of colony-forming units (CFU) per milliliter.

Challenge of PMN with *B. dermatitidis*. In most experiments, PMN (0.1 ml of culture containing 5×10^6 cells per ml of CTCM) were dispensed into round-bottom microtest plate wells (Linbro, Flow Laboratories, Hamden, Conn.), 0.1 ml of *B. dermatitidis* (5,000 CFU/ml of CTCM) was added, and the plate was centrifuged at $25 \times g$ for 5 min. In other experiments, the number of PMN per well or the number of CFU of *B. dermatitidis* was varied. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2–air. In addition plastic tubes (12 by 75 mm) (no. 2054; Falcon Plastics) containing the same proportions of cells and fungi were incubated at 37°C on a shaker at 200 rpm or on a rotating (8 rpm) rack.

Challenge of PMN with *Candida albicans*. The candidacidal assay of Lehrer (6) was used to test the fungicidal activity of PMN. Giemsa-stained cytocentrifuge preparations of cocultured PMN and *C. albicans* (1:1) were used to determine the percentage of phagocytized *C. albicans* that were killed. Concurrent cocultures of PMN were challenged with V (1:1), and the reduction of the number of CFU was assessed as described above.

Challenge of monocytes or macrophages with *B. dermatitidis*. After aspiration of the medium from adherent cell cultures, the cells were challenged with 0.2 ml of *B. dermatitidis* (2,500 CFU/ml of CTCM). Cocultures were incubated at 37°C in a humidified incubator with a 5% CO_2–air atmosphere for different periods of time, e.g., 2, 24, or 72 h, before determination of the number CFU per well.

Measurement of the number of CFU per microtest plate well. The number CFU of *B. dermatitidis* in cocultures was measured as described previously (2). Briefly, well contents were removed with a Pasteur pipette and placed into 15 ml of distilled water, and each well was washed five times with 0.2 ml of distilled water. The culture contents and washings were diluted to 20 ml. After we vigorously mixed the diluted fluid for 20 s, we distributed 1 ml on a blood agar plate. After 4 days at 37°C, the number of CFU per plate was recorded, and the number of CFU per well was calculated. The percent reduction of CFU, or inhibiton of replication, was calculated by the following formula: [1 − CFU of the experimental culture/CFU of the control culture (medium)] × 100. Negative values of percent reduction of CFU, therefore, represent stimulation or enhancement of replication.

Hemacytometer counts of MCU and total number of cells per culture. The 19 ml of diluted well contents and washings remaining after removal of the portion for CFU enumeration was centrifuged (10 min, $900 \times g$), and 17 ml of the supernatant was carefully removed from each tube. The pellets from triplicate cultures were then pooled in a 15-ml conical centrifuge tube. After centrifugation as described above, all but 0.1 ml of supernatant was removed, and the multicellular units (MCU) (an aggregate of yeast cells) of *B. dermatitidis* resuspended in this volume were counted with a hemacytometer. When MCU were counted, the number of cells per MCU was also tabulated, so that the average number of cells per MCU and the total number of cells per well could be calculated.

Statistical analyses. Analyses were performed by using the Student *t* test.

RESULTS

Effect of monocytes on *B. dermatitidis* in vitro. Peripheral blood mononuclear cells which adhered to plastic (90% monocytes) were cocultured with V and AV. At 2 h, no significant differences were noted between cocultures and controls (11 to 13% enchancement of replication in cocultures). After 24 h, the number of CFU per well in cocultures was found to be significantly ($P < 0.05$) less than that of controls (V or AV alone). The replication of both V (35%) and AV (38%) was inhibited in cocultures (Fig. 1). When the culture time was extended to 72 h, inhibition of replication was maintained. Although AV (63%) was inhibited more than V (45%), this difference was not significant in these experiments (Fig. 1). In other experiments, monocytes from another donor inhibited the replication of V (26 to 39%) and AV (33%) in 24-h cocultures (Table 1). These results show that peripheral blood monocytes could restrict the replication of *B. dermatitidis*.

