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Failure of Bacterial Lipopolysaccharide To Elicit a Cytostatic Effect on *Plasmodium vinckei petteri* in C3H/HeJ Mice

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Malarial parasites, *Plasmodium vinckei petteri*, taken from lipopolysaccharide (LPS) high-responder (C3H/HeJGiFWeHi) mice which had been injected 7 to 8 h previously with either *Escherichia coli* LPS B or LPS W incorporated the purine nucleotide precursor hypoxanthine more slowly in an in vitro assay than parasites taken from saline-injected controls. In contrast, malarial parasites taken from LPS low-responder C3H/HeJ mice after injection of either LPS B or LPS W did not show reduced levels of hypoxanthine incorporation. These differing results with LPS high- and low-responder mouse strains demonstrated that the cytostatic effect on the parasites seen in the high-responder strain was not due to the direct action of LPS and implied that the cytostasis was mediated via host lymphoreticular cells. Furthermore, the failure of LPS B, a lipid A-associated protein-containing LPS preparation, to elicit a cytostatic effect on *P. vinckei petteri* in C3H/HeJ mice suggested that the LPS-induced effector mechanisms acting against malarial parasites may be similar to those reported against bacteria and tumors.

Malarial parasites (*Plasmodium vinckei petteri*) taken from CBA mice which were injected with bacterial lipopolysaccharide (LPS) incorporate less hypoxanthine in vitro than parasites from controls (16). This indicated reduced synthesis of nucleic acid by the parasites (17). Furthermore, this experimental evidence implied that a soluble factor (or factors) whose release was induced by LPS was responsible for the cytostatic effect on parasites. Since these findings may provide a biochemical explanation for the protective action of LPS in malaria infections (11, 12), for the protection against *Plasmodium* spp. and other hemoprotozoa afforded animals pretreated with reticuloendothelial stimulants such as BCG and *Corynebacterium parvum* (4, 5), and for the parasite crisis of some malarial infections (14, 19), their potential significance is considerable. To understand the mechanisms involved more fully, we made use of histocompatible LPS high-responder (C3H/He) and LPS low-responder (C3H/HeJ) mouse strains. The usefulness of this mouse model to study the mechanisms behind the diverse host responses to LPS is well established from other systems (13); its basis lies in the refractoriness of the C3H/HeJ strain to most effects of LPS.

Our present results suggested that host lymphoreticular cells were involved in the expression of the cytostatic effect on the malarial parasite *P. vinckei petteri*. In addition, they implied a similarity between the antiparasite mechanism described here and those acting against tumors and bacteria.

MATERIALS AND METHODS

Mice. Inbred, CBA/H and histocompatible C3H/HeJGifWehi (LPS high-responder) and C3H/HeJ (LPS low-responder) mouse strains were used when 5 to 12 weeks old. All were females, except that one experiment (Table 1, experiment 7) used C3H/HeJ male mice. The CBA mice were bred at the John Curtin School of Medical Research. Our C3H/HeJ stock originated from Jackson Laboratories (Bar Harbour, Maine) 2 years ago and has subsequently been bred at the John Curtin School. The C3H/HeJGifWehi (henceforth referred to as C3H/He) mice were obtained from the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.

Parasite. A lethal strain of *P. vinckei petteri* was maintained by serial passage in CBA mice. Mice were used 4 to 5 days after an intraperitoneal injection of 1.5×10^6 parasites. Parasitemias of individual mice were determined by counting a minimum of 100 erythrocytes in Giemsa-stained thin blood smears. Parasitemias of pooled blood were determined by counting a minimum of 400 erythrocytes.

LPS. *Escherichia coli* serotype O128:B12 LPS B (trichloroacetic acid extracted) and *E. coli* serotype O55:B5 LPS W (phenol-water extracted) were obtained from Difco Laboratories, Detroit, Mich., and stored in saline at −20°C at a concentration of 1 mg/ml. For in vivo use, this preparation was subsequently diluted with saline so that the required dose was contained in 200 μl.

