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# PMN chemotactic factor produced by glass-adherent cells in the acute inflammation caused by *Paracoccidioides brasiliensis*

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**Summary.** Intraperitoneal inoculation of BIO.A mice with *P. brasiliensis* induces an acute inflammatory infiltrate in which 40–50% of the cells are PMN leucocytes. Previous depletion of serotonin, prostaglandin, histamine and complement does not alter the course of inflammation. Complement-derived factors appear to have no active participation in the process since C5-deficient mice depleted or not by Cobra venom factor (CoF) show the same kind of cellular influx. On the other hand, peritoneal cells incubated (6 h) with the fungus release a soluble factor that induces *in vivo* an active chemotaxis of PMN cells when inoculated i.p. The factor has the following characteristics: a) it is produced by adherent cells; b) it is protein in nature; c) its production is inhibited by incubation of peritoneal cells with 10 μg/ml puromycin and d) it has a molecular weight less than 15 000 daltons, as determined by gel filtration through a Sephadex G-75 column.

Keywords: *Paracoccidioides brasiliensis*, PMN chemotaxis, inflammation, chemotactic factor, macrophages

The mechanisms responsible for the accumulation of polymorphonuclear leucocytes (PMN) at sites of inflammation are not fully understood. The components C5a (Fernandez *et al.* 1978) and C567 (Ward 1968) produced by complement activation are well known to have chemotactic activity. The peptide N-formyl-methionyl-leucyl-phenylalanine is the best studied chemotactic factor (Schiffmann *et al.* 1975; Showell *et al.* 1976). Other chemotactic agents such as histamine (Clark *et al.* 1975), eosinophil chemotactic factor of anaphylaxis (ECF-A) (Goetzl & Austen 1975), leucotriene B4 (Ford-Hutchinson *et al.* 1981; Goetzl & Pickett 1981), polypeptides (Hunninghake *et al.* 1978) and proteins (Kaplan *et al.* 1972) also have been characterized.

Previously, in a study of complement participation in the acute inflammatory reaction produced by *Paracoccidioides brasiliensis*, we verified that although the yeast phase of this fungus activates the complement alternative pathway *in vitro*, this system does not participate in the PMN influx *in vivo* (Calich *et al.* 1979). Both normal (BIO.A) and C5-deficient mice (AKR), previously depleted of complement by treatment with cobra venom factor (CoF), did not show significant differences in PMN accumulation at the subcutaneous sites of fungus inoculation.

The present work was undertaken to determine the participation of several mediators and cells in PMN chemotaxis produced by intraperitoneal inoculation of *P. brasiliensis* in mice. It was shown that a glass-adherent cell population was responsible for the production of a PMN chemotactic factor of low molecular weight, probably a peptide. This could explain, at least in part, the unusual PMN influx observed during the inflammatory process produced by yeast cells.

## Materials and methods

*Fungus.* The yeast phase of *P. brasiliensis* (isolate 18) was grown for 7 days in Fava-Netto's medium (Fava-Netto 1955) at 37°C. Cell suspensions were washed three times in phosphate-buffered saline (PBS) pH 7.2, counted in an haemocytometer and adjusted to $2 \times 10^6$ cells/ml.

*Mice.* Inbred B10.A, C3H/He, C3HeB/Fe, AKR and outbred adult female mice (20–25 g body weight) were originally obtained from Jackson Laboratory Bar Harbor, Maine.

*In vivo chemotactic assays.* Unless otherwise stated adult female B10.A mice were used *P. brasiliensis* suspension (0.5 ml) containing $1 \times 10^6$ cells was inoculated intraperitoneally in mice. Peritoneal washings were done 6, 12, 24 and 48 h later with 3.0 ml of PBS–heparin (5 units/ml). For each determination, groups of five mice were used; control mice were injected with 0.5 ml of PBS and the peritoneal cavities washed 6 or 12 h later. Total cell numbers were determined with a haemocytometer. Differential counts were performed on fixed and stained cell suspensions using 0.05% crystal violet in 3% acetic acid, or in smear preparations stained with Giemsa.