Analysis of monocyte inhibition of *B. dermatitidis* replication. Microscopic examination of cocultures showed that V was buried in clumps of monocytes. Inhibition of replication, as measured by the reduction of the number of CFU, was not due to differences between clumping

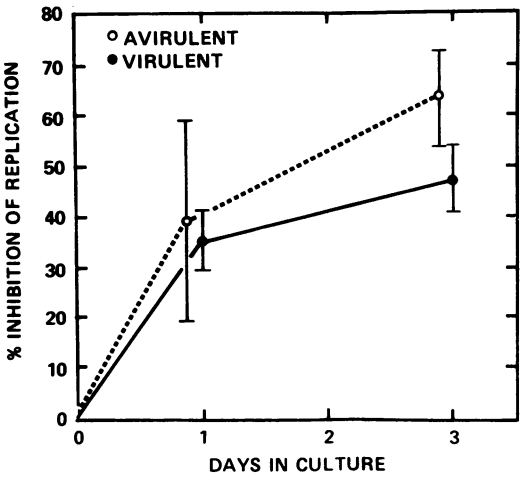

FIG. 1. Inhibition of *B. dermatitidis* replication by monocytes. The mean percent inhibition of replication ± SD (three experiments) of AV and V by monocytes from donor 1 is given. Mean numbers of CFU ± SD for three experiments at zero hour are as follows: V, 424 ± 201; AV, 339 ± 59. The mean numbers of CFU of the control cultures and those of the experimental cultures are as follows (for V and AV, respectively). At 24 h: 2,682 ± 482 and 1,730 ± 165; 1,975 ± 1,007 and 1,170 ± 708. At 72 h: 19,190 ± 4,289 and 10,453 ± 1,388; 38,403 ± 9,564 and 13,149 ± 2,522.

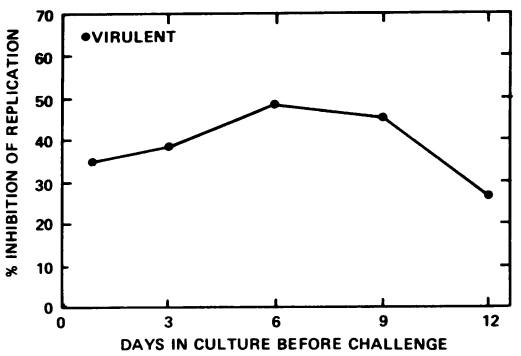

FIG. 2. Effect of time in culture on monocyte activity. The percent inhibition of replication of V in 24-h cocultures by monocytes cultured for 1, 3, 6, 9, or 12 days before challenge is shown.

(number of cells per CFU) in control cultures and clumping in cocultures. Counts performed with a hemacytometer showed no difference between control and experimental cultures in the mean number of cells per MCU. Furthermore, reduction in the number of CFU per well correlated with reduction in the total number of cells per well. When V was cultured in the supernatant from a 24-h culture of monocytes or V or a monocyte-V coculture, it replicated as well during a 24-h study period in all three supernatants as it did in fresh medium (data not shown). Therefore, no evidence was obtained which indicates that monocytes elaborated soluble factors that would inhibit replication of V or that monocytes and V depleted the culture medium of nutrients necessary for the replication of V.

Effect of time in culture on monocyte activity. Peripheral blood monocytes can be cultured in vitro for extended periods of time; however, they undergo dramatic changes (8). These changes are not only morphological (fivefold increase in size) but also functional, e.g., increased tumoricidal (10) and cytotoxic (7) activities. We tested the ability of monocytes to inhibit the replication of V after the monocytes had been in culture for 1, 3, 6, 9, or 12 days. Triplicate control cultures and cocultures were studied at these times, and the 24-h percent inhibition of replication of V was determined. The results from these experiments are given in Fig. 2. The ability of cultured monocytes to inhibit replication of added V increased with time in culture from 35% (1 day) to 48% (6 days) and then declined to 25% after 12 days (Fig. 2). Monocyte morphology changes which increased with time in culture were those described by Musson et al. (8). Briefly, by day 7, cultured

TABLE 1. Inhibition of *B. dermatitidis* replication by monocytes in vitro

Expt[a]	Strain	No. of CFU/well[b]	Inhibition (%)	*P* value
1	V	4,833 ± 234		
	Monocytes + V	2,993 ± 966	39	<0.05
2	V	4,226 ± 280		
	Monocytes + V	3,140 ± 380	26	<0.02
3	AV	3,600 ± 470		
	Monocytes + AV	2,420 ± 121	33	<0.02

[a] Triplicate determinations of the challenge dose per well were made at zero hour. Experiment 1, 433 ± 100 CFU; experiment 2, 646 ± 40 CFU; experiment 3, 386 ± 100 CFU.

[b] Results are expressed as means ± SDs for triplicate cultures after 24 h.

