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Structural Similarities among Malaria Toxins, Insulin Second Messengers, and Bacterial Endotoxin

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Malaria toxin causes hypoglycemia and induction of tumor necrosis factor. Extracts of parasitized erythrocytes which were coeluted and copurified with one of the two subtypes of mammalian insulin-mimetic inositolphosphoglycans similarly induced fibroblast proliferation in the absence of serum. In addition, induction of tumor necrosis factor in macrophages by malaria toxin and by lipopolysaccharide from *Escherichia coli* was enhanced by pretreatment of these toxins with α-galactosidase. Thus, parasitized erythrocytes contain both soluble inositolphosphoglycan-like insulin second messengers and endotoxin-like lipidic molecules.

Since the severity of clinical manifestations of malaria is correlated with the presence of tumor necrosis factor (TNF) in the circulation (8, 13, 15), components of parasitized erythrocytes which induce its production and cause hypoglycemia (25) are customarily referred to as toxins (11). The TNF-inducing activity is associated with a phospholipid (1, 3) and is inhibited by inositol monophosphate (2). The hypoglycemia associated with malaria correlates with hyperinsulinemia (6). Toxin preparations also synergize with insulin in stimulating lipogenesis in adipocytes in vitro (26).

Insulin second messengers. Insulin second messengers are derived from membrane-associated glycosylphosphatidylinositol (GPI) (7). Inositolphosphoglycans (IPGs) are released outside the cell by the action of a phospholipase which is activated following receptor ligation by insulin. A family of structures with A- and P-type subfamilies which are functionally and chemically distinct exists, and their release is tissue specific (14). The A-type mediators mimic the lipogenic activity of insulin on adipocytes, whereas the P-type mediators mimic the glycogenic activity of insulin on muscle. Both A- and P-type mediators are mitogenic when added to fibroblasts in serum-free medium.

To see if parasitized erythrocytes contain IPG-like activities, they were extracted as described previously for rat and bovine tissues (14, 17). Female CD1 mice (Charles River Ltd., Margate, Kent, United Kingdom) infected with *Plasmodium yoelii* YM (from A. Holder, National Institute of Medical Research, London, United Kingdom) were bled, and washed erythrocytes (RBCs) containing more than 80% schizonts of *P. yoelii* were incubated at 37°C overnight at 2×10^8 cells per ml in 50 ml of Earle's balanced salt solution (Life Technologies Ltd.); uninfected RBCs at 2×10^9/ml were used as a control. They were then dried in a rotary evaporator and extracted by being boiled in 50 mM formic acid containing 1 mM EDTA and 1 mM β-mercaptoethanol and then centrifuged at 29,500 × *g* for 90 min. The supernatant was treated with charcoal (10 mg/ml) on ice and recentrifuged. The clear supernatant was diluted to 5 mM formic acid, adjusted to pH 6.0 with 10% NH_4OH, and shaken overnight with AG1X8 (formate form) resin (Bio-Rad, Hemel Hempstead, United Kingdom). The resin was then poured into a chromatography column and washed with water and 1 mM HCl (2 bed volumes of each). It was eluted with 10 mM HCl (5 bed volumes), yielding P-type IPG, followed by 50 mM HCl (5 bed volumes), yielding A-type IPG. The two fractions were adjusted to pH 4.0 with 10% NH_4OH and then dried, and each was dissolved in 200 μl of BSS and adjusted to pH 7.0. Rat liver after insulin infusion was also extracted as a positive control, and the presence of A-type (50 mM HCl eluate) and P-type (10 mM HCl eluate) IPGs was confirmed by their biological activities (14).

The two fractions obtained from erythrocytes were assayed for their ability to support proliferation of epidermal growth factor receptor-transfected T17 fibroblasts (gift from I. Varela-Nieto) in the absence of serum (18). Cells (10^4 per well) were grown overnight in 96-well plates in Dulbecco's modified Eagle's medium (DMEM [Life Technologies Ltd., Paisley, Scotland]) containing 10% fetal calf serum and 10 μg of ciprofloxacin (Ciproxin; Bayer, Newbury, Berkshire, United Kingdom) per ml, washed free of serum, and maintained in serum-free DMEM for a further 24 h. They were then incubated, in triplicate, with serial dilutions of the erythrocyte-derived fractions in serum-free DMEM, DMEM with 10% fetal calf serum, and P- and A-type mediators from rat liver (diluted 1/40) as controls. After 18 h, cells were pulsed with [^{3}H]thymidine (1 μCi per well; Amersham, Buckinghamshire, United Kingdom) for 4 h, detached by trypsinization, and harvested, and DNA-incorporated radioactivity was determined.

