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Functional and Phenotypic Changes in Human Lymphocytes after Coincubation with *Leishmania donovani* In Vitro

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In this paper we describe functional and phenotypic changes in T cells after in vitro coincubation of peripheral blood mononuclear cells (PBMC) and *Leishmania donovani* parasites at different parasite/peripheral blood mononuclear cell ratios. The phytohemagglutinin (PHA)-induced lymphoproliferative response was reduced by the coincubation, and at the maximal parasite/peripheral blood mononuclear cell ratio used (7.5:1), the average response was less than 40% of the response in the absence of parasites. The cause of the reduction in lymphoproliferation is not clear, but it requires live parasites. Interleukin-1 production was unaffected, the levels of soluble interleukin-2 receptor in supernatants were not changed by the coincubation, and the addition of exogenous interleukin-2 failed to revert the suppressive effect of the parasites. In addition to the reduction in lymphocyte proliferation, phenotypic lymphocyte changes were observed. Cell surface expression of the CD3 antigen, which is part of the CD3–T-cell receptor complex, was significantly reduced with increasing parasite/peripheral blood mononuclear cell ratios; the reduction was general in the sense that the parasites caused a shift in the fluorescent intensities of anti-CD3 labeled cells toward lower values, without affecting the distribution pattern. In contrast, the parasites altered the CD25 (interleukin-2 receptor) expression on PHA-stimulated cells from a homogenous CD25-positive population to two populations, one small and without CD25 expression and the other, larger population with only a slight reduction in size and CD25 expression. In addition to the changes in expression of surface antigens, a general reduction in the size of PHA-stimulated lymphocytes after coincubation with the parasites was observed. The data presented thus suggest that the inhibition of the proliferative response to PHA by live *L. donovani* in vitro is associated with early processes in lymphocyte activation. Further studies on the inhibitory phenomena described may be of potential significance in the investigation of the suppressive mechanisms in human visceral leishmaniasis.

Leishmania donovani, the causative agent of human visceral leishmaniasis, is an obligate intracellular hemoflagellate parasite living in the phagolysosomes of macrophages. Patients suffering from visceral leishmaniasis are characterized by aberrant immune functions. High levels of circulating antibodies are being produced; most of these are unspecific, and the remainder confer little, if any, protection. Furthermore, the patients show markedly depressed cellular immune responsiveness, as demonstrated by the absence of delayed-type hypersensitivity reactions to leishmanial antigen (14, 15) and the inability of peripheral blood mononuclear cells (PBMC) to respond to antigen by either lymphoproliferation or cytokine production (7–9). The mechanisms responsible for the observed immunosuppression are far from fully elucidated but probably involve functional changes in monocytes and macrophages as well as lymphocytes.

In the present report we describe functional and phenotypic changes in peripheral blood T lymphocytes after coincubation with *L. donovani* parasites in vitro.

MATERIALS AND METHODS

Mononuclear cells. PBMC were isolated from heparinized venous blood by density centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). Upon isolation, the PBMC were stored in liquid nitrogen until use. On the day of use, the PBMC were quickly thawed to 37°C and washed three times in RPMI 1640 supplemented with 10% heat-inactivated pooled human serum, 58.4 μg of L-glutamine per ml, 20 IU of penicillin per ml, and 20 μg of streptomycin per ml (RPMI-S). Cell viability upon thawing was >90%, determined by trypan blue exclusion. All donors used in this study were healthy adults without known prior exposure to *Leishmania* species.

Parasites. The *L. donovani* Kituri strain Kenya (NBL 274) was grown as promastigotes in vitro in medium 199 supplemented with 20% heat-inactivated fetal calf serum and 50 μg of gentamicin per ml (M199-S), at 27°C.

Before use in experiments, stationary-phase parasites were washed once in RPMI-S and suspended at 1×10^7, 2×10^7, and 3×10^7 cells per ml. Parasite supernatants were produced by incubating promastigotes in microwell plates (Nunc, Roskilde, Denmark) at the above concentrations at 37°C in 5% CO_2 for 4 days. Plates were then centrifuged, and supernatants were harvested.

