IDENTIFICATION AND CHARACTERISATION OF A LIPOPHOSPHOGLYCAN LIKE MOLECULE FROM A PATHOGENIC STRAIN OF

Entamoeba histolytica-RAHMAN

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microbes - life that modulates life

SCHOOL OF ENVIRONMENTAL SCIENCES

CERTIFICATE

The research work embodied in this dissertation titled "Identification and characterisation of a lipophosphoglycan like molecule form a pathogenic strain of *Entamoeba histolytica*-Rahman" has been caried out in the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

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LIST OF ABBREVIATIONS

APS	Ammonium Per Sulphate
CPM	counts per minutes
°C	Degree Celsius
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
GPI	Glycosyl-phosphatidylionsitol
Hepes	(N-[2-Hydroxyethyl]piperazine-N'-(2-ethane Sulphonic acid])
kb	kilo base
kDa	kilo Dalton
LPG	lipophosphoglycan
McAb	Monoclonal Antibody
ND	Not Determined
PBS	Phosphate Buffered Saline
PI-PLC	Phosphatidylinositol-specific Phospholipase C
PPO	2,5 Diphenyloxazole
SDS	Sodium dodecyl Sulphate
TBS	Tris Buffered Saline
TEMED	N,N,N',N',Tetramethyl ethylene diamine
Tris	Tris-hydroxymethyl amino methane

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INTRODUCTION

1.1 General features of *Entamoeba histolytica*

Entamoeba histolytica, a protozoan parasite which causes amoebiasis in humans is classified as -

Family	-	Entamoebidae
order	-	Amoebida
class	-	Lobosea
Subphylum	-	Sarcodina
Phylum	-	Sarcomastigophora

Though ubiquitous in distribution, this parasite is more prevalent in tropical and subtropical regions of the world.. Over 10% of the world population is infected with *E. histolytica*, of which about 10-15% suffer from symptomatic amoebiasis (Walsh 1986). The majority of *E. histolytica* infections are asymptomatic, in which the parasite lives as a commensal and does little harm to the host. However, in about 10% of the infected individuals, the parasite invades mainly the intestinal epithelial cells causing ulceration which may lead to severe amoebic dysentry. The mode of infection is predominantly through ingestion of *E. histolytica* cysts from human faeces contaminating food and water, or by direct fecal-oral contact. Occasionally the amoeba spreads through blood vessels to other organs like liver, inducing an abscess which could be fatal. In rare cases, the parasite can even spread to the lungs and the brain. Extraintestinal infection is the major cause of death in amoebiasis. The disease is widely spread in developing countries due to inadequate sanitary conditions.

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1.1.1 Life cycle of *E. histolytica*

E. histolytica exists in two forms, (a) - the vegetative trophozoite and (b) - the infective cyst. Cysts are a relatively resistant form which are shed continually in the stool of an infected individual. These are quadrinucleated and can survive outside the host for weeks to months in moist environments at temperatures not exceeding 55°C.

1.1.2 Morphology of *E. histolytica*

As observed by light microscopy the trophozoites are uninucleate and range in size from 10 - 60 μm. They show finger like pseudopodia (cytoplasmic extrusions) which are involved in motility. The cytoplasm is finely granular. Granulation is much more pronounced in axenic cells. Cysts are spherical in shape and range in size from 10 - 20 μm (usually 12 - 15 μm). Immature cysts (seen occasionally) contain 1 or 2 nuclei, whereas the mature cyst has four nuclei. The cytoplasm has chromatoid bodies (elongated bars with bluntly rounded ends) and contains glycogen, usually in diffuse form.

Ultrastructure of the *E. histolytica* cell as revealed by electron microscopy shows the following features:

Surface structure: As the trophozoites are deformed easily by environmental stress it is difficult to determine in fixed preparations their true dimensions and shape. Living trophozoites remain motile at 37°C in an isoosmotic

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environment but cease to be motile and round up as the temperature is lowered below 37°C. The *E. histolytica* cell surface (axenic culture), as studied by Scanning Electron Microscopy, is wrinkled. Transient structural specialisations can be grouped as evaginations and invaginations.

Evaginations: Hyaline pseudopodia (lobopodia) and cytoplasmic protrusions appear as smooth surfaces. Besides these, filopodia (needle like) and Uroid are also seen. The Uroid is a posterior, shelf-like region of the cytoplasm of actively motile trophozoites from where a large number of small and large filopodia arise.

Invaginations: Micropinocytic vesicles (0.1 μm dia.) appear as minute surface openings seen together with large and small endocytic vesicles. Large endocytic vesicles (phagocytic, 10 μm dia.) occur more commonly in monoxenic amoebae whereas in axenic cultures the smaller openings are more common. Filopodia are virtually absent in axenic cultures. Different strains show differences in the structural features (Gonzalez-Robles and Martinez-Palomo 1983).

Surface coat: A fuzzy glycocalyx coat is present on the *Entamoeba* surface (Siddiqui and Rudzinska 1965). The glycocalyx is least conspicuous in axenically grown trophozoites and much thicker on monoxenic or human host derived amoebae (Lushbaugh and Miller 1988). The glycocalyx is composed of acidic mucopolysaccharides, glycoproteins, or

glycolipids with a high negative charge. The glycocalyx does not appear to contain sialic acid, as in many mammalian cells. It does contain antigens that react with antiamoebal antibodies (Lushbaugh and Miller 1988). A glycocalyx layer has also been found in other protozoa like *Leishmania*.

1.1.3 Metabolic pathways

Although a comprehensive study is lacking, yet certain unusual pathways in *E. histolytica*, have been discovered (Weinbach 1988). Carbohydrates are the main energy source as with many other protozoan parasites. However, in *E. histolytica* only glucose and galactose are used. As yet there is no evidence for lipids as an energy source. There is practically no data on protein and nucleic acid metabolism. Though microaerophilic, it does utilize oxygen. The end products of glycolysis are ethanol and acetate. The ratio of these products are different under anaerobic (3:1) and aerobic (1:3) conditions. Other significant observations are listed below :

- the parasite lacks lactate dehydrogenase. Pyruvate is anaerobically converted to ethanol and carbon dioxide and aerobically to acetate either by pyruvate oxidase using inorganic orthophosphate (P_i) or by pyruvate oxidase (CoA-acetylating), a novel enzyme.

- Pyrophosphate (PP_i) replaces ATP in several glycolytic reactions and its intracellular concentrations are unusually high.

- although it contains abundant glycogen, glycogen synthetase is absent.

- a functional tricarboxylic acid cycle is missing.

- heme proteins involved in electron transport chain\s are absent.

- inhibitors and uncouplers of oxidative phosphorylation, e.g. antimycin, cyanide or azide, do not affect the respiration of *E. histolytica*.

- a Ca^{2+}-dependent ATPase associated with the surface membrane of *E. histolytica* has been demonstrated which requires micro molar amounts of Ca^{++} (McLaughlin and Muller 1981).

- a nicotinamide nucleotide trans hydrogenase catalyses hydrogen exchange in the reaction: $NADH + NADP^+ ->$ $NAD^+ + NADPH$.

- glutathione(GSH) is present in unusually low amount. The enzymes that utilize GSH are absent (Fahey et al. 1984).

- A superoxide dismutase removes O_2^-, if formed during aerobic metabolism and converts it to H_2O_2. In the absence of catalase it is not known how H_2O_2 would be detoxified.

1.1.4 Molecular biology

Work on the molecular biology of *E. histolytica* was initiated only 7-9 years ago. Basic information regarding the number of chromosomes, ploidy level and the organization

of chromosomal DNA are not available for this organism. Recent studies on chromosome structure of *E. histolytica* by Torres-Guerrero et al. (1991) demonstrated nucleosome-like particles with an average diameter of 10 nm. Apart from chromosomal DNA, there is a large amount of an extrachromosomal circular DNA (EhR1) present in the nucleus of *E. histolytica*. This episome is 25 kb in size, encodes two rRNA transcription units, (Bhattacharya et al. 1989; Huber et al. 1989), and is present in 200 copies per genome of *E. histolytica* HM-1:IMSS. So far there is no report describing presence of a copy of this plasmid in the chromosome. Some of the other major genes that have been analyzed are the 170 kDa surface lectin (Tannich et al. 1991c;Mann et al. 1991), 27 kDa cysteine protienase (Tannich et al. 1991b), actin (Edman et al. 1987; Huber et al. 1987), ferredoxin (Huber et al. 1988), superoxide dismutase (Tannich et al. 1991d) and a calcium-binding protein (Prasad et al. 1992). Some of them like lectin, cysteine proteinase and actin are present in multiple copies. So far a number of polymorphic loci have been found in *E. histolytica* (Huber et al.1989; Bhattacharaya et al.1992; Sehgal et al.1993) and all these regions come from EhR1. EhR1 also encodes a number of repeated sequences. Some of these repeated DNA sequences have potential to be used as diagnostic probes for *E. histolytica* (Garfinkel et al. 1989; Mittal et. al.in press). The mechanisms controlling gene expression in *E. histolytica*

are not yet known.

1.1.5 Pathogenicity of *E. histolytica*

There are no reliable reports regarding the actual role of host factors in pathogenicity except the requirement of resident bacterial flora in causing amebic lesions (Anaya-Velazquez and Padilla-Vaca 1992). A number of factors that govern host-parasite relationships include host factors like nutrition, gut environment and genetic make-up; and parasite factors like variation in parasitic strains and levels of lectins, proteases, granules etc.

Brumpt (1925) proposed the presence of two morphologically indistinguishable species, the nonpathogenic "*E. dispar*" and the pathogenic "*E. dysenteriae*". Support for this view came from isoenzyme analysis of several enzymes which showed that pathogenic strains (P) of amoeba have distinct isoenzyme patterns as compared to nonpathogenic (NP) strains (Sargeaunt et al. 1978). Different isolates were grouped according to the electrophoretic mobility of the enzymes (banding pattern in a starch gel) and each group was described as a zymodeme (Zym=enzyme, deme=population). The P and NP strains are also antigenically different as shown by reactivity towards monoclonal antibodies (Strachan et al. 1988). More recently P and NP strains have been reported to display genetic differences as shown by RFLP (Tannich et al. 1989; Tannich and Barchard 1991a) recombinant DNA probes (Garfinkel et al.

1989) and riboprinting (Clark and Diamond 1991). The level of genetic difference among these strains suggests that P and NP. amoeba could be classified as two distinct species as originally suggested by Brumpt.

1.1.5.1 Molecular mechanisms of pathogenesis: Molecular mechanisms regulating pathogenesis in amoeba are not very clear. However, it is thought that *E. histolytica* needs to make contact with target cells before its cytolytic activity can be expressed. Invasion of both intestinal and extraintestinal tissues would require the help of a number of hydrolytic enzymes. Several molecules and enzymes have been identified that mediate contact with target cells and enzymes that are secreted by the cells. Some of these molecules are described below :

<u>Pore forming proteins</u>: Amoebapore, a protein from *E. histolytica* that can form pores in artificial membranes, was identified some years ago (Lynch et al. 1982; Young et al. 1982). This protein could also depolarize the membrane of human lymphocytes and macrophages (Young et al. 1982; Rosenberg et al. 1989). It is likely that this protein may be responsible for potent cytolytic activity of pathogenic *E. histolytica*.

<u>Adherence receptors</u>: There are a number of molecules which have been reported to be involved in the recognition of target cells. The galactose\N-acetyl galactosamine-specific lectin has been studied most extensively. Monoclonal

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antibodies to the 170 kDa galactose-specific lectin prevent adherence, contact dependent cytolysis and complement-mediated lysis of amoeba. It may also be involved in attachment of amoeba to colonic mucosal layer (Petri et al. 1987; Ravdin et al. 1985).

Proteolytic Enzymes: The cysteine protienases and the collagenases are the major proteolytic enzymes identified in *E. histolytica* (De Meester et al. 1991 ; Reed et al. 1989). These are believed to be involved in the degradation of matrix proteins such as collagens, laminin and fibronectin thus helping the amoeba to invade tissues. The pathogenic isolates secrete larger amounts of the cysteine proteinases compared to their nonpathogenic counterparts (Tannich et al. 1991c; Keene et al. 1990). Munoz et al. (1982) showed a direct correlation between collagenase activity and the degree of virulence of different strains.

1.1.6 Immune response to *E. histolytica*

Very little is known about the immune response elicited by *E. histolytica* in commensal state. This is mainly due to absence of a true animal model as none are true hosts of *E. histolytica*. Old world monkeys are regularly infected by *E. histolytica* and it has adjusted to its system (Miller and Bray 1966). Asymptomatic infection could stimulate low level antibody response, but peripheral blood lymphocytes fail to proliferate when challenged with *E. histolytica* (Trissl 1982). This response is mainly against the secreted

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products of the amoebae.

Here I describe only what is known about the immune response in invasive amoebiasis.

1.1.6.1 Humoral immune response: High antibody levels are observed in invasive cases which increase with the severity of the disease, but are ineffectual in containing the disease (Pittman and Pittman 1973). The circulating antibody specific for *E. histolytica* can be detected for a long time, may be years, after treatment (Reed et al. 1983). The human serum kills the trophozoite by complement activation, both by classical and alternate pathways. Pathogenic strains from patients are resistant to complement while nonpathogenic ones are susceptible. Resistance to complement may be a prerequsite for invasive disease (Reed et al. 1983).

