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Effect of Nifedipine on Calcium Status and Chemiluminescence Response of Phagocytes During *Plasmodium berghei* Infection in Mice

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Abstract—The macrophages and neutrophils from nifedipine-treated mice, both *Plasmodium berghei*-infected and uninfected, showed suppressed capacity to generate oxygen free radicals as compared with untreated controls. Nifedipine treatment did not affect resting state free calcium levels in these cells. But the rise in intracellular calcium levels of macrophages and neutrophils following *P. berghei* infection was significantly less ($P < 0.05$) in nifedipine-treated mice as compared with untreated groups at various parasitaemia levels. Probably this reflects a more potent effect of nifedipine on these cells in the depolarized state. Similarly, the rise in intracellular calcium levels of these cells following formyl-Met-Leu-Phe (fMLP) stimulation was also significantly less in nifedipine-treated groups than in untreated controls at different parasitaemia levels. A positive correlation between this fMLP-stimulated rise in calcium levels and the chemiluminescence response of macrophages and neutrophils was observed in nifedipine-treated and untreated groups at various parasitaemia levels. Thus the respiratory-burst responses of these cells during *P. berghei* infection depend on the calcium homeostasis in the cells. The disturbances of the calcium-regulating mechanisms by nifedipine treatment resulted in subnormal phagocytic cell responses which lead to more severe and rapidly fatal *P. berghei* infection in these animals.

The phagocytic macrophages and neutrophils spearhead the cellular immune responses against malaria. Allison & Eugui (1982) suggested that these effector cells upon activation, either by T-cells or by phagocytosis of parasite or its products, release reactive oxygen intermediates. This, in turn, is believed to cause intraerythrocytic death of the parasite and appearance of crisis forms. The capacity of macrophages to produce free oxygen radicals has been found to correlate with the ability of mice to recover from lethal *Plasmodium berghei* infection (Brinkmann et al 1984; Li & Li 1987).

Interaction of macrophages and neutrophils with specific and non-specific stimuli initiates responses such as aggregation, degranulation and respiratory-burst activity. Changes in ion movements across the plasma membrane are integral to the process of signal transmission. Calcium ions act as second messengers in phagocytic leucocytes as in other cell types. It has been proposed that different ligand-receptor systems activate a common calcium-translocating mechanism, thus acting as an amplifying factor in neutrophil activation (Korchak et al 1984). Agents which can modulate Ca^{2+} fluxes are likely to alter various physiological functions of macrophages and neutrophils. The calcium-channel antagonists, being widely used in the management of cardiovascular disorders, are known to impair Ca^{2+} entry into excitable and non-excitable cells. Further, the action of these calcium-channel blockers, on the macrophage and neutrophil physiology in-vitro is well documented (Wright et al 1985; Azuma et al 1986). Although the clinical relevance of these in-vitro observations is not clear, Pennington et al (1986) suggested the possibility that such impaired macrophage and neutrophil function is associated with increased risk of certain infections and it can be speculated that one of the pharmacological effects of these calcium antagonistic drugs might be predisposition to infection.

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We have observed that nifedipine administration to mice results in depressed chemiluminescence response of macrophages and neutrophils. Further, *P. berghei* infection in these nifedipine-treated mice was more severe and lethal, exhibiting significantly shorter prepatent, patent and survival periods along with early peak parasitaemia (unpublished observations). The mechanism of action of such calcium-channel blockers remains debatable. The present study was undertaken to explore the possible role of disruption of calcium fluxes in immune effector cells, such as macrophages and neutrophils, in the causation of more severe *P. berghei* infection in mice as a result of drug administration.

Materials and Methods

Parasite

Plasmodium berghei, strain NK-65, originally obtained from Central Drug Research Institute, Lucknow, India, was used in this study.

Animals

Four to five week-old Swiss albino mice, 15–20 g, were used throughout the study in all experimental groups.

Drug administration

Nifedipine (Sigma) was dissolved in the minimum quantity of ethanol and diluted in 0·9% NaCl (saline) without exposure to light. The animals were dosed orally (0·015 mg kg^{-1} day^{-1}) (Nalini 1988). On the 15th post-treatment day, the mice were infected intraperitoneally with 10^3 parasitized

erythrocytes. The drug treatment was continued during the course of infection. An equal number of infected animals receiving ethanol saline in place of nifedipine constituted the control group. The infection was monitored from day 1 post-infection in both groups. Five to six animals in each group were killed at 0–1%, 5–10%, 15–25% and more than 40% parasitaemia. Each experiment was performed six times.

