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REQUIREMENT FOR LIPOPOLYSACCHARIDE FOR ENHANCED *IN VITRO* SUPEROXIDE PRODUCING COMPETENCE IN MACROPHAGES FROM NORMAL AND MALARIA (*PLASMODIUM CHABAUDI*) INFECTED MOUSE SPLEEN

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Nucleated cells from mouse spleens were suspended in RPMI-1640 medium and plated in polystyrene tissue culture dishes with or without lipoplysaccharide (LPS, endotoxin) and incubated at 37°C for 3 h in a humid incubator under 5% CO_2. Adherent spleen cells obtained from uninfected control or *Plasmodium chabaudi*-infected mice showed negligible superoxide (O_2^-) production *in vitro*, in response to triggering with phorbol myristate acetate (PMA). However, O_2^- release increased in a concentration-dependent fashion as LPS was introduced above a threshold of about 10 ng ml. This LPS dependence is at least 100 times more than that required by mouse peritoneal macrophages and 10^6 times greater than the requirement reported for human monocytes. These findings emphasize the differences among the three mononuclear cells, and also suggest that LPS could prove useful in the isolation and culture of other metabolically competent cells.

KEY WORDS: Lipopolysaccharide, superoxide, macrophages, malaria infection.

INTRODUCTION

The importance of the spleen in the intravascular clearance of plasmodial,[1] babesial,[2] and bacterial[3,4] infections has been emphasized. The defence function of the spleen results from both humoral and cell-mediated immune responses,[4] following from integration of interactions between the various types of cells (macrophages, T- and B-lymphocytes, etc.) making up this organ, and the invading pathogen. The central role of mononuclear cells in these events has also been highlighted.[4-6]

In recent years many of the major insights into the underlying mechanisms of these immune responses have benefitted from the application of tissue and cell culture techniques which involve the isolation of specialized cells from the spleen and other organs of the reticuloendothelial system. In order to isolate functionally-competent cells for *in vitro* assays in tissue culture, it has become traditional to adopt a number of enzymatic and non-enzymatic treatment of tissues.[7-11]

Reported herein are data consistent with the fact that reasonable *in vitro* competence of O_2^- production can be achieved in adherent spleen cells (macrophages) isolated from normal control and from malaria (*P. chabaudi*) infected mice only if these cells are exposed to high endotoxin (lipopolysaccharide, LPS) concentrations (above 10 ng/ml) during the period of incubation for adherence. This LPS requirement is several orders of magnitude greater than that earlier reported for mouse peritoneal macrophages[12] and human blood monocytes.[13]

* Work performed in the Laboratories of Professors B.M. Babior and D.J. Wyler at the New England Medical Center Hospitals, Boston, U.S.A., while the author was a Public Health Service International Research Fellow, Fogarty International Center, N.I.H., Bethesda, U.S.A.

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MATERIALS AND METHODS

Animals and Malaria Parasites

Strain C57 BL/6 female black mice (free from virus infection) were purchased from Charles River Breeding Laboratories Inc., Wilmington, Massachusetts at 16–18 g. These were used within not more than two months thereafter. CBA female mice were obtained from Jackson Laboratories at 5 weeks old and used as soon as obtained. *Plasmodium chabaudi* maintained by passaging through rats (the kind gift of Dr. Ross Mikkelson of the Division of Radiobiology, New England Medical Center Hospital, Boston, USA) was passaged through mice by intraperitoneal injection of 5×10^6 parasitized erythrocytes. The parasites were always used for infection after the second passage (at least) when the parasitemia developed reproducibly well, reaching up to at least 50% at peak.

Reagents, Media and Chemicals

Hanks Balanced Salts Solution (HBSS) without phenol red, and Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Grand Island Biochemicals (GIBCO) New York, while HBSS with phenol red, and RPMI-1640 were supplied by the media laboratory of the Cancer Institute, Tufts University Medical School, Boston. Endotoxin (*Salmonella enteritidis* lipopolysaccharide) type IV, phorbol-myristate acetate (PMA) and cytochrome c type VI were purchased from Sigma Chemical Company, St. Louis, Missouri. Foetal Calf Serum (FCS) was Hyclone brand. All other chemicals and reagents were of the highest purity available and were obtained from standard sources.

Tissue Culture Ware

Plastic 24-well (16 mm diameter-well) culture dishes and other tissue culture ware were supplied by Corning.

