Killing of Blood-Stage Plasmodium falciparum by Lipid Peroxides from Tumor Necrosis Serum

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The multiplication of Plasmodium falciparum in culture, as measured by [3H]hypoxanthine incorporation, was inhibited in a dose-dependent manner by rabbit tumor necrosis serum. The regimen by which tumor necrosis serum is produced caused significant increases in the levels of triglycerides and lipid peroxides, with the latter being indicated by the level of malondialdehyde in the serum. When tumor necrosis serum was depleted of lipoproteins by aerosil (fumed silica), no parasiticidal activity remained, and when it was separated by ultracentrifugation, more than 70% of the parasiticidal activity was found in the lipoprotein fraction. This suggests that lipid peroxides may account for most of the parasiticidal activity in tumor necrosis serum but that a nonlipid toxic factor may also be present.

Tumor necrosis serum (TNS) is generated when animals are treated with Mycobacterium bovis BCG or Propionibacterium acnes (formerly Corynebacterium parvum) followed 14 days later with bacterial lipopolysaccharide (LPS) and then are bled 2 h later (4). This serum, which contains tumor necrosis factor and increased levels of numerous other molecules, has been shown to kill blood-stage malaria parasites in vitro (11) and in vivo (22); this effect in vitro is not due to tumor necrosis factor, as demonstrated by using the recombinant molecule (13, 23). However, the conditions under which this serum is made could implicate reactive oxygen intermediates (ROI). It has been shown in vitro that mitogenic stimulation of peritoneal macrophages 14 days after in vivo treatment with BCG gives an optimal hydrogen peroxide release (19) and that hydrogen peroxide is cytotoxic to blood-stage malaria parasites in vitro (6). However, hydrogen peroxide and other ROI have very short half-lives and are unlikely to be responsible for the in vitro effect of TNS; moreover, their effects would be blocked by antioxidants, which is not the case for the parasiticidal activity of TNS (10). However, lipid peroxides, which can be formed by reacting with ROI, are unaffected by antioxidants (18). Lipid peroxidation has the effect of stabilizing the reactive oxygen groups and thus creates a cytotoxic molecule with a much longer half-life. Also, many by-products of low molecular weight are formed; one is malondialdehyde (MDA) (17). This molecule can be used as an indicator of lipid peroxidation (2). Indeed, it has been shown that low-molecular-weight products are formed when Plasmodium vinckei-infected erythrocytes are exposed to oxidative stress (3) and that these products are toxic to the parasite when tested in vitro (5).

In this study, we have investigated the lipoprotein fraction of rabbit TNS to see whether lipid peroxidation occurs and whether these lipid peroxides are cytotoxic to Plasmodium falciparum in vitro.

MATERIALS AND METHODS

Culture of P. falciparum. The Ugandan strain of P. falciparum (Palo Alto) (7) was maintained in human O− erythrocytes at a hematocrit of 5% in a standard culture system (25) in RPMI 1640 medium containing 10% heat-inactivated human A+ serum, supplemented with 25 mM HEPES (N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid), 0.2% NaHCO3, and 40 U of streptomycin per ml (complete medium) in an atmosphere of 5% CO2, 5% O2, and 90% N2. Cultures were synchronized by treatment with 5% sorbitol (16).

Rabbit sera. Normal rabbit serum was obtained from rabbits that had fasted overnight. TNS was produced as previously reported (22). Briefly, 5 × 109 live M. bovis BCG organisms were injected intravenously. Two weeks later, after an overnight fast, the animals were given 80 μg of bacterial endotoxin (Escherichia coli; Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom) and 1.5 to 2 h later were bled out. The blood was allowed to clot overnight, and the serum was stored at −20°C before use.

Measurement of lipid peroxidation in serum. The following substances were added to 1 ml of fresh serum in a glass container: 5 μl of 0.3% butylated hydroxytoluene (to prevent peroxidation catalyzed by iron), 2 ml of solution containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25% NaCl. The container was then placed in boiling water for 15 min. The sample was cooled and centrifuged at 1,000 × g for 15 min, after which the optical density of the supernatant was measured at 535 nm, and the concentration of MDA was calculated by using an extinction coefficient of 1.56 × 105 M−1 cm−1. The amount detected in phosphate-buffered saline (PBS) after the treatment was taken as the zero value in each assay (2).

Measurement of triglycerides. Triglycerides were measured by using an enzymatic method (kit 676519; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and an Encore chemical system (Baker Instruments Group, Allentown, Pa.).

Depletion of lipoproteins by aerosil. Aerosil (fumed silica; Sigma Chemical Co., St. Louis, Mo.) was used to deplete serum of lipoproteins (21). A 20-mg quantity of aerosil was added to 2 ml of serum (protein concentration, 7.5%; pH 7.2 to 7.8). After the serum was stirred in a water bath at 45°C for 4 h, the serum was cooled and centrifuged at 2,500 × g for 20 min. The supernatant was decanted and dialyzed against PBS—5 mM glutathione (reduced) (GR) before testing.
Separation of lipoproteins by ultracentrifugation. Serum dialyzed against PBS–5 mM GR was adjusted to a density of 1.21 g/ml with NaBr–5 mM GR (ρ = 1.51 g/ml) solution. A 1-ml quantity of NaBr–5 mM GR (1.21 g/ml) was layered on top of the serum preparation, which was then centrifuged for 44 h at 5°C in a 50 Ti rotor at 105,000 × g. After centrifugation was completed, the top 1-ml lipoprotein-containing fraction was removed from each sample. The serum protein pellet was then resuspended in the remaining volume. All samples were dialyzed extensively against PBS–5 mM GR and concentrated with polyethylene glycol (M₉, 20,000; BDH, Poole, England) as necessary to adjust volumes to the original serum volume.