Parasite viability after incubation at 37°C in 5% CO_2 for 4 days in the absence of PBMC was tested by using the parasites for inoculation of new routine cultures in M199-S at 27°C. Parasite infectivity was tested by coincubation of promastigotes and PBMC (2:1) at 37°C in 5% CO_2 for 4 days and confirming promastigote invasion and amastigote multiplication by daily microscopy of Giemsa-stained slides. The parasites were viable and infective by these criteria.

Parasite inhibition assay. All experiments were done in 96-well round-bottomed microwell plates (Nunc). In experiments with parasites, wells contained 75 μl of PBMC (1.33×10^6/ml), 25 μl of promastigote suspension, and 20 μl of phytohemagglutinin (PHA) (300 μg/ml). In experiments with parasite supernatants, wells contained 10 μl of PBMC (1×10^7/ml), 90 μl of promastigote supernatant, and 20 μl of PHA

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(300 μg/ml). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 days and pulsed with 20 μl of [^{3}H]thymidine (1.85 MBq/ml; Dupont, NEN Research Products, Boston, Mass.) per ml 24 h before termination of the assay. Cultures were harvested onto glass fiber filters, and the incorporation of radiolabel into DNA was determined by liquid scintillation spectrometry. All tests were done in triplicate.

In experiments aimed at restoring the proliferative response, 10 or 100 U (final concentration) of exogenous recombinant interleukin-2 (IL-2) per ml was added to the cultures.

Cytokine measurements. Supernatants from cultures run in parallel to the parasite inhibition cultures were assayed for IL-1β, IL-2, and soluble IL-2 receptor. Levels of IL-1β (Interleukin-1 beta ELISA kit; Cistron Biotechnology, Pine Brook, N.J.) and soluble IL-2 receptor (CellFree IL-2R ELISA kit; T Cell Sciences, Cambridge, Mass.) were measured after 24 h. Levels of IL-2 (InterTest-2 ELISA kit; Genzyme Corp., Boston, Mass.) were measured after 48 h of coincubation.

Fluorescence-activated cell sorting analyses. For analysis of PBMC by fluorescence-activated cell sorting, 600 μl of PBMC, 200 μl of promastigote suspension, and 160 μl of PHA (all concentrations as above) were incubated in 24-well multi-dish plates (Nunc) for 3 days as above. After two washes in phosphate-buffered saline (pH 7.2) containing 3% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), samples were labeled at 4°C with anti-CD18 (DakoPatts, Glostrup, Denmark) stained by phosphatidylethanolamine-conjugated anti-kappa light chain (Becton-Dickinson and Co., Mountain View, Calif.), and either fluorescein isothiocyanate-conjugated anti-CD3 (Becton-Dickinson) or fluorescein isothiocyanate-conjugated anti-CD25 (DakoPatts). After labeling, samples were washed twice in cold phosphate-buffered saline–3% fetal calf serum and analyzed on a FACStar fluorescent cell sorter. A total of at least 10,000 cells, filtered to exclude debris, were analyzed.

Statistical analyses. The Page test for ordered alternatives was used to test the null hypothesis that the mean results of experiments were independent of doses of coincubated parasites or parasite supernatants versus the alternative hypothesis that there was a dose-dependent suppression. $P(L)$ values of <0.05 were considered significant.

RESULTS

Lymphocyte proliferation. Figure 1 shows the effect on PHA-induced lymphoproliferation of coincubation of PBMC with live stationary-phase leishmanial parasites at various concentrations. Coincubation with live *L. donovani* caused a significant [$L = 207$, $n = 7$, $P(L) < 0.01$] parasite-dose-dependent suppression of the mitogenic lymphocyte response.

In three experiments with parasites that had been killed before the assay by heating (56°C, 30 min), or boiling (100°C, 5 min), no significant inhibition was seen ($L = 83$ and $L = 81$, respectively) (Fig. 1).

To test the possibility that the observed inhibition might be caused by depletion by the parasites of a nutritional constituent necessary for the lymphoproliferative response, promastigotes were incubated in RPMI-S for 4 days at the same concentrations and conditions as in the direct inhibition experiments. After the incubation, the supernatants were used for cultivation of PBMC as described above. In these experiments no significant inhibition of the PHA-induced lymphoproliferative response related to the parasite concentration during the preassay conditioning was seen ($L = 161$, $n = 6$) (Fig. 2).