Medium. RPMI 1640 (GIBCO Diagnostics, Madison, Wis.) was used with the following additions per liter: 2 g of $NaHCO_3$, 5.96 g of HEPES (*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Sigma Chemical Co., St. Louis, Mo.), 0.65 g of NaCl, 40 mg of gentamicin sulfate (Schering Corp. U.S.A., Kenilworth, N.J.), and 2 g of essentially fatty acid-free bovine albumin (Sigma). The pH was adjusted to 7.4.

Blood glucose determinations. Blood glucose levels in whole blood were determined semiquantitatively by the use of Dextrostix (Ames Co., Elkhart, Ind.) reagent strips. These strips are graduated to give readings of 0, 25, 45, 90, 130, 175, and 250 mg/dl.

Preparation of blood for culture. Mice under ether anesthetic were bled from an incision in the axilla into heparin (50 U/ml of blood). Blood from each group of mice was pooled. The plasma was removed, and after three washes the cells were suspended in medium to twice the original volume. Leukocytes were removed by passage through columns of cellulose powder CF11 (Whatman, Inc., Clifton, N.J.). After smears of the pooled leukocyte-free blood were made, the erythrocytes were suspended in medium to a concentration of 10^9/ml.

Measurement of parasite synthesis of nucleic acid. The incorporation of [^{3}H]hypoxanthine (specific activity, 1.2 Ci/mmol; concentration, 1 mCi/ml; Radiochemical Centre, Amersham, Bucks, U.K.) was used as a measure of the synthesis of nucleic acid by parasites. The incorporation of hypoxanthine was measured over six time points (zero time, 5 min, 10 min, 20 min, 30 min, and 1 h). The hypoxanthine was added to cultures as a 0.408 mM solution made up from equal concentrations of labeled and unlabeled hypoxanthine in phosphate-buffered saline. Round-bottom microwells (96-well microtitration trays; Linbro Titertek, Hamden, Conn.) were used for all experiments.

The procedure was essentially the same as that described previously (16): 25 μl of medium was delivered into each well, five for each treatment. To this was added 100 μl of the 10^9-erythrocytes/ml suspension. Finally, 20 μl of the hypoxanthine solution was added before incubation at 37°C. After incubation, trays were immediately centrifuged at 850 × *g* for 2 min at 4°C in a Beckman TJ-6 centrifuge. The pellets were retained and, after washing three times, were suspended in 100 μl of cold medium. A 50-μl sample from each well was then spotted onto 3-mm filter disks (Whatman). The filters were dried and then washed four times in 10% trichloroacetic acid, followed by three washes in ethanol (all at 4°C). After again being dried, the disks were placed in xylene-PPO (2,5'-diphenyloxazole) scintillation fluid for counting in a Packard Tri-Carb liquid scintillation counter. A counting time of 10 min per sample was used.

Results are expressed as mean counts per minute ± standard error of five replicates. Statistical comparisons were done by using a variance-stabilizing transformation for Poisson variables (the square root transformation) (2) and applying a paired *t*-test to the data.

RESULTS

Parasitized blood collected after injection of LPS W. Infected mice were divided into two equal groups (of six to eight animals) with parasite densities as similar as possible and deprived of food. One group received 20 μg of LPS W intravenously, and the other received 200 μl of saline by the same route. Both groups of mice were bled out 7 to 8 h later, and after removal of leukocytes, the rates of hypoxanthine incorporation by parasitized erythrocytes were determined as described above. This experiment was done with both C3H/He and C3H/HeJ mice.

We found that parasites taken from LPS high-responder mice (C3H/He) after the injection of LPS W incorporated significantly less hypoxanthine than parasites from saline-treated controls (Fig. 1; Table 1, experiments 1 and 2). After 1 h in culture, the incorporation of hypoxanthine by the parasitized erythrocytes from LPS W-injected C3H/He mice was more than 43% lower than by similar cells from saline-injected control mice. In contrast, parasitized erythrocytes from LPS low-responder mice (C3H/HeJ) treated with either LPS W or saline incorporated similar amounts of hypoxanthine (Fig. 2; Table 1, experiments 3 and 4). These counts of incorporated hypoxanthine probably represent true incorporation by parasites, since similarly processed uninfected blood showed negligible incorporation over a 1-h interval (16). This is also depicted in Fig. 1, where the lower graph represents counts per minute ± standard error for uninfected blood from both LPS W- and saline-treated groups. CBA mice were used in the experiments with uninfected blood.