Isolation of Spleen Cells and Purification of Spleen Adherent Cells

The experimental mouse was sacrificed by narcosis with CO_2 and the spleen immediately excised and immersed in 0.5 ml HBSS in a 50 ml Corning polypropylene centrifuge tube. From this point on, all operations were carried out on ice. The spleen was teased up in this polypropylene centrifuge tube using small curved scissors, and then passed through 20-gauge spinal needle at least ten times and then through a 23-gauge spinal needle at least 3 times. The resulting cell suspension was mixed with more HBSS before the cells were obtained by centrifugation at 4°C and at 800 g for 15 min. The red blood cells were lysed by the addition of 8 ml of warm (37°C) Tris/NH_4Cl pH 7.4 (0.83% NH_4Cl/0.17 M Tris. HCl, pH 7.4), then vortexing and incubating at 37°C for 3 min. This was then diluted to about 35 ml with HBSS and centrifuged again as above. These cells were re-suspended to about 20×10^6 per ml in RPMI-1640 medium supplemented with penicillin (100 iu/ml)/streptomycin (100 μg/ml), and 2 mM L-glutamine. They were then plated in 16 mm diameter wells (Corning 24-well polystyrene dishes) at 1.5×10^6 nucleated cells per well in a total volume of 400 μl with heat inactivated FCS supplemented to a final concentration of 10%, and with or without LPS as desired. For the purposes of counting adherent cells,

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larger Corning polystyrene tissue culture dishes (60 mm diameter) were used and the cells plated at the same cell density and other concentrations kept the same as in the 16 mm wells.

These plates were then incubated for 3 h in a humid, 5% CO_2 incubator at 37°C, after which the adherent cells were purified by washing off the non-adherent cells three times with warm (37°C) phenol red-free HBSS. These adherent cells were then assayed for O_2^- generation while still adherent to the bottom of the wells. By the criterion of trypan blue exclusion[14] these cells were routinely 96–100% viable.

Latex Phagocytosis

The fraction of the total spleen cells in suspension which phagocytosed latex particles gave an idea of the number of the spleen cells which were macrophages. This indirect approach was necessitated by the fact that it was difficult to obtain the accurate value of the actual number of the adherent cells which were macrophages.

Uniform latex beads (1% polystyrene, diameter 1.091 μ) from Sigma were washed 4 times in PBS and diluted 1:100 in RPMI-1640 medium. This 1:100 latex bead suspension was then added to the spleen cells to a final ratio of 50 μl of 1:100 dilution latex to 5×10^5 nucleated cells. 250 μl of this suspension should contain 2.5×10^5 nucleated cells. The suspension was incubated at 37°C in a humid 5% CO_2 incubator for 2 h. At the end of this incubation, 250 μl of the suspension was centrifuged on to a slide using a cytocentrifuge (Shandon, model SCA-0031). The slide was stained using SP (American Scientific Products) Diff-Quick stain. Cells containing beads were identified and counted as macrophages.

Superoxide Generation by Adherent Cells

Superoxide release from the adherent cells was assayed by the cytochrome c reduction procedure of Babior *et al.*[16] modified in this case for adherent cells. On to adherent cells derived from 1.5×10^6 spleen nucleated cells in each 16 mm well (see earlier) was layered (after 3 times washing with colorless HBSS) 200 μl colorless HBSS followed by 20 μl of 10 mg/ml cytochrome c in PBS, and then the reaction was triggered with 10 μl of 2 μg/ml phorbol myristate acetate (PMA). Each well was prepared in duplicate and incubated at 37°C in a 5% CO_2 humid incubator for 60 min. For each assay, the contents of two (duplicate) 16 mm diameter wells were mixed (460 μl total volume) and the amount of reduced cytochrome c determined in the microcuvette of a Cary 118 double beam spectrophotometer scanned between 575 nm and 525 nm. The absorbance peak at 550 nm was obtained and the extinction coefficient for cytochrome c (0.0183 A_{550}/nmole/ml) at 550 nm was used to calculate the amount of cytochrome c reduced. This was equivalent to O_2^- produced.

