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# Blockade by antimacrophage serum of the migration of PMN neutrophils into the inflamed peritoneal cavity

GLORIA E.P. de SOUZA and S.H. FERREIRA

Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, S.P. Brazil

*Abstract*

**The effect of rat antimacrophage serum (rAMS) was tested on the influence of normal or thioglycollate-stimulated macrophage populations of the rat peritoneal cavity on the migration of polymorphonuclear neutrophils (PMN) induced by carrageenin, heterologous serum (rabbit) and sheep red blood cells. The rAMS used did not cross-react with PMN or lymphocytes nor did it affect circulating white cells, complement levels or lysed PMN present in the inflammatory exudate. It did, however, give a positive immunofluorescence reaction with resident and stimulated macrophages. The rAMS inhibited macrophage function as tested by sheep red blood cell phagocytosis *in vivo* and release of a PMN chemotactic factor(s) *in vitro*. Thioglycollate-stimuated peritoneal cavities showed an increased macrophage population and responded with increased PMN migration when challenged with heterologous serum or carrageenin, as compared with control rats. The presence of rat antimacrophage antibodies inhibited PMN migration induced by heterologous serum, sheep red blood cells and carrageenin. It is concluded that resident macrophages participate in the control of PMN migration to the site of an acute inflammation by acting as 'alarm cells' and triggering several defence mechanisms which ultimately protect the host from injurious stimuli.**

## *Introduction*

There is mounting evidence associating resident macrophages with the migration of polymorphonuclear neutrophils (PMN) to an inflamed site. KAZMIEROWSKI et al. [1] first noted the importance of alveolar macrophages for PMN appearance into the broncho-alveolar lavage fluid of primates and showed that stimulated alveolar macrophages released a low molecular weight factor which specifically stimulated PMN chemotaxis. The ability of harvested alveolar macrophages from several species, including man, to generate PMN chemotactic factor(s) has been confirmed using a variety of stimuli [2–13]. In addition to these *in vitro* observations HUNNINGHAKE et al. [2] showed that phagocytosis of heat-killed *Staphylococcus aureus* by guinea-pig resident alveolar macrophages generated a factor that, when injected intratracheally, increased the absolute number of PMN in broncho-alveolar lavage fluid. Chemotactic activity was also found in the lavage fluid after intratracheal injections of irritants [2, 13]. The generation of chemotactic factor(s) for PMN is not restricted to alveolar macrophages since TONO-OKA et al. [14] showed that blood monocytes generated a similar activity either by simple adhesion to a dish surface or when stimulated by *Salmonella abbortus* lipopolysaccharide and anti-$\beta_2$ microglobulin serum. Involvement of macrophages in the migration of PMN into the peritoneal cavity in response to lipopolysaccharides (LPS) was shown in RUSSO [15]. Using a strain (C3H/HeJ) of mouse resistant to bacterial LPS he observed an increased migration of PMN into the abdominal cavity in response to LPS after repopulation with macrophages derived from a LPS-sensitive histocompatible strain (C3HeB/FeJ). Our laboratory became interested in control of PMN migration by macrophages after we observed a very reduced population of PMN in the inflammatory lesions in rat paws induced by dead *M. tuberculosis* in animals chronically treated with rAMS [16].

The aim of the present investigation was to determine the effect of treatment with rAMS against resident or thioglycollate-stimulated macrophage populations of the peritoneal cavity on

the migration of PMN induced by three different inflammatory stimuli: carrageenin, heterologous serum and sheep red blood cells. In addition we have investigated the effect of rAMS on the release, by harvested peritoneal macrophages, of a factor which stimulates PMN migration when injected into the peritoneal cavity of rats.

