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FINAL REPORT

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for research entitled

Identification of Mammalian Target Cell Molecules Involved in Attachment and Stimulation of Attack by *Entamoeba histolytica*.

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FINAL REPORT.

1. Brief Summary.

Objective: The scientific objective of this research has been to identify molecules on the surface of mammalian target cells of Entamoeba histolytica which are involved in attachment of the target cells and stimulation of attack by this protozoan parasite.

Significance: The etiology of Amebiasis, a human tissue invasive disease caused by the protozoan parasite, Entamoeba histolytica, is not understood. The development of immunological or chemotherapeutic means of prevention of Amebiasis requires greater knowledge of the molecules involved in the attack stimulating interaction between the parasite and the human host cells. The research conducted with this AID grant is the first which has been directed toward the identification of mammalian cell surface molecules which trigger attack by the amoeba. The information obtained will provide the basis for procedures to investigate the molecular mechanism of target cell attack by E. histolytica, as well as for immunological methods which will be used to aid the search for a vaccine against Amebiasis.

Results: The objectives of this research were achieved. Using human red blood cells (RBC) as a mammalian target cell model, it was determined that the mammalian target cell surface molecules responsible for stimulating parasite attack are lipids. Furthermore, it was determined that the phospholipid, phosphatidylserine, which is present in the RBC membranes, and digalactyl, diacyglyceride, a plant lipid which may bind to E. histolytica via a galactose sensitive lectin, could stimulate attack almost as effectively as whole target cells. A major outcome of this work has been the establishment of a collaborative relationship between the Principal Investigator's laboratory and that of Dr. Sen-Itiroh Hakamori, Program of Biochemical Oncology and Membrane Research, Fred Hutchinson Cancer Research Center, Departments of Pathobiology, Microbiology and Immunology, University of Washington, Seattle, Washington. Dr. Hakamori is a world authority on the structure and distribution of cell membrane lipids, particularly the glycosphingolipids of the human red blood cell. Together with Dr. Hakamori's laboratory we will continue this study to further delineate the specific mammalian cell membrane lipids required for attachment and stimulation of attack.

The results from these AID supported studies have been described in a full length manuscript which has been reviewed and accepted for publication after revisions by the journal, Infection and Immunity. Furthermore, aspects of this research have been presented at two national and one international scientific meeting during and following the grant period. The text of the manuscript and abstracts of the meeting presentations provide the body of the enclosed detailed report.

Value of the Research Findings: Knowledge of the the structure of the target cell surface molecules which stimulate E. histolytica attack provides a means to study the mechanism of parasite attack stimulated by these target molecules. Furthermore, the identity of the molecules on the amoeba surface with which the target cell ligands interact and which are essential to the attack mechanism can be identified by immunological and other methods now that the target cell ligands have been identified. It is conceivable that one of the latter amoeba-associated molecules could serve as a vaccine to induce immunoprotection against Amebiasis.

2. Detailed Report.

The detailed statement of the results of the research conducted with the support of this AID Grant can be best described by the scientific manuscripts and presentations which have resulted from the investigations. A full length manuscript

has been submitted and reviewed by the journal, Infection and Immunity. The editors have returned the manuscript with reviewers' comments and have indicated that the paper will be published in that journal after completion of revisions and additional experiments recommended by the reviewers. The additional experiments suggested have already been completed and are described in the abstracts included in this report. These experiments were planned to be the subject of a second manuscript, but will now be included in the revised initial manuscript. The revised paper will be resubmitted to Infection and Immunity within the coming month. To expedite submission of this final report the original manuscript is provided.

Research results described in abstracts referred to below was presented at the annual meeting of the American Society of Biological Chemists held in Washington, D.C., 8 - 12 June 1986, at the Xth International Seminar on Amebiasis in Mexico City, 5 - 9 October 1986. The most recent results will be presented at the annual meeting of the American Society of Cell Biology to be held 7 - 11 December 1986 in Washington, D.C.

2. A. Text of manuscript to be published in Infection and Immunity.

STIMULATION OF ENTAMOEBA HISTOLYTICA ATTACK BY TARGET CELL MEMBRANE LIPIDS.

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Abstract

Contact with a variety of mammalian target cells stimulates actin polymerization and target phagocytosis by Entamoeba histolytica. Using human RBC as a target model and a spectrofluorimetric assay of rhodamine-phalloidin binding to quantify actin polymerization, we sought to identify the target cell ligand which triggers the amoeba cytoskeleton response. We found that liposomes prepared from chloroform:methanol extracts of protein-depleted RBC membranes stimulated amoeba actin polymerization as effectively as whole target cells or plasma membranes. Synthetic liposomes, prepared from phosphatidylcholine, sphingomyelin and cholesterol, did not. Encapsulated fluorescent dye was internalized by amoebae 50- times faster from RBC membrane liposomes than from the synthetic liposomes. Rhodamine-phalloidin fluorescence, DIC and scanning electron microscopy revealed that the target cell membrane liposomes triggered rapid formation of gross endocytic invaginations, rimmed with polymerized actin, over the surface of the amoebae. Our results suggest that contact-mediated stimulation of E. histolytica actin polymerization is intitiated by interaction of a lipid or lipid-associated ligand of the RBC membrane with an amoeba receptor, and that phagocytic attack upon the target is a major consequence of this signal.

Introduction

The mechanism of host tissue invasion by the protozoan parasite, Entamoeba histolytica, is not clear. Lytic enzymes (7,12,18,22) and membrane perforating proteins (19,28) have been suggested as partially responsible. Destruction of mammalian cells in vitro is contact dependent (11,21,24) and requires action of the amoeba actin cytoskeleton (13,26,17). We have demonstrated stimulation of amoeba actin polymerization, which signals the initiation of phagocytic attack, at sites of target cell attachment (2). This and other evidence (20,23,27) support the view that mechanical factors may be important in the tissue destructive process.

Contact with non-cellular particles does not trigger significant amoeba actin polymerization (2), suggesting that the cytoskeleton response is dependent upon recognition of specific ligands on the target cell by amoeba surface receptors. Using human RBC as a model, we are seeking the identity of such ligands. Here we report evidence that ligand activity resides in the lipid fraction of the target cell plasma membrane. Specifically, we have found that protein-depleted liposomes prepared from RBC membrane lipid extracts, but not synthetic liposomes, trigger amoeba actin polymerization to the same extent as do whole RBC or isolated RBC plasma membranes. Furthermore, amoebae accumulated encapsulated fluorescent dye 50-times faster from RBC liposomes than from synthetic liposomes, suggesting that the cytoskeleton response reflected recognition-specific endocytosis of the target cell-derived vesicles. This was supported by fluorescence and scanning electron micrographs, which showed that challenge with the target cell liposomes stimulated the formation of gross endocytic stomata over the surface of the amoebae.

In addition to initial evidence of the chemical nature of a natural ligand which stimulates phagocytic attack by Entamoeba, the RBC membrane liposome preparation provides a biochemically inert, cell-free stimulus with which to initiate, and thereby investigate, molecular events within the amoeba which constitute and regulate this attack response. Also, the recognition specific liposomes may find application in studies of other aspects of Entamoeba cell biology or in the search for means to control amebiasis.

Materials and Methods

Amoebae. E. histolytica, strain HM1-IMSS, was cultured in TYI-S33 medium (6). For experiments, trophozoites in mid-growth phase were centrifuged at 200 X g for 2 min and resuspended in their own medium at 10^6 cells/ml.

Preparation of RBC Fractions. Human Type A (+) RBC from freshly drawn or outdated bank blood were washed three times with cold 15 mM potassium phosphate- buffered saline, pH 6.3 (PBS). For Pronase (Boeringher-Manheim, Indianapolis, IN) treatment of whole RBC, 150 mM NaCl containing 5mM $CaCl_2$ was substituted for PBS in the last wash. The packed cells were suspended in five volumes of this solution containing 1 mg/ml Pronase, incubated for two hr at 37°C, and washed three times with cold PBS. RBC plasma membranes (ghosts) were prepared by hypotonic lysis of packed RBC with 10 volumes of cold, 5 mM potassium phosphate, pH 8.0, and washed three times by centrifugation (12,000 x g, 15 min) through cold, 20-fold diluted PBS. For Pronase treatment of membranes, the procedure used with whole cells was followed with the following variations: the final membrane wash was with a 1/20 dilution of the NaCl/$CaCl_2$ solution; the membranes were not diluted before addition of Pronase; they were sonicated 1 min after addition of Pronase, but before the incubation for two hr at 37°C. The Pronase treated membrane fragments were used to challenge amoebae without further treatment. Untreated membranes were sonicated before being used to challenge amoebae.

Incubations, Fixation and Fluorescent Staining of Amoebae, and Quantitation of Polymerized Actin. Interactions of amoebae with challenge preparations were carried out following the procedures described previously (2). For measurements of the stimulation of actin polymerization, amoebae were allowed to interact with whole target cells or membranes for 4 min and with membrane fragments or liposomes for 1 min, before fixation. Fixation, detergent extraction and rhodamine-phalloidin staining of amoebae for fluorescence microscopy, and for methanol extraction of bound rhodamine-phalloidin to quantitate polymerized actin spectrofluorimetrically, were also accomplished as described earlier (2).