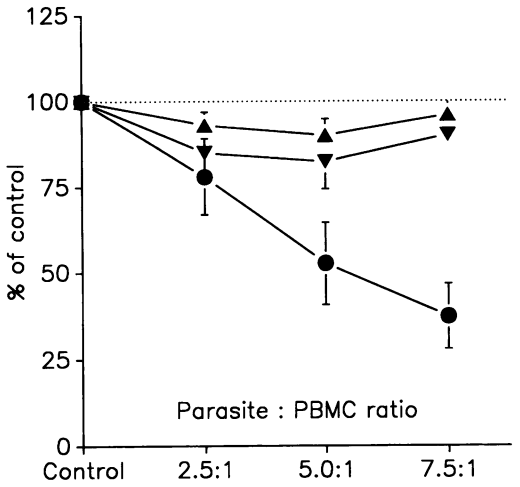

FIG. 1. Effect of *L. donovani* on the PHA-induced lymphoproliferative response of PBMC. Symbols: ●, live parasites ($n = 7$); ▲, parasites killed by heating to 56°C for 30 min ($n = 3$); ▼, parasites killed by heating to 100°C for 5 min ($n = 3$). Results are given as percentages of incorporation of [^{3}H]thymidine in the absence of parasites (means ± standard errors of the means).

Exogenous IL-2 (10 or 100 U/ml) added to the culture in two experiments failed to remove the inhibitory effect of the parasite coincubation but produced a slight general increase in [^{3}H]thymidine incorporation (Fig. 2).

L. donovani parasites incorporated [^{3}H]thymidine after 4 days in culture at 37°C, but always less than 5% of the incorporation of radiolabel by the lymphocytes.

Cytokine measurements. Supernatant levels of IL-1 were 310 to 1,500 pg/ml, levels of soluble IL-2 receptor were 190

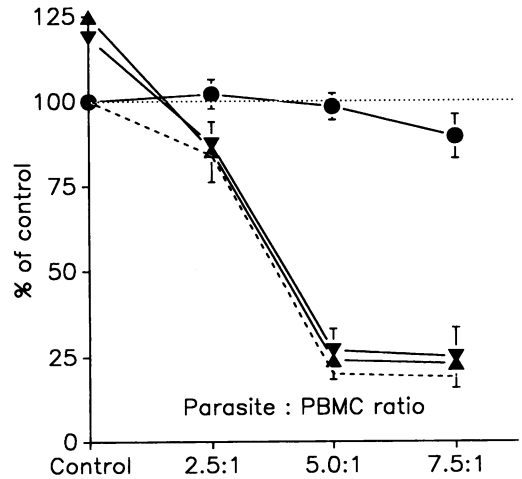

FIG. 2. Effect of soluble factors on the PHA-induced lymphoproliferative response of PBMC. Symbols: ●, supernatants conditioned by previous culture of various numbers of *L. donovani* for 4 days ($n = 6$); ▲, live parasites plus 10 U of IL-2 per ml ($n = 2$); ▼, live parasites plus 100 U of IL-2 per ml ($n = 2$). Incorporation in the corresponding two experiments with live parasites in the absence of IL-2 is indicated by a dashed line for reference. Results are given as percentage of incorporation of [^{3}H]thymidine in the absence of parasites (means ± standard errors of the means).

TABLE 1. Relative changes in phenotypic parameters after PHA stimulation of PMBC in the presence of increasing amounts of *L. donovani* parasites

Parasite/PMBC ratio	Relative change (% of value in absence of parasites; mean ± SEM; $n = 5$)				
	CD18-positive cells			CD25-positive cells	
	Mean CD3 fluorescence[a]	Mean cell volume[a] (FCS[b])	% CD25-positive cells[a]	Mean CD25 fluorescence	Mean cell volume[a] (FSC)
2.5:1	106.2 ± 15.0[c]	94.9 ± 6.3	97.0 ± 4.8	99.5 ± 13.3	98.5 ± 3.8
5.0:1	79.2 ± 19.1[c]	87.2 ± 5.4	92.0 ± 5.5	105.4 ± 11.3	88.7 ± 4.9
7.5:1	52.2 ± 6.2	81.7 ± 5.8	75.9 ± 7.5	90.5 ± 12.8	90.4 ± 3.8

[a] Reduction significant at $P = 0.01$.
[b] FSC, Forward scatter signal.
[c] $n = 4$.

to 780 U/ml, and levels of IL-2 were generally below or just above the detection limit of the assay (approximately 1 U/ml). No pattern could be detected relating supernatant levels of these cytokines to the inhibition of lymphoproliferation seen (data not shown).

