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Stimulators of tumour necrosis factor production released by damaged erythrocytes

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SUMMARY

We sought to characterize factors released by sonicated human erythrocytes that stimulate peripheral blood mononuclear cells (PBMNC) to release tumour necrosis factor-α (TNF). This response is not inhibited by polymyxin B, indicating that contaminating lipopolysaccharide (LPS) is not responsible. When erythrocyte lysates are fractionated by reverse-phase chromatography using a gradient of *n*-propanol on Sep-Pak® C18 cartridges, the TNF-inducing activity elutes as a single peak. The erythrocyte-derived TNF-inducing activity is unaffected by digestion with proteases but is destroyed by mild base hydrolysis or digestion by lipases, indicating that compounds containing ester-linked acyl chains may be essential. These properties are similar to those of TNF stimulators that we have previously identified in erythrocytes infected with malaria parasites, except that the TNF-inducing activity per cell is about 200 times higher in parasitized erythrocytes than in uninfected erythrocytes. Lipase-digested erythrocyte lysates inhibit the TNF-inducing factors of both normal and malaria-infected erythrocytes, suggesting that lipase digestion creates partial structures which compete with active components for macrophage receptors. Such receptors may recognize a common structure that contains an inositol monophosphate (IMP)-like component, as IMP also inhibits the TNF response to erythrocyte-derived factors and to parasite lysates whereas it does not affect the response to LPS. We conclude that lysed erythrocytes release specific cytokine-inducing factors that may contribute to the fever response to non-infectious tissue injury.

INTRODUCTION

Episodes of widespread cellular damage are commonly associated with generalized symptoms such as fever, as well as local inflammation. Although cell damage accompanied by fever is usually observed in the context of infection or immunological disturbance, it also occurs in malignancy and when tissues are damaged by severe trauma or vascular occlusion. Apart from those situations where an infectious or immune process is clearly involved, the mechanism of the fever response to cell injury is poorly understood.

It appears that all fevers, irrespective of their primary cause, are ultimately mediated through endogenous pyrogens such as tumour necrosis factor-α (TNF), interleukin-1 (IL-1) and IL-6.[1] This raises the question of whether cell damage directly stimulates the production of endogenous pyrogens. Damaged cells release much debris including intracellular mediators and possibly cytokines that might stimulate other cells to release endogenous pyrogens. However it is also possible that cellular injury results in the release of specific factors that signal tissue damage and thereby elicit an appropriate host response. We have previously reported that TNF-inducing factors are released by malaria parasites when they rupture out of host erythrocytes.[2] In this paper we report that damaged human erythrocytes themselves stimulate human mononuclear cells to release TNF, and that this can be attributed to specific TNF-inducing factors which we have partially characterized.

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MATERIALS AND METHODS

TNF stimulation assays

Heparinized blood from healthy adult volunteers was mixed with an equal volume of saline and peripheral blood mononuclear cells (PBMNC) were isolated on Lymphoprep (Nyegaard, Oslo, Norway). They were washed three times, resuspended in RPMI containing 1% A+ human serum, and dispensed into flat-bottomed 96-well microtitre plates at 1×10^5 cells/well. After allowing cells to adhere for 1 hr at 37°, the samples to be tested were added. In some assays non-adherent cells were removed by gentle washing and then inhibitors were added followed by stimulants, all diluted in RPMI. As positive controls for TNF stimulation we used titrations of lipopolysaccharide (LPS) from *Escherichia coli* 0·55:B5 or lipoteichoic acid from *Streptococcus pyogenes*

(Sigma, Poole, UK). The plates were incubated overnight in 5% CO_2 at 37° and the supernatants were harvested and assayed for TNF by enzyme-linked immunosorbent assay (ELISA) as previously described.[3] In brief, microtitre plates (Nunc Maxisorb, Roskilde, Denmark) were coated with a murine monoclonal antibody to human TNF (CB0006; Celltech, Slough, UK) in carbonate buffer. After washing wells with phosphate-buffered saline plus 0·2% Tween-20 (PBS-T), samples were added. The plates were then incubated for 2 hr, washed with PBS-T, incubated for 1 hr with rabbit anti-human TNF IgG (Endogen, Boston, MA), washed with PBS-T, and then incubated for 45 min with goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase. After a final wash with PBS-T, *p*-nitrophenylphosphate in diethanolamine buffer was added and colour development was measured at 405 nm. TNF concentrations were estimated with reference to recombinant human TNF standards which were included on each plate.