Isolation of neutrophils and peritoneal macrophages

The blood was collected from the axillary vein of anaesthetized mice in heparin (20 int. unit mL^{-1}). For isolating neutrophils, the blood was subjected to dextran sedimentation (mol. wt 65 000 Da BDH) followed by layering on top of lymphoprep (Nyegaard and Co, Norway, density 1·077). The neutrophils were separated according to the method described by Boyum (1968). The contaminating erythrocytes were removed by hypotonic lysis using 0·3% saline. Peritoneal exudate cells were collected by lavaging the peritoneal cavity using heparinized RPMI-1640 (Gibco Labs, Green Island). The cell concentration was adjusted to 10^6 cells mL^{-1} for both neutrophils and macrophages.

Chemiluminescence response

Oxidative metabolism and extent of free-oxygen radical generation by neutrophils and macrophages was measured based on luminol-dependent chemiluminescence. Latex beads were used as stimulant to elicit oxidative burst (Cheung et al 1984). Briefly, 1×10^6 cells mL^{-1} were incubated in RPMI-1640 supplemented with 10% foetal calf serum for 45 min at 37°C in an atmosphere of 5% CO_2. The cells were pelleted at 450 *g* for 5 min, washed once with minimum essential medium (MEM) (pH 7·4) and the resulting pellet was suspended in MEM without indicator. The chemiluminescence response was measured in a luminometer (Berthold, LB-9500C). The values were recorded as counts min^{-1} to study the status of macrophage and neutrophil activation.

Measurement of cytosolic free calcium

Fluorescent dye FURA-2AM (Sigma) was used to measure cytosolic free calcium (McCarthy et al 1989). Macrophages/neutrophils (10^6) were suspended in morpholino propanesulphonic acid (MOPS) buffer (pH 7·4) supplemented with 1% bovine serum albumin (BSA) and 1 mM $CaCl_2$. The loading of cells with FURA-2 was with a final concentration of 0·5 μM, for 45 min at 37°C. The cells were then washed and resuspended in the same buffer without the dye. The fluorescence of cell suspension, representing the basal intracellular calcium was measured in a spectrofluorometer (Kontron, SFM-25) as relative fluorescence (340 nm excitation 500 nm emission). These cells were then stimulated with 10 nM formyl-Met-Leu-Phe (fMLP) in the presence of 1 mM $CaCl_2$ and the fluorescence was monitored until a steady-state level was attained.

Statistical analysis

The results of various parameters were compared by Student's *t*-test. Coefficient of correlation was applied to determine the correlation between the parameters.

Results

Chemiluminescence

The response exhibited by macrophages and neutrophils from nifedipine-treated mice was significantly lower ($6{\cdot}0 \pm 0{\cdot}7 \times 10^3$ and $20{\cdot}7 \pm 4 \times 10^3$ counts min^{-1} per 10^6 cells, respectively) in comparison with the untreated control animals (Fig. 1). The neutrophils isolated from nifedipine-treated and untreated mice showed significantly higher activation potential during the early phase of infection (0–1 and 5–10% parasitaemia) as compared with neutrophils from uninfected mice ($P < 0{\cdot}001$). In neutrophils from drug-treated animals this declined significantly as it also did in untreated controls during the later phase of infection (15–25 and 40% parasitaemia). However, the quantum of response in drug-treated, infected mice was considerably lower than that in untreated infected mice (Fig. 1).

The peritoneal exudate cells separated at various parasitaemia levels also exhibited comparable responses with those observed with neutrophils. The macrophages from the untreated, *P. berghei*-infected group showed maximum respiratory-burst activity at 5–10% parasitaemia followed by a decline at higher levels. In contrast, the macrophages from nifedipine-treated mice displayed much lower chemiluminescence response, the peak activity being observed at 0·1% parasitaemia, followed by a diminution of response at higher parasitaemia. Moreover the time taken to exhibit their peak activity was prolonged in both untreated and nifedipine-treated mice as compared with that of neutrophils (data not shown).

