*Clin. exp. Immunol.* (1986) **66**, 681–690

# Fungicidal activity of murine inflammatory polymorphonuclear neutrophils: comparison with murine peripheral blood PMN

E. BRUMMER, J. G. McEWEN & D. A. STEVENS *Division of Infectious Diseases, Department of Medicine, Santa Clara Valley Medical Center, and Institute for Medical Research, San Jose, and the Department of Medicine, Stanford University Medical School, Stanford, California*

*(Accepted for publication 17 July 1986)*

## SUMMARY

A characteristic of inflammation is the influx of polymorphonuclear neutrophils (PMN) from peripheral blood to the inflammatory reaction. We report on metabolic alterations and alterations in fungicidal activity in PMN elicited intraperitoneally with different inflammatory agents. The fungicidal activity of murine peripheral blood PMN (PB-PMN) against phagocytosable *Candida albicans* and nonphagocytosable *Blastomyces dermatitidis* was compared to that of murine inflammatory PMN. PMN elicited with sodium caseinate exhibited enhanced killing of *B. dermatitidis* ($93 \pm 3\%$) compared to PB-PMN ($38 \pm 11{\cdot}7\%$). In contrast, thioglycollate medium elicited PMN had significantly less ability to kill *B. dermatitidis* ($3 \pm 5\%$) than PB-PMN. Incubation of caseinate PMN with thioglycollate medium for 1 h significantly reduced their ability to kill *B. dermatitidis* (95% vs 25%). This effect was not due to cytotoxicity of thioglycollate medium for PMN. Candidacidal activity of inflammatory PMN (caseinate or proteose peptone-elicited) was not significantly greater than that of peripheral blood PMN. However, inflammatory PMN had significantly greater candidacidal activity than thioglycollate-elicited PMN.

**Keywords** neutrophils neutrophil activation neutrophil depression fungicidal activity

## INTRODUCTION

Polymorphonuclear neutrophils (PMN) are potent microbicidal phagocytes; nevertheless, a few microorganisms withstand their attack. For example, the bacteria *Nocardia asteroides* (Filice, 1983) and *Brucella abortus* (Riley & Robertson, 1984) resist killing by human peripheral blood PMN. We have shown that the yeast form of the fungus *Blastomyces dermatitidis* is not consistently killed by human PMN (Brummer & Stevens, 1982; unpublished results). In some instances this resistance may be attributed to microbial catalase (Filice, 1983), poor stimulation of the oxidative burst (Riley & Robertson, 1984; Brummer, Beaman & Stevens, 1985) or inhibition of degranulation (Riley & Robertson, 1984); in others, the mechanism is unknown. Recently, it has been shown that under certain conditions the microbicidal activity of PMN can be increased (Cross & Lowell, 1978; Van Epps & Garcia, 1980; Brummer & Stevens, 1984; Brummer, Sugar & Stevens, 1984; Brummer *et al.*, 1985a). In some of these situations lymphokines generated by stimulation of lymphocytes with

Correspondence: Dr E. Brummer, Department of Medicine, Santa Clara Valley Medical Center, 751 S. Bascom Avenue, San Jose, CA 95128, USA.

Presented in part to the 1985 Annual Meeting, American Association of Immunologists.

mitogens (Cross & Lowell, 1978; Brummer & Stevens, 1984) or antigen (Brummer & Stevens, 1984) were implicated in enhancement of PMN microbicidal activity.

Recent reports indicate that acute (4 to 6 h) inflammatory PMN induced intraperitoneally with sterile irritants, e.g. 10% proteose peptone or 2% glycogen (Czuprynski, Henson & Campbell, 1984), nonviable *Corynebacterium parvum* (Lichtenstein *et al.*, 1984), or streptococcal preparation OK 432 (Watabe *et al.*, 1984), had enhanced bactericidal and tumoricidal activity compared to thioglycollate-elicited PMN. However, comparisons with peripheral blood PMN were not made. The purpose of the present study was to investigate this phenomenon by comparing the metabolic and fungicidal activity of peritoneal inflammatory PMN elicited with several sterile irritants to unstimulted circulating (peripheral blood) PMN as well as thioglycollate-elicited PMN. Our data show that PMN elicited with sodium caseinate had a significantly greater ability to kill nonphagocytosable *B. dermatitidis* than did peripheral blood PMN. In contrast, thioglycollate-elicited PMN had less fungicidal activity than peripheral blood PMN. Killing of *B. dermatitidis* by PMN correlated with the magnitude of the oxidative burst when stimulated with *B. dermatitidis*.

## METHODS AND MATERIALS

*Animals.* Pathogen-free BALB/cByJIMR male mice (Institute for Medical Research, San Jose, CA) 8 to 12 weeks of age were used in these experiments.