Extraction of Membrane Lipids. RBC membrane preparations were lyophilized, and lipids were extracted by shaking the residue for 15 min at room temperature with a

volume of chloroform:methanol (2:1 v/v) equal to the volume of packed membrane fragments before lyophilization. Insoluble material was collected by filtration, extracted again, and removed by filtration. The combined filtrates were dried at 45° C under a stream of air. The residue was dissolved in choloroform:methanol, filtered, dried at 45° C to a constant weight, redissolved in choloroform:methanol and stored at 4° C.

Preparation of Liposomes. A volume of the chloroform:methanol extract representing a known volume of packed RBC membrane fragments was dried as above in a small glass tube. A volume of PBS equal to the orginal volume of packed membranes was added and the mixture sonicated for 5 min in ice. The resulting suspension contained a heterogeneous size distribution of multilaminar vesicles, the largest of which were approximately 3 um in diameter, but most of which were 1 um or less.

Synthetic liposomes were prepared with commercial lipids (Sigma Chemical Co., St. Louis, MO) and contained phosphatidylcholine: sphingomyelin: cholesterol (40:40:20, w/w) . The lipid components were dissloved together in chloroform:methanol, dried and resuspended as liposomes as described above. The final concentration of the synthetic liposomes was the same by weight as that of RBC liposomes. The size distribution of the synthetic liposomes appeared similar to that of the RBC liposomes.

Liposomes were loaded with carboxyfluorescein (CF) (Molecular Probes, Eugene, OR) by first dissolving the fluorescent dye (5mg/ml) in the PBS used for sonication of the dried lipid extracts. CF loaded liposomes were separated from unencapsulated dye by elution with PBS from a column of Sephadex G-200 through which a suspension of non-fluorescent RBC liposomes had first been passed to block lipid adsorption sites (18). The CF content of the eluted liposomes was determined by measurement of the fluorescence (493 nm/515 nm) of a 50 ul aliquot of the vesicles solubilized in 1.5 ml of 0.1% Triton X-100 in PBS. The loading efficiency of RBC and synthetic liposomes was similar (4% - 6% of the total CF).

Measurement of Endocytosis. Erythrophagocytosis was measured as previously described (2). For measurement of liposome endocytosis, suspensions of amoebae were prepared and challenged as described above with 1/10 vol of CF-loaded liposomes. At selected times 3 ml of cold PBS was added to terminate endocytosis. The cells were then washed twice by centrifugation with cold PBS (2 min each at 500 X g). The amoeba pellet was solubilized in 1.5 ml 0.1% Triton X-100 in PBS, and the internalized CF fluoresence was quantitated as above.

Microscopy. Differential interference contrast (DIC) (Nomarski), fluorescence and scanning electron microscopy were accomplished as described previously (2).

Electrophoresis. SDS PAGE of RBC membrane fractions through 3% to 22% linear gradient gels was performed by the method of Laemmli (16). Gels were stained with Commassie Blue. Densitometric recordings were made with an LKB Ultrascan laser densitometer. Protein was assayed by the method of Bradford (4).

Results

By DIC and fluorescence microscpoy of rhodamine-phalloidin stained cells, we have observed contact-associated *E. histolytica* actin polymerization and target phagocytosis in response to challenge with a variety of mammalian cell types in addition to RBC. These have included cultured Friend erythroleukemia, Chinese hamster ovary and 3T3 cells, and freshly isolated parietal and chief cells of the rabbit gastric mucosa and epithelial cells of the ileal and colonic mucosae. It appears that most mammalian cells, including some from tissues destroyed in amebic infections, are capable of stimulating contact-mediated amoeba cytoskeleton activation and phagocytic attack.

Although not a tissue of consequence in the pathogenesis of amebiasis, we selected human RBC to initiate a search for an amoeba attack stimulating ligand because of easy availability, the wealth of knowledge of RBC membrane structure and methodology and, particularly, because, in contrast to other mammalian cells, RBC contain little rhodamine-phalloidin-sensitive actin. The last property permitted use of the rhodamine-phalloidin assay to detect ligand activity without interference from target cell actin.

Stimulation of Amoeba Actin Polymerization by RBC Fractions. RBC treated with Pronase under conditions which remove most of the exposed peptides on the membrane outer surface (3) triggered actin polymerization and were phagocytized to the same extent as whole cells (data not shown). Whole membranes (ghosts), prepared by hypotonic lysis of both untreated and Pronase-treated RBC, stimulated actin polymerization to about the same extent, and somewhat more effectively than whole cells at the same concentration (Table I). Although RBC membranes were barely visible by light microscopy following fixation and Triton X-100 extraction, their phagocytosis was evident from amoeba morphology and the patterns of Rhodamine- phalloidin staining of actin at the sites of attachment (not shown).

Following sonication in the presence of 1 mg/ml Pronase (to facilitate access of the protease to membrane proteins) and incubation for 2 hr at 37° C, only traces of Commassie Blue sensitive material were detected in gels following SDS PAGE, and this was all located at the position of the low molecular weight tracking dye (Fig. 1). Sonicated, untreated membranes showed a protein profile typical of RBC membranes (9). The protein-depleted membrane fragments stimulated actin polymerization as well as the untreated fragments (Table I). When compared on the basis of packed volume, membrane fragments showed a more rapid loss of stimulatory activity upon dilution of the challenge suspension than did whole RBC (data not shown). However, this was not due to destruction of a stimulating ligand during protease treatment, since the same loss occurred with the untreated as with the Pronase-treated membrane fragments.

Liposomes prepared from total lipid extracts of lyophilized, protein-depleted membrane fragments stimulated amoeba actin polymerization as well as the membrane fragments themselves (Table I). Peptides were not detectable in the RBC lipid extract, either by assay of total protein or by electrophoresis (Fig. 1). Lipids were also extracted from lyophilized, whole RBC membranes, and the insoluble residue resuspended by sonication in PBS. This suspension, mostly insoluble membrane protein, did not stimulate amoeba actin polyerization when used as a challenge (not shown). Our conclusion from these results was that lipid or lipid-associated structures of the RBC plasma membrane serve to stimulate contact dependent amoeba actin polymerization. We have no evidence that surface peptides or peptide conjugates serve as stimulatory ligands; at least, they are not essential.

Synthetic liposomes, formulated with phosphatidylcholine, sphingomyelin and cholesterol, did not stimulate amoeba actin polymerization when used as a challenge at the same or twice the concentration (by total weight) of the RBC liposomes (Table I). Therefore, it is not lipids or lipid vesicles, per se, which activate amoebae. Perhaps the most obvious structural difference between the RBC membrane and synthetic liposomes is the presence of exposed oligosaccharides of glycosphingolipids in the former. These might contain the ligand activity. However, in agreement with evidence of others that E. histolytica does not discriminate between the ABO blood groups for atttachment (17,26), liposomes prepared from type O RBC membranes stimulated actin polymerization as effectively as liposomes from type A RBC (Table I). Therefore, the terminal N-acetylgalactosamine and galactose antigens of the A and B blood groups are not essential for ligand activity.

Specific Endocytosis of RBC Liposomes. Selective endocytosis of RBC membrane liposomes was demonstrated by comparison of the uptake of carboxyfluorescein encapsulated in RBC liposomes and synthetic liposomes (Fig. 2A). The average initial rate of internalization of fluorescent dye from the target cell liposomes in five separate experiments was more than 50-times that from synthetic liposomes. Endocytosis, rather than surface binding, was confirmed by fluorescence microscopy of amoebae which had been incubated with fluorescent RBC liposomes (Fig. 2B). Net internalization of fluorescence reached a maximum approximately 3 hr following challenge, at which time amoebae contained 15% of the added fluorescence. We have not yet determined whether the plateau reflects an equilibrium between endocytosis and exocytosis of the dye, as has been shown to occur during fluid pinocytosis by E. histolytica (1), or represents actual cessation of liposome uptake.

The time course of the actin response to RBC liposomes is also shown in Figure 2. The average cell content of polymerized actin rose within 2 min to a maximum of approximately twice that in unstimulated cells and returned to close to the unstimulated level over the following 60 min. After 60 min the rate of endocytosis of RBC liposomes was less than 10% of that during the first 4 min following challenge. If the elevated cellular content of polymerized actin following challenge is directly related to the the rate of phagocytosis, then phagocytosis of RBC liposomes beyond 60 min would be maintained by an amount less than 10% above the basal level; this is within the error range of the actin assay. Thus, as was the case with whole cells (2), amoeba actin polymerization in response to the RBC liposome challenge most probably reflects cytoskeleton mobilization primarily for phagocytosis of the target vesicles.

When amoebae were challenged with RBC liposomes 1 min ahead of a 4 min challenge with whole RBC, adherence of the latter was inhibited 53% +/- 0.13% (SEM) and phagocytosis was inhibited 85% +/- 0. 11% (SEM). These results are consistent with, but do not prove, competition between the liposome and whole target cell ligands for binding to the same sites on the amoebae .

