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Malarial parasites induce TNF production by macrophages

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SUMMARY

Mouse peritoneal macrophages incubated with erythrocytes infected with non-lethal or lethal variants of *Plasmodium yoelii* or with *P. berghei*, in the presence of polymyxin B to exclude the effects of any contaminating endotoxin, secreted a cytotoxic factor into the supernatant that was shown to be tumour necrosis factor (TNF). No differences were observed in the ability of the three types of parasite to induce TNF production, which was maximal in the range of 0·2–5 infected erythrocytes per macrophage. TNF production was equivalent to that induced by lipopolysaccharide (LPS) and was enhanced by pretreatment of the macrophages with interferon-gamma (IFN-γ) or with indomethacin. Culture media containing parasite products also induced macrophages to secrete TNF. The activity withstood boiling and was inhibited by malaria-specific antisera. Since heat-stable antigens are present in the circulation of patients with malaria, they may induce the secretion of TNF, a mediator of endotoxic shock, which could contribute to the pathology of the disease.

INTRODUCTION

Tumour necrosis factor (TNF), a secretory product of activated macrophages, is a mediator of inflammation and various immunological reactions that can cause a wide range of changes in a variety of cell types (reviewed by Beutler & Cerami, 1986). There is evidence that early in malaria it may play a protective role, in that administration of recombinant TNF to infected mice inhibits the multiplication of both liver and blood stage parasites (Schofield *et al.*, 1987; Taverne *et al.*, 1987; Clark *et al.*, 1987). However, it is also likely that many of the pathological effects seen in malaria are mediated by TNF (reviewed by Clark, 1987). In particular, Grau *et al.* (1987) have demonstrated that mice can be prevented from developing cerebral malaria by specific antibody against TNF, while TNF was detected in the serum of mice with neurological complications. Its presence has also been reported in sera from patients infected with *Plasmodium falciparum* (Scuderi *et al.*, 1986) and in mice infected with *P. vinckei* (I. A. Clark and G. Chaudri, manuscript in preparation). Furthermore, spleen cells from mice infected with lethal and non-lethal parasites acquire cytotoxic activity that is mediated by TNF (Taverne, Treagust & Playfair, 1986).

TNF appears to be a membrane-associated protein (Decker, Lohmann-Matthes & Gifford, 1987) whose release can be triggered by various molecules, principally of microbial origin, of which the most active is bacterial lipopolysaccharide (LPS). This agent greatly increases the rate of production of mRNA and causes transcription and secretion of TNF (Beutler *et al.*, 1986), which acts as an important mediator of endotoxicity.

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Endotoxin-like activity has been detected by the Limulus lysate assay in the serum of patients infected with *P. falciparum* (Tubbs, 1980; Usawattanakul *et al.*, 1985), of mice infected with *P. berghei* (Tubbs, 1980) and in lysates of erythrocytes infected with *P. berghei* (Felton, Prior & Spagna, 1980). Recently, P.H. Jacobsen, L. Baek and S. Jepsen (manuscript submitted for publication) reported that affinity-purified soluble antigens of *P. falciparum* also reacted in the Limulus lysate assay. Furthermore, Hotez *et al.* (1984) have shown that a saponin lysate of *P.berghei*-infected erythrocytes, like endotoxin, induces mouse peritoneal cells to release a substance which suppresses lipoprotein lipase activity and which may be TNF.

We report here that several species of malarial parasites and their soluble antigens induce the release of TNF from mouse peritoneal macrophages in conditions excluding any effect of contaminating endotoxin. Since macrophages are activated in malaria to be capable of secreting TNF (Taverne *et al.*, 1986) and TNF activity is found in the serum if infected mice are given endotoxin (Clark *et al.*, 1981; Taverne, Depledge & Playfair, 1982), it is likely that the parasites themselves and their circulating antigens induce the secretion of TNF during infection.

MATERIALS AND METHODS

Mice

Six-week-old outbred females were used (Tuck No 1, A. Tuck & Sons, Battlesbridge, Essex).