*Treatment of mice.* B10.A and AKR mice were complement-depleted by CoF (Cordis Labs, Miami, Fla.) according to Cochrane *et al.* (1970). C3 levels were determined by single radial immunodiffusion (Mancini *et al.* 1965). Serial dilutions of a normal standard mouse serum (undiluted, 1:2, 1:4 and 1:8) were placed in each plate containing specific anti-mouse C3 and 10 mM EDTA. C3 levels were expressed as the percentage of normal standard mouse serum.

The influence of several non-specific mediators of inflammation on the acute phase of *P. brasiliensis* infection was studied using B10.A mice previously treated with several drugs. The drugs and dosage used were: methysergide (Sandoz Labs, Brazil) 6 mg/60 kg/12 h, s.c.; methylprednisolone (Upjohn Prods Farmac, Brazil) 50 mg/kg/24 h, i.m., heparin (Roche Labs, Brazil) 5 U/animal/12 h, s.c.; diphenhydramine (Parke-Davis Labs, Brazil) 2 mg/kg/6 h, i.m.; and indomethacin 10 mg/kg/8 h, oral. The first dose of methylprednisolone and heparin was given 4 h, diphenhydramine and indomethacin 2 h and methysergide 24 h before *P. brasiliensis* inoculation.

*Resident peritoneal cell suspensions.* Peritoneal cells were aseptically collected after i.p. injection of 3 ml PBS. The cell suspensions were washed three times in PBS–glucose (4 g/l) and adjusted to a concentration of $1 \times 10^6$/ml. Cell viability, assessed by trypan blue exclusion test, was always greater than 95%.

*Enriched cell suspensions.* Resident peritoneal cells kept in RPMI medium (Flow Laboratories, England) containing 20% heat-inactivated fetal calf serum (Flow Laboratories, England) were allowed to adhere (30 min) to Falcon plastic dishes. The non-adherent peritoneal cells were aspirated from the supernatant after repeated washings. The remaining adherent cells were detached from culture dishes by treatment with 12 mM lidocaine hydrochloride (Astra Pharmaceutical Co., Worcester, Mass.) according to Rabinovich & De Stefano (1976). The non-adherent and adherent cell fractions consisted of 90% lymphocytes and 94% macrophages, re-

spectively, as judged by morphological criteria.

All cell suspensions were washed twice and adjusted to $0.7 \times 10^6$ cells/ml using a PBS–glucose solution. Cell viability, assessed by the trypan blue exclusion test, was greater than 95%.

*Preparation of chemotactically active supernatants.* Equal volumes and concentrations of resident or enriched populations of peritoneal cells were mixed and incubated 6 h at 35°C in a Dubnoff shaker with *P. brasiliensis.* Cells were removed by centrifugation (1000 *g* for 15 min at 4°C) and supernatants filtered on a Millipore filter (pore: 0.22 μm). Control preparations for these experiments included supernatants from peritoneal cells incubated without *P. brasiliensis* and supernatants of *P. brasiliensis* incubated without peritoneal cells. One millilitre of each supernatant and respective controls were injected i.p. into five B10.A mice which were killed 6 h later. Peritoneal washings and cellular evaluation were made as previously stated.

In some experiments, peritoneal cell suspensions and *P. brasiliensis* were incubated in the presence of 10 μg/ml of puromycin (Sigma Chemical Co., St Louis, Mo.) to evaluate the effect of this drug on the chemotactic activity of the supernatants.