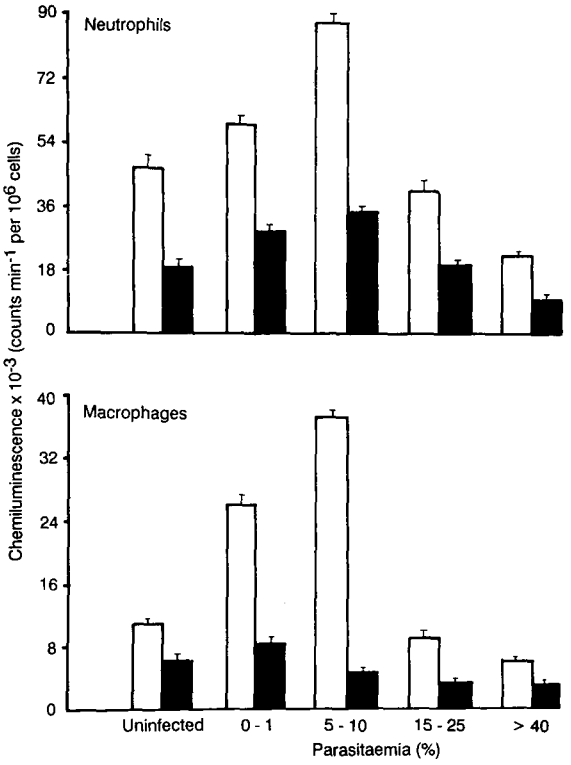

FIG. 1. Peak chemiluminescence responses in neutrophils and peritoneal macrophages from nifedipine-treated (■) and untreated (□) *P. berghei*-infected mice at various parasitaemia levels.

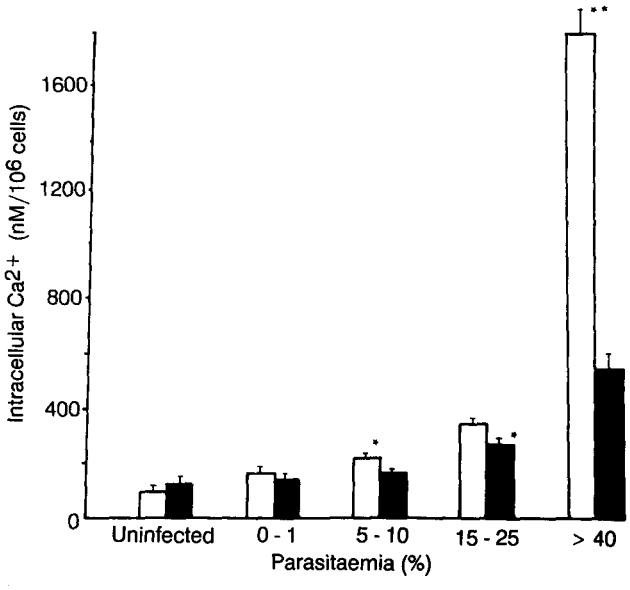

FIG. 2. Intracellular Ca^{2+} in neutrophils from nifedipine-treated (■) and untreated (□) infected mice at various parasitaemia levels. $*P<0{\cdot}05$, $**P<0{\cdot}001$.

Intracellular Ca^{2+}

Before infection, baseline intracellular Ca^{2+} in the phagocytic leucocytes from both nifedipine-treated and untreated control mice remained unaltered (Figs 2, 3). Following infection, an increase in Ca^{2+} with rise in parasite load in both drug-treated and untreated mice was noticed. However, this was significantly lower ($P<0{\cdot}01$) in nifedipine-treated animals compared with controls. Stimulation with fMLP evoked a twofold rise in ionic calcium which was maintained up to 5–10% parasitaemia (Figs 4,5). This declined at higher parasite loads in both groups, but the decline was significantly greater ($P<0{\cdot}01$) in the nifedipine-treated group.

Chemiluminescence-response and changes in cytosolic Ca^{2+}

To delineate the role that calcium might play in triggering the chemiluminescence response, we looked for correlation between changes in Ca^{2+} after priming with fMLP and peak responses of phagocytes at various parasitaemia levels. A positive correlation was observed between the two in both macrophages and neutrophils isolated at different parasitaemia levels in drug-treated as well as control groups (Tables 1, 2).

Discussion

The mononuclear phagocytes and neutrophils play a pivotal role in determining the immune responses responsible for protection against malaria parasites (Allison & Eugui 1983). Oxidant stress is detrimental to the intraerythrocytic development of plasmodium (Clark et al 1983). The biphasic chemiluminescence response of phagocytes observed in our study is in agreement with the findings of Brinkmann et al (1984) and Li & Li (1987) who reported that with lethal *P. berghei* infection, after initial enhancement of free oxygen radical production there was inhibition of oxidative burst activity. The immunosuppression seen during acute infection is also attributed to high parasite burden which probably brings about a degree of immunotolerance (Morakote & Justus 1988). Even though the macrophages and neutrophils expressed some degree of stimulation, those from nifedipine-treated mice invariably exhibited suppressed capacity to generate oxygen free radicals. Their decreased activation potential suggests the in-vivo effect of nifedipine during the course of malaria infection.