Polymerized Actin Patterns and Surface Topography of Stimulated Amoebae. Patterns of polymerized actin characteristic of E. histolytica before and after challenge with RBC liposomes are shown in Figure 3. In suspension at 25° C most trophozoites showed faint rhodamine-phalloidin staining of the cortical actin mesh (Fig. 3A). 15 sec following challenge with RBC liposomes a punctate pattern of polymerized actin sites, roughly reflecting the size distribution of the challenge particles, was observed about the periphery of many amoebae (Fig. 3B). Such patterns were rarely seen 30 sec or longer after challenge. Then, polymerized actin appeared as bright waves (Fig. 3C) or rings (Fig. 3D) underlying gross membrane convolutions and what appeared to be endocytic invaginations (Figs. 3 E, F) over the surface of stimulated cells. The changes in membrane topography following challenge are shown more clearly in the scanning electron micrographs of Figure 4. We infer from the membrane and underlying actin patterns that contact with the target vesicles first stimulated actin polymerization specifically at the sites of initial interaction, and that this triggered a more generalized endocytic response by the amoebae.

Discussion

The results of this study support the conclusion that contact-dependent actin cytoskeleton activation and accompanying phagocytic attack of RBC by E. histolytica is stimulated by recognition specific binding of lipid or lipid-associated components of the target cell plasma membrane to the amoeba surface. All detectable protein was eliminated during the preparation of the RBC membrane liposomes, with little, if any, loss of ligand activity. Therefore, it seems unlikely that the attack response was stimulated specifically by target cell surface peptides or structures attached to them. The failure of synthetic liposomes to stimulate sig-

nificant amoeba cytoskeleton activation or endocytosis, compared to the target cell membrane liposomes, eliminates a non-specific response to lipids or lipid vesicles, per se, as the basis of stimulation. A plausible candidate for the ligand is a carbohydrate moiety of one or more of the glycolipids found in the RBC plasma membrane. Indeed, inhibition of adherence of mammalian cells to E. histolytica by N-acetylgalactosamine (26), galactose (25) and N-acetylglucosamine (13) has been cited as evidence that binding of an amoeba lectin to these sugars on the surface of target cells may mediate attachment of amoebae to target tissues. This question will be addressed in future studies. However, the terminal N- acetylgalactosamine and galactose residues of the A and B blood groups are not required for activation of the cytoskeleton, since liposomes from type O RBC were as effective as those from type A cells.

We have observed vigorous phagocytic action by E. histolytica upon a variety of mammalian cells with associated amoeba actin polymerization. A major reason that a target cell model more closely related to one of consequence in amebic infections was not chosen from among these for this study was to avoid interference by the polymerized actin of those cells with the assay of amoeba actin. (RBC contain little rhodamine-phalloidin sensitive actin (2).) Applying the knowledge gained in this study, it is now plausible to prepare actin-free plasma membrane liposomes from other mammalian cells to determine whether ligand activity is associated with the membrane lipid fraction of target cell membranes generally.

As was the case with whole RBC (2), the present experiments indicated that most, if not all, of the newly polymerized actin stimulated by challenge with RBC liposomes was involved in the formation, growth and contraction of numerous endocytic stomata over the surface of the stimulated cells. Changes in orientation of elements of the cytoskeleton take place in cytotoxic lymphocytes during contact-mediated attack of target cells (14). These changes include polymerization of actin and localization of talin, a protein involved with binding of actin to cell membranes (5), at the sites of target cell contact (15). It has been proposed that these changes reflect the involvement of the cytoskeleton in the secretion of cytolytic substances (10) and in the protection of the effector cell membrane against these toxins (15). Secreted cytotoxic substances have been suggested by some as the effectors of target cell destruction by E. histolytica (7,12,18,19,22,28). If so, the amoeba cytoskeleton may be required to serve functions analagous to those proposed for killer lymphocytes. However, the major activity of the amoeba cytoskeleton following contact appears to be phagocytic attack upon the target cell. This makes sense in view of the presumed goal of the parasite - ingestion of nutriments.

In addition to initial evidence of the chemical nature of a natural ligand which stimulates phagocytic attack by Entamoeba, the RBC membrane liposome preparation provides a biochemically inert, cell-free stimulus with which to initiate, and thereby investigate, molecular events within the amoeba which constitute and regulate this attack response. Also, the recognition-specific liposomes may find application in studies of endocytosis or other aspects of Entamoeba cell biology, or in the search for means to control amebiasis (e.g., targeted drug delivery).

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TABLE I

Stimulation of E. histolytica Actin Polymerization by RBC Fractions

Challenge*	Relative Polymerized Actin Content#	
None	1.00 +/- 0.15	(10)
Whole RBC	1.81 +/- 0.40	(6)
Membranes from Untreated RBC	2.11 +/- 0.12	(6)
Membranes from Pronase Treated RBC	2.52 +/- 0.28	(4)
Untreated Membrane Fragments	2.02 +/- 0.14	(6)
Pronase Treated Membrane Fragments	1.96 +/- 0.29	(5)
RBC Membrane Liposomes	1.76 +/- 0.15	(5)
Synthetic Liposomes	1.18 +/- 0.09	(6)
Type O RBC Membrane Liposomes	1.95	(2)

* Type A RBC used except where otherwise indicated.

Relative increase in rhodamine-phalloidin fluorescence units bound per 10^5 amoebae compared to unchallenged controls in each experiment. Amoebae were challenged with whole cells and membranes for 4 min (2), and with other preparations for 1 min. Values are means +/- SEM of results of the number of experiments shown in parentheses. The mean polymerized actin content of challenged amoebae was significantly greater than that of controls ($P < 0.005$) for all challenges except synthetic liposomes ($P > 0.05$).

Figure Captions

Figure 1. Densitometric scans of Commassie blue-stained SDS gels of RBC membrane fractions. Untreated membranes (A) displayed a typical protein profile, numbered here in the conventional manner (9). Pronase treated membrane fragments (B) and chloroform:methanol extracts prepared from these (C) revealed no detectable protein bands. Lanes B and C were loaded with twice the amount of sample as lane A, based on the original volume of membranes used to prepare each fraction.

Figure 2. Time course of RBC membrane liposome-stimulated actin polymerization (O) and endocytosis of RBC membrane liposomes (O) and synthetic liposomes comprised of phosphatidylcholine, sphingomyelin and cholesterol (V). Actin polymerization was measured as methanol-extracted rhodamine-phalloidin fluorescence and endocytosis was measured as fluorescence released by Triton X-100 from internalized carboxyfluorescein-loaded liposomes. The rate of RBC liposome endocytosis estimated from the segmental slopes of this curve was over 10-times greater between 0 and 4 min than it was between 60 and 120 min. These curves are typical of those obtained in 4 experiments. The inset displays amoebae washed and glutaraldehyde-fixed 60 min following challenge with carboxyfluoresein-loaded RBC liposomes. 600 X. In mounts of live cells the internalized fluorescence was seen to be within vesicles, some containing several fluorescent particles.

Figure 3. The appearance of rhodamine-phalloidin stained actin in E. histolytica trophozoites before (A) and at 15 sec (B) and 30 sec (C - E) following challenge with RBC membrane liposomes. Unchallenged amoebae displayed relatively uniform cortical polymerized actin (accentuated sometimes at spots) about the cell periphery (A). 15 sec following challenge some cells displayed a punctate pattern of sites of polymerized actin roughly the size of the challenge liposomes (B). However, by 30 sec polymerized actin in all stimulated cells was organized in (usually) closed waves or rings under the surface. That the polymerized actin outlined gross convolutions of the surface membrane is evident in the DIC micrograph (E) taken in the same plane of focus as the fluorescence micrograph in D. A, 500 X; B,E, and F, 1500 X; C and D, 1000 X.

4. Scanning electron micrographs of E. histolytica trophozoites before (A) and 30 sec following (B) challenge with RBC membrane liposomes. Unchallenged amoebae displayed a relatively uniform surface topography, some cells displaying what appear to be extended pseudopods or blebs. The latter may be artifacts of processing, but were rarely seen on stimulated cells. Surface topographies of stimulated cells representative of those with the actin staining patterns seen in Figure 3 are shown in B. 1000 X.