Extracts from parasitized but not from uninfected RBCs supported cell proliferation, the activity of the 10 mM eluate (P-type IPG) greatly exceeding that of the 50 mM eluate (A-type IPG) (Fig. 1). This activity was not directly related to their content of organic phosphate as determined by standard molybdate assay. Thus, the 10 mM eluate from parasitized RBCs contained 7.6 nmol/μl, compared with 28 nmol/μl in the 50 mM eluate. The uninfected RBC fractions contained 5.3 and 13.4 nmol/μl, respectively. The comparable fractions from rat liver contained 13.2 and 27.6 nmol/μl. The 10 mM fraction also stimulated pyruvate dehydrogenase phosphatase activity (data not shown). Proliferative activity was only found in the 50 mM eluate at the lowest dilution. Neither fraction induced TNF. The smaller amount of A-type activity supports the original observation that most toxin preparations had no direct effect on lipogenesis in adipocytes (26). Since the aqueous extracts we employed would not have contained lipid-soluble GPI

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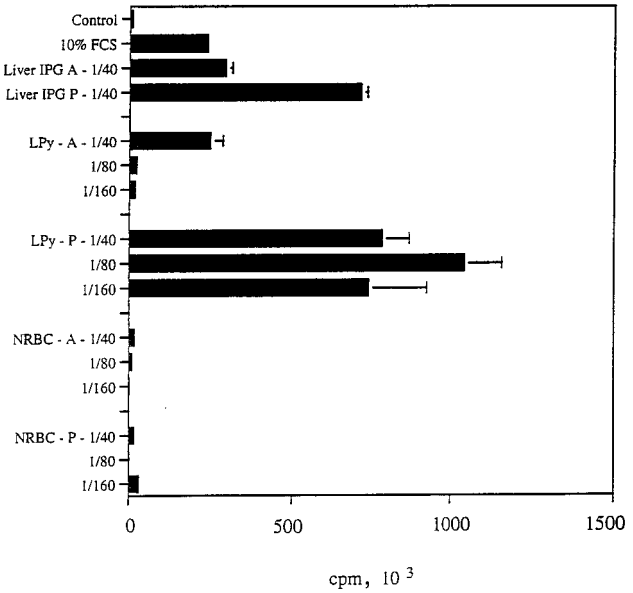

FIG. 1. IPG activity of malaria toxin assessed by proliferation of fibroblasts transfected with the human epidermal growth factor receptor. Cells were incubated in the absence of serum (control) or with serial dilutions made from the IPG-containing solutions of RBCs infected with *P. yoelii* (LPy) or normal RBCs (NRBC). As positive controls, 10% fetal calf serum (FCS) and A-type and P-type IPG, obtained from rat liver as described previously (14), were included (10 μl of stock solution represents the amount of mediator recovered from 800 mg (wet weight) of liver). Values are means ± standard deviations of triplicate determinations.

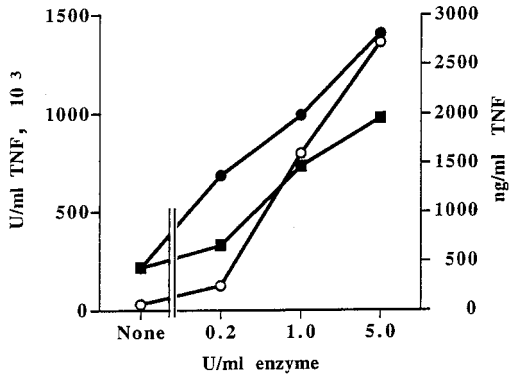

FIG. 2. Typical enhancement of the TNF-inducing ability of malaria toxin by treatment with α-galactosidase from two different sources. Serial dilutions of an extract of parasitized RBCs which (undiluted) induced 435 ng of TNF per ml as determined by ELISA and which induced 30,400 U/ml in the bioassay were incubated at pH 6.0 at 37°C overnight with a series of concentrations of α-galactosidase from Sigma or from Oxford GlycoSystems, boiled, neutralized, and then tested on macrophages for TNF induction by bioassay and ELISA. (Means of duplicate determinations are shown.) The amount of TNF that would have been induced by the original undiluted extract was then calculated. ○, units per milliliter by bioassay (enzyme from Sigma); ●, nanograms per milliliter by ELISA (enzyme from Sigma); ■, nanograms per milliliter by ELISA (enzyme from Oxford GlycoSystems).

structures, our results cannot be compared directly with those described by Schofield and Hackett (22).