*Materials and methods*

**1. Preparation of rat antimacrophage serum (rAMS) and normal rabbit serum (NRS)**

Peritoneal exudate cells were obtained from Wistar rats 4 days after an i.p. injection of 10 ml of 3% thioglycollate (Difco). The cells were harvested in Eagle's medium pH 7.4 (MEM, Difco), containing 5 U/ml of herapin, placed in Erlenmayers and incubated at 37°C for 1 h. Adherent cells were washed three times with heparinized (5 U/ml) phosphate-buffered saline (hPBS) [17] and finally scraped using a rubber policeman. The resuspended cells are washed three times by centrifugation with hPBS and the concentration was adjusted to 1–5 × $10^6$ macrophages/ml. The viability of the cells as tested by 1% eosin Y [18] was 90–95%. New Zealand rabbits were treated weekly for 8 weeks by injecting 2 ml of the cell suspension subcutaneously into the back. For the two initial injections the cell suspensions were emulsified with an equal volume of Freund's complete adjuvant (Difco). The animals were bled 7–10 days after the last injection. The sera were complement-inactivated by heating to 56°C for 30 min, subsequently adsorbed with rat red blood cells (1 vol of the washed red cells with 5 vol of antiserum, for 2 h at 4°C) and acetone tissue powder of several organs (brain, spleen, heart, lung, liver and kidney) for 24 h at 4°C and then sterilized (Millipore filter, 0·2 $\mu$). Control sera consisted of normal rabbit serum similarly treated (NRS, normal rabbit serum).

**2. Characteristics of the rAMS**

The sera were tested for the following:

(a) Indirect immunofluorescence reaction [19, 20] using anti-rabbit globulin conjugated with fluorescein isothiocyanate (Wellcome, London) was tested in macrophages, spleen lymphocytes [21] and a predominantly PMN population obtained from peritoneal exudates induced by carrageenin (Marine Colloids, USA).

(b) Haemolytic activity. Rat red cells were incubated with rAMS (1/20 dilution) with or without guinea-pig complement [22, 23].

(c) Total and differential blood white cell counts and serum complement consumption [22, 23]. For these tests blood was used from tolerant rats treated for 5 days with 0.1 ml, i.p. of either NRS or rAMS.

(d) Lysis of PMN present in inflammatory exudates. The number and viability of the PMN were estimated in the peritoneal exudates 15 min after an injection of either rAMS or NRS (0.1 ml of serum diluted in 5 ml saline) in cavities treated with carrageenin (1 mg) 4 h earlier.

**3. Collection of Peritoneal or pleural cells**

After exsanguination of the rats the cells were harvested from normal or stimulated cavities by injection of 5 ml or 10 ml of hPBS into pleural or peritoneal cavities respectively. Only a fraction of the injected fluid was withdrawn for cell counts (4 ml for pleural and 5 ml for peritoneal cavities). The results are expressed in number of cells/ml of collected fluid.

**4. Total and differential cell count**

Pleural or abdominal washings were placed in plastic tubes and washed twice with hPBS and total cell counts were performed immediately using standard white blood cell pipettes, diluting fluid and a Neubauer chamber. For differential counting a cell pellet was obtained by centrifugation and resuspended in 1 ml of hPBS containing 5% of serum albumin (human or bovine). For all concentrations a simple technique was devised. A piece of Whatman chromatographic paper was fixed to a glass slide using strong sellotape. A hole (4 mm diameter) was previously made through the sellotape and the chromatographic paper. One or two hundred microlitres (containing 3 × $10^3$ to $10^4$ cells) were then added to the well. The fluid was slowly absorbed and the cells evenly adhered to the glass surface not covered by the paper. The paper was then allowed to dry and the sellotape together with the paper were removed. The cells were stained using the method of Rosenfeld and the differential count (100 cells) was made under light microscopy.

**5. Experimental design**

Male Wistar rats weighing 170–200 g were used.

(a) *Effect of 5 days treatment with rAMS on PMN migration induced by carrageenin.* Rats born 24 h previously were made tolerant [24] to rabbit serum by an i.p. injection of 0.1 ml of NRS. The efficacy of this treatment was tested by gel-immunodiffusion [25] in animals receiving prolonged treatment (21 days) with NRS. Tolerant rats received a daily injection s.c. of 0.3 ml of either rAMS or NRS for 5 days after which peritoneal cells were harvested. In another group PMN migration was induced by i.p. administration of 1 mg of carrageenin, 30 min after the last injection of the sera. The cells wece harvested 4 h after the carrageenin injection.

(b) *Effect of a single i.p. administration of rAMS on PMN-migration induced by carrageenin.* Control (NRS) or rAMS (0.1 ml of sera diluted in 2 ml of saline) was given intraperitoneally 30 min before carrageenin injection (100 $\mu$g diluted in 2 ml of saline i.p.). Cell migration was measured 4 h after carrageenin injection in 11 rats.