Erythrocyte preparations

Venous blood was collected from healthy donors and separated on Lymphoprep as already described. After removing the PBMNC layer the erythrocyte pellet was washed three times, resuspended at 1×10^9 cells/ml in RPMI, and stored at 4° for up to 2 weeks. Three different types of erythrocyte preparation were used.

(1) Washed erythrocytes were sonicated using a Lucas Dawe ultrasonicator for 30 seconds on constant cycle at full power.

(2) Washed erythrocytes were lysed by the addition of 0·01% saponin for 2 hr at 4°. Lysed cells were centrifuged at 10 000 *g*, and the pellet was washed repeatedly in sterile PBS until a haemoglobin-free membrane preparation was achieved.

(3) Washed erythrocytes were lysed by the addition of 0·5% TX-114 in PBS and incubated on ice for 1 hr with vortexing at intervals of 10 min. Cellular debris was removed by an initial centrifugation at 1000 *g* for 10 min at 4°. The supernatant was collected, warmed to 37° overnight and centrifuged at 10 000 *g*. The pellet was collected and washed with prewarmed sterile PBS.

The erythrocyte membrane preparations from (2) or (3) were diluted 1 in 10 in PBS containing 5% *n*-propanol and applied to C18 Sep-Pak® cartridges (Millipore, Harrow, UK). Cartridges were washed with PBS containing 5% *n*-propanol and erythrocyte lysates were fractionated by reverse-phase chromatography under a gradient of 5–80% *n*-propanol in PBS. The true *n*-propanol concentration of each fraction was calculated from refractometer readings compared to known standards. Fractions were subsequently dried in a Jouan evaporator, and resuspended to a standard volume in PBS before testing.

Plasmodium falciparum *preparations*

Plasmodium falciparum was cultured in human erythrocytes of blood group O using RPMI-1640 supplemented with 2 g/l glucose and 10% human serum, essentially as described by Trager & Jensen.[4] Cultures containing mature trophozoites and schizonts at ~10% parasitaemia were centrifuged at 10 000 *g* for 10 min and the supernatant removed. The pellet was lysed by the addition of 4 volumes of sterile endotoxin-free water, diluted a further 1 : 10 in PBS, and stored at −20° prior to testing. In some experiments we used partially purified preparations of these lysates, from which the non-stimulatory components had been removed by washing in PBS and methanol/chloroform/water (8 : 4 : 3) (manuscript in preparation). The parasite strain used was R29, a laboratory line derived from the Brazilian isolate IT 14/4/25.

Extraction with acidified methanol : chloroform : water

Samples (0·5 volumes) were dissolved in 1·9 volumes of a methanol/chloroform mixture (2 : 1) with 0·43 M HCl. After vortexing, a further 0·6 volumes of chloroform and then 0·6 volumes of water were added. The mixture was again vortexed, allowed to stand at room temperature and then centrifuged at 3000 *g* for 10 min. The upper (methanol–water) phase, the lower (chloroform) phase and the methanol–chloroform interface were collected separately, dried under vacuum and resuspended in PBS before testing.

Enzymatic and chemical treatments

Proteases. Samples were incubated for 24 hr at 37° in 100 μg/ml pronase E (Sigma) and then boiled. To minimize the risk of endotoxin contamination, all preparations were then mixed with polymyxin B-agarose (Sigma) centrifuged and sterilized by filtration.

Lipases. Samples were diluted in PBS and incubated overnight at 37° with 5 U/ml of mixed lipases (triacyl glycerol lipase and acyl hydrolase; Sigma) bound to agarose. This digestion was terminated by the removal of enzyme by centrifugation at 10 000 *g*. Digested samples were filtered before use.

NaOH deacylation. Samples were diluted in 0·05 M NaOH and incubated at 56° for 2 hr. The reaction was terminated by neutralization with 1 M HCl.