*Gel filtration of active supernatants on G-75 Sephadex.* Molecular sieve chromatography of active supernatants was performed on G-75 Sephadex (Pharmacia Fine Chemicals) using a $1.5 \times 50$ cm column and 0.85% saline buffered with 0.04 M phosphate (pH 7.4) as the eluant. Fractions of 1.5 ml were collected, and elution of protein was estimated spectrophotometrically at 280 nm (Uvicord II 8.300 LKB). The chemotactic activities of the peaks were measured by injecting 1 ml of undiluted eluates into the peritoneal cavity of B10.A mice (groups of five mice per eluate). For molecular weight estimates columns were calibrated with ovalbumin (45 000 daltons) bovine serum albumin (66 000 daltons) and lysozyme (15 000 daltons).

*Statistical analysis.* Mean, standard error and unpaired *t*-tests were used in analysis of data. For significance, the value for *P* was less than 0.01.

## Results

### *Peritoneal PMN leucocyte influx in different strains of mice*

In order to determine whether PMN chemotaxis produced by *P. brasiliensis* was a general phenomenon, we inoculated yeast cells of this fungus into mice of different strains. A volume of 0.5 ml of a cell suspension containing $1 \times 10^6$ *P. brasiliensis* was inoculated i.p. into inbred B10.A, AKR, C3H/He, C3HeB/Fe and outbred adult female mice. Peritoneal washings were done 6, 12, 24 and 48 h later with PBS–heparin. Total and differential counts were performed as described in the materials and methods section.

Previous kinetic studies showed that the highest PMN influx occurs 12 h after fungus inoculation. Table 1 shows the PMN influx 12 h after *P. brasiliensis* inoculation and, as can be seen, the different strains all displayed strong chemotaxis for PMN leucocytes. B10.A, C3H/He and C3HeB/Fe mice showed 40–46% of PMN in peritoneal washings, and outbred and AKR mice a lower percentage (19–22%). These differences were not statistically significant.

### *Influence of different mouse treatments on the PMN chemotaxis*

The influence of the complement system on PMN chemotaxis was investigated using normal and C5-deficient mice previously treated with CoF. The decrease of C3 levels in serum of mice pretreated with CoF as compared to normal C3 levels was $89.3 \pm 1.9\%$ at 12 h after infection. Table 2 shows the peritoneal PMN influx in normal and CoF-

**Table 1.** Peritoneal PMN influx in several strains of mice 12 h after i.p. inoculation of $1 \times 10^6$ *Paracoccidioides brasiliensis*

| Mice strain | Total cells ($\times 10^4$) | % PMN | Total PMN ($\times 10^4$) |
|---|---|---|---|
| B10.A | 811±73.4 | 40±3.1 | 326±48.6 |
| AKR | 879±149.5 | 19±4.0 | 163±42.8 |
| C3H/He | 717±68.9 | 46±4.5 | 335±54.0 |
| C3HeB/Fe | 915±134.2 | 44±0.9 | 409±62.6 |
| Outbred | 1205±207.6 | 22±3.6 | 260±54.0 |

Results are mean±SEM from five mice.

**Table 2.** Peritoneal PMN influx in normal and CoF-treated B10.A and AKR mice, 12 h after i.p. inoculation of $1 \times 10^6$ *Paracoccidioides brasiliensis*

| Mice and treatment | Total cells ($\times 10^4$) | % PMN | Total PMN ($\times 10^4$) |
|---|---|---|---|
| B10.A | 811±73.4 | 40±3.1 | 326±48.6 |
| B10.A+CoF | 767±218.0 | 32±13.0 | 389±132.0 |
| AKR | 879±149.5 | 19±4.0 | 163±42.8 |
| AKR+CoF | 421±48.6 | 37±6.0 | 162±39.0 |

Results are mean±SEM from five mice. CoF, cobra venom factor.

treated B10.A and AKR mice. No significant difference in PMN influx was observed.

The influence of several non-specific mediators of inflammation on the acute phase of *P. brasiliensis* infection was analysed using B10.A mice treated with various drugs (methylsergide, methylprednisolone, heparin, diphenhydramine and indomethacin). No significant difference was observed among the several groups of mice studied (Table 3). This experiment showed that inhibition of histamine, serotonin, prostaglandins and the coagulation system does not alter the PMN influx into the peritoneal cavity of mice. The use of high doses of corticosteroid also did not change the kinetics of PMN leucocyte migration.

