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Enhanced Oxidative Burst in Immunologically Activated but Not Elicited Polymorphonuclear Leukocytes Correlates with Fungicidal Activity

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Polymorphonuclear neutrophils (PMN) induced locally in immune mice by intraperitoneal injection of antigen exhibit enhanced fungicidal activity compared with PMN elicited with thioglycolate. The mechanism of the differences in these PMN populations was studied. Sublethal infection was used to produce immunity to *Blastomyces dermatitidis*. A correlation was sought between the ability of PMN to kill, or not kill, *B. dermatitidis* and the production of the oxidative burst, as measured by luminol-enhanced chemiluminescence (CL). Although elicited PMN cocultured with *Candida albicans* produced a burst of CL and were candidacidal, killing did not occur when PMN were cocultured with *B. dermatitidis*. Lack of killing of *B. dermatitidis* by elicited PMN correlated with lack of stimulation of a brisk oxidative burst. In contrast to elicited PMN, PMN induced by *B. dermatitidis* antigen responded to this fungus with a burst of CL and a significant reduction of inoculum CFU (80%). Furthermore, these PMN when cocultured with *C. albicans* produced an enhanced burst of CL, and killing was enhanced compared with that by elicited PMN, e.g., 86 versus 58%. The CL burst and killing of *B. dermatitidis* by antigen-induced PMN was abrogated in the presence of catalase, implying a critical role for hydrogen peroxide. Partial but significant depression of CL and killing in the presence of dimethyl sulfoxide, a hydroxyl radical scavenger, identified hydroxyl radical, or its metabolites, as a toxic product(s) responsible for a significant fraction of fungicidal activity. These results indicate that the metabolic activity and microbicidal activity of PMN can be altered (enhanced) at the site of an immunological reaction and thus could constitute an important factor in resistance.

Evidence has accumulated that indicates that polymorphonuclear neutrophils (PMN) are functionally a heterogenous population (24, 25). Furthermore, the metabolic activity of PMN has been reported to be enhanced during gram-negative bacterial infections (12, 18), elicitation in immune mice with antigen (5), or treatment in vitro with supernatants from either mitogen (4, 7, 11) or antigen-stimulated lymphocyte cultures (4). Enhancement of PMN metabolic activity associated with gram-negative bacterial infections was measured by increased reduction of nitro-blue tetrazolium (12, 18) or by chemiluminescence (CL) (2) upon interaction with opsonized zymosan. The ability of PMN to generate an oxidative burst upon interaction with microbes has been shown to correlate generally with microbicidal activity (6, 8, 13). However, this correlation is not absolute (10), as the lack of correlation may be due to either mechanisms possessed by some microorganisms for neutralizing toxic products of the oxidative burst, e.g., catalase or superoxide dismutase (9, 16), or nonoxidative microbicidal mechanisms possessed by PMN (14).

Recently, it was reported that PMN induced locally in *Blastomyces dermatitidis*-immune mice by intraperitoneal (i.p.) injection of killed *B. dermatitidis* had enhanced candidacidal activity and also could kill *B. dermatitidis* (5), although *B. dermatitidis* yeasts are too large to be ingested by individual PMN. In contrast, thioglycolate-elicited PMN from immune or nonimmune mice, or PMN obtained from nonimmune mice by injection of killed *B. dermatitidis*, failed to kill *B. dermatitidis* but killed *Candida albicans*.

The purpose of the present study was to identify differences between elicited and antigen-induced (activated) PMN that would correlate with their ability to kill, or not kill, *B. dermatitidis*. We measured the ability of PMN to generate an oxidative burst while interacting with *B. dermatitidis* in vitro.

MATERIALS AND METHODS

Animals. Sendai virus-free BALB/cByJIMR (Institute for Medical Research, San Jose, Calif.) male mice (8 to 12 weeks of age) were used throughout these experiments. Mice were bled as previously described (5). Sera from uninfected mice are referred to as normal mouse sera. Mice become resistant to *B. dermatitidis* challenge 4 weeks after resolution of subcutaneous infection (40,000 CFU of yeast form *B. dermatitidis* divided in two dorsal sites) (17). Sera from such mice contain specific antibody (17) and are referred to as immune mouse sera.

