PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 326–330 (1980)

The Role of Macrophages in the Chemotactic Response of Polymorphonuclear Leukocytes to Bacterial Lipopolysaccharides (40871)[1]

MOMTCHILO RUSSO

Departamento de Microbiologia e Immunologia, Instituto de Ciencias Biomedicas, Universidade de Sao Paulo, Sao Paulo, Brazil

Abstract. C3H/HeJ mice do not respond to the toxic and immunological properties of LPS whereas histocompatible C3HeB/FeJ do respond. The unresponsiveness of C3H/HeJ strain is genetically determined and occurs primarily at the cellular level. In the present study, the author investigated the peritoneal PMN influx induced after i.v. LPS administration in C3H/HeJ recipients that received i.p. different C3HeB/FeJ or C3H/HeJ peritoneal cells. It is shown that a significant PMN migration into the peritoneal cavity does occur after i.v. LPS administration only in C3H/HeJ recipients previously inoculated with C3HeB/FeJ unseparated or adherent peritoneal cells. The peritoneal PMN influx after i.v. LPS injection was not observed in C3H/HeJ recipients that received i.p. C3HeB/FeJ nonadherent peritoneal cells or C3H/HeJ unseparated peritoneal cells. Our data suggests that the peritoneal chemotactic signal is triggered after the interaction of LPS with the adherent peritoneal cell population.

The mechanisms responsible for the accumulation of polymorphonuclear leukocytes (PMN)[2] at sites of inflammation are not well understood. The early migration of PMN in inflammatory exudates induced by gram-negative bacterial lipopolysaccharides (LPS) is through to depend on the activation of the fifth component (C5) of the complement system (C) (1). However, in C5 deficient strains of mice, a delayed but still significant influx of PMN is observed after i.p. LPS administration (1, 2).

The concept that the inflammatory response to LPS is under genetic control has been advanced by the discovery of a unique strain of mice (C3H/HeJ), that does not present several of the LPS induced responses observed in other strains. Thus, C3H/HeJ mice are highly resistant to endotoxin shock (3), induce a poor immune response to LPS (4, 5), are refractory to macrophage activation (6–8), and show decreased colony stimulating activity (CSA) response to endotoxin (9, 10).

The present study was prompted by an observation noted while working with C3H/HeJ recipients that received i.p. C3HeB/FeJ histocompatible peritoneal cells and challenged i.v. with LPS. It was found that the recipients peritoneal cavity was densely populated by PMN cells. This finding was rather surprising since it is known that mice receiving a single i.v. LPS injection experience only small increases in the number of neutrophils in their peritoneal fluid (11).

This observation raised the question whether the chemotactic stimuli induced by LPS arises from its interaction with cells.

To evaluate this possibility C3H/HeJ recipients were injected i.p. with different C3HeB/FeJ cell types and the peritoneal chemotactic response after i.v. LPS administration quantified.

C3HeB/FeJ donors were selected because contrasting with the C3H/HeJ mice, they are susceptible to LPS and there are no measurable histocompatibility differences between donor and acceptor strains (12).

Evidence obtained with the experiments described herein, suggest that macrophages

[1] This work was supported by Grant from Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (78/1101) and NIH Grants CA 15565 and AI 10969 to Dr. M. Rabinovitch.

[2] Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; LPS, bacterial lipopolysaccharides, C, complement system; PBS, phosphate-buffered saline; i.v., intravenous or intravenously; i.p., intraperitoneal or intraperitoneally.

0037-9727/80/070326-05$01.00/0

Copyright © 1980 by the Society for Experimental Biology and Medicine.
All rights reserved.

play a role in the starting signal of extravascular neutrophilia induced after LPS injection.

Materials and methods. Animals. Genetically inbred female C3H/HeJ and C3HeB/FeJ mice, 20–25 g body weight were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Endotoxin. LPS from *Salmonella abortus* equi Lot:613234, phenol–water extraction methods, from Difco Laboratories, Detroit, Michigan, was used throughout the experiments.

Irradiation. Mice were irradiated with 720 rads in an acrylic plastic box by a Gammator M^{137} cesium source (Radiation Machinery Corp., Parrisppany, N.J.) delivering 630 rads/min.