*Production of chemotactically active supernatants by interaction of peritoneal cells and P. brasiliensis*

The participation of cellular components of the peritoneal cavity in PMN chemotaxis was then studied. Total or enriched populations of peritoneal cells were incubated 6 h with *P. brasiliensis*; 1 ml of each supernatant was injected into the peritoneal cavity of normal B10.A mice. Six hours later the mice were killed, their peritoneal cavity washed and cells counted. Table 4 shows the PMN chemotaxis induced by the supernatants produced after incubation of total peritoneal cells with the fungus and by their respective controls. Only the supernatant obtained after interaction of peritoneal cells with *P. brasiliensis* produced a massive PMN influx. Statistical analysis, at a 1% level of significance, confirmed the chemotactic activity of this supernatant.

The role of enriched populations of peritoneal cells (that is glass-adherent and non-adherent mononuclear cells) in the production of active supernatants was subsequently investigated. An active supernatant was produced only when adherent cells were

**Table 3.** Peritoneal PMN influx in normal and drug-treated B10.A mice, 12 h after i.p. inoculation of $1 \times 10^6$ *Paracoccidioides brasiliensis*

| Mice treatment | Total cells ($\times 10^4$) | % PMN | Total PMN ($\times 10^4$) |
|---|---|---|---|
| Normal | 811±73.4 | 40±3.1 | 326±48.6 |
| Methylsergide | 1104±204.5 | 53±2.2 | 604±134.6 |
| Methylprednisolone | 818±127.4 | 51±2.2 | 420±74.8 |
| Heparin | 836±110.8 | 38±3.1 | 314±39.6 |
| Diphenhydramine | 1213±182.4 | 41±0.9 | 499±83.3 |
| Indomethacin | 921±239.6 | 46±3.5 | 423±111.5 |

Results are mean±SEM from five mice.

**Table 4.** Peritoneal PMN influx in B10.A mice inoculated with supernatants obtained from the interaction *in vitro* of total peritoneal cells (TPC) and *Paracoccidioides brasiliensis* (Pb)

| Supernatant* obtained from: | Total cells ($\times 10^4$) | % PMN | Total PMN ($\times 10^4$) |
|---|---|---|---|
| TPC+Pb | 1970.4±59.4 | 56.2±2.1 | 1108.2±56.3** |
| TPC | 1863.6±103.1 | 30.2±3.4 | 562.4±70.8 |
| Pb | 992.4±110.4 | 19.2±2.7 | 194.8±40.1 |
| PBS | 814.8±104.0 | 23.8±1.8 | 195.0±33.0 |

Results are mean±SEM from five mice.

*Supernatants were obtained after incubation 6 h at 37°C of $1 \times 10^6$ TPC with $1 \times 10^6$ Pb in PBS–glucose (4 g/l). A volume of 1.0 ml of each supernatant (experimental and controls) was injected i.p. into groups of five mice and cellular influx evaluated 6 h later.

**Significantly different from control groups ($P<0.01$).

incubated with *P. brasiliensis* (Table 5). The supernatants obtained from non-adherent cell populations in the presence of fungus showed the same activity as controls.

Supernatants obtained after incubation of total peritoneal cells and glass-adherent cells without *P. brasiliensis* also showed some chemotactic activity. This probably was due to some non-specific activation of macrophages, but the activity of these supernatants was never as great as that obtained by incubation of peritoneal cells with fungus.

*Effect of puromycin on the production of chemotactically active supernatants*

The effect of 10 μg/ml puromycin (an inhibitor of protein synthesis) on production of the chemotactic factor was analysed. It significantly decreased the chemotactic activity of supernatants obtained from peritoneal cells incubated with the fungus (Table 6). All controls confirmed the experimental data.