We also noted that infected C3H/He mice which had been given LPS W exhibited all of the signs of endotoxicity when exsanguinated: lethargy, hypothermia, ocular inflammation, diarrhea, and profound hypoglycemia. None of these features was seen in the saline controls or in any experiments with C3H/HeJ mice. Blood glucose values are given in Table 2 (experiments 1–4). Uninfected CBA mice given 20 μg of LPS W showed ruffled fur, lethargy, and diarrhea, but their blood glucose levels were the same as those of the saline-infected controls (Table 2, experiment 9).

Parasitized blood collected after injection of LPS B. The experimental procedures when LPS B was injected were as described above for LPS W. Parasites from C3H/He mice injected with LPS B incorporated significantly less hypoxanthine over a 1-h period than parasites from saline-injected controls. After 1 h in culture, intra-erythrocytic *P. vinckei petteri* from LPS-injected C3H/He mice had incorporated 37 to 59% less hypoxanthine than *P. vinckei petteri* from saline-injected controls (Table 1, experiments 5 and 6). These results were similar to those found after the injection of LPS W into this strain. LPS B also elicited the signs of

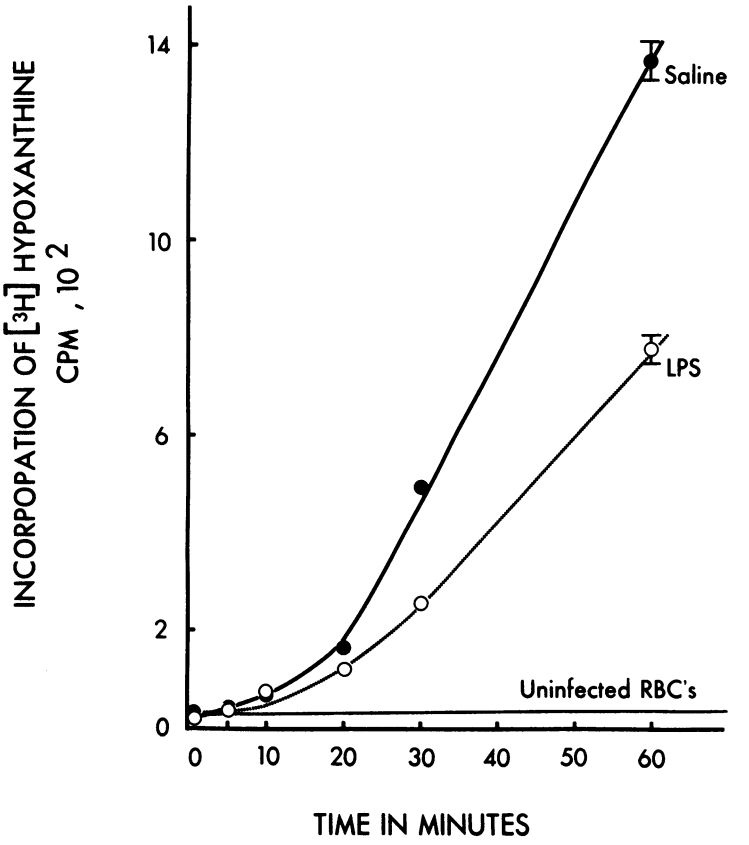

FIG. 1. Time course of hypoxanthine incorporation by parasites taken from LPS high-responder C3H/He mice 7 to 8 h after the injection of LPS W or saline. Incorporation of hypoxanthine by uninfected erythrocytes from CBA mice under similar conditions is also shown. Data are expressed as counts per minute ± standard error (most standard errors too small to be shown).