Peritoneal cell suspensions. Resident peritoneal cells were aseptically collected after i.p. injection of 3 ml Ca^{2+}, Mg^{2+}-free PBS. The cell suspensions were incubated in PBS supplemented with 20% heat inactivated fetal bovine serum for 30 min at room temperature in 60-mm Falcon tissue culture dishes. The nonadherent peritoneal cells were aspirated from the supernatant after repeated washing. The remaining adherent peritoneal cells were detached from culture dishes by treatment with 12 m*M* Lidocaine hydrochloride (Astra Pharmaceutical Co., Worcester, Mass.) for 10 min at room temperature followed by 10 jets of PBS using a syringe (M. Rabinovitch, personal communication). The purity of nonadherent and adherent cell fractions consisted of 80% lymphocytes and 89% macrophages, respectively, as judged by morphological criteria.

All cell suspensions were washed twice (900 rpm for 10 min) and adjusted to the desired concentration. The cell viability assessed by trypan blue exclusion test was greater than 95%.

Experimental Protocol. Group I (nonirradiated recipients). C3H/HeJ nonirradiated recipients were inoculated i.p. with 2.0×10^6 unseparated peritoneal cells obtained from C3HeB/FeJ or C3H/HeJ strains. Twelve hours later the recipients received an i.v. challenge of 20 μg of LPS. Six and twenty four hours after the LPS injection the animals were sacrificed with chloroform and their peritoneal cavities washed with 3 ml of Ca^{2+}, Mg^{2+}-free PBS for total and differential cell counts. Control groups did not receive LPS.

Group II (irradiated recipients). C3H/HeJ recipients were lethally irradiated (720 rads) and 48 hr later divided in four experimental subgroups that received i.p., respectively, (IIa) 2.0×10^6 C3HeB/FeJ unseparated peritoneal cells, (IIb) 0.5×10^6 C3HeB/FeJ adherent peritoneal cells, (IIc) 0.5×10^6 C3HeB/FeJ nonadherent peritoneal cells, and (IId) 2.0×10^6 C3H/HeJ unseparated peritoneal cells. Three days after the cell transfer, the irradiated recipients were challenged i.v. with 20 μg of LPS and 24 hr later the animals were sacrificed and total and differential counts quantified.

Total cell numbers in the peritoneal washings were determined with a hemocytometer. Differential counts were performed on fixed and stained cell suspensions utilizing 0.05% crystal violet in 3% acetic acid or in brush preparation stained with Giemsa.

Results. Production of chemotactic activity by adoptive transfer of peritoneal cells after i.v. LPS administration. Previous findings had suggested that C3HeB/FeJ peritoneal cells transferred i.p. to C3H/HeJ recipients induce an i.p. PMN influx after i.v. LPS administration. The initial experiments were carried out to confirm this observation.

Nonirradiated C3H/HeJ recipients (Group I) were inoculated i.p. with 2.0×10^6 unseparated peritoneal cells from C3HeB/FeJ or C3H/HeJ strains and 12 hr later, the recipients were injected i.v. with 20 μg of LPS. Six and twenty four hours after LPS. Six and twenty four hours after LPS injection the total and differential leukocytes count of the peritoneal exudate was quantified. Control groups did not receive LPS. As can be seen in Table I, the transferred C3HeB/FeJ unseparated peritoneal cells induced a significant influx of PMN into the peritoneal cavity of C3H/HeJ recipients after LPS challenge. The i.p. migration of inflammatory cells reached its maximum

value at 6 hr with a slight decrease at 24 hr. Control groups, not injected with LPS, or C3H/HeJ recipients that received C3H/HeJ unseparated peritoneal cells showed only a minor increase in the PMN content of their peritoneal cavity (Table I).

Results obtained above confirm that the C3HeB/FeJ peritoneal cell population mediate the i.p. influx of PMN observed in the C3H/HeJ recipients after systemic LPS administration.

In order to explore in more detail what type of peritoneal cell are responsible for the production of chemotactic activity and to minimize the recipients immunological response, experiments were performed in irradiated recipients which received i.p. different peritoneal cell types (Group II).

C3H/HeJ recipients were lethally irradiated (720 rads) and 48 hr later divided in four subgroups which received i.p. (IIa) 2.0 $\times$ 10^6 CeHeB/FeJ unseparated peritoneal cells, (IIb) 0.5 $\times$ 10^6 C3HeB/FeJ adherent peritoneal cells, (IIc) 0.5 $\times$ 10^6 C3HeB/FeJ nonadherent peritoneal cells, and (IId) 2.0 $\times$ 10^6 C3H/HeJ unseparated peritoneal cells. Three days after the cell transfer, the irradiated recipients were challenged with 20 μg of LPS i.v. and 24 hr later the total and differential leukocyte count of the peritoneal exudate quantified.