Inhibition studies

Erythrocyte-derived TNF-inducing factors were obtained by pooling active fractions eluted from C18 Sep-Pak cartridges and digested with lipases as outlined above. Digested preparations were filtered and tested for their ability to inhibit macrophage stimulants. Other inhibitors tested included polymyxin B sulphate, phosphatidylinositol (sodium salt, PI), inositol-1-monophosphate (IMP), mannose, mannose-1-phosphate, glucosamine, glucosamine-1-phosphate, galactose, galactose-1-phosphate, galactosamine, and galactosamine-1-phosphate which were all obtained from Sigma.

RESULTS

Sonicated erythrocytes stimulate TNF secretion by PBMNC

We have previously shown that erythrocytes lysed by freezing or hypotonic lysis stimulate little or no TNF production.[5,6] The present experiments show that sonicated erythrocytes stimulate high levels of TNF production by autologous mononuclear cells. This response was dose dependent, and was not observed for untreated erythrocytes or sonicated control media (Fig. 1). No TNF secretion was observed when PBMNC were incubated with untreated erythrocytes of different ABO type, confirming that blood group incompatibility was not responsible for TNF secretion (data not shown).

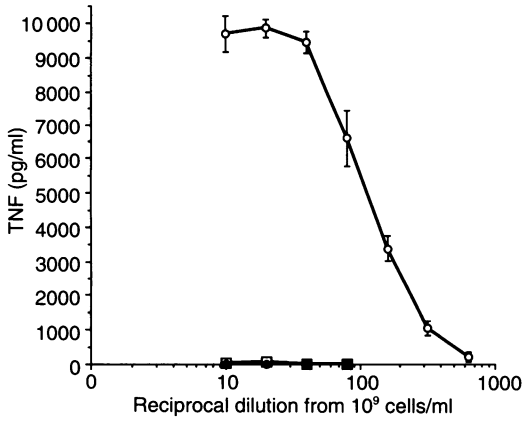

Figure 1. Sonicated human erythrocytes stimulate the secretion of TNF from PBMNC. Mean yields of TNF ± SD of triplicate wells incubated with sonicated autologous erythrocytes (○), untreated autologous erythrocytes (●) or sonicated RPMI controls (□) are shown from a representative experiment. Similar results were obtained in four different experiments.

Extraction and fractionation of erythrocyte-derived TNF-inducing factors

To discover if TNF-inducing activity could be recovered from erythrocytes by methods other than sonication, we also lysed erythrocytes with saponin or with the detergent TX-114 as described in the Materials and Methods. The membrane fraction of these lysates were solubilized with an acidified mixture of methanol/chloroform/water. When such mixtures were made biphasic by the addition of water, TNF-stimulating activity was recovered from the alcohol–water phase and at the interface of this biphasic mixture, while no activity was found in the chloroform phase (Fig. 2).

Membrane preparations isolated either by saponin lysis, or by TX-114 lysis, were resuspended in 5% *n*-propanol in PBS

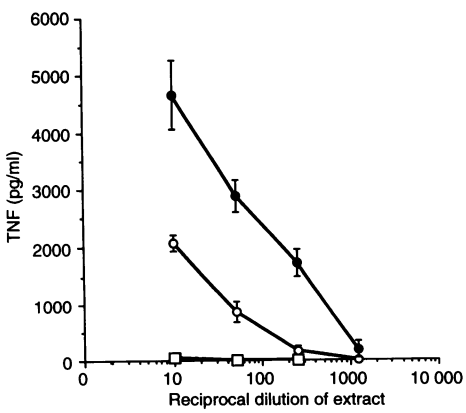

Figure 2. TNF induction by the detergent fraction of a TX-114 erythrocyte lysate, after extraction of this fraction in a biphasic mixture of acidified methanol/chloroform/water. Values given are the mean amount of TNF ± SD secreted, from triplicate wells incubated with dilutions of the alcohol–water phase (●), the chloroform phase (□), and the interface between the two (○). These data are representative of six experiments: similar results were found for saponin-lysed erythrocytes.