RESULTS

Figure 1 shows the linearity of the O_2^- release with the number of spleen cells plated at 10^3 ng LPS/ml added during the adherence stage. For all experiments, 1.5×10^6 spleen nucleated cells were plated in each well (i.e., 3.0×10^6 spleen nucleated cells per two wells). In the lower curve of Figure 2 are embodied the data showing that when spleen adherent cells isolated from normal healthy mice are triggered with

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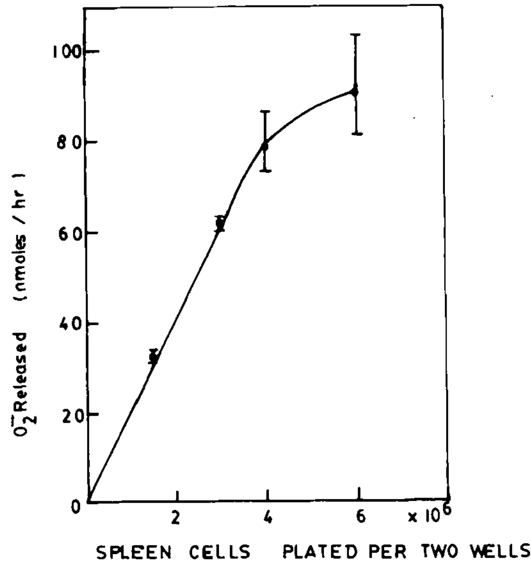

FIGURE 1 Linearity of Superoxide (O_2^-) generation as a function of number of total nucleated spleen cells plated. Spleen nucleated cells were isolated, adherent cells obtained, and O_2^- generation after triggering with phorbol myristate acetate (PMA) assayed as given in "Methods and Materials" in the presence of 10^3 ng LPS/ml, and varying numbers of spleen cells as indicated.

Each point is the mean of three separate experiments except for that at 1.5 × 10^6 spleen cells which is mean of two experiments. The bar indicates the range of the least and highest values.

PMA, the amount of O_2^- liberated is exceedingly small (about 15 nmole O_2^- per hour per 3 × 10^6 spleen nucleated cells) if LPS (endotoxin) was excluded from the medium during the 3 h incubation of the total spleen cells for adherence. On inclusion of endotoxin in the suspension of total spleen cells during the incubation for adherence, the level of O_2^- liberated continues to remain low until a threshold level of about 10 ng LPS/ml. Beyond this concentration, there is a dramatic increase in the O_2^- released on PMA triggering, this beginning to stabilize at about 45–50 nmole O_2^- per hour per 3 × 10^6 spleen nucleated cells plated, when the LPS concentration is about 10^5 ng/ml. This represents a three-fold increase of O_2^- released.

The upper curve of Figure 2 represents cells from a malaria infected spleen (24 hours post-infection). This represents a typical behaviour. The data (not given here) at various other times post-infection are similar and are the subject of a separate paper (Eze, M.O., in preparation). Here again (upper curve of Figure 2), the threshold LPS concentration required during adherence is 10 ng/ml. However, the baseline level of O_2^- at zero LPS is elevated (to about 30 nmole O_2^- per hour per 3 × 10^6 spleen nucleated cells) by this stage of the infection. In this case, the enhancement by LPS starts at about 10 ng LPS/ml and reaches about 90 nmole O_2^-/hr/3 × 10^6 spleen nucleated cells, representing a three-fold increase also, at 10^5 ng LPS/ml. Figure 3 incorporates the data for normal healthy spleen cells from C57 BL/6 and CBA female mice. Endotoxin produced similar effects on the O_2^- release from both samples. Thus, this observation is not unique to C57 BL/6 mice.

When adherent cells were prepared without LPS and freed from other (non-adherent) cells, and then were incubated with LPS, there was no enhancement of the