Parasites

P. yoelii 17X, its lethal variant YM (obtained from Dr Walliker, Edinburgh University, as described by Freeman & Holder,

1983) and *P. berghei* Anka were used. Mice were infected i.v. with 10^4 parasitized erythrocytes and parasitaemia was determined from blood films stained with Giemsa.

Parasite soluble antigens

Mice with high parasitaemia were bled by cardiac puncture, the erythrocytes washed, suspended in RPMI at a concentration of 10^8 parasitized cells per ml and incubated for 24 hr at 37° in an atmosphere of 5% CO_2 in air. Supernatants were centrifuged, passed through a 0·2 μm millipore filter (Flow Laboratories, Irvine, Ayrshire) and stored at 4° or at −20°.

Antigen

Antiserum against the lethal variant of *P. yoelii* was kindly provided by Mr J. B. de Souza of this department. It was obtained from mice that had been vaccinated with a Triton X-100 lysate of parasitized erythrocytes, injected with saponin as adjuvant, challenged and bled 5 days after recovery (Playfair & de Souza, 1986). Rabbit antiserum against recombinant murine TNF made by Biogent was kindly provided by Dr W. Fiers of Rijksunniversiteit, Gent, Belgium.

Peritoneal cells

Cells were obtained by peritoneal lavage of normal mice or of mice given 1 ml of fetal calf serum (FCS; Gibco Ltd, Paisley, Renfrewshire) i.p. 24 hr previously or 2 mg i.p. of killed *Propionibacterium acnes* (*Corynebacterium parvum*; Wellcome Research Laboratories, Beckenham, Kent) 7–12 days previously or infected more than 3 weeks earlier with 10^7 BCG bacilli (Glaxo) (Evans Medical Ltd, Greenford, Middlesex) and boosted 3 days previously. Washed cells were suspended in 5% FCS in RPMI-1640 (Flow Laboratories), and counted using acridine orange and ethidium bromide, and the number of macrophages present was determined morphologically from cytocentrifuge preparations stained with Giemsa. Cell suspensions were adjusted to a known number of viable macrophages per millilitre and 0·1-ml volumes were dispensed into wells of 96-well microtitre plates (Nunc, Roskilde, Denmark) and incubated alone or with indomethacin (1 μg/ml) (Sigma, Poole, Dorset) or recombinant mouse interferon-gamma (rIFN-γ) (Genentech, kindly provided by Dr G. R. Adolf, Ernst-Boehringer Institut, Vienna) for 3 hr at 37° in an atmosphere of 5% CO_2 in air. Non-adherent cells were then removed by washing, the medium replaced with the various stimulants to be tested and the plate incubated overnight. Next day, supernatants were collected and stored at 4° for at most 3 days, or at −20°, before they were assayed for TNF.

TNF assay

TNF was assayed colorimetrically by its cytotoxicity for mouse L929 cells (kindly provided by Dr N. Matthews, Welsh National School of Medicine, Cardiff) as described previously (Taverne *et al.*, 1984) except that serial dilutions of supernatants were tested in duplicate in medium containing 5 μg/ml actinomycin D (Sigma). One unit is defined as that causing 50% cell destruction.

Other reagents

Polymyxin B sulphate was obtained from Sigma and lipopolysaccharide (LPS W from *Escherichia coli* 055:B5) from Difco Laboratories, Detroit, MI.

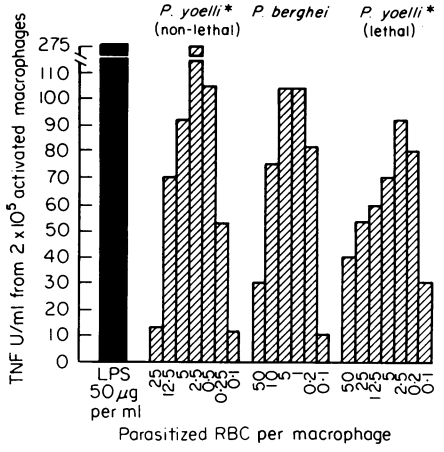

Figure 1. Induction of TNF release by parasitized erythrocytes. Macrophages obtained from mice infected with BCG were incubated overnight with different numbers of parasitized erythrocytes and the supernatants were assayed for TNF; means of duplicates are shown. At least three preparations of each parasite were tested and the results of a typical experiment are illustrated. * Diluted in normal mouse erythrocytes to give a constant ratio of 50 erythrocytes per macrophage.