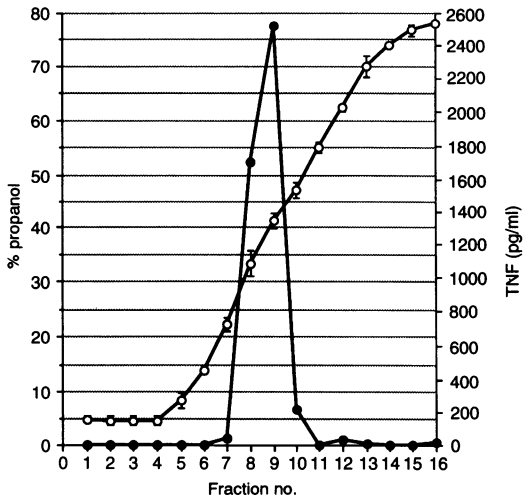

Figure 3. Isolation of erythrocyte-derived TNF-inducing factors by reverse-phase chromatography. Saponin-lysed or TX-114-lysed erythrocytes were suspended in 5% *n*-propanol, applied onto C18 Sep-Pak cartridges, and eluted with a 5–80% gradient of *n*-propanol. Closed circles (●) show the mean TNF response to fractions from four runs (two saponin lysates and two TX-114 lysates) each of which was tested in duplicate wells. Mean ± SD concentrations of *n*-propanol (○) were determined by refractometer readings compared to known standards.

and applied to C18 Sep-Pak cartridges. The TNF-inducing activity was retained on the cartridges at low concentrations of *n*-propanol, but eluted in a single peak at *n*-propanol concentrations of between 30 and 40%. Fractions were tested in duplicate wells and the average amount of TNF secretion for four experiments (two using saponin lysates and two TX-114 lysates) is shown in Fig. 3.

Activity is not the result of LPS contamination

For most of the following experiments we used preparations of saponin-lysed erythrocytes that had been partially purified on C18 Sep-Pak cartridges, and it was important to show that the TNF-inducing activity of these preparations was not the result of contaminating LPS. Addition of 100 U/ml of polymyxin B to the media resulted in the complete inhibition of TNF responses to LPS (Fig. 4a). In contrast 100 U/ml of polymyxin B had no effect on the induction of TNF by erythrocyte-derived factors (Fig. 4b). In other experiments, samples were pretreated with 1 μg/ml of polymyxin B bound to agarose: this totally inhibited the TNF-inducing activity of 1 μg/ml of LPS but did not affect TNF secretion induced by erythrocyte-derived factors (data not shown).

The activity of erythrocyte-derived TNF-inducing factors is dependent on a lipid component

To investigate the nature of the TNF-inducing erythrocyte factors, fractions eluted from C18 Sep-Pak cartridges were treated with either 100 μg/ml of pronase E, 5 U/ml of mixed lipases, or 0·05 M NaOH. Pronase-digested erythrocyte factors stimulated similar amounts of TNF as did undigested preparations. These digested samples contained undetectable levels of protein when analysed on sodium dodecyl sulphate–polyacrylamide gel electrophoresis SDS–PAGE and stained

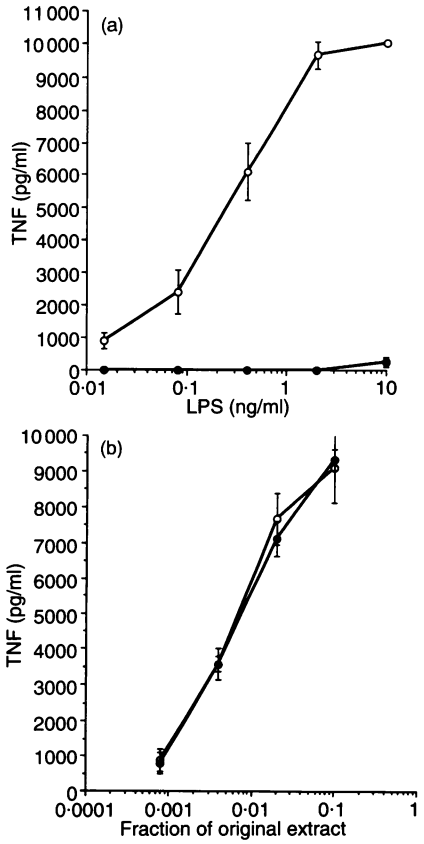

Figure 4. Polymyxin B inhibits TNF secretion induced by LPS but not by factors derived from saponin-lysed erythrocytes that had been partially purified on C18 Sep-Pak cartridges. Mean amounts of TNF ± SD secreted from triplicate wells incubated with dilutions of (a) LPS or (b) erythrocyte-derived factors, in the presence (●) or absence (○) of 100 U/ml of polymyxin B.

with silver nitrate (Amersham Quick Silver, Amersham, UK). Control preparations of pronase alone did not stimulate TNF production. These results suggest that proteins do not form an essential part of the TNF-inducing moiety. In contrast, the TNF-inducing activity of erythrocyte-derived factors was destroyed by mild alkaline hydrolysis or by digestion with mixed-lipases suggesting that the active moiety of these factors contain ester-linked acyl chains (Table 1).

