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Tumor Necrosis Factor Does Not Induce *Plasmodium falciparum* Crisis Forms†

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Mouse and rabbit sera from animals treated with *Mycobacterium bovis* BCG and lipopolysaccharide contained tumor necrosis factor (TNF) and induced malaria parasite crisis forms. However, neither purified mouse- nor recombinant DNA-produced human TNF induced crisis forms in cultured *Plasmodium falciparum*. Furthermore, rabbit polyclonal and mouse monoclonal antibodies against human TNF did not block the parasite inhibitory activity of human malaria crisis form factor serum from Sudan.

Crisis forms are malarial organisms whose intraerythrocytic development has been retarded, resulting in moribund or morphologically and physiologically abnormal parasites in the absence of pharmacologic interference. The mechanism for crisis form induction is not known, but since crisis forms were first described by Taliaferro and Taliaferro as occurring in *Plasmodium brasilianum* infections of *Cebus* monkeys undergoing severe immunologic crisis, i.e., the abnormal parasites appeared within erythrocytes concomitantly with a rapid decrease in parasitemia during a severe infection, these investigators attributed the appearance of the parasites to immunologic intervention (13). Once described, crisis forms were routinely reported as occurring in murine and simian malaria infections. A few years ago, Clark and his co-workers reported that mice whose cell-mediated immune responses were hyperstimulated by infections with *Mycobacterium bovis* BCG followed by injections of lipopolysaccharide (LPS) were also protected from potentially severe malarial infections, which were resolved by an unknown mechanism that resulted in the appearance of crisis forms (2, 3). Because the BCG-LPS treatment was known to stimulate the production of numerous mononuclear cell secretions, the most thoroughly characterized being the monokine, tumor necrosis factor (TNF), it was postulated that malaria crisis forms had resulted from the actions of TNF (3, 15). Numerous subsequent studies with serum from BCG-LPS-treated animals (known as tumor necrosis serum [TNS]) known to contain TNF demonstrated inhibition by TNS of malarial parasites both in vitro and in vivo (14, 15, 17), thus adding support for the hypothesis that intracellular killing of malaria parasites was induced by TNF. Finally, polyclonal antibody against semipurified TNF was used to inhibit the antiparasitic activity of TNS, providing what appeared to some investigators to be the definitive evidence needed to establish the antiplasmodium activity of TNF (4). The report of these results, with similar reports cited above, precipitated reviews in which TNF was specifically identified as the cause of malaria crisis forms (9, 10, 16). Indeed, Playfair et al. (9) mentioned that, pending results with recombinant TNF, the best evidence was that TNF, and not some other component, was responsible for malaria crisis forms.

With the demonstration that human serum from malarious areas of Sudan contains a yet unidentified, nonimmunoglobulin component that induces crisis forms in *Plasmodium falciparum* in vitro (5, 6), the importance of crisis forms in the immune response of falciparum malaria has been established. The purification and characterization of this serum component, termed crisis form factor (CFF), has obvious implications for the development of effective malaria immunoprophylaxis. Since it has been repeatedly stated in the literature that TNF induces malarial crisis forms, we have attempted to confirm whether TNF is the same as CFF by using cultures of *P. falciparum* to test both serum-purified and gene-cloned preparations of TNF for intraerythrocytic parasite inhibition. Notwithstanding the previous report that human serum containing CFF had no demonstrable TNF activity (1), definitive evidence that TNF and CFF are different factors has not yet been presented.

Assays for TNF activity involve cultivation of a sensitive cell line, such as ML929, with titrations of the test sample followed by observations for cytolysis by morphologic examination of stained cells (11) or by measurement of the release of [^{3}H]thymidine from prelabeled cells (8). Measurements of TNF activity are reported in units per milliliter, where a unit represents the dilution factor which results in cytolysis of 50% of the target cells. For example, "250,000 U of TNF" indicates that the sample must be diluted 250,000 times to reach a 50% toxicity level in the assay. The assay for *P. falciparum* crisis form induction requires that highly synchronous ring-stage parasite cultures be cultivated for 40 h in the presence of the test solutions, with retardation of intraerythrocytic development being determined by microscopic examination of Giemsa-stained thin films or by measurements of incorporation of [^{3}H]hypoxanthine into parasite nucleic acids (7). Highly inhibitory malaria-immune serum can reduce parasite development by more than 90% when used in parasite cultures at a 6.25% (vol/vol) concentration (6, 7). In the experiments reported here, we used the morphologic and radiometric assays outlined above for ML929 (TNF) and *P. falciparum* (CFF) assays, respectively.