(c) *Effect of heterologous serum (NRS), rAMS and carrageenin in normal and thioglycollate-stimulated peritoneal cavity.* Thioglycollate treatment was as described in Section 1. Four days later treated animals were injected intraperitoneally with heterologous serum (normal rabbit serum, NRS), rAMS (0.1 ml added to 4.9 ml of saline) or carrageenin. The peritoneal cells were harvested 2 to 4 h after i.p. treatment.

(d) *Effect of rAMS on the phagocytosis and PMN migration induced by sheep red blood cells.* The sera (rAMS or NRS, 0.3 ml diluted in 3 ml saline) were given i.p. 30 min before the intraperitoneal injection of sheep fresh red blood cells (10 × $10^8$ cells in 2 ml of sterile saline). The peritoneal cavities were washed 30 min or 2 h after the injection of red cells. The total count was made in the washings which were subsequently centrifuged. The cell pellet was then submitted to a hypotonic shock (incubation with 3 ml of distilled water for 20 sec followed by the restoration of isotonicity by addition of 1 ml of 3.6% NaCl) to lyse non-phagocytosed red cells and the same routine for differential cell count was followed. Phagocytosis was evaluated by counting 200 cells

and the results are expressed as percent of macrophages showing ingested red cells.

(*e*) *Inhibition by rAMS of the in vitro release by macrophages of a factor which stimulates PMN migration in vivo.* Two experiments were performed. In the first, peritoneal resident macrophages from 15 rats were harvested with 10 ml of Eagle's medium pH 7.4 without heparin and divided into the following three treatment groups: PBS alone or PBS containing NRS or rAMS (diluted to a final concentration of 1:80). The cells ($3 \times 10^7$ cells/8 ml per 250 ml Erlenmayer) were allowed to adhere for 1 h at 37°C. The supernatants were discarded. The supernatants of the three groups contained an equivalent number of non-adherent cells. The monolayers were washed three times with PBS. After 1 h of incubation in PBS the monolayers were scraped and the cell suspensions were removed. There was no difference in the number of adhered macrophages among the three treatments. The cell suspensions were then centrifuged and the supernatants sterilized (Millipore 0.2 $\mu$) and kept at 20°C overnight. An equal volume (3 ml) obtained from each treatment was injected into the abdominal cavity of test rats ($n = 5$). In the second experiment macrophages were harvested from the peritoneal cavity with 10 ml of RPMI-1640 medium (Difco) and allowed to adhere for 1 h. The cell monolayers were washed 5 times with the medium and cultured for 24 h in medium supplemented with $NaHCO_3$ (2 g/l). L-arginine (0.2 g/l), folic acid (12 mg/l), asparagine (3.6 mg/l), HEPES (2.38 g/l), 2 mercapto-ethanol (2 $\mu$l/l, v/v), L-glutamine (20 g/l), garamicine (40 mg/l), and 20% heat-inactivated foetal calf serum. After being cultured the cell monolayers were again washed 5 times with the medium without calf serum and glutamine and incubated with the medium alone or containing NRS or rAMS (1:80). After 1 h of incubation (37°C) the monolayers were washed with PBS (5 times) and incubated for 1 h in the same buffer. The monolayers were then treated as in the first experiment.

## *Results*

### 1. Characteristics of the rAMS

Using serial dilution of sera, resident or stimulated macrophages reacted positively the indirect immunofluorescence reaction with rAMS (1:640) while lymphocytes or PMN reacted to a dilution of 1:20. The rAMS did not cause haemolysis, consumption of serum complement or changes in the blood differential white cell counts. The rAMS did not affect the number and viability of PMN present in the carrageenin-induced exudates.

### 2. Effect of 5 days treatment with rAMS on PMN migration induced by carrageenin

Subcutaneous administration of rAMS for 5 days reduced the number of resident macrophages in the abdominal cavity (Fig. 1). There was also a significant diminution of the number of lymphocytes ($9.39 \pm 1.35$ to $2.8 \pm 0.7 \times 10^5$ cells/ml), not shown in Fig. 1. The rAMS significantly reduced the PMN migration induced by carrageenin (Fig. 1).