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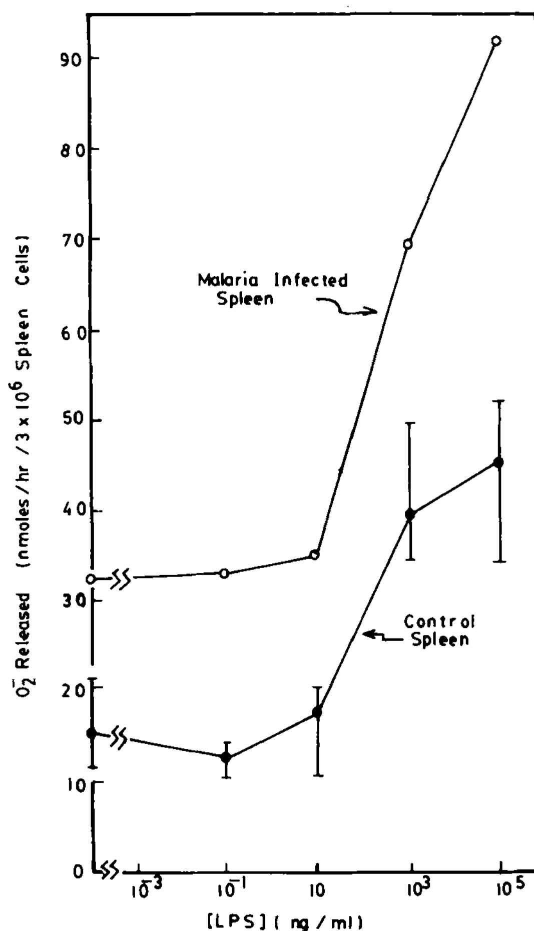

FIGURE 2 Effect of Endotoxin on O_2^- release from control and malarial spleen cells. Spleen adherent cells from either control or malaria-infected mice were obtained, and while maintaining the number of nucleated spleen cells plated at 3×10^6 per two wells, the O_2^- was assayed as given in Figure 1, but at varying concentrations of LPS. For the control (lower curve) each point is the mean of four separate experiments. The bar is the range of least and highest values. Each point in the upper curve (Malaria infected spleen) is the result of one experiment only.

O_2^- released (data not presented here). Thus, the LPS effect required the presence of all the spleen cell types (B- and T-cells inclusive). This behaviour contrasts sharply with that reported by Pabst and Johnston, Jr.[12] for mouse peritoneal macrophages for which the incubation, after adherence, with the required very low levels of LPS produced the enhancement effect.

In Table 1, the density of adherent spleen cells in the presence or absence of LPS at 10^3 ng/ml is shown for malaria-infected and normal healthy mice. Adherent cell density is depicted as the mean number of adherent cells per low power field of view of a NIKKON (Biological Microscope, LABOPHOT) phase contrast microscope. There is only a slight increase in the adherence of malarial spleen cells in the presence

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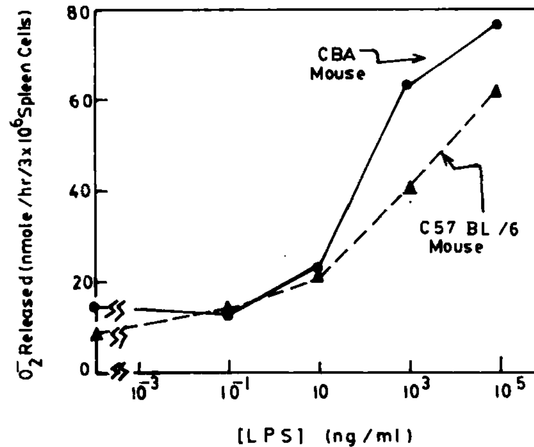

FIGURE 3 Effects of endotoxin concentration on O_2^- release by spleen macrophages from C57 BL/6 female mouse and CBA female mouse. Spleen adherent cells were obtained from either C57 BL/6 or CBA mouse and the O_2^- assays carried out as given in Figure 1. Each point for each mouse represents one determination.

of 10^3 ng LPS/ml (13–40% increase only). For control spleen cells, there is in fact a slight decrease in adherence in the presence of 10^3 ng LPS/ml compared to adherence in the absence of LPS. Thus, the enhancement of O_2^- release (250–300%) in both malaria-infected and control spleen adherent cells due to 10^3 ng LPS/ml (see Figure 2) cannot be due to increased adherence of the spleen cells at that concentration of endotoxin.

The presence of LPS, as can be seen in Table 2, has no appreciable effect on latex phagocytosing ability of either the malaria infected or the control spleen cells. Malaria infection, however enhances latex phagocytosis by spleen cells several fold at the various times post-infection investigated.

TABLE I

Effect of 10^3 ng LPS/ml on adherence of spleen cells isolated from control mice and mice at various stages of malaria infection. The spleen cells were isolated and adherence carried out as detailed in "Materials and Methods" in the presence (+LPS) or absence (−LPS) of *Salmonella enteritidis* lipopolysaccharide at 10^3 ng/ml. Each value is the mean ± standard error of the mean (SEM) of the number (in parentheses) of fields of view counted for each mouse. Each control represents the mean ± SEM for three separate spleens.