Table 1. TNF induction by erythrocyte-derived factors

	Reciprocal dilution of erythrocyte-derived factors		
	10	50	250
Undigested preparation	2726 ± 150	2300 ± 149	676 ± 207
Pronase digested	2850 ± 239	2261 ± 104	893 ± 159
Lipase digested	36·7 ± 34	5 ± 9·3	0
Deacylated	19·7 ± 21	2 ± 3·4	0

TNF (pg/ml) secreted PBMNC stimulated by erythrocyte-derived factors, partially purified on C18 Sep-Pak cartridges, before and after treatment with either proteases, mixed lipases, or 0·5 M NaOH. Mean amounts of TNF ± SD secreted from triplicate wells are shown.

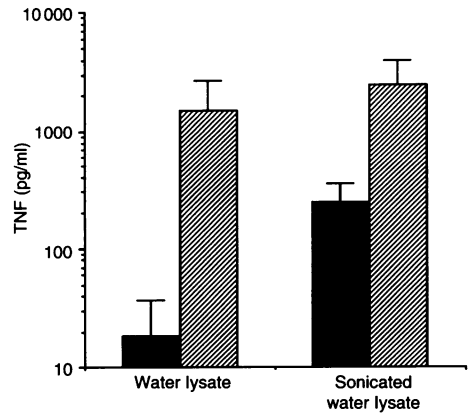

Figure 5. Amounts of TNF induced by preparations of lysed erythrocytes from a single donor, diluted to the equivalent of 10^6 lysed cells/microtitre well, comparing control erythrocytes (■) with erythrocytes infected at 5% parasitaemia with *P. falciparum* (▨). Values given are mean TNF production ± SD in response to six aliquots of each preparation, tested in duplicate.

Comparison between the TNF-inducing activity of *P. falciparum*-infected and non-infected erythrocytes

We have previously shown that *Plasmodium*-infected erythrocytes stimulate TNF secretion.[5] This raised the possibility that TNF induction by malaria parasites might simply be because of endogenous erythrocyte-derived factors that become exposed during parasitization. We therefore tested water lysates from uninfected erythrocytes and erythrocytes infected with the R29 strain of *P. falciparum* at 5% parasitaemia for their ability to stimulate TNF production. The erythrocytes came from a single donor and lysates were added to PBMNC at the equivalent of 10^6 lysed cells per microtitre well, i.e. 10^7/ml. Considerably more TNF was produced in response to lysates of parasitized erythrocytes than lysates of uninfected cells. Even after the lysates were sonicated, parasitized erythrocytes stimulated much greater amounts of TNF than uninfected erythrocytes (Fig. 5).

Inhibitory activity of lipase-digested erythrocyte-derived factors

We have previously noted that when malarial lysates are digested with mixed lipases, they not only lose their ability to induce TNF but they also acquire the ability to inhibit the TNF-inducing activity of undigested lysates.[7] To explore this further, we took a pool of erythrocyte-derived TNF-inducing factors that had been partially purified by fractionation on C18 Sep-Pak cartridges as described above, and digested this with mixed lipases. These lipase-digested preparations were then diluted 1:5 in medium and incubated with an equivalent dilution of undigested erythrocyte preparations. It was found that the lipase-digested material inhibited the TNF response to factors derived from normal or parasitized erythrocytes, but did not affect the TNF response to 5 ng/ml LPS (Fig. 6). Control preparations consisting of mixed lipases incubated in the absence of substrate were not inhibitory.