RESULTS

In preliminary experiments in which macrophages were incubated with LPS, we confirmed that the amount of TNF produced varied not only with the concentration of LPS but also with the state of activation of the macrophages, that is, whether they were obtained from normal mice or from mice injected with *P. acnes* or BCG. Sometimes highly activated macrophages released some TNF when incubated in medium alone, without stimulants, perhaps because they were stimulated *in vivo* or during manipulation: this background activity has been subtracted from all figures quoted here. In order to enable comparisons to be made between experiments done with macrophages of different activity, a control concentration of LPS known to give maximal yield of TNF was included in all experiments. In a pilot experiment, 5 μg/ml of polymyxin B was shown to inhibit all TNF induction from activated macrophages incubated with 5 μg/ml of LPS. Therefore, to exclude the possibility that traces of contaminating endotoxin might contribute to TNF production, all other stimulating agents were diluted in medium containing 1 μg/ml of polymyxin B.

Induction of TNF release by parasitized erythrocytes

Incubation of activated macrophages with varying numbers of erythrocytes infected with non-lethal or lethal parasites was found to induce the release of cytotoxic activity into the supernatants in a dose-dependent manner. When a polyclonal antiserum against murine recombinant TNF was incubated with a parasite-induced supernatant in the tumour cell assay, all the cytotoxicity was blocked, indicating that it was due to TNF. No activity was found when macrophages were incubated with uninfected mouse erythrocytes at a ratio of 50 erythrocytes to one macrophage, or with sheep erythrocytes. The greatest activity was observed with ratios of 0·2–5 parasites per macrophage for the three types of parasite tested (Fig. 1) and all induced TNF release to about the same extent. In some experiments the amount of TNF produced was similar to that

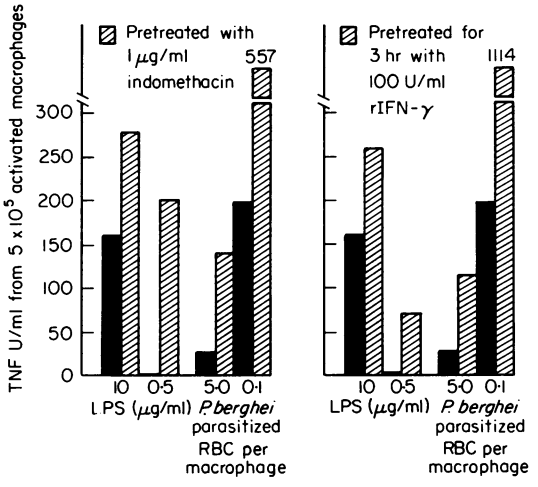

Figure 2. Enhancement of parasite-induced TNF production by pretreatment of macrophages with (a) 1 μg/ml indomethacin and (b) 100 U/ml IFN. Cells were treated for 3 hr and washed before overnight incubation with parasitized erythrocytes: means of duplicate assays for TNF are shown. Results of a typical experiment are shown, which illustrate consistently reproducible effects. Hatched bars = pretreated cells.

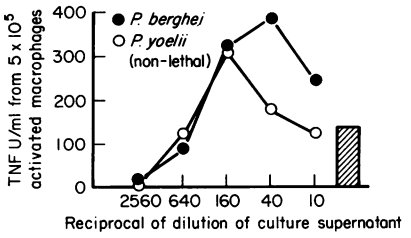

Figure 3. Induction of TNF release by boiled supernatants of parasite cultures. The points are means of duplicates. At least three samples of supernatants of cultures of the three types of parasite have been tested and representative results for *P. berghei* (●) and *P. yoelii* (non-lethal) (○) are illustrated. Bar = 1 μg/ml of LPS.