*Fungi. Blastomyces dermatitidis* (ATCC 26199), an isolate shown to be virulent in mice (Brass *et al.*, 1982), was used throughout these studies. Another isolate, ATCC 26198, was also tested. Yeast form *B. dermatitidis* from 72 h liquid medium cultures was used to inoculate blood agar plates (BAP). Seventy two to 96 h growth of *B. dermatitidis* on BAP was used to prepare inocula for challenging leukocyte cultures. Multicellular units per ml were counted with a haemacytometer, and colony forming units (c.f.u.) per ml determined by plating dilutions on BAP. *Candida albicans*, isolates Sh27 and H12, were grown without shaking in yeast nitrogen base broth (Difco Laboratories, Detroit, MI) at 35°C. Growth from 3 to 4 day cultures was washed twice in saline, counted with a haemacytometer, and c.f.u. per ml determined by plating 1 ml of appropriate dilutions on BAP.

*Media and reagents.* Dulbecco's phosphate buffered saline (PBS), minimal essential medium (MEM), RPMI-1640, heat inactivated fetal bovine serum (FBS), penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml) were purchased from GIBCO Laboratories, Grand Island, NY. Complete tissue culture medium (CTCM) consisted of RPMI-1640, 10% FBS (vol/vol), and 100 U penicillin and 100 μg streptomycin per ml. Histopaque 1077 (density 1.077), dextran 500 K, and Luminol were obtained from Sigma Chemical Co., St Louis, MO. Thioglycollate liquid media: BACTO-B256 (Difco) and broth with haemin, vitamin K and $CaCO_3$ (Clinical Standards Laboratories, Carson, CA) were used in these experiments. Glycogen was obtained from Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH. Sodium caseinate and proteose peptone were purchased from Difco Laboratories.

*Peritoneal exudate cells (PEC).* PEC were induced by intraperitoneal injection of 1 ml of 10% proteose peptone, 2% glycogen, 2% sodium caseinate, or thioglycollate broth. Four hours later, peritoneal exudate cells were collected by repeated lavage of the peritoneum of each mouse with a total of 10 ml of MEM containing 10 U/ml of preservative-free heparin (American Scientific Products, McGraw Park, IL). PEC were washed once in MEM, suspended in CTCM and counted with a haemacytometer.

*Polymorphonuclear neutrophils (PMN).* PEC were fractionated by density gradient centrifugation on Histopaque 1077. They were layered over 3 ml of Histopaque in a 15 ml plastic centrifuge tube and centrifuged at 400 ***g*** for 30 min at room temperature. Pelleted cells were highly enriched for inflammatory PMN. Peripheral blood PMN were obtained as follows: (a) layering heparinized blood (30 U/ml) diluted 2:1 in saline over an equal volume of Histopaque 1077; (b) centrifugation at 400 ***g*** for 20 min; (c) suspension of pelleted erythrocytes and PMN in an equal volume of saline; (d) mixing suspended pelleted cells with an equal volume of 3% (w/v) Dextran 500 in saline; (e)

sedimentation at 1 *g* for 1 h at 37°C; (f) collection of buffy coats and pelleting of cells by centrifugation (400 *g*, 10 min); (g) suspension of pelleted cells in 10 ml of 0·85% $NH_4Cl$ to lyse contaminating erythrocytes; (h) washing of treated cells with MEM by centrifugation followed by suspension in CTCM.

*Differential counts.* Pelleted cells were suspended in a drop of FBS and microscope slide smears prepared. Dried smears were stained using the Diff Quik staining protocol (American Scientific Products). Two hundred cells per slide were counted at ×900 magnification, and the percentage of PMN, lymphocytes and monocyte-macrophages recorded.

*Fungicidal assay.* PEC or PMN enriched fractions in CTCM were dispensed in quadruplicate, 0·1 ml of $5 \times 10^6$ cells/ml per flat bottom MicroTest plate well (Falcon 3072, Becton Dickinson Co., Oxnard, CA). These cells were challenged with 0·1 ml of *B. dermatitidis* (5,000 c.f.u./ml) or *C. albicans* (10,000 c.f.u./ml) in CTCM. This was followed by addition of 0·02 ml of fresh mouse serum to each co-culture and control culture. After incubation for 2 h at 37°C in 5% $CO_2$–95% air, cultures were harvested and c.f.u. per culture determined as previously described (Brummer & Stevens, 1984; Brummer *et al.*, 1984). Briefly, well contents were removed and placed in 7 ml of distilled water, then wells washed repeatedly with distilled water. Pooled culture contents and washings (total, 10 ml) were plated on BAP (1 ml/BAP), and c.f.u. per culture determined after incubation of *C. albicans* for 2 days and *B. dermatitidis* for 4 days at 37°C. The percent reduction of inoculum CFU was calculated by the formula $(1 - (\text{co-culture c.f.u./inoculum c.f.u.})) \times 100$. As previously described (Brummer & Stevens, 1982), reduction of c.f.u. in co-cultures was not due to clumping, e.g. the mean number of fungal cells per multicellular unit in washings, as determined microscopically, was similar in control and experimental cultures.