Fluorescence-activated cell sorting analyses. Analyses of phenotypic changes after 3 days of PHA stimulation were done by fluorescence-activated cell sorting. To secure the exclusion of extracellular parasites from analyses, all samples were gated to include only CD18-positive cells. The CD18 antigen is part of the leucocyte function-associated antigen LFA-1 (CD11/CD18), a molecule which is present on almost all leucocytes. Parasites were not labeled by anti-CD18.

A generalized reduction was seen in the amount of CD3 on the surface of PBMC incubated with *L. donovani*, which was significantly related to the parasite dosage [$L = 118$, $n = 4$, $P(L) < 0.01$]. Table 1 shows the reduction in mean fluorescence, and Fig. 3A shows results from a typical experiment to illustrate the fact that, although a shift toward lower fluorescent intensities was seen, the shape of the fluorescence intensity curves remained essentially unchanged. The CD3 antigen is associated with the T-cell receptor complex CD3-TcR. CD3 was present on approximately 70% of resting PBMC, rising to about 85% after 3 days of PHA stimulation. The average fluorescence intensity of unstimulated PBMC was about 60% of the intensity of PHA-stimulated cells.

In addition to the reduction in CD3 expression, a change in the surface expression of the CD25 antigen was noted. In contrast to the observations with CD3, PHA-stimulated PBMC changed from one homogenous CD25-expressing population to two populations, one that expressed the antigen and another smaller population that did not (Fig. 3B). This change resulted in a reduced percentage of CD25-positive cells that were significantly related to increasing numbers of parasites [$L = 141$, $n = 5$, $P(L) = 0.01$]. When looking exclusively at the CD25-positive cells, the mean fluorescence was almost unchanged; the reduction was not significant ($L = 129$, $n = 5$), although a significant reduction in mean cell volume, evaluated as forward scatter signal, was seen [$L = 143$, $n = 5$, $P(L) < 0.01$] (Table 1). The CD25 antigen is a component of the IL-2 receptor and was nearly absent on resting T cells but present on most PHA-activated T cells by day 3.

The forward scatter signal reduction in CD25-positive cells was also seen when all CD18-bearing cells were examined. There was a general reduction in mean cell volume significantly correlated to the parasite/PBMC ratio [$L = 145.5$, $n = 5$, $P(L) < 0.01$] (Table 1). Figure 3C shows results from a typical experiment.

DISCUSSION

Human visceral leishmaniasis is a disease associated with severe immunosuppression of the patient. Cellular responses to specific leishmanial antigen are reduced in vivo (14, 15) as well as in vitro (6). Some controversy exists as to whether in vitro proliferative responses to nonleishmanial antigens and to mitogens are likewise affected (7, 9) or not (3–6).

To investigate the immunosuppressive effects of *L. donovani* parasites, we conducted a series of experiments to examine whether the presence of these parasites would influence the PHA-induced responses of lymphocytes from donors never exposed to the disease. That flagellate parasites of humans can suppress PHA-induced responses has previously been reported in the case of *Trypanosoma cruzi* (1). We found that the PHA-induced lymphoproliferative response was reduced by coincubation with *L. donovani*, resulting in about 60% reduction at the maximal parasite/PBMC ratio used (7.5:1), compared with the response in the absence of parasites. The reduction was seen only when live parasites were used, suggesting that some metabolic mechanism was responsible, either in the parasites themselves or in the monocytes and macrophages infected by them, subsequently subversing lymphocyte functions. Simple nutritional depletion of media by the parasites as the cause of inhibition seems unlikely, since media conditioned by cultivation of parasites for 4 days before assay were not inhibitory to proliferation.