*Preliminary characterization of the chemotactic factor*

Three protein fractions were eluted by Sephadex G-75 gel fractionation of active supernatants obtained from the interaction of mouse peritoneal cells and *P. brasiliensis*. Protein content of pooled eluates was estimated by the folin–phenol reagent. Peak III (which contained the lowest amount of

**Table 5.** Peritoneal PMN influx in B10.A mice inoculated with supernatants obtained from the interaction *in vitro* of glass-adherent (G-Ad PC) and non-adherent (N-Ad PC) peritoneal cells with *Paracoccidioides brasiliensis* (Pb)

| Supernatant* obtained from: | Total cells ($\times 10^4$) | % PMN | Total PMN ($\times 10^4$) |
|---|---|---|---|
| G-Ad PC+Pb | 607.8±70.8 | 62.2±3.6 | 373.0±38.2** |
| G-Ad PC | 544.2±143.1 | 23.0±2.3 | 129.8±36.1 |
| N-Ad PC+Pb | 335.4±49.9 | 23.6±3.0 | 79.8±18.8 |
| N-Ad PC | 470.4±11.9 | 30.4±3.5 | 142.6±15.8 |
| Pb | 363.0±40.0 | 28.4±1.7 | 101.0±7.0 |
| PBS | 346.8±19.6 | 24.4±0.9 | 84.8±5.8 |

Results are mean±SEM from five mice.

*Supernatants obtained 6 h after interaction of peritoneal cells ($0.7 \times 10^6$) and fungus ($0.7 \times 10^6$). A volume of 1.0 ml of each supernatant was inoculated i.p. into groups of five B10.A mice. Cellular influx was estimated 6 h later.

**Significantly different from control groups ($P<0.01$).

**Table 6.** Puromycin effect on the chemotactic factor production by the interaction *in vitro* of total peritoneal cells (TPC) and *Paracoccidioides brasiliensis* (Pb)

| Supernatant obtained from | Total cells ($\times 10^4$) | % PMN | Total PMN ($\times 10^4$) |
|---|---|---|---|
| TPC+Pb | 1365.2±258.5 | 44.8±1.3 | 628.2±141.3* |
| TPC+Pb+Pur† | 655.2±86.3 | 20.2±1.3 | 133.6±20.8 |
| TPC | 1365.4±130.2 | 15.0±0.8 | 200.8±12.5 |
| TPC+Pur | 941.2±160.4 | 15.7±2.2 | 151.0±31.3 |
| Pb | 1018.2±145.0 | 10.8±0.8 | 105.8±8.1 |
| Pb+Pur | 1014.2±175.0 | 13.8±1.5 | 140.2±25.5 |
| Pur | 807.6±126.3 | 13.6±1.1 | 112.2±23.2 |
| PBS | 369.6±51.0 | 12.8±0.8 | 46.2±7.2 |

Results are mean±SEM from five mice.

*Significantly different from control groups ($P<0.01$).

†Puromycin, final concentration 10 μg/ml.

protein) was able to mount a PMN chemotactic response when injected i.p. into B10.A mice (Fig. 1). This fraction eluted with a molecular weight of less than 15 000 daltons. Peaks I and II showed the same activities as controls (supernatant of either peritoneal cells or *P. brasiliensis* incubated on their own).

## Discussion

Products of complement activation have PMN leucocyte chemotactic activity (Fernandez *et al.* 1978; Ward 1968). Previously we demonstrated that *P. brasiliensis* is able to activate the alternative pathway of the complement system (Calich *et al.* 1979); the