*Treatment with anti-mouse granulocyte antibody.* Monoclonal antibody specific for murine granulocytes was a gift from Robert Coffman, DNAX, Palo Alto, CA. Specificity of this antibody for PMN was determined by DNAX and confirmed by studies here (Brummer, Sugar & Stevens, 1985b). Further studies showed, for example, fungicidal activity of PMN was abolished when treated with anti-PMN antibody plus complement; however, macrophage killing of *Candida parapsilosis* was not affected. Briefly, 0·5 ml of $5 \times 10^6$ cells/ml were incubated with 0·5 ml of a 1:30 dilution of antibody in MEM at 4°C for 1 h, cells were pelleted by centrifugation, and resuspended in 3·5 ml of Low-Tox rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY) diluted 1:10 in MEM. After incubation at 37°C for 1 h, cells were pelleted by centrifugation and resuspended in CTCM for use in fungicidal assays described above.

*Chemiluminescence.* To detect the products of oxidative metabolism, subsequent to interaction of PMN with fungal particles in CTCM plus fresh mouse serum, a luminol method was employed (Allen & Loose, 1976). Luminol was dissolved in dimethyl sulphoxide (Eastman Kodak, Rochester, NY) to form a stock solution of 10 mg/ml, and was diluted in PBS to obtain a working solution of 80 μg/ml. Briefly, 0·1 ml of PMN ($10 \times 10^6$/ml PBS), 0·1 ml luminol (80 μg/ml), 0·05 ml of fresh mouse serum, 0·15 ml of PBS and 0·1 ml of killed fungal cells ($10 \times 10^6$/ml PBS) were combined at room temperature. Photon emission was measured in a scintillation counter (Mark II, Nuclear Chicago, Chicago, IL) at room temperature with windows set on 'manual' and levels at L–infinity. The manual setting permitted rapid counting of samples, and the counts per min (ct/min) were calculated using the counting time ($\leq 0{\cdot}2$ min) registered by the scintillation counter.

*Peripheral blood and serum.* Mice were anaesthetized with ether, a pouch of skin was formed between a front leg and body torso by dissection, the brachial artery severed, and blood collected with a pasteur pipette. When blood was used as a source of PMN it was heparinized (30 U/ml) upon collection. Fresh mouse serum was collected from clotted blood and was shown to have complement activity in a cytoxicity assay, e.g. anti-mouse T cell serum plus fresh mouse serum vs murine thymocytes.

*Endotoxin assay.* Limulus amebocyte lysate (E-Toxate, Sigma Chemical Co.) was used to measure endotoxin in irritants injected to elicit peritoneal exudate cells. The E-Toxate kit protocol was followed using the supplied endotoxin reference standard.

*Statistics.* Comparisons between groups were analysed by Student's *t*-test, with significance assumed to be $P < 0{\cdot}05$.

## RESULTS

*Fungicidal activity of peripheral blood PMN.* A population of peripheral blood cells enriched, as described in Methods, for murine PMN (80·7±9·2% PMN, 19·0±9·2% small lymphocytes) had significant fungicidal activity. Fungal inoculum c.f.u. were reduced 38·0±11.7% (mean of five experiments±s.d.) in the case of *B. dermatitidis* (ATCC 26199), and 84·0±7·1% for *C. albicans* (Sh27) ($P<0·001$ for both). When co-cultures were examined microscopically, c.f.u. of *B. dermatitidis* were found to be surrounded by numerous PMN. On the other hand, co-cultures containing purified small lymphocytes (99%), isolated from between the mononuclear cell layer and the erythrocyte pellet, and similar to those contaminating PMN preparations, did not show clumping around *B. dermatitidis* or killing of *B. dermatitidis*. When other isolates of *B. dermatitidis* (ATCC 26198) and *C. albicans* (H12) were tested against murine PMN they were killed to a similar extent.

*Yield and composition of PEC elicited by various irritants.* The yield and composition of PEC elicited with thioglycollate medium, sodium caseinate, or proteose peptone 4 h after injection were similar (Table 1). By contrast, glycogen elicited the fewest PEC per mouse ($6±1\times10^6$), and these were less enriched for PMN (53±3%) (Table 1).

*Yield and PMN composition of fractionated PEC (PMN preparations).* When 4 h PEC were fractionated by density gradient centrifugation, highly enriched (85 to 95%) PMN populations were obtained (Table 2). The yield (percentage of applied cells recovered) was greatest (53%) with caseinate elicited PEC and the least (8·5%) with proteose peptone elicited PEC (Table 2). These partially purified preparations are hereafter referred to as PMN preparations.

*Killing of* B. dermatitidis *by PEC and PMN preparations elicited with various irritants.* PEC elicited with proteose peptone, glycogen or caseinate markedly reduced inoculum c.f.u. of *B. dermatitidis*, 60 to 95%, compared to 11% for thioglycollate elicited PEC (glycogen PEC and caseinate PEC, $P<0·02$ and $<0·01$, respectively, vs thioglycollate PEC) (Fig. 1). When compared to killing by peripheral blood PMN (38·0±11·7%), only significantly ($P<0·001$) greater killing was achieved by glycogen and caseinate elicited PEC. Caseinate or proteose peptone PMN preparations also had significantly enhanced ability to kill *B. dermatitidis* (50 to 80%) compared to 3±5% for thioglycollate elicited PMN ($P<0·01$, $P<0·05$ respectively). However, when killing by elicited PMN preparations was compared with peripheral blood PMN, only killing by caseinate PMN preparations ($P<0·01$), was significantly greater (Fig. 1). In contrast, thioglycollate PMN preparations had significantly ($P<0·01$) lower fungicidal activity against *B. dermatitidis* (3%) compared to peripheral blood PMN.