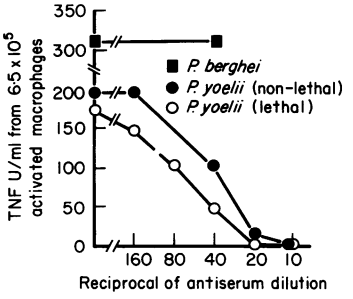

Figure 4. Inhibition by antiserum against lethal *P. yoelii* of inducing activity of boiled culture supernatants. The points are means of duplicates. Three different serum samples were tested with the same results. The sample illustrated was tested twice, but results from one experiment are shown. (○) Lethal *P. yoelii* diluted 1/40; (●) non-lethal *P. yoelii* diluted 1/100; (■) *P. berghei* diluted 1/40.

obtained with LPS. Like LPS, parasites induced greater secretion from both normal and activated macrophages, which had been pretreated with IFN; results from a typical experiment are shown (Fig. 2b). Prostaglandins decrease the amount of TNF secreted by macrophages (Kunkel *et al.*, 1986) and pretreatment with indomethacin to inhibit synthesis of prostaglandins also enhanced TNF induction by parasitized erythrocytes (Fig. 2a), often resulting in the same yield of TNF from normal macrophages as obtained from untreated macrophages activated *in vivo* (not shown).

Induction of TNF release by soluble products of parasites

Parasitized erythrocytes that had been fixed with formaldehyde still induced secretion of TNF, though usually less than unfixed preparations, and the observation that fewer than one infected red blood cell (RBC) per macrophage was effective suggests that a soluble product may be involved in induction. Therefore, tests were done with culture media from parasites incubated overnight in various conditions. These showed that soluble products from the three sorts of parasite induced release of TNF that could be similar to that produced by 1 μg/ml of LPS, that their activity increased proportionately with the number of parasitized erythrocytes cultured, up to concentrations of 10^8 per ml, and that greater activity was obtained if the parasites were kept in medium consisting of RPMI-1640 alone rather than with serum. TNF induction was still observed, to about the same degree, with parasite culture medium that had been boiled for 5 min. The results illustrated are of representative titrations of samples of boiled culture medium harvested from 10^8 erythrocytes per millilitres infected with either *P. yoelii* or *P. berghei* (Fig. 3). Less induction was usually observed at the highest concentrations and end-points occurred at about 1/1000 dilution. None of the parasite culture supernatants were themselves cytotoxic for the tumour cells. Supernatants from the same number of uninfected erythrocytes incubated similarly did not induce macrophages to secrete TNF.

Inhibition by malaria-specific antisera of TNF induction by parasite products

When antiserum against lethal *P. yoelii* was incubated with boiled culture medium from erythrocytes infected with this parasite or with the non-lethal *P. yoelii* before addition to the macrophages, TNF production was inhibited in a dose-related fashion. Normal mouse serum had no inhibitory effect. The results of titrations of one batch of antiserum are shown (Fig. 4). It did not inhibit the activity of a similar boiled supernatant prepared from *P. berghei*-infected cells, nor that of LPS. Similarly, an IgG fraction separated from hyperimmune serum against the non-lethal *P. yoelii* blocked the activity of a homologous boiled supernatant but not of one prepared from *P. berghei* (not shown).

DISCUSSION

Our results show clearly that erythrocytes infected with malarial parasites induce activated macrophages to release TNF, that the parasites need not be alive, that some of their soluble products are also effective and that some (or all) of these are heat-stable.

The highest concentrations of parasitized erythrocytes were noticeably less effective at inducing TNF release than some lower ones, and this was also true of parasite culture supernatants, whether or not they had been boiled, though it was not observed with LPS. One explanation may be that some other parasite antigens, which are also released into the culture medium, inhibit the capacity of the heat-stable products to stimulate macrophages, or actually block the release of TNF. Since parasite growth is not synchronized we have not yet correlated the synthesis of the active soluble products with a particular stage of the life cycle. Indeed, some of the variability encountered between experiments in the amount of TNF obtained may have been due to the variable composition, in terms of developmental stages, of preparations of parasitized erythrocytes, either used to stimulate macrophages directly or cultured to prepare active supernatants. Some variability is also due to differences in the responsiveness of macrophage populations.