Before the availability of gene-cloned TNF, we obtained from the laboratory of L. Old, Sloan-Kettering Institute, New York, N. Y., semipurified TNF extracted from sera of mice treated with *M. bovis* BCG LPS. Notwithstanding the fact that the intact BCG-LPS-treated mouse serum readily induced crisis forms in cultured *P. falciparum* and that semipurified TNF was 200-fold more potent against ML929 cells than was the intact serum, semipurified mouse TNF

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was not active against the malaria parasites. We concluded from these experiments that BCG-LPS-treated mouse TNS contained CFF as well as TNF, since the serum was both antiparasitic and tumoricidal, and that although the TNF-directed chromatography increased the antitumor activity, it resulted in the loss of CFF. With the recent availability of human gene-cloned TNF, we tested two preparations for *P. falciparum* crisis form activity in vitro. The first sample, a gift from L. Old, was produced by Genentech and contained 10^6 U of TNF per ml (assay confirmed by us). This preparation, tested against the parasites at concentrations of up to 250,000 U of TNF per ml, was not inhibitory to *P. falciparum* in vitro. The second TNF preparation (code named PAC 4-D) was kindly provided by its producer, Asahi Chemical Co. of Japan, which also provided us with mouse monoclonal and rabbit polyclonal antibodies against its gene-cloned human TNF. We assayed the PAC 4-D preparation against cultures of ML929 cells and found that the company estimate of 500,000 U of TNF per ml was conservative, the actual concentration being nearly twice that amount.

Because the preparation of PAC 4-D contained 1% gelatin to stabilize it, we prepared 1% gelatin (Knox, unflavored, low LPS) in phosphate-buffered saline and used this solution as a control diluent. Thus, we compared the intraerythrocytic development of *P. falciparum* in the presence of 250,000, 125,000, and 62,500 U of PAC 4-D in RPMI 1640 medium containing 5% (vol/vol) pooled human serum and in a medium containing the 1% gelatin diluted to the same concentration as that found in PAC 4-D. Parasite development from highly synchronous rings to multinucleated schizonts was unaffected by the presence of TNF (PAC 4-D), as determined by Giemsa-stained thin films and the incorporation of [^{3}H]hypoxanthine into parasite nucleic acids (7). These negative results notwithstanding, the possibility existed that TNF might somehow have been inactivated by the parasite culture conditions (perhaps being adsorbed to the erythrocytes or otherwise impaired); therefore, we repeated the inhibition experiments, this time collecting the medium from the parasite cultures after the 40-h crisis form assay and then assaying for TNF with ML929 cells. Controls for this experiment were PAC 4-D dilutions kept at 4°C during the 40 h of the parasite cultivation. There was no significant loss of anti-ML929 cell activity by PAC 4-D when it was used in medium to culture the parasites. Hence, our experiments demonstrated that TNF did not inhibit the development of *P. falciparum* in vitro, even when used at 250,000 U/ml. Furthermore, parasite culture conditions did not significantly reduce the potency of TNF, because it maintained its anti-ML929 cell activity after 40 h of incubation with parasite-infected erythrocytes. Some reports have suggested that TNF activity is enhanced by the presence of alpha and beta interferons (12). Accordingly, we repeated our experiments using interferons with TNF, and again, there was no evidence of antiplasmodial activity.

According to the protocols we received from Asahi Chemical Co., equal volumes of the monoclonal or polyclonal antibodies mixed with PAC 4-D would neutralize the TNF activity of the preparation. Our experience suggested that under such conditions, the antibodies reduced TNF activity by 50%, confirming our earlier observation that PAC 4-D was probably more concentrated than the company estimated. Notwithstanding the fact that both the mouse monoclonal and rabbit polyclonal antibodies abolished the ML929 cell activity of PAC 4-D, these antibodies had no effect on reducing the antiparasitic activity of human CFF-containing serum. Moreover, human CFF-containing serum, which could reduce parasite development by 50% when used at 6.25% serum concentration (equivalent to 16 U of CFF activity, by the same standards as those applied to TNF activity), had no inhibitory activity against ML929 cells, as reported previously (1).

In summary, despite the fact that TNS obtained from mice or rabbits stimulated with *M. bovis* BCG and LPS to induce cytokine secretion contains factors which are cytotoxic to ML929 cells (TNF activity) and inhibit intraerythrocytic malarial parasite development (CFF activity), these two activities are apparently due to different molecules, because serum-purified TNF, being highly toxic to ML929 cells, was not active against the parasites. Furthermore, our experiments have demonstrated that human serum collected from individuals living in malarious regions of Sudan induces crisis forms in *P. falciparum* in vitro, but is not inhibitory to ML929 cells (1). More direct evidence has now been obtained by culturing the parasites at a concentration 250,000 times that needed for human gene-cloned TNF to induce 50% ML929 cell toxicity without measurable effects on the parasite development. In other words, 250,000 U of TNF had no inhibitory activity against *P. falciparum*, and 16 U of CFF had no activity against ML929 cells. Antibodies against the gene-cloned product abolished its ML929 cell activity, but they did not affect the ability of human CFF-containing serum to induce crisis forms in *P. falciparum*. These latter observations are in conflict with those reported by Haidaris et al. (4), but their antibodies were developed against semipurified TNS, which probably still contained CFF. For these reasons, we believe that CFF is not human or mouse TNF.

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