*Killing of* C. albicans *by PEC and PMN elicited with various irritants.* In contrast to *B. dermatitidis*, *C. albicans* was easily killed by thioglycollate elicited PEC and PMN preparations, 40 and 77% respectively (Fig. 2). Furthermore, candidacidal activity of thioglycollate PMN preparations (77±3%) was not significantly different from that of peripheral blood PMN

**Table 1.** Differential counts and yield of peritoneal exudate cells after injection of irritants

| Irritants | Differential counts | | | Yield |
|---|---|---|---|---|
| | PMN | Lymphocytes | Mono-Mφ | PEC × $10^6$ |
| Thioglycollate (2) | 75±8* | 19±7 | 6±1 | 21±9† |
| Caseinate (3) | 80±9 | 17±7 | 2±2 | 15±2 |
| Proteose peptone (2) | 62±9 | 19±7 | 6±1 | 14±0·1 |
| Glycogen (2) | 53±3 | 38±1 | 9±4 | 6±1 |

* Mean percentage±s.d., number of experiments in parentheses.
† Mean±s.d. of peritoneal exudate cells (PEC) per mouse.

**Table 2.** Differential counts and yield of PMN fraction from peritoneal exudate cells after injection of irritants

| Irritants | Differential counts | | | Percent recovery of PMN from PEC |
|---|---|---|---|---|
| | PMN | Lymphocytes | Mono-Mϕ | |
| Thioglycollate (2) | 85±13* | 9±7 | 6±6 | 16±3† |
| Caseinate (3) | 95±2 | 4±1 | 0·3±0·5 | 53±4 |
| Proteose peptone (2) | 90±6 | 9±4 | 2±2 | 8·5±9 |

* Mean percentage±s.d., number of experiments in parentheses.

† Mean percentage±s.d. of PMN recovered from peritoneal exudate cells (PEC).

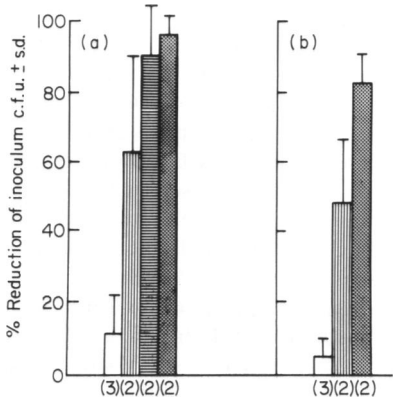


**Fig. 1.** Killing of *B. dermatitidis* by peritoneal exudate cells or PMN elicited with various irritants. The mean percent reduction of inoculum c.f.u. ± s.d. from the mean by (a) PEC or (b) PMN collected 4 h after injection i.p. with 1 ml of (□) thioglycollate broth, (■) 10% proteose-peptone, (■) 2% glycogen, or (■) 2% sodium caseinate. The number of experiments is indicated by the number in parentheses.

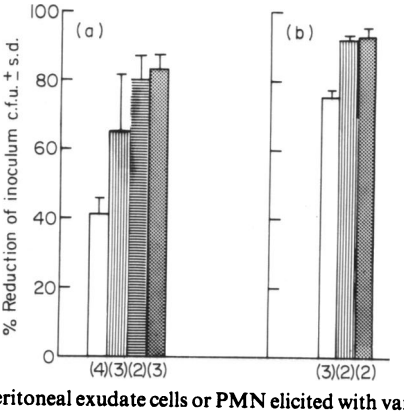


**Fig. 2.** Killing of *C. albicans* by peritoneal exudate cells or PMN elicited with various irritants. The mean percent reduction of inoculum c.f.u ± s.d. from the mean by (a) PEC or (b) PMN collected 4 h after injection i.p. of 1 ml of thioglycollate broth; 10% proteose-peptone, 2% glycogen, or 2% sodium caseinate. The number in parentheses indicates the number of experiments. Symbols as Fig. 1.

**Table 3.** Effect of anti-PMN antibody plus complement on fungicidal activity of PEC

| | Treatment of PEC | | |
|---|---|---|---|
| *B. dermatitidis* vs | MEM+MEM | MEM+C′ | Anti-PMN+C′† |
| Glycogen PEC | 79%* | 85% | 33% |
| Proteose peptone PEC | 60% | ND | 0% |
| Caseinate PEC | 63% | 62% | 30% |

* Percent reduction of inoculum c.f.u. after 2 h at 37°C.

† Anti-PMN+C′ vs controls; $P<0{\cdot}001$, $<0{\cdot}01$, $<0{\cdot}02$ for glycogen PEC, proteose peptone PEC and caseinate PEC, respectively.

ND, Not done.