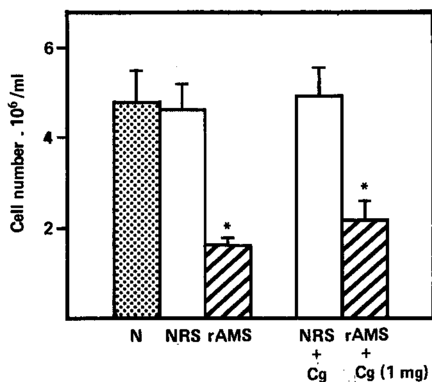


*Figure 1*
Effect of rAMS administration for 5 days on PMN migration induced by injection of carrageenin in the abdominal cavity of rats. The rAMS and control NRS were injected (s.c. 0.3 ml daily) for 5 days. The bars on the left show the number of resident macrophages in naive rats (N), those pretreated with control normal rabbit serum (NRS) and with antimacrophage serum (rAMS). The bars on the right show the number of PMN present in the cavity 4 h after being challenged with carrageenin i.p. (Cg 1 mg/animal). The results are expressed as the number of cells/ml of peritoneal wash fluid. The bars represent the means of 6–7 animals ± SEM. $^*P < 0.05$.

### 3. Effect of a single i.p. administration of rAMS on carrageenin-induced PMN migration

Control NRS did not affect the PMN migration induced by carrageenin (100 $\mu$g) into naive cavities. The control migration ($3.4 \pm 0.22 \times 10^6$ cells/ml) was significantly reduced by pretreatment of the cavities with rAMS ($1.2 \pm 0.3 \times 10^6$ cells/ml).

### 4. Effect of heterologous serum and carrageenin in thioglycollate-stimulated peritoneal cavities

Figure 2 shows that TG-stimulated cavities have an increased number of macrophages and lymphocytes (not shown in the figure) and respond with a greater emigration of PMN to carrageenin challenge. The number of other cell types (eosinophils, mast cells) was not altered by TG treatment. TG-stimulated animals ($n = 8$) responded with increased migration of PMN ($4.7 \pm 0.8 \times 10^6$ cells/ml) to normal rabbit serum (heterologous serum) as compared with naive animals ($2.5 \pm 0.3 \times 10^6$ cells/ml), not shown in Fig. 3).

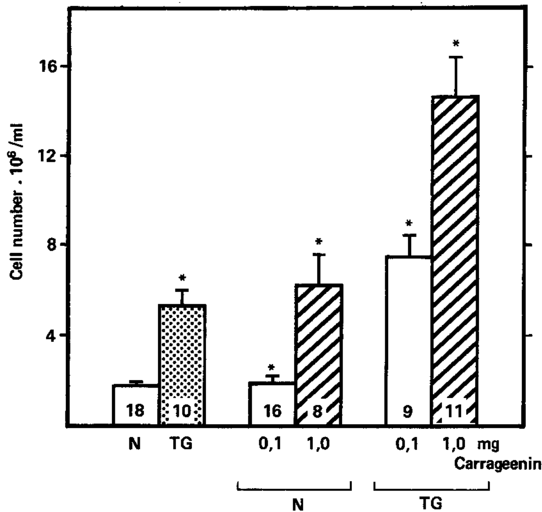


*Figure 2*
Increased migration of PMN induced by two different doses of carrageenin in thioglycollate-treated animals. The two columns on the left indicate the number of macrophages in normal (N) and thioglycollate (TG)-stimulated peritoneal cavities. The other two blocks of columns show the migration induced in the peritoneal cavity 4 h after the administration of carrageenin in N and TG-stimulated animals, respectively. The bars represent the means ± SEM. The number of animals is indicated in each bar. $^*P < 0.05$.

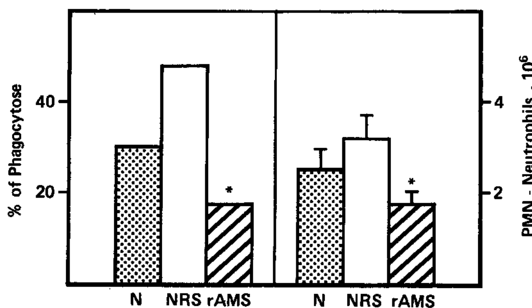


*Figure 3*
Effect of rat antimacrophage serum (rAMS) on the phagocytosis by resident macrophages and PMN migration to the peritoneal cavity induced by an injection of sheep red blood cells. The left panel shows the percentage of phagocytosing macrophages and the right panel shows the number of PMN present in the cavity 2 h after challenge with the sheep red blood cells. The bars for naive (N), normal rabbit sera (NRS) and rAMS-treated animals represent the mean of the results using 7–9 animals. $^*P < 0.05$.