Fungi. *B. dermatitidis* (ATCC 26199) was grown in a defined liquid culture medium at 35°C (5). Blood agar plates (BAP) were inoculated with log-phase liquid culture growth. After 3 days at 35°C, growth on BAP was used as the inoculum. Units of *B. dermatitidis* were counted with a hemacytometer, and CFU were determined by plating 1 ml of an appropriate dilution in quadruplicate on BAP. Yeast form *B. dermatitidis* was obtained from cultures rendered nonviable by prolonged storage (more than 2 years) at −70°C. Washed, packed cells were quantitated on a wet-weight basis, and 1 mg/ml was equivalent to 2×10^7 cellular units per ml. *C. albicans* (Sh 27 strain) was grown for 3 to 4

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days in yeast nitrogen base broth (Difco Laboratories, Detroit, Mich.) at 32°C from stock cultures maintained on Sabouraud agar under water at 4°C. These preparations yielded only yeast forms, without hyphae or pseudohyphae. *C. albicans* was washed twice in saline and counted in a hemacytometer, and CFU were determined by plating on BAP. Washed *C. albicans* was killed by heating for 1 h at 60°C.

Media and reagents. Dulbecco phosphate-buffered saline (PBS), minimal essential medium, RPMI 1640 medium, penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and heat-inactivated fetal bovine serum were purchased from GIBCO Laboratories, Grand Island, N.Y. Complete tissue culture medium consisted of RPMI 1640 medium, 10% (vol/vol) fetal bovine serum, and 100 U of penicillin and 100 μg of streptomycin per ml. Metrizamide [2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose (Sigma Chemical Co., St. Louis, Mo.) was made as a 35.5% solution in distilled water, which was then isotonic. Required concentrations of metrizamide for gradients were made by diluting the stock solution in PBS. Luminol (Sigma) was dissolved in dimethyl sulfoxide (DMSO; Eastman Kodak Co., Rochester, N.Y.) to form a stock solution of 10 mg/ml and was diluted in PBS to obtain a working solution of 40 μg/ml.

PMN. Peritoneal exudate cells (PEC) enriched for PMN were induced in normal or immune mice with 1 ml of thioglycolate broth (Clinical Standards Laboratories, Carson, Calif.) i.p. 4 h before harvest. It has been shown in mice that thioglycolate-elicited PMN have biochemical and microbicidal properties similar to those of peripheral blood PMN (3). Elicited PMN were further purified by centrifugation of PEC on metrizamide gradients (14.5% over 15.5%) at 400 × *g* for 20 min as previously described (5). In selected experiments, cytocentrifuge preparations of gradient pelleted cells were stained with Wright's stain, 200 cells per slide were examined, and the numbers of PMN, lymphocytes, and monocytes-macrophages were recorded. In these studies, elicited PMN-enriched pellet-1 cells consisted of 73% PMN, 22% lymphocytes, and 5% monocytes-macrophages.

PMN obtained by centrifugation in similar fashion from PEC (55% PMN, 16% monocytes-macrophages) induced in *B. dermatitidis*-immune mice by injection of 0.5 mg of nonviable *B. dermatitidis* i.p. 24 h earlier were termed activated PMN (5). The activated PMN-enriched layer (pellet-1 cells) consisted primarily of PMN (63%), lymphocytes (28%), and monocytes-macrophages (9%). It was shown previously that treatment of this population of cells with rat anti-murine granulocyte-specific monoclonal antibody (a kind gift from Robert Coffman, DNAX, Palo Alto, Calif.) plus Low-Tox M rabbit complement (Accurate Chemical Co., Westbury, N.Y.) completely abrogated (70 to 0%) killing of *B. dermatitidis* (5). We also demonstrated that PEC from immune mice treated in this way reduced killing of *C. albicans* from 83 to 19%. This reduced *C. albicans* killing by the remaining cells was similar to the 15% killing by untreated peritoneal cells (<2% PMN) obtained from normal mice with heparinized minimal essential medium, further suggesting that monocyte-macrophage function is resistant to the monoclonal antibody.