(84±7%). Similarly, candidacidal activity of proteose peptone or caseinate PMN preparations (93±3%) was not significantly different from that of peripheral blood PMN. The only population of cells that had significantly less ability to kill *C. albicans* than peripheral blood PMN was thioglycollate PEC (84±7% vs 40±5%, $P<0{\cdot}01$).

*Effect of anti-PMN antibody plus complement on fungicidal activity of PEC and PMN.* When PEC elicited with various irritants were treated with anti-PMN antibody plus complement, killing was significantly reduced in all cases (Table 3). Killing by proteose peptone PEC was completely abrogated, whereas killing by glycogen PEC and caseinate PEC was reduced from 79 to 33% and 63 to 30% respectively. These findings suggest that cells other than PMN in glycogen and caseinate PEC, but not proteose peptone PEC, also have ability to kill *B. dermatitidis*, e.g. macrophages. In contrast, previous studies (Brummer & Stevens, 1984; Brummer *et al.*, 1984; 1985b) have shown that the lymphocytes and monocytes-macrophages present in PMN preparations did not contribute to fungicidal activity. In the present study this was confirmed: candidacidal activity of thioglycollate PMN preparations was 55% after treatment with MEM plus complement, 52% after treatment with anti-PMN antibody plus MEM, but 0% after anti-PMN plus complement.

*Chemiluminescence.* An explanation was sought for the enhanced capacity of caseinate PMN to kill *B. dermatitidis* as opposed to peripheral blood and thioglycollate PMN preparations. One possible explanation for these differences in fungicidal activity was the ability to generate an oxidative or respiratory burst when interacting with *B. dermatitidis*. This was tested by measuring the products of the respiratory burst in a luminol-enhanced chemiluminescence system. We found that caseinate PMN preparations interacting with *B. dermatitidis* produced a brisk oxidative burst (Fig. 3). Thioglycollate PMN preparations failed to produce such a reaction. In another experiment peripheral blood PMN or caseinate or thioglycollate PMN preparations interacted with *B. dermatitidis*. The results showed that peripheral blood PMN produced a brisk oxidative burst by 15 and 30 min (32,550 and 22,591 ct/min, respectively), but not as high as caseinate PMN preparations (57,788 ct/min and 57,212 ct/min, respectively). Thioglycollate PMN preparations failed to generate such bursts.

Moreover, enhanced candidacidal activity of caseinate PMN preparations correlated with a higher and more prolonged oxidative burst compared to that of thioglycollate PMN preparations (Fig. 3). The thioglycollate PMN preparation-candida data confirm earlier chemiluminescence studies (Brummer *et al.*, 1985b). Differences between the chemiluminescent response to *C. albicans* and *B. dermatitidis*, as demonstrated here, were described earlier (Brummer *et al.*, 1985).

These results demonstrate a correlation between fungicidal activity of caseinate PMN preparations and peripheral blood PMN for *B. dermatitidis* and the magnitude of an oxidative burst as measured by chemiluminescence.

*Effect of thioglycollate medium on PMN.* Thioglycollate liquid media, Clinical Standards (CS) and Difco Fluid Medium (B 256), are complex mixtures of yeast extract, pancreatic digest of casein, dextrose, sodium chloride, L-cystine, thioglycollic acid, agar, and Eh indicators (e.g. resazurin).

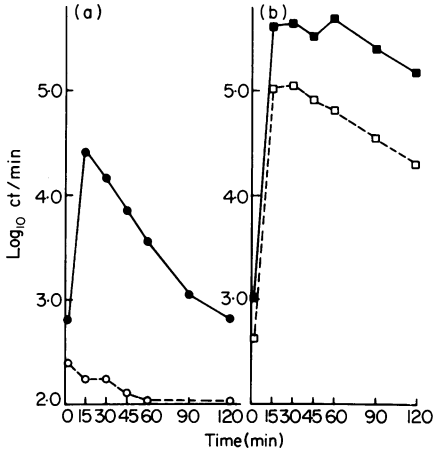


**Fig. 3.** Chemiluminescence produced by PMN interacting with (a) *B. dermatitidis* or (b) *C. albicans*. The log (to base 10) counts/min resulting from various intervals of interaction of (●, ■) sodium caseinate elicited PMN with *B. dermatitidis* or *C. albicans* and (○, □) thioglycollate broth elicited PMN with *B. dermatitidis* or *C. albicans* are given.

**Table 4.** Effect of thioglycollate medium on fungicidal activity of caseinate elicited PMN

| | Treatment* | c.f.u. ± s.d. | Percent reduction of inoculum c.f.u. |
|---|---|---|---|
| *B. dermatitidis* (Bd) inoculum | — | 526 ± 70† | — |
| Bd + no PMN | None | 540 ± 62‡ | 0 |
| Caseinate PMN + Bd | CTCM | 25 ± 17 | 95 |
| | Thioglycollate exudate fluid | 43 ± 17 | 92 |
| | Thioglycollate medium | 390 ± 17 | 25§ |
| Thioglycollate PMN + Bd | CTCM | 490 ± 7 | 7 |
| | Casein exudate fluid | 477 ± 6 | 9 |
| | Caseinate | 490 ± 26 | 7 |

* Purified PMN were incubated for 1 h at 37°C, then pelleted by centrifugation, the supernatants aspirated and the cells suspended in CTCM or the other fluids shown.