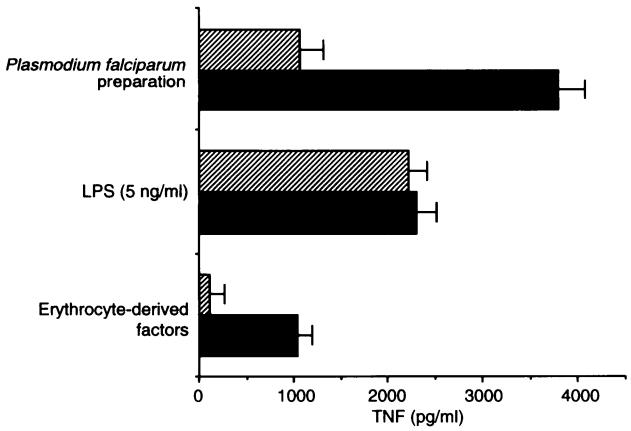

Figure 6. Lipase-digested erythrocyte-derived factors inhibit the induction of TNF by factors derived from both parasitized and normal erythrocytes, but do not affect TNF induction by 5 ng/ml LPS. A preparation of erythrocyte membranes was partially purified on C18 Sep-Pak cartridges, and then digested with mixed lipases. Figure shows the TNF response to components of uninfected erythrocytes, malaria-infected erythrocytes, or LPS in the presence (▨), or absence (■) of this lipase-digested preparation. Data are means ± SD of triplicate wells in a representative experiment.

Inhibitory activity of PI and IMP

The TNF-inducing activity associated with malaria-infected erythrocytes is inhibited by PI and IMP.[7] In the present experiments, PI and IMP caused a dose-dependent inhibition of TNF production in response to erythrocyte-derived factors, but had no effect on the induction of TNF by LPS (Fig. 7). The amount of these compounds required to reduce TNF production by 50% was typically around 1 μg/ml. This inhibition of erythrocyte-derived TNF-inducing factors by IMP was specific, in that mannose, galactose, glucosamine, galactosamine or their mono-phosphorylated derivatives were not inhibitory at concentrations of up to 200 μg/ml (Table 2).

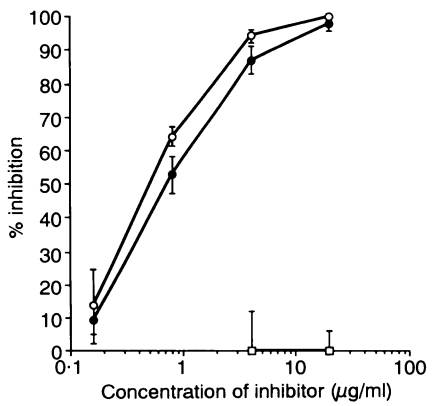

Figure 7. Dose-dependent inhibition of TNF secreted by PI, IMP and inositol. Average amounts of TNF, expressed as a percentage inhibition of control preparations incubated in medium only, secreted from PBMNC incubated with erythrocyte membrane preparations partially purified on C18 Sep-Pak cartridges, in the presence of PI (●), IMP (○) or inositol (□). Means ± SD are shown from triplicate wells in a representative experiment: these results were repeated in another four experiments.

Table 2. Inhibitory effect of phosphorylated sugars on the TNF response to factors derived from uninfected and malaria-infected erythrocytes

Inhibitor	Uninfected erythrocytes	Malaria-infected erythrocytes
Medium	5652 ± 456	8522 ± 801
Inositol monophosphate	121 ± 124	110 ± 99
Mannose	6054 ± 188	8842 ± 449
Mannose-1-phosphate	5682 ± 380	8727 ± 497
Glucosamine	5855 ± 240	8412 ± 766
Glucosamine-1-phosphate	6592 ± 505	8312 ± 442
Galactose	6086 ± 895	8480 ± 603
Galactose-1-phosphate	5634 ± 588	8325 ± 313
Galactosamine	5640 ± 354	8632 ± 848
Galactosamine-1-phosphate	5674 ± 914	9361 ± 620

Mean amounts of TNF (pg/ml, ± SD) secreted from triplicate wells of PBMNC stimulated by partially purified factors derived from uninfected or malaria-infected erythrocytes, in the presence of 200 μg/ml of various saccharides or phosphorylated saccharides. Similar results were obtained on three other occasions.