TABLE 1. In vitro incorporation (at 1 h) of [^{3}H]hypoxanthine by intra-erythrocytic malarial parasites (*P. vinckei petteri*) after the injection of the host with LPS W or LPS B

Expt no.	Mouse strain	No. of mice/ group	Treatment[a]	Parasitemia (%) before injection	Parasitemia (%) of pooled blood (ex column)[b]	Incorporated hypoxanthine at 1 h (cpm ± SE)	Level of significance (*P*)
1	C3H/He	6	Saline	13	21	1,368 ± 40	<0.001
		6	LPS W	13	20	773 ± 25	
2	C3H/He	7	Saline	24	37	3,868 ± 40	<0.001
		7	LPS W	24	27	817 ± 33	
3	C3H/HeJ	7	Saline	46	42	6,053 ± 248	>0.05
		7	LPS W	46	43	6,313 ± 162	
4	C3H/HeJ	8	Saline	17	28	4,796 ± 167	>0.05
		8	LPS W	17	30	4,555 ± 60	
5	C3H/He	7	Saline	5	11	1,336 ± 59	<0.001
		7	LPS B	5	10	551 ± 15	
6	C3H/He	6	Saline	4.3	4.0	224 ± 6	<0.001
		6	LPS B	4.4	4.7	141 ± 6	
7	C3H/HeJ	7	Saline	24	27	2,586 ± 70	<0.001
		7	LPS B	24	28	3,544 ± 155	
8	C3H/HeJ	7	Saline	20	30	1,977 ± 77	<0.001
		7	LPS B	20	26	2,356 ± 62	

[a] LPS dose, 20 μg/mouse.
[b] Parasitemias of the effluent erythrocytes off of the cellulose columns.

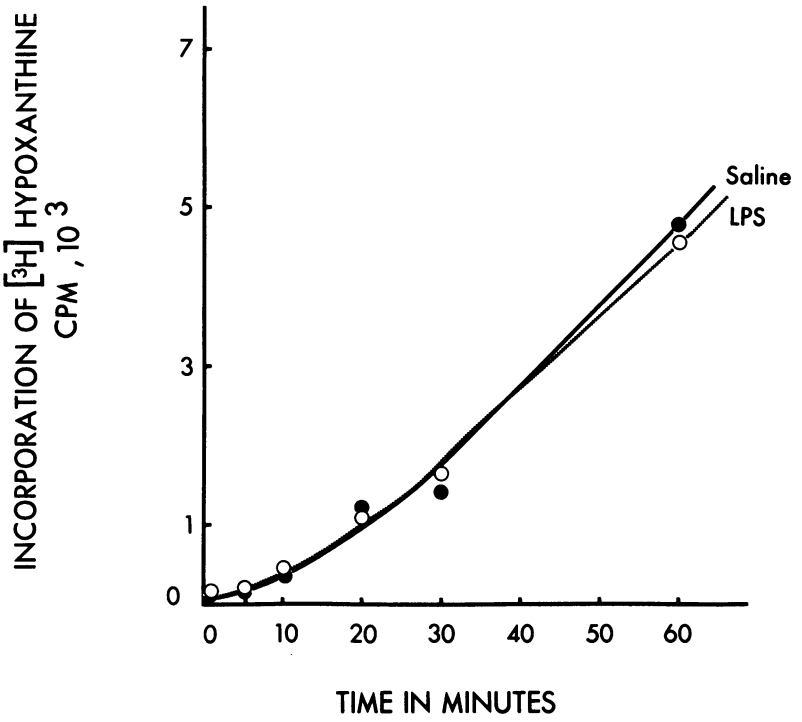

FIG. 2. Time course of hypoxanthine incorporation by parasites taken from LPS low-responder C3H/HeJ mice 7 to 8 h after the injection of LPS W or saline. Data are expressed as in Fig. 1.