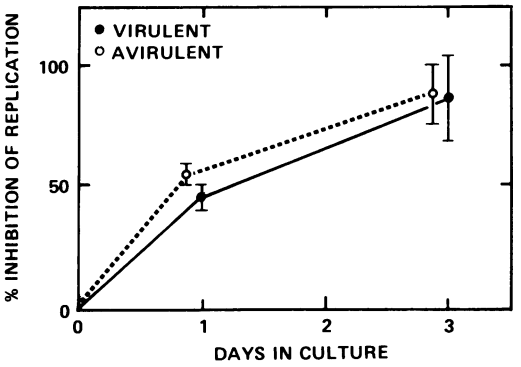

FIG. 3. Inhibition of *B. dermatitidis* replication by monocyte-derived macrophages. The mean percent inhibition ± SD (two experiments) of AV and V by macrophage monolayers is shown. Macrophages were derived from monocytes by culturing for 9 days. Mean numbers of CFU ± SD for two experiments at zero hour are as follows: V, 730 ± 240; AV, 646 ± 122; the mean numers of CFU of the control cultures and those of the experimental cultures are as follows (for A and AV, respectively). At 24 h: 4,436 ± 1,211 and 2,479 ± 603; 4,290 ± 1,400 and 1,889 ± 504. At 72 h: 24,166 ± 5,067 and 4,506 ± 2097; 35,783 ± 10,206 and 4,199 ± 1,649.

cells were fivefold larger than monocytes and were more spread in their attachment. These results show that monocytes which have undergone differentiation and maturation in vitro (monocyte-derived macrophages) are effective in inhibiting the replication of V in cocultures.

Effect of monocyte-derived macrophages on *B. dermatitidis* in vitro. Macrophage monolayers, derived from monocytes by in vitro culturing for 9 days, were challenged with V or AV. After 24 h, the number of CFU per well in cocultures was found to be significantly less ($P < 0.01$) than that of the controls. Replication of V and AV was inhibited 48 and 55%, respectively (Fig. 3). When the coculture time was extended to 72 h, the inhibition of replication of both V and AV increased 85 and 88%, respectively (Fig. 3). These results indicate that macrophage-like cells are especially effective in restricting the replication of *B. dermatitidis*, particularly in 72-h cocultures.

Effect of PMN on *B. dermatitidis* in vitro. When PMN were cocultured with V, A, or AV for 2 h, no significant increase or decrease in the number of CFU per well, compared with that of control cultures (*B. dermatitidis* alone), was recorded (Fig. 4). However, when the coculture time was extended to 24 h, a significant ($P < 0.02$) enhancement of replication of V (45%) and A (25%) but not AV (18%) was measured (Fig. 4). Enhancement of V replication was increased over that of A or AV ($P > 0.05$). Enhancement of *B. dermatitidis* replication continued to increase with time in culture. At 72 h, replication of all three strains was enhanced further, as follows: V, 68%; A, 62%; and AV, 65% (Fig. 4).

In a second set of experiments, replication of V and AV was also enhanced in 24-h cocultures with PMN from another donor (Fig. 5). A significant ($P < 0.01$) enhancement of V (48%) but not AV (15%) replication was recorded (Fig. 5). In this set of experiments, the difference was maintained even in 72-h cocultures (Fig. 5). When PMN and V were incubated for 24 h in flat-bottom wells or in tubes that were rotated or shaken, the enhancing effect of PMN on replication of V was also seen. A 2-h incubation of PMN with *C. albicans* or V in rotated tubes resulted in 30% of the ingested *C. albicans* being killed but, again, no reduction in the number of CFU of V (8% enhancement).

Relationship between increased number of CFU and hemacytometer counts of MCU. We thought it possible that the increased number of *B. dermatitidis* CFU in the presence of PMN could have been due to the number of MCU generated with fewer cells per unit being greater than that seen in control cultures. This possibility was tested by counting the number of MCU per well with a hemacytometer as well as the number of cells per MCU in experimental and control cultures. The number of CFU per well was determined concurrently.

The results from this experiment are shown in Table 2. Although the numbers of CFU per well in control (2,520 ± 120) and experimental (5,060

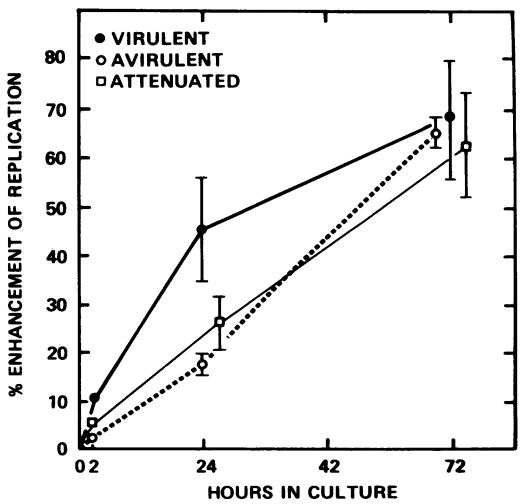

FIG. 4. Enhancement of *B. dermatitidis* replication by PMN. The mean percent enhancement of replication ± SD (two experiments) of V, A, and AV by PMN from donor 1 is shown.