The changes of cytosolic free Ca^{2+} following stimulation with a variety of chemotactic factors have paradoxical effects on the extent of free oxygen radical production by macrophages and neutrophils. The resting state free Ca^{2+} observed in the present study is within the range of normal values (100–200 nM). A significant exponential increase in intracel-

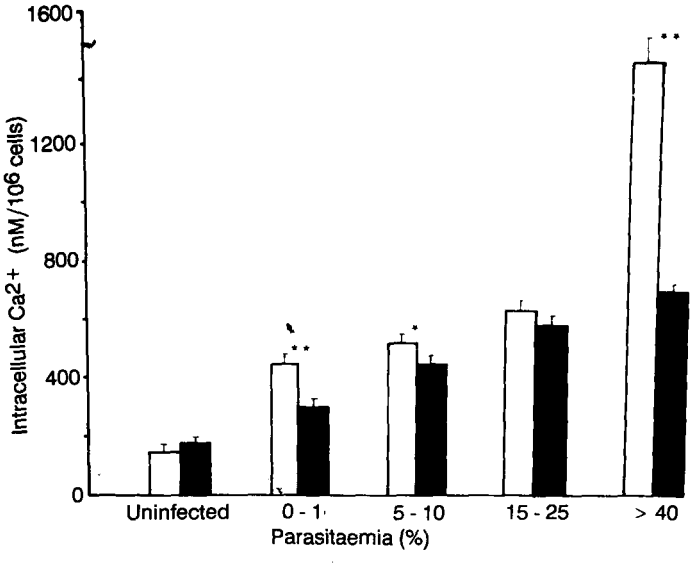

FIG. 3. Intracellular Ca^{2+} in macrophages from nifedipine-treated (■) and untreated (□) infected mice at various parasitaemia levels. $*P<0{\cdot}05$, $**P<0{\cdot}001$.

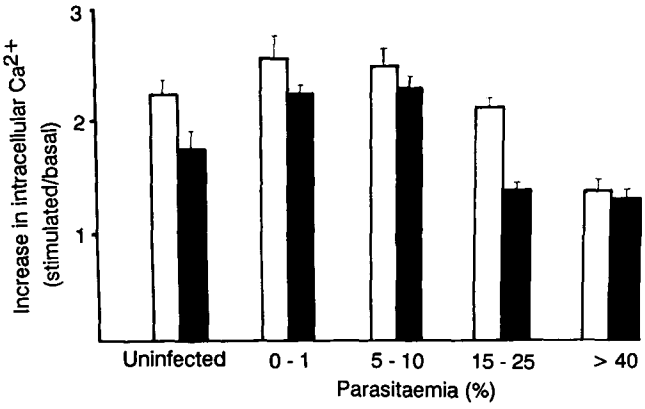

FIG. 4. Rise in intracellular Ca^{2+} on fMLP stimulation in neutrophils from nifedipine-treated (■) and untreated (□) infected mice at various parasitaemia levels.

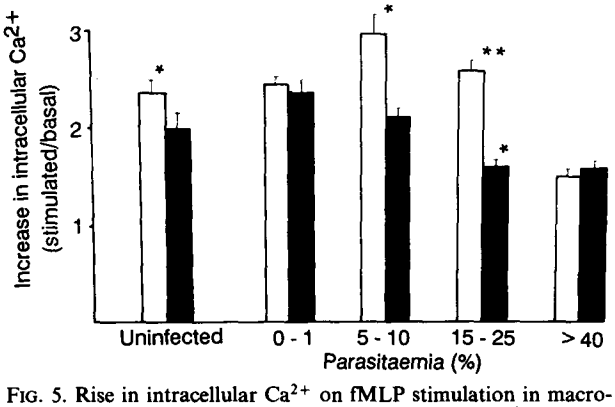

FIG. 5. Rise in intracellular Ca^{2+} on fMLP stimulation in macrophages from nifedipine-treated (■) and untreated (□) infected mice at various parasitaemia levels. $*P<0.05$, $**P<0.001$.

lular Ca^{2+} of phagocytes following infection with *P. berghei* was observed at different parasitaemia levels studied. This finding is in agreement with the finding of a considerable increase in Ca^{2+} occurring in macrophages (Young et al 1984) and neutrophils (Korchak et al 1984; Lew et al 1984) following phagocytosis of opsonized particles and immune complexes. Repeated stimulation of phagocytes by soluble and particulate parasite products as well as circulating immune complexes might be the reason for the consistent rise in intracellular Ca^{2+}.