Cocultures of PMN and fungi. PMN (0.1 ml of 5 × 10^6/ml of complete tissue culture medium) were dispensed into MicroTest plate wells (flat-bottom) (Linbro; Flow Laboratories, Hamden, Conn.), and then *C. albicans* (0.1 ml of 10,000 CFU/ml of complete tissue culture medium) or *B. dermatitidis* (0.1 ml of 5,000 CFU/ml of complete tissue culture medium) was added. After fresh mouse serum (0.02 ml) was added to each coculture, they were incubated at 37°C for 2 h in 5% CO_2–95% air. Cultures were harvested with distilled water as previously described (5) and plated on BAP. CFU per culture were determined by counting CFU per plate after 2 days (*C. albicans*) or 4 to 5 days (*B. dermatitidis*) of incubation at 35°C. Percent reduction of inoculum CFU was calculated by: [1 − (coculture CFU/inoculum CFU)] × 100.

CL. To detect the products of oxidative metabolism subsequent to challenge of PMN with fungal particles in fresh serum, the luminol method of Allen and Loose was used (1). Briefly, 0.1 ml of PMN (10 × 10^6/ml of PBS), 0.1 ml of luminol solution (10 to 80 μg/ml), 0.05 ml of fresh mouse serum, 0.15 ml of PBS, and 0.1 ml of fungi (10 × 10^6/ml of PBS) were combined and kept at room temperature. When catalase (Sigma), prepared as described previously (5), or DMSO was added (0.15 ml) to the mixtures, PBS was omitted. Photon emission was measured in a scintillation counter (Mark II; Nuclear-Chicago Corp., Des Plaines, Ill.) at room temperature with the windows set on "manual" and levels at L-∞. The manual setting permitted rapid counting of samples, and the counts per minute were calculated by using the counting time (≤0.2 min) registered by the scintillation counter.

RESULTS

Determination of conditions satisfactory for measuring CL. Since many factors influence the magnitude of luminol-enhanced measurement of PMN CL (8, 19), preliminary experiments were done to determine acceptable conditions. The number of PMN (10^6), amount of fresh serum (10%), temperature (20 to 22°C), volume of luminol (0.1 ml), and volume of the reaction mixture (0.5 ml) were held constant, but the concentrations of luminol and microorganisms were varied (Fig. 1). The magnitude of CL measured was dependent upon the concentration of luminol. For example, PMN interacting with *C. albicans* in 16 μg of luminol per ml gave $10^{4.4}$ cpm compared with $10^{3.3}$ cpm in the presence of 2 μg of luminol per ml. Similarly, PMN interacting with *B. dermatitidis* (10^5) also had a 10-fold increase in counts per minute when the luminol concentration was increased eightfold. When the luminol concentration was held constant (2 μg/ml), the magnitude of CL in the PMN mixture depended upon the concentration of *B. dermatitidis* added, e.g., 10^6 elicited a 10-fold greater response than did 10^5, $10^{2.3}$ versus $10^{1.2}$ cpm. These results suggested that a mixture of 0.1 ml of 10 × 10^6 PMN per ml of PBS, 0.1 ml of 80 μg of luminol per ml, 0.1 ml of fungal particles (2 × 10^6 to 10 × 10^6/ml of PBS), 0.05 ml of fresh mouse serum, and 0.15 ml of PBS would be satisfactory for obtaining responses in the range of 10^3 to 10^5 cpm, thus minimizing base-line counts per minute.