Inhibition by hyperimmune serum against the lethal and non-lethal *P. yoelii* demonstrated that induction of TNF release is mediated by parasite antigens specifically, and not by putative traces of endotoxin. Furthermore, although peritoneal macrophages from C_3H/HeJ mice (which are genetically less responsive to LPS) secreted 10 times less TNF than cells from outbred mice in response to a given concentration of LPS, they produced more TNF when stimulated with the heat-stable products of *P. berghei* and lethal *P. yoelii* (our unpublished work). A number of other agents, including mycobacteria (Matthews, 1982; Rook *et al.*, 1987), zymosan (Matthews, 1982), Sendai virus (Aderka *et al.*, 1986) and some mitogens (Nedwin *et al.*, 1985) are also known to trigger secretion of TNF. Whether or not these antigens act by way of the same receptor as LPS is yet to be determined.

The close resemblance between the pathological features of malaria and endotoxaemia led Clark (see 1987 review) to postulate that parasites possess endoxin-like activity and subsequently led Tubbs (1980) and Felton *et al.* (1980) to test parasitized erythrocytes for activity in the Limulus lysate assay. Tubbs also tested sera from patients with malaria, as did Usawattanakul *et al.* (1985), who concluded that the presence of endotoxin-like activity bore no relationship to Gram-negative bacteriaemia but was related to parasitaemia and was probably derived from the parasites themselves or from the gut. It seems likely that many of the clinical changes in both conditions are mediated by TNF and that in malaria, in view of our results, parasite products may be responsible for inducing its release from macrophages. These are known to be activated as a consequence of infection (Taverne *et al.*, 1986), probably through the action of IFN-γ, and are capable of yielding TNF if stimulated by LPS (Clark *et al.*, 1981; Taverne *et al.*, 1982), and we report here that treatment of macrophages with IFN-γ increases the amount of TNF produced after stimulation with parasitized erythrocytes. Although in man the association of TNF with cerebral malaria is still unclear, the presence of TNF does appear to be associated with cerebral malaria in CBA mice infected with *P. berghei* (Grau *et al.*, 1987). Since, however, mice of other strains infected with this lethal parasite do not develop cerebral malaria and antibody against TNF did not protect those CBA mice, which did not develop early neurological symptoms, from dying later with high parasitaemia, induction of TNF release by the parasite does not appear to be the sole cause of its lethality. Indeed, we found that erythrocytes infected with non-lethal or lethal parasites, and their soluble products, induced TNF secretion equally well *in vitro*. Perhaps the normal mechanisms that limit the damaging effects of TNF *in vivo* are less effective in lethal infections, or there may be less, or less inhibitory, antibody made against the soluble antigens than in non-lethal infections.

Although we have not yet identified the particular antigens that can induce release of TNF, experiments are in progress to see if media from cultures of *P. falciparum* act similarly. In their ability to withstand boiling, the stimulatory antigens of these rodent parasites TNF resemble the diverse, strain-specific system of S-antigens of *P. falciparum*, first described in the serum of patients with malaria (Wilson *et al.*, 1969). S-antigens are associated with the development of the late trophozoite and are released at schizogony (Wilson & Bartholomew, 1975); they have since been characterized (Winchell, Ling & Wilson, 1984) and one has been cloned (Coppel *et al.*, 1983). Heat-stable antigens have also been found in the serum of mice infected with *P. yoelii* and *P. berghei* (Taylor *et al.*, 1986). It is well known that fever is associated with schizogony, and it is interesting to note that both the pyrogenic mediators, IL-1 and TNF, are macrophage products whose synthesis and release can be stimulated by LPS and, we would propose, by circulating parasite antigens released at schizogony; yields of both are known to be enhanced from macrophages activated by IFN-γ.

Although their function is unknown, the antigenic diversity of S-antigens probably reflects strong immune pressures that must be of positive value to the host, and Anders (1986) has suggested that the presence of such antigens in the circulation could promote parasite survival by diverting antibody from damaging the parasite itself. Alternatively, we would suggest that early in infection and at low concentrations secretion of TNF induced by such antigens may promote host survival (Taverne *et al.*, 1987; Clark *et al.*, 1987), whereas later on the potentially harmful effects of excess production of TNF may be limited by the circulating antibody, as suggested by our results obtained with hyperimmune serum (see Fig. 4). If this is so, these antigens may be useful as disease-preventing vaccines.

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