Structural components of the malaria toxin. Structural components of malaria toxin which affect activity have been inferred from enzymatic and chemical treatments. TNF induction by malaria toxin is unaffected (3) by treatment with dilute HONO. This resistance to HONO distinguishes the active portion of lipidic TNF-inducing malaria toxin from GPI anchors of proteins which, like IPG, are degraded by HONO, because of the presence of a non-*N*-acetylated hexosamine residue. Glycoconjugates from parasites, such as lipophosphoglycans attached to the membrane via GPI anchors (16) and the glycan moiety of GPI-anchored proteins (27), contain α-galactose residues. Likewise, bacterial lipopolysaccharide (LPS) contains α-galactose residues (20). α-D-Galactosidase has also been shown to alter the antigenicity of an antigen in *Plasmodium falciparum* culture medium (12) which induces TNF (23). To see if the TNF-inducing activity of malaria toxin contained α-galactose residues, we treated both the malaria toxin and LPS with a preparation of α-galactosidase.

Mice bred here [(CBA × BALB/c)F_1 or (CBA × C57Bl)F_1] were infected with *P. yoelii* YM or *Plasmodium berghei* ANKA. Parasitized RBCs (10^8/ml) in BSS were incubated at 37°C overnight and then disrupted by freezing and thawing, digested overnight with 250 μg of pronase E (Sigma) per mg of protein (determined by Bio-Rad assay), boiled, treated with 25 μg of polymyxin B agarose (Sigma) per ml to eliminate endotoxin, filtered through a 0.2-μm-pore-size filter (Sartorius AG, Göttingen, Germany), and stored at 4°C (24). TNF induction from thioglycolate-induced peritoneal macrophages and enzyme-linked immunosorbent assays (ELISAs) for murine TNF were described previously (24). Polymyxin B (5 μg/ml) was included in all experiments with malaria toxin to exclude effects of contamination with LPS. A more sensitive one-plate procedure for TNF production and assay was also used (5). Cytotoxicity assays for TNF were done with L929 cells (23). Green coffee bean α-D-galactosidase was obtained from two sources (Sigma, Poole, Dorset, United Kingdom, and Oxford GlycoSystems, Ltd., Abingdon, Oxford, United Kingdom).

A significant increase in TNF induction in macrophages was reproducibly observed when more than a dozen different extracts of RBCs infected with *P. yoelii* and three infected with *P. berghei* (but not uninfected RBC controls that did not induce TNF) were pretreated with α-D-galactosidase. A typical titration showing dose-dependent enhancement of TNF induction by one sample of malaria toxin treated with increasing amounts of α-galactosidase is shown in Fig. 2. The amount of TNF enhancement by any concentration of α-galactosidase depended on toxin potency, with higher dilutions of toxin always showing greater enhancement. No TNF was induced from macrophages incubated with only 2 U of enzyme per ml, and no enhancement occurred with boiled enzyme, again excluding any contribution from contaminating LPS, which is heat stable. Macrophages treated with 2 U of enzyme per ml for 1 h at 37°C and then stimulated with toxin or 2 or 10 ng of LPS per ml did not secrete more TNF than untreated cells.

The α-galactosidase hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside (αPNP-Gal) is optimal at pH 6.0 (4, 9) and undetectable at pH 8.0 (9), as we confirmed, but enhancement of TNF induction by malaria toxin was greatest at pH 8.0 (Fig. 3). The TNF-inducing activity of LPS was also enhanced with a pH profile similar to that of the toxin, indicating a homologous structure-function relationship. The degree of enhancement also varied with the concentration of LPS, and at higher concentrations, enhancement was also detectable at pH 6.0.

The most likely explanation for the lack of activity against αPNP-Gal at pH 8.0 is the presence of an α-galactosidase activity which does not hydrolyze this substrate. This was supported by the observation of batch-to-batch variability and the fact that the stability of the TNF-enhancing activity differed from that of the αPNP-Gal activity. Although all enzyme preparations were first dialyzed and adjusted to the same activity