TABLE 2. Effect of LPS on blood glucose levels in *P. vinckei petteri*-infected mice

Expt no.[a]	Mouse strain	Blood glucose before injection[b] (mg/dl)	Treatment[c]	Blood glucose 7–8 h after injection (mg/dl)
1	C3H/He	130 (90/130–130/175)	Saline	90 (45–90)
			LPS W	0 (0–25)
2	C3H/He	130 (90–130)	Saline	45/90 (45/90–90)
			LPS W	0 (0–25)
3	C3H/HeJ	90 (45/90–130)	Saline	130 (90–130)
			LPS W	130 (45–130)
4	C3H/HeJ	90/130 (90–175)	Saline	90/130 (45–130)
			LPS W	90 (45–130)
5	C3H/He	90 (45/90–130)	Saline	45 (45–90)
			LPS B	0 (0–25)
6	C3H/He	90	Saline	45 (45–90)
			LPS B	25 (25–45/90)
7	C3H/HeJ	175 (90–175)	Saline	130 (90–130)
			LPS B	90
8	C3H/HeJ	130 (90–130)	Saline	90
			LPS B	90 (45–90/130)
9	CBA[d]	130 (130–130/175)	Saline	90 (45–90)
			LPS W	90 (45–90)

[a] Experiment numbers correspond to those in Table 1.
[b] Values given were those shown by most animals at time of testing; range is given in parentheses.
[c] LPS dose; 20 μg/mouse.
[d] Uninfected.

endotoxicity in these mice, including hypoglycemia (Table 2, experiments 5 and 6).

In contrast, parasites taken from C3H/HeJ mice treated with LPS B actually incorporated more hypoxanthine than saline-injected controls (Fig. 3; Table 1, experiments 7 and 8). Incorporation after 1 h was 27% higher in one experiment and 16% higher in the other. In one experiment, one or two of the C3H/HeJ mice injected with LPS B appeared slightly ruffled, but there was no diarrhea, and blood glucose values were not appreciably different from those of saline-treated controls (Table 2, experiments 7 and 8).

DISCUSSION

We have shown in an in vitro assay that intraerythrocytic *P. vinckei petteri* parasites taken from LPS high-responder C3H/He mice after the injection of LPS W incorporate considerably less of the purine nucleotide precursor hypoxanthine than do saline-injected controls (Fig. 1; Table 1, experiments 1 and 2). This cytostatic effect on parasite metabolism was not obtained when the parasites were taken from LPS low-responder C3H/HeJ mice after similar treatment (Fig. 2; Table 1, experiments 3 and 4). *P. vinckei petteri*-infected C3H/HeJ mice also exhibited none of the toxic consequences of LPS W injection, whereas these features were fully displayed in similarly infected and treated C3H/He mice (e.g., Table 2, experiments 1–4). It is known that C3H/HeJ mice are resistant to in vivo and in vitro effects of LPS W because of their inability to respond to lipid A (1, 9, 20). Furthermore, it has been shown that the unresponsiveness of this mouse strain to LPS resides primarily in its lymphoreticular cells (13). The results of our experiments with LPS W and these two histocompatible mouse strains thus imply that host cells (macrophages, lymphocytes, or both) are involved in the mediation of the cytostatic effect on malarial parasites. In addition, they support our previous conclusions from experiments with CBA mice (16) that the cytostasis was not due to the direct action of LPS, but to a soluble mediator whose release was LPS induced.

Since C3H/HeJ mice show some responsiveness (e.g., mitogenicity and polyclonal B cell activation) to preparations of LPS which contain the protein-rich component known as lipid A-associated protein (8), we examined the ability of one such preparation, LPS B, to elicit an antiparasite response in these mice. We found that whereas LPS B was effective in producing cytostasis of parasites in LPS high-responder C3H/He and CBA mouse strains (Table 1, experiments 5 and 6; 16), it was unable to evoke this response in C3H/HeJ mice (Table 1, experiments 7 and 8). This finding with LPS B suggested that the cellular requirements for the induc-