Activated versus elicited PMN CL. When PMN were mixed with killed *C. albicans* in the presence of fresh serum, activated PMN produced a higher and more prolonged level of CL than did elicited PMN, e.g., $10^{5.1}$ versus $10^{4.7}$ cpm at 15 min and $10^{4.4}$ versus 10^3 cpm at 120 min (Fig. 2). The CL profile of activated PMN plus *C. albicans* was depressed when heat-inactivated fetal bovine serum was substituted for fresh serum. The initial burst of CL was severely depressed ($10^{5.1}$ versus $10^{3.6}$ cpm), even below that of elicited PMN in fresh serum, and peak CL was delayed until after 90 min of incubation. These results indicate that activated PMN produced an enhanced oxidative burst compared with that

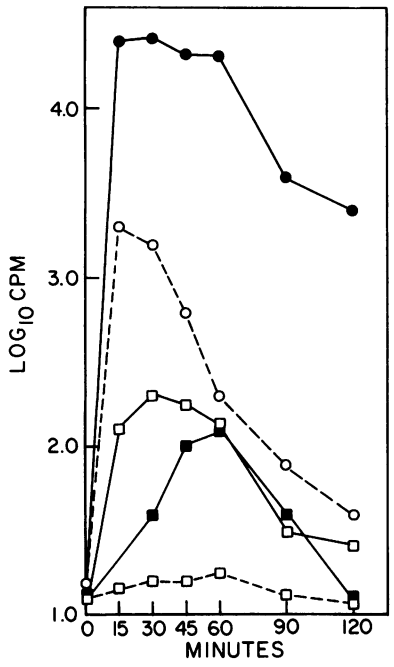

FIG. 1. Effect of concentrations of luminol or fungal particles on CL produced by interaction with activated PMN. Given are counts per minute from *C. albicans* (2×10^5) and PMN (10^6) with luminol at 16 μg/ml (●) or 2 μg/ml (○). Also shown are counts per minute from PMN and *B. dermatitidis* (10^6) with 2 μg of luminol per ml (□—□), *B. dermatitidis* (10^5) with 2 μg of luminol per ml (□---□), and *B. dermatitidis* (10^5) with 16 μg of luminol per ml (■). Total culture volume, 0.5 ml.

produced by elicited PMN and suggest that fresh serum is required for optimal CL.

CL generated by activated versus elicited PMN plus *B. dermatitidis*. Since we previously reported that activated PMN, but not elicited PMN, killed *B. dermatitidis* (5), we tested the ability of activated versus elicited PMN to produce CL upon interaction with *B. dermatitidis*. We found that activated PMN produced a 10-fold greater CL burst than did elicited PMN after 15 min of incubation (Fig. 3). Furthermore, enhanced CL was measured up to 60 min of incubation (Fig. 3). PMN elicited by thioglycolate in immune mice did not differ in their production of CL (data not shown) or fungicidal activity (5) from PMN elicited with thioglycolate in nonimmune mice. These results clearly demonstrate a correlation between the production of CL and the ability of activated PMN to kill *B. dermatitidis*.

The timing of CL by activated PMN is similar to and slightly in advance of the time course of killing (5), which is near maximal at 1 h and is maximal in 2 h.

Effect of immune versus normal serum on CL from PMN plus *B. dermatitidis*. The importance of specific antibody in the generation of CL by PMN was investigated by interacting PMN and *B. dermatitidis* in fresh immune or nonimmune serum (Fig. 3). CL generated by activated PMN plus *B. dermatitidis* was essentially the same in the presence of fresh immune as in the presence of fresh nonimmune serum. Furthermore, immune serum compared with nonimmune serum did not enhance the production of CL by elicited PMN plus *B. dermatitidis* (Fig. 3). These results argue against a critical role for specific antibody in the oxidative burst generated by the interaction of PMN and *B. dermatitidis*.

Effect of catalase on the generation of CL by activated PMN plus *C. albicans*. To determine the importance of hydrogen peroxide in the complex reactions of the oxidative burst and CL, activated PMN were mixed with *C. albicans* in the presence or absence of catalase. Catalase (20,000 U/ml) completely abrogated the generation of a CL burst (Fig. 4). Heated catalase did not have this effect (data not shown). These findings indicate that hydrogen peroxide is produced by the interaction of activated PMN and *C. albicans* and that efficient removal of H_2O_2 negates generation of the burst of CL. When these same cells were incubated with viable *C. albicans* with or without catalase, killing (86%) of *C. albicans* by activated PMN was abrogated in the presence of catalase (Table 1). Taken together, these results show that hydrogen peroxide is an essential product in the generation of CL and production of candidacidal activity during the interaction of activated PMN and *C. albicans*.