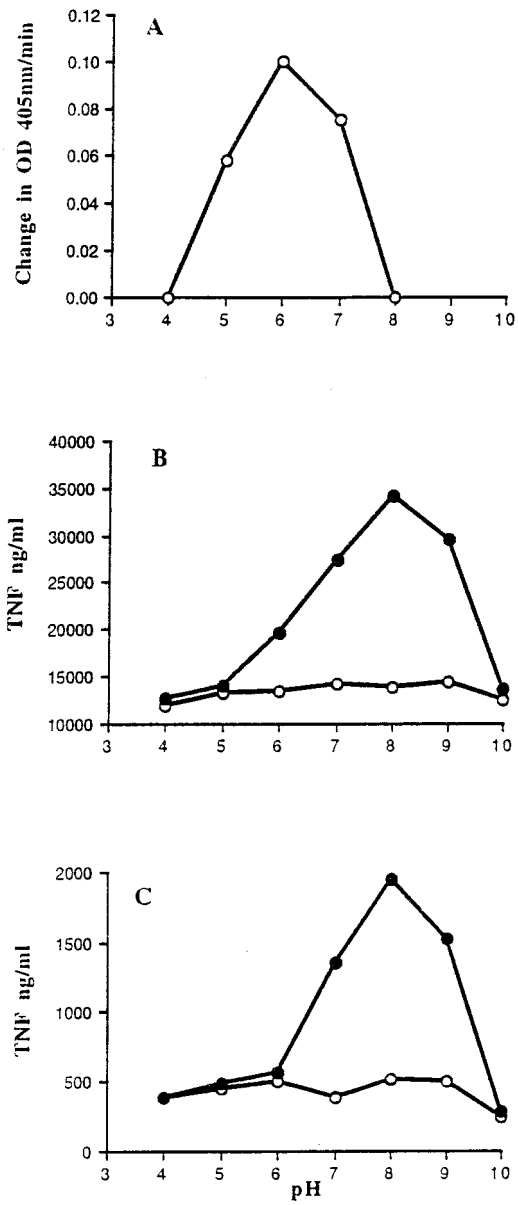

FIG. 3. Enhancement of TNF-inducing activity of malaria toxin and of LPS at different pHs compared with specific α-galactosidase activity. OD, optical density. Results from one of two experiments are shown. (A) Rate of reaction of 0.04 U of α-galactosidase per ml at 37°C with αPNP-Gal as substrate as determined over the first 3 min. (B and C) Malaria toxin from *P. yoelii* diluted 1/10 (B) and 50 ng of LPS per ml (C) were incubated overnight at 37°C with 2 U of α-galactosidase per ml and then boiled and tested on macrophages for TNF induction, as assayed by ELISA. Means of duplicate experiments are shown. ○, samples incubated in buffer only, boiled, and then adjusted to neutral pH in RPMI 1640 medium as pH controls; ●, samples incubated with enzyme.

against αPNP-Gal, batches from different sources enhanced TNF activity to different degrees (as illustrated in Fig. 2). TNF-enhancing capacity deteriorated with storage at 4°C, although activity against the αPNP-Gal substrate did not. No differences were visible in a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis mini-gel system (Pharmacia, Uppsala, Sweden), with 4.5 μg of protein, between batches which did or did not enhance TNF activity. Apart from bovine serum albumin, all preparations contained three bands with sizes of 33, 29, and 27 kDa; 28- and 36.5-kDa isoforms were previously described (4). No β-galactosidase activity was detectable against βPNP-Gal at pH 6.0, nor did 0.8 U of β-galactosidase (Sigma, grade X from *E. coli*) per ml at any pH from 4 to 8 affect malaria toxin or LPS activity.

It is commonly found that not all exoglycosidase activities can be monitored with a PNP surrogate substrate. For example, while the α-(1,2)-, α-(1,3)-, and α-(1,6)-mannosidase activities of jack bean α-mannosidase can be assayed with PNP-mannoside, the α-(1,2)-mannosidase activity from *Aspergillus phoenicis* cannot, and only natural substrates can be used (10). This property is normally referred to as the aglycon specificity of an exoglycosidase. Further experiments are necessary to confirm that an α-galactosidase causes the enhancement.

The presence in parasitized erythrocytes of a soluble mediator which may have structural features homologous to the P-type IPG second messenger contrasts with the almost exclusive presence of A-type mediators from pathogenic mycobacteria (21). These mediators are pleiotropic and are released by a number of hormones and growth factors other than insulin (19). For example interleukin-2, adrenocorticotropin, insulin-like growth factor 1, epidermal growth factor, transforming growth factor β, and nerve growth factor all stimulate the hydrolysis of membrane-associated GPI and release IPG extracellularly. Clearly the existence of mediators in malaria which mimic those derived from mammalian tissue may lead to the elucidation of new parasitic pathogenic mechanisms.

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