Review Article

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Glycobiology of *Leishmania donovani*

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***Leishmania donovani*, the causative organism of visceral leishmaniasis (VL) is one of the deadliest of the entire known *Leishmania* species. This protozoan parasite displays immense adaptability to survive under extremely harsh conditions. Cell surface glycoconjugates play a pivotal role in parasite virulence and infectivity. This review mainly highlights on the importance of these molecules and their reported roles with special emphasis on *L. donovani* sialobiology. The recently evolved information reported by our group regarding the identification and characterization of sialoglycans and their possible mode(s) of acquisition as also the detailed identification, characterization of anti-O-acetylated sialic acid (anti-OAcSA) antibodies and their emerging biological roles, notably as molecules that may aid in host defense against the pathogen has been vividly discussed in this review.**

Key words Anti-O-acetylated sialic acid antibodies - complement pathway - *Leishmania donovani* - lipophosphoglycan - O-acetylated sialic acid - phosphoglycan - proteophosphoglycans - sialic acid - visceral leishmaniasis

Leishmaniasis is endemic in about 88 countries and is responsible for the annual loss of 2.4 million disability adjusted life-years and over 59,000 deaths[1]. The visceral form of the disease is mainly caused by *Leishmania donovani*, *L. chagasi*, or *L. infantum*, and 500,000 new cases of visceral leishmaniasis (VL) occur each year[2]. Approximately 50 per cent of the world's cases of VL occur in the Indian subcontinent, and about 90 per cent of Indian patients with VL live in Bihar[3].

Considering the wide global distribution and consequent burden of leishmaniasis, research efforts are presently being directed towards discovering novel molecular determinants on the parasite surface. These molecules may help us in gaining an insight into their role in host pathogen interaction as also whether such interactions could be exploited to target parasite death.

Leishmania parasites while shuttling between intermediate carriers and vertebrate hosts encounter extremely harsh conditions and their survival strategies are designed to keep invaders at bay. Their survival strategies frequently involve the participation of glycoconjugates that form a protective barrier against hostile environment. In fact, a common feature of parasite cell surface architecture is the presence of an elaborate and highly decorative glycocalyx that allows the parasite to interact with and respond to its external environment. Cell membrane bound carbohydrates and sugars play a key role in parasite survival and proliferation. Most

of these specialized molecules are members of a family of phosphoglycans while others are a family of glycoinositol phosholipids[4].

Glycoconjugates of *Leishmania donovani*

Throughout their life cycle, *Leishmania* survive and proliferate in highly hostile environments and have evolved special mechanisms that enable them to endure these adverse conditions. To protect themselves from such harsh conditions one of the adaptive mechanisms includes the production of a dense cell surface glycocalyx composed of lipophosphoglycan (LPG), glycosylinositol phospholipids, or GIPLs[5,6] and secreted glycoconjugates, proteophosphoglycan (PPG)[7-9], and secreted acid phosphatase (sAP)[10-12].

Lipophosphoglycan (LPG)

The cell surface of leishmania promastigotes predominantly comprises of LPGs. It is localized over the entire parasite surface, including the flagellum. Found in all species of *Leishmania* that infect humans, it is composed of four domains, (*i*) a 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol lipid anchor, (*ii*) a glycan core, (*iii*) Gal(b1,4) Man (a1)-PO_4 backbone repeat units, and (*iv*) an oligosaccharide cap structure[13,14]. Structural analysis of LPG from different species has revealed complete conservation of the lipid anchor, the glycan core, and the Gal (β1, 4) Man (α1)-PO_4 backbone of repeat units. The distinguishing features of LPG are in the variations in the carbohydrate chains that branch off the main backbone and in the cap structures[15]. The C3 hydroxyl of the repeat unit Gal is the site of most side chain modifications. The LPG of *L. donovani* from Sudan does not possess any side chains, whereas the *L. donovani* LPG from India possesses one to two β-Glc every four to five repeat units[16]. The most common *L. donovani* cap is the branched trisaccharide Gal (β1,4) [Man (α1,2)] Man (α1).

LPG serves as the ligand for binding to lectins in the sandfly midgut, and thus structural variations correlate with infectivity and transmission by various sandfly species. Structural modifications are observed as the parasite progresses through various life stages. *L. donovani* attaches to its natural sandfly vector's midgut via the LPG cap structure, which terminates in a b-linked Gal and a-linked Man. Although both are required for binding, there is no information on the putative receptor or lectin. As the procyclic promastigotes undergo metacyclogenesis, the number of repeat units doubles from approximately 15 to 30[17]. This is believed to result in a conformational change that masks the terminal cap sequence and thus allows the parasite to detach from the midgut and migrate anteriorly.

The number of LPG molecules expressed by the intracellular amastigotes is substantially downregulated. A number of functions have been implicated for LPG in the mammalian host. In the blood stream, LPG prevents complement-mediated lysis by preventing insertion of the C5b-9 membrane attack complex into the promastigote membrane. It serves as a ligand for receptor-mediated endocytosis by the macrophage via complement receptors as well as the mannose receptor. Inside the macrophage LPG inhibits protein kinase C and the microbicidal oxidative burst as well as phagosome-endosome fusion[14] (Table I).

GIPLs

The GIPLs are a major family of low molecular weight glycolipids synthesized by Leishmania parasites, which are not attached to either proteins or polysaccharides[18-20,4]. These are expressed in very high copy numbers, approximately 10^7 copies per cell on both promastigote and amastigote surfaces. There are three major lineages of GIPLs that are expressed to different levels in different species or developmental stages. Based on the pattern of their glycan head groups they are classified as type I (analogous to protein GPI anchors and based on the structure Manα1,6 Manα1,4GlcNα1,6-PI), type II (analogous to LPG anchors and based on the structure Manα1,3Manα1,4GlcNα1,6-PI), or hybrid (contains features of both and based on the Manα1,6(Manα1,3)Manα1,4GlcNα1,6-PI motif). The lipid components of the hybrid and type I GIPLs are rich in alkyl-acyl-PI with shorter (C18:0) alkyl chains. The type II GIPLs are more

Table I. Major glycoconjugates of *Leishmania donovani*: occurrence and possible biological roles

Glycoconjugates	Promastigote	Amastigote	References
LPG (Structure composed of phosphatidyl(myo)inositol lipid anchor, glycan core, Gal(β1,4)Man(α1)-PO_4 backbone repeat units oligosaccharide cap structure)	Number of LPG molecules bear direct correlation with infectivity, during metacyclogenesis the carbohydrate repeat units doubles from approx 15 to 30. LPG prevents complement-mediated lysis of the promastigote and serves as a ligand for receptor-mediated endocytosis by the macrophage	Number of LPG down-regulated. LPG of amastigotes inhibits protein kinase C and the microbicidal oxidative burst as well as phagosome-endosome fusion	13, 14, 17
GIPLs (Protein free glycolipids)	Present. Have a role in macrophage invasion	Major constituents of the amastigote surface. Involved in modulating signaling events in the macrophage such as NO synthesis and the oxidative burst.	4, 18, 19, 20, 21, 22, 23, 24, 25
gp63 (Glycoprotein, zinc metalloprotease)	Major cell surface glycoprotein found on entire surface. Serves as a ligand for the macrophage receptor via complement components protect against complement mediated lysis.Proteolytically cleaves host macromolecules	Expressed in lower level found in the flagellar pocket	26, 27, 28, 29
sAP (glycosylated proteins)	Secreted from the flagella	Not reported	7, 8, 10, 30, 31, 32
PPG (Proteophosphoglycans)	GPI-anchored cell associated filamentous form termed mPPG The gel-like matrix, formed by these interlocking filaments, traps the parasites in the sandfly anterior gut	Secrete their own non filamentous form termed aPPG.aPPG is believed to contribute to the formation of the parasitophorous vacuole, thus participating in the maintenance of infection.Activate the complement system via the mannose-binding pathway	8, 9, 11, 32, 33, 34, 35, 36
Phosphoglycan (hydrophilic phosphoglycan consisting of capped oligosaccharide repeat units but minus the GPI anchor and the glycan core	Present function not yet defined.	Not reported	13
Sialoglycans (sialic acid derivatives)	Sialic acid derivatives present, but Neu 5Gc absent the O acetylated forms activate the classical complement pathway.	Sialic acid derivatives present. Neu5Gc present. Role not yet known.	37, 38, 68

LPG, lipophosphoglycans; GIPLs, glycosylinositol phospholipids; gp63, glycoprotein63; sAP, secreted acid phosphatase; GPI,glycosylphosphatidylinositol

heterogeneous and contain longer alkyl chains (C24:0 or C26:0).