Numerous antigens, recognized by human immune serum, have been identified. The most prominent among these are, a 110 kDa surface adhesin, a 125 kDa surface antigen, a 28 kDa secreted protien, a 170 kDa surface lectin (Petri et al. 1987), a 96 kDa integral membrane protien (Harris et al. 1978) and lipophosphoglycan (LPG) (Bhattacharaya et al. 1992). *E. histolytica* plasma membrane undergoes high turnover. As a result the cells can repeatedly aggregate, ingest and endocytose antibody bound to their surface without any loss of viability (Aust-Kettis et al. 1981). This may explain the lack of protection due to humoral immune response.

1.1.6.2 Cell mediated immune response: All depressors of cell mediated immunity increase the severity of the invasive disease (Salata and Ravdin 1986) which implies a role for cellular immune response in the control of disease. Cytotoxic T cells and activated macrophages can kill *E. histolytica* (DeLeon 1970). Reinfection of patients resulting in amoebic liver abscess (ALA) is very rare (Salata et al. 1986) which suggests development of some sort of immunity towards *E. histolytica*. Treated ALA patients have lymphocytes which show a specific proliferative response to amoebic antigen preparations (Salata and Ravdin 1985).

A peculiar feature of amoebic invasive disease is the absence of scar tissue after recovery which implies lack of macrophages at the site. Amoebae seem to release a factor that inhibits locomotion of monocytes. The macrophages found around the liver abscess can not kill amoebae *in vitro* (Trissl 1982). Amoebic protiens are chemoattractants for neutrophils. Virulent amoebae kill PMNs, monocytes, macrophages and T cells with no loss in viability.

1.1.7 Cell surface molecules of *E. histolytica*

The amoebic plasma membrane has been shown to contain about 12-18 glycoprotiens (Aley et al. 1980). A number of these cell-surface associated plasma membrane protiens have recently been characterized mainly using molecular biological techniques. Prominent among these are :

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Lectins, a N-acetyl glucosamine inhibitable lectin (Kobiler and Mirelman 1980), a N-acetyl galactosamine inhibitable lectin (Ravdin et al. 1985) and a 220 kDa poly N-acetylglucosamine inhibitable lectin (Rosales-Encina et al. 1987). In all of these cases adhesion was completely inhibited by the respective sugars. A 110 kDa adhesin molecule has been described on amoebic cell surface. Adherence, phagocytosis and cytopathic killing were inhibited by monoclonal antibodies specific to this molecule. A 96 kDa integral membrane protien, present in high amounts in pathogenic strains and low levels or absent in non pathogenic strains has also been observed (Harris et al. 1978).

Amoebae have phospholipase A and C, lyso phospholipase and sphingomyelinase. Phospholipase A is of two types, a membrane bound Ca^{++} dependent and a Ca^{++} independent (Van der Bosch 1980). Collagenolytic activity of E. histolytica has been linked to its virulence (Gadasi and Kessler 1983). A group of protienases, differentiated on the basis of their size have been identified (Orstoa-Saloma et al. 1989). A low molecular weight proteinase is present on plasma membrane and degrades collagen types 1 and 2.

1.2 Lipophosphoglycan (LPG)

Protozoan parasites have a remarkable ability to avoid destruction in the hostile environments they encounter, including the blood stream. This is mainly due to the

protection conferred by specialized molecules on the cell surface. The structure-function relationship of these molecules are begining to be understood. One such molecule is a novel glycoconjugate - lipophosphoglycan(LPG).

Existence of LPG is well documented in *Leishmania*. It was first reported in *L. donovani* by Turco et al. in 1984 and subsequently in a number of other species of *Leishmania* (Turco and Descoteaux 1992). LPG has also been reported in *Trichomonads* (Singh 1993) and *E. histolytica* (Stanley et al. 1992; Bhattacharaya et al. 1992).

1.2.1 Structure of LPG

The structure of LPG as understood today is mainly based on the study of LPG from *Leishmania* promastigotes. LPG is the major glycoconjugate localized all over the surface including the flagellum (King et al. 1987), and each cell contains several million molecules of LPG (McConville and Blackwell 1991). LPG consists of four domains:

a. The Lipid anchor: The polysaccharide portion of LPG is anchored to an unusual phospholipid derivative 1-O-alkyl-2-phosphatidyl(myo)inositol (Orlandi and Turco 1987). LPG can be hydrolyzed by bacterial Phosphatidylinositol-specific Phospholipase C (PI-PLC), producing 1-O-alkyl glycerol and the entire polysaccharide chain as products.

b. The phosphosaccharide core: To the inositol of the lipid core is attached the phosphosaccharide core region. The

glycan core consists of an unacetylated glucosamine, two mannoses, a galactose-6-phosphate, a galactopyranose and a galactofuranose. The presence of galactofuranose is extremly unusual in eukaryotic glycoconjugate as the furanose is internal to the chain. Nitrous acid treatment cleaves the hydrophobic tail (lipid anchor) by attacking glucosamine-inositol linkage producing the entire phosphopolysaccharide chain as product (Zamze et al. 1988).

c. The repeating units: The salient feature of LPG is the repeating phosphorylated saccharide region. All LPG molecules reported so far contain multiple units of a backbone structure of $Po4^- - 6Gal(\beta 1,4)Man(\alpha 1)$. A noteworthy feature of the backbone is the 4-O substitution of the mannose residue, which is not present in any other known eukaryotic glycoconjugate. The substitution of galactose residue varies from species to species. The number and the size of the repeating units per LPG molecule depends on the developmental stage and the species. The repeating units can be depolymerized by mild acid which cleaves $sugar-1-Po4^-$ bond.

d. The cap: The LPG terminates at the non reducing end with one or several small neutral oligosaccharides containing galactose and\or mannose. Again the cap structure and the branching in it varies from species to species.

The structure of LPG derived from three species of *Leishmania* is shown in figure 1.1.

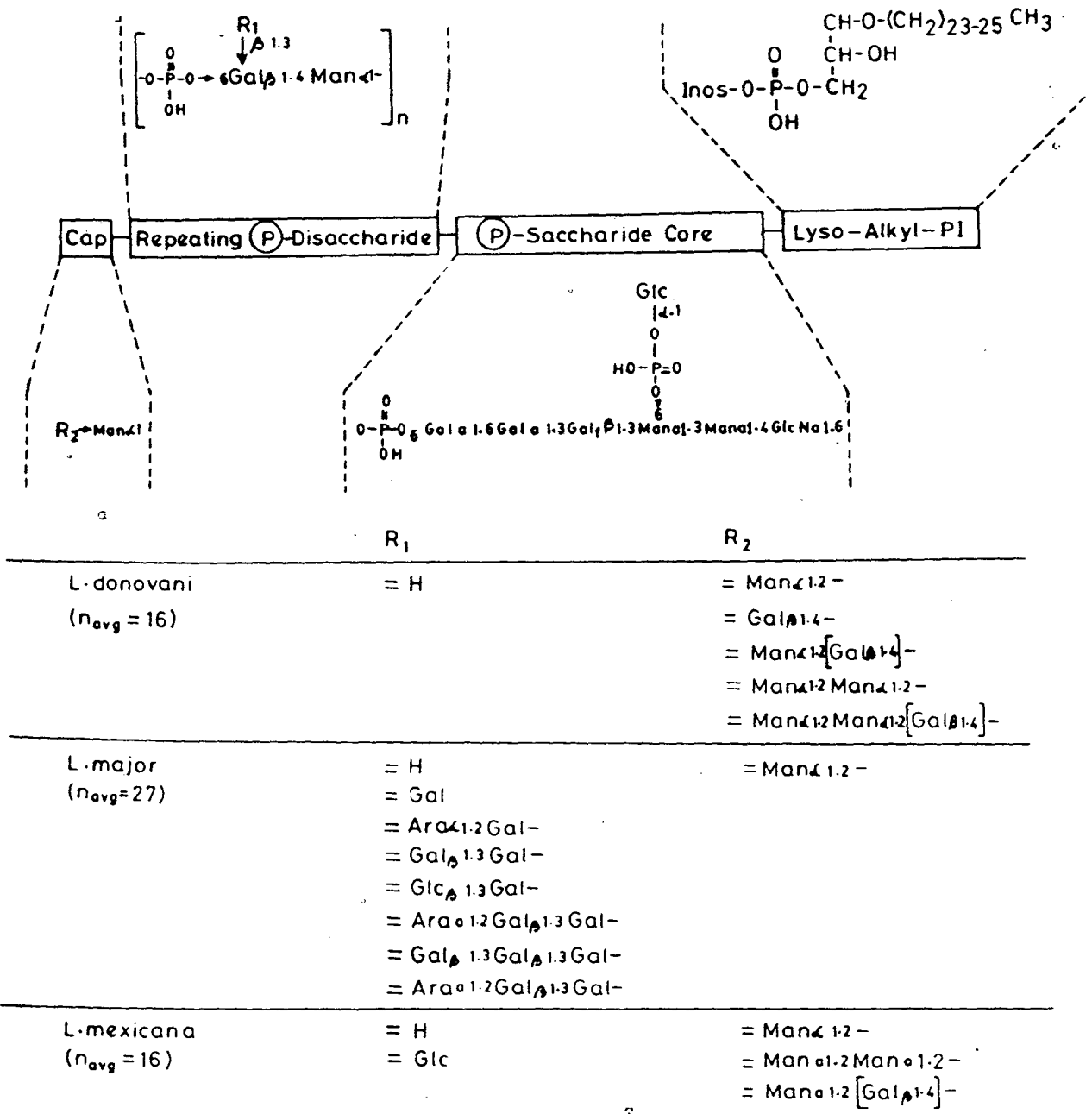|	R_1	R_2
L·donovani $(n_{avg} = 16)$	= H	= Manα1.2 –
		= Galβ1.4 –
		= Manα1.2[Galβ1.4] –
		= Manα1.2 Manα1.2 –
		= Manα1.2Manα1.2[Galβ1.4] –
L·major $(n_{avg} = 27)$	= H	= Manα1.2 –
	= Gal	
	= Araα1.2Gal–	
	= Galβ1.3Gal–	
	= Glcβ1.3Gal–	
	= Araα1.2Galβ1.3Gal–	
	= Galβ1.3Galβ1.3Gal–	
	= Araα1.2Galβ1.3Gal–	
L·mexicana $(n_{avg} = 16)$	= H	= Manα1.2 –
	= Glc	= Manα1.2Manα1.2 –
		= Manα1.2[Galβ1.4] –

Fig. 1.1: Structures of LPG from three species of *Leishmania* promastigotes, grown in log to late-log phase of growth. (Turco and Descoteaux 1992).

1.2.1.1 The tertiary structure of LPG: The immunochemical structure and surface arrangement of *L. donovani* LPG using monoclonal antibody was determined by Tolson et al. (1989) and by Turco et al.(1987) using physico-chemical techniques (Fig. 1.2A and 1.2B). The model shows the orientation of LPG on the cell surface and the accessbility of repeating phosphorylated disaccharide and phosphosaccharide core epitopes (Fig.1.2A). The main structural difference between PI anchored protiens and LPG is the conventional diacylated PI for protiens and a Lysoalkyl-PI for LPG (Fig.1.2B).

1.2.2 Biosynthesis of LPG

Since LPG is essential for survival of the parasite, detailed knowledge of LPG biosynthesis can provide specific targets in the search for efficaceous drugs.

The PI anchor region of LPG which possesses a lyso-1-O-alkyl PI anchor is synthesized probably via the formation of a 1-O-alkyl-2 acyl PI precurssor (Wassef et al.1985). The suggested pathway of LPG biosynthesis in *Leishmania* is shown in Fig.1.3. It is believed that the galactose and mannose residues of the LPG repeating units of *L. donovani* are added by their respective nucleotide-sugar donors sequentially and directly to LPG and these are assembled in the Golgi (Bates et al. 1990). An analysis of the capping oligo saccharide structures of various *Leishmania* LPG reveals that all but one contain a Man(α1,2)Man(α1)at the reducing end. It is

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Fig. 1.2 A: Schematic representation of the immunochemical structure of *L.donovani* LPG and its arrangement at the cell surface. (A) PO_4-6Galβ1,4Man repeating disaccharide (exposed epitope); (B) phosphosaccharide core (hidden epitope); (C) lyso-alkyl-phosphatidylinositol anchor; (D) glycosyl-phosphatidylinositol-anchored membrane proteins. (Tolson et al. 1989).