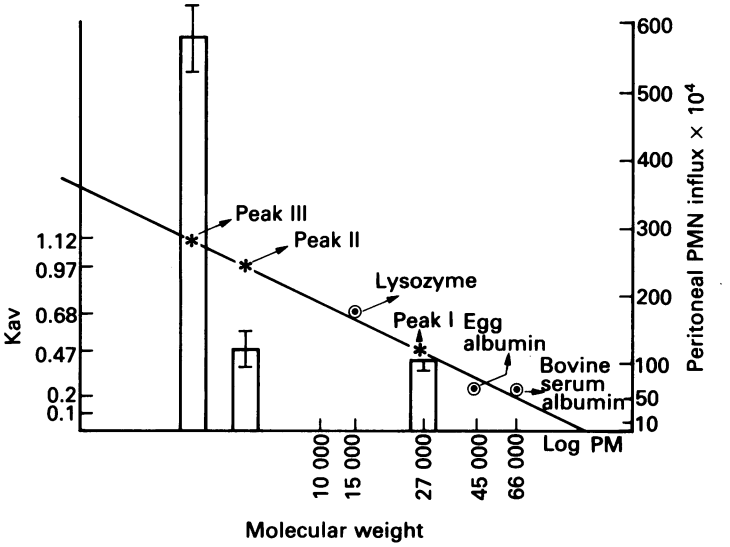


Fig. 1. Chemotactically active supernatants obtained after interaction of total peritoneal cells with *P. brasiliensis* (6 h at 37°C) were fractionated on a Sephadex G-75 calibrated column. As can be observed, only peak III displayed an intense chemotactic activity. Also shown are the Kav of proteins with known molecular weights.

accumulation of PMN at sites of fungus inoculation therefore was attributed to this phenomenon. The present work shows that the complement system does not play an important role in the PMN influx induced by intraperitoneal inoculation of *P. brasiliensis* in mice. The PMN inflammatory response did not differ significantly between normal and C5-deficient mice, and previous depletion of complement system by cobra venom factor did not alter the acute inflammatory exudate.

Chemotactic factors derived from serum and plasma have been identified as products of Hageman factor activation and kinin generating systems (Gallin & Wolff 1975). In addition to these mediators, histamine (Clark *et al.* 1975), ECF-A (Goetzl & Austen 1975), leukotriene B4 (Ford-Hutchinson *et al.* 1981; Goetzl & Pickett 1981) and bacterial derived factors (Schiffmann *et al.* 1975; Showell *et al.* 1976) are all chemotactic. The participation of several other soluble inflammatory agents was investigated in our model. The previous depletion or inhibition of histamine, serotonin, prostaglandins, leukotrienes and coagulation system did not alter PMN influx into the peritoneal cavity of mice inoculated with *P. brasiliensis*.

Mouse peritoneal cells incubated for 6 h with *P. brasiliensis* release a soluble factor that induces active chemotaxis for PMN when inoculated i.p. in Bio.A mice. This factor is produced by adherent cells and its production or release is inhibited by puromycin. A preliminary characterization of the chemotactic factor by sephadex-gel filtration showed an active peak with an apparent molecular weight lower than 15 000 daltons. Several other investigators, working with different models, have described small peptides with chemotactic activity for PMN leucocytes. Lynn *et al.* (1978) characterized two peptides which are synthesized and liberated by neutrophils in the rat pleural cavity, as the major chemotactic agents in experimental pleural inflammation. Another peptide previously isolated from phagocytosing neutrophils was described by Spilberg *et al.* (1976). Cells of the mononuclear phago-

cytic system also were reported to produce chemotactically active peptides. Hunninghake *et al.* (1978) described a neutrophil chemotactic factor of low molecular weight (<5000 daltons) generated by guinea-pig alveolar macrophages. This was produced after phagocytosis of heat-killed *Staphylococcus aureus*.

The present study with another experimental model agrees with previous reports that peptides play an important role in the chemotaxis of polymorphonuclear leucocytes *in vivo*. As in the case of chemotactic responses of PMN leucocytes to bacterial LPS (Russo 1980) our results suggest that macrophages probably provide the starting signal for extravascular neutrophilia. The generation of this chemotactic factor strongly supports the view that peptides are the major chemotactic mediators *in vivo* and provides evidence that peritoneal macrophages serve as initiators of host defense by secreting a potent mediator of the acute inflammatory response.

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