Table II shows that a significant i.p. PMN migration after i.v. LPS administration occurred only when the C3H/HeJ recipients were inoculated i.p. with C3HeB/FeJ unseparated or adherent peritoneal cells. Neither C3HeB/FeJ nonadherent peritoneal cells nor C3H/HeJ unseparated peritoneal cells were able to mediate the LPS induced PMN migration response in C3H/HeJ irradiated recipients.

These experiments again confirm that the peritoneal cell population of the C3HeB/FeJ strain transferred to the C3H/HeJ recipients, is responsible for the i.p. inflammatory response induced after i.v. LPS administration, and adds further evidence that the adherent peritoneal cells (probably macrophages) are necessary for an effective chemotactic response triggered by LPS.

Discussion. The results reported in this paper indicate that the C3HeB/FeJ unseparated or adherent peritoneal cells transferred to C3H/HeJ recipients mediate the production of chemotactic activity after i.v. LPS administration.

From our experiments we concluded that most likely the PMN chemotactic stimuli arises from the interaction of LPS with the population of adherent peritoneal cells (mainly macrophages). This appears to be corroborated by the observation that 0.5 $\times$ 10^6 C3HeB/FeJ adherent peritoneal cells were as effective as 2.0 $\times$ 10^6 C3HeB/FeJ unseparated peritoneal cells and by the fact that 0.5 $\times$ 10^6 C3HeB/FeJ nonadherent cells were almost ineffective in mediating the i.p. PMN influx. Furthermore, cell transfers with spleen cell population that contains less than 10% of macrophages,

TABLE I. INTRAPERITONEAL LEUKOCYTE RESPONSE OF C3H/HeJ MICE TO i.v. ENDOTOXIN AFTER i.p. CELL TRANSFER OF C3HeB/FeJ OR C3H/HeJ PERITONEAL CELLS[a]

Peritoneal cells transferred from	Time after LPS (hr)	Dose of LPS (μg)	Total leukocytes in exudates ($\times 10^6$)	Percentage of neutrophils	P value
C3HeB/FeJ	6	None	10.82 ± 0.18	14.00 ± 1.31	$P < 0.001$
C3HeB/FeJ	6	20	9.62 ± 0.55	41.00 ± 3.27	
C3HeB/FeJ	24	None	10.06 ± 0.50	6.60 ± 1.52	$P < 0.001$
C3HeB/FeJ	24	20	10.44 ± 1.33	35.2 ± 1.46	
C3H/HeJ	6	None	11.88 ± 1.05	17.20 ± 1.77	NS
C3H/HeJ	6	20	11.42 ± 1.74	21.00 ± 2.24	

[a] Groups of five nonirradiated C3H/HeJ mice were injected i.p. with 2.0 $\times$ 10^6 C3HeB/FeJ or C3H/HeJ unseparated peritoneal cells and 12 hr later challenged i.v. with 20 μg of LPS. Six and twenty four hours after the LPS injection, the animals were sacrificed and the peritoneal exudates quantified by total and differential leukocyte count. Values represent the mean ± SEM. P values were determined from the t statistic for significance of differences of the mean (unpaired) for each set of results.

TABLE II. INTRAPERITONEAL LEUKOCYTE RESPONSE OF IRRADIATED C3H/HeJ MICE INJECTED i.p. WITH DIFFERENT CELL TYPES TO i.v. ENDOTOXIN[a]

Peritoneal cells transferred from	Number and cell type	Dose of LPS (μg)	Total leukocytes in exudates ($\times 10^6$)	Percent of neutrophils	Number of mice
C3HeB/FeJ	Unseparated 2.0×10^6	None	1.80 ± 0.09	0.71 ± 0.47	7
C3HeB/FeJ	Unseparated 2.0×10^6	20	2.60 ± 0.33	47.11 ± 2.26	18
C3HeB/FeJ	Adherent 0.5×10^6	20	0.70 ± 0.06	45.67 ± 3.35	6
C3HeB/FeJ	Nonadherent 0.5×10^6	20	0.70 ± 0.11	2.75 ± 1.18	4
C3H/HeJ	Unseparated 2.0×10^6	20	2.00 ± 0.38	2.00 ± 0.92	4

[a] C3H/HeJ recipients were lethally irradiated and 48 hr later injected i.p. with whole or fractionated peritoneal cells as indicated in the first and second column (left). The recipients were challenged with 20 μg of LPS i.v. 3 days after the cell transfer and 24 hr later, the mice were sacrificed and the peritoneal exudates quantified by total and differential leukocyte count. Mean ± SEM.

also were not able to mediate the i.p. PMN influx (13).

