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Virulence attenuation of a UDP-galactose/*N*-acetylglucosamine β1,4 galactosyltransferase expressing *Leishmania donovani* promastigote

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Abstract Protozoan parasites of the genus *Leishmania* are the causative agent of leishmaniasis, a disease whose manifestations in humans range from mild cutaneous lesions to fatal visceral infections. Human visceral leishmaniasis is caused by *Leishmania donovani*. Long-term culture *in vitro* leads to the attenuation of the parasite. This loss of parasite virulence is associated with the expression of a developmentally regulated UDP-Galactose/*N*-acetylglucosamine β 1–4 galactosyltransferase and galactose terminal glycoconjugates as determined by their agglutination with the pea nut agglutinin (PNA). Thus, all promastigotes passaged for more than 11 times were 100% agglutinated with PNA, and represent a homogeneous population of avirulent parasites. Identical concentrations of PNA failed to agglutinate promastigotes passaged for ≤5 times. These PNA^- promastigotes were virulent. Promastigotes passaged from 5 to 10 times showed a mixed population. The identity of populations defined by virulence and PNA agglutination was confirmed by isolating PNA^+ avirulent and PNA^- virulent clones from the 7th passage promastigotes. Only the PNA^+ clones triggered macrophage microbicidal activity. The PNA^+ clones lacked lipophosphoglycan. Intravenous administration of [^{14}C] galactose-labeled parasite in BALB/c mice resulted in rapid clearance of the parasite from blood with a concomitant accumulation in the liver. By enzymatic assay and RT-PCR we have shown the association of a UDP-Galactose/*N*-acetylglucosamine β1,4 galactosyltransferase with only the attenuated clones. By immunofluorescence we demonstrated that the enzyme is located in the Golgi apparatus. By western blot analysis and SDS-PAGE of the affinity-purified protein, we have been able to identify a 29 KDa galactose terminal protein from the avirulent clones.

Keywords UDP-Galactose/*N*-acetylglucosamine β1–4 Galactosyltransferase · Galactosylation · Avirulent *Leishmania donovani* promastigotes

Abbreviations

GalT	UDP-Galactose/*N*-Acetylglucosamine β1.4 Galactosyltransferase
LD	*Leishmania donovani*
V-LD	virulent *Leishmania donovani* clone
A-LD	avirulent *Leishmania donovani* clone
Mϕ	macrophage
PNA	pea nut agglutinin
Ab	antibody
mAb	monoclonal antibody
RB	respiratory burst

Introduction

Human visceral leishmaniasis, caused by *Leishmania donovani* (LD), is fatal if left untreated. The variability of the clinical features observed in Leishmaniasis results from both the parasite biodiversity [7, 9, 19, 27, 35] and the

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genetic and immunologic status of the host [27, 28, 34, 51]. Depending on the interactions between specific parasite molecules and specific host immune compartments, the disease can result in either host death or recovery [50]. The "invasive" antigenic determinants of the virulent phenotype trigger an immune response resulting in a symptomatic phase, while "protective" determinants interact with the immune system to effect a clinical cure. Differential expression of the determinants alone by parasites may have a profound impact on parasite transmission or pathology. However, to date, biological markers to account for parasite biodiversity remain largely undetermined, though a number of parasite-derived factors have been shown to play an important role in virulence and the outcome of the disease. For a better understanding of the pathology of visceral leishmaniasis (VL), parasite virulence needs to be better understood.

Population genetic analysis performed on Leishmania spp. basically favors the hypothesis of a clonal population structure. Since parasite populations in naturally infected hosts are likely to be composed of multiclonal associations, it has been suggested the virulence of individual parasite clones may influence both the course of the disease and the phenotype of the immune response which develops during infection [36]. Studies of clones of *L. major* derived from a single isolate have revealed the presence of parasite lines, which differ in virulence after prolonged passage in culture [63]. However, neither the genes nor the genetic mechanisms involved in the rapid loss of virulence have been identified although changes in abundant surface molecules such as LPG and gp63 have been variably noted by many workers. Sacks *et al*. [60] have shown that during metacyclogenesis, the PNA binding site of *Leishmania major* promastigotes was masked by altered glycosylation of LPG. Our earlier work with LD promastigotes had suggested that there was probably an association between the loss of parasite virulence with the expression of galactose terminal glycoconjugates and a developmentally regulated β1,4 galactosyltransferase (GalT). In order to better understand these relationships, expression of galactose terminal glycoconjugates, β1,4 GalT, and parasite virulence were assessed in a panel of isogenic PNA^+ (pea nut agglutinin) and PNA^- parasite clones.

Materials and methods

Animals, parasites, and animal infection

4 to 6 weeks old BALB/c mice (irrespective of sex), reared in the institute (Indian Institute of Chemical Biology) facility were used, with prior approval of the animal ethics committee of the Institute. *Leishmania* strain UR6 (MHOM/IN/1978/UR6) was originally isolated from the bone marrow of a kala-azar patient and has been maintained in Ray's modified medium for more than 30 years in our laboratory [54]. *L. donovani* strain AG83 (MHOM/IN/83/AG83) was maintained in complete M 199 medium as described before [18]. For infection, mice were inoculated with 2×10^7 promastigotes in 0.2 ml saline through the tail vein [46]. Splenic and hepatic parasite burden in infected animals were determined as described by Stauber [68], and results were expressed as a mean parasite number ± standard deviation.

Cloning of parasites by limiting dilution

Parasites from a mixed population of 7th passage were cloned by the limiting dilution method. 100 µl aliquots of parasites were diluted to a final concentration of 1 cell/ml per well of 'U' bottom 96-well culture plate. M-199 culture medium (100 µl) containing 20% FCS was added to each well to make a final culture volume of 200 µl. During the next 2 weeks, 50 µl of medium was removed from each well and fresh 50 µl medium was added to each well. Proliferating clones could be seen by microscopy and were maintained in 24-well plates with less than 1×10^6 parasites/well. After 2 weeks, aliquots from each were assessed for viability, PNA agglutinating ability and virulence.

Pea nut agglutinin mediated agglutination of parasite clones

Agglutination assays were performed as described [60] in 96-well, flat-bottomed microtiter plates containing 1×10^8 parasites/ml. Agglutination inhibition assays were done at room temperature by using 100 µg/ml of PNA and 10 mM D-Galactose.

Cell Surface Radio Immuno (CS-RIA) assay of LD clones

The CS-RIA was carried out with the clones essentially by the previously described method [45]. The optimal dilution for 1° Ab and 2° Ab giving the best results were found to be 1:10,000 (stock 10 mg ml^{-1}) and 1×10^6 cpm, respectively. A similar experiment was carried out with nonspecific IgM as the control.

Labelling of PNA or antibody with FITC

PNA or GalT antibody was directly labeled with Fluorescein Isothiocyanate (FITC) using the Pierce EZ-Label™-FITC Protein Labelling KIT.

β galactosidase treatment

Galactosidase treatment was performed using 2×10^6 *Leishmania* clonal parasites. Treatment with α-galactosidase

(G8507, Sigma-Aldrich, St.Louis,) was carried out using 90 mU of enzyme and *p*-nitrophenyl α-D-galactopyranoside as a positive control. Treatment with positionaly specific β1–4 galactosidase (G-0413, Sigma-Aldrich, St.Louis,), used 9 mU of enzyme and *p*-nitrophenyl β-D-galactopyranoside as the positive control. For all enzyme treatments both the treated sample and an identical negative control were worked up in tandem using the manufacturer's recommended conditions.

To add back ^{14}[C]galactose, the degalactosylated A-LD parasites were cultured in medium containing 0.05 μmol UDP[^{14}C] Galactose (1.17×10^6 cpm/, μmol).

Blood clearance of A-LD parasites

Blood clearance was studied in BALB/c mice injected intravenously with 1×10^7 ^{14}[C] labeled A-LD parasites containing 1,260 cpm/100 μl. Blood samples were collected at the indicated time points before killing the animals. Livers (1.2 to 1.5 gm) were excised, washed in saline and digested in 30% KOH (2 ml), and counted in a liquid scintillation counter.

Galt (UDP-Gal/GlcNAc-R β1,4 galactosyltransferase) assay

GalT activity was determined in reaction mixture containing the following components in a final volume of 0.1 ml: 10 mmol *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (pNPG) [2] or 200 mg of the protein substrates [4], or 0.05 μmol of acceptor glycosphingolipids; 0.25 μmol $MnCl_2$; 10 μmol sodium cacodylate/HCl buffer, pH 7.2; 0.05 μmol UDP[^{14}C] Galactose (1.17×10^6 cpm/, μmol) and 0.05 to 0.1 μg of enzyme protein. The mixtures were incubated for 2 h at 37°C, and the reaction was stopped with 3.0 μmol EDTA (pNPG or protein) or 10 μl chloroform/methanol (2:1, *v*/*v*; glycolipid). The reaction mixture was spotted on Whatman 3MM paper. Ascending chromatograms were developed in 1% sodium tetraborate for the protein substrates and in chloroform–methanol–water (60:35:8, *v*/*v*) for glycolipid substrates [4]. The radioactivities of appropriate areas of each chromatograms were determined quatitatively by liquid scintillation counting.

Immunofluorescence

Promastigotes of *L. donovani* strain AG83 (1×10^7 cells ml^{-1}) passaged for various numbers of times, AG83 amastigotes or UR6 promastigotes were suspended in PBS containing 5% fetal calf serum (PBSS). FITC labelled Pea Nut Agglutinin (PNA-FITC) was added to the cell suspension (at 1:100 dilution) and incubated at room temperature for 30 min. Cells were washed in PBSS thrice and fixed in paraformaldehyde (2%). The fluorescence of the fixed cells were analysed using a FACS Calibur (BD Biosciences).

Assessment of parasite virulence

Promastigotes of *L. donovani* clones (2×10^7 cells) were injected into the tail vein of 4-week old female BALB/c mice. After 12 weeks, the mice were sacrificed and prints of their spleen and liver cells were stained using Giemsa. The number of parasites per 10^6 infected cells was determined by direct counting.

Measurement of Reactive Oxygen Species (ROS)

The splenic macrophages (Mϕ), obtained from BALB/c mice were allowed to adhere to 22 mm glass coverslips as described earlier [18]. Three hours after plating, coverslips were washed with warm RPMI-1640 to remove non-adherent cells. Cells were infected with 1×10^7 LD clonal populations at a parasite/Mϕ ratio of 10:1. Cultures were incubated at 37°C for another 6 h in 5% CO_2. Free parasites were then removed by repeated washing, and the cultures were incubated for up to 3 days under the same conditions. The culture was then incubated with 0.05% Nitroblue Tetrazolium (NBT, Sigma-Aldrich) for 20 min, in 5% CO_2, at 37°C for optimum initiation. The coverslips were stained with safranine and the percentage of NBT reduction in cytoplasm assessed by light microscope (Leica). Microscopic fields distributed throughout the slides were randomly selected and all phagocytes in each particular field were examined. Superoxide $\left(O_2^-\right)$ is detected by the reduction of NBT to an insoluble form (formazan). Reduction in the number of NBT + zymogen-ingested macrophages was taken as the positive control.

Measurement of NO

The splenic Mϕs were infected with clonal population as above. The culture supernatant was analyzed for the presence of nitrite $\left(NO_2^-\right)$ by Griess Reagent as described before [3]. Measurement of nitrite was an indication of NO produced by these cells.

RNA isolation and RT-PCR of β1,4 galactosyltransferase

RNA from the clonal promastigote populations, AG83 amastigotes, or UR6 promastigotes (5×10^6 cells) was isolated with Trizol™ reagent (Invitrogen Life Technologies) as described [68]. For cDNA synthesis, 1 μg of total RNA was then reverse transcribed with MMLV reverse transcriptase and β1,4 galactosyltransferase specific reverse primer at 37°C for 1 h. The oligonucleotide

sequences of the PCR primers used for the experiments are: 5′-end primer: 5′-ctgcaccccagcccagacgg-3′; 5′-cggtcat catccccttttaga-3′ and 3′-end primer: 5′-ccaaagtagccagca taggg-3′, as follows; denaturation 94°C/45 s, annealing, 52°C/30 s and extension 72°C/1 min 45 s, for 30 cycles, in a thermocycler (Perkin Elmer model 9700) with a hot start at 94°C for 7 min in a final volume of 50 µl. Level of α-tubulin expression, used as the house-keeping gene, was compared with expression of β1,4 galactosyltransferase for each PCR reaction.

Localization of Galt enzyme by confocalmicroscopy

The Golgi and ER of live *L. donovani* promastigotes were visualized with the Golgi marker BODIPY-TR [77] and ER marker BIP [21]. GalT was visualized with anti-human β1,4 galactosyltransferase (a kind gift from Prof. S Basu, University of Notre Dame). Cells were washed in serum-free medium supplemented with 1.8% de-fatted BSA, fixed by the addition of 2% paraformaldehyde (8 min on ice) and permeabilized by incubating in 0.5% (w/v) Triton X-100 (5 mins/4°C). Cells were blocked with BSA-supplemented serum free medium for 30 min and incubated with BSA-conjugated BODIPY-TR, BIP or FITC-labeled GalT antibody in blocking solution for 30 min at 4°C. Cells were washed three times and the BIP labeled cells were incubated for 10 min with the PE-conjugated secondary antibody. Cells were immobilized for fluorescence microscopy by mounting under poly-L-lysine coated coverslips. Samples were examined in a confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

Affinity chromatography on PNA-sepharose columns

Galactose terminal protein was purified by affinity chromatography on PNA-sepharose column and subsequently analysed by SDS-PAGE and western-blot analysis.

Statistics

Experiments were repeated at least three times, and the representative data from one set of these experiments is presented. In each experiment at least six animals were used in each group. The extent of variation between experiments was within 5%.

Results

Isolation and characterization of parasite clones

Our earlier studies had demonstrated that serial culture of *L. donovani* promastigotes was associated with a gradual decrease in parasite infectivity, resulting in complete attenuated parasites in long-term cultured promastigotes [18]. Lectin binding studies demonstrated the presence of galactose terminal glycoconjugates in the attenuated parasites. To correlate the gradual expression of galactose terminal proteins with loss of parasite virulence in serially passaged parasites, flow cytometric analysis with the galactose-binding lectin, PNA, was carried out (Fig. 1). PNA binding occurred with parasites that were passaged for seven (P7) times or more. While P7 to P10 parasites showed the presence of both PNA^+ and PNA^- promastigotes (Fig. 1a), amastigotes and P2-P4 promastigotes were 100% PNA^- and virulent. The percentage of PNA positive promastigotes increased gradually with increasing numbers of *in vitro* passages, with the long passaged promastigotes (≥11) showing homogeneous 100% PNA^+ promastigote population. This was consistent with the fact that, prolonged passaging resulted in avirulent parasites.

Analysis of lectin agglutinating characteristics and virulence pattern of *Leishmania donovani* clones

Based on the above observation, it was important to determine whether the two heterogeneous P7 populations (PNA^+/PNA^-) represented developmentally distinct populations with respect to infectivity. Thus PNA-agglutinated promastigotes were separated from PNA unagglutinated free promastigotes by sedimentation through 50% FBS on the basis of PNA binding [60] and the discrete populations were used to infect BALB/c mice. The heterogeneous population of P7 PNA^+/PNA^- promastigotes was further cloned by limiting dilution, and 20 different clones were obtained that exhibited disparate growth characteristics falling into fast growing (11 clones, doubling time 9 h) that were PNA^- (termed as virulent LD promastigotes or V-LD) and slow growing (9 clones, doubling time 18 h) clones that were 100% PNA^+ (designated as attenuated LD promastigotes or A-LD). Trypan blue exclusion showed that all 20 clonal populations remained viable. While all of the 11 fast-growing PNA^- clonal population were able to infect BALB/c mice ($1.9\pm0.3\times10^7$ parasites/10^6 spleen cells; $2.2\pm0.5\times10^6$ parasites/10^6 liver cells), 9 PNA^+ clonal population failed to establish infection in BALB/c mice (Fig. 2).

Galactosidase treatment

In order to confirm the association of terminal galactosylation with loss of parasite virulence, A-LD parasites were treated with α or β galactosidase. Coffee bean α-galactosidase did not produce any change in the virulence pattern of A-LD. However, treatment with

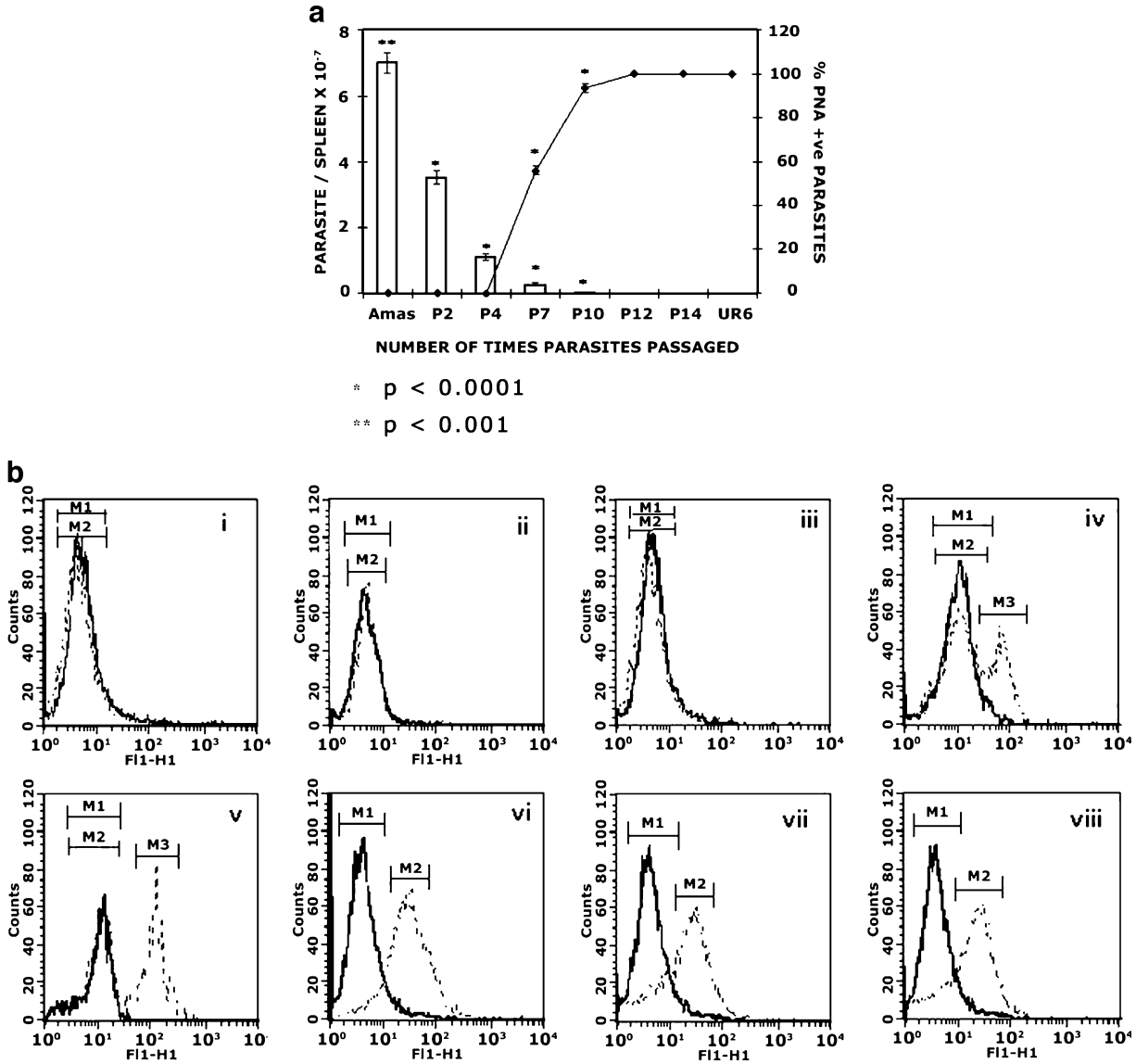

Fig. 1 The relative virulence of serially passaged promastigotes versus expression of PNA specific glycans. **a** Mice were inoculated through the tail vein at day 0 with 2×10^7 UR6 promastigotes or AG83 amastigotes or promastigotes subcultured for various length of time. Animals were sacrificed on day 90 and splenic parasite burden (*bars*) was calculated as described in "Materials and methods". Values shown are mean ± SD of six animals per group. **b** The expression of PNA specific glycans of serially passaged promastigotes. *i–viii* shows flowcytometric analysis of FITC labeled PNA binding to LD promastigotes and amastigotes (*dotted lines*). *i–iii* shows histogram overlay of PNA binding to amastigote (*i*), P2 (*ii*) and P4 (*iii*) AG 83 promastigote (Peak M2) matching the histogram plots of the matching unstained cells (*solid line*, M1). The histogram overlay of PNA binding to P7 (*iv*) and P10 (*v*) shows two peaks; one representing a population (M2) of PNA^- promastigotes that overlays unstained cells (Peak M1) and a second population (M3) of PNA^+ promastigote. *vi–viii* show a positive shift in the overlay image of PNA binding to P12 (*vi*), P 14 (*vii*) and UR6 (*viii*) promastigotes (*dotted lines*, peak M2) and corresponding unstained cells (*solid line*, Peak M1). The % of PNA^+ cells corresponding to the LD promastigotes passaged for different number of times and LD amastigotes estimated from the dot plots are correlated to parasite virulence [**a** (filled triangles)]. The data represents mean ± SD of three independent experiments

commercially available position-specific β 1,4 galactosidases produced significant changes in the virulence profile of the A-LD. The galactosidase treated A-LD promastigotes were used to infect BALB/c mice. Once the terminal galactose was removed from the A-LD, the galactose minus (Gal^-) promastigotes were able to infect mice (Fig. 3).

Clearance of A-LD parasite from blood and appearance in Liver

Following intravenous administration in BALB/c, most of the ^{14}[C] galactose labelled parasites were cleared rapidly from the blood. During that time, ~92% labelled parasites could be recovered from the liver (Fig. 4). Clearance of A-

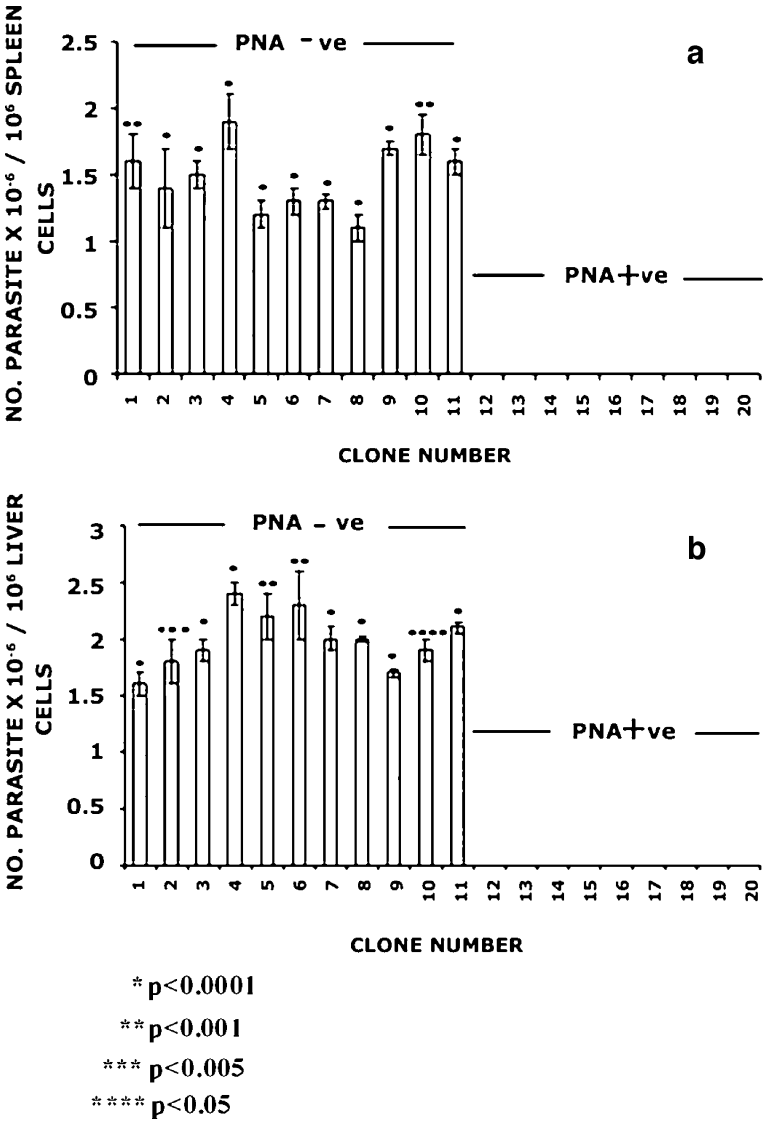

Fig. 2 Virulence of PNA^+ and PNA^- LD clones. Animals were divided into 20 groups, with 6 animals in each group. One clone was used to infect the 6 animals of a group. Animals were infected through the tail vein at day 0 with 2×10^7 promastigotes. Animals were sacrificed on day 90 and splenic (**a**) and liver (**b**) parasite burden was calculated as described in "Materials and methods". Values represent mean ± SD from three replicate experiments

LD was inhibited on prior treatment of parasites with 100 μg PNA.

Concurrent infection with clones

As P5 to P10 promastigotes showed the presence of both PNA^+ and PNA^- promastigotes (20, 50, 80, and 90% of P5, P7, P9, and P10 promastigotes bound to PNA respectively), we tried to reconstitute these multiclonal parasite populations *in vitro*, and examined the course of *in vivo* infection in BALB/c mice. Mice injected simultaneously with the virulent and avirulent clones, at a virulent to avirulent ratio of 3:1 (P5) or 1:1 (P7), developed a virulent type of infection with high levels of parasite burden in liver and spleen. With a virulent to avirulent ration of 1:3 (P9) or 1:9 (P10), a controlled infection profile a with parasite burden persisting at low to moderate levels in the spleen and liver was observed. Concurrent infection with clonal populations indicated that virulent clones had a competitive advantage, and the presence of virulent clones in the inoculums always resulted in an infective profile (Table 1).

Stability of the A-LD clones

It was observed that the virulent clones displayed decreased virulence after longitudinal propagation *in vitro*; to correlate these virulent changes we recloned the P7 virulent- cloned promastigotes and monitored their virulence behavior. Once again we could divide the clones into the PNA^- V-LD clones, and the PNA^+ A-LD clones. Attenuated parasites are known to regain their virulence by *in vivo* animal passage, but since the PNA^+ A-LD clones failed to establish an infection in BALB/c mice, there was no reversion *in vivo*. The stability of the attenuated clones was established by

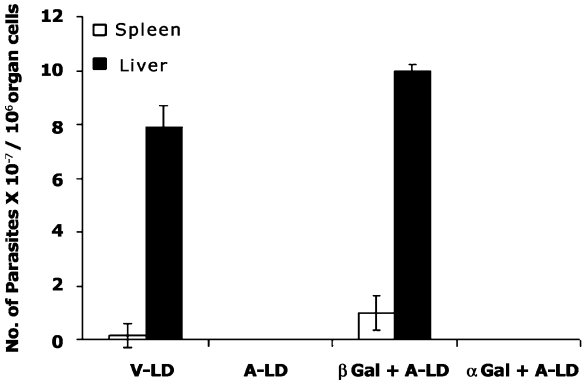

Fig. 3 Parasite burden in liver and spleen of BALB/c mice after infection with β galactosidase treated A-LD parasites. Animals were infected with 2×10^6 V-LD, A-LD and α or β galactosidase treated A-LD parasites. Thirty days post infection, animals were sacrificed and spleenic and liver parasite burden was calculated as mentioned in "Materials and methods". Data represents mean ± SD for ten animals

culturing the PNA^+ A-LD clones *in vitro* for more than 27 times with the attenuation remaining stable.

Status of LPG in *Leishmania* clones

The status of LPG in A-LD and V-LD was analyzed by CS-RIA using mAb (CA7AE) specific to LPG. There is a report that mAb CA7AE binds to LPG of LD and *L. major* [70]. It was observed that CA7AE bound to V-LD (specific count 7749±321), but failed to bind to A-LD (specific count 0) or to the LPG mutant R2D2 (specific count 234±54).

Superoxide and nitrite generation *in vitro* in Mϕs in response to clonal promastigote populations

Nitrite and superoxides are two macrophage-derived oxidants critical in controlling *Leishmania* infection [29, 37, 38, 47, 73]. An inverse relationship exists between LD virulence and its ability to stimulate Mϕ RB response. Virulent LD parasites are known to inhibit the LPS-mediated Mϕ respiratory burst activity (RB) [11], where attenuated parasites stimulated the production of nitrite and superoxides [18, 45]. Since, it has been reported that LPG inhibits macrophage RB activity [10, 41], and since attenuated A-LD parasites lack LPG, we became interested in studying the ability of A-LD to induce burst activity in macrophages. It was observed that while the PNA^- V-LD clonal populations inhibited both the Mϕ RB activity and nitric oxide production, since the virulence of the attenuated PNA^+ A-LD parasites are reverted on removal of the terminal galactose by β galactosidase treatment, we became interested in studying the Mϕ stimulating ability of these parasites. As expected, the agalacto A-LD was unable to stimulate the Mϕ RB activity. Avirulent A-LD clonal populations stimulated both (Fig. 5).

PNA agglutination, expression of β1–4 galactosyltransferase and attenuation

Our earlier work had suggested a direct correlation between the terminal galactosylation of proteins to loss of parasite infectivity [18]. The existence of two opposite modulatory effects of A-LD and V-LD on Mϕ RB activity and nitric oxide production, along with the modification of surface carbohydrates of V-LD, evolving into acquisition of galactose terminal determinants in the A-LD (as demonstrated by preferential agglutination of A-LD by PNA), led us to search for a galactosyltransferase responsible for these modifications. Assay of galactosyltransferase (GalT) in both V-LD and A-LD, showed the presence of a highly active GalT in the A-LD. This enzyme could transfer galactose from UDP [^{14}C]Gal equally well to the glycoprotein substrates, asialo agalcto α_1- acid glycoprotein, and asialo aglacto fetuin, and its natural substrate, total proteins extracted from the virulent clones (Table 2). It could also transfer [^{14}C] Gal to the glycolipid substrate $nLcOse_3Cer$, indicating the presence of a UDP-Gal/GlcNAc β1,4 galactosyltransferase [2, 4]. Oligonucleotide primers were designed using the Primer 3 programme with known β1,4 Galactosyltransferases as the template. RT-PCR performed on total RNA isolated from PNA^- virulent and PNA^+ avirulent parasite clonal populations resulted in a single amplification product from PNA^+ attenuated clonal promastigotes only (Fig. 6). No amplification product could be detected from AG83 amastigotes or virulent clonal populations. The 360 bp product was purified and sequenced (Gen Bank Accession No. EF159943). It was observed that this

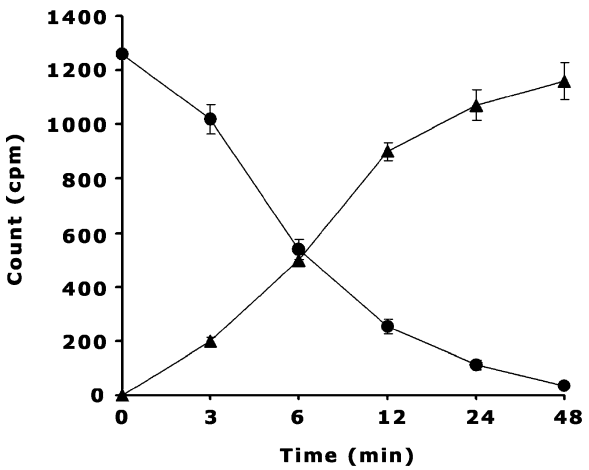

Fig. 4 Blood clearance of 14[C] labeled A-LD promastigotes. Each mice was injected intravenously with 1×10^7 ^{14}[C] labeled A-LD parasites containing 1260 cpm. Blood was collected at time intervals between 3 and 48 min prior to killing of the animals and excision of liver. Radioactivity was counted in each sample. Each point represents the mean value from three experiments and the bars represents standard deviation. *Filled circles*, blood; *filled triangles*, liver

Table 1 Concurrent infection with LD clones

		Parasite burden in BALB/c mice immunized with V-LD: A-LD in the ratios					
		1:0	0:1	1:1	3:1	1:3	1:9
Parasite Count (Mean ± SD) in 10^6	Spleen cells	$(1.5\pm0.23)\times10^7$	–	$(1.4\pm0.55)\times10^7$	$(0.8\pm0.18)\times10^7$	$(0.01\pm0.03)\times10^7$	0.005×10^7
	Liver cells	$(2\pm0.51)\times10^6$	–	$(0.9\pm0.19)\times10^6$	$(1\pm0.22)\times10^6$	$(0.004\pm0.0011)\times10^6$	$(0.0001\pm0.0001)\times10^6$

BALB/c mice were injected at day 0 with 10^7 promastigotes of V-LD and A-LD clones simultaneously in the indicated ratio as mentioned in the experimental procedures. Animals were sacrificed on day 90 and splenic and hepatic parasite burden was calculated as described in "Materials and methods". Data represents mean ± SD for ten animals.
V-LD, *L. donovani* virulent clone; A-LD, *L. donovani* avirulent clone.

sequence showed a high degree of homology to a sequence located on *L. major* Friedlin strain, chromosome 13 in Genedb data base with a homology to other known β1,4 Galactosyltransferases. The 5′3′Frame2 predicted a protein sequence obtained using the ExPASY translation tool showed a significant degree of sequence homology to other known β1,4 Galactosyltransferases and to a hypothetical protein present in chromosome 13 locus P214.35/0690 of *L. major* by WU-Blast2 search (data not shown). Remarkably, this 360 bp amplification of β1–4 Galactosyltransferases could also be observed from a known avirulent, attenuated *Leishmania* strain-UR6 that has been maintained for more than 3 decades in our laboratory.

Intracellular localization of the GalT in *Leishmania*

Since, the β1–4 Galactosyltransferases enzyme from the attenuated parasite clones showed a high degree of homology to human β1,4 Galactosyltransferases, we used the FITC-labeled human UDP-Gal/GlcNAc β1,4Galactosyltransferase antibody to immunostain the leishmanial β1–4GalT. By using the Golgi-specific marker, BSA-conjugated BODIYP-TR, we could show the presence of the β1,4GalT enzyme in the Golgi by co-localization study (Fig. 7).

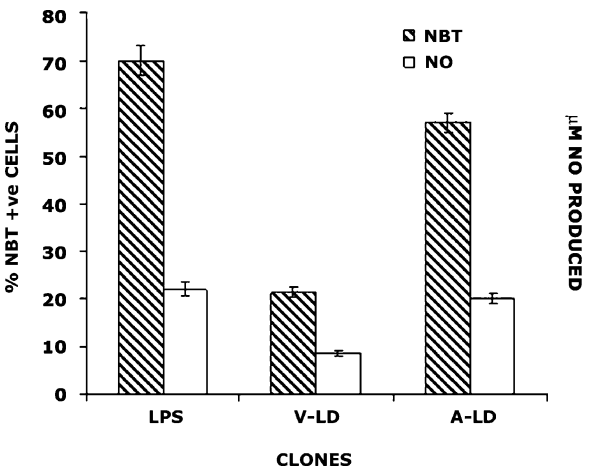

Fig. 5 Measurement of Reactive oxygen species and Reactive nitrogen intermediates by cultured BALB/c splenic macrophages infected with AG83 clones. Reactive oxygen species and Reactive nitrogen intermediates produced in infected macrophages were compared to LPS mediated Reactive oxygen species and Reactive nitrogen intermediate production in uninfected macrophages and are the average of a triplicate assay

PNA-sepharose affinity chromatography

Since A-LD lacks LPG despite binding to PNA, we looked for the presence of other galactose terminal glycoconjugates in A-LD by PNA affinity chromatography. A 29 KDa protein (Fig. 8) was purified from the A-LD promastigotes by affinity chromatography on PNA-sepharose column. Presence of terminal galactose was confirmed by western blot analysis using biotinylated PNA (Fig. 8b). The PNA binding was stable when stored at −70°C.

Discussion

Glycans are increasingly recognized for their information content, ranging from roles in protein folding, pathogen recognition on host cells, innate and acquired immunity, and intracellular trafficking [8, 14, 16, 22, 31, 39, 66, 71, 72]. Glycosylation is the most common and ubiquitous form of post translational modification of proteins with over half of all proteins estimated to contain one or more glycan chains [1, 52, 75]. Since complex carbohydrates can be linked in a number of different ways forming a variety of spatial conformations, they may contain an informational content far surpassing those imparted by proteins or nucleic acids alone. Advances in the field of lectinomics portray these oligosaccharides as code words in their own right. Because they are central to all biosynthesis process involving sugars, glycosyltransferases and glycosidases form an integral part of the glycomics network.

In Leishmania, glycosylation disorders have been correlated to virulence phenotypes [26]. The life cycle of

Table 2 Acceptor specificity of Galactosyltransferase of A-LD

Acceptor substrate	nmol [^{14}C]Gal Incorporated/mg protein/hour	
	V-LD*	A-LD*
P-NPG (10 nmol)	0.09	13.50
SA$^-$Gal$^-$α1AGP (200 mg)	nd	30.76
SA$^-$Gal$^-$Fetuin (200 mg)	nd	27.45
nLcOse3Cer (0.05 μmol)	nd	21.36
nLcOse4Cer (0.05 μmol)	nd	nd
V-LD Total Protein (100 mg)	nd	29.77
A-LD Total Protein (100 mg)	nd	nd

nd, not detectable; *nLcOse$_3$Cer*, neolactotriosylceramide; *nLcOse$_4$Cer*, neolactotetraosylceramide
*Source of Galactosyltransferase enzyme

Leishmania spp includes sequential development of invertebrate promastigotes from a non-infective procyclic form to an infective metacyclic form [17, 59]. The common phenomenon of loss of virulence with frequent subcultures [47, 58, 61, 62, 69] has also been attributed to metacyclogenesis potential [17]. Metacyclic promastigotes are characterized by modifications of the major cell surface glyconjugate of the promastigote, LPG, resulting in down regulation of PNA agglutinated promastigotes. Though Sacks [60] observed a difference in PNA agglutinibility in *L. major* promastigotes during differentiation, no such difference was observed in *L. donovani* promastigotes. Besides LPG, various other molecules playing an important part in the biology and infectivity of Leishmania parasites have also been identified [6, 20, 43, 44, 53, 67, 76]. The present work indicates that virulence attenuation is accompanied by the expression of a UDP-Galactose/GlcNAc β1,4 galactosyltransferase and stage-specific galactose terminal glycoconjugates (Gal-GCs) in *L. donovani* promastigotes.

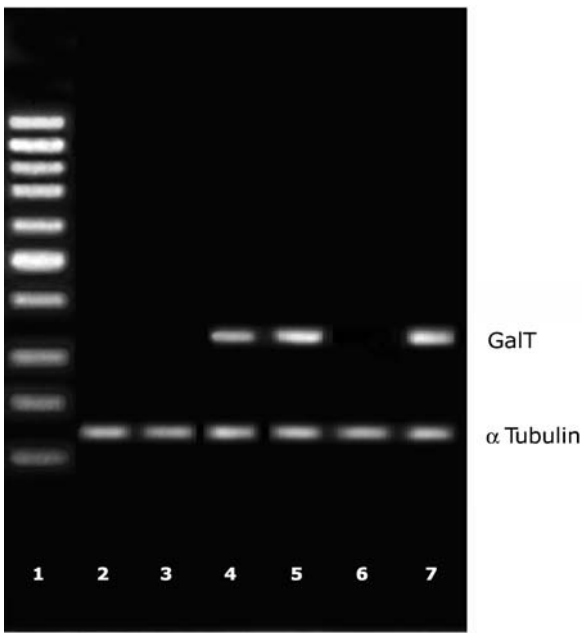

Fig. 6 Expression of β1,4 galactosyltransferase transcript in L. donovani. Total RNA samples were examined by RT-PCR for the expression of a house keeping gene control α tubulin and β1,4 GalT. (*1*) 1 kb DNA ladder, (*2* and *3*) Two representative Virulent LD clonal populations, (*4* and *5*) Two representative Avirulent LD clonal populations, (*6*) AG 83 amastigote and (*7*) UR6 promastigote

Longitudinal propagation of LD promastigotes leads to a gradual loss of parasite virulence with an upregulation of Galactose terminal glycoconjugates. Different expression levels of Gal-GCs define a sequential development of LD promastigotes from a virulent to an avirulent stage. The population dynamics of promastigotes, distinguished on the basis of agglutination with PNA, appeared to be closely coordinated with changes in the infectivity, the relative virulence of these promastigotes being inversely proportional to the relative proportion of PNA specific glycan contents of the promastigotes. The direct correlation between the terminal galactosylation and loss of promastigote virulence was indicated by the purification of PNA$^-$ virulent and PNA$^+$ avirulent parasites from a mixed population of P7 promastigotes.

The identification of stage specific antigens is useful as they might represent functionally important molecules with respect to infectivity [13, 30, 32, 40]. We tried to address this question by studying several virulent and avirulent promastigote clones. By limiting dilution, 11 PNA- and 9 PNA+ clones were isolated. The PNA$^-$ clones were found to be virulent, while the PNA$^+$ clones were found to be avirulent. The decrease in virulence that accompanies frequent subcultures of freshly transformed promastigotes was also observed in cloned virulent populations. Similar results were also observed by Sacks *et al.* [17]. The progressive loss of virulence in a continuously cultured, mixed population was probably due to selection, as the initial mixture of a stable virulent clone and a stable avirulent clone eventually yielded a totally avirulent promastigote population. However, in a reconstituted mixed population, the virulent clones had a dominant profile and the relative expression of the virulence profile probably controls the outcome of the disease. Though there are reports that attenuated parasites can regain their original virulence by *in vivo* passage in BALB/c mice [17], the attenuated clonal populations failed to induce any

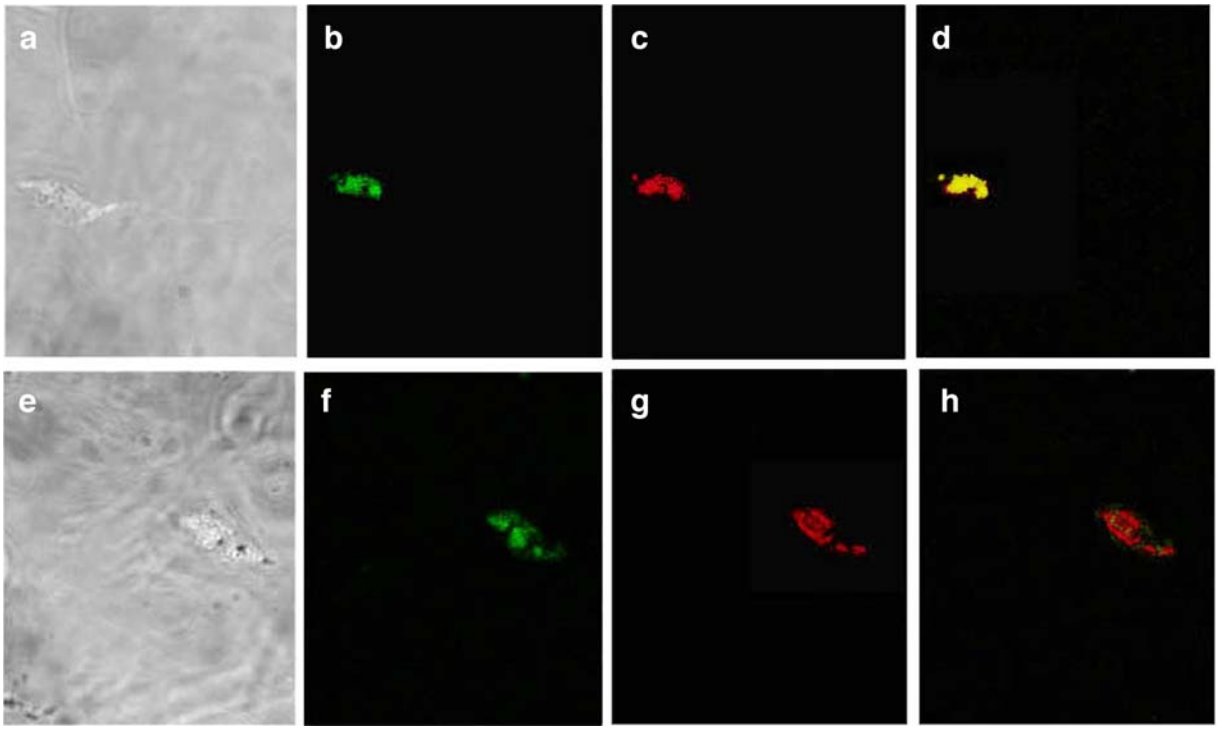

Fig. 7 β1–4 GalT is detected at the Golgi and not ER. A-LD parasites were fixed, permeabilized and immunostained with (**a**–**d**) the Golgi marker BODYP-TR (*red*) and anti β1–4 GalT-FITC (*green*) or [e–h] with the ER marker anti BiP antibody (*red*) and anti β1–4 GalT-FITC (*green*) prior to being analyzed with confocal microscope. Anti-BiP was detected using goat anti rat IgG-PE (*red*). (**a**) Bright field (*BF*) image of a A-LD parasite. The same cell stained for GalT (**b**) and Golgi (**c**). (**d**) Intense colocalization of immunostaining (*yellow*) by GalT and Golgi in the same cell. (**e**) BF image of another A-LD parasite. The same cell stained for GalT (**f**) and BiP (**g**). There was no colocalization between GalT and BiP (**h**)

infection in BALB/c mice and the clones remained stable for an extended period (27 passages) of *in vitro* culture.

It is reported that activated macrophages kill ingested leishmania parasites by secreting reactive oxygen species and/or reactive nitrogen intermediates [24, 48, 49, 55, 65]. Attenuated LD promastigotes are also known to induce superoxide generation, [18, 45], where as virulent Leishmania spp. parasites are known to inhibit it [7, 45]. The clones were further characterized by their ability to trigger macrophage microbicidal activity. In the present study we saw that the A-LD clones behaved as typical attenuated parasites and triggered both the macrophage RB activity and nitric oxide production while the virulent clones failed to do so.

The search for the molecular basis for these galactosylation changes in *L. donovani* were wide ranging because of the different factors involved in the biosynthesis of these complex carbohydrates. This could be due to a lack of the donor of galactose, UDP-galactose, as has been reported for galactosemia [25]; to a decreased import of UDP-galactose into the Golgi apparatus; or to a defective transfer of galactose by galactosyltransferase; or the ability to synthesize the appropriate substrate; or the acquisition of a galactosidase or sialidase. The presence of abundant surface and secreted molecules, such as lipophosphoglycan (LPG) and proteophosphoglycans (PPGs), containing extensive galactose in the form of phosphoglycans (PGs) based on (Gal–Man–PO_4) repeating units indicate the presence of functional galactosylation machinery in the virulent leishmania parasites. This is further supported by the recent report of the presence of two functionally divergent UDP-Gal nucleotide sugar transporters in *L.major* [12]. On the other hand though attenuated parasites lack LPG, they contain a normal level of related PPGs and PGs [44, 67].

Sialic acid residue, which is closely related to antigen masking, is most commonly linked to the penultimate galactose (Gal) or *N*-acetylgalactosamine (GalNAc) on glycan branches. The exposure of galactose by hydrolysis of sialic acid containing glycoconjugates or the excision of galactose by the action of β-galactosidase on cell-surface glycoconjugates would result in either the exposure of underlying galactose residues or removal of it and this alone would account for the glycosylation changes observed in *L. donovani* parasites. However, until this time date no sialidase/trans sialidase activity has been reported in leishmania promastigotes [5]. The only glycosidase activities reported in *L. donovani* are α glucosidase [33] and Chitinase activity [64]. No other glycosidase has been

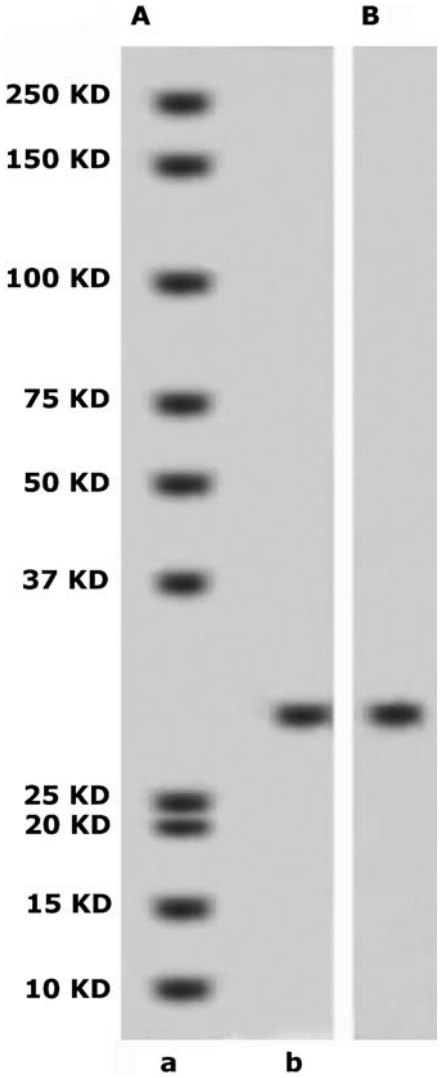

Fig. 8 (**a**) SDS-PAGE in 10% gel of galactose terminal protein prepared by affinity chromatography on PNA-Sepharose column (*A*). Bands were stained with silver nitrate. Positions of molecular weight markers are indicated (*B*). (**b**) Western blot analysis of galactose terminal protein with biotinylated PNA

reported in *L. donovani* [15]. Also, we could not detect any galactosidase or sialidase activity in A-LD or V-LD. To address the question, whether these changes in galactosylation pattern reflected the differential levels of expression of the enzyme responsible for their biosynthesis, we looked for the presence of galactosyltransferase in these parasites. We could detect a UDP-Gal/GlcNAc-R β1–4 GalT activity in the attenuated clones. This enzyme transfers galactose from UDP-Gal equally well to terminally exposed GlcNAc residues on glycoproteins and glycolipids. It could also transfer galactose to the synthetic substrate, pNP-GlcNAc and its endogenous substrate, the total protein extract from virulent clones. It did not transfer any [^{14}C] galactose to the total protein extract from attenuated parasite clones. This clearly demonstrated the presence of the UDP-Gal-GlcNAc-β1,4 GalT enzyme in the attenuated clones and presence of acceptor substrates in the virulent clones. RT-PCR using UDP-Gal/GlcNAc β1,4 galactosyltransferase-specific primers revealed a single 360 bp mRNA only in the avirulent clone. Expression of β1–4 GalT in the attenuated atypical Leishmania parasite UR6 indicated that the association of the enzyme with virulence attenuation is probably a general phenomenon. The 360 bp product showed 77% sequence identity with *L. major* Friedlin strain, chromosome 13. This product also showed a high degree of sequence identity with other known β1,4 GalTs. The predicted translated protein was homologous to other known β1,4 GalTs and a hypothetical protein present in chromosome 13 of *Leishmania major*. It is worth while to mention that the galactosyltransferase associated with LPG mutants is a galactofuranosyltransferase [57].

To address the question, whether this galactosylation of precursor glycoconjugates of the virulent parasites is implicated in parasite virulence, we removed the terminal galactose from the avirulent clones by β-galactosidase treatment. Removal of terminal galactose resulted in the conversion of the avirulent clones into highly infective virulent parasites, indicating a direct correlation between the terminal galactosylation and virulence attenuation. The rapid clearance of ^{14}[C] galactose labeled A-LD from circulation, with concomitant appearance of labeled parasite in the liver, could be explained by the recognition of terminal galactose by the hepatic galactose receptors [23, 42, 56].

It is a well-known fact that some glycosylation reactions occur in the lumen of the ER; others, in the lumina of the *cis*-, *medial*-, or *trans*-Golgi cisternae. Galactosyltransferase is localized to the Golgi cisternae. Co-localization of GalT with Golgi marker BODIPY-TR in A-LD confirmed the presence of the GalT enzyme in the attenuated parasites only.

Since glycosyltransferases are largely responsible for generating cell surface glycoconjugate structural diversity, the presence of a 29 KDa protein from the PNA$^+$ attenuated clones was found to parallel closely the up-regulation of the UDP-Gal/GlcNAc β1,4 GalT. This is an extremely important event because availability of such a glycan with terminally linked galactose is responsible for uptake and destruction of the parasite in the liver. However, the properties and structure of the asialoglycoprotein for the liver receptor [74] is not yet known yet.

In summary our results show that the loss of virulence of *L. donovani* promastigotes on longitudinal propagation is associated with the expression of a developmentally regulated UDP-Gal/GlcNAc β1,4 GalT resulting in the stage specific expression of a 29 KDa galactose terminal protein.

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