Crawford et al. (8) found that human monocytes infected with *Leishmania tropica* showed reduced IL-1 production compared with that of uninfected controls when they were challenged with *Staphylococcus epidermidis*, a potent IL-1 inducer, whereas Cillari et al. (7) found LPS-induced IL-1 production in vitro to be normal in PBMC obtained from visceral leishmaniasis patients compared with that in healthy controls. We did not observe any reduction in IL-1 levels that could be related to the degree of infection of the monocytes.

It has been shown that lymphocytes from visceral leishmaniasis patients produce little or no IL-2 after antigenic or mitogenic stimulation in vitro (5, 7), but to our knowledge no reports exist concerning IL-2 production after artificial *L. donovani* infection of unexposed human PBMC in vitro. In a similar system, with *Trypanosoma cruzi*, Beltz et al. (2) found no decrease in ability to produce IL-2 after exposure to the parasites. Measurements of supernatant levels of IL-2 produced no evidence for the idea that the faulty proliferative response in our experiments was caused by lack of this mediator, and experiments aimed at removing the parasite-

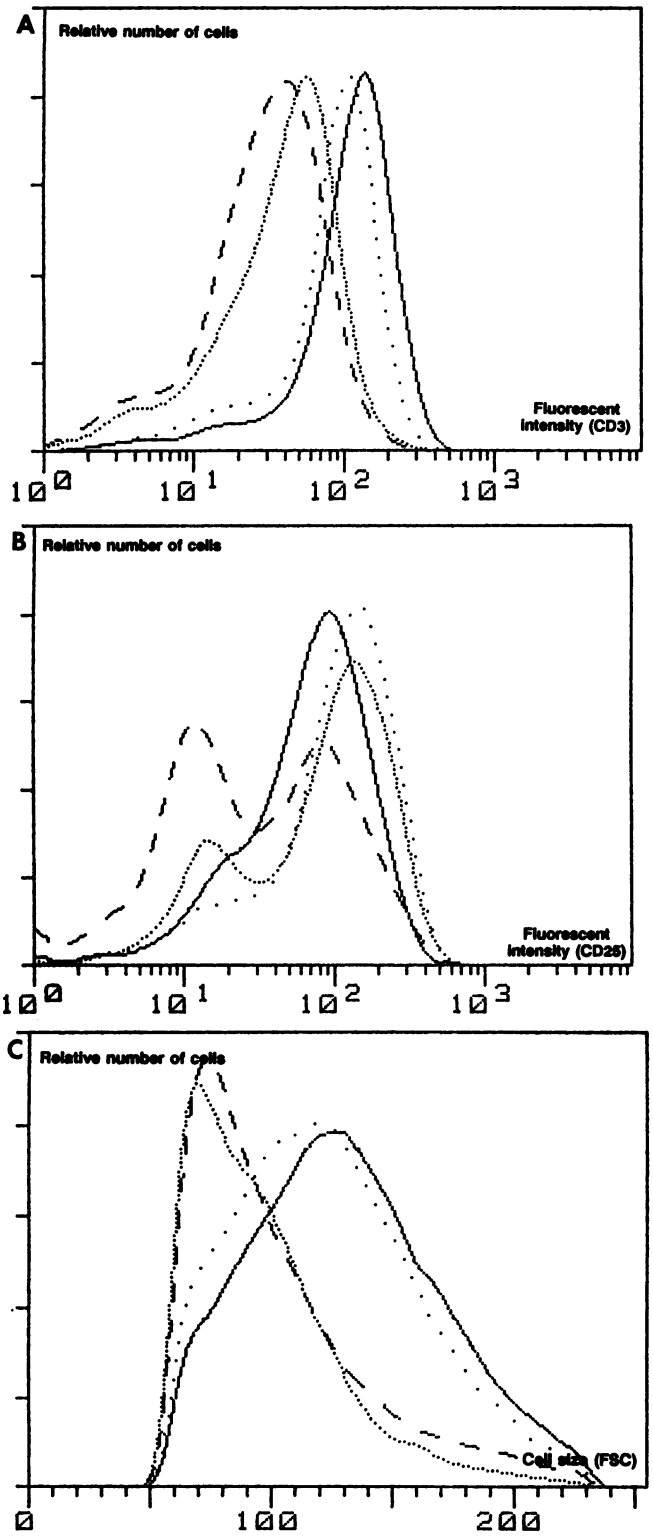

FIG. 3. Phenotypic changes in lymphocytes after coincubation with increasing numbers of *L. donovani*. (A) CD3 surface expression. (B) CD25 surface expression. (C) Cell volume (forward scatter signal). Abscissa, Arbitrary units of either fluorescence intensity (A, B) or forward scatter signal (C). Ordinate, Relative numbers of cells. Solid lines, no parasites; wide dotted lines, parasite/PBMC ratio of 2.5:1; narrow dotted lines, parasite/PBMC ratio of 5.0:1; dashed lines, parasite/PBMC ratio of 7.5:1. Results of a typical experiment are given.

induced inhibition of the proliferative response by the addition of exogenous IL-2 failed to do so.

Clues to the suppressive mechanisms involved may lie in the observation that coincubation with parasites resulted in a reduced surface expression of antigens of importance to lymphocyte function.

The CD3 antigen, which is strongly associated with the T-cell receptor, is a molecule of crucial importance in the major histocompatibility complex-restricted T-cell activation by accessory cells, and modulation of CD3 expression has been shown to result in loss of responsiveness of T cells to activation by PHA (12). It is unknown how *L. donovani* could affect the lymphocyte expression of CD3, and hence responsiveness, but it could be through some sort of monokine signaling. Media conditioned by parasite cultivation before the assay did not inhibit the lymphoproliferative response to PHA, making it unlikely that a soluble mediator such as an excreted factor (13) from the parasites themselves was responsible. The reduction in CD3 expression was general in the sense that it did not lead to a change in the shape of the distribution of CD3 fluorescence intensities. It could be argued that this reduction was merely a reflection of the reduced cell volumes, and hence surface membrane area, which was also seen as a consequence of the parasite coincubation. However, PHA-stimulated cells were larger than nonstimulated PBMC, even in the presence of the maximum parasite/PBMC ratio used, whereas CD3 fluorescence was lower, suggesting a parasite-induced loss of this antigen. Several mechanisms could be responsible for such a loss. One possibility is proteolytic cleavage by parasite proteins with enzymatic activity, such as GP63.