At present, little can be said about the chemical nature of the true chemotactic factor or the mechanism by which the chemotactic activity mediated by macrophages is generated. It will be interesting to learn whether the chemotactic factor is produced directly by a cell product or indirectly, by the interaction of the cell, or its products, with humoral factors.

The importance of C5 in the generation of complement dependent chemotactic activity *in vitro* as well as *in vivo* is recognized (1, 14). However, it is unlikely that the chemotactic activity mediated by macrophages observed in our experiments is due to the production of C5a, since LPS were administered i.v. and so generating a C5a chemotactic factor in the circulation and not in the peritoneal cavity. Furthermore, animals from C5 deficient strains or decomplemented by cobra venom factor were shown to exhibit a normal chemotactic response mediated by macrophages (M. Russo, unpublished observations).

There is a possibility that the recipients were repopulated with C3HeB/FeJ PMN or other precursor cells present among the donor peritoneal population and that these cells would be responsible for the chemotactic response to LPS rather than the transferred macrophages. This appears to be unlikely in the light of experiments in which 1.0×10^6 C3HeB/FeJ bone marrow cells (rich in PMN and precursor cells) were injected i.p. but no evidence of PMN migration could be observed (13).

A number of studies using different inflammatory agents have demonstrated a cell derived chemotactic factor(s) (15–18), but the relationship between the LPS induced macrophage mediated chemotactic activity and the cell derived chemotactic materials produced by other agents is unclear at the present.

The findings presented here clearly established the requirement of adherent peritoneal cells for the production of chemotactic activity and suggest that macrophage response to LPS is responsible for the initiation of the chemotactic response.

Thanks to Dr. W. Dias da Silva and Dr. Maro Mariano for the thoughtful review of the manuscript. The advice of Dr. S. H. Ferreira and support of Dr. M. Rabinovitch are gratefully acknowledged.

1. Snyderman, R., Phillips, J. K., and Mergenhagem, S. E., J. Exp. Med. **134**, 1131 (1971).
2. Sultzer, B. M., Infect. Immunol. **5**, 107 (1972).
3. Sultzer, B. M., Nature (London) **219**, 1253 (1968).
4. Watson, J., and Riblet, R., J. Exp. Med. **140**, 1147 (1974).
5. Coutinho, A., Moller, G., and Gronowicz, E., J. Exp. Med. **142**, 253 (1975).
6. Chedid, L., Parrant, M., Damais, C., Parant, F.,

Juy, D., and Galelli, A., Infect. Immunol. **13**, 722 (1976).
7. Bianco, C., and Edelson, P. J., Fed. Proc. **36**, 1263 (1977).
8. Ruco, L. P., and Meltzer, M., J. Immunol. **120**, 329 (1978).
9. Russo, M., and Lutton, J. D., J. Cell Physiol. **92**, 303 (1977).
10. Apte, R. N., and Pluznik, D. H., J. Cell Physiol. **89**, 313 (1976).
11. Frumman, G. J., R.E.S. **12**, 62 (1972).
12. Watson, J., and Riblet, R., J. Immunol. **114**, 1462 (1975).
13. Russo, M., "Proc. Future Trends in Inflammation IV," in press.
14. Mergenhagen, S. E., Snyderman, R., Gewurz, H., and Shin, H. S., Curr. Topics Microbiol. Immunol. **50**, 37 (1969).
15. Ryan, G. B., and Hurley, J. V., Brit. J. Exp. Pathol. **47**, 530 (1966).
16. Borel, J. F., Int. Arch. Allergy Appl. Immunol. **39**, 247 (1970).
17. Zigmond, S. H., and Hirsh, J. G., J. Exp. Med. **137**, 387 (1973).
18. Kazmierowski, J. A., Gallin, J. I., and Reynolds, H. Y., J. Clin. Invest **59**, 273 (1977).

Received November 13, 1978. P.S.E.B.M. 1980, Vol. 164.