† c.f.u. ± s.d. of quadruplicate cultures at 0 h.

‡ c.f.u. ± s.d. of quadruplicate cultures after 2 h at 37°C.

§ Significantly different from CTCM control, $P < 0{\cdot}001$.

They have been used extensively to induce 3 to 5 day peritoneal exudates as a source of macrophages, and used more recently for induction of copious 4 to 6 h exudates as sources of inflammatory PMN. Our finding that thioglycollate PMN preparations had significantly decreased fungicidal activity against *B. dermatitidis*, compared to peripheral blood PMN, suggested that thioglycollate medium may contain substances detrimental to fungicidal activity of PMN against *B. dermatitidis*. We tested this hypothesis by incubating caseinate PMN preparations, which have enhanced fungicidal activity relative to peripheral blood PMN, with thioglycollate medium or thioglycollate peritoneal exudate fluid for 1 h at 37°C (Table 4). Thioglycollate and caseinate peritoneal exudate fluids were obtained by lavaging the peritoneum with 1 ml of MEM 4 h after injection of irritants i.p. Treatment with thioglycollate, but not thioglycollate exudate fluid,

significantly reduced the ability of caseinate PMN to kill *B. dermatitidis*. Thioglycollate treatment was not cytotoxic for PMN as judged by trypan blue dye exclusion. These data support the hypothesis that thioglycollate medium contains non-cytotoxic factor(s) which depress the ability of caseinate PMN to kill *B. dermatitidis*.

*Endotoxin content of various irritants.* The endotoxin content of various irritants was postulated as an explanation for their differing abilities to induce peritoneal exudate PMN with enhanced fungicidal activity. Consequently, thioglycollate medium, proteose peptone and caseinate were tested for endotoxin levels. It was found that thioglycollate medium contained the most endotoxin, greater than 10 but less than 100 ng/ml; whereas, proteose peptone and caseinate contained less endotoxin, greater than 1 but less than 10 ng/ml. These results did not suggest that endotoxin was a factor in irritants that could solely account for the effects measured.

## DISCUSSION

The data presented here indicate that murine peripheral blood PMN have a significantly greater ability to kill *B. dermatitidis* than do thioglycollate-elicited PMN preparations. This observation was made possible by recently devised methods for isolating sufficient numbers of murine peripheral blood PMN (Shellhaas, J.L., Am. Soc. Micro. Annual Meeting, Abstract B71, p. 30, 1985). However, these types of experiments are still hampered by the fact that mice have substantially fewer circulating PMN (15% of leukocytes) than, for example, do humans (33–75% of leukocytes) or guinea pigs ($\geq$85%) (Wright, 1960; Robinson, Karnovsky & Karnovsky, 1982). The PMN yield per mouse was $0{\cdot}77 \pm 0{\cdot}41 \times 10^6$.

We found that, depending on the eliciting agent, inflammatory PMN elicited with various irritants had enhancd or decreased ability to kill *B. dermatitidis* compared to peripheral blood PMN. Thioglycollate elicited PMN preparations had severely depressed ability to kill *B. dermatitidis* compared to peripheral blood PMN. Furthermore, thioglycollate medium treatment *in vitro* depressed the ability of caseinate PMN preparations to kill *B. dermatitidis*. Further studies are in progress to determine the identity of suppressive factor(s) in thioglycollate medium. On the other hand, caseinate-elicited PMN preparations had significantly greater capacity to kill *B. dermatitidis* than did peripheral blood PMN. The enhanced fungicidal activity of caseinate PMN preparations ($93 \pm 3$%) was similar to that of immunologically activated PMN (PMN elicited i.p. as a result of an immune reaction) ($73 \pm 4$%) described previously (Brummer *et al.*, 1984; 1985a, b). By contrast, the ability of proteose peptone PMN preparations to kill *B. dermatitidis* was slightly greater, but not significantly different, from that of peripheral blood PMN.

Although some factors capable of activation of inflammatory PMN or peripheral blood PMN have been shown, e.g. chemotactic factors (Issekutz, Lee & Gigger, 1979; Van Epps & Garcia, 1980), lymphokines (Cross & Lowell, 1978; Inoue & Sendo, 1983; Brummer *et al.*, 1984; Brummer & Stevens, 1984), and other factors (Gasson *et al.*, 1984; Vadas *et al.*, 1984), further understanding of the mechanisms is needed. A role for acute reactive proteins of inflammation on PMN function in fungal infection has also been suggested (Drouhet *et al.*, 1981).