Cytotoxicity assay. All samples for the cytotoxicity assay were extensively dialyzed against PBS–5 mM GR. Doubling dilutions of the test samples were made in PBS–5 mM GR in 50-μl volumes in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) in triplicate. Synchronized cultures of *P. falciparum* at the ring stage were adjusted to a parasitemia of between 0.5 and 1% at a hematocrit of 5% in complete medium, and 50-μl volumes were added to the test samples.

The plates were incubated at 37°C in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. At 24 h, tritiated hypoxanthine (0.4 μCi per well) was added to each well. At 48 h, the plates were harvested and incorporation was measured in a Tri-Carb 574 counter (Packard Instrument Co., Inc., Rockville, Md.).

Results were calculated as the mean percent inhibition of hypoxanthine uptake (± standard error of the mean) and compared with values for controls in which parasites were cultured in 50% PBS–5 mM GR in place of the test serum. From the dose-response curves produced, the concentration of samples that resulted in a 50% parasite growth inhibition was calculated. These concentrations were used to compare the killing of *P. falciparum* by the various serum samples.

**RESULTS**

Rabbits were injected either with BCG or LPS alone or with BCG and LPS. Only the combination of BCG followed by LPS triggered the production of significant parasiticidal activity (Fig. 1). Serum samples from rabbits given BCG alone or LPS alone were unable to produce 50% parasite killing, even at a dilution of 1:2. The parasiticidal activity of each serum sample was measured here as the dilution resulting in a 50% inhibition of parasite growth, as measured by [³H]hypoxantine incorporation.

In order to test whether the parasiticidal activity was related to the lipoproteins, the concentrations of triglycerides were measured in the sera. There was a significant increase in triglycerides (P < 0.01) following the administration of BCG or LPS or both (Table 1). This was not due to dietary factors as all animals were fasted overnight before they were bled. However, there was no direct relationship between the levels of triglycerides and parasite killing by the serum.

MDA was measured next to give an indication of the oxidation state of the lipoproteins. TNS had the highest level of MDA (P < 0.01) and the level of MDA was also elevated in LPS serum (Table 1). No increase was seen in BCG serum. This suggests that lipid peroxidation, rather than simply a high concentration of lipid, may be involved in parasite killing. There was no correlation between the triglyceride concentration and the MDA concentration.

To test more directly for the involvement of lipoproteins, a method was required to deplete the serum of these molecules. Aerosil (fumed silica) almost exclusively removes lipoproteins (21). This treatment entirely depleted the small level of activity in normal rabbit serum and almost completely depleted the activity in TNS (Fig. 2). This suggests that the activity of normal rabbit serum is not due simply to the inability of a human parasite to thrive in the serum of a

**TABLE 1. Effects of BCG and LPS, alone and combined, in rabbits**

<table>
<thead>
<tr>
<th>Rabbit treatment</th>
<th>Serum level (mean ± SEM [n])</th>
<th>% Serum (mean ± SEM [n]) to inhibit parasite growth by 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Triglycerides (nM)</td>
<td>MDA* (μM)</td>
</tr>
<tr>
<td>Control</td>
<td>0.53 ± 0.06 (8)</td>
<td>2.43 ± 0.34 (12)</td>
</tr>
<tr>
<td>LPS</td>
<td>1.82 (2)</td>
<td>3.31 (2)</td>
</tr>
<tr>
<td>BCG</td>
<td>1.21 ± 0.090 (3)</td>
<td>2.36 ± 1.03 (3)</td>
</tr>
<tr>
<td>TNS</td>
<td>1.55 ± 0.620 (7)</td>
<td>5.62 ± 0.290 (8)</td>
</tr>
</tbody>
</table>

* MDA is an indicator of lipid peroxidation.
* * P < 0.01 compared with control sample.

FIG. 1. Inhibition of *P. falciparum* growth by serum samples from rabbits treated with BCG alone (●), LPS alone (○), BCG followed 14 days later by LPS (■), or serum samples from two normal rabbits (□). The data given are from a single experiment in which all sera were tested at the same time. The point used to compare parasiticidal activity in experiments is the dilution of serum required to inhibit parasite growth by 50% (—). Each datum point is the mean ± standard error of the mean for the sample tested in triplicate.