DISCUSSION

These investigations stemmed from our observation that sonicated erythrocytes stimulate high levels of TNF production by human mononuclear cells, whereas intact and water-lysed erythrocytes are non-stimulating. As vigorous sonication could cause biochemical modifications to cellular components, we also studied saponin-lysed erythrocytes and TX-114 extracts of erythrocyte membranes, and TNF-inducing factors with similar properties were identified in each of these preparations. Strenuous efforts were made to avoid any contamination of the erythrocyte preparations. We showed that the TNF-inducing activity of erythrocyte lysates was not owing to bacterial LPS by the use of polymyxin B, a small peptide antibiotic which binds to the lipid A portion of LPS and inhibits its biological activity.[8] Erythrocyte-derived TNF-inducing factors did not bind to polymyxin B agarose, and soluble polymyxin B did not affect their TNF-inducing activity. After partial purification on C18 Sep-Pak cartridges, these TNF-inducing factors were further characterized in a number of experiments. Their activity was resistant to digestion by proteases, indicating that protein is not essential for the induction of TNF. The activity was destroyed both by digestion with mixed lipases and by mild base hydrolysis, suggesting that the active moiety contains ester-linked acyl chains.

We have previously reported some similar properties for the TNF-inducing factors released by malaria parasites when they rupture out of host erythrocytes.[2] Specifically, their activity is destroyed by lipase digestion or by mild alkaline hydrolysis;[9] they are recovered from the alcohol–water phase and from the interface of a biphasic mixture of acidified methanol/chloroform/water; and they are eluted from C18 Sep-Pak cartridges by similar concentrations of *n*-propanol (manuscript in preparation). This raised the question of whether malaria infection, sonication and detergent lysis all cause TNF induction by exposing the same erythrocyte-derived factors.

However our present data show that a *P. falciparum*-infected erythrocyte contains the capacity to stimulate approximately 200 times as much TNF as a non-infected erythrocyte, even after extensive sonication. This indicates that the TNF-inducing activity of malaria-infected erythrocytes cannot be simply explained by erythrocyte lysis *per se*, and is more likely owing to the synthesis of new TNF-inducing factors or to biochemical modification of erythrocyte components.

Erythrocyte-derived TNF-inducing factors eluted from C18 Sep-Pak cartridges and digested with lipases did not induce TNF, but they were found to inhibit the TNF-inducing factors of both normal and malaria-infected erythrocytes. The inhibitory effect was specific, in that the TNF response to LPS was not inhibited. It is possible that the lipase-digested compounds act as partial structures which inhibit macrophage receptor binding by the TNF-inducing factors. If so, this would imply that the TNF-inducing factors of both normal and malaria-infected erythrocytes interact with the same macrophage receptor, and that this is different from the LPS receptor.

Our finding that the TNF-inducing factors of both normal and malaria-infected erythrocytes were inhibited by IMP, but not by other phosphorylated saccharides, suggests that part of the active moiety may have an IMP-like structure. Glycosylphosphatidylinositol (GPI) anchors are associated with several erythrocyte membrane proteins including CD59, decay-accelerating factor and acetylcholinesterase,[10] and they are also actively synthesized by malaria parasites. Molecules containing GPI have been proposed as mediators of TNF induction in malaria, based on observations that TNF induction by malaria-infected erythrocytes depends on a phospholipid component[9] and that the GPI-anchored merozoite surface proteins of *P. falciparum* possess TNF-inducing activity.[11] However we have found no evidence that TNF secretion is inhibited by glucosamine or mannose, which have been noted to inhibit cell-signalling events attributed to GPI-anchors in other systems.[12] Furthermore we have tested a number of purified GPI-anchored antigens from other sources and none was found to stimulate TNF secretion (unpublished data). Thus if a GPI moiety is responsible for the TNF induction by lysed erythrocytes or malaria parasites, it probably has an unconventional structure. Other IMP-like molecules that might be responsible for macrophage activation are the phosphorylated phosphatidylinositols, which have a second messenger function in many cells including erythrocytes. There is evidence that malaria parasites can independently synthesize components of the phosphoinositide pathway.[13]

Endogenous pyrogens such as TNF are highly likely to be responsible for the fever response to non-infectious as well as infectious diseases: for example, elevated TNF levels have been found in the sera of patients with acute myocardial infarction.[14] In some instances, damaged cells may themselves release TNF or other cytokines that indirectly promote the fever response, but there may also be specific markers of cell damage that stimulate macrophages to release TNF and other inflammatory mediators. Our data indicate that damaged erythrocytes release such factors, which could possibly have an important role initiating the host response to tissue injury. Further work is needed to determine their molecular structure and their distribution in other cell types.

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