Not much is known about the functions of the GIPLs. The use of the mannose receptor in parasite attachment to the macrophage suggests that the mannose-rich GIPLs may play a role in macrophage invasion. Because the levels of LPG and the major promastigote surface protease, gp63, are dramatically downregulated, the GIPLs are the major constituents of the amastigote surface and are presumably involved in protecting the parasite from

environmental hazards as well as playing some role in parasite-host interactions, especially in the mammalian stage. In fact, there is evidence that GIPLs are involved in modulating signaling events in the macrophage such as NO synthesis and the oxidative burst[21-24]. There are recent data to show that enzymes involved in glycosylphosphatidylinositols (GPI) biosynthesis are essential for parasite virulence[25], thus emphasizing the importance of protein-free GPI glycolipids in parasite viability.

gp63

gp63 is the major cell surface glycoprotein of *Leishmania* promastigotes with 500,000 copies per cell and accounting for 1 per cent of all cellular proteins. In amastigotes gp63 is expressed to a lower level, and the bulk of it is found in the flagellar pocket as opposed to covering the entire surface, as in promastigotes[26]. It is a 63-kDa zinc metalloprotease and is anchored to the cell surface via a myristic acid containing gpI anchor. The amastigote gp63 subpopulation found in the flagellar pocket lacks a membrane anchor. The crystal structure has been solved and found to contain an active site structural motif found in other zinc proteases that may aid design of specific inhibitors[27]. Primary sequence analysis has shown that gp63 contains three potential glycosylation sites[28]. The glycans are biantennary high mannose-type, and some bear a terminal Glc in α1,3 linkage. The two major structures found in all promastigote species examined are $Man_6GlcNAc_2$ and $GlcMan_6GlcNAc_2$. In amastigotes the structures are more variable, and in *L. donovani* there appear to be no N-linked glycans. The presence of terminal Glc in the gp63 glycan is highly unusual with respect to oligomannose structures found in glycoproteins. Whether the stage-specific changes in glycan structure affect parasite infectivity and development is unknown.

The importance of gp63 in parasite life cycle is not well defined. gp63 has been shown to be proteolytically active against a number of substrates and thus may be involved in degradation of host macromolecules. It may also serve as a ligand for the macrophage receptor via complement components and protect the parasite against complement mediated lysis[29].

Secreted glycoconjugates

In addition to cell surface LPG and GIPLs, *Leishmania* secrete a family of heavily glycosylated proteins and proteoglycans that are important for parasite virulence. Most of these express glycans that are similar in structure to those found on LPG, notably the Gal-Man-PO_4 repeat unit motif. The structural features of secreted acid phosphatase, phosphoglycan and proteophosphoglycan are briefly outlined below[30].

sAP: With the exception of *L. major*, all *Leishmania* promastigotes secrete sAP from the flagellar pocket, their chief secretory organelle[10,31,32]. The secreted glycoproteins and proteoglycans tend to form distinct macromolecular complexes found both in the flagellar pocket as well as the culture media. Old World species, such as *L. donovani*, *L. tropica*, and *L. aethiopica* secrete mono- or oligomeric sAPs, whereas the South American species, such as *L. mexicana*, *L. braziliensis*, and *L. amazonensis* secrete sAPs that aggregate into large pearl-like filamentous polymers[7,8,30]. The sAPs are encoded by multiple genes that have very high levels of sequence identity, even within different species. The *L. donovani* sAP peptides are heavily glycosylated on C-terminal serine/threonine-rich domains. The glycans are phosphodiester-linked to serine residues and commonly consist of the 6Gal(β1,4)Man (α1-)PO_4 repeat units found on LPG. The average number of repeat units is 32. The target sites of phosphoglycosylation are not random, rather, they are composed of repetitive motifs, with modifications on select serine residues.

PPG

Proteophosphoglycans (PPG) identified to date in promastigotes include filamentous PPG or fPPG and a putative GPI-anchored cell-associated form or mPPG[8,11,32]. Amastigotes secrete their own nonfilamentous and stage-specific form termed aPPG[33]. The filamentous form, fPPG, is secreted by promastigotes of all *Leishmania* species and forms a highly viscous mesh within which the parasites lie embedded. Compositionally fPPG consists of 95 per cent phosphoglycans, with an abundance of serine, alanine, and proline in the peptide component. Over

80-90 per cent of the serine residues are phosphoglycosylated with short Gal-Man-PO_4 repeats attached via phosphodiester bonds, which are terminated by small oligosaccharide cap structures[9]. Although there is no direct evidence for the function of fPPG, it is believed that the gel-like matrix, formed by the interlocking filaments, traps the parasites in the sandfly anterior gut. Further, it has been hypothesized that the presence of the parasite plug deters the ingestion of a second blood meal, thereby encouraging the sandfly to probe several hosts and in the process improve the chances of transmission[34].

Within the macrophage, aPPG is believed to contribute to the formation of the parasitophorous vacuole, thus participating in the maintenance of infection in the mammalian host[35,36]. Amastigote PPG is believed to activate the complement system via the mannose-binding pathway by virtue of the large number of potential mannose-binding lectin-binding sites. There is also evidence that PPG may contribute to the binding of *Leishmania* to host cells and may play a role in modulating the biology of the infected macrophage at the early stage of infection.

Phosphoglycan

Culture supernatants of *Leishmania* promastigotes contain a hydrophilic phosphoglycan consisting of capped oligosaccharide repeat units identical to those found on LPG, but minus the GPI anchor and the glycan core[13]. The structure precludes the possibility of phosphatidylinositol-specific phospholipase C (PI-PLC)-mediated release from LPG, rather it is thought to have been released from the flagellar pocket via exocytosis.

Sialoglycans on *L. donovani*

The topography of *Leishmania* parasites with regard to their sialoglycan profile remains a poorly investigated area and it is only recently that the status of sialoglycans on *L. donovani* promastigotes as well as amastigotes has been reported from our laboratory[37,38].

Sialic acids typically present as terminal residues on glycoproteins and glycolipids are known to play a significant role in the mediation of many biological phenomena involving cell-cell and cell-matrix interactions by either reacting with specific surface receptors or masking other carbohydrate recognition sites[39,40].

Sialic acids are a structurally complex family of nine-carbon polyhydroxy amino ketoacid of N- and *O*-substituted derivatives of neuraminic acid, a monosaccharide commonly referred to as N-acetylneuraminic acid or Neu5Ac[41]. It is the most abundantly available monosaccharide present as the terminal residue of cell surface sugar chains. Its strategic terminal position provides it accessibility, reflected in its regulation of a multitude of cellular and molecular interactions[39]. Over 50 different modifications of sialic acid are generated following substitution of the amino group by an acetyl or glycolyl group and one or more hydroxyl groups by methylation or esterification with acetyl, lactyl, sulphate or phosphate groups[40].

However, the most frequently occurring modification (over 18) is *O*-acetylation at position C-7/8/9 to form *N*-acetyl-7/8/9-O-acetylneuraminic acid respectively generating a family of *O*-acetylated sialoglycoconjugates or *O*-AcSGs[41].

To address the status of sialoglycans on the protozoa, several analytical, biochemical and Immunological methods were performed in our laboratory. Interestingly, The chromatogram of the parasite promastigote exhibited well-resolved peaks that coincided with that of sialic acid and one, co-migrating with 9-Oacetylated sialic acid, which resembles 7.7 per cent of total sialic acids. The cells contained about 800 ng of sialic acid in 2 x 10^9 cells corresponding to 7 x 10^5 molecules of sialic acid per cell (Table II). The total amount of sialic acids and derivative quantified on the amastigotes was 658 ng/ 1.6x10^9 cells of which the majority is only sialic acid corresponding to 385ng. The number of copies of sialic acid residues per cell was thus found to be 12.8 x 10^5. This data were further confirmed with mass spectrometry analysis of trimethylester derivatives of the parasite promastigote sample, sialic acid was clearly detected in the parasite as evident from the mass fragmentography of sialic acid having

Table II. Quantitative analysis of sialic acids on *Leishmania donovani* by high pressure liquid chromatography (HPLC) analysis

Derivative	Promastigotes*	Amastigotes**
	ng/$2x10^9$ cells	ng/$1.6x10^9$ cells
Number of sialic acid molecules/ cells	$7x10^5$	$12.8x10^5$
Neu5Gc	Could not be detected	175
Neu5Ac	800	385
Neu5Gc9Ac	Could not be detected	12
Neu5Ac9Ac	7.7 per cent of total sialic acid	4

*Adapted from Chatterjee *et al, Glycobiology*, 2003 (Ref. 37)
**Adapted from Chava *et al, J Biol Chem*, 2004 (Ref. 38)

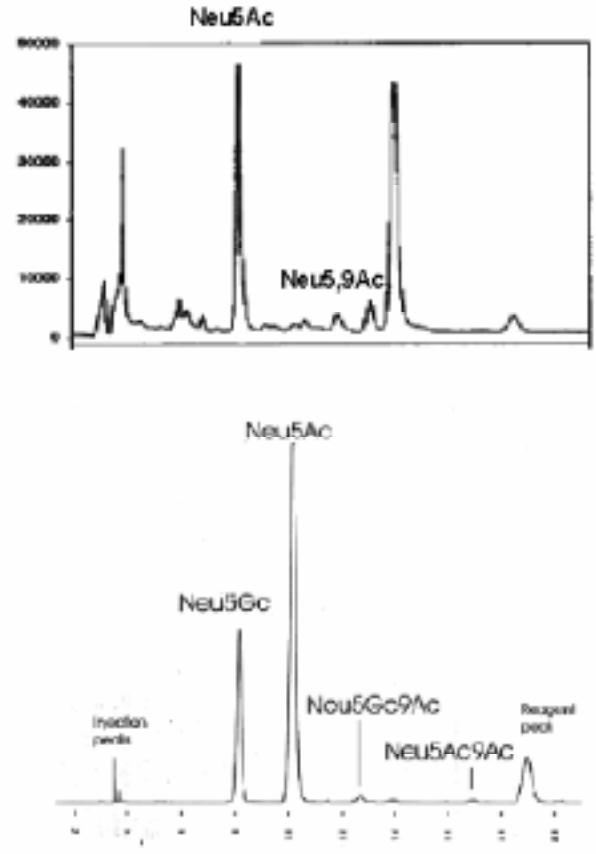

Fig. 1. Fluorimetric high performance liquid chromatography (HPLC) analysis of sialic acid (Neu5Ac) and its derivatives on *Leishmania donovani* promastigotes and amastigotes.
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fragment ions (m/z) at 668, 624, 478, 400, 317, and 298, respectively[37]. Mass spectrometric analysis also confirmed the presence of sialic acids on amastigotes by mass fragmentography, principally being sialic acid and Neu5Gc showing fragment ions (m/z).

Although the presence of Neu5Ac and Neu5Gc was detectable on *L. donovani* amastigotes both by HPLC and mass spectroscopy, Neu5Gc was not observed on promastigotes by similar analysis[37]. Although Neu5Gc is a major sialic acid derivative in most mammals (including our closest evolutionary relatives, the great apes[42]), it is thought to be absent in healthy humans[43]. Considering the wide differences in Neu5Gc expression in certain parasitic diseases[44], it is important to check the status of this sugar and its functional relevance in *L. donovani*; such studies are ongoing. HPLC analysis also demonstrated that amastigotes have a 2.0 fold higher copy number of Neu5Ac than promastigotes[38] (Fig. 1).

The surface density of sialoglycoconjugates present on *L. donovani* promastigotes was examined further by flow cytometric analysis using two sialic acid-binding plant lectins, Sambucus nigra agglutinin (SNA) and Maackia amurensis agglutinin (MAA), that recognize α2-6 and α2-3 sialylgalactosyl residues, respectively[45,46]. Low binding with MAA and high amounts of SNA binding indicated the predominance of α2-6 linked sialylglycotopes on the parasite promastigote. Corroborative evidence for the presence of α2-3 and α2-6 linked sialoglycans on the *L. donovani* promastigotes was provided further by the binding of various recombinant sialic acid binding lectins (Siglecs). Siglecs, members of the immunoglobulin superfamily, bind to sialic acids and are mainly expressed by cells of the haematopoietic system[47]. Although the binding pattern appeared complex, most Siglecs tested showed some degree of binding (Table III). Siglecs exhibit widely differing preferences for sialic acid linkage to subterminal sugars. For example, CD22/Siglec-2 binds only to α2-6-linked sialic acids, whereas sialoadhesin/Siglec-1 prefers α2-3-linked sialic acids and Siglec-5 binds both linkages. The presence of α2-6 linked sialic acids on the parasite cell surface was consistent with their

Table III. Lectin binding patterns of *Leishmania donovani*

Probe	Linkage specificity	Occurrence	Binding (%)	
			Promastigotes	Amastigotes
SNA	α 2-6	*Sambucus nigra* agglutinin (SNA)	72.0 ± 14.3	87.7 ± 6.5
MAA	α 2-3	*Maackia amurensis* agglutinin (MAA)	41.0 ± 6.3	27.24 ± 3.4
Achatinin-H	9-*O*AcSA α 2-6 GalNAc	*Achatina fulica* snail	44.3 ± 3.4	49.3 ± 4.5
Siglec-1	α 2-3 > α 2-6	Macrophage	17.3 ± 3.7	48.0 ± 2.7
Siglec-2	α 2-6 >> α 2-3	B cell	50.5 ± 11.4	54.0 ± 3.5
Siglec-5	α 2-3 = α 2-6	Neutrophils, myeloid cells	31.4 ± 3.7	60.0 ± 2.6
Siglec-7	α 2-6 > α 2-3	NKcells, monocytes	19.0 ± 4.3	61.0 ± 3.1
Siglec-8	α 2-3	Eosinophils	28.65 ± 4.2	65.0 ± 3.7
Siglec-10	α 2-3 = α 2-6	Myeloid cells	32.5 ± 1.5	40.0 ± 2.2

Mean ± SD of positive cells as determined by flow activated cell sorter (FACS) analysis using sialic acid binding probes
Adapted from Chava *et al*, *J Biol Chem*, 2004 (Ref. 38)
NK, natural killer

binding to Siglec-2/CD22, known to require sialic acids α2 α6 linked to Galb1 a 4GlcNAc sequences for recognition[48]. Further, the lower binding with Siglec-1 and Siglec-8 that prefer α2-3-linked sialic acids points toward the predominance of α2-6 linked sialic acids. Taken together, these results support the idea that sialic acids, both α2-3 and α2-6 linked, are present on *Leishmania* promastigotes.

A similar pattern of binding was also observed with *Leishmania* amastigotes. In general, the binding of siglecs was 2-3 fold higher in amastigotes than promastigotes. More importantly, siglec 1 which is abundantly present on macrophages showed an almost three-fold higher binding with amastigotes than promastigotes. It remains to be investigated whether these sialoglycans are playing a major role in the infectivity and intracellular survival of the parasite.

Molecular characterization of sialoglycans present on the promastigote parasite surface, was examined by their reactivity towards two plant lectins, SNA and MAA by western blotting. Using SNA, the presence of three sialoglycoproteins corresponding to 123, 90, and 70 kDa were identified on parasite membranes. As compared to 90 and 70 kDa, the expression of 123 kDa was much weaker. In case of MAA, five sialoglycans were identified that corresponded to 130, 117, 106, 70, and 61 kDa[37] (Fig. 2A).

Western blotting of amastigote membrane glycoproteins with SNA demonstrated the presence of two sialoglycoconjugates 164 kDa and 150 kDa. Similarly, binding of MAA demonstrated the presence of five distinct sialoglycans corresponding to molecular masses of 188, 162, 136, 137 and 124 kDa. Interestingly, the sialoglycans adsorbed from serum onto promastigotes are different from those present on amastigotes. This raises the possibility that during transformation to the amastigote form, parasites acquire a new array of sialoglycans onto their surface (Fig. 2B).

Futher, to examine the possible presence of *O*-Acetylated derivative of sialic acid on the cell surface of *L. donovani* promastigotes and amastigotes Achatinin-H, a snail lectin with defined specificity towards 9-*O*-AcSA, was used as a probe. The selective binding of Achatinin-H with promastigotes was observed through agglutination, enzyme linked immunosorbant assay as also by flow cytometric analysis (Fig. 3). The presence of 9-*O*-acetylated determinants on *L. donovani* promastigotes was also confirmed using the CD60b-specific monoclonal antibody UM4D4, whose epitope has been, defined as 9-*O*-acetylated ganglioside GD3 and related structures[49].

To further characterize the *O*-acetylated sialoglycoproteins present on *L. donovani* promastigote membranes, western blotting was

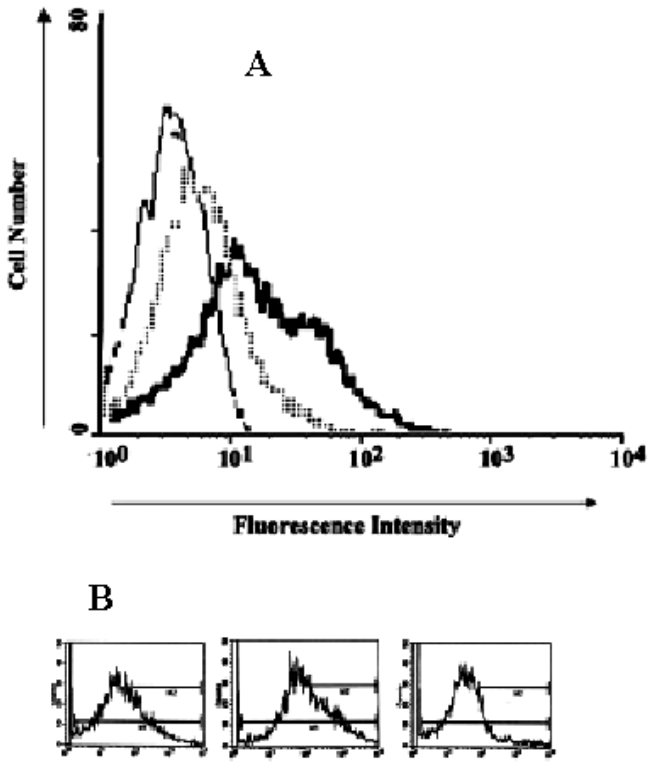

Fig. 2. Flow cytometric analysis of cell surface sialoglycans on *Leishmania donovani* promastigotes (**A**) using Achatinin-H before (light gray dashed line) and after esterase treatment (bold line) as compared to control (solid thin blak line) and amastigotes (**B**) control vs using Achatinin-H before and after esterase treatment.
[Reproduced from (Ref. 37 & 38) with permission of the publishers, Oxford University Press and Walter de Gruyter GmbH & Co press].

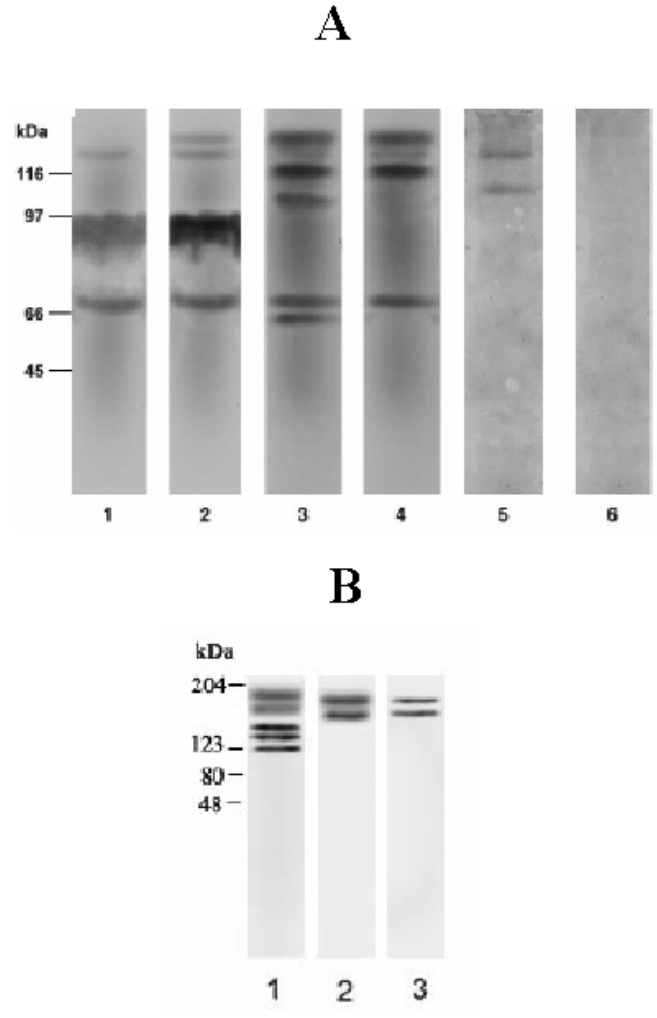

Fig. 3. Western blot analysis of sialoglycoproteins present on *Leishmania donovani* (**A**) Molecular characterization of sialoglycoproteins present on *L. donovani* promastigotes were electrophoresed (7.5% SDS PAGE) and following transfer onto nitrocellulose membranes were incubated with SNA (lane 1) or MAA (lane 3) or Achatinin-H (lane 5). Similarly Western blot was carried out to demonstrate the binding of SNA (lane 2), MAA (lane 4) and Achatinin-H (lane 6) to medium M199 containing 10 per cent fetal calf serum. (**B**) Amastigote membrane was electrophoresed (10%SDS PAGE) and transferred onto nitrocellulose. The membranes were incubated with MAA (lane 1) or SNA (lane 2) or Achatinin-H (lane 3). [Reproduced from (Ref. 37 & 38) with permission of the publishers, Oxford University Press and Walter de Gruyter GmbH & Co press].

performed. Achatinin-H bound to two *O*-acetylated sialoglycoproteins corresponding to 123 and 109 kDa. Similarly, two O-acetylated sialoglycoproteins corresponding to 164 and 150 kDa could be detected on the amastigote cell surface upon blotting with Achatinin-H[37,38] (Fig. 2A, B).

Biosynthetic pathways of glycoconjugates

Leishmania synthesize a range of mannose-rich glycoconjugates that form the cell surface glycocalyx or are secreted. These glycoconjugates comprise GPI-anchored glycoproteins, the GPI-anchored lipophosphoglycan, the glycoinositolphospholipids (GIPLs) and the PPGs described above. Biosynthesis of all these macromolecules depends directly or indirectly on the availability of GDP-mannose. As mentioned above, several of these molecules are considered virulence factors, and parasites lacking them cannot survive in macrophages or mice.

A prerequisite for the biosynthesis of glycoconjugates in *Leishmania*, like in other eukaryotes, is the conversion of monosaccharides to activated sugar nucleotides and dolicholphosphate derivatives. The activation of mannose involves phosphomannomutase (PMM), GDP-Man pyrophosphorylase (GDP-MP) and dolicholphosphate-Man synthase (DPMS). GDP-MP is a critical enzyme in the mannose biosynthetic pathway. The consecutive action of PMM and GDP-MP transforms Man-6-PO_4 to GDP-Man which is essential for glycoconjugate synthesis in eukaryotes. The gene encoding GDP-MP is a single copy gene expressed in both parasite life cycle stages. Deletion of the GDP-MP leads to the loss of virulence as reflected by survival in macrophages or mice.

The first distinct step in GPI biosynthesis is the generation of N-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI) from UDP-GlcNAc and specific PI substrate (different PI pools are used for GPI anchor, LPG and GIPLs pathway in the parasite) catalyzed by the GPI-N-GlcNAc-transferase (GPI-GnT), the GlcNAc-PI is then N-deacylated to form glucosaminyl phosphatidylinositol (GlcN-PI). This has been proposed that the synthesis of GlcN-PI occurs on the cytoplasmic side of endoplasmic reticulum (ER) and then these intermediate GlcN-PI and/or GlcN-acyl-PI translocate to the luminal side (mediated by a putative "flippase") where first three mannosyl residues are transferred from Dol-P-Man[50].

The most striking feature of LPG structure is the variable phosphoglycan (PG) domain composed of [6-Gal (β1, 4) Man (1α-PO_4) repeats linked together by phosphodiester groups. The PG repeats are signature motif of phosphoglycan family of molecules expressed both in the promastigote (lipid linked phosphoglycan such as LPG) and amastigote (protein linked phosphoglycan PPG) phase of the parasite. The biosynthesis of PG repeats occurs inside the golgi (after the pre-assembled GPI core is translocated from ER to golgi) and involve a set of putative initiating and elongating Man-1α-PO_4-transferases (iMPT and eMPT respectively). These MPTs are unique to *Leishmania* parasite and are capable of transferring intact Man-a-phosphate (and not just the Man) from the GDP-Man nucleotide sugar donor. Interestingly a unique GDP-Man transporter (GMP antiporter) has recently been identified in *Leishmania* golgi vesicles[51]. The biosynthetic assembly and trafficking of PG repeats and involvement of unique MPTs and GDP-Man-transporter are interesting target for synthesis, conformation and inhibitor design[51,52].

Detection of these sialic acids raises the obvious question regarding the mechanism(s) adopted by the parasite to acquire these terminal sugar molecules. No biosynthetic machinery for sialic acid has been elucidated in *Trypanosoma* parasites possessing sialic acids on their cell surface[53]. Barring a few bacteria, biosynthesis of sialic acids is restricted to multicellular organisms, the key enzyme being UDP-GlcNAc 2-epimerase, which catalyzes the first step of this pathway and shows a strong feedback inhibition[54]. Therefore, it follows that, if *L. donovani* should have its own sialic acid biosynthesis, expression of UDP-GlcNAc 2-epimerase activity would be necessary. The accumulated data from our laboratory clearly showed that *L. donovani* has no UDP-GlcNAc 2-epimerase activity and consequently does not possess a machinery for sialic acid biosynthesis[37]. Trypanosomal parasites possess trans-sialidases, which enable them to transfer glycosidically linked sialic acids from the environment (*e.g.*, serum sialoglycoconjugates onto parasite surface molecules)[55]. However, among *Leishmania* species, the presence of such trans-sialidases has not been demonstrated[56]. The presence of serum trans-sialidases is still a matter of debate, and it would be interesting to analyze whether such trans-sialidases are operative in leishmaniasis, accounting for parasite sialylation. Alternatively, another approach that the parasite might well utilize is ecto-sialyl transferases or serum sialyl transferases that would catalyze the transfer of sialic acid from the nucleotide sugar donor CMP-sialic acid onto acceptor glycoconjugates[57]. However, such enzymatic reactions would require the presence of CMP-sialic acid, whose presence in serum of VL patients is yet to be substantiated. Another option that the parasite may adopt is to acquire sialic acid from the growth medium either by transglycosylation or by incorporation of serum components to the

parasite polyanionic lipophosphoglycan, LPG/ proteophosphoglycan- rich cell surface[58,59]. However, this maybe ruled out because the Western blotting showed discrete glycoprotein bands and not a smear (10 to 60 kDa) characteristic of lipophosphoglycan[60]. Parasitologists are limited by lack of availability of genomic data on protozoa, and therefore the search for genes possibly involved in the biosynthesis, activation or transfer of sialic acids in protozoa still remains unanswered[40]. Our studies indicate that there is a direct transfer, that is, adsorption of certain sialoglycoproteins from culture medium onto the parasite surface, as binding of SNA and MAA to parasite membranes and culture medium demonstrated the presence of analogous sialoglycans[37]. This was reconfirmed with the decreased binding of SNA and MAA to parasites when cultured in decreasing concentrations of FCS. Our investigations therefore demonstrate that the parasite is "borrowing" sialoglycans from the culture medium through simple adsorption to possibly compensate for the deficient sialic acid[37]. It may be speculated that incorporation of serum sialoglycoconjugates onto the parasite surface might be related to the membrane architecture, and they are accordingly adsorbed under different stimuli or stress conditions[57]. However, what remains to be investigated is whether these adsorbed components of FCS are transferred wholly or partially following fragmentation by cellular enzymes or are degraded otherwise.

Humoral responses of glycoconjugates of *L. donovani*

Carbohydrate epitopes in parasitic infections are also known to produce profound humoral response in the host. The overall importance of glycan-specific antibodies in protection against infection or in the pathological nature of the infection is still not clear, and only a relatively modest amount of information is available about specific structures or expression of relevant glycan antigens. This section of the review highlights some of attempts made from our laboratory in discovering antibodies directed against these sialoglycotopes which can cause complement mediated death of the *Leishmania* promastigote.

Antibodies directed against sialoglycans, their importance and role

Antibodies directed against carbohydrate epitopes are reported to be present in the human host in high titres. VL patients contain elevated levels of IgM and IgG antibodies directed against 9-*O*acetylated sialoglycoconjugates (9-*O*-AcSG)[61]. These affinity purified antibodies bound to 9-*O*AcSGs epitopes of *L. donovani* promastigotes as detected through Immunoflurescence microscopy. Remarkably, immunofluorescence microscopic analysis revealed a strong binding of this purified IgM with the cytosol of *L. donovani* promastigotes. This binding appears to be specific, because Achatinin-H, a snail lectin with binding specificity toward 9-*O*-AcSA a2,6GalNAc, also bound to promastigote cytosolic glycotopes, and the binding of anti-*O*-AcSGIgM is decreased after preincubation with Achatinin-H[61] (Fig. 4). Because these purified anti-*O*-AcSG antibodies showed significant binding to *L. donovani* promastigotes, the biological relevance of these disease-specific antibodies was next investigated. As it is known that enhanced levels of *O*-AcSA glycotopes can be correlated with an increased sensitivity towards complement lysis. Therefore, the biological relevance of these antibodies, with regard to activation of the classical complement pathway (CP), was investigated[62].

The disease process of leishmaniasis is initiated by promastigote inoculation into the host macrophage[62], whereby the host defense responds by activating its complement system, culminating in cleavage of the third complement component (C3) and followed, ultimately, by the lytic pathway[63-65]. This promastigote-C3 opsonization is mediated mainly by 3 pathways—namely, the classical pathway (CP), the alternate pathway (AP), and the lectin-mediated pathway, leading to the formation of a cytolytic membrane attack complex (C5b-9). The involvement of anti-leishmanial IgM, a complement activator minimally present in normal human serum (NHS), causes parasite agglutination, CP activation, and parasite killing[66,67,] Additionally, parasite-specific IgG induces lysis of *Leishmania* and *Trypanosoma* organisms[67]. Anti-*O*-AcSGs, even under normal physiological conditions, could trigger CP mediated

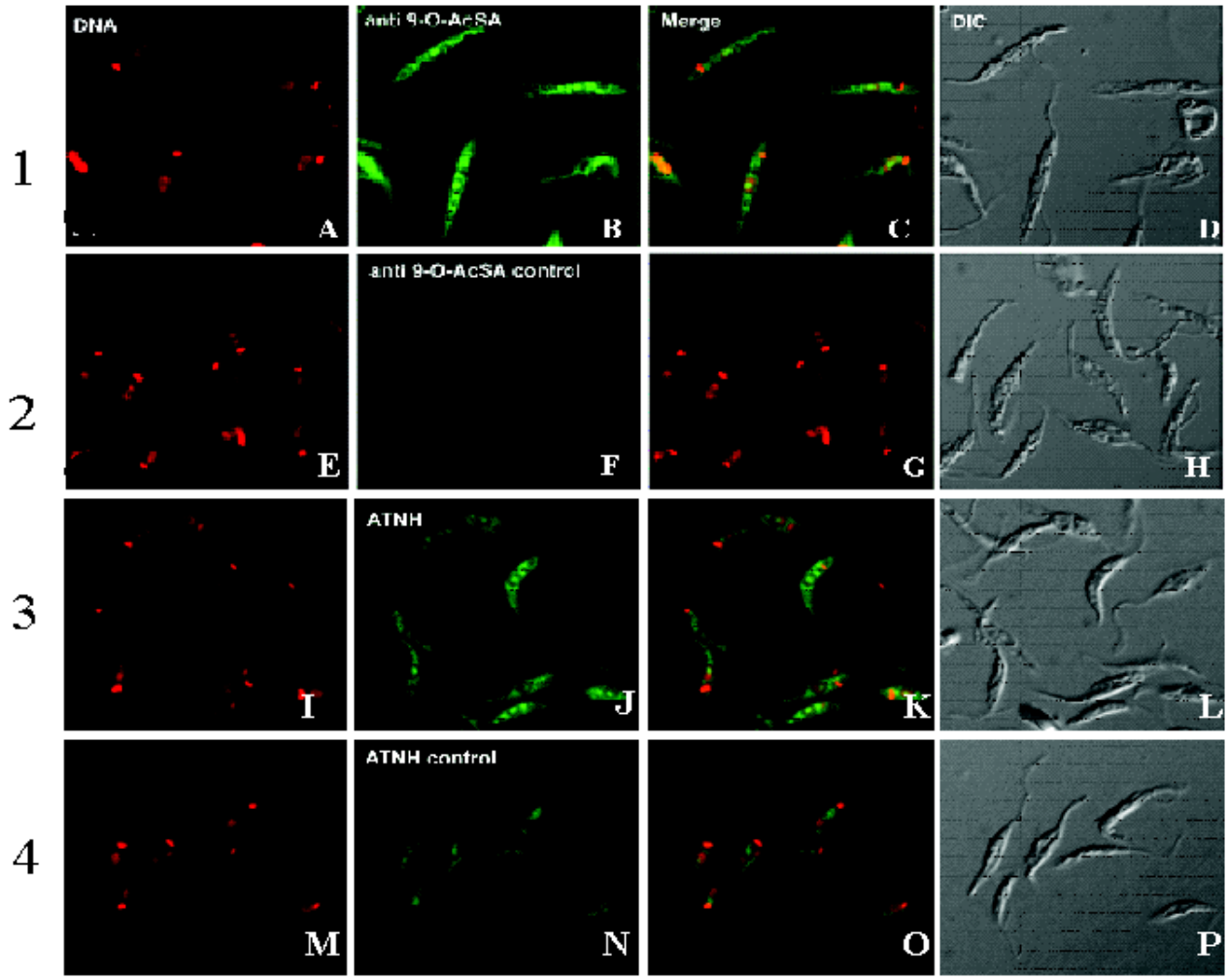

Fig. 4. Fluorescence microscopic analysis of anti-*O*AcSG IgM binding to *Leishmania donovani* promastigote. (Panel 1) Represents binding of anti-*O*-AcSG IgM with *L. donovani*. **A**, Red fluorescence represents DNA binding. **B**, Green fluorescence represents anti-*O*-AcSG binding. **C**, The merged image represents the localization of anti-*O*-AcSG binding in promastigotes. **D**, Represents the differential interference contrast (DIC) image of the cells in view. (Panel 2) Control set for anti-*O*AcSG binding. Cells were preincubated with Achatinin-H, whose lectinogenic glycotope has been previously defined as 9-*O*-AcSA, linked to subterminal GalNAc through an α2-6 linkage, followed by addition of FITC-labeled anti-*O*-AcSG. The (**E**) red and (**F**) green fluorescence represents DNA and anti-*O*-AcSA binding, respectively. **G**, The merged image represents the localization of anti-*O*-AcSA binding in promastigotes. (**H**) represents the DIC image. (Panel 3) Represents binding of Achatinin-H with *L. donovani*. **I**, Red and (**J**) green fluorescence represents DNA and Achatinin-H binding. **K**, The merged image represents the localization of binding of Achatinin-H in promastigotes. **L**, Represents the DIC image. (Panel 4) The control set for Achatinin-H binding. **M**, Red and (**N**) green fluorescence represents DNA binding and Achatinin-H binding. **O**, The merged image represents the localization of Achatinin-H binding in promastigotes. **P**, represents the DIC image.
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C3 (CP-C3) deposition on promastigotes, causing their lysis, whereas other complement pathways were demonstrated to play a negligible role (Fig. 5).

Anti-*O*-AcSGs from both healthy donors and patients with VL elicited C3 deposition as early as 3 min, which triggered parasite lysis, as demonstrated by use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and corroborated by the high rate of uptake of PI[62] (Fig. 6). Analysis of complement activation by mannan-binding lectin and C-reactive protein demonstrated their negligible contribution during the 3-min time frame. Anti-*O*-AcSGs were thus

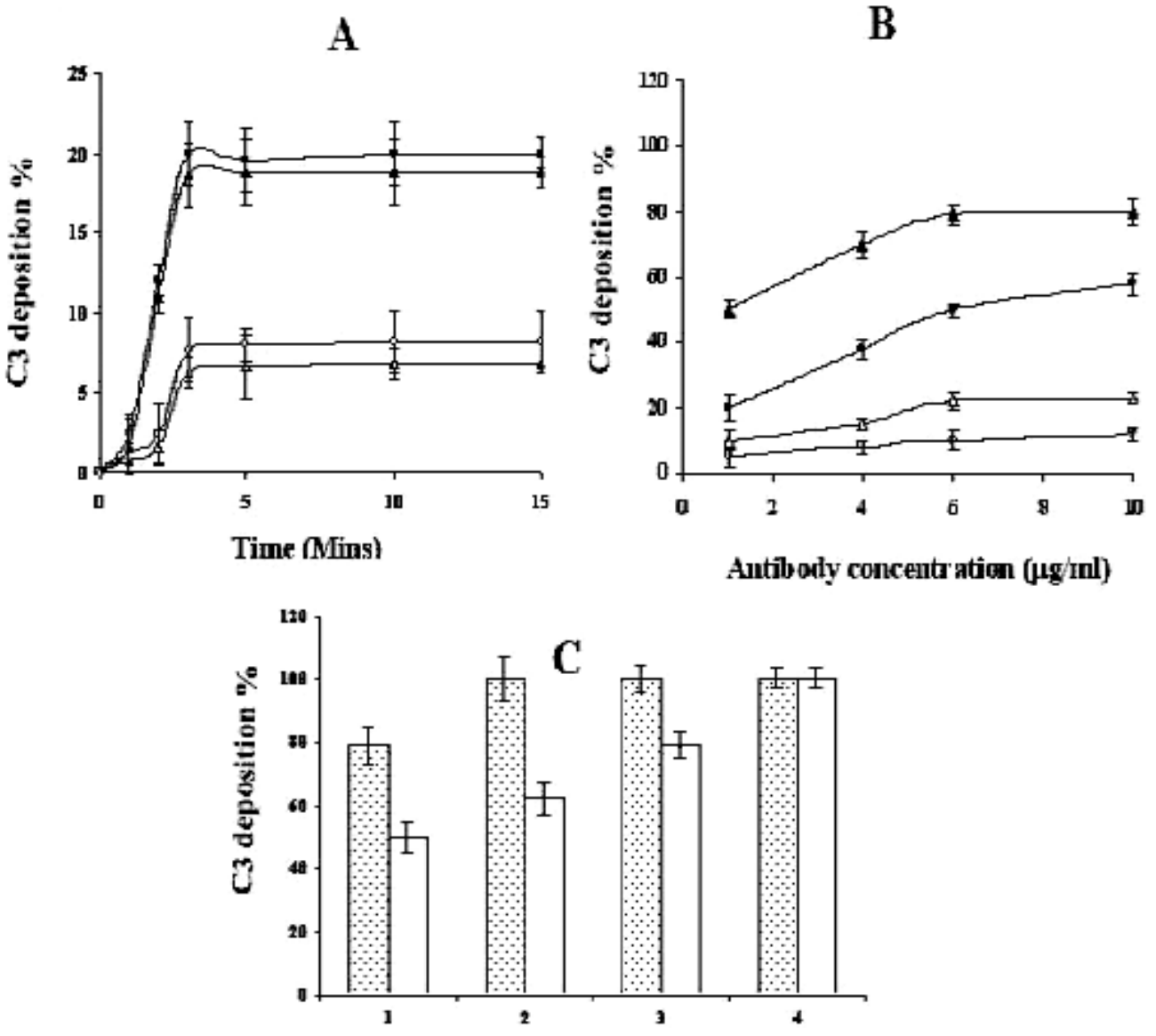

Fig. 5. Classical complement pathway activation induced by anti–*O*-acetylated sialoglycoconjugate (AcSG)normal human serum (NHS) vs. that induced by total antibodyNHS. The parasites were incubated in presence of Ads-NHS (25%) as a source of complement and several complement activators. The amount of C3-deposition was measured by using ^{125}I-anti-C3mAb. (**A**) A fixed concentration (6 µg/ml) of different antibodies namely, Anti-*O*-AcSGIgM$_{NHS}$ (l), anti-*O*-AcSGIgG$_{NHS}$ (▲), total IgM$_{NHS}$ (o) and total IgG$_{NHS}$ (Δ) was selected to study C3-deposition as induced by them individually at different time periods. (**B**) Comparison of C3-deposition within 3 minutes as triggered by Anti-*O*-AcSGIgM (▲, Δ) and anti-*O*-AcSGIgG (l, o) purified from sera of VL patients (▲, l) and NHS (Δ, o) in different concentrations. (**C**) Comparison of C3-deposition within 3 minutes as triggered by 6 µg/ml of anti-*O*-AcSGIgM$_{VL}$ (⁘) and anti-*O*-AcSGIgG$_{VL}$ (□) in four strains MHOM/IN/83/AG83 (1), MHOM/IN/90/GE1 (2), NS1(3) and NS2 (4) isolated from VL patients.
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identified as an important source of CP activation under normal physiological conditions, suggesting that they play a role in conferring host protection against parasite infection.

Previous reports have demonstrated that total IgM antibodies present naturally in NHS are a source of CP activation[65]. However, we have reported that anti-*O*-AcSGNHS is 3-fold more potent than are total

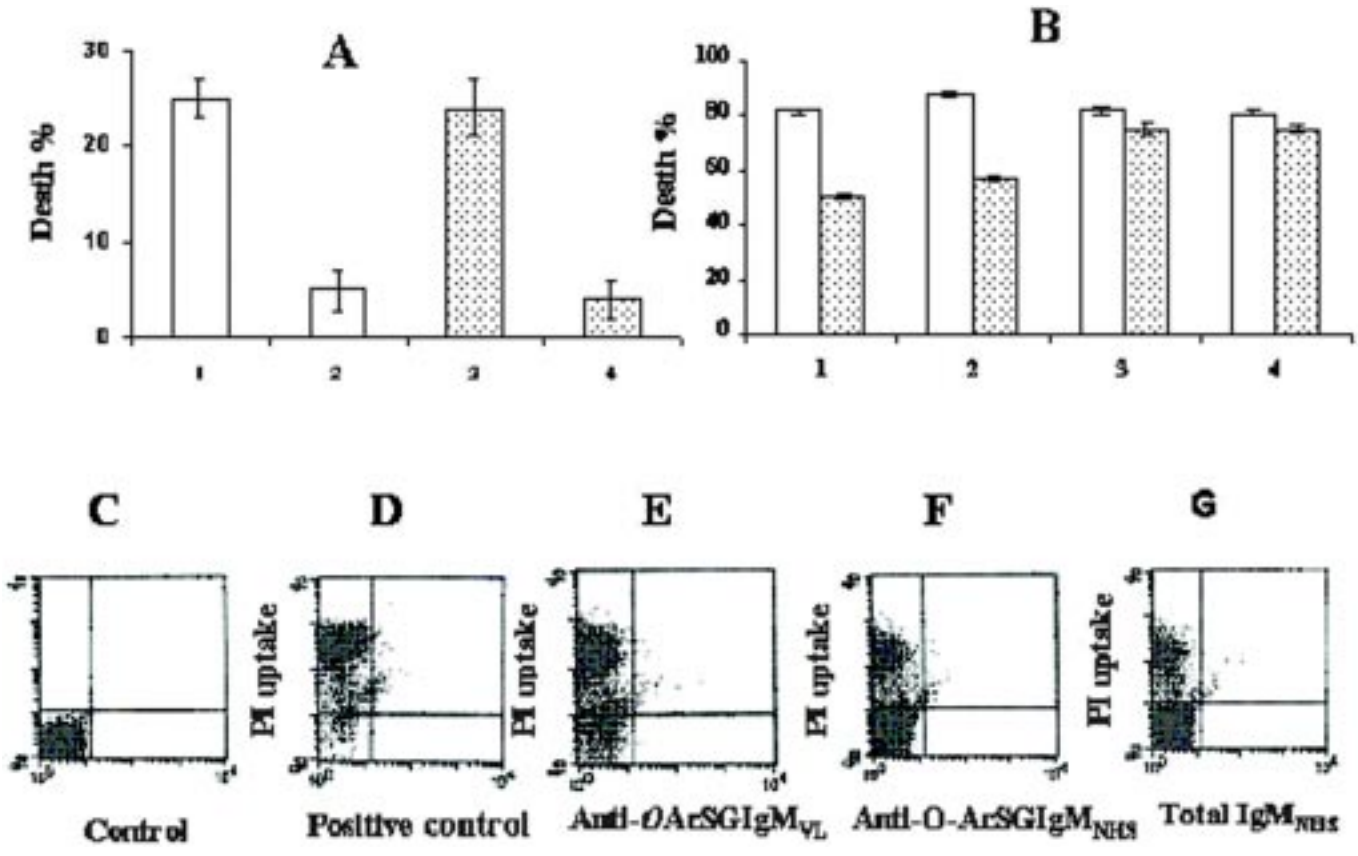

Fig. 6. Induction of promastigote lysis due to C3 deposition induced by anti–*O*-acetylated sialoglycoconjugate (AcSG). (**A**) A representative profile of cell death (%) as detected by MTT assay using 6 µg/ml of purified anti-*O*-AcSGIgM$_{NHS}$ (1) and anti-*O*-AcSGIgG$_{NHS}$ (3) vs. total antibodies$_{NHS}$ IgM (2) and IgG (4) in the presence of Ads-NHS (25%) within three minutes at 37°C. The cells were washed and incubated for another 3 hours at 37°C with MTT (100 µg/50 µl) and processed. (**B**) Comparison of cell death in MHOM/IN/83/AG83 (1), MHOM/IN/90/GE1 (2), NS1(3) and NS2 (4) isolated from VL patients using fixed concentration of 6 µg/ml of different antibodies anti-*O*-AcSGIgM$_{VL}$ (□) and anti-*O*-AcSGIgG$_{VL}$ (⬚). (**C-G**) Promastigote lysis was also analyzed by uptake of PI through Flow cytometric analysis as triggered by Ads-NHS (25%) along with 6 µg/ml of purified anti-O-AcSGIgM$_{VL}$ (E), anti-O-AcSGIgM$_{NHS}$ (**F**) and total IgM$_{NHS}$ (**G**) as compared to absence of complement activators (C) and maximum PI uptake (97%) after methanol : acetone treatment (D).
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antibodies in NHS, demonstrating, for the first time, that natural anti-*O*-AcSG is one of the major triggers of CP activation and promastigote opsonization[62].

Interestingly, in elicitation of C3 deposition on promastigotes, purified disease-specific anti-*O*-AcSG IgM$_{VL}$ and anti-*O*-AcSG IgG$_{VL}$ antibodies (6 µg/ml) were 5-fold more potent than anti-*O*-AcSG IgMNHS and anti-*O*-AcSG IgGNHS antibodies (Fig. 6B). The enhanced presence of 9-*O*-AcSG containing the 9-*O*-AcSAα2-6GalNAc glycotope, on the parasite surface, has been reported elsewhere[37,38,68] and has been corroborated here by the high rate of binding of anti-*O*-AcSG antibodies to promastigotes. Therefore, the enhanced presence of 9-*O*-AcSGs on parasites corroborates their increased susceptibility to complement lysis. Previous reports have shown that, irrespective of their linkage specificity, 9-*O*-AcSGs present on the surface of murine erythrocytes and murine erythroleukaemia cells contribute significantly to their susceptibility to lysis by activation of the AP[69]. Further investigations from our group have shown that, in mammalian erythrocytes, the complement lysis induced via the AP correlates more significantly with linkage-specific 9-*O*-AcSAα2-6GalNAc[70]. This correlation has been extended to erythrocytes from patients with VL[70].

Sialic acids are critical determinants of parasite protection against attack by the host complement system[71]. The removal of sialic acid by treatment with neuraminidase is known to increase MBL binding, with a subsequent increase in MBL mediated complement-dependant cell cytolysis, in *Neisseria meningiditis*[72]. Sialylation is also known to protect *N. gonorrhoeae* from MBL-activated complement killing[73] and confer protection to both epimastigote and trypomastigote forms by hindering the binding of lytic anti-galactose antibodies[74]. Thus, it may be envisaged that the host induces enhanced 9-*O*-acetylation on the parasite, thus generating anti–9-*O*-AcSG titres that, in turn, induce parasite lysis via CP activation. The role that 9-*O*-AcSG glycotopes on parasites play in mediating complement activation was further corroborated by *O*-acetylesterase-treated parasites, which resulted in the removal of *O*-acetyl moiety from sialic acid. These treated cells, when incubated with anti-*O*-AcSG IgM and anti-*O*-AcSG IgG, caused a significant reduction of C3 deposition. Quantitation of the number of C3-bound molecules per cell revealed that, in triggering the activation of C3 deposition, anti-*O*-AcSG IgMVL was 2.5-fold more potent than was anti-*O*-AcSG IgGVL[62]. Interestingly, although total antibodies in NHS are capable of inducing cell lysis, as confirmed by use of MTT assay, however, they were 3-fold less potent than anti-*O*-AcSG$_{NHS}$, establishing the critical role of these glycotope-specific antibodies. Further, anti-*O*-AcSG–induced death, compared with that induced by total antibodies in NHS, revealed a much higher population of necrotic cells, as confirmed by the massive uptake of PI. The importance of 9-*O*-AcSGs was further demonstrated when promastigotes grown in serum-free medium were found to be incapable of undergoing anti-*O*-AcSG-mediated complement lysis; however, they became susceptible to lysis when they were transferred to medium supplemented with FCS, the source of this interesting 9-*O*-AcSG[62]. The susceptibility to lysis, even in medium supplemented with low FCS (5%), confirmed that these glycotopes play important *in vivo* roles in complement activation. In contrast to CP, within the 3 min time frame, AP, CRP and MBL have a negligible effect, and no cell death could be detected during this time, as confirmed by use of MTT assay[62]. A comparative analysis distinctly revealed that the C3 deposition (mean ± SEM) triggered by anti-*O*-AcSGVL (94% ± 5% by IgM and 72% ± 10% by IgG) was maximal, compared with that triggered by CRP (22% ± 9%), MBL (24% ± 5%), or AP (27% ±1.4%) (Fig. 7)[65].

The above study demonstrated, for the first time, the biological role that these *O*AcSG-specific antibodies play in host protection. Thus, a vivid analysis of the differential recognition of antigenic glycoconjugates and antibodies is required for comprehension of the immune response in VL.

Perspective

The Identification of novel molecular determinants on *L. donovani* monitoring their mode of acquisition and their subsequent characterization will provide insight into the disease biology of visceral leishmaniasis. Proteomics of these newly identified molecules will lead to the development of future vaccine candidate.

The influence of these molecules in triggering host responses via the production of high titres of anti-*O*-AcSA antibodies have also been reported[61,62]. Therefore, identification of these antibodies and probing for their detailed mechanism of their interaction may help us in identifying novel anti-

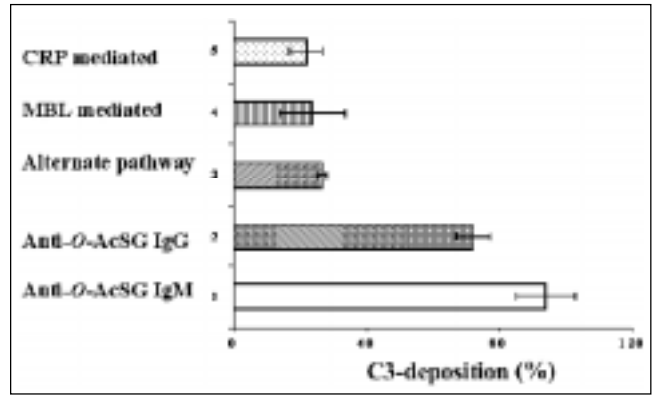

Fig. 7. A comparative analysis of C3-deposition on promastigotes by all the complement pathways as induced by the different complement activators namely, CRP (□) 50 µg/ml, MBL (▒) 20 µg/ml, AP (▨), anti-*O*-AcSG$_{VL}$ IgM (▧) 6 µg/ml or IgG (□) 6 µg/ml within 3 min.
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leishmanial molecules which may subsequently have therapeutic application. A detailed understanding of sialoglycans on *L. donovani* and their subsequent interaction with the host will help to foster innovative new strategies for diminishing the mortality and morbidity caused by this pathogen.

The current problem in Indian leishmaniasis is the increasing unresponsiveness to first-line treatment with pentavalent antimonial drugs, namely sodium antimony gluconate[37]. In view of the alarming increase in antimonial unresponsiveness, it would be interesting to study whether surface sialoglycans of antimonial resistant parasites are differentially adsorbed. They would then potentially serve as molecular targets helpful in distinguishing drug-responsive from drug unresponsive patients and allowing development of new drug strategies. In the future, it will be important to find whether these sialylated determinants, especially the 9-*O*-acetylated derivative, are present in other *Leishmania* strains, whether their expression is stage-specific, and what is the machinery that regulates sialylation and *O*-acetylation of the *Leishmania* parasites. The potential payback is a greater understanding of the endogenous roles of sialoglycans that may be relevant with regard to the host-parasite relationship.

The metabolism of *O*-AcSA is under the control of two groups of enzymes, *O*-acetyltransferases and 9-*O*-acetylesterases[75]. *O*-acetyltransferases are difficult to purify, and attempts to clone their genes have failed in isolating the true 9-*O*-acetyltransferase cDNA, accounting for the limited information regarding expression of *O*-AcSA[76]. In this context it is worthwhile to consider whether such a system exists in *L. donovani* amastigotes; such studies are underway. Parasitologic research is limited by the lack of availability of genomic data on protozoa and therefore the search for genes possibly involved in the biosynthesis, activation or transfer of sialic acids in protozoa has to be continued in the future.

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