FIG. 2. Inhibition of *P. falciparum* growth by TNS and effect of depleting serum lipoproteins with aerosil. The values were calculated as in the legend to Fig. 1. Abbreviations: 45°C, Serum incubated for 4 h at 45°C; A, serum after treatment with aerosil at 45°C; Dil⁻¹, dilution⁻¹; NRS, normal rabbit serum. Each bar is the mean ± standard error of the mean for four to six experiments. Symbols: *, P < 0.001 compared with normal rabbit serum; **, not significant compared with TNS; ***, P < 0.001 compared with TNS.
different species. This depletion was not due to the incubation of serum at 45°C for 4 h as shown in Fig. 2.

As a result of the nature of aerosol, it was not possible to recover the lipoproteins for testing. Instead, lipoproteins were preparatively separated by density gradient ultracentrifugation at a density of 1.21 g/ml. Figure 3 shows that the lipoproteins in TNS are toxic to the parasite (P < 0.01). The activity of the nonlipid fraction of TNS was reduced to a level not significantly different from that of normal rabbit serum. Thus, the depletion of lipids, while significantly reducing parasite killing, was not as effective as aerosol treatment, which removed essentially all activity. This may suggest the presence of two parasiticidal molecules, one of which is lipoprotein in nature.

**DISCUSSION**

We have demonstrated that the administration of BCG followed by LPS leads to a rise in the concentration of serum triglycerides in rabbits and also to an increase in lipid peroxidation, as indicated by the thiobarbituric acid test. The elevated level of triglycerides is partly due to the BCG infection, as previously shown (24), but the LPS injection increases this level still further. These changes can be accounted for by several mechanisms. LPS can cause release of lipoproteins from various cell types. Also, this regimen causes the release of tumor necrosis factor, which in turn can block the action of lipoprotein lipase, thus blocking triglyceride clearance from the serum (15). LPS can also interfere with the activation of triglyceride clearance enzymes. It has been shown that the concentration of serum triglycerides can increase significantly within 2 h of LPS injection alone (14).

It is perhaps not surprising, then, that lipid peroxidation has occurred in TNS when the treatment is also optimal for macrophage activation (19). LPS alone can activate macrophages but not as well as treatment with BCG and LPS; therefore, some lipid peroxidation would be expected in the LPS serum, but it was not as high as in TNS (Table 1).

The results shown in Table 1 suggest that lipid peroxidation is not solely involved because the LPS serum should then have activity intermediate between that of TNS and normal rabbit serum, which it clearly does not. However, the lipoprotein fraction of TNS was undoubtedly more toxic than that of normal rabbit serum (Fig. 3). If this effect were simply due to the triglyceride concentration, one would expect the BCG and LPS sera to be as toxic as TNS. There was no correlation between triglyceride concentration and the percent serum required to inhibit parasite growth by 50%.

If the aerosol results and the ultracentrifugation results are taken together, it is clear that there is another parasiticidal factor present which also binds to aerosol, suggesting a factor with high hydrophobicity. This molecule may also be present in normal rabbit serum but is either in a less toxic condition or at a lower concentration than in TNS. Preliminary data from chromatographic separations do support the idea of two cytotoxic fractions, one a lipoprotein fraction and a low-molecular-weight fraction (data not shown).

Lipid peroxides have been implicated in a number of diseases and have been shown to be cytotoxic to various cell types. They could kill cells in several ways. Since the parasites do not have a de novo triglyceride synthesis pathway (20), they must obtain all their lipid requirements from the host serum. If oxidized lipids are taken up by the parasite, they may have a direct cytotoxic effect on the parasite, for example, in cross-linking proteins. However, they are more likely to destabilize the cell membrane, in particular the erythrocyte membrane, which could then lead to parasite death without necessarily destroying the erythrocyte; this is quite possible as no significant hemolysis was seen in the cytotoxicity assays. It has been shown that in a malaria infection, the lipid content of the erythrocyte membrane can rise by up to 600% (1). Of the lipids, linoleic acid in particular increases. This is an unsaturated fatty acid which is readily oxidized. Therefore, linoleic peroxides are likely to be readily taken up by the parasitized erythrocytes. As well as causing membrane destabilization, the peroxides can initiate further oxidation in the presence of oxygen, especially in the presence of ferric ions, which act as a catalyst; both of these are available within the erythrocyte. The erythrocyte does have antioxidant pathways which would prevent this occurrence, but the potential oxidative capacity of TNS may be too much for the system. This threshold concept could explain why LPS serum is not parasiticidal, if the oxidative capacity of the LPS serum were within the antioxidant capacity of the erythrocyte.

If the erythrocyte were damaged enough, it would not support parasite growth. This process would be semiselective for the parasitized erythrocytes because parasitized erythrocytes would take up more lipid as mentioned above. There would be some effect on normal erythrocytes but not enough perhaps to hinder normal erythrocyte function, although there might be an effect on reinvasion by the parasites.

In summary, we have demonstrated that the direct parasiticidal effect of rabbit TNS can be attributed to an increase in lipid peroxides, with the probability that a second nonlipid parasiticidal molecule is also present. These molecules, then, must be added to the growing list of non-antibody-mediated anti-plasmodial molecules, which already includes eosinophil products (26), orosomucoid (9), polymine oxidase (8), and crisis forming factor (12). We are currently investigating whether our nonlipid factor is related to one of these molecules.
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