Fig. 1.2 B: Schematic representation of proteins, such as trypanosomal VSGs (Ferguson et al. 1985), and LPG anchored in membranes. I, inositol; G, glycan core; P, phosphate; E ethanolamine. In this schematic representation, all 16 phosphorylated disaccharide units are illustrated as a ribbon-like, consecutive sequence. However, it is possible that one or more of the units are attached to a second site in the heptasaccharide core (G). (Turco et al. 1987).
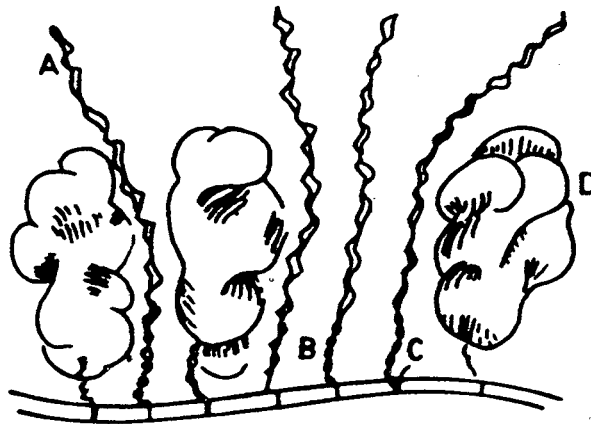
Protein   Lipophosphoglycan
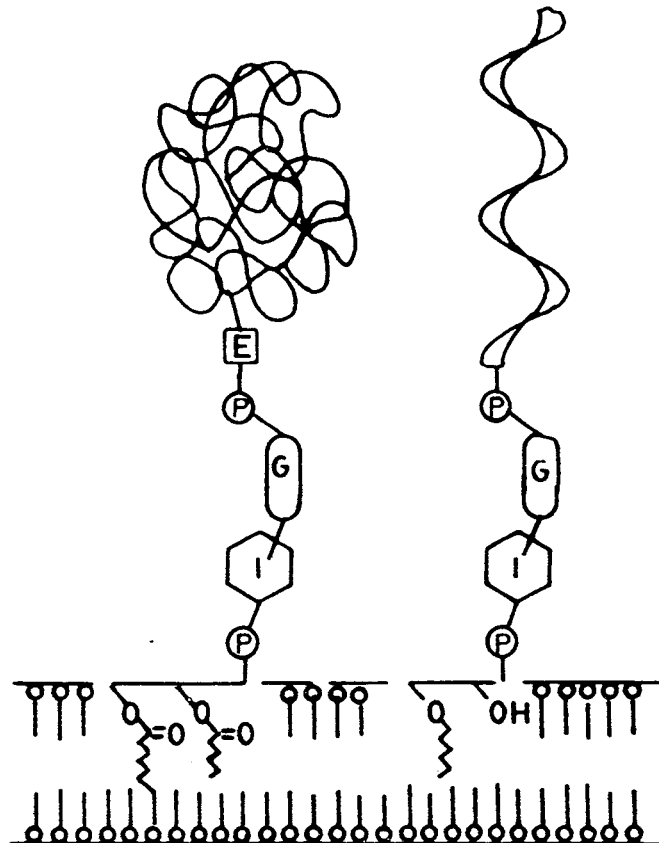
Fig. 1.3: Proposed pathway of assembly of the repeating units and capping oligosaccharides of the *L.donovani* LPG. The core structure is Gal(α1,6)Gal(α1,3)Gal$_f$(β1,3)Man(α 1,3)Man(α 1,4)GlcN(α 1,6) and PI is lyso-I-O-alkylphosphatidylinositol. The sequence of monosaccharide addtion is not yet known. (Turco and Descoteaux 1992).

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                 Core ── PI

                    │  ┌GDP─Man
                    │  │                    Initiation of polymerization
                    ▼  └►GMP

        Man─P─ Core ── PI

                    │  ┌UDP─Gal
                    │  │                    Completion of initial
                    ▼  └►UDP                disaccharide unit

     Gal─Man─P─ Core ── PI

                    │  ┌[UDP-Gal+GDP─Man]$_n$
                    │  │                    Polymerization
                    ▼  └►[UDP+GMP]$_n$

 (Gal─Man─P)$_n$─Gal─Man─P─ Core ── PI

                    │  ┌GDP─Man
                    │  │                    Initiation of cap formation
                    ▼  └►GMP

 Man─P─(Gal─Man─P)$_n$─Gal─Man─P─ Core ── PI

                    │  ┌UDP─Gal/GDP─Man
                    │  │                    Cap formation
                    ▼  └►UDP/GDP

