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Incubation of Trypanosome-Derived Mitogenic and Immunosuppressive Products with Peritoneal Macrophages Allows Recovery of Biological Activities from Soluble Parasite Fractions

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This report describes further attempts to define the nature of the parasite product(s) responsible for the extensive changes in lymphoid tissue in mice during infection with *Trypanosoma brucei*. As previously described, potent mitogenic and immunosuppressive effects are induced by a trypanosome-derived crude membrane fraction in vivo. There was no enrichment in these activities when purified parasite surface membranes were used. Mitogenic activity can be recovered from soluble trypanosome material only when it is incubated with peritoneal macrophages before transfer into syngeneic recipients. Thus, by encouraging association with a critical target cell, soluble parasite products can be studied, and their active components can be separated by conventional methods. Preliminary fractionation of high-spin trypanosome supernatant over Sepharose 4B confined the mitogenic activity to the high-molecular-weight fraction, which is a macromolecular complex of proteins, glycoproteins, and lipid. Extracted lipid from this material was able to significantly suppress a primary immunoglobulin G anti-sheep erythrocyte response. The activity was periodate sensitive and pronase resistant. The use of macrophages in vitro may be a general method whereby important biological activities lost as a result of fractionation procedures can be recovered and the active components studied in greater detail.

The effects on immune function caused by African trypanosomes have received considerable attention. Laboratory infection of a murine host results in a transient nonspecific augmentation of immunological activity, as seen in the increased levels of priming with T-dependent or -independent antigens (12, 13, 16) and in the increase of background plaque-forming cells (PFC) to, for example, trinitrophenyl-coupled sheep erythrocytes (TNP-SRBC) (12). This is followed by a profound suppression of immune response to both parasite- (24) and nonparasite-related antigens (7, 12, 13, 16, 20). The severity of trypanosome-induced immunodepression has been linked to parasite virulence and the ultimate failure of the murine host to control infection (24, 25).

The kinetics and gross features of these lymphoid cell changes have been reported (6); however, the mechanisms involved in such events are not yet clear. Infection is associated with a distinct mitogenic phase, which acts on T-, B-, and null lymphoid cell populations in spleen and bone marrow (17, 19). The immunodeficiency which follows has been associated with suppressor splenic macrophages (26) and T-cells (7, 13), a functional depletion of B- and T-cells in the spleen (2), and a direct inhibitory effect of trypanosome-derived substances (1).

Of crucial importance in the study of these phenomena is the nature of the trypanosome-derived stimulus responsible for these changes and the mechanism of interaction between this material and the components of the immune system. Our previous work has demonstrated the ability of relatively small doses of killed trypanosomes or a subcellular crude membrane fraction to mimic the mitogenic and immunosuppressive effects of live parasites on the host in vivo (5).

In this study, we describe further attempts to define the nature of the parasite product(s) responsible for the extensive changes in lymphoid tissue. We present data concerning the activity of purified trypanosome plasma membranes, the fractionation and partial characterization of an active "soluble" trypanosome product, and the

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possible role of macrophages in the action of this material in vivo.

MATERIALS AND METHODS

Mice. (CBA/H × C57BL/6) F_1 female mice bred under specific-pathogen-free conditions at the National Institute for Medical Research, London, were used at 4 to 7 months of age. Animals were age matched within experiments and maintained under minimal disease conditions.