Days Post-Infection	Number of adherent Spleen Cells per Low Power Field of View (Mean ± SEM)			
	Malaria-Infected Spleen Cells		Control Spleen Cells	
	−LPS	+LPS (10^3 ng/ml)	−LPS	+LPS (10^3 ng/ml)
9	176 ± 11 (8)	247 ± 21 (4)		
14	126 ± 21 (8)	142 ± 26 (9)	199 ± 18 (21)	178 ± 13 (25)
17	148 ± 11 (8)	172 ± 31 (8)	(three spleens)	(three spleens)

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DISCUSSION

The data presented here (Figure 2) reveal that treatment with high concentrations of LPS during incubation for adherent cells is absolutely required in order for mouse spleen macrophages to manifest *in vitro* respiratory burst activity monitored here as O_2^- release. For control mouse spleen macrophages, this O_2^- generation in presence of 10^3 ng LPS/ml is usually at least three times higher than in the absence of LPS. It is unlikely that the possibility of contaminating LPS in the media and buffers has any role to play in these observations. This is because, as can be seen in Figure 2, the concentration of LPS required as threshold is very high.

Macrophages from spleens of malaria-infected mice also exhibit the same qualitative dependence on LPS, requiring LPS for O_2^- release. The quantitative pattern of this behaviour is, however, the subject of another report (Eze, M.O., in preparation). Normal spleen cells from CBA female mice gave essentially similar results as for C57 BL/6 female mice.

Pabst and Johnston Jr.[12] earlier demonstrated that while mouse resident peritoneal macrophages can generate O_2^- on triggering with phorbol myristate acetate (PMA) and in the absence of an agent like endotoxin (LPS) or muramyl dipeptide, low concentrations of LPS ($\leqslant 10$ ng/ml) enhanced O_2^- release. However, at high concentrations (above 100 ng/ml) LPS inhibited this reaction. These workers,[13] later showed that human blood monocytes require even lower concentrations of LPS (about 10^{-5} ng/ml) before generation of appreciable O_2^- is possible.

In the present investigation, and consistent with the report of Pabst and Johnston,[12] mouse peritoneal macrophages showed very little but concentration dependent inhibition (data not shown). These differences in the requirement for LPS and the magnitute of response to it thus reveal major differences among these three types of monounclear cells. LPS may also prove useful in the isolation and tissue culture of cells competent in other vital metabolic reactions which may otherwise exhibit latency in the absence of LPS treatment. This is even more so for membrane-bound processes since LPS is thought to exert its effect on the membrane.[16]

Collagenase type IV from Sigma also gave similar effects (data not presented). This sounds a note of caution that this enzyme (or a contaminant therein) may indeed be doing more than the function it is presumed to be performing during its use in tissue

TABLE II

Percent of total nucleated spleen cells phagocytosing latex particles in suspension. The spleen cells were isolated and the % of them which phagocytosed latex particles in suspension determined as given in "Methods and Materials" in the presence (+LPS) or absence (−LPS) of 10^3 ng LPS/ml. Each value is the mean ± SEM of the number (in parentheses) of fields of view counted for each mouse. Each control value is the mean ± SEM for five separate spleens.

Days Post-Infection	% Total Spleen Cells Phagocytosing Latex Particles (MEAN ± SEM)			
	Malaria Infected Spleen Cells		Control Spleen Cells	
	−LPS	+LPS (10^3 ng/ml)	−LPS	+LPS (10^3 ng/ml)
5	5.8 ± 1.5 (21)	5.0 ± 1.3 (2)		
7	9.1 ± 1.8 (17)	9.9 ± 2.7 (18)		
9	11.6 ± 1.8 (12)	10.9 ± 1.9 (12)	3.3 ± 0.8 (83)	2.8 ± 0.5 (81)
11	5.4 ± 1.6 (19)	12.2 ± 3.1 (15)	(five spleens)	(five spleens)
14	8.1 ± 1.9 (28)	9.2 ± 2.2 (17)		

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culture. Thus, while it can be used for disrupting tissue for cells, its possible non-specific effects should first be scrupulously eliminated. This buttresses the suspicions expressed by others.[7,14,17]

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