In the uninfected state, the Ca^{2+} levels of phagocytes from drug-treated mice remained similar to those from untreated animals. Following infection, even though a rise in Ca^{2+} was also seen in phagocytes from nifedipine-treated mice, it was markedly lower than in untreated infected animals. Presumably, this reflects a more potent effect of nifedipine on macrophages and neutrophils in the depolarized state.

The fMLP-mediated rise in Ca^{2+} observed during ongoing infection is biphasic. The ability of phagocytes to respond optimally to fMLP was impaired at higher parasitaemia levels, and we suggest that at lower antigenic load, phagocytes remain capable of optimal stimulation, while with increasing parasitaemia, the cells become desensitized or exhausted due to repeated stimulation. Similar results were obtained by Lee et al (1989) where repeated stimulation of phagocytes was performed in-vitro. The ability of phagocytes to respond to fMLP at higher parasitaemia levels was further compromised in nifedipine-treated animals. The chemiluminescence response in drug-treated animals was found to be significantly lower at higher parasitaemia levels, suggesting the effect of drug is Ca^{2+}-mediated.

From the results of our study, it appears that the quantum of respiratory-burst activity depends on the rise in intracellu-

Table 1. Correlation of neutrophil chemiluminescence response with rise in calcium after fMLP stimulation at various parasitaemia levels.

Untreated *P. berghei* infected	Rise in Ca^{2+} [a]	Chemiluminescence response (counts min^{-1})	
0	2·277 ± 0·120	47932·4 ± 1913·52	
0–1%	2·5943 ± 0·185	59147·4 ± 1050·6	
5–10%	2·5030 ± 0·152	89093·4 ± 2152·52	r = 0·724135
15–25%	2·1467 ± 0·068	34599·6 ± 1962·16	$P<0.01$
>40%	1·376 ± 0·125	21376·0 ± 420·669	
Nifedipine-treated *P. berghei* infected			
0	1·7683 ± 0·19	20747·8 ± 1413·09	
0–1%	2·2723 ± 0·052	28857·2 ± 753·89	
5–10%	2·335 ± 0·148	34429·4 ± 1047·1	r = 0·8817
15–25%	1·4225 ± 0·049	19068 ± 973·34	$P<0.01$
>40%	1·3242 ± 0·072	9093·4 ± 298·12	

$$a = \frac{Ca^{2+} \text{ on fMLP stimulation}}{\text{basal } Ca^{2+}}$$

Table 2. Correlation of macrophage chemiluminescence response with rise in calcium after fMLP stimulation at various parasitaemia levels.

Untreated *P. berghei* infected	Rise in Ca^{2+a}	Chemiluminescence response (counts min^{-1})	
0	2·3893 ± 0·127	10725·2 ± 908	
0–1%	2·4415 ± 0·042	26075·8 ± 1108·81	
5–10%	2·977 ± 0·2334	37203·2 ± 800·86	r = 0·7839
15–25%	2·6025 ± 0·0918	8360·6 ± 492·29	$P < 0·01$
>40%	1·5189 ± 0·0428	5260·4 ± 252·6	
Nifedipine-treated *P. berghei* infected			
0	2·0655 ± 0·170	6005·2 ± 735·2	
0–1%	2·383 ± 0·136	7893 ± 350·55	
5–10%	2·1312 ± 0·11	3890·8 ± 204·78	r = 0·6325
15–25%	1·611 ± 0·488	2598·2 ± 218·09	$P < 0·01$
>40%	1·6084 ± 0·0334	2375·8 ± 66·69	

$$a = \frac{Ca^{2+} \text{ on fMLP stimulation}}{\text{basal } Ca^{2+}}$$

lar Ca^{2+}, which is maintained by critically balanced Ca^{2+} fluxes. With the impairment of these mechanisms during disease or following treatment with calcium antagonists, a suboptimal expression of phagocyte responses could occur, which in turn might prove detrimental to the host during an infection episode.

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