Although caseinate PMN preparations killed significantly more *B. dermatitidis* than did peripheral blood PMN, both killed *C. albicans* to a comparable degree. This may reflect difficulties in killing multicellular units of *B. dermatitidis* (8 to 10 micron diameter cells, usually 2 to 4 cells per unit) compared to phagocytosable *C. albicans*. Our data support the concept that the activated state of PMN can best be demonstrated when challenged with a difficult target (non-phagocytosable), such as *Naegleria fowleri* (Ferrante & Mocatta, 1984) or tumour cells (Inoue & Sendo, 1983; Lichtenstein *et al.*, 1984; Watabe *et al.*, 1984).

The findings of differences in killing ability between elicited and peripheral blood in this study could be explained by (a) effect of the elicitant directly or indirectly on elicited cells, (b) different distribution of PMN subpopulations (Klempner & Gallin, 1978) between peripheral and elicited intraperitoneal populations, or (c) effect of exposure of elicited cells to the intraperitoneal milieu and subsequent purification as compared to the effect of purification of the peripheral PMN. The experiments with added thioglycollate suggest the first of these possibilities is relevant to at least some of the findings we report, but further studies are now indicated to address unresolved

questions. In contrast to the present study, few studies have compared blood PMN and exudate PMN, for any functions, in the same species (Robinson *et al.*, 1982; Zimmerli *et al.*, 1984). The previously published data suggested functional differences between such populations (Scott & Cooper, 1974; Wilton, Renggli & Lehner, 1977; Wright & Gallin, 1979; Takamori & Yamashita, 1980; Wandall, 1982; Zimmerli *et al.*, 1984). Where, specifically, microbicidal function has been examined, both equivalence (Bell *et al.*, 1972) of the two types of PMN, and depression of exudate PMN (Hellum & Solberg, 1977), have been reported. However, it is important to compare cells of the same species, though this has not always been done previously, since functional differences in peripheral PMN between species have been shown (Zimmerli *et al.*, 1984). We also have reported no consistent killing of *B. dermatitidis* by peripheral human PMN (Brummer & Stevens, 1982; unpublished results), in contrast to the present murine PMN data. Our previous studies (Brummer *et al.*, 1984) suggested that other intraperitoneal elicitants besides thioglycollate, e.g. killed fungi injected into nonimmune hosts, yield PEC with impaired ability to kill *B. dermatitidis* (but not *C. albicans*) compared to that demonstrated for peripheral blood PMN in this study.

Our findings presented here, and the unresolved issues mentioned, suggest that caution should be exercised in using the activity of elicited PMN as a baseline for murine PMN. This is not only true for fungicidal activity against *B. dermatitidis*, but may also be true for other nonphagocytosable targets of PMN, such as tumor cells (Inoue & Sendo, 1983; Lichenstein *et al.*, 1984; Watabe *et al.*, 1984). Not only are there differences depending on the elicitant (Czuprynski *et al.*, 1984; present study), but even differences in functional activity with time after the elicitant is injected (Brummer & Stevens, 1984; Czuprynski *et al.*, 1984).

This work was supported by contract N00014-83-K-0018 from the Office of Naval Research, US Navy.

## REFERENCES

ALLEN, R.C., & LOOSE, L.D. (1976) Phagocytic activation of luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. *Biochem. Biophys. Res. Comm.* **69,** 245.

BELL, M.L., CLAY, R.C., HOWE, C.W. & RUTENBERG, A.M. (1972) Antibacterial properties of human inflammatory leucocytes. A comparison with leucocytes derived from peripheral blood. *RES. J. Reticuloendothel. Soc.* **11,** 167.

BRASS, C., VOLKMANN, C.M., KLEIN, H.P., HALDE, C.J., ARCHIBALD, R.W.R. & STEVENS, D.A. (1982) Pathogen factors and host factors in murine pulmonary blastomycosis. *Mycopathol.* **78,** 129.

BRUMMER, E. & STEVENS, D.A. (1982) Opposite effects of human monocytes, macrophages and polymorphonuclear neutrophils on replication of *Blastomyces dermatitidis* in vitro. *Infect. Immun.* **36,** 297.

BRUMMER, E. & STEVENS, D.A. (1984) Activation of murine polymorphonuclear neutrophils for fungicidal activity with supernatants from antigen-stimulated immune spleen cell cultures. *Infect. Immun.* **45,** 447.

BRUMMER, E., SUGAR, A.M. & STEVENS, D.A. (1984) Immunological activation of polymorphonuclear neutrophils for fungal killing: studies with murine cells and *Blastomyces dermatitidis* in vitro. *J. Leuk. Biol.* **36,** 505.

BRUMMER, E., BEAMAN, L. & STEVENS, D.A. (1985a) Killing of endospores, but not arthroconidia, of *Coccidioides immitis* by immunologically activated polymorphonuclear neutrophils. In: *Coccidioidomycosis*, Proc. 4th International Conference on Coccidioidomycosis (ed. by H.E. Einstein & A. Catanzaro) p. 201. National Foundation for Infectious Diseases, Washington DC.

BRUMMER, E., SUGAR, A.M. & STEVENS, D.A. (1985b) Enhanced oxidative burst in immunologically activated, but not elicited, polymorphonuclear leukocytes correlates with fungicidal activity. *Infect. Immun.* **49,** 396.