The local cell contribution of TG treatment was tested by injecting the pleural cavity with TG and inducing migration in the abdominal cavity. TG treatment increased the pleural macrophage population from $1.8 \pm 0.98 \times 10^6$ to $7.43 \pm 0.89 \times 10^6$ cells/ml but there was no difference in PMN migration induced by carrageenin injected in the abdominal cavity (pleural TG-treated $7.9 \pm 0.7 \times 10^6$ cells/ml, naive animals $6.2 \pm 1.4 \times 10^6$ cells; $n = 8$).

### 5. Effect of rAMS on the migration induced by heterologous serum in normal and thioglycollate-stimulated peritoneal cavity

NRS injected in normal abdominal cavities causes, after 4 h, PMN migration ($2.5 \pm 0.3 \times 10^6$ cells/ml; $n = 14$) which is significantly reduced ($1.1 \pm 0.2 \times 10^6$ cells/ml; $n = 16$) by the presence of rat antimacrophage antibodies. The rAMS also reduced in TG-stimulated rats the PMN migration induced by control NRS (from $2.7 \pm 0.5 \times 10^6$ cells/ml to $1.0 \pm 0.2 \times 10^6$ ml; $n = 8$) measured 2 h after the administration of the sera. This reduction was not statistically significant when measured 4 h after the i.p. injections (from $4.7 \pm 0.8 \times 10^6$ to $3.1 \pm 0.5 \times 10^6$).

### 6. Effect of rAMS on the phagocytes and PMN migration induced by sheep red blood cells

Injection of sheep red blood cells into the abdominal cavity leads to phagocytosis by macrophages and to PMN migration (Fig. 3). This figure shows that administration of rAMS inhibited the phagocytosis and PMN migration. The inhibition of the phagocytosis by rAMS was more pronounced 30 min after adinistration of the sheep red blood cells, when the PMN migration to the cavity was negligible (not shown in the figure). At this time the percentage of phagocytosing macrophages in animals treated with NRS was reduced from 65% to 4% in rAMS-treated rats.

### 7. Inhibition of rAMS of the in vitro release by macrophages of a factor which stimulates PMN migration in vivo

Figure 4 shows that incubation of cultured and non-cultured peritoneal cell monolayers releases into the supernatant a factor which, when injected into the peritoneal cavity, induced PMN migration. The PMN stimulating activity present in the supernatant was significantly increased when these cells were treated with NRS and was strongly inhibited by rAMS. The culture monolayers showed a more homogeneous macrophage population (98%) than non-cultured monolayers (60–70%).

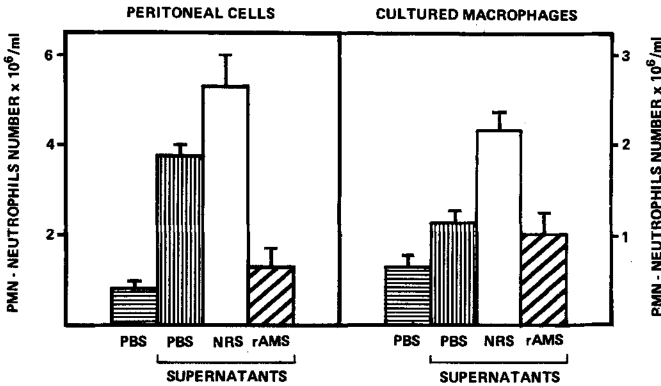


*Figure 4*
Inhibition by rat antimacrophage serum (rAMS) of the *in vitro* release by adhered peritoneal cells (left panel) or cultured macrophages (right panel) of a factor which stimulates PMN migration when injected in the peritoneal cavity of rats. The columns show the PMN migration induced by the injection i.p. into cat peritoneal cavities of 3 ml of supernatant obtained from the incubation of macrophage monolayers without treatment (PBS) or pretreated with control heterologous serum (NRS) or rAMS. The first bar from the left is the PMN migration induced by an injection of PBS alone. The bars represent the means and SEM of 5 animals in the left panel and 8–10 rats in right panel.