FIG 1

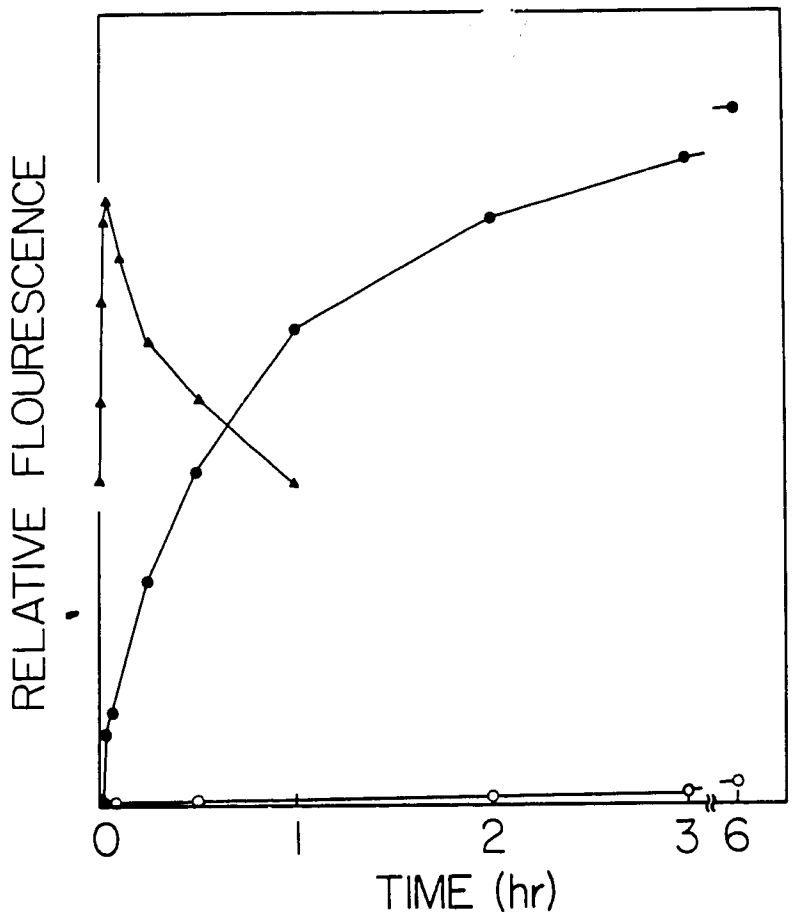

FIG 2

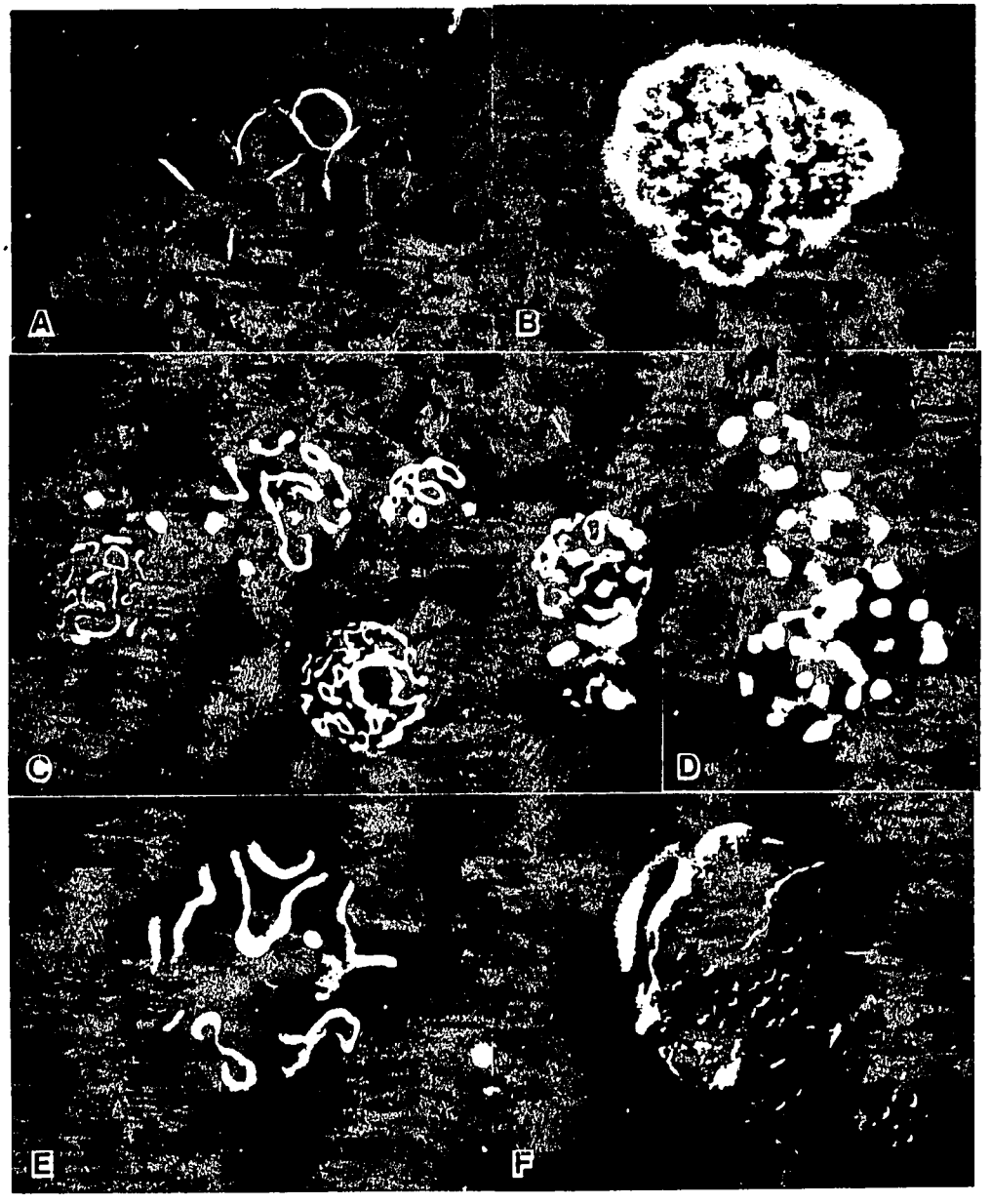

FIG 3

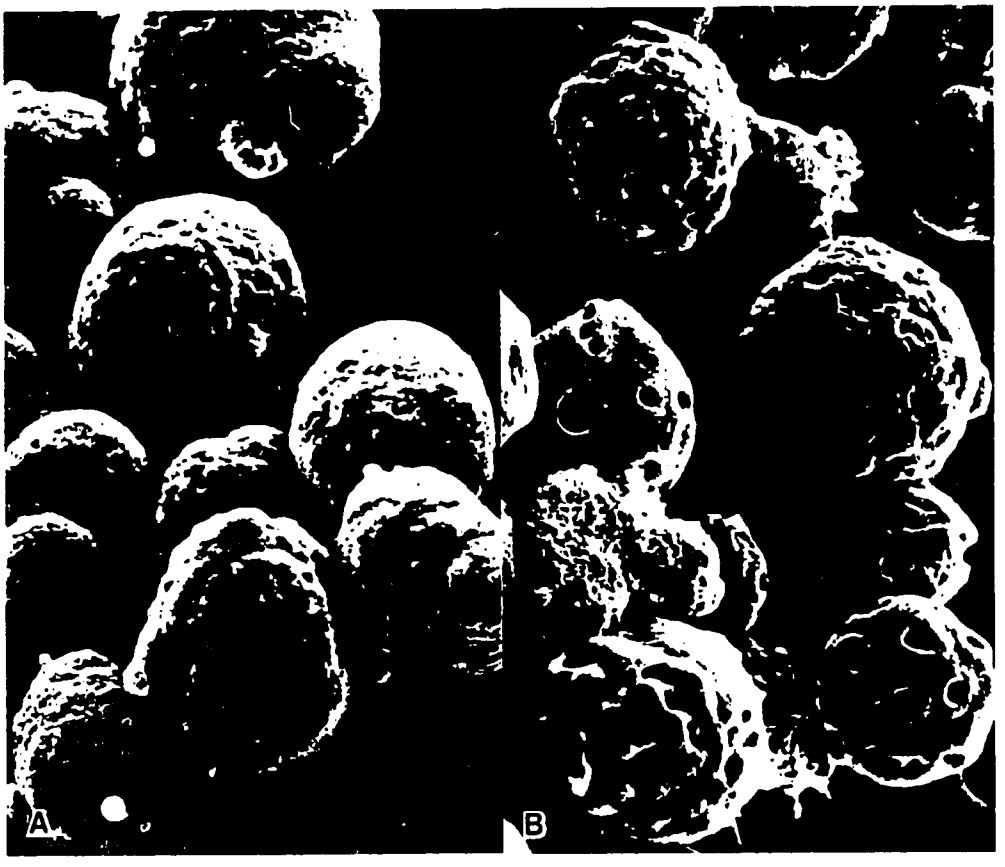

FIG 4

2. C. Presentation at the annual meeting of the American Society of Biological Chemists, 8 - 12 June 1986.

The research which was presented as a poster at this meeting describes the AID supported work included in the manuscript above. Since the scientific information is presented in the maunuscript, it is not necessary to reproduce the abstract here. support from AID was acknowledged in both the abstract and on the poster displayed at the meeting.

2. D. Presentation at the Xth International Seminar on Amebiasis. Mexico City, 5 - 10 October 1986.

The work described in the above manuscript as well as more recent studies to be included in the revised manuscript to be published in Infection and Immunity was presented at this seminar. The following text, which is abstracted from that presentation only the the more recent experiments not described in the above manuscript but which will be included in it.

We have prepared liposomes from a variety of commercially available fatty-acyl-glycerol and ceramide based lipids and tested the ability of these to stimulate the amoeba actin response. The results are shown in Table II below.

STIMULATION of ACTIN POLYMERIZATION and PHAGOCYTOSIS by SYNTHETIC LIPOSOMES.