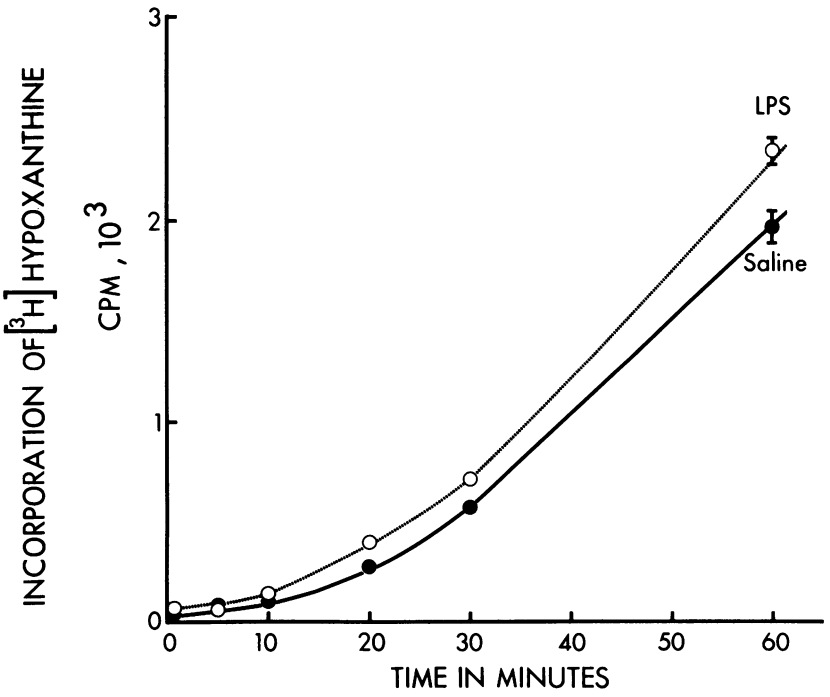

FIG. 3. Time course of hypoxanthine incorporation by parasites taken from LPS low-responder C3H/HeJ mice 7 to 8 h after the injection of LPS B or saline. Data are expressed as in Fig. 1.

tion of a parasite cytostasis may be similar to those required for LPS-induced tumor cytotoxicity and LPS-induced protection against bacteria, since LPS B is also ineffective in C3H/HeJ mice in these two systems (3, 9). For instance, in the tumor study (9), C3H/HeJ mice pretreated with BCG and injected with either LPS W or B did not produce serum which was cytotoxic for tumor cells, whereas such serum was readily produced in the LPS high-responder strain. Similarly, LPS W or B injected into LPS low-responder C3H/He Orl mice could not protect against the bacterium *Klebsiella pneumoniae*, yet both of these preparations protected in the histocompatible high-responder strain (3).

The serum factor causing the lysis of tumor cells has been identified as a heat-stable entity with a molecular weight of 60,000; it appears to be identical to tumor necrosis factor (10). Whether this factor is involved in the cytostasis of malarial parasites has still to be determined. Certainly, its reported inhibition of tumor cells and of other rapidly dividing cells (e.g., bone marrow [18]) as well as its presence in the serum of parasite-infected and LPS-injected mice (6) suggest that it is a likely candidate. Furthermore, since macrophages appear to be both the source of the antitumor activity (10) and part of the antibacterial effect (7, 15), it is likely that they may also be the source of the mediator(s) of our antiparasite effect. We hope to be able to demonstrate this with experiments now in progress.

An additional finding of this study was that parasites from C3H/HeJ mice injected with LPS B showed higher than normal levels of hypoxanthine incorporation (Fig. 3; Table 1, experiments 7 and 8). This effect on parasite metabolism was not seen when C3H/HeJ mice were injected with LPS W (Fig. 2; Table 1, experiments 3 and 4). It is feasible that the lipid A-associated protein moiety of LPS B, although unable to elicit a cytostatic effect on parasites in this strain, could have induced the release of a substance which promoted parasite growth; this interpretation is consistent with the observed dichotomy in the responses of C3H/HeJ mice to LPS B (8).

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ADDENDUM IN PROOF

The recently published findings of Taverne et al. (Infect. Immun. 33:83–89, 1981) support the main conclusions of this study.

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