        Man
         │
 Gal─Man─P─(Gal─Man─P)$_n$─Gal─Man─P─ Core ── PI
```
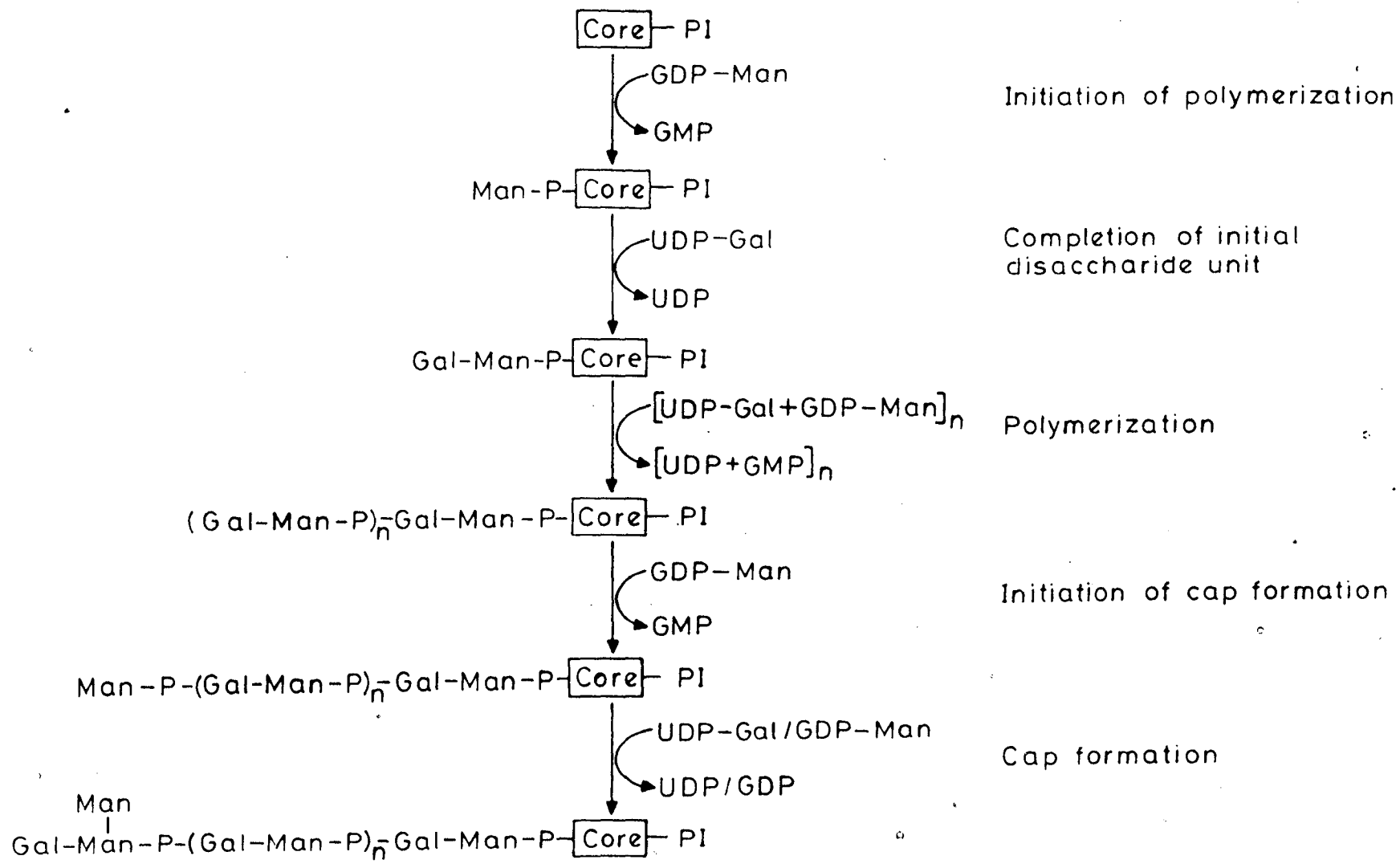
therefore, speculated that there exists an enzyme like Man(α 1,2) Mannosyltransferase. The activity of this putative enzyme would result in the signal for cessation of LPG elongation with the formation of a chain terminating Man(α 1,2)Man- containing cap oligosaccharide.

1.2.3 Functions of LPG

The uniqueness of the overall structure of LPG and its highly unusual domains indicate that it might have several important functions. LPG has a role in Sand fly-*Leishmania* interaction, as one of the major changes that occurs during metacyclogenesis is sequential structural modification of LPG (Sacks et al. 1985).

In the blood stream of the host, the LPG prevents complement activation and confers resistance to complement-mediated damages by inhibiting C_3 binding to promastigotes (Russel 1987). The glycoprotien gP_{63} (Bouvier et al. 1987) and LPG (Handman and Goding 1985; Wilson and Hardin 1988) are considered to be parasitic ligands for macrophages. These molecules are thought to be involved in the entry of the parasite into macrophages (Handman and Goding 1985).

Several intracellular functions have been attrbuted to LPG. Some of them are listed below in the table :

Table:1.1

Function	Reference
Intracellular survival in host phagolysosome	Alexender & Vickerman 1975. Chang & Dwyer 1976.
Chelator of Calcium	Eilman et al. 1985.
Inhibitor of protien kinase C and oxidative burst	Buchmuller-Rouiller & Mauel 1987. Pearson et al. 1982. McNeely & Turco 1990.
Inhibitor of chemotaxis	Frankenburg et al. 1990. Frankenburg et al. 1992.
Scavenger of toxic Oxygen metabolites	Chan et al. 1989.
Inhibitor of IL-1 production	Reiner 1987.
Modulator of TNF receptors	Green et al. 1990.
Inhibitor of C-fos gene expression	Descosteaux & Matlashewski 1989.

1.2.4 The LPG of E. histolytica

The chemical extraction of a lipopeptidoglycan from E. histolytica was reported by Isibasi et al. in 1982. However, this molecule was not characterized. The presence of LPG in E. histolytica was demonstrated in 1992 by our group and Stanley et al. 1992 (Stanley et al. 1992; Bhattacharaya et al. 1992.) The glycoconjugate, appeared as two polydisperse bands on SDS-PAGE with average molecular weight range of 110 kDa and 45 kDa and contained sugars, phosphate, lipids but no peptides. It has a hydrophobic tail attached by PI linkage. Just like the LPG of Leishmania, amoebic LPG also

has sugar-phosphate repeating groups and one of the repeating groups in the strain HM-1:IMSS is Galβ1-4Man, similar to that present in *L. donovani*. The glycoconjugate is believed to be the major surface molecule and is involved in recognition of target cells by *E. histolytica*. Treatement with a specific McAb inhibited cytotoxicity of amoeba and adhesion to chinese hamster ovary cells. Moreover, amobeic LPG also displays polymorphic epitopes suggesting that it may have structural polymorphism similar to that present in *Leishmania* (Jaffe et al. 1990). The LPG was also recognized by human immune sera from invasive amoebiasis patients. This recognition is mediated through carbohydrate domains. These observations suggest that the LPG of *E. histolytica* contains strain-specific, polymorphic epitope and that LPG is immunogenic in human.

Aim of the present work

As it is evident from the above discussion, the LPG of *E. histolytica* is a specialized molecule on the cell surface. It may be involved in host-pathogen interaction in amoebiasis. Our group has reported inter strain variation in the reactivity of *E. histolytica* strains to anti-LPG antibody (Prasad et al. 1992). As a step further it was of interest to see if any LPG-like molecule existed in *E. histolytica* strain, Rahman and if it did exist, to characterize it.

MATERIALS

All strains used in this study were obtained from Dr.L.S. Diamond, N.I.H.,(USA). Biochemicals, enzymes and nitrocellulose membrane were obtained from various companies like Sigma, United States Biochemicals, Fluka, (U.S.A.), Boehringer Mannheim (Germany), and Indian manufacturers. [^{3}H]-labeled sugars and [^{32}P]-labeled orthophosphate were obtained from Amersham (UK) and BARC (India), respectively. X-ray films were bought from Hindustan Photographic Company (Indu, India) and Kodak (USA). The medium components were from DIFCO and BBL (USA). Adult bovine serum was procured from BIOFULIDS (USA).

2.1 Organism and Culture conditions

E. histolytica strains, Rahman clone 1, HM-1:IMSS clone
6 and 200:NIH clone 2, were maintained axenically by
continuous subculturing in TYI-S-33 medium at 35°C as
described (Diamond et al. 1978).

2.2 Sera and Antibodies

2.2.1 Human Patient Sera: Human immune sera were obtained
from clinically proven amoebiasis patients from AIIMS, (New
Delhi). These patients were diagnosed by the attending
consulting clinicians and confirmed by either stool
examination or by serology. The healthy control serum was
obtained in the United States from a person with no known
history of amoebiasis.

2.2.2 Polyclonal anti-LPG antibody: Rabbit antibody against
pronase-digested E. histolytica LPG (aLPG.HM1) raised by
Prasad et al. (1992) was used. The pre-bleed of the rabbit
was used as control serum.

2.3 Prepration of cultures

The cultures were maintained by continuous subculturing
as mentioned before. The subculturing was carried out every
72 hrs. 2-4 drops of a fully grown culture (mid to late log

phase) was used as inoculum after chilling the tube for 5 min on ice water and mixing gently. The growth of *E. histolytica* was monitored by microscopy daily. The tubes were kept standing or inclined in an incubator at 35°C.

2.4 Metabolic labeling and extraction

Actively growing cells ($2\text{-}5\text{x}10^6$/ml) were incubated with [^{3}H]-labeled sugars, such as, (^{3}H)galactose (100-200 μCi/ml), (^{3}H)glucose (100-200 μCi/ml), (^{3}H)mannose (100-200 μCi/ml) in glucose free TYI-33 medium or (^{32}P)orthophosphate (0.5 mCi/ml) in phosphate free TYI-33 medium for 1.5 hrs at 36°C. The cells were then washed with PBS#8 (K_2HPO_4 0.11%,$KH_{2\ 4}$ 0.37%, NaCl 0.95%, pH 7.2,)three times at 4°C. The cells were then extracted sequentially with chloroform/methanol (3:2) 5 parts and one part of 4 mM $MgCl_2$, 4 mM $MgCl_2$, 5 parts of chloroform/methanol/water (10:10:3) and one part of chloroform/ methanol (1:1) and chloroform/methanol/water (10:10:3). The pellet was then extracted with solvent E [water/ethanol/diethylether/ pyridine/ammonium hydroxide (15:15:5:1:0.017)] three times. The extracted material was dried under nitrogen to reduce the volume and finally in a centrifugal evaporator under reduced pressure for complete drying. The dried material was resuspended in a small volume of 0.1 M NaCl, 0.1 M acetic acid, briefly sonicated and passed through a phenyl-Sepharose column for further purification.

2.5   Phenyl-Sepharose   chromatography

Phenyl-Sepharose chromatography was carried out as described by Bhattacharaya et al. (1992). The solvent E extracted material was passed through phenyl-Sepharose CL 4B (Pharmacia, Sweden). The columns were washed sequentially with 0.1 M acetic acid containing 0.1 M NaCl, 0.1 M acetic acid, water and solvent E. 0.5 ml fractions were collected and radioactivity determined by liquid scintillation counting. The material eluted with solvent E was dried in a centrifugal evaporator and used for further experiments. In the case of gradient elution with n-Propanol, a gradient of n-Propanol (5-50%) in 0.1 M NaCl containing 0.1 M acetic acid was applied to the column.

2.6   Sodium   Dodecyl   Sulphate-Poly   Acrylamide   Gel Electrophoresis (SDS-PAGE)