CROSS, A.S. & LOWELL, G.H. (1978) Stimulation of PMN leukocyte bactericidal activity by supernatants of activated human mononuclear cells. *Infect. Immun.* **22,** 502.

CZUPRYNSKI, C.J., HENSON, P.M. & CAMPBELL, P.A. (1984) Killing of *Listeria monocytogenes* by inflammatory neutrophils and mononuclear phagocytes from immune and nonimmune mice. *J. Leuk. Biol.* **35,** 193.

DROUHET, E., DUPONT, B., PELLETIER, M. & GIROUD, J.P. (1981) Acute non-specific inflammatory reactions and protection against experimental *Candida albicans* infection. *Agents and Actions.* **11,** 629.

FERRANTE, A. & MOCATTA, T.J. (1984) Human neutrophils require activation by mononuclear leukocyte conditioned medium to kill the pathogenic free-living amoeba, *Naegleria fowleri. Clin. exp. Immun.* **56,** 559.

FILICE, G.A. (1983) Resistance of *Nocardia asteroides* to oxygen dependent killing by neutrophils. *J. inf. Dis.* **148,** 361.

GASSON, J.C., WEISBART, R.H., KAUFMAN, S.E., CLARK, S.C., HEWICK, R.M., WONG, G.G. & GOLDE, D.W. (1984) Purified human granulocyte-macrophage colony stimulating factor: direct action on neutrophils. *Science* **226,** 1339.

HELLUM, K.B. & SOLBERG, C.O. (1977) Human leucocyte migration. Studies with an improved skin chamber technique. *Acta. Path. Micro. Scand.* (Sect. C) **85,** 413.

INOUE, T. & SENDO, F. (1983) In vitro induction of cytotoxic polymorphonuclear leukocytes by supernatants from Con A stimulated spleen cell cultures. *J. Immun.* **131,** 2508.

ISSEKUTZ, A.C., LEE, K. & GIGGER, W.D. (1979) Enhancement of human neutrophil bactericidal activity by chemotactic factors. *Infect. Immun.* **24,** 295.

KLEMPNER, M.S. & GALLIN, J.I. (1978) Separation and functional characterization of human neutrophil subpopulations. *Blood* **51,** 659.

LICHTENSTEIN, A.K., KAHLE, J., BEREK, J., & ZIGHELBOIM, J. (1984) Successful immunotherapy with intraperitoneal *Corynebacterium parvum* in a murine ovarian cancer model is associated with the recruitment of tumor-lytic neutrophils into the peritoneal cavity. *J. Immunol.* **133,** 519.

RILEY, L.K. & ROBERTSON, D.C. (1984) Ingestion and intracellular survival of *Brucella abortus* in human and bovine polymorphonuclear leukocytes. *Infect. Immun.* **46,** 224.

ROBINSON, J.M., KARNOVSKY, M.L. & KARNOVSKY, M.J. (1982) Glycogen accumulation in polymorphonuclear leukocytes, and other intracellular alterations that occur during inflammation. *J. Cell. Biol.* **95,** 933.

SCOTT, R.B. & COOPER, L.V.W. (1974) Leucocyte glycogen response in inflammatory exudates. *Br. J. Haematol.* **26,** 485.

TAKAMORI, K. & YAMASHITA, T. (1980) Biochemical properties of polymorphonuclear neutrophils from venous blood and peritoneal exudates of rabbits. *Infect. Immun.* **29,** 395.

VADAS, M.A., NICOLA, N., LOPEZ, A.F., METCALF, D., JOHNSON, G. & PEREIRA, A. (1984) Mononuclear cell-mediated enhancement of granulocyte function in man. *J. Immunol.* **133,** 202.

VAN EPPS, D.E. & GARCIA, M.L. (1980) Enhancement of neutrophil function as a result of prior exposure to chemotactic factor. *J. clin. Invest.* **66,** 167.

WANDALL, J.H. (1982) Function of polymorphonuclear neutrophilic leucocytes. *Acta. Path. Micro. Scand. (Sect. C)* **90,** 7.

WATABE S., SENDO, F., KIMURA, S. & ARAI, S. (1984) Activation of cytolytic polymorphonuclear leukocytes by in vivo administration of a streptococcal preparation, OK432. *J. nat. Cancer Inst.* **76,** 1365.

WILTON, J.M.A., RENGGLI, H.H. & LEHNER, T. (1977) The role of Fc and C3b receptors on phagocytes by inflammatory polymorphonuclear leukocytes in man. *Immunol.* **32,** 955.

WRIGHT, D.G. & GALLIN, J.I. (1979) Secretory response of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. *J. Immunol.* **123,** 285.

WRIGHT, H.P. (1960) General properties of blood: the formed elements. In: *Medical Physiology and Biophysics.* (ed. by T.C. Ruch & J.F. Fulton) p. 502. W.B. Saunders, Philadelphia.

ZIMMERLI, W., LEW, P.D., COHEN, H.J. & WALDVOGEL, F.A. (1984) Comparative superoxide-generating system of granulocytes from blood and peritoneal exudates. *Infect. Immun.* **46,** 625.