Trypanosome preparation. *Trypanosoma brucei* subsp. *brucei* S42, clone NIM-2, known to cause fatal trypanosomiasis in mice within 30 days (7), was passaged for 5 days in lethally irradiated mice (900 $R^{50}C^{o}$). All subsequent procedures were carried out at 4°C unless otherwise stated, and samples were either tested immediately for in vivo activity or stored at −70°C in the presence of 1 mM phenylmethylsulfonyl fluoride. Parasites were separated from whole heparinized blood by centrifugation at 800 × *g* for 10 min. The trypanosome fraction remaining on the erythrocyte interphase was collected, suspended in 3 volumes of Krebs glucose, and separated from remaining erythrocytes by passage through DEAE-52 cellulose equilibrated in Krebs glucose (14). Purified trypanosomes were washed once in Krebs glucose and suspended in homogenization buffer (pH 7.4) containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 250 mM sucrose, 0.15 mM $MgSO_4$, 1 mM phenylmethylsulfonyl fluoride, and 100,000 IU of Aprotonin (Sigma Chemical Co.) per ml at 1 ml per 6 × 10^9 trypanosomes. The parasites were stored at −70°C, and 95% were disrupted by rapid thawing followed by forced passage through a 27-gauge needle and homogenization in a glass tissue homogenizer in the presence of no. 11 Ballotini glass beads. This suspension was centrifuged at 12,000 × *g* for 15 min to remove nuclei, kinetoplasts, and large debris. The supernatant was then subjected to high-speed centrifugation at 50,000 to 150,000 × *g* for 45 min, and the pellet was suspended in phosphate-buffered saline (PBS). Unless otherwise stated, protein determinations were performed by the Lowry method (15).

For further fractionation, the 50,000 × *g* supernatant was concentrated by ultrafiltration with an Amicon PM10 membrane under N_2 pressure.

Gel filtration was performed on a Sepharose 4B chromatography column (40 by 1.5 cm) equilibrated in PBS at 4°C and monitored by absorbance at 280 nm. Fractions from individual peaks (I through IV) and control effluents before peak I were tested for in vivo activity or stored at −70°C.

Enzyme treatment. Sepharose 4B peak I fractions were treated with 40 mM sodium metaperiodate in 50 mM sodium acetate (pH 4.5) for 12 h at 4°C followed by dialysis against PBS for 6 h at 4°C. Peak I controls were treated as above but in the absence of sodium metaperiodate.

For pronase treatment, peak I samples were incubated at 37°C with 10% (wt/wt) pronase (Sigma) or PBS for 90 min and then tested for in vivo activity.

Preparation of plasma membranes. *T. brucei* plasma membranes were prepared by the method of Rovis and Baekkeskov (22). Purified trypanosomes (10^{10}) were suspended in 80 ml of homogenization buffer and equilibrated in a nitrogen bomb at 250 lb/in^2 with constant stirring for 30 min at 4°C. The suspension was released dropwise from the bomb into 10 mM EDTA to a final concentration of 1 mM EDTA. The released material was completely disrupted as determined by electron microscopy. A microsomal fraction was collected by high-speed centrifugation (100,000 × *g* for 90 min) after removal of the larger organelles at 100 × *g* (15 min) and 12,000 × *g* (15 min). The pellet was washed five times with 1 mM phenylmethylsulfonyl fluoride–1 mM $MgCl_2$ (pH 8.2) and dialyzed for 3 h against the same buffer. The suspension was layered onto 3.5 ml of 26% (wt/vol) 40,000-molecular-weight (40K) dextran prepared in this buffer and centrifuged at 230,000 × *g* for 3 h at 4°C. Electron microscopy confirmed that the material remaining at the interface represented a homogeneous plasma membrane fraction and the sedimenting material represented a heterogeneous fraction containing internal membranes. Both were washed in PBS before in vivo testing or storage.

Macrophage transfer. Peritoneal macrophages were harvested into Ca^+- and Mg^+-free PBS 3 days after intraperitoneal (i.p.) injection of 2.5 ml of 10% proteose peptone solution. The cells were washed once in PBS, suspended in soluble trypanosome fractions or PBS at 10^7 cells per ml, and left for 15 min at room temperature. Then 3 × 10^6 cells plus fractions were injected i.p. (0.4 ml per mouse). The effect of transfer on the background immunoglobulin M (IgM) splenic PFC response to TNP-SRBC, prepared as described by Rittenberg and Pratt (21), was assayed by the slide modification of the Jerne technique (9) 4 days after injection of the fractions. To study the enhancement of primary antibody responses, we primed mice with 10^7 SRBC i.p. at the time of injection of parasite fractions. For immunosuppresive effects, 4 × 10^7 to 6 × 10^7 SRBC were given i.p. 4 days after the macrophage transfer.

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed in the presence of 0.1% sodium dodecyl sulfate. Samples were reduced by boiling in the presence of 3% mercaptoethanol, 1 M urea, and 2% sodium dodecyl sulfate and run on 7.5% polyacrylamide gels. Gel tracks were stained for proteins with 0.2% Coomassie blue. Binding of ^{125}I-labeled concanavalin A was performed by the method of Gurd and Evans (11) and assessed by autoradiography. For binding of anti-variant-specific glycoprotein antiserum, gels were fixed in methanol–acetic acid–water (8:1:11, vol/vol) overnight and then washed in PBS containing 1% bovine serum albumin for 8 to 12 h with several changes. Gels were incubated for 2 h with the IgG fraction of protein A-purified rabbit anti-NIM-2 variant-specific surface glycoprotein antiserum raised against conventionally purified NIM-2 surface antigen (8), washed for 3 h with frequent changes of PBS–1% bovine serum albumin, and incubated with a ^{125}I-labeled $F(ab')_2$ fraction of goat anti-rabbit immunoglobulin antiserum for 12 h. The gels were then washed extensively and developed for autoradiography. Antiserum dilutions were performed in PBS–1% bovine serum albumin, and all incubations were at 37°C with gentle rocking.

Lipid analysis. Trypanosome fractions were extracted in chloroform–methanol–water (1:2:0.6) with shaking for 5 min, brought to a final ratio of 2:1:0.8 to give a two-phase system, and shaken for a further 10 min.

This mixture was centrifuged at 700 × g for 15 min, and the lower chloroform layer was removed. The aqueous phase was reextracted by the addition of 2 volumes of chloroform and shaking for 10 min. The chloroform phase was removed after centrifugation, and the procedure was repeated. The pooled chloroform extracts were evaporated under vacuum, reconstituted in 1 ml of chloroform, and assayed for the presence of lipid by thin-layer chromatography. Silica gel thin-layer chromatography plates (Merck Sharp & Dohme; 0.25-mm gel depth) were activated at 100°C for 1 h before use. Sample extracts and purified standards were applied and separated with chloroform–methanol–water (14:6:1) or petroleum ether (BP 40/60)–ether–glacial acetic acid (73:25:2) solvent systems. Plates were stained for phospholipids and other components by charring with 50% H_2SO_4 at 150°C. All reagents used were analytically pure grade.

RESULTS

Mitogenic activity of trypanosome plasma membranes. Previous studies with subcellular fractions of *T. brucei* suggested that the mitogenic activity of the parasite in vivo was confined to a crude membrane fraction sedimenting between 12,000 and 150,000 × g. In the current series of experiments, the method of Rovis and Baekkeskov (22) was used to purify plasma membranes from *T. brucei* to further characterize active membrane components. The mitogenic activity of a crude membrane pellet prepared by nitrogen cavitation was comparable with the activity previously observed with a similar fraction prepared from freeze-thawed homogenates (5). Such a pellet derived from 10^8 trypanosomes increased background PFC against TNP-SRBC more than fourfold (Table 1, experiment A) and contained vesicles derived from the fragmentation of both external and internal membranes. The isolation of plasma membranes on the basis of their unique internal fixed charge and buoyant density was achieved by high-speed centrifugation on magnesium-containing dextran. By electron microscopic analysis, the plasma membrane vesicles obtained were found to be free of microtubules and organelles. When the vesicles were tested for their effect on background PFC in vivo, there was no enrichment in the activity of the plasma membrane fraction relative to the internal membrane fraction (designated the endoplasmic reticulum). Although both fractions had demonstrable effects, the heterogeneous endoplasmic reticulum fraction retained the more potent activity whether injected as derived from equal numbers of trypanosomes (experiment A) or as equal amounts of protein (experiment B). Mitogenic activity does not appear to be a unique property of the parasite surface membrane.

Enhancing effect of soluble trypanosome fractions after interaction with macrophages. As previously observed (5), the injection of a trypanosome-derived soluble fraction, either high-spin supernatant or detergent-solubilized membranes, did not result in the enhancement of background PFC to TNP-SRBC when compared to the untreated membrane pellet (Table 2, experiment A). However, it has recently been shown that peritoneal macrophages after the ingestion of trypanosomes in vivo are capable of mediating mitogenic and immunosuppressive activities independent of free parasites (10). The lack of stimulatory activity by the soluble trypanosome fraction alone is probably due to an inadequate uptake of the material by macrophages in vivo. By pretreatment of peritoneal

TABLE 1. Trypanosome membrane fractionation: in vivo effect on background PFC to TNP

Fraction[a]	Trypanosome equivalents (expt A)	Protein yield (mg) per 10^{10} trypanosomes (expt B)	Protein equivalent (μg) (expt B)	Anti-TNP PFC per spleen[b] (±SE)	Stimulation index[c]
Expt A					
PBS	—[d]			1,355 ± 212	—
12,000 to 150,000 × g pellet	10^8			6,480 ± 76	4.4
Plasma membrane (pure)	10^8			4,520 ± 508	3.3
Endoplasmic reticulum	10^8			5,700 ± 559	4.0
Expt B					
PBS		—	—	480 ± 245	—
12,000 to 150,000 × g pellet		6.5	100	5,934 ± 562	12.4
Plasma membrane (pure)		2.0	100	3,024 ± 411	6.3
Endoplasmic reticulum		1.2	100	6,180 ± 1,058	12.8

[a] PBS or membrane fractions suspended in PBS were injected i.p. (0.2 ml per mouse).
[b] Assayed 4 days after administration of test fractions against TNP-SRBC. Values are arithmetic means, five mice per group.
[c] Stimulation index = anti-TNP PFC per spleen (test fraction)/anti-TNP PFC per spleen (PBS).
[d] —, Not applicable.

TABLE 2. Effect on background PFC to TNP of trypanosome subcellular fractions: influence of cotransfer with peritoneal macrophages

Fraction	Macrophage transfer[a]	Anti-TNP PFC per spleen[b] (±SE)	Stimulation index[c]
Expt A			
PBS	−	520 ± 80	—[d]
150,000 × *g* pellet from 10^8 trypanosomes	−	6,272 ± 782	12.0
150,000 × *g* supernatant from 5 × 10^8 trypanosomes	−	592 ± 98	1.1
150,000 × *g* pellet, deoxycholate treated[e]	−	1,260 ± 181	2.4
Expt B			
PBS	+	608 ± 54	—
150,000 × *g* pellet from 10^8 trypanosomes	+	8,560 ± 381	14.1
150,000 × *g* supernatant from 5 × 10^8 tryponosomes	+	5,424 ± 554	9.0

[a] A total of 3 × 10^6 peritoneal macrophages were incubated with each fraction for 15 min before transfer i.p.
[b] Assayed 4 days after administration of test fractions against TNP-SRBC. Values are arithmetic means, five mice per group.
[c] See Table 1, footnote *c*.
[d] —, Not applicable.
[e] Disrupted with 0.5% sodium deoxycholate in 10 mM Tris buffer (pH 8.0).

macrophages with soluble trypanosome fractions in vitro, we sought to manipulate the fate of injected material so as to encourage association with this potentially crucial target cell type. Peptone-elicited peritoneal macrophages were incubated at room temperature for 15 min with a high-speed supernatant fraction derived from 5 × 10^8 parasites, and this mixture was injected into syngeneic mice i.p. This resulted in a strong stimulation with a ninefold increase in background PFC (Table 2, experiment B).

To further define the active parasite component, the parasite supernatant was subjected to chromatography on Sepharose 4B. The elution profile of a high-speed supernatant prepared from 2 × 10^{10} trypanosomes is shown in Fig. 1. Pooled fractions of the four protein peaks were individually tested for in vivo activity by injecting material derived from 10^9 trypanosomes per mouse after in vitro incubation with peritoneal macrophages. The most potent stimulatory activity was confined to a high-molecular-weight material (peak I) eluting at or near the exclusion volume of the column (Table 3). In terms of protein, this corresponded to approximately 20-fold enrichment in the parasite activity relative to the whole supernatant. To test whether this enhancement could be mediated by a high-speed supernatant of any nucleated cell, we prepared a chicken erythrocyte soluble fraction for comparison. Although stimulation by the parasite-derived supernatant was relatively low in this case, no augmentation of background PFC was observed with the chicken erythrocyte material obtained from a similar number of cells.

Polyclonal activation induced during infection with African trypanosomes is accompanied by nonspecific enhancement of immune responsiveness if antigen is given within the first few days of infection (12, 13). The gross immunological consequences of in vivo exposure to macrophages pretreated with peak I are similar to those induced by living parasites during active infection. (Fig. 2). In mice primed with a suboptimal dose of SRBC simultaneously with an injection of parasite fraction, there was a 3-fold and 11-fold increase in the IgM and IgG PFC against SRBC, respectively.

Immunosuppression by peak I, a complex of lipid and proteins. Because peak I showed the biological activity, we wished to further define its biochemical properties. Soluble parasite fractions were therefore analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine the protein composition of the active material (Fig. 3). In comparison with the heterogeneous nature of the untreated high-speed supernatant, the Sepharose 4B peak I showed only four protein bands (a through d), with approximate molecular weights of 84K, 71K, 64K, and 50K. This pattern was observed for both reduced and nonreduced samples and suggests that the high-molecular-weight peak I fraction is composed of a complex of these proteins and perhaps lipid or other material. Binding of ^{125}I-labeled concanavalin A in situ was observed with gel bands a, b, and d, indicating that these polypeptide species are glycoproteins. After the gel was overlaid with ^{125}I-IgG antibody to NIM-2 variant glycoprotein, components b and d were shown to bind the antibody (Fig. 3). On a Coomassie blue-stained gel, band d comigrated with purified NIM-2 surface glycoprotein, which for this trypanosome clone has a molecular weight of 50K (C. Clayton, Ph.D. thesis, National Institute for Medical Research, 1978). Compo-

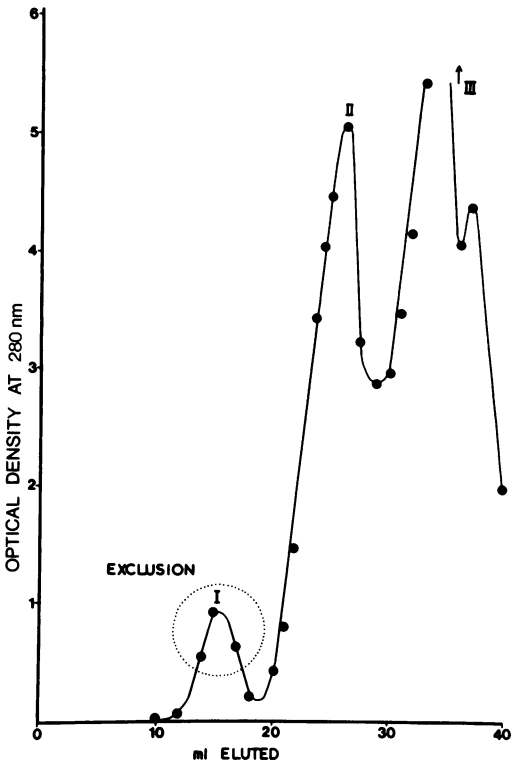

FIG. 1. Sepharose 4B gel filtration profile of trypanosome-derived soluble fraction. The column was loaded with 2 to 4 ml of 150,000 × *g* supernatant derived from 2 × 10^{10} trypanosomes. Fractions were collected at the rate of 2 to 3 ml/h.

nent b of higher molecular weight (71K) appears to be related to or a precursor of the isolated surface glycoprotein; when b and d are eluted from the gel by metaperiodate treatment, both gel components bound to the rabbit anti-glycoprotein antibody in a solid-phase radioimmunoassay (manuscript in preparation). To date, it has not been possible to isolate the 71K glycoprotein in sufficient quantity or purity to test for its in vivo activity.

To examine whether lipid was present in peak I, this fraction was extracted in chloroform–methanol–water, and the organic solvent phase was assayed by thin-layer chromatography. This fraction contained lipids comigrating with standard preparations of sphingomyelin, phosphatidylcholine, cholesterol, fatty acids, and possibly phosphatidyl ethanolomine.

Preincubation of macrophages with this extracted lipid before transfer into mice suppressed the IgG response by 60% when SRBC challenge was given 4 days after the macrophage transfer (Fig. 4). It had been reported previously that lipid extracted from autolyzed African trypanosomes stimulated spleen cell proliferation in vitro (3). Our most active fraction (peak I) therefore appears to be a complex of some trypanosome surface proteins and lipid. Treatment of macrophages with unextracted peak I before transfer also causes immunosuppression when antigen is given 4 days after injection. In the particular experiment illustrated, the suppression of IgG was only 34%. Perhaps the lower suppression by peak I compared with its lipid extract was due to the fact that the amount of peak I used was derived from 60% fewer parasites than the lipid fraction. A lipid rather than protein nature of the active parasite component would explain our findings after pronase or periodate treatment of peak I. The mitogenic activity of peak I in conjunction with macrophages was not reduced by pronase treatment, which, if anything, increased the polyclonal stimulation observed (Table 4). However, more than 80% of the activity of peak I was destroyed by periodate treatment. Further studies are required to ascertain whether pure lipid components are responsible for the macrophage-mediated stimulation of lymphoid cell proliferation and the subsequent defect in immune responsiveness.

DISCUSSION

We previously demonstrated that the immune dysfunction which occurs during African trypanosome infection in mice determines to a great extent the course and outcome of infection (24, 25). We tried to mimic the lymphoid disturbances seen during infection with killed trypanosomes and their fractions to identify the components which might be responsible for these effects. Crude membrane material derived from relatively few trypanosomes is able to induce potent mitogenic and immunosuppressive effects in mice when administered in vivo (5) and in cultures of human peripheral blood leukocytes (M. E. Selkirk, B. M. Ogilvie, and T. A. E. Platts-Mills, Clin. Exp. Immunol., in press). Our initial observations that soluble trypanosome-derived fractions were relatively inactive led us to pursue a more careful examination of the active components contained within the heterogeneous membrane preparations being studied. Trypanosome surface membranes were successfully separated, but they were found not to be enriched for mitogenic activity over the more heterogeneous internal membranes when tested either as protein equivalents or as derivatives of equivalent numbers of parasites. We conclude that mitogenic activity is not an exclusive property of the trypanosome surface membrane.

A series of related experiments (10) designed to elucidate the cellular targets of trypanosome-induced activation and immune suppression

TABLE 3. Effect on background PFC to TNP of trypanosome- or chicken erythrocyte-derived soluble fractions

Fraction[a]	Protein injected per mouse (μg)	Anti-TNP PFC per spleen[b] (±SE)	Stimulation index[c]
PBS	—[d]	2,450 ± 451	—
Chicken erythrocyte supernatant	ND[e]	2,570 ± 333	1.0
Trypanosome supernatant	500	5,360 ± 185	2.2
Sepharose 4B peak:			
I	27	10,933 ± 394	4.5
II	140	4,787 ± 927	1.9
III	175	3,080 ± 1,633	1.2
IV	100	3,267 ± 339	1.3

[a] Mice received 10^9 trypanosome equivalents of Sepharose 4B fractions or 4×10^8 equivalents of trypanosome- or chicken erythrocyte-derived 50,000 × *g* supernatant. Test fraction or PBS was incubated with peritoneal macrophages, and 0.4 ml of this suspension was injected i.p. per mouse.

[b] Assayed 4 days after administration of test fractions against TNP-SRBC. Values are arithmetic means, five mice per group.

[c] See Table 1, footnote *c*.

[d] —, Not applicable.

[e] ND, Not determined.

prompted us to return to soluble trypanosome fractions as a source of active material. The observation that macrophages, after the uptake of parasites in vivo, could themselves transfer mitogenic and immunosuppressive activities independent of free trypanosomes suggested that the inactivity of soluble compared with particulate trypanosome fractions after in vivo administration might have been due to an inadequate uptake of soluble material by a crucial intermediary cell type. By preincubation of the high-spin soluble fraction with elicited syngeneic mouse peritoneal cells in vitro, we were able to uncover mitogenic activity, presumably by enhancing the association of the soluble fraction with a critical target cell. With this assay system, fractionation of active soluble components by conventional methods became feasible. By gel filtration of the whole supernatant through Sepharose 4B, a 20-fold protein enrichment in parasite mitogenic activity was obtained in the high-molecular-weight peak I. This fraction was also able to induce enhancement or suppression of a primary antibody response, the particular

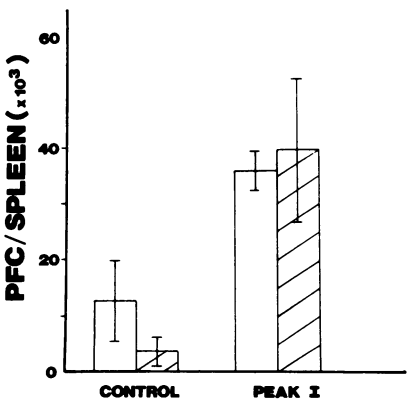

FIG. 2. Enhancement of primary anti-SRBC PFC in spleen by Sepharose 4B peak I. Each mouse received i.p. 3×10^6 peritoneal macrophages in PBS (control) or in peak I soluble fraction (10^9 trypanosome equivalents; 45 μg of protein). Mice were primed simultaneously with 10^7 SRBC, and anti-SRBC PFC was assayed 6 days later. Open bars, IgM; crossed bars, IgG. Values represent the arithmetic means plus or minus the standard error of six mice per group.

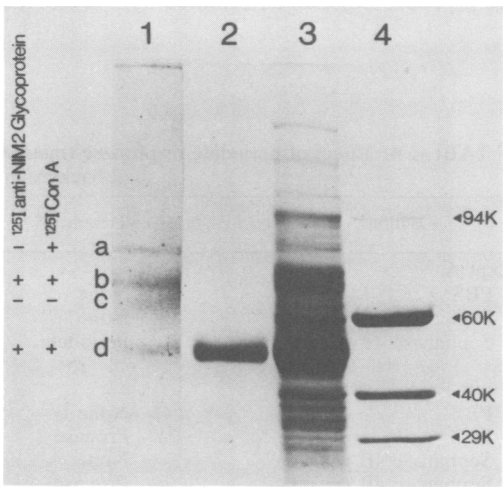

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile and lectin-antiserum binding characteristics of soluble trypanosome fractions. Coomassie blue-stained gel: track 1, Sepharose 4B peak I (60 μg); track 2, NIM-2 glycoprotein standard; track 3, 150,000 × *g* supernatant (200 μg); track 4, molecular weight markers.

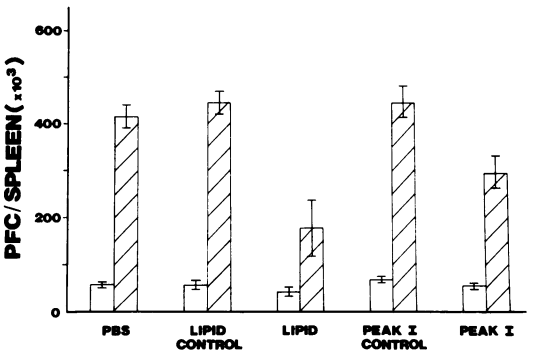

FIG. 4. Suppression of primary anti-SRBC spleen PFC by Sepharose 4B peak I and peak I-derived lipid. Controls were pre-peak I column effluent and its lipid extract. Macrophages plus fractions were transferred i.p. 4 days before priming with 6×10^7 SRBC. Anti-SRBC PFC was assayed 6 days later. Open bars, IgM; crossed bars, IgG. Values represent the arithmetic means plus or minus the standard error of six mice per group.

effect depending on the timing of treatment and antigen challenge. Thus, all of the immunological consequences of active infection, i.e., polyclonal activation, immunoenhancement, and immunosuppression, are mimicked by a high-molecular-weight soluble parasite fraction in vivo.

This fraction contains a complex of four detectable Coomassie blue-staining proteins which are dissociated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Three of these are glycoproteins, as determined by ^{125}I-labeled concanavalin A binding. One of these glycoproteins comigrates with the conventionally purified variant surface antigen (8) of clone NIM-2 and binds ^{125}I-labeled anti-NIM-2 glycoprotein antibody. Curiously, one other of the glycoproteins with a molecular weight of approximately 71K also binds the anti-NIM-2 antibody which was raised against the purified 50K glycoprotein. The conventionally purified antigen has consistently been unable to induce either polyclonal activation or immunosuppression, even when presented in association with macrophages. We did consider the interesting possibility that the activity may in fact lie in the variant surface antigen, but only in its precursor form containing hydrophobic sequences (4) which the higher-molecular-weight antibody-binding glycoprotein might represent. However, the pronase resistance of the activity and our failure to absorb out the activity on an anti-NIM-2 immunoabsorbant speak against this possibility.

In light of the work by Assoku et al. (3) linking mitogenicity in high-density cultures with *T. congolense*-derived saturated fatty acids, it was of interest to learn from preliminary thin-layer chromatography that our active fraction from *T. brucei* also contained lipid, including fatty acids. We therefore tested a lipid extract of this material for immunological activity in vivo, in this case for the ability to induce generalized immunosuppression. The extracted lipid from peak I was able to significantly suppress a primary IgG antibody response to SRBC and is therefore consistent with the suggestion of Assoku et al. that immunosuppression in African trypanoso-

TABLE 4. Effect of periodate or pronase treatment of Sepharose 4B peak I fraction on enhancement of background PFC to TNP

Fraction[a]	Treatment[b]	Anti-TNP PFC per spleen[c] (±SE)	Stimulation index[d]
Expt A			
PBS	None	1,500 ± 111	—[e]
Sepharose 4B peak I	None	13,383 ± 3,103	8.9
Sepharose 4B peak I	Periodate	2,400 ± 220	1.6
Expt. B			
PBS	None	1,440 ± 255	—
PBS	Pronase	1,090 ± 160	0.8
Sepharose 4B peak I	None	5,325 ± 758	3.7
Sepharose 4B peak I	Pronase	10,200 ± 2,242	7.1

[a] After treatment, control or trypanosome fractions were incubated with 3×10^6 peritoneal macrophages and transferred in 0.4 ml per mouse.

[b] See text.

[c] Anti-TNP PFC was assayed 4 days after administration of test fractions. Values are arithmetic means, five mice per group.

[d] See Table 1, footnote *c*.

[e] —, Not applicable.

miasis may be associated with the immunosuppressive effects of trypanosome-generated B-cell-mitogenic free fatty acids. It should be noted that, in the current study, we fractionated whole organisms rather than autolysates and assayed for activity, including immunosuppressive activity directly, in vivo. The periodate sensitivity of the activity suggests carbohydrate involvement. In this regard, other lipid species other than free fatty acids, and in particular glycolipids, have been found to be mitogenic in vitro for normal spleen cells (23) and can inhibit a primary in vitro SRBC response (18). Such activity by trypanosome-derived glycolipids has not been reported, and we are currently pursuing this question with *T. brucei*.

The question of lipopolysaccharide contamination contributing to our results is an important one. As evidence that this is not the case, we note that samples from the column did not show activity before or after peak I, suggesting that we are not getting shedding of lipopolysaccharide from the column. In addition, as the resolved peaks II and III were not active, lipopolysaccharide is unlikely to have been loaded onto the column in the whole supernatant because it would not be expected to come off the column cleanly in peak I, but would be distributed into other fractions.

A more difficult problem is the possibility that the confinement of mitogenic activity to the high-molecular-weight soluble fraction is a reflection once again of the requirement for macrophage uptake for activity to be expressed. Thus, only the macromolecular complex contained in the peak I fraction might exhibit activity not because the other fractions are lacking in the appropriate molecules, but because they are still lacking an appropriate form with which to present them, i.e., as aggregated molecules or in association with membrane structures. Our attempts to circumvent this problem by incorporating soluble trypanosome fractions into liposomes have not been successful.

These experiments emphasize the difficulties inherent in trying to isolate biologically active molecules. The separation of these molecules away from the physical context in which they are normally found can drastically affect their potential activities. We described one method by which the mitogenic and immunosuppressive activities of a normally inactive soluble trypanosome fraction can be recovered after in vitro incubation with peritoneal macrophages. This method has facilitated the preliminary separation and characterization of the active species involved and should permit further work on those trypanosome products which appear to play a central role in the pathogenesis of the disease.

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