Radiolabeled samples (LPG based on hexose) were analyzed by discontinuous SDS-PAGE under reducing conditions according to Laemmli (1970). The separating gel was 10% acrylamide in 1.5 M Tris-Cl pH 8.8, 0.1% (w/v) SDS, 0.04% (w/v) APS and TEMED. The stacking gel was 4% acrylamide in 0.5 M Tris-Cl pH 6.8, 0.1% (w/v) SDS, 0.04% (w/v) APS and TEMED. Samples were prepared by diluting with an equal volume of 2X SDS-PAGE sample buffer [125 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue] and incubating in a

boiling water bath for 4 min. The samples electrophoresed at 80 v for 2 hrs and 120 v till the bromophenol blue dye front reached 1-1.5 cm from the bottom of the gel.

2.6.1 Flurography and Autoradiography: Gels containing [^{3}H] labeled-samples were flurographed according to Laskey and Mills (1975). The gel was first saturated with DMSO for 1 hr followed by incubation for 3 hrs with 20% PPO (w/v) made in DMSO. It was then washed with cold water to remove excess PPO and DMSO. The dried gels were exposed to X-ray films. Gels containing [^{32}P]-labeled materials were dried and exposed to X-ray films in presence of intensifying screens.

2.7 Mild acid treatment

LPG was hydrolyzed by mild acid as described by Turco et al. (1984). Mild acid conditions of 0.02 M HCl, 5 min at 100°C were used to depolymerize the molecule as phosphorylated saccharide repeat units from the carbohydrate core region. The reaction was stopped by rapid cooling and neutralization of pH with 1 M NaOH. The samples were dried under a stream of nitrogen.

2.8 PI-PLC treatment

PI-PLC digestion was carried out as per Low et al. (1987). The LPG was resuspended in 200 μl of 25 mM Hepes, pH 7.4, 0.1% Chaps, 5 mM EDTA and incubated with 0.1 unit of PI-PLC from *Bacillus thruingiensis* (Boehringer-Mannheim,

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Germany) at 37°C for 16 hrs.

2.9 Nitrous acid deamination

Deamination of LPG was carried out as described by Zamze et al. (1988). The LPG was resuspended in 0.1 M sodium acetate (pH 4.0) containing 0.25 M sodium nitrite and incubated at 42°C for 2-4 hrs. The reaction was stoped by rapid cooling and neutralization of pH with 1 M NaOH. The samples were dried under a stream of nitrogen.

2.10 Paper Chromatography

Paper chromatography of LPG was performed as described by Turco et al. (1984). Aliquots of [^{3}H]galactose-labeled and purified LPG were depolymerized as indicated. The samples were chromatographed on paper (Whatman no. 1) for 16 hrs in n-butyl alcohol; pyridine; water (6:4:3). Quantification of radioactivity was carried out by cutting 1 cm strips of the paper after chromatography, soaking the strips in 0.6 ml of 0.1% SDS followed by radioactivity determination using a scintillation counter in presence of scintillation fluid. Standard sugars were galactose (monosaccharide), lactose (disaccharide) and raffinose (trisaccharide). These were detected by staining with orcinol-sulphuric acid (raffinose) and alkaline silver nitrate (galactose and lactose).

2.11 DE-52 Cellulose Chromatography

This was carried out essentially as described by Turco

et al. (1984). The radiolabeled LPG was treated with mild acid and neutralized with 40 mM NH_4OH. The digested sample was dried by evaporation under nitrogen and resuspended in 1 mM Tris-Cl, pH 8.0. This was applied to a column of DE-52 cellulose (0.5cm X 1 cm) equilibrated in 1 mM Tris-Cl, pH 8.0. Fractions of 0.5 ml were collected. After the sixth fraction, a gradient of NaCl (0-0.1 M) in 1 mM Tris-Cl, pH 8.0, was applied to the column. The amount of radioactivity in each fraction was determined by scintillation counting.

2.12 Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out according to Bhattacharya et al. (1990). 50 µg of LPG (based on hexose) in PBS was used to coat each well of a microtiter plate (Costar, USA) overnight at $4^{o}C$. Nonspecific sites were blocked with 3% (w/v) gelatin in TBS (20 mM Tris-Cl pH 7.5, 500 mM NaCl) for 1 hr at room temperature. All incubations with antibodies were carried out for 2 hrs at room temperature in 1% gelatin-TBS. The wells were washed thourghly with TBS after each treatment. aLPG.HM-1 or human patient sera were used as first antibody. The amount of bound first antibody was detected using appropriate alkaline phosphatase-labeled second antibody using p-nitrophenylphosphate as substrate. OD was measured at 411 nm.

2.13 Western blotting

Prewarmed purified LPG samples were treated with

preheated 2xSDS-PAGE sample buffer (Laemmli 1970). Polyacrylamide gels (10%) were used for all separations. Typically 0.5 µg of LPG (based on hexose) was used for each analysis. After separation, the molecules were transferred to nitrocellulose membrane electrophoretically according to Towbin et al. (1979). The transfer was carried out at 400 ma for 4 hrs at 4°C in Tris-glycine buffer containing 20% methanol. After transfer, the membranes were incubated overnight in 3% gelatin at room temperature to block the non specific binding sites. The immunoreactive molecules were located using either aLPG.HM-1 or human patient sera and alkaline phosphatase-conjugated anti-Rabbit or anti-human immunoglobulins (Bio-Rad, USA) as second antibody. Colour development utilized Nitro Blue Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate as substrate.

2.14 Hexose estimation

Total hexose of LPG samples were estimated as per Wingler (1955) using orcinol-sulphuric acid method. 1.6% orcinol in water and 60% sulphuric acid (w/v) were mixed in 1:7.5 ratio to make the working reagent. The working reagent was added to suitably diluted LPG samples and kept at 80°C for 20 min. It was cooled to room temperature and OD measured at 540 nm. Glucose 0.1-5.0 ug was used as standard. All measurements were done in triplicate.

RESULTS

3.1 Rahman has a Glycoconjugate

E. histolytica strain, Rahman, was metabolically labeled with [^{3}H]galactose and a glycoconjugate was extracted following the protocol used earlier for extraction of LPG from *Leishmania* and *E. histolytica* strains, HM-1:IMSS and 200:NIH. The glycoconjugate was chromatographed on phenyl-Sepharose to see if it was hydrophobic in nature. As shown in figure Fig. 3.1A nearly 40-70% of the material bound to the column which could only be eluted with hydrophobic solvent (solvent E). When this portion was rechromatographed on the same column all of it could only be eluted with solvent E (Fig. 3.1B), indicating that the material purified was essentially hydrophobic in nature. The nature of the hydrophobic glycoconjugate was further analyzed by hydrophobic chromatography on phenyl-Sepharose using an increasing gradient of n-Propanol for elution. The bound material eluted as a major peak between 30-35% n-Propanol (Fig.3.2) similar to LPG of *Leishmania* and *E. histolytica* suggesting that it could also be an LPG-like glycoconjugate.

The purified Rahman glycoconjugate was analyzed by SDS-PAGE along with LPG molecules of HM-1:IMSS and 200:NIH. Rahman glycoconjugate appeared as a single diffuse band between the molecular weight range of 45-66 kDa (Fig.3.3, lane 3). The patterns obtained with HM-1:IMSS and 200:NIH *i.e.*two diffuse bands of average molecular weight range 110
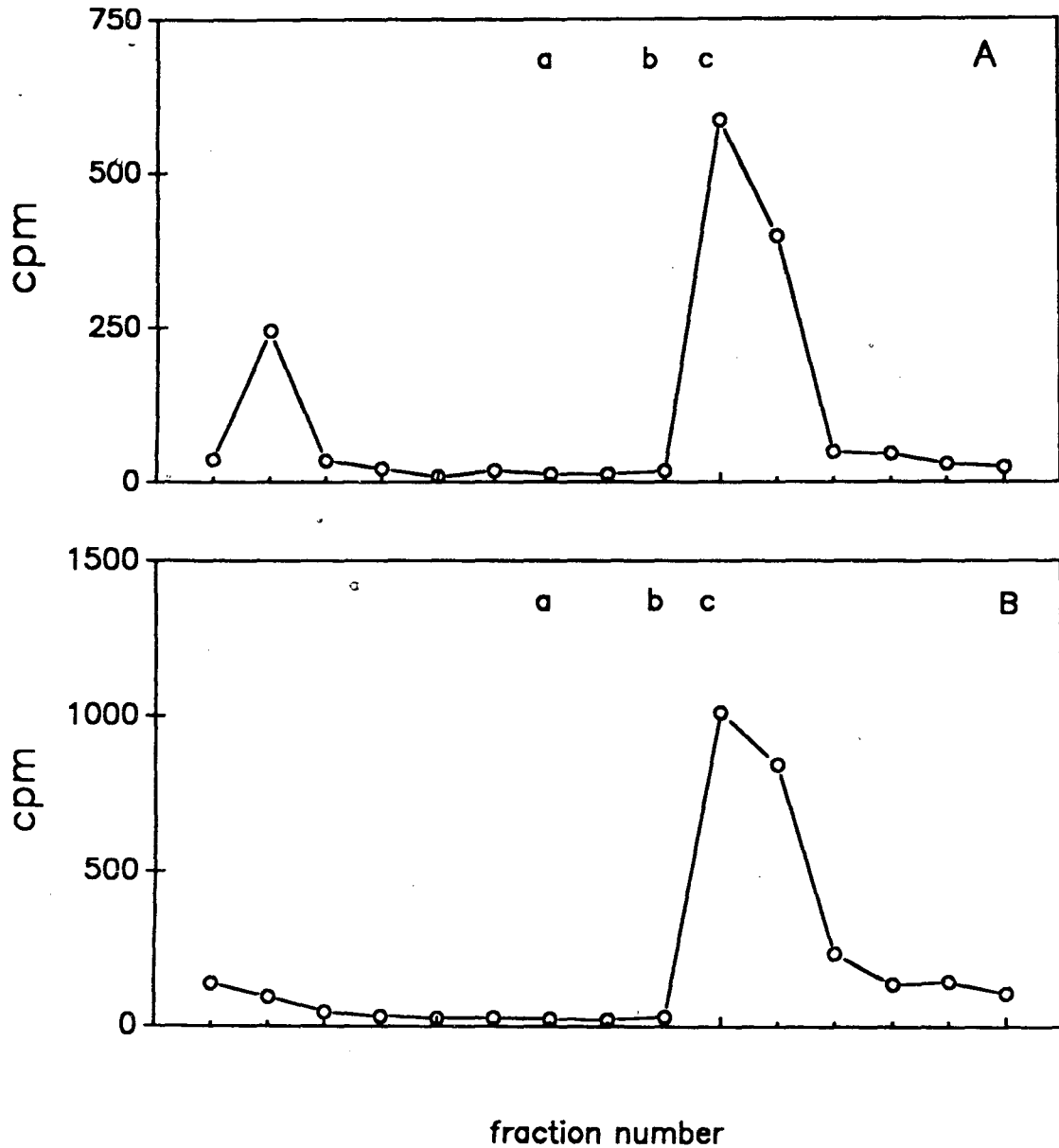
Figure 3.1. Phenyl-Sepharose chromatography. [^{3}H]galactose-labeled glycoconjugates were extracted from cells and fractionated on a phenyl-Sepharose column as described in section 2.5. The purified material,in 0.1M NaCl, 0.1M acetic acid, was allowed to bind to the phenyl-Sepharose column. The column was washed first with 0.1M NaCl in 0.1M acetic acid and then sequentially eluted with 0.1M acetic acid (a), water (b) and solvent E (c). Panel A: Rahman Solvent E extract. Panel B: Solvent E fractions of panel A rerun on the column.