Effect of DMSO or catalase on CL produced by activated PMN plus *B. dermatitidis*. Hydroxyl radical and hydrogen peroxide scavengers were used to determine the respective contributions of these products of oxidative metabolism in the generation of CL during the interaction of activated PMN and *B. dermatitidis*. Removal of hydrogen peroxide from the system by catalase (20,000 U/ml) resulted in no burst of CL (Fig. 5). In contrast, scavenging of hydroxyl radicals by DMSO (300 mM) only blunted the burst of CL ($10^{3.7}$ versus 10^3 cpm) after 15 min of incubation (Fig. 5). These results show that removal of hydrogen peroxide, an early product of the oxidative burst, has a greater effect on CL than does removal of hydroxyl radicals (one of the terminal products) by DMSO at the maximum concentration previously used (21), a concentration effective in hydroxyl radical scavenging. When activated PMN were cocultured with viable *B. dermatitidis* and catalase, PMN fungicidal

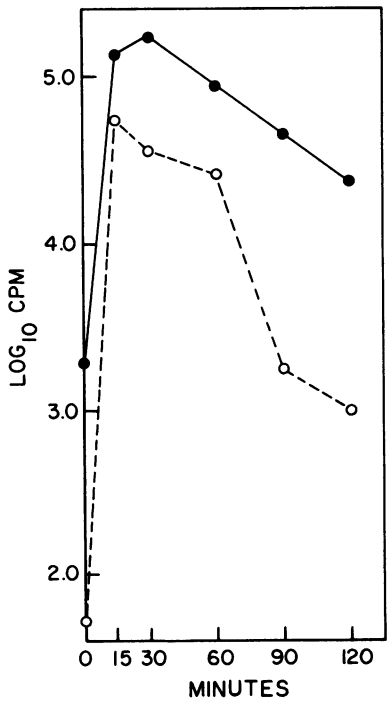

FIG. 2. CL produced by interaction of *C. albicans* (10^6), luminol (8 μg), and PMN. Shown are counts per minute produced by *C. albicans* plus activated (●) or elicited (○) PMN.

activity was completely abrogated, e.g., 80 versus 9% (Table 1). Heated catalase did not have this effect (data not shown). In contrast, the fungicidal activity of activated PMN was only partially ablated (80 versus 58%) in the presence of DMSO. This difference (80 versus 58%) was statistically significant ($P < 0.01$), indicating that the residual levels of hydroxyl radicals or other metabolites (e.g., HOCl) contribute to the total fungicidal activity.

It is noted that elicited PMN, unlike activated PMN, did not show a burst with *B. dermatitidis* but instead showed a lower gradual sustained generation of CL (Fig. 3 and 5).

Effect of treatment with anti-granulocyte monoclonal antibody plus complement. Treatment of pellet-1-activated PMN with rat anti-mouse granulocyte monoclonal antibody plus complement abrogated the burst of CL when these cells were stimulated with *C. albicans* in 10% fresh serum. PMN treated with minimal essential medium plus complement made a good response to such stimulation compared to anti-granulocyte antibody-treated cells, e.g., $10^{3.5}$ versus $10^{2.5}$ cpm after 30 min of incubation. These results indicate that production of the oxidative burst required cells that were sensitive to anti-mouse granulocyte antibody plus complement.

DISCUSSION

We have shown that elicited and activated PMN differ in their ability to produce a burst of CL when interacting with *C. albicans* or *B. dermatitidis*, and these differences parallel their fungicidal activity. The magnitude of the CL response, rather than the increment of CL over base line, appears to correlate with the fungicidal activity of activated PMN. Similarly, differences in bactericidal activity and production of CL between resting and stimulated human peripheral blood PMN have been reported by Van Epps and Garcia (25), with a different stimulus. Our findings are consistent with that report, and our results extend the scope of activating stimuli to those generated in an immunologically specific reaction and extend the enhanced microbicidal activity to include fungal pathogens.