In a previous study Cillari et al. reported unchanged CD25 expression after PHA stimulation of PBMC from visceral leishmaniasis patients compared with that of healthy controls (7). In the present study, coincubation with parasites led to a significant reduction in the percentage of T cells expressing CD25 after PHA stimulation, although the majority of T cells had essentially normal levels of CD25 expression despite some reduction in size. Thus the parasites appeared to induce a change in CD25 expression that was quite different from the effect seen on CD3 expression and resulted in a splitting of the T cells into two subpopulations, one expression CD25 the other not.

Several possibilities exist in the interpretation of the above findings in the light of the reduced lymphoproliferative response. A reduction in CD25 expression, albeit slight, could contribute to the impaired response to PHA, although a panel of anti-CD25 antibodies failed to effectively block PHA-mediated lymphocyte activation (10). Alternatively, the reduction in the frequency of CD25-positive cells might be of little consequence, and the cause of T-cell anergy could be functional damage to the expressed IL-2 receptors. This impairment could either be in the p75 molecule, which together with CD25 forms a heterodimer IL-2 receptor with high affinity for IL-2, or in a part of CD25 affecting IL-2 binding but not anti-CD25 binding. Finally, the cause of the failing proliferative response may be unrelated to changes in the IL-2 receptor altogether.

After the incubation with parasites, the lymphocytes changed from one homogenous CD25-expressing population to two populations; one expressing the antigen normally and the other essentially without expression. This indicates that the lymphocyte populations are differentially affected by the coincubation.

Although very high levels of soluble IL-2 receptors in serum have been described in visceral leishmaniasis patients

(11), we did not find a significant increase in the levels of soluble IL-2 receptors in supernatants that could be taken as evidence for pathological receptor shedding.

The finding that the general size of PHA-stimulated PBMC was reduced by coincubation with the parasites could be taken as evidence that whatever the inhibitory mechanism is, it affects very early processes in the T cell-activating sequence preceding the blast formation.

The in vitro model described in this report might be useful in attempts to elucidate the mechanisms involved in the immunosuppression seen in visceral leishmaniasis.

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