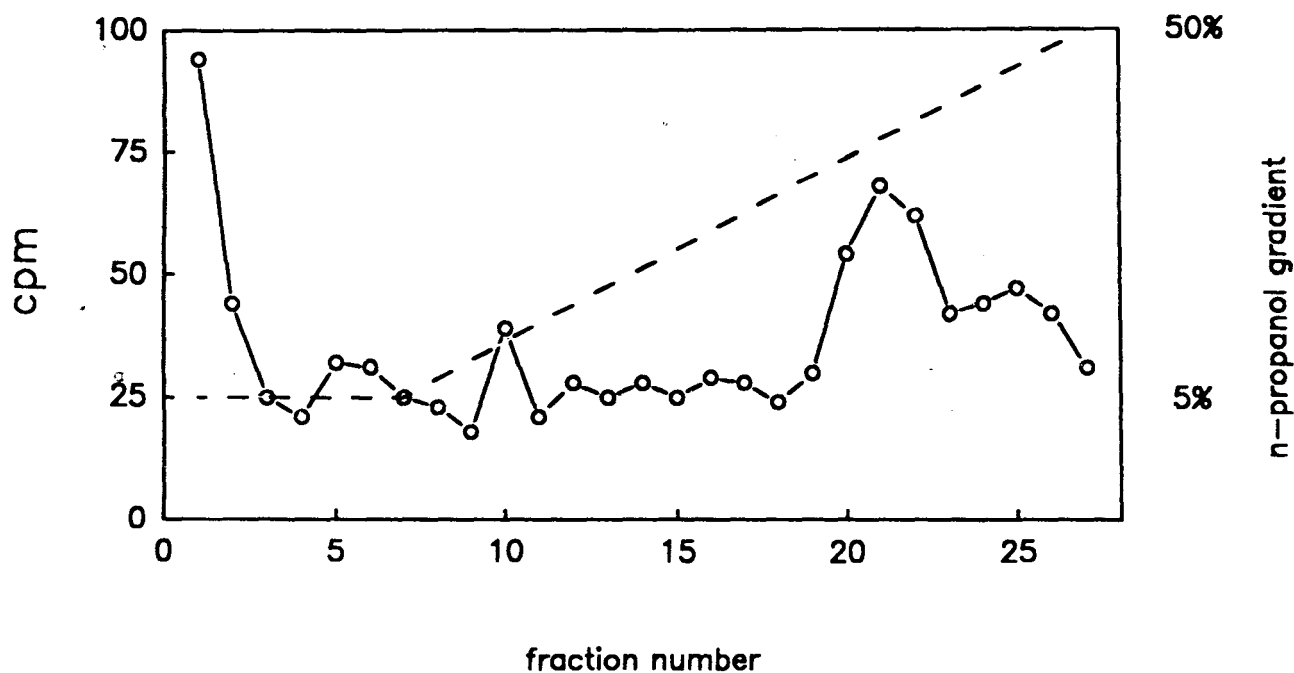
Figure 3.2. n-Propanol elution of the purified glycoconjugate. [^{3}H]galactose-labeled glycoconjugates were extracted from cells and fractionated on a phenyl-Sepharose column as described in section 2.5. The purified material,in 0.1M NaCl, 0.1M acetic acid\ 5% n-propanol was allowed to bind to the phenyl-Sepharose column. The bound material was eluted with a gradient of .n-propanol (5-50%) in 0.1M NaCl, 0.1M acetic acid.
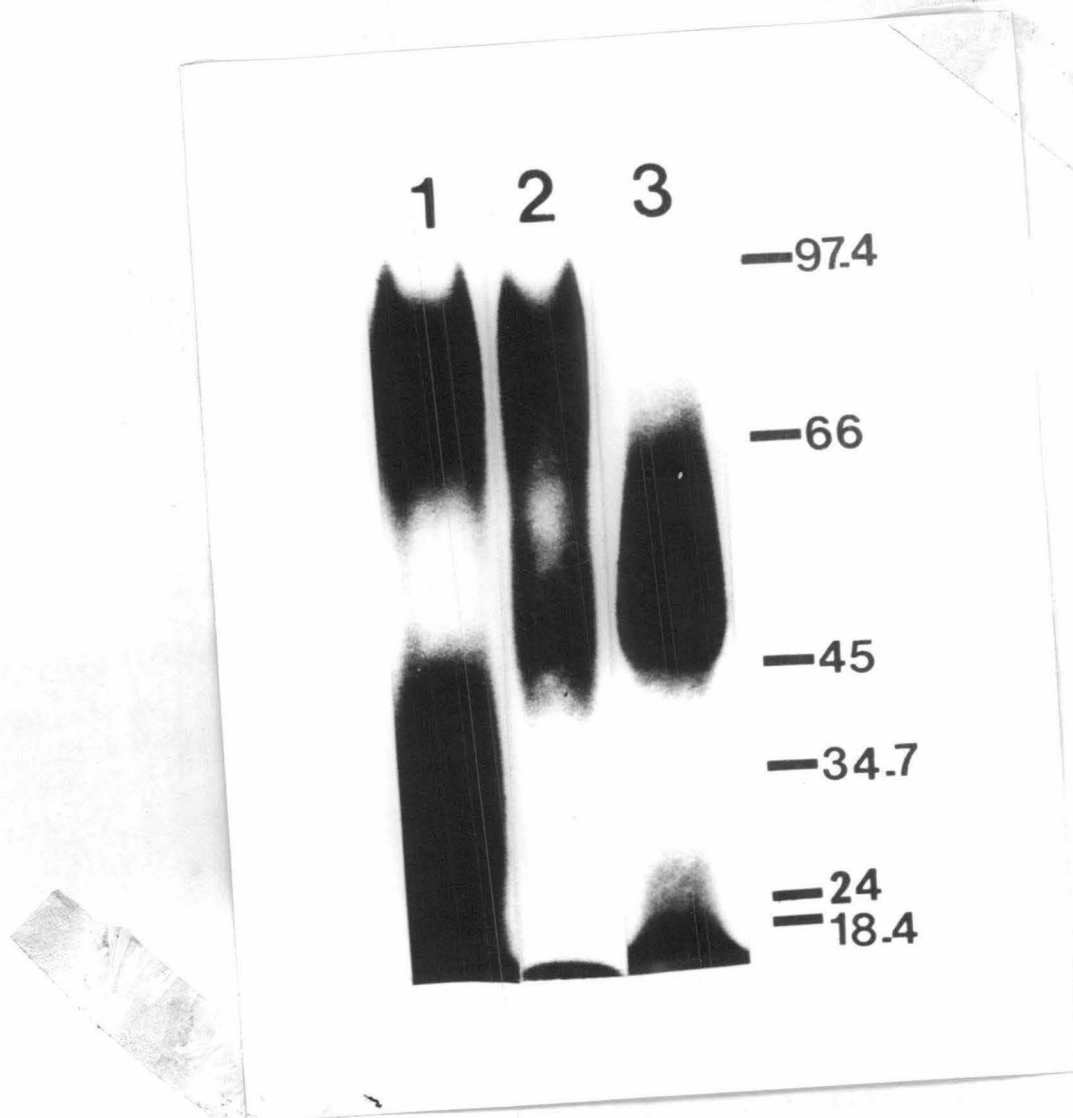
Figure 3.3. Gel profile of radiolabeled, purified glycoconjugate. Cells from different strains of *E. histolytica* were labeled with [³H]galactose, sequentially extracted with different solvents followed by phenyl-Sepharose chromatography as described in section 2.2 and 2.3. The purified material (approximately 8,000-10,000 cpm) was subjected to 10% SDS-PAGE followed by fluorography. Lane 1,HM-1:IMSS; lane 2, 200:NIH; lane 3, Rahman. Molecular weight markers (kDa) are shown on the right.

and 45 kDa were different from that of Rahman.

The presence of glucose and mannose in the glycoconjugate was tested by metabolically labeling Rahman cells with [^{3}H]glucose and [^{3}H]mannose and analyzing the product by SDS-PAGE. The Rahman glycoconjugate did incorporate these sugars though the relative incorporation of different sugars into the glycoconjugate was different (Fig.3.4A, lanes 1-3). The maximum incorporation was observed with galactose while the minimum with mannose. This data indicates the relative amount of different sugars in the glycoconjugate.

The presence of phosphate groups in the glycoconjugate was demonstrated by metabolically labeling Rahman cells with [^{32}P]orthophosphate. SDS-PAGE profile of the purified glycoconjugate showed a single diffuse band (Fig. 3.4B, lane 1) of molecular weight range 45-66 kDa. The pattern was very similar to that of [^{3}H]sugar-labeled glycoconjugate suggesting that the incorporation took place into the same molecule.

All the characteristics shown by the glycoconjugate, extracted and purified from Rahman, indicate that it is hydrophobic in nature containing sugars and phosphate and may be similar to LPG observed in *E. histolytica* strain, HM-1:IMSS.

3.2 The glycoconjugate is an LPG molecule

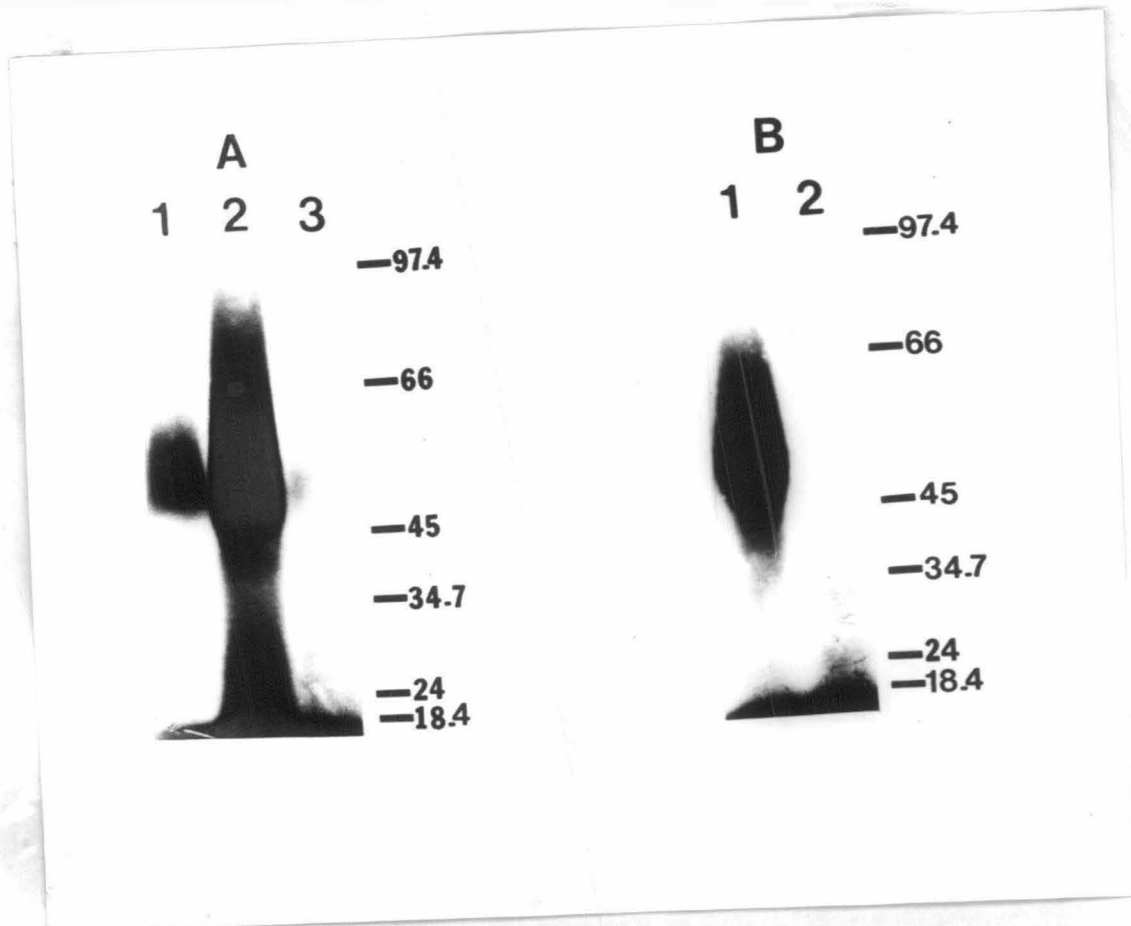Mild acid depolymerization is an established criterion

Figure 3.4. Gel profile of Rahman glycoconjugate radiolabeled with different sugars and phosphate. Cells were metabolically labeled with either a radiolabeled sugar or orthophosphate. The glycoconjugates were purified and analyzed by SDS-PAGE followed by fluorography or autoradiography depending upon the isotope used. **A:** lane 1, [³H]glucose; lane 2, [³H]galactose; lane 3, [³H]mannose. **B:** lane 1, [³²P]orthophosphate-labeled glycoconjugate; lane 2, mild acid treatment of the material in lane 1.

used to identify LPG molecules and was used to confirm the identity of the Rahman glycoconjugate. The [^{32}P]orthophosphate-labeled glycoconjugate was treated with mild acid (0.02N HCl, 5min, 100°C) and analyzed on SDS-PAGE. The molecule was completely degraded suggesting that it carries repeating sugar-phosphate groups just like a typical LPG molecule (Fig. 3.4B, lane 2).

To further confirm the above observation, [^{3}H]galactose labeled glycoconjugate was similarly treated with mild acid and chromatographed on phenyl-Sepharose. While the control (purified glycoconjugate without mild acid treatment) eluted only with solvent E (Fig.3.5A) after mild acid treatement about 60% of the radioactivity was found in the aqueous portion indicating degradation of the molecule (Fig.3.5B).

All LPG molecules known so far contain a GPI anchored lipid tail. It would be of interest to see if the LPG-like molecule of Rahman also has a GPI anchored lipid moiety. This was demonstrated by digestion of the glycoconjugate by PI-PLC and analysis of the product by phenyl-Sepharose chromatography. The purified, untreated glycoconjugate (control) was retained on the column and could only be eluted with solvent E (Fig. 3.6A). After digestion with PI-PLC, 40-50% of the material did not bind to the column, and came out in the acetic acid\ sodium chloride eluent (Fig.3.6B) showing that the glycoconjugate did contain a GPI anchor. This was further confirmed by deamination of
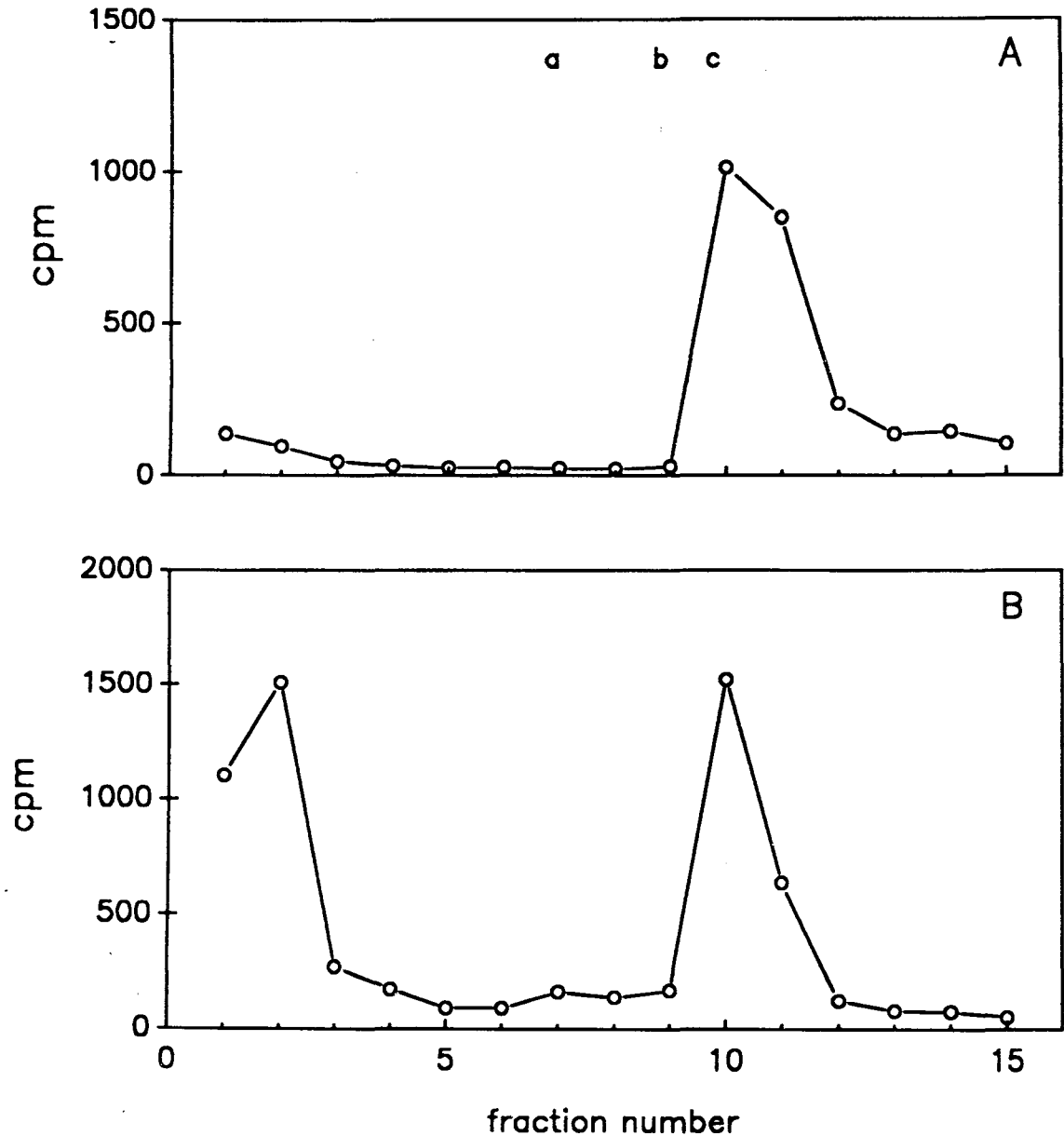
Figure 3.5. Phenyl-Sepharose chrmatography of mild acid treated glycoconjugate. Purified [^{3}H]galactose-labeled glycoconjugate was depolymerized by mild acid and analyzed by phenyl-Sepharose chromatography. Untreated (Panel A); after mild acid treatment (Panel B). Column washes with 0.1M acetic acid, water and Solvent E are indicated by a, b and c, respectively.
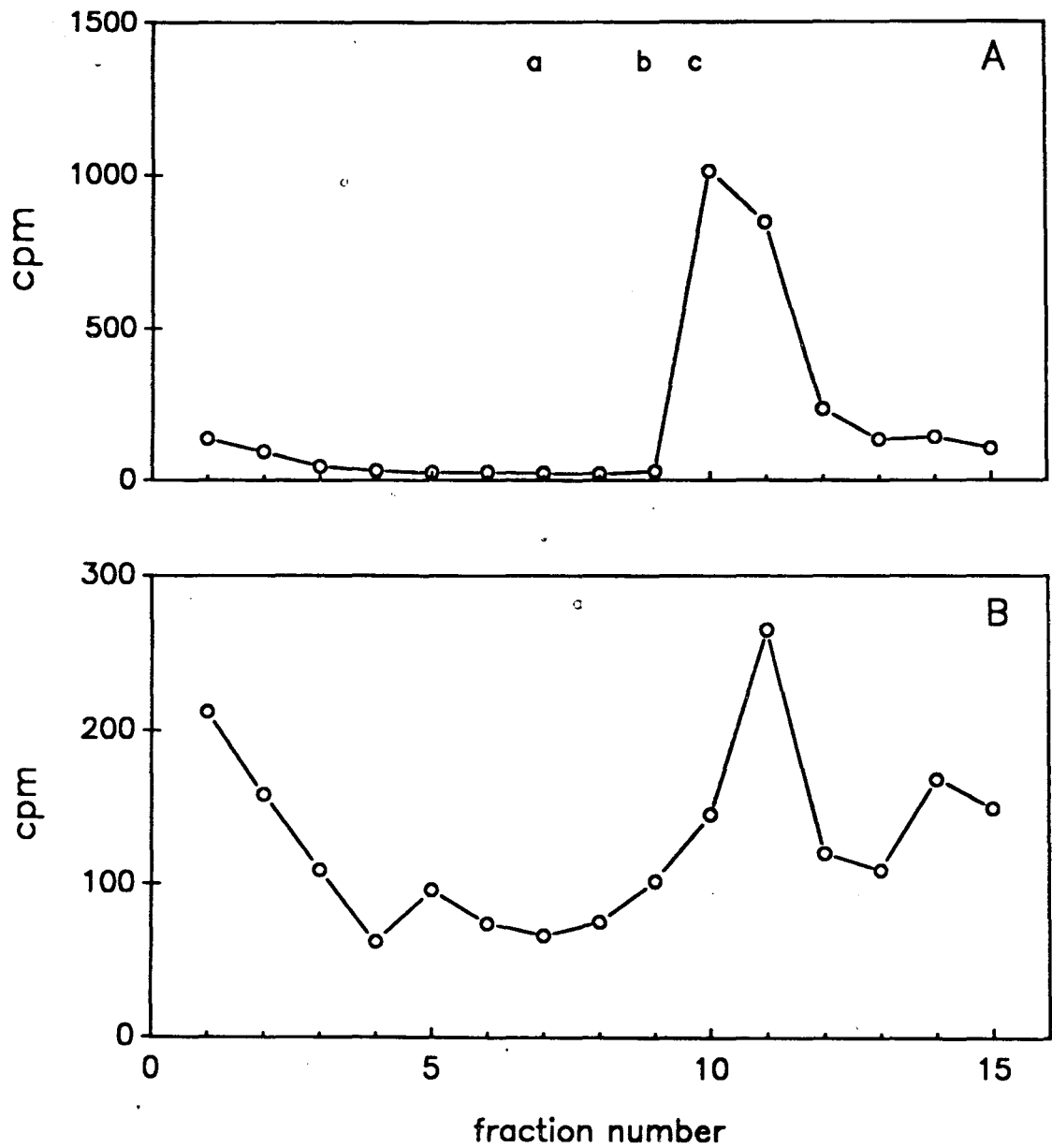
Figure 3.6. Phenyl-Sepharose chromatography of the PI-PLC treated glycoconjugate. Purified [^{3}H]galactose-labeled glycoconjugate was digested with PI-PLC and analyzed by phenyl-Sepharose chromatography. Undigested (panel A); PI-PLC digested (panel B). Column washes with 0.1M acetic acid, water and Solvent E are indicated by a, b and c, respectively.

glucosamine-inositol linkage by nitrous acid, which results in cleavege of the hydrophobic tail. In absence of deamination the glycoconjugate bound to phenyl-Sepharose column (Fig.3.7A) while there was no detectable binding after deamination (Fig. 3.7B).

All these characteristics displayed by the glycoconjugate confirm that the extracted and purified molecule from Rahman is indeed a LPG molecule.

3.3 Rahman LPG has oligosaccharide cap

Intact LPG or phosphorylated carbohydrate groups, generated by mild acid depolymerization, normally do not migrate in paper chromatography (see section 2.10). Under the conditions used in this study only dephosphorylated or neutral products of mild acid digestion would show migration. On paper chromatography the untreated sample, Rahman LPG, remained at the origin, as expected (Fig.3.8A), while in the mild acid treated LPG samples there were two spots (Fig.3.8B). The relative levels of these spots varied with different preparations of LPG. In general the spot at fraction six was found to be the major spot comigrating with the disaccharide standard, whereas the monosaccharide standard comigrated with the other spot at position thirteen. This indicated that the mild acid treatment of Rahman LPG generated disaccharides as a major neutral sugar molecule along with monosaccharides. When HM-1:IMSS LPG was similarily analyzed, there was no migration suggesting that
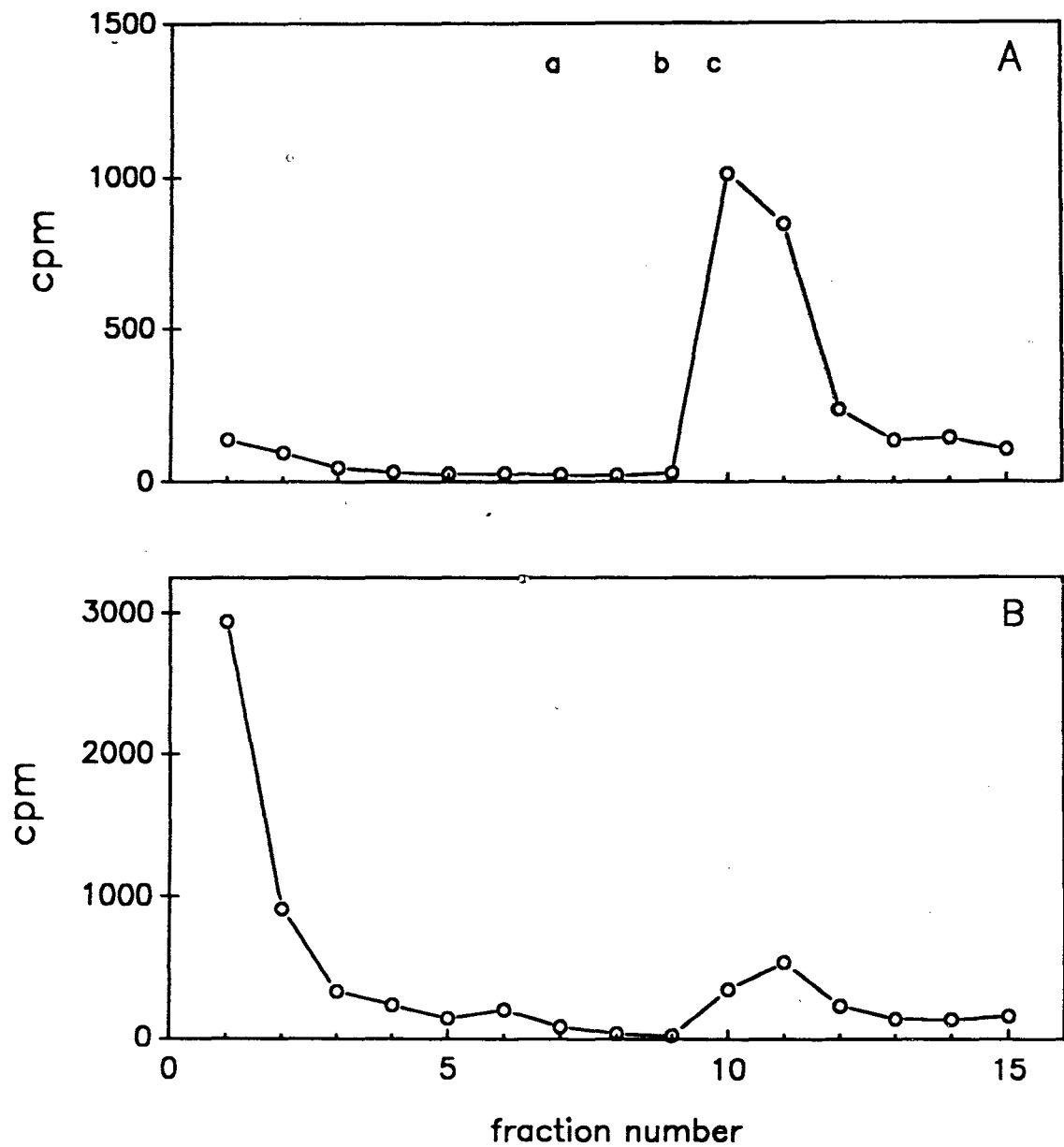
Figure 3.7. Phenyl-Sepharose chromatography of the nitrous acid treated glycoconjugate. Purified [^{3}H]galactose-labeled glycoconjugate was treated with nitrous acid and analyzed by phenyl-Sepharose chromatography. Untreated (panel A); deaminated with nitrous acid (panel B). Column washes with 0.1M acetic acid, water and Solvent E are indicated by a, b and c, respectively.

Figure 3.8. Paper chromatography of [^{3}H]galactose-labeled fragments generated by mild acid hydrolysis. Panel A: Rahman LPG without any treatment. Panel B: Rahman LPG after mild acid hydrolysis. Panel C: HM-1:IMSS LPG after mild acid hydrolysis. [The standards used were raffinose (T,trisaccharide), lactose (D,disaccharide) and galactose (M,monosaccharide).]

