The Antiretroviral Lectin Cyanovirin-N Targets Well-Known and Novel Targets on the Surface of *Entamoeba histolytica* Trophozoites\[†‡

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*Entamoeba histolytica*, the protist that causes amebic dysentery and liver abscess, has a truncated Asn-linked glycan (N-glycan) precursor composed of seven sugars (Man5GlcNAc2). Here, we show that glycoproteins with unmodified N-glycans are aggregated and capped on the surface of *E. histolytica* trophozoites by the antiretroviral lectin cyanovirin-N and then replenished from large intracellular pools. Cyanovirin-N caps the Gal/GalNAc adherence lectin, as well as glycoproteins containing O-phosphodiester-linked glycans recognized by an anti-proteophosphoglycan monoclonal antibody. Cyanovirin-N inhibits phagocytosis by *E. histolytica* trophozoites of mucin-coated beads, a surrogate assay for amebic virulence. For technical reasons, we used the plant lectin concanavalin A rather than cyanovirin-N to enrich secreted and membrane proteins for mass spectrometric identification. *E. histolytica* glycoproteins with occupied N-glycan sites include Gal/GalNAc lectins, proteases, and 17 previously hypothetical proteins. The latter glycoproteins, as well as 50 previously hypothetical proteins enriched by concanavalin A, may be vaccine targets as they are abundant and unique. In summary, the antiretroviral lectin cyanovirin-N binds to well-known and novel targets on the surface of *E. histolytica* that are rapidly replenished from large intracellular pools.

*Entamoeba histolytica* causes amebic dysentery and liver abscess in the developing world (10, 20, 29). We are interested in *E. histolytica* glycoproteins containing Asn-linked glycans (N-glycans) for numerous reasons. *E. histolytica* makes an N-glycan precursor that contains 7 sugars (Man5GlcNAc2-PP-dolichol) rather than 14 sugars (Glc3Man9GlcNAc2-PP-dolichol) made by most animals, plants, and fungi (21, 31, 44). *E. histolytica N*-glycans are aggregated and capped on the surface of *E. histolytica* trophozoites by the antiretroviral lectin cyanovirin-N and then replenished from large intracellular pools. Cyanovirin-N caps the Gal/GalNAc adherence lectin, as well as glycoproteins containing O-phosphodiester-linked glycans recognized by an anti-proteophosphoglycan monoclonal antibody. Cyanovirin-N inhibits phagocytosis by *E. histolytica* trophozoites of mucin-coated beads, a surrogate assay for amebic virulence. For technical reasons, we used the plant lectin concanavalin A rather than cyanovirin-N to enrich secreted and membrane proteins for mass spectrometric identification. *E. histolytica* glycoproteins with occupied N-glycan sites include Gal/GalNAc lectins, proteases, and 17 previously hypothetical proteins. The latter glycoproteins, as well as 50 previously hypothetical proteins enriched by concanavalin A, may be vaccine targets as they are abundant and unique. In summary, the antiretroviral lectin cyanovirin-N binds to well-known and novel targets on the surface of *E. histolytica* that are rapidly replenished from large intracellular pools.

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*E. histolytica* trophozoites by the antiretroviral lectin cyanovirin-N, which is specific for α-1,2-linked mannos, present on unprocessed N-glycans, binds *E. histolytica* N-glycans and forms aggregates or caps on the surface of *E. histolytica* trophozoites (1, 25, 31, 44, 45). *E. histolytica* glycoproteins are also capped by the plant lectin concanavalin A (ConA), which has a broader carbohydrate specificity (mannose and glucose) than cyanovirin-N (3, 16, 18, 19). Heavy subunits of the Gal/GalNAc lectin, the most important *E. histolytica* vaccine candidate, have 7 to 10 potential sites for N-linked glycosylation (32, 39, 43). Inhibition of N-glycan synthesis results in Gal/GalNAc lectins that are unable to bind to sugars on host epithelial cells.

Carbohydrates appear to be an important target on the surface of *E. histolytica* as anti-proteophosphoglycan (PPG) monoclonal antibodies bind to O-phosphodiester-linked glycans and protect animal models from amebic infection (6, 33, 35, 40, 48). Lectin affinity columns are a powerful method for enriching unique parasite glycoproteins that may be identified by mass spectrometry (MS) of tryptic fragments (17, 55). For example, we recently used the plant lectin wheat germ agglutinin to dramatically enrich glycoproteins with short N-glycans of *Giardia* (42).