LIPOSOME CHALLENGE	REL. ACTIN POLYM.	REL. PHAGO-CYTOSIS	"FOOD CUPS" FORM	
NONE	1.00		-	
RBC MEMBRANE	1.96	45	++	P <.001
PHOSPHATIDYL SERINE	1.53	31	+	P <.01
Di-Gal DIGLYCERIDE	1.47		+	P <.01
PHOSPHATIDYL INOSITOL	1.20		(+)	P >.05
PHOSPHATIDIC ACID	1.18		(+)	"
PHOSPHATIDYL ETHANOL.	1.12		-	"
PHOSPHATIDYL CHOLINE	1.10	1	-	"
SPHINGOMYELIN	1.06		-	"
Gal-CEREBROSIDE	1.06		-	"
DIACYL GLYCEROL	1.00		-	"

Amoebae challenged with phosphatidylserine liposomes or liposomes formulated with a plant lipid, diGalactose diglyceride contained significantly greater polymerized actin than unchallenged controls, but less than amoebae challenged with RBC membrane liposomes in the same experiment. Phosphatidylinositol and phosphatidic acid liposomes did not stimulate a statistically significant increase in polymerized actin, but amoebae challenged with these phospholipids were observed by fluorescence microscopy to bear more actin rimmed food cups than were the controls, so they were probably weakly stimulatory. No stimulation was observed with phosphatidylethanolamine or phosphatidylcholine of with either of the sphingolipids tested, sphingomyelin and Galactose Cerebroside.

The stimulatory lipids share in common the diacyglycerol moiety, suggesting the possibility of cytoskeleton stimulation involving Protein Kinase C activation. However, neither octoyl, acyl glycerol, the diacyl glycerol which stimulates protein kinase C in some whole cell systems, nor phorbol ester (which is not shown) stimulated the amoebae. Stimulation by digalactose diglyceride, which does not occur in the RBC membrane may have been facilitated by binding of the the galactose residues to the amoeba GalNAc/Galactose lectin. However, the stimulatory activity probably

derives from a non-carbohydrate structure since phosphatidylserine contains no carbohydrate and galactose cerebroside showed no activity.

Dr. Sen-Itiroh Hakamori at the Fred Hutchinson Cancer Research Center, University of Washington has agreed to collaborate with us and to supply selected RBC glycolipids which will be constructed into liposomes to determine the role of carbohydrates in the cytoskeleton response.

2. C. Abstract of work to be presented at annual meeting of the American Society for Cell Biology, 7 - 12 December 1986.

This work which, has been accepted for presentation as a poster at the ASCB meeting, expands upon the earlier studies and describes the beginning experiments to detect antibodies which inhibit the amoeba attack response. Portions of the research described will also be included in the manuscript to be published in Infection and Immunity.

Lipids in the Contact-Dependent Attack Response of Entamoeba histolytica. G. B. Bailey, C. N. Nokkaew and D. B. Day. Morehouse School of Medicine and Atlanta University, Atlanta, GA. 30310.

Destruction of mammalian cells by E. histolytica is contact-dependent, involves net polymerization of amoeba actin, and is accompanied by phagocytic attack upon the target (Bailey et al. 1985. J Exp Med 162:546). We are seeking the identity of the target cell molecules responsible for stimulation of these responses. Protein-depleted liposomes prepared from human RBC membranes stimulated amoeba actin polymerization and endocytosis as effectively as whole target cells. Synthetic liposomes prepared from phosphatidylserine (PS) and cholesterol (80:20), and to a lesser extent phosphatidic acid:cholesterol vesicles, also stimulated rapid polymerization of amoeba actin. Similarly formulated liposomes containing phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, sphingomyelin or oleic acid did not. Serine was not stimulatory alone and did not inhibit stimulation by PS liposomes. Antisera to amoeba plasma membranes, which inhibited target cell liposome-stimulated cytoskeleton activation, had no detectable effect on the response to PS liposomes. These results suggest that contact-dependent attack by E. histolytica is mediated by more than a single target cell membrane molecule, and involves specific target membrane lipids. (Supported by grants from NIH and USAID.)

3. Names and positions of the investigators who contributed to the research efforts.

Gordon B. Bailey, Ph.D.	Professor of Biochemistry Morehouse School of Medicine
Diane B. Day, B.S.	Research Assistant, Department of Biochemistry Morehouse School of Medicine
Cumnuan Nokkaew, M.Sc., D.V.M.	Graduate Student, Department of Biology Atlanta University
Carolyn C. Harper, M.Sc.	Graduate Student, Department of Biology Atlanta University

_____ Date:_____

Gordon B. Bailey, Ph.D.
Principal Investigator

Proposal to

The Agency for International Development

from

Morehouse School of Medicine
Atlanta, Georgia 30314

Identification of Mammalian Target Cell Molecules Involved in Attachment and Stimulation of Attack by *Entamoeba histolytica*.

Gordon B. Bailey, Ph.D.
Professor of Biochemistry
Principal Investigator

25 March 1985

Gordon B. Bailey, Ph.D.
Principal Investigator

Walter W. Sullivan, Ph.D.
Associate Vice President for
Sponsored Programs

Proposals for research which overlaps aspects of the investigations described here were submitted to a private foundation on 7 March 1985 and will be submitted to the NIH by 1 June 1985. No support for this research has yet been awarded.

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B. Relevance of the Proposed Work to the Mission of A.I.D.

1. Statement of the Problem.

Amebiasis is a major cause of morbidity and mortality in tropical developing countries. A clinical means of disease prevention awaits a clearer understanding of the nature of interaction of the causative organism, Entamoeba histolytica with the mammalian target cells it attacks and of the mechanism of cytopathogenicity. The objective of this research is to identify specific molecules involved in the interaction of E. histolytica with target cells. The information obtained will further the understanding of the mechanism of Amebiasis and could be of value in the development of immunoprotective agents against this parasitic disease.

According to estimates of the World Health Organization (1) as many as 480 million people in Africa, Asia, Central and South America are infected with Entamoeba histolytica each year. Of these, 35 to 50 million exhibit clinical symptoms of disease. As many as 110 thousand people die of Amebiasis annually. Although this mortality estimate places Amebiasis among the greatest parasitic disease killers, the morbidity rate presents an even more profound determent to advancement by affected third world countries. Each ill individual experiences from 5 to 7 debilitating days per incidence of amoebic infection. Given that adults of working age are among those at greatest risk (2), the economic losses to these already poor countries caused by debilitation can be measured in the billions of dollars. If developing nations are to approach the status of developed countries, ways must be found to prevent Amebiasis as well as the other important tropical infectious diseases.

Infection by Entamoeba results from ingestion of cysts in drinking water or food which has become contaminated with fecal matter from other infected individuals. Prevention of Amebiasis can be accomplished effectively by improvement in sanitation practices. However, this requires improved living standards and education of the affected populations, which, realistically, can not be achieved until the causes of poverty, including infectious disease are abated. This vicious circle demands that medical as well as social means of disease protection be sought. A goal of this study is to obtain information which will aid the development of a vaccine against Amebiasis.

2. Developing Country Participation in the Proposed Research.

None.

3. Environmental Effects of the Proposed Experiments.

None.

C. Scientific Aspects of Proposed Work.

1. The Problem to be Addressed.

The induction of immunoprotection through vaccination has been the most effective means to conquer infectious diseases. In cases of Amebiasis circulating antibodies are detected (3), and there is evidence of acquired immunity in humans recovering from severe hepatic infection or amoebic colitis

(4). Extracts of E. histolytica trophozoites possess antigenic activity. However, there has been little progress toward isolation of a specific antigen which might be used to induce immunity to Amebiasis.

1a. Research Objectives of this Project.

The immediate objective of this project is to identify the molecular constituent(s) of human red blood cell (rbc) required for attachment and stimulation of attack by E. histolytica.

A procedure we have developed (5, accompanying manuscript, App A) to detect activation of the attack mechanism of E. histolytica in response to attachment of target cells and structurally altered targets will be employed as an assay to detect the substances sought. The human red blood cell (rbc) has been chosen for this study because it binds and is attacked vigorously by Entamoeba, and because more is known of the membrane molecular architecture and composition of rbc than of other mammalian cell types. The information obtained with rbc will provide insight to aid the identification of analogous components of cells more closely related to those attacked in vivo by this parasite.

1b. Longer Range Research Objectives.

The identified molecules (ligands) from rbc or other mammalian target cells will be used to identify their specific molecular receptor(s) on the amoeba surface. When isolated, these can be tested for their ability to elicit a protective immune response in animals. Alternatively, monoclonal antibodies to the target cell ligand molecules themselves may be tested as immunoprotective antigens, since these antibodies will bear idiotypes resembling antigenic determinants of the amoeba receptor(s).

2. Summary of Previous Work Leading to this Study.

Achievement of the goals of this study would be aided by an understanding of the mechanism of E. histolytical cytopathogenicity. However, the cellular mechanism of destruction of mammalian target cells by this pathogen remains unexplained. Hydrolytic enzymes (6), enterotoxins (7) ionophores (8), hormones (9) lectins (10) or lectin substrates (11) are among the amoeba-associated molecular components suggested as possibly having a role in the parasite's attack strategy. Some things seem certain, at least in in vitro systems: Destruction requires contact of the amoeba with the target (12). And operation of the amoeba motility apparatus is essential - more than for movement to the target - since the events of the killing process are blocked by the polymerized actin destabilizer, cytochalasin (13). Phagocytosis, which depends upon action of the actin cytoskeleton, virtually always accompanies contact destruction, and phagocytic competence also has been correlated with virulence (14). Attachment of target cells to amoebae has been proposed to occur by binding of an amoeba surface lectin(s) (receptor) to a target carbohydrate(s) (ligand) (10). However, this has not been established.

Recently, we have been successful in demonstrating and quantitating a rapid polymerization of actin in trophozoites at the sites of target cell attachment immediately following challenge (5, App.A). The characteristics of this response indicated that attachment and activation of the amoeba cytoskeleton by rbc is a receptor-mediated process. Red cells were used as the targets in

this study since, compared to Entamoeba, they contain relatively small amounts of actin, and, therefore, did not interfere with the quantitation of amoeba actin. However, the same qualitative observation of the cytoskeleton response was made when larger, nucleated (Friend erythroleukemia) cells were used as targets. The cytoskeleton response by amoebae following target cell attachment is one of the earliest detectable events indicative of attack and is triggered by binding of the amoeba surface receptor to a target ligand.

The procedures we have developed can be used to investigate further the receptor-mediated interaction between trophozoites and target cells and to aid identification of specific molecule(s), both of the target and of the amoeba, responsible for attachment and stimulation of attack.

We propose to use non-ionic detergent extraction and selective enzyme treatment of whole rbc or rbc membranes (ghosts) to prepare cytoskeleton components and specifically altered surface structures. The ability of the altered cells or isolated components from them to stimulate the actin polymerization response directly or to inhibit the responses by amoebae to whole target cells (or ghosts) will be used as an assay to seek molecular structures involved in target cell attachment, amoeba cytoskeleton activation and subsequent phagocytosis. If this is successful, we plan to use the information gained to identify comparable molecules of target cells more closely related to those attacked in vivo. Thereafter, it may be possible to use these molecules to identify and isolate the trophozoite receptors to which they bind. Such knowledge would be of value in explaining the mechanism of Entamoeba cytopathogenicity and in seeking immunoprotective means for disease prevention.

2a. Preliminary Observations and their Implications.

At the time of this writing we have conducted several preliminary experiments of the sort indicated above to assess the feasibility of the proposed study. RBC have been subjected to treatments which remove specific cellular components, and the altered structures have been assayed for their ability to stimulate actin polymerization and, where observable, phagocytosis by E. histolytica trophozoites. The results of these initial experiments, summarized below, demonstrated that the approach I propose is feasible and provided intriguing initial information.

Description of Preliminary Experiments.

The assay procedures used in these preliminary experiments to quantitate actin polymerization and phagocytosis by amoebae are described in detail in the accompanying manuscript (5, App. A).

Stimulation by RBC Ghosts: RBC membranes (ghosts) were prepared by hypotonic lysis of whole rbc, washed thoroughly with the hypotonic buffer and resuspended at a concentration approximating that used for assays with whole rbc (5). Trophozoites were challenged with the ghosts for 3 min at 25° C. Although the ghosts were not visible by light microscopy, fluorescence microscopy of rhodamine-phalloidin stained amoebae revealed that they had attached to the trophozoites, stimulated amoeba actin polymerization and were phagocytized as aggressively as were whole rbc. Quantitation of bound rhodamine-phalloidin indicated a 2.15-fold stimulation of amoeba actin polymerization compared to unchallenged controls. Thus empty cell membranes are as active as whole cells

Stimulation of E. histolytica actin polymerization by human red blood cells lysed and extracted with 0.1% Triton-X 100. The Triton-X depleted rbc skeletons are not visible in the light micrograph (left), but their presence is revealed by the fluorescent patches of rhodamine-phalloidin stained actin over the surface of the amoeba trophozoite (right). Attachment of rbc to trophozoites triggers polymerization of amoeba actin and phagocytosis of the target cells. Thus, the rbc skeletons must retain the molecule(s) responsible for attachment and stimulation of this attack response by the amoebae.

in stimulating attack by the parasite.

Effect of Pronase Treatment: Fresh washed and packed human rbc were diluted 1/10 in normal saline containing 1 mM $CaCl_2$ and 1 mg/ml pronase and incubated for 30 min at 37°C (15). This treatment removes virtually all of the exposed outer surface peptides, most of which are glycopeptides (16). The treated cells were washed to remove enzyme and the products of hydrolysis, and tested for their ability to attach to trophozoites and stimulate actin polymerization and phagocytosis compared to untreated rbc. In two separate experiments the response to the pronase treated cells was markedly greater than that to untreated rbc as shown by the data in the table below.

Challenge	Relative Amount of Amoeba Polym. Actin		Adherence (rbc/amoeba)		Phagocytosis (rbc/amoeba)	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
None	1.00	1.00	—	—	—	—
Untreated rbc	2.68	1.40	3.24	4.10	3.36	3.96
Pronase treated rbc	5.31	2.11	10.48	7.86	8.28	6.26

Effect of Triton-X 100 Extraction: Extraction of rbc ghosts with 0.5% Triton-X 100 in low ionic strength buffer (μ = 0.03) removes all integral membrane proteins and glycerolipids and leaves a spectrin-based cytoskeleton shell and associated glycolipids (17). Initial tests using these cytoskeletons as a challenge for amoeba attack have been inconclusive. Although there was evidence by fluorescence microscopy of stimulation of polymerization of trophozoite actin and phagocytosis at some contact sites, this activity was not as extensive as when less radically depleted preparations were used. A significant increase of the amount of amoeba actin was detected in only one of three experiments conducted. One complication in these experiments is that shells prepared in this manner aggregate when they are washed by centrifugation. Thus few discrete particle remnants of the ghosts were available for attack. This may have affected the behavior of the amoebae when challenged by these preparations. Means to overcome the aggregation problem are in hand and are described in the Experimental section.

When rbc membranes were extracted with a lower Triton-X concentration at higher ionic strength (0.1% Triton in phosphate buffered saline), they appeared visibly membrane depleted - they were invisible with Nomarski optics and faint rings under phase - but retained their shape through washings. Although these cytoskeletons were not visible attached to trophzoites following challenge, it was clear upon rhodamine-phalloidin staining of actin that they had adhered in abundance, triggered the actin polymerization response and were phagocytized vigorously (photographs on the following page). The Triton extracted cells appeared as stimulatory as the ghosts, but the activity of these two preparations has not yet been directly compared.

These experiments must be repeated, accompanied by tests (e.g., electrophoresis of products) to determine the extent to which integral membrane proteins are removed by the intermediate Triton extractions. Our tentative conclusion is that extraction with high concentrations of Triton probably removes a membrane

component involved in binding and triggering attack by amoebae. However, the possibility of direct or indirect interaction of trophozoites with elements of the rbc cytoskeleton has not been eliminated.

Interpretation of Preliminary Experiments.

Principally these experiments demonstrate the feasibility of the proposed approach. They also provide initial information concerning trophozoite receptor specificity and suggest directions for further experiments.

Amoeba surface lectin(s) possibly with specificities for N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) (10), both of which are found in the surface glycoproteins of the A+ rbc used, have been proposed to be involved in adhesion of target cells. Thus, we anticipated that binding, stimulation of actin polymerization and subsequent phagocytosis of the rbc might be diminished by the pronase and Triton treatments. Pronase treatment enhanced the response, and after mild Triton extraction, which we would expect to have removed significant amounts of intergal glycoprotein, the response appeared qualitatively as vigorous as with untreated membranes.

Thus, it appears that exposed (glyco)peptides are not essential as ligands for trophozoite binding and attack activation, and that their removal may better expose the binding site. The glycoproteins are high in terminal N-acetylneuraminic acid (NeuNAc) residues as well as containing a variety of neutral sugars. Neither pronase nor Triton treatment removes membrane glycosphingolipids which contain most of the same oligosaccharides found on the glycoproteins (but represent a smaller percentage of the total surface carbohydrates of rbc (18)). Thus, amoeba attachment may still occur via lectin - sugar interaction, but to glycolipid rather than to glycoprotein residues.

It is not the case that E. histolytica possesses no binding specificity and is endocytotically stimulated by any particle. Our data with pronase and with latex beads (5) demonstrate the ability of trophozoites to discriminate between surface structures. Perhaps trophozoites possess a broad binding specificity, and can bind and be stimulated by a variety of biomolecular structures. If this is so, it is worth knowing and can be established by the experiments proposed. Another intriguing possibility is that amoeba receptors interact directly or through associated molecules (glycolipids ?) with the rbc cytoskeleton upon contact. This notion is particularly attractive since it has been discovered that virtually all non-erythroid cells possess cytoskeleton proteins exhibiting considerable antigenic overlap with rbc cytoskeleton molecules (19). Thus, it is possible that E. histolytica, which responds to virtually any mammalian cell type, could interact with the same molecule(s) in all cells. Interaction with the target cytoskeleton would also explain the ability of trophozoites to deform target cell shape so dramatically upon binding (5).

3. Experimental Design.

The specific objectives of this study, the identification of target cell molecule(s) which bind to E. histolytica and trigger the attack process, are planned to be accomplished in approximately one year. In this period I propose to:

- confirm preliminary observations indicating that E. Histolytica trophozoites

bind and exhibit a greater cytoskeleton and endocytotic response to human rbc stripped of surface glycopeptides than to untreated rbc.

- complete experiments to quantitate the ability of rbc cytoskeleton shells extracted maximally with Triton-X 100 to stimulate actin polymerization by trophozoites.

- based on the results of the above experiments, determine by selective removal of rbc membrane constituents with enzymes or by extraction with Triton-X 100 (monitored by analysis of the products) what surface molecules are required (or not required) for binding and stimulation of the cytoskeleton and endocytotic response by trophozoites.

- test specific molecules or molecular fractions from rbc (suggested by the results of the above experiments) for their ability to inhibit the cytoskeleton and endocytotic response of trophozoites to whole rbc or rbc ghosts.

- covalently couple specific molecules from rbc membranes to inert particles, or in the case of water insoluble molecules (e.g., glycolipids) prepare liposomes, and assess the ability of these to stimulate actin polymerization and phagocytosis by trophozoites or to inhibit the attack upon whole rbc or ghosts.

- extend these observations to nucleated cells related to the cell types attacked by E. histolytica in vivo in order to identify analogous ligands on these cells.

3a. Experimental Rationale.

Human rbc have been chosen as the target cells for this study because of the extensive knowledge of erythrocyte membrane composition and the abundance of established methods for isolation, analysis and characterization of rbc molecules. It is our assumption that E. histolytica trophozoites bind to and are stimulated to attack other mammalian cells by interaction with the same or similar molecular structures as will be found for rbc. Thus, the results obtained with erythrocytes will be applicable to a search for surface ligands of cells more closely related to those attacked in vivo by the parasite.

The strategy of the search for specific molecules or molecular fractions responsible for binding and stimulation of attack will be as follows: First, structurally altered rbc or rbc ghosts which remain insoluble following treatment will be used to challenge trophozoites, and their ability to stimulate a response by the amoeba cytoskeleton will be assessed. If they stimulate a response, the stimulatory ligands must have remained associated with the cellular remnants through the treatment. When the ability of the modified cells or membranes to stimulate attack is lost by treatment, the crucial ligands must have been removed or destroyed by the treatment. In this event the extracts (hydrolysates), after removal of detergent or hydrolytic enzymes, will be evaluated for their ability to inhibit the trophozoite response to untreated rbc or ghosts. In addition, identified soluble components may be assessed for their ability to stimulte attack by amoebae after coupling to particles (or reconstitution with carrier lipids, if a nonpolar ligand is indicated). The last approach is proposed because stimulation of an attack response is more direct evidence that a molecular fraction serves as an attack stimulating ligand than is the ability to inhibit

a response. We propose that coupling to an insoluble support will be necessary to generate stimulatory activity by soluble ligands. This is based on our assumption that extensive polymerization of amoeba actin at the site of target cell contact requires polyvalent interaction of ligands with receptors, and therefore, will probably not occur when amoebae are challenged with a ligand in solution. We feel it more likely that a response can occur if the ligands are first immobilized by attachment to a particle and the particle presented as a target possessing multiple binding sites.

The initial experiments will be conducted using cytoskeleton shells prepared by extraction with high concentrations of Triton-X 100 at low ionic strength, since these possess the minimum molecular structure. If these cytoskeletons trigger actin polymerization by trophozoites, we can presume that they possess molecular components which serve as ligands for amoeba attack receptors. If the amoebae do not respond, this will imply that essential ligands were lost or altered in the extraction. In this case, we will assess the inhibitory activity of the extracts and return to selective enzymatic degradation of surface components to identify the essential ligands.

We recognize the possibility that interaction between E. histolytica and target cells may require a specific supramolecular organization on the target cell surface. If this is the case it may not be possible to identify a single stimulatory ligand. However, it should at least be possible to delineate a minimal membrane organization and classes of molecules required for activity. This information will be valuable in itself and will aid the design of further experiments.

Determination of the efficacy of treatments and analysis of preparations will be accomplished by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) for proteins, including glycoproteins, and high performance thin-layer chromatography (HPTLC) for lipoid substances. Specific structural analysis of carbohydrate structures, if these are implicated as ligands, is not planned for this stage of the study. Some structural information will be implied from the specificity of the treatment and knowledge of the nature of the glycoprotein or glycolipid fractions acted upon.

3b. Methods.

Assays of attachment, amoeba actin polymerization and phagocytosis will be conducted as described in the accompanying manuscript (5, Appendix A).

Triton-X 100 extraction: RBC ghost will be prepared and these will be extracted with 0.5 % Triton at low ionic strength which produces cytoskeleton shells containing essentially only spectrin, actin, syndein, and band 4.1 with associated glycolipids (17). Triton prepared shells retain a roughly circular shape until centrifuged, whereupon they aggregate (17). We would prefer to work with individual cell skeletons. Therefore, the shells will be lightly fixed with formaldehyde or glutaraldehyde before isolation by centrifugation in order to preserve them as discrete spheres. We have determined using partial cytoskeletons prepared with 0.1% Triton that fixation does not destroy the functional domains of the target ligand, since the fixed cells stimulated a distinct actin polymerization response by trophozoites.

Enzymatic Treatments: Chymotrypsin, trypsin and pronase, in that order, release increasingly more of rbc surface peptides (20). Since we found that the most drastic treatment not only did not diminish, but enhanced rbc effectiveness in stimulating attack by trophozoites, little may be learned from experiments involving more limited hydrolysis of surface molecules with the other proteases. Thus, enzymatic alterations will focus on determining the role of oligosaccahrides in attachment and stimulation of attack.

Neuraminidase, B-galactosidase, N-acetylglucosaminidase and endo-B-galactosidase and possibly other glycosidases, separately or sequentially, will be used to selectively remove portions of the oligosaccharide chains (21) of surface glycolipids from whole rbc after these have been stripped of pronase sensitive glycopeptides. Ghosts will be prepared from the degraded cells and used to challenge amoebae. We will look for loss in the ability of the sequentially degraded cells to attach, stimulate actin polymerization and be phagocytized by trophozoites. The known specificities of these enzymes and the known structures of the major surface oligosaccharides they act upon (21) will allow us to infer which surface carbohydrates may be involved in binding and stimulating amoeba attack. In combination, these enzymes are capable of removing most of the carbohydrate coat from the surface of erythrocytes(21).

Analysis of Extracts and Hydrolysates: Triton-X 100 extracts and protease and glycosidase hydrolysates will be retained, particularly those generated in treatments which cause loss of stimulatory activity by the cellular structure treated. Triton extracts will be dialyzed or passed over molecular exclusion gels to remove detergent. After concentration the biomolecular fractions will be tested for their ability to inhibit interaction between trophozoites and whole rbc or ghosts. Enzyme hydrolysates will be dialyzed to separate the products of hydrolysis from enzymes; after concentration and reconstitution, the dialysates will be tested for their ability to inhibit attack by trophozoites. Reconstitution with carrier lipids (22) may be required for suspension of nonpolar substances. Published procedures will be used to isolate any specific molecules or molecular fractions (e.g., neutral glycolipids) which may be indicated as ligands (23) and these will be tested similarly for inhibitory effects.

Coupling of Molecules to Particles: Established methods (24) will be used to couple proteins to commercially available activated Sepharose, polystyrene or polyacrylamide beads of appropriate size. Lipids dissolved in nonpolar solvents also will be mixed with hydrophobic bead suspensions and dried to coat the beads with hydrophobic or amphipathic test ligands. The coated beads will be dispersed by sonication after suspension in aqueous media for testing.

Analytical procedures: Proteins including glycoproteins will be analyzed by SDS PAGE (25) and (glyco)lipids by HPTLC (23). Carbohydrates will be detected with the periodic acid Schiff stain. Other anticipated procedures are standard.

3c. Resources and Environment.

Space.

The principal investigator presently occupies approximately 800 sq ft of individual laboratory space. In addition he has ready access to a variety of shared spaces and facilities including fine instrument lab, heavy equipment room, warm room, cold room, biolgical kitchen, sterilization facilities, isotope handling and counting areas, dark room, animal facilities, shop and storage space.

Equipment.

The following major instruments and equipment are available for ready use either in the PI's own laboratory or in shared facilities: Compound and inverted research microscopes with phase, differential interference contrast (Nomarski) and fluorescence optics and cine, 35 mm, polaroid, video recording capabilities; heat and filter sterilization equipment, -85 C freezers, incubators, CO2 incubators, shakers, baths, biohazard hoods, fume hoods, electronic cell counter/sizer, refrigerated high speed and preparative ultracentrifuges with numerous heads, UV/VIS recording spectrophotometers, variable wavelength recording fluorescence spectrophotometer, flame photometer, one and two-dimensional slab and tube electrophoresis, electrofocusing and transblotting equipment; liquid chromatography (LC), high performance liquid chromatography (HPLC) and fast peptide liquid chromatography (FPLC) with UV/VIS detectors and fraction collectors; liquid scintillation beta and gamma spectrometers, osmometer, sonicator, scanning and transmission electron microscopy services, fluorescence flow cytometry services, histological services, computer facilities, including word processing, statistics and graphics, PROPHET system access; and a variety of routine equipment.

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D. Qualifications of the Principal Investigator.

Dr. Gordon B. Bailey is currently a Professor of Biochemistry at the Morehouse School of Medicine. Previously he served for ten years in the development of medical education and medical research programs in Thailand. He has first hand knowledge of the health problems faced by the peoples of developing countries and the devastating effects of tropical parasitic diseases. It was while serving in Thailand that Dr. Bailey initiated his research on Amebiasis. Although develping country participation in the research proposed here is not feasible or essential, Dr. Bailey is anxious to reinitiate collaborative research with investigators in these countries. It is hoped that the studies proposed will lead to such an opportunity.

Dr. Bailey is experienced in the conduct of all of the major techniques proposed in this project. The only procedures he has not personally undertaken are the immobilization of proteins on particles and the reconstitution of lipids in liposomes. However, it is not anticipated that these techniques will present major problems. All equipment and instruments for the proposed experiments are available (see Resources and Environment), except as requested in the Budget section .

Biographical Sketch.

Gordon B. Bailey, Ph.D.
Professor of Biochemistry

Education.

Ph.D. (Biochemistry) 1966. Univ. of Florida school of Medicine.
MA (Botany/Chemistry) 1961. University of Massachusetts.
BA (Botany) 1956. Brown University.

Appointments.

Professor, Department of Biochemistry, Morehouse School of Medicine, Atlanta, Georgia. 1976 - present.

Consultant, The World Health Organization. 1975 - 1976.

Deputy Director (1975 - 1976) and Visiting Professor (1973 - 1976), Anemia and Malnutrition Research Center, and Visiting Professor (1973 - 1976), Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Assistant Professor, Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri, 1973 - 1976.

Field Staff, The Rockefeller Foundation and Visiting Professor, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand. 1966 - 1973.

Publications on Entamoeba.

Bailey, G. B. Locomotion and chemotaxis by E. histolytica. in Amebiasis: Human infection by Entamoeba histolytica, Ravdin, J. I. ed., Wiley and Sons, Inc. New York. in preparation.

Bailey, G. B., D. B. Day and J. W. Gasque. Rapid polymerization of Entamoeba histolytica actin induced by interaction with target cells. Submitted to J. Exp Med. (manuscript enclosed, Appendix A).

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MOREHOUSE
SCHOOL OF MEDICINE

Department of Biochemistry

17 April 1985

Mr. Floyd O'Quinn
S & T/RUR
Agency for International Development
Building 309 SA-18
Washington, DC 20523

Dear Mr. O'Quinn:

Pursuant to my telephone conversation with Dr. J. S. Prince on 15 April 1985 and in accordance with his sugestion I have prepared a revised budget for my recently submitted proposal to A.I.D. titled "Identification of Mammalian Target Cell Molecules Involved in Attachment and Stimulation of Attack by Entamoeba histolytica". This revised budget and a budget justification are enclosed.

The original budget was the minimum required to accomplish the proposed work. The revised budget is an optimal budget which would provide more adequately the needs for the project and greatly speed the work. It represents the suggestions of Dr. Prince in the addition of salary for the PI and travel support for the Research Assistant. However, I have also added several items of Equipment and increased the amount for Consumables on the Supplies line. Acquisition of these material items would greatly facilitate the investigation and shorten the time required to achieve the Research Objectives. Attached to the revised budget is a detailed justification for all items and a breakdown of the travel costs requested.

I would be happy to answer any further questions you may have. Thank you for consideration of my proposal.

Sincerely yours,

Gordon B. Bailey

Gordon B. Bailey, Ph.D.
Professor
(404) 752-1691/1503

Enclosures.

cc: Dr. W. W. Sullivan

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APR 23 1985
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E. Budget Information and Estimates.

1. Estimated total Funding Requested.

(a) Direct costs: $58334.
(b) Indirect costs: $29662.

TOTAL: $87996.

2. Contributions or Cost-sharing from other sources. (None)

3. Detailed Budget.

Salaries:

	Effort	Salary	Fringe Benefit	Total
Gordon B. Bailey, PhD, PI	20%	12190.	2438.	14628.
Diane B. Day, BSc, Res. Asst.	40 hr/wk	19000.	3610.	22610.
		31190.	6048.	37238.

Student Lab Helper (10hr/wk @ $4.5 x 48 wks) 2160.

Travel:
PI and Research Asst. to Xth Intl Seminar on Amebiasis $804. X 2 (See budget justification for breakdown) 1608.

Equipment:		
Thin Layer Chromatography Equipment	850.	
Electrophoresis Cell	1150.	
Cooling Water Bath	1350.	
Refrigerated Benchtop Centrifuge	1840.	
Electronic Automatic Pipettor	960.	
Microcomputer/Printer/Modem	6628.	12778.

Supplies:		
Culture Media	150.	
BioReagents	1000.	
Electrophoresis/Chromatographic Reagents	300.	
Photographic Supplies	100.	
Consumables	2000.	3550.

Other:
Equipment maintenance and publication costs. 1000.

Indirect Costs:
95.1% of salaries. 29662.

TOTAL: $ 87996.

E. 1. Budget Justification.

Salaries: Fringe benefit rate, 20%; Hiring a student lab helper would relieve the Research Assistant and PI from time consuming, trivial routine lab tasks, principally washing lab glassware. Hourly rate conforms to institutional policy. Indirect costs and Fringe Benefits are not applicable for the lab helper.

Travel: Travel support is requested for the PI and Research Assistant to attend the Xth International Seminar on Amebiasis scheduled in Mexico city from 22 through 25 September 1985. This is the most comprehensive forum in the world for communication of basic and clinical studies of E. histolytica. PI's laboratory will present progress on the studies proposed herein at this meeting. Cost breakdown is as follows: RT economy airfare, $369.; Per diem, Mexico City, 5 days at $69./day ($345.); Transfers, taxis, tips, etc., $90. total.

Equipment:

Thin Layer Chromatography equipment. This is essential for analysis of glycolipid preparations. Costs include tanks, prepared plates and reagents.

Electrophoresis Cell. PI presently has one electrophoresis cell, but has power sources for running two units simultaneously. Purchase of a second unit would allow running two units simultaneously thus halving the time required for electrophoretic analyses. Costs are for Bio Rad Protean II or equivalent.

Cooling Water Bath. Both the assay of amoeba interactions with rbc and electrophoresis runs are conducted at different temperatures below ambient. PI has one cooling water bath in his lab. Acquisition of a second bath would make it possible to conduct assays and run electrophoresis at the same time, a major time-saver. Cost is for Lauda RC 20B or equivalent.

Refrigerated Bench Top Centrifuge. At present chilled centrifugations of rbc, a frequent procedure, must be conducted in a shared lab on a different floor of the building. Acquisition of this relatively inexpensive piece of equipment would greatly speed cell preparations. Cost is for IEC clinicool or equivalent.

Electronic Automated Pipettor. Fixed volume micropipettes are available for this work. However, this variable automated unit would shorten aliquoting time and speed up several assay procedures to be conducted frequently in this study. Cost is for Rainin EDP and accessories.

Microcomputer. The PI presently owns a limited cabability personnel computer purchased privately and uses this extensively for manuscript and proposal preparation (word processing), data storage and retrieval and some statistical analyses. His efficiency and productivity would be enormously increased if a computer and certain peripherals were available at the laboratory. Analysis of the quantitative data and preparation of the graphs appearing in the manuscript accompanying this proposal were accomplished on the PC of the type requested belonging to a colleague. Unfortunately, this unit is rarely available for the PI's use. With the modem it would be possible to communicate with and utilize the data analysis and graphics capabilities of the PROPHET SYSTEM terminal installed in the Atlanta University Center and also to communicate with an existing PC in an adjacent lab to utilize its sophisticated plotter. PI also

requires a more advanced PC which has a more comprehensive data management capability for maintaining and searching literature files. Office space in the lab is availble to house this unit. Costs are for IBM PC-XT or equivalent with some additional boards, monitor, letter quality printer and modem.

Supplies: Bioreagents include the various enzymes, fluorescent tags and other biologicals required. In the revised budget the Consumables cost request is doubled. These include many items of plasticware which have been washed and reused in the past for the sake of economy. Increasing this budget item will make it possible to discard most disposable items after use, saving time and reducing the risk of contamination in future experiments.

Other: Maintenance costs include microscope cleaning, biohazard hood inspection and filter replacement, water purification filter changes. At present other major equipment maintenance costs are borne by the institution.

Indirect Costs: This is the current rate approved for Morehouse School of Medicine.