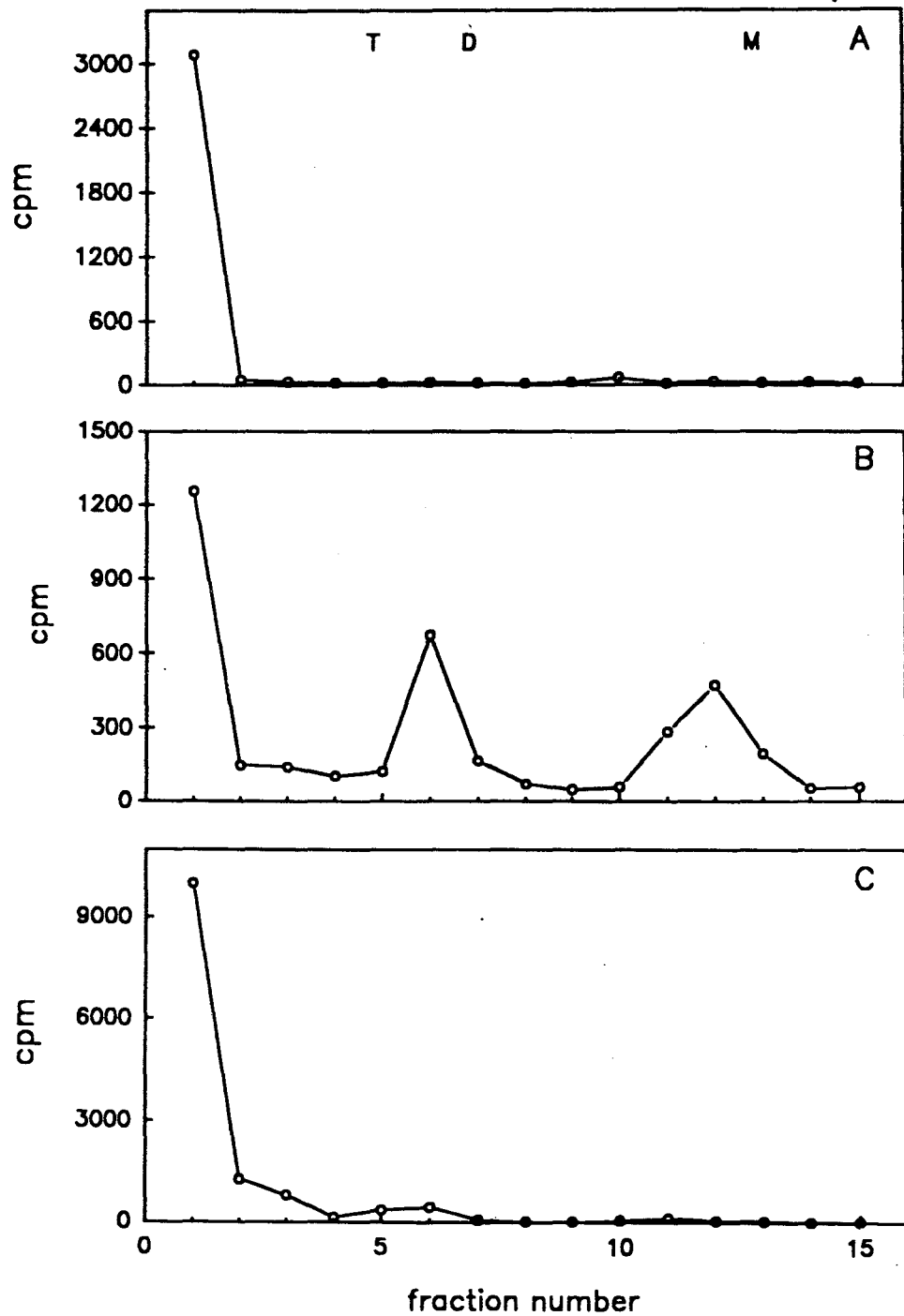the mild acid treatment did not generate any detectable amount of neutral sugar (Fig.3.8C). This was further confirmed by separating the neutral sugar molecules by DE-52 ion exchange chromatography after mild digestion (see section 2.11) .The fraction that did not bind to the column was analyzed by paper chromatography. The same two spots at fraction six and fourteen were found to be present (Fig.3.9). There was also a saccharide fraction which eluted at 0.5M sodium chloride (Fig.3.10). It is likely to contain phosphorylated saccharide units. These results suggest the presence of capped oligosaccharide groups containing galactose groups only in Rahman LPG.

3.4 Rahman LPG shares common epitopes with HM-1:IMSS LPG

The results shown above indicate major structural differences between LPG derived from HM-1:IMSS and Rahman. In order to show if they have any crossreactvity, a polyclonal antibody, raised against HM-1:IMSS LPG (aLPG.HM-1), was used in ELISA. The Rahman LPG was also recognized by the same antibody and the binding was comparable to that with HM-1:IMSS LPG (Table 3.1). This was further confirmed by Western blots using the same polyclonal antibody. The antibody also immunostained the Rahman LPG (Fig.3.11, lane 2). The pattern obtained with this antibody and LPG of HM-1:IMSS was similar to that obtained with other polyclonal antibodies (Fig.3.11, lane 1) (Prasad et al. 1992).

The above results clearly indicate that while HM-1:IMSS

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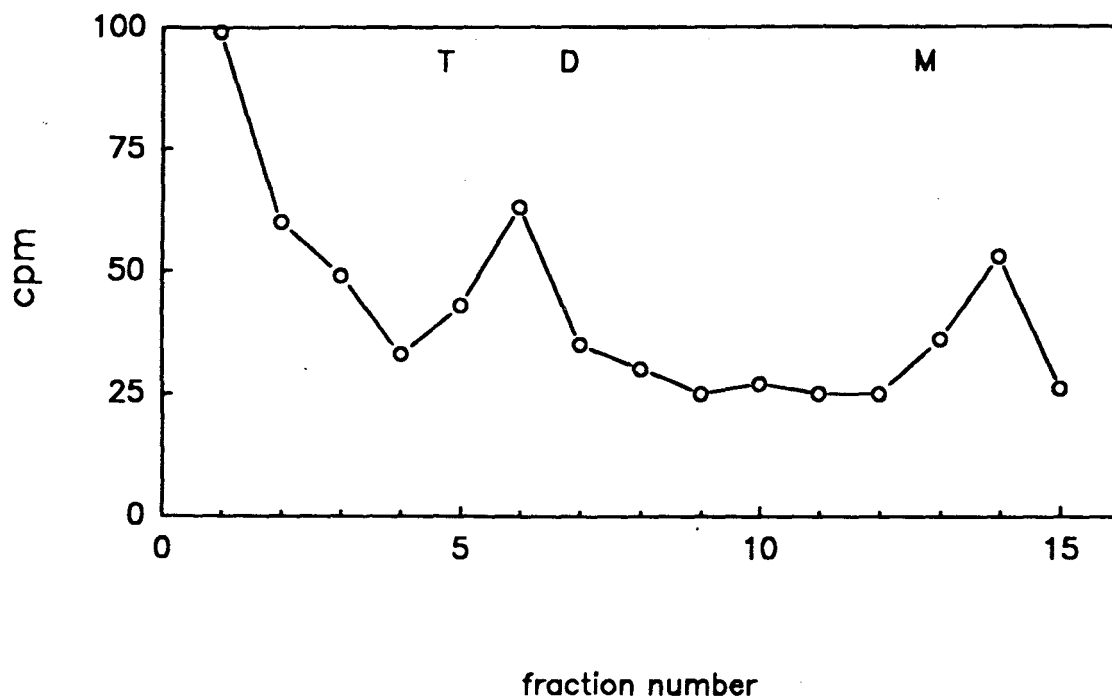
Figure 3.9. Paper chromatography of the unbound fraction of DE-52 ion exchange chromatography. [^{3}H]galactose-labeled purified LPG was treated with mild acid and chromatographed on a DE-52 column. The unbound fraction was pooled, dried and chromatographed on paper (Whatman no.1). [The standards used were raffinose (T,trisaccharide), lactose (D,disaccharide) and galactose (M,monosaccharide).]
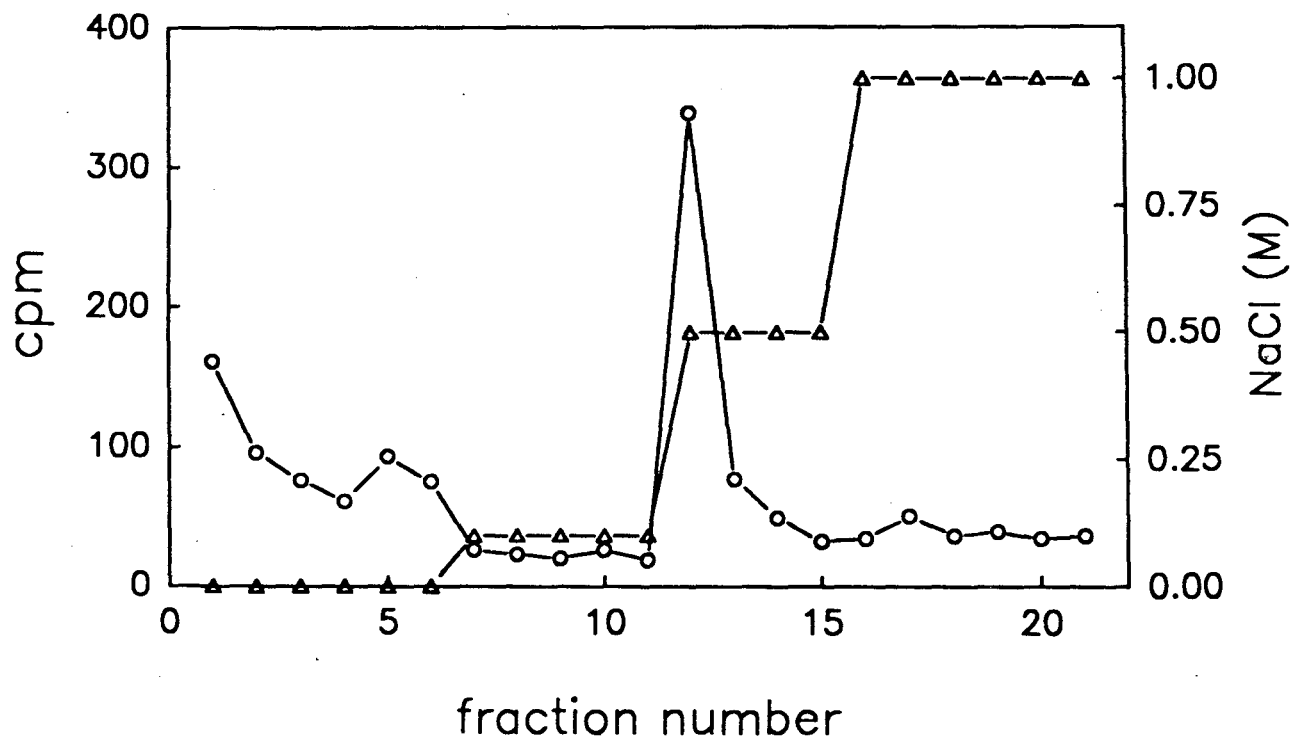
Figure 3.10. DE-52 ion exchange chromatography of the mild acid treated LPG. [^{3}H]galactose-labeled purified LPG was treated with mild acid , dried, resuspended in 1mM Tris-Cl, pH 8.0 and applied to a DE-52 cellulose column equilibrated with 1mM Tris-Cl, pH 8.0. Fractions of 0.5 ml were collected. After the fifth fraction the column was washed with 1mM Tris-Cl containing 0.1M, 0.5M and 1.0M NaCl, respectively. (o--o--o) mild acid treated LPG (Δ--Δ--Δ) NaCl gradient.

Table: 3.1

ELISA for recognition of Rahman and HM-1:IMSS by polyclonal anti-LPG antibody.

Antigen concentration	OD_{411}	
	Rahman	HM-1:IMSS
5 µg	0.834(0.011)	0.397(0.005)
2 µg	0.775(0.031)	0.289(0.001)
1 µg	0.274(0.002)	0.193(0.003)
500 ng	0.181(-0.007)	0.157(0.014)
200 ng	0.089(-0.009)	0.028(-0.006)

Different amounts of purified LPG (based on hexose) was coated in each well and dried. The wells were washed extensively before use. The primary antibody used was aLPG.HM1 (1:1000). The secondary antibody was anti Rabbit IgG conjugated to alkaline phosphatase (1:1000). The colour was developed using p-nitrophenylphosphate as substrate. The values shown within brackets were obtained by using prebleed serum (control).
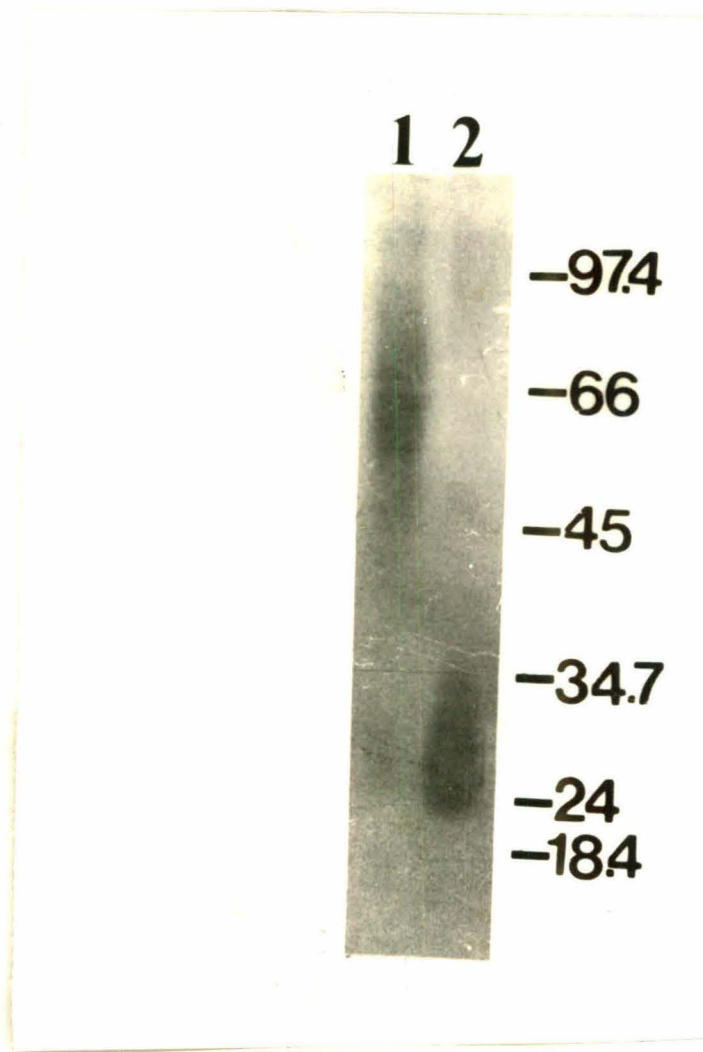
Figure 3.11. Western blot analysis. Purified LPG of HM-1:IMSS and Rahman were separated on a 10% polyacrylamide gel before transferring onto a nitrocellulose membrane. Immunostaining was carried out using aLPG.HM1 (1:1000) and anti-Rabbit IgG conjugated with Alkaline phosphatase. Lane 1, HM-1:IMSS and lane 2, Rahman.

LPG has at least one distinct epitope absent in Rahman LPG, the two share common epitopes detectable by polyclonal antibodies.

3.5 Rahman LPG recognized by human immune sera

The strain Rahman has originated from an invasive amoebiasis patient in Bangladesh. Since the LPG derived from Rahman was found to be structurally different from HM-1:IMSS, it was of interest to find out if patient sera collected from New Delhi would also recognize this. The anti Rahman LPG titre of human immune sera was determined by ELISA (Table 3.2) and were found to be comparable to the LPG of HM-1:IMSS.

Table: 3.2

ELISA for recognition of Rahman and HM-1:IMSS LPG by different Human immune sera.

Human sera	Dilution	OD_{411}	
		Rahman LPG	HM-1:IMSS LPG
Patient 1	400	0.624	0.294
	800	0.351	ND
	1600	0.127	ND
	6400	0.034	0.025
Patient 2	400	0.847	0.484
	800	0.557	ND
	1600	0.303	ND
	6400	0.204	0.139
Patient 3	400	0.880	0.448
	800	0.657	ND
	1600	0.320	ND
	6400	0.090	0.070
Patient 4	400	1.001	0.910
	800	0.737	ND
	1600	0.497	ND
	6400	0.213	0.300
Control	400	0.061	0.003
	800	0.050	ND
	1600	0.008	ND
	6400	0.007	0.001

1.0 µg of purified LPG (based on hexose) was coated in each well and dried. The wells were washed extensively before use. The secondary antibody was Goat anti human IgG conjugated to alkaline phosphatase (1:1000). The colour was developed using p-nitrophenylphosphate as substrate.

Surface molecules of parasites play a major role in host-pathogen relationship. Thus, in recent years the emphasis has been on the identification and characterization of novel surface molecules of parasites. A number of surface molecules of *E. histolytica* have also been characterized. Functional significance of majority of these are not known. However, a few of these have been implicated in the recognition of target cells before cytolysis. Among these molecules, the 170 kDa galactose/N-acetyl galactosamine binding lectin has been characterized in much more detail (Petri et al. 1989, Tannich et al. 1991c).Other lectins, a N-acetyl glucosamine inhibitable (Kobiler and Mirelman 1980) and a 220 kDa poly-N acetyl glucosamine inhibitable lectin (Rosales-Eniona et al. 1987) have also been reported. It is presumed that some of these molecules, whose functional involvement has mostly been demonstrated *in vitro*, also participate in cytopathic activity associated with amoebic virulence.

Apart from surface proteins/glycoproteins, *E. histolytica* also contains a surface glycolipid-lipophosphoglycan (Stanley et al. 1992, Bhattacharya et al. 1992). LPG is also present in the promastigote form of *Leishmania* sp. (Turco et al. 1984; Turco and Descoteaux 1992) and Trichomonads (Singh 1993). Among these three genera, LPG of *Leishmania* has been characterized in much more detail. Thus, the complete structure of LPG from a

number of species of *Leishmania* have been reported (Turco and Descoteaux 1992). All the LPG known so far show same characteristic features such as presence of a GPI anchor, core polysaccharide, repeating sugar-phosphate unit and cap oligosaccharide. In addtion polymorphism in the LPG structure has been observed to different extent in all the three genera. Interspecies differences have been observed in *Leishmania* (Turco and Descoteaux 1992) and *Trichomonas* (Singh 1993). Polymorphism may be present in the repeating units or the capped oligosaccharide structures.

Interstrain differences among pathogenic isolates of *E. histolytica* were found to be in LPG as reported from our laboratory, (Prasad et al.1992). This interpretation was based on the inability of an LPG-specific McAb, 2D7.10, to recognize pathogenic *E. histolytica* strains, 200:NIH and Rahman, whereas it recognized all the other pathogenic *E. histolytica* strains tested. It is likely that the absence of McAb, 2D7.10, binding may be due to absence of LPG in these strains. The results presented in this study and by Prasad et al.(1992) suggest that the non reactive strains do synthesize LPG-like molecules. The conclusion is based on the following experiments :

1. The Rahman glycoconjugate can be metabolically radiolabeled with [^{3}H]-labeled sugars and [^{32}P]orthophosphate and can be extracted using the same protocol used earlier.

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2. It is hydrophobic in nature as it binds to phenyl-Sepharose.

3. The Rahman glycoconjugate can be depolymerized with mild acid treatment indicating that it carries repeating sugar-phosphate groups just like a typical LPG molecule.

4. It can also be delipidified with PI-PLC and deaminated with nitrous acid suggesting that it contains a GPI anchored lipid tail.

All these features of Rahman glycoconjugate are exhibited by all LPG-like molecules so far identified. Thus it appears that LPG-like glycoconjugate is present in all strains of *E. histolytica*. However, the structural organization of the molecule may differ in each strain.

Different preparations of LPG of a given strain give slightly different SDS-PAGE patterns as indicated earlier (Bhattacharya et al. 1990). These variations may be due to the presence of a group of molecules rather than a single molecular species. Thus, minor variations in the patterns may not indicate any gross difference in the structure of LPG. However, SDS-PAGE profiles of LPG from strains HM-1:IMSS, 200:NIH and Rahman indicated that the Rahman glycoconjugate was very different from the other two. This was further confirmed by analyzing the product of the mild acid hydrolysis by paper chromatography. When HM-1:IMSS LPG was subjected to this analysis, no small (smaller than

pentasaccharide) neutral sugars could be detected as all the [^{3}H]galactose-labeled material stayed at the origin. Similar analysis of Rahman LPG showed distinct peaks, moving along with disaccharide and monosaccharide standards. This was also confirmed by separation on a DE-52 column followed by paper chromatography. The unbound fraction showed the same two spots at fraction six and fourteen. This suggests presence of relatively large proportions of the total carbohydrate groups as capped structures in Rahman. These observations suggest major structural differences between LPG derived fom the two pathogenic strains of *E. histolytica* HM-1:IMSS and Rahman. Major structural differences have been observed in different species of *Leishmania*, e.g. *L. donovani* and *L. major* (Jaffe et al. 1990). The LPG of *L. donovani* is essentially a linear molecule with a single capped structure whereas there are a number of branched structures present in *L. major*. Such differences in LPG among the different strains of the same species have not been reported. Moreover, multiple capped structures have also not been seen in other LPG.

Although the LPG of HM-1:IMSS (Stanley et al. 1992, Bhattacharya et al. 1992) shows major structural difference from that of Rahman LPG, antigenically both share common epitopes. This was evident from the ELISA and Western blot analysis using a polyclonal antibody raised against HM-1:IMSS LPG. The nonpolymorphic structural features may be

involved in essential functions carried out by this molecule. The role of LPG in pathogenesis of amoebiasis and\or colonization by *E. histolytica* has not yet been investigated, barring a single report indicating its involvement in target cell recognition (Stanley et al. 1992).

Similarly the recognition of Rahman LPG by human patient sera from New Delhi, at levels comparable to those of HM-1:IMSS, shows the geographical spread of the epitope present in Rahman. This is of interest as Rahman originated from a patient of invasive amoebiasis in Bangladesh whereas HM-1:IMSS was isolated in Mexico. These findings further strenghten the idea that all amoebic LPG may share common structural features which may be involved in host recognition.

SUMMARY

The protozoan parasite *Entamoeba histolytica* causes amoebiasis in humans. Though it is distributed all over the world, it is endemic in tropical areas. Over 10% of the world population is infected with *E. histolytica*. Most of amoebic infections are asymptomatic. However, about 10-15% of the infected individuals develop symptomatic amoebiasis leading to severe amoebic dysentery. In rare cases it invades extra intestinal organs, through the blood vessels, like liver, lungs and brain. It exists in two forms - the vegetative trophozoite and the infective cyst. *E. histolytica* has certain unusual pathways and lacks many of the enzymes, for example, lactate dehydrogenase, glycogen synthetase and catalase. It also lacks some key metabolic pathways such as a functional tricarboxyilic acid cycle. Basic information regarding the number of chromosomes, ploidy level and the organization of the chromosomal DNA are not available.

Several surface molecules have been identified and characterized in *E. histolytica*, including the 170 kDa galactose/N-acetyl galactosamine inhibitable lectin, which has been studied in much detail. A glucosamine and a poly N-acetyl glucosamine inhibitable lectins are also present. Besides surface protein/glycoproteins a novel glycoconjugate - lipophosphoglycan (LPG) has also been observed in *E. histolytica*. Existence of LPG on the surface of *Leishmania*

promastigotes is well documented. All the LPG molecules identified so far show some common features like - presence of a glycosyl-phosphatidylinositol (GPI) anchored lipid tail; core polysaccharide, repeating sugar-phosphate units and capped oligosaccharides and depolymerization by mild acid. Polymorphism in LPG has been observed in different genera and species.

An earlier study from our laboratory showed inter strain differences in *E. histolytica*. This was based on the inability of a monoclonal antibody (McAb) raised against the LPG of *E. histolytica* strain, HM-1:IMSS, to react with the strains, Rahman and 200:NIH. To determine if a structurally distinct LPG existed in Rahman, [^{3}H]sugar and [^{32}P]orthophosphate-labeled glycoconjugates were electrophoresed through Sodium dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic pattern in Rahman showed a single diffuse band while that of HM-1:IMSS and 200:NIH showed two distinct bands. A number of experiments including depolymerization with mild acid, deamination with nitrous acid and delipidification with phosphatidylinositol specific phospholipase C (PI-PLC) suggest that the Rahman glycoconjugate is indeed a LPG-like molecule but distinctly different from the LPG of HM-1:IMSS. Thus, while LPG molecules from the two strains share common epitopes, they are clearly distinct from each other.

Paper chromatography of mild acid-treated LPG from Rahman and HM-1:IMSS revealed the presence of neutral trisaccharides and monosaccharides in Rahman but not in HM-1:IMSS. This suggests that the Rahman LPG may contain large number of capped oligosaccharides. This was also confirmed by DE-52 ion exchange chromatography, where the unbound fraction also showed the same two saccharides on paper chromatography.

The crossreactivity of Rahman LPG with a polyclonal antibody raised against LPG of HM-1:IMSS was checked by ELISA and Western blots and with patient sera from New Delhi by ELISA. The results indicated that though structurally different the LPG from HM-1:IMSS and Rahman share common epitopes and may be involved in similar functions in the pathogenesis of amoebiasis.

In this work we have shown that the *E. histolytica* strain, Rahman does contain a LPG-like molecule which is different from LPG of HM-1:IMSS and 200:NIH. The LPG of Rahman contains neutral capped oligosaccharide structures. It cross reacts with a polyclonal antibody raised against LPG of HM-1:IMSS and also with human immune sera indicating the LPG from two species may not be absolutely different and may be involved in similar functions.

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