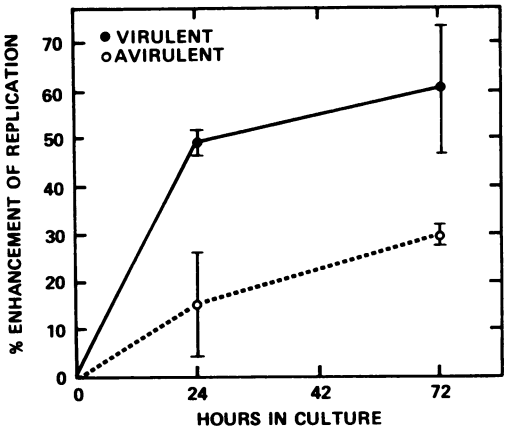

FIG. 5. Enhancement of *B. dermatitidis* replication by PMN. The mean percent enhancement of replication ± SD (two experiments) of V and AV by PMN from donor 2 is shown.

± 300) cultures were slightly less than the number of visually determined MCU per well in control (3,007) and experimental (5,700) cultures, the percent increase of the numbers CFU and MCU per well were in close agreement: 50 and 48%, respectively. Furthermore, the average numbers of cells per MCU in control and experimental cultures were similar: 4.26 and 4.16, respectively. These results indicate that the increase in the number of CFU per well in experimental cultures represented enhancement of replication and was not due to more MCU with fewer cells per unit.

Effect of PMN concentration on enhancement of *B. dermatitidis* replication. When various numbers of PMN were cocultured with V, A, or AV for 24 h, the percent enhancement of replication depended on the number of PMN present (Fig. 6). Lower concentrations of PMN, e.g., 5×10^3 or 5×10^4, did not significantly enhance replication of V, but a concentration of 5×10^5 did ($P < 0.01$) (Fig. 6). Similar results were obtained in another dose-response experiment, in which 0.5 $\times 10^5$, 1×10^5, 5×10^5, and 10×10^5 PMN per well enhanced the replication of V by 20, 30, 48, and 43%, respectively. In a second experiment, V was cocultured for 24 h with various numbers of viable PMN or PMN killed by undergoing two cycles of freezing and thawing. The results of this experiment are given in Fig. 7. Either viable or killed PMN enhanced the replication of V to the same extent in this system (Fig. 7). In another experiment, PMN lysates enhanced replication of V by 40%, compared with a 43% enhancement effected by intact PMN (5×10^5 PMN per well). These results indicate that in cocultures, PMN stimulated replication of *B. dermatitidis* in a dose-dependent manner and that the viability of PMN was not a requirement for the achievement of this effect.

DISCUSSION

We have reported here that human monocytes, especially monocyte-derived macrophages, significantly restricted the replication of *B. dermatitidis* in vitro. This inhibition of replication was similar to that reported for activated murine macrophages (2). Our findings suggest that these types of phagocytic cells play a similar role in vivo and thereby constitute an important factor in resistance during the early stages of *B. dermatitidis* infection or its early stages of dissemination to new sites. On the other hand, our in vitro findings did not determine the in vivo role that monocytes or macrophages have in the killing and eradication of *B. dermatitidis*. Other elements of the immune system, e.g., specific antibody, lymphokines, and complement, may be necessary adjuncts in killing in vivo or in demonstrating killing in vitro.

Microscopic examination of cocultures showed that MCU of *B. dermatitidis* were completely embedded in or covered with monocytes or macrophages. This accumulation of phagocytic cells over MCU of *B. dermatitidis* suggested that the cells migrated to the MCU. Experiments showed that the release of soluble inhibitory factors into or the depletion of nutri-

TABLE 2. Comparison of numbers of CFU, MFU, and *B. dermatitidis* cells per culture

Strain	No. of:			
	CFU/well[a]	MCU/well[b]	Cells/MCU	Cells/well[c]
V[d]	2,520 ± 120	3,007	4.26	12,809
PMN[e] + V	5,060 ± 300	5,700	4.16	23,712

[a] Results are expressed as means ± SDs for triplicate cultures after 24 h. Enhancement of replication, 50%.
[b] Enhancement of replication, 48%.
[c] Enhancement of replication, 46%.
[d] Mean of triplicate determinations of the challenge dose per well at zero hour, 480 ± 120 CFU.
[e] 10^6 PMN per well.

ents from the culture medium by monocytes in cocultures did not account for the inhibition of replication observed. Although the "walling off" of *B. dermatitidis* by monocytes or macrophages correlated with the inhibition of replication, the exact mechanism whereby inhibition of replication occurred remains to be determined.

Early histological observations suggest that PMN are ineffective in inhibiting *B. dermatitidis* in vivo and that "the bulk of the work is performed by macrophages" (3). Although others have reported the killing of *B. dermatitidis* by PMN in short-term cultures (9), we were not able to confirm this in our assay system. Clumping of fungal cells during assay for survivors in an earlier study (9), a possibility not examined in that study, could account for the decline in cells, which was interpreted as killing. We found that, in contrast, PMN, unlike monocytes and macrophages, significantly enhanced the replication of *B. dermatitidis* in 24-h cocultures. This novel in vitro finding raises the possibility that PMN may have a similar effect in vivo and instead of helping to control *B. dermatitidis* infection may actually exacerbate it. Even killed PMN enhanced the replication of *B. dermatitidis* in vitro, and this suggested that PMN contain a substance(s) that stimulates replication of the pathogen. The observation that dead PMN are commonly observed during microscopic examination of purulent blastomycotic lesions in vivo (1) is noteworthy. Identification of this substance(s) from PMN and the mechanism whereby it stimulates replication of *B. dermatitidis* await further investigation.

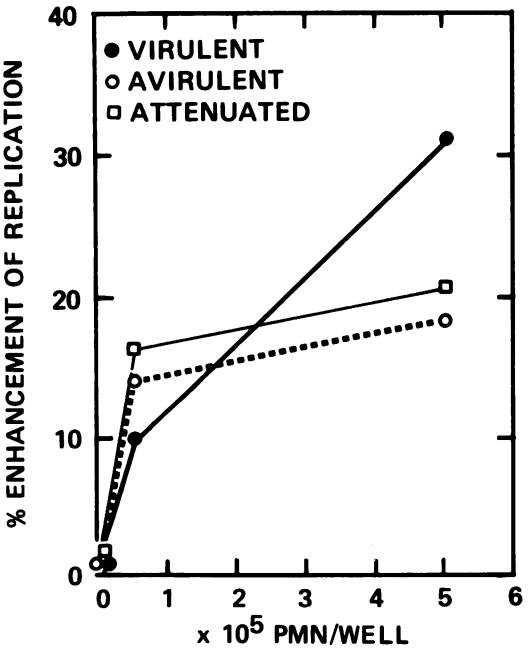

FIG. 6. Effect of PMN concentration on enhancement of *B. dermatitidis* replication. The mean percent enhancement of replication of V, AV, and A in 24-h cocultures by 0.05×10^5, 0.5×10^5, and 5×10^5 PMN is shown.

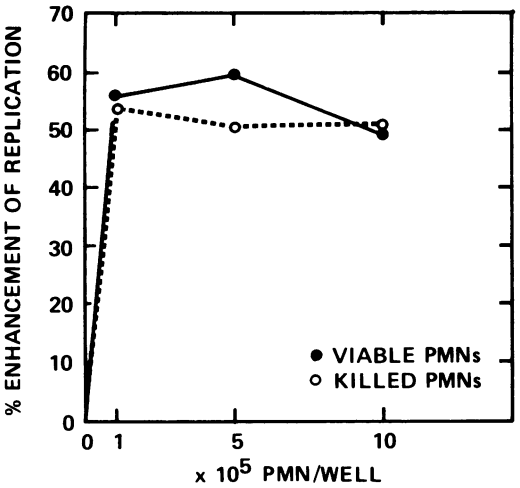

FIG. 7. Effect of killed PMN on enhancement of *B. dermatitidis* replication. The percent enhancement of replication of V in 24-h cocultures by viable and killed PMN is depicted.

Even though the virulence of V, A, and AV was defined for mice, human PMN significantly enhanced the replication of V more than that of A or AV in vitro. However, since the virulence of V for humans is not known, the significance of these in vitro findings in terms of virulence for humans is difficult to evaluate. Of interest is the observation that murine spleen cells enriched for PMN by centrifugation on Lympholyte-M (Cedarlane Laboratories, Accurate Chemicals, Westbury, N.Y.) also enhanced the replication of V more than that of A or AV in cocultures (unpublished data).

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