An earlier study showed that purified PMN obtained 24 h

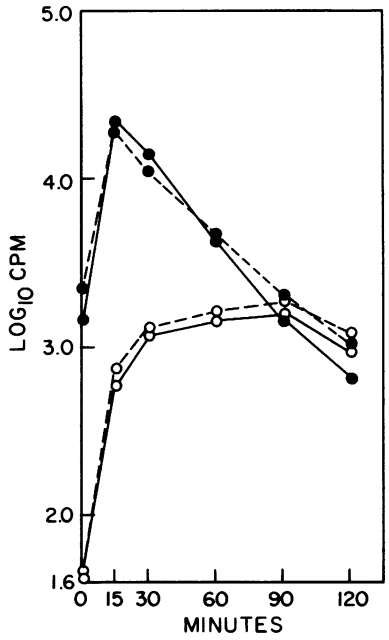

FIG. 3. CL produced by interaction of *B. dermatitidis* (10^6), luminol (8 μg), and PMN. Given are counts per minute generated by interaction of *B. dermatitidis* and activated PMN in 10% fresh immune serum (●——●) or normal serum (●-----●). Also shown are counts per minute produced by interaction of *B. dermatitidis* and elicited PMN in 10% fresh immune serum (○——○) or normal serum (○-----○) under the same conditions.

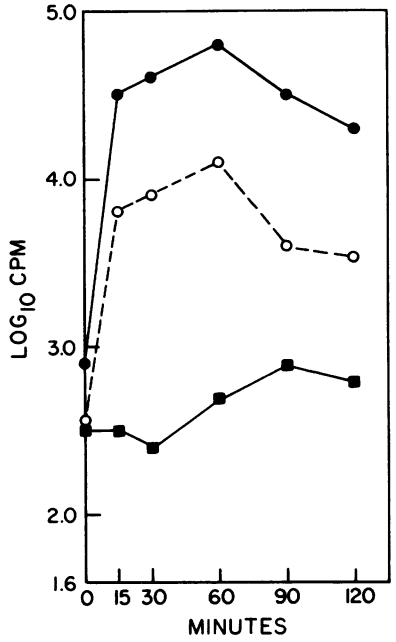

FIG. 4. Effect of catalase on CL produced by interaction of *C. albicans* (10^6), luminol (1 μg), and PMN in 10% fresh mouse serum. We show counts per minute generated by *C. albicans* plus activated PMN (●), elicited PMN (○), or activated PMN with 20,000 U of catalase per ml (■).

TABLE 1. Effect of catalase or DMSO on killing of *C. albicans* or *B. dermatitidis* by activated PMN

Fungus	CFU[a] per well	% Reduction of CFU	P[b]
C. albicans			
Inoculum (0 h) +	660 ± 47		
Medium	790 ± 78	0	NS
Elicited PMN[c]	280 ± 26	58	<0.001
Activated PMN[d]	90 ± 18	86	<0.001
Activated PMN + catalase[e]	717 ± 20	0	NS
B. dermatitidis			
Inoculum (0 h) +	550 ± 67		
Medium	590 ± 120	0	NS
Elicited PMN[c]	465 ± 72	8	NS
Activated PMN[d]	103 ± 10	80	<0.001
Activated PMN + 300 mM DMSO	237 ± 58	58	<0.001
Activated PMN + catalase[e]	460 ± 30	9	NS

[a] Mean CFU ± standard deviation from the mean of quadruplicate cultures.
[b] NS, not statistically significant ($P > 0.05$).
[c] PMN from thioglycolate-elicited PEC.
[d] PMN from PEC elicited in immune mice by antigen.
[e] Catalase at 20,000 U/ml.

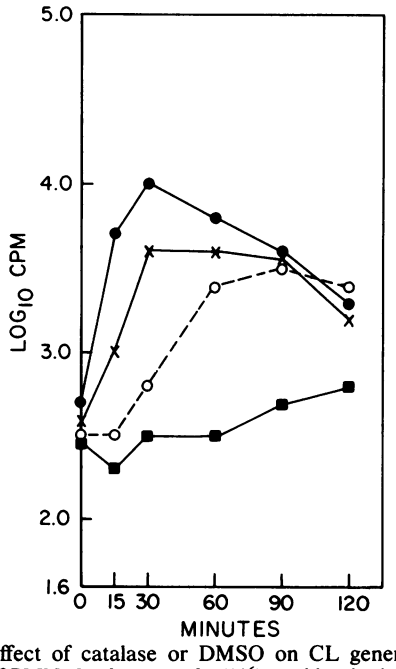

FIG. 5. Effect of catalase or DMSO on CL generated by the interaction of PMN, *B. dermatitidis* (10^6), and luminol (8 μg) in 10% fresh mouse serum. Shown are counts per minute produced by *B. dermatitidis* plus activated PMN alone (●), with 300 mM DMSO, or with 20,000 U of catalase per ml (■). Also shown are counts per minute generated by elicited PMN plus *B. dermatitidis* (○) under the same conditions.

after thioglycolate in normal mice do not kill *B. dermatitidis* (as was also the case with those obtained 4 h after thioglycolate in these studies) and that i.p. injection of *B. dermatitidis* in nonimmune animals fails to elicit cells that kill *B. dermatitidis* (5). We also showed that lymphocytes are ineffective and that lymphocytes or monocytes, or both, are nonenhancing to PMN killing in this in vitro system during the coculture period (5).

After stimulation, enhanced production of CL by activated PMN compared with elicited PMN resembles the differences between CL generated by PMN from patients with active bacterial infections compared with that generated by PMN from healthy individuals (2). The elevated activity of PMN from patients, e.g., CL, chemotaxis, and phagocytosis, was linked to the infection because activity of PMN returned to normal after the infection cleared and during convalescence (2). Elevated activity of PMN was not associated exclusively with bacterial infections as was previously postulated (18) but has been observed in viral and fungal infections as well as neoplasms (12). Although activation of PMN has been associated with disease in patients, possibly related to immunological reactions, our recent reports (4, 5) provide direct experimental evidence for a mechanism for activation to occur secondary to an immunological reaction.

We previously showed that killing of *B. dermatitidis* by activated PMN was significantly enhanced almost equally by fresh normal or fresh immune mouse serum, indicating that complement was critical but antibody was not (5). We report here that normal serum was as effective as immune serum in the generation of the CL burst by the interaction of activated PMN and *B. dermatitidis*.

It previously was reported that *B. dermatitidis* is sensitive to the products of the cell-free hydrogen peroxide-myeloperoxidase-halide system (22) as well as to the products of oxidative metabolism inhibitable with catalase (5). Here, we confirmed and extended these studies by showing that the burst of CL generated by the interaction of activated PMN with *B. dermatitidis* or *C. albicans*, which correlates with killing, was abrogated in the presence of catalase. These findings differ to some extent from those reported by Repine et al. (21), in which catalase significantly inhibited CL, but not killing, in a human PMN plus *Staphylococcus aureus* system. However, this discordance may reflect differences in human versus activated murine PMN, or susceptibility of yeasts versus *S. aureus* to certain oxygen radicals generated.

Experimental results reported here may support the latter possibility in that DMSO, a hydroxyl radical scavenger (21), appears less effective than catalase in inhibiting CL and killing. We cannot exclude the possibility that the effect of DMSO could be on the fungus, making it resistant to killing, though we do not think this likely. Our findings suggest that eliminating hydrogen peroxide, an early product of oxidative metabolism, has a more profound inhibitory effect than does the scavenging of hydroxyl radicals, a later product. Significant killing by the generation of hydroxyl radicals during the interaction of activated PMN and *B. dermatitidis* confirms the susceptibility of *B. dermatitidis* to hydroxyl radicals generated in the hydrogen peroxide-ferrous iron-halide system (23) and is consistent with toxicity for other fungi of hydroxyl radical (15) or its metabolites (20).

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