*Discussion*

Rats tolerant to rabbit serum showed a reduction in the number of macrophages and lymphocytes in the peritoneal cavities after 5 days of s.c. treatment with rAMS and exhibited a significant reduction of PMN migration when challenged with carrageenin. This result is in line with our previous observation that prolonged treatment with rAMS reduced PMN migration into lesions induced in rat paws by a preparation of dead *M. tuberculosis* [16]. The reduced PMN migration after rAMS treatment could not be a consequence either of a change in the circulating white cell population or of a consumption of serum complement. We have no explanation why our serum did not alter plasma serum complement. Because of the concomitant reduction of lymphocytes the diminution of resident macrophages could not be strictly associated with the reduced PMN migration induced by carrageenin. It is, however, improbable that lymphocytes play any major role in the control of PMN migration since it has been demonstrated that stimulated lymphocytes, in contrast with stimulated monocytes, do not release PMN chemotactic factors [14, 26]. On the other hand, the diminution of peritoneal lymphocytes by rAMS could be secondary to the diminution of macrophages since these cells are known to release chemotactic factors for lymphocytes [6, 12, 27].

In this paper we have also shown that rabbit serum containing antimacrophage antibodies (rAMS) administered to a normal peritoneal cavity significantly reduced the PMN migration evoked by carrageenin, heterologous serum (rabbit normal serum) and sheep red blood cells. The rAMS also reduced the PMN migration induced by heterologous sera in cavities in which macrophage population was increased with thioglycollate. Thus, the rAMS seems to affect both resident and stimulated macrophage populations. This effect may reflect an already described impairment of macrophage function [28, 29] by rAMS which prevents the release of a putative PMN chemotactic factor(s). In fact *in vivo* rAMS strongly inhibited sheep red blood cell phagocytosis by resident macrophages and significantly reduced PMN migration which may reflect a blockade of the release of a chemotactic factor during phagocytosis. It is well established that phagocytosing alveolar macrophages release PMN chemotactic factor(s) [2, 5]. These factor(s) are also released into the incubating fluid by simple adhesion of the macrophages or monocytes to a glass surface [5, 14]. Furthermore, as we have shown in this paper, rAMS caused a significant reduction of the PMN chemotactic activity expontaneously generated *in vitro* by monolayers of glass adhered macrophages.

The participation of macrophages in the control of PMN migration could be also appreciated when the number of peritoneal cells was increased by previous treatment of the cavities with thioglycollate. In this instance

carrageenin or heterologous sera challenge produced a much greater cell migration than in naive animals. A systemic non-specific effect of thioglycollate treatment upon cell migration could be ruled out since similar treatment of the pleural cavity, which provoked an equivalent increase of macrophage population, did not affect carrageenin-induced migration of PMN in the abdomonal cavities.

Although it is plausible that a phlogogenic stimulus can directly active plasma systems or injure local cells with liberation of chemotactic factors, the experiments described in this paper stress the importance of the resident macrophages in modulating PMN migration. Furthermore, it seems that this is a general property of macrophages and not peculiar to the alveolar macrophages. It was beyond the scope of this work to define the biochemical nature of this factor(s) which has been carefully reviewed by REYNOLDS [30] for alveolar macrophages. It is thought that the major chemotatic activity is due to a peptide.

In summary, our rAMS appears to be specific for peritoneal macrophages since it did not cross react with PMN or lymphocytes, as shown by the indirect immunofluorescence reaction, nor did it affect circulating white cells or emigrated PMN, but it did reduce macrophage functions. This rAMS blocked *in vitro* the release of a chemotactic factor(s) and reduced the PMN accumulation in the peritoneal cavity induced by three different stimuli namely carrageenin, sheep red blood cells and heterologous serum. The growing understanding of the role of macrophages in commanding PMN migration to the site of acute inflammation supports our concept that resident macrophages act as 'alarm cells' triggering several defense mechanisms which ultimately protect the host from an injurious stimulus [31].

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