The goal of the present studies was to explore further the interaction of the antiretroviral lectin cyanovirin-N with *E. histolytica* trophozoites in *vitro*. Questions asked included the following: Are *E. histolytica* glycoproteins with N-glycans replenished on the plasma membrane after capping with cyanovirin-N? What is the effect of cyanovirin-N capping on other amebic virulence factors and/or vaccine candidates (e.g., the
Gal/GalNAc lectin and PPG)? Is capping by cyanovirin-N mediated by actin, as described for capping by the Gal/GalNAc lectin and ConA? What is the effect of the cyanovirin-N on amebic phagocytosis of mucin-coated beads, a surrogate assay for virulence? Which trophozoite glycoproteins are potential targets of cyanovirin-N (identified by mass spectrometry of lectin-enriched *E. histolytica* proteins)? Are any of them potential vaccine candidates?

**MATERIALS AND METHODS**

**Fluorescence microscopy.** Logarithmic-phase trophozoites of the genome project HM1 strain of *E. histolytica* were chilled to release adherent organisms, concentrated by low-speed centrifugation, and washed in chilled phosphate-buffered saline (PBS) (29). For surface labeling, trophozoites were incubated for 30 min at 4°C in cyanovirin-N labeled with either Alexa Fluor 488 (green) or Alexa Fluor 585 (red) (1, 31). Cyanovirin-N-labeled trophozoites were washed three times in PBS and then fixed for 10 min at 4°C in 2% paraformaldehyde in 100 mM phosphate, pH 7.4. For capping experiments, trophozoites labeled with cyanovirin-N were warmed to 37°C for 15 min prior to fixation.

To determine whether N-glycans are replenished on the surface of trophozoites capped with cyanovirin-N, we treated capped and fixed organisms with PBS containing 2% bovine serum albumin (BSA) to quench free aldehydes and then labeled them with cyanovirin-N conjugated to a different Alexa Fluor dye. To demonstrate actin filaments, we permeabilized capped and fixed organisms with 0.15% Triton X-100, washed, and then stained them with 0.1 mg/ml phalloidin conjugated to Alexa Fluor 488 for 1 h at 4°C (18). To determine whether cyanovirin-N capping other *E. histolytica* antigens, we incubated capped and fixed *E. histolytica* with an Alexa Fluor-labeled mouse monodonal antibody to the Gal/GalNAc lectin (a generous gift of William Petri) (32, 39). Alternatively, capped and fixed *E. histolytica* organisms were incubated with an Alexa Fluor-labeled mouse monoclonal antibody to the *E. histolytica* PPG (a generous gift of Michael Duchêne) (33).

For internal labeling with cyanovirin-N, we fixed *E. histolytica* trophozoites for 10 min at 4°C, and Triton X-100 was added to a final concentration of 0.1% for 1 min. Cells were gently pelleted by centrifugation, washed with PBS–2% BSA, and then incubated with cyanovirin-N, as described above. Similar methods were performed for labeling the surface and interior of *E. histolytica* with anti-Gal/GalNAc antibodies and for determining whether Gal/GalNAc lectins are replenished on the parasite surface after capping.

The nuclei of *E. histolytica* cells labeled with cyanovirin-N or the anti-Gal/GalNAc antibody were stained with 0.1 µg/ml 4',6'-diamidino-2-phenylindole (DAPI), SlowFade antifade solution (Invitrogen) was added, and organisms were visualized with a DeltaVision Deconvoluting microscope (Applied Precision, Issaquah, WA) with channels for each fluorochrome. Images were taken at a primary magnification of ×100 and deconvolved using Applied Precision’s SoftWoRx software.

**Phagocytosis of mucin-coated spheres.** Assays for *E. histolytica* phagocytosis of mucin-coated spheres were performed, as described previously (18). Briefly, *E. histolytica* trophozoites (10⁵/ml) were incubated with microspheres (10⁷/ml) in E. mucin-coated spheres were performed, as described previously (18). Briefly, 

**Mass spectrometry.** Mass spectra of *E. histolytica* proteins was performed using two different methods, as two different mass spectrometers were used. For the linear trap quadrupole (LTQ) ProteomX ion trap mass spectrometer (Thermo Finnigan) present at the Boston University Proteomics Core Facility, *E. histolytica* peptides were prepared and analyzed using methods that were essentially the same as those used to identify peptides from the *E. histolytica* cyst wall (54) or from lectin affinity preparations of *Giardia* glycoproteins (42, 56). In addition, some samples were run on a similar Thermo Finnigan mass spectrometer at the Cancer Center at the Massachusetts Institute of Technology (MIT). Mass spectra were compared to tryptic digests of *E. histolytica* proteins predicted from whole-genome sequencing using SEQUEST, GPM (The Global Proteome Machine Organization [www.thegpm.org]) open source software, or Mascot software (13, 22, 23).

Two-dimensional protein gels were simulated from mass spectrometry data using GPM, where the position of each protein was determined by its predicted pl and mass, not including posttranslational modifications, and the size of the spot was proportional to the number of observed ions corresponding to that protein. These two-dimensional gels highlighted relative abundances of secreted and plasma membrane proteins (defined by either an N-terminal ER-targeting sequence or a transmembrane helix) [TMH] (24, 36) versus nucleocytoplasmic proteins (defined by the absence of these features). The Excel files in the supplemental material each show the merged results of four mass spectrometric experiments using Mascot software. Proteins previously identified as hypothetical because they showed no homology to other eukaryotic proteins were assigned simple names based upon their topology (e.g., unique nucleocytoplasmic protein, unique secreted protein, unique type 1 membrane protein, unique glycosylphosphatidylinositol [GPI]-anchored protein, etc.). GPI anchors were predicted using the algorithms of Eisenhaber et al. (15). Where there seemed a good match in the nonredundant (NR) database as demonstrated by a high score with BLASTP (2), we renamed the *E. histolytica* protein (e.g., “cytoseine protein” or “disulfide isomerase” rather than “conserved hypothetical protein”)

To identify occupied N-glycan sites, we used a two-dimensional chromatographic approach. A peptide mixture was prepared from tryptic digests of enriched *E. histolytica* glycoproteins were treated with PNGaseF to remove N-glycans and to convert Asn to Asp. PNGaseF-treated peptides and an untreated control were separated using strong cationic exchange (SCX) chromatography prior to Nano-flow reversed-phase high-performance liquid chromatography (HPLC)-coupled tandem mass spectrometry (MS/MS). SCX chromatography was performed on a Beckman Coulter ProteomeLab PF2D using a PolySulfoethyl A column. The buffers used were the following: buffer A, 7 mM KH₂PO₄, pH 2.65, 30% acetonitrile (ACN; vol/vol); buffer B, 7 mM KH₂PO₄, 350 mM KCl, pH 2.65, 30% ACN (vol/vol); buffer C, 50 mM KH₂PO₄, 500 mM NaCl, pH 7.5. Peptides were separated using a linear gradient from 0% to 70% of buffer B in 30 min, from 70% to 100% of buffer B in 10 min, and then 100% of buffer B for 6 min. The flow rate used was 0.5 mℓ/min. Thirteen 2-min fractions were collected. Each fraction was dried to eliminate ACN before LC-MS/MS analysis.

LC-MS/MS was performed using a nanoAcuity ultra-performance liquid chromatography (UPLC) capillary system (Waters Corp., Milford, MA), coupled to an LTQ-Orbitrap hybrid mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a TriVersa NanoMate ion source (Advion, Ithaca, NY). Sample concentration and desalting were performed online using a nanoAcuity UPLC trapping column (180 µm by 20 mℓ; packed with 5-µm, 100-A-pore-size Symmetry C₁₈ material; Waters Corp.) at a flow rate of 15 µmℓ/min for 1 min. Separation was accomplished on a nanoAcuity UPLC capillary column (100 µm by 100 mm; packed with 1.7-µm, 130-A-pore-size bridged ethyl hybrid [BEH] C₁₈ material; Waters Corp.). A linear gradient of A and B buffers (buffer A, 3% ACN-0.1% formic acid [FA]; buffer B, 97% ACN-0.1% FA) from 7% to 45% buffer B over 124 min was used at a flow rate of 0.5 µmℓ/min to elute peptides into the mass spectrometer. Columns were washed and reequilibrated between LC-MS/MS experiments. Electrospray ionization was carried out at 1.7 kV using the nanoeMate, with the LTQ heated capillary set to 150°C.

Mass spectra were acquired in the Orbitrap in the positive-ion mode over the range of m/z 300 to 2,000 at a resolution of 60,000. Mass accuracy after internal calibration was within 4 ppm. Simultaneously, tandem MS spectra were acquired using the LTQ for the five most abundant, multiply charged species in the mass spectrum with signal intensities of >5,000 noise levels. MS/MS collision energies were set at 35%, using helium as the collision gas, and MS/MS spectra were acquired over a range of m/z values dependent on the precursor ion. Dynamic exclusion was set such that MS/MS for each species was acquired a maximum of twice. All spectra were recorded in profile mode for further processing and analysis.
and protein assignments were conducted using Mascot to search against the Entamoeba histolytica database employing an error window of 6 ppm on the precursor ions and 0.6 Da on the fragment ions. Table 1 shows occupied N-glycan sites as shown by PNGaseF treatment and mass spectrometry.

**RESULTS**

*Entamoeba histolytica* glycoproteins are capped by the antiretroviral lectin cyanovirin-N and then replenished from large intracellular pools. Cyanovirin-N, which labels α,1,2-linked mannose residues in unprocessed N-glycans, evenly stains the surface of *E. histolytica* trophozoites either kept at 4°C to prevent capping or fixed prior to labeling (see Fig. S1 in the supplemental material). Glycoproteins containing N-glycans are replenished on the surface of *E. histolytica* trophozoites when cyanovirin-N-labeled trophozoites are warmed to 37°C (Fig. 1A and C, large arrows). Subsequent labeling of fixed parasites with cyanovirin-N conjugated to a different Alexa Fluor dye shows that many glycoproteins containing N-glycans are replenished on the surface of *E. histolytica* trophozoites away from the cap (Fig. 1B and C, small arrows). Similarly, Gal/GalNAc lectins that are capped by a monoclonal antibody are replenished on the surface of *E. histolytica* away from the cap (Fig. 1D; see also Fig. S1 in the supplemental material).

The source of the new glycoproteins with N-glycans on the *E. histolytica* surface after capping is likely the large intracellular pool of glycoproteins containing N-glycans. These glycoproteins are clearly visible on cyanovirin-N labeling of fixed and permeabilized *E. histolytica* trophozoites (Fig. 1G). Cyanovirin-N binds to a reticular or membrane pattern to glycoproteins of permeabilized *E. histolytica*. In contrast, the anti-Gal/GalNAc lectin monoclonal antibody labels the membranes and the contents of numerous secretory vesicles throughout the *E. histolytica* trophozoite (Fig. 1H). A model for the replenishment of capped *E. histolytica* surface glycoproteins from large intracellular pools is shown in Fig. 1I and discussed below.

**Cyanovirin-N caps the Gal/GalNAc lectin and glycoproteins recognized by an anti-proteophosphoglycan antibody.** Consistent with the presence of 7 to 10 N-glycan sites on each heavy subunit of the Gal/GalNAc lectin, cyanovirin-N caps the Gal/GalNAc lectin (Fig. 2A to C). The presence of anti-Gal/GalNAc lectin antibody labeling in areas away from the cap (Fig. 2B and C) is consistent with spontaneous replenishment of the Gal/GalNAc lectin from the large intracellular pools concurrent with the capping event. Cyanovirin-N also caps glycoproteins recognized by the anti-PPG antibodies (Fig. 2D to F). There is binding of the anti-PPG in areas away from the cap (Fig. 2E), consistent with replenishment of the PPG from large intracellular pools (data not shown).

**Cyanovirin-N inhibits phagocytosis by *Entamoeba* trophozoites of mucin-coated beads.** Filamentous actin, which is labeled by the fungal toxin phalloidin, is important for amebic motility, capturing, and phagocytosis. Actin filaments accumulate in the region of the cyanovirin-N induced cap (Fig. 2G to I), as has been shown for caps by the plant lectin ConA and by the monoclonal antibody to the Gal/GalNAc lectin.

Cyanovirin-N inhibits phagocytosis of mucin-coated beads by *E. histolytica* trophozoites, a surrogate assay for amebic virulence (Fig. 3A to C). While untreated *E. histolytica* trophozoites phagocytose 43 ± 22 (mean ± standard deviation [SD])
mucin-coated beads, cyanovirin-N-treated trophozoites phagocytose 9 ± 11 (mean ± SD) beads (P < 0.005). The inhibition of phagocytosis by cyanovirin-N is comparable to that caused by overexpression of a dominant negative p21\textsuperscript{rac} mutant that interferes with localization of actin filaments during phagocytosis (18).

*E. histolytica* membrane and secreted proteins are dramatically enriched by affinity chromatography with ConA. Lectin affinity chromatography was performed with ConA-Sepharose because glycoproteins can be eluted with excess α-methyl mannoside. In contrast, glycoproteins bound to cyanovirin-N–Sepharose may only be eluted with SDS that introduces nonspecifically bound contaminants. Western blotting showed that cyanovirin-N conjugated to horseradish peroxidase binds to *E. histolytica* glycoproteins that were enriched by ConA affinity chromatography (Fig. 4A). In contrast, cyanovirin-N no longer binds to *Entamoeba* glycoproteins treated with PNGaseF to remove N-glycans (Fig. 4A). These results confirm that cyanovirin-N is binding only to *E. histolytica* N-glycans.

In the absence of ConA affinity chromatography, the vast majority (87%) of 302 *E. histolytica* proteins identified by mass spectrometry of tryptic peptides are nucleocytosolic (Fig. 4B and C, labeled blue). For example, when proteins are listed by their Mascot score, there are 41 nucleocytosolic proteins before the first secreted protein, a cysteine proteinase (see Excel file S1 in the supplemental material). While they are not the focus of the present study, nucleocytosolic proteins (many of which have greater than 50% peptide coverage) include en-
zymes involved in fermentation, glycolysis, and protein synthesis as well as chaperones and cytoskeletal proteins.

Following ConA affinity enrichment, the majority of \textit{E. histolytica} proteins identified (52%) were membrane or secreted, as shown by the presence of N-terminal signals and/or transmembrane helices (Fig. 4B and C, labeled red). For example, 25 of the 30 proteins with the highest Mascot scores are secreted or membrane proteins rather than nucleocytoplasmic proteins (see Excel file S2 in the supplemental material). These glycoproteins (many of which have greater than 50% peptide coverage) include well-characterized virulence factors such as all three subunits of the Gal/GalNAc adherence lectin, as well as lysosomal proteases and phosphatases (see Excel file S2) (7, 10, 29, 32, 39). ER chaperones, protein disulfide isomerases, peptidyl-prolyl cis-trans isomerases, and calreticulin are all abundant. Of particular interest for discovery of potential vaccine candidates are 27 unique type 1 membrane proteins and six unique GPI-anchored proteins (see the FASTA file in the supplemental material). In the absence of information with regard to the location of any of these unique proteins, in the Excel files in the supplemental material the proteins with TMHs were arbitrarily assigned to the plasma membrane while proteins with an N-terminal signal peptide and no TMHs were assigned to the lysosome.

\textit{E. histolytica} glycoproteins with occupied N-glycan sites include numerous proteins implicated in amebic pathogenesis. ConA-enriched glycoproteins were treated with PNGaseF, and peptides in which the predicted Asn was converted to Asp were identified by a shift in mass of +0.984 Da (Table 1). These modified peptides (32 total), which represent occupied N-glycan sites, are absent from \textit{E. histolytica} proteins that have not been treated with PNGaseF. Glycoproteins (26 total) with occupied N-glycan sites include numerous well-characterized virulence factors and/or vaccine candidates (heavy and intermediate subunits of the Gal/GalNAc lectin, serine and cysteine peptidases, and a receptor kinase) (Table 1) (8, 10, 32). Other
glycoproteins with occupied N-glycan sites include 17 unique proteins that are secreted, membrane associated, or GPI anchored. Because some of these unique E. histolytica proteins with occupied N-glycan sites are both short and abundant (e.g., EHI_077530 is 206 amino acids long with 56% peptide coverage and EHI_161040 is 180 amino acids long with 42% peptide coverage), it is likely that they would make good vaccine candidates. A list of unique E. histolytica glycoproteins is shown in the FASTA file in the supplemental material.

**DISCUSSION**

**Capping is more complex than previously supposed.** While actin-mediated capping of amebic proteins has been described (3, 16, 18, 19, 50), this is the first demonstration, to our knowledge, of replenishment of surface antigens from large intracellular pools. This process is shown in the model in Fig. II, where the precip surface antigens are shown in green, and the precap internal pool of antigens is shown in red. During capping of the green antigens by cyanovirin-N or antibodies to the Gal/GalNAc lectin, the red antigens move from internal pools to cover the parasite surface. Because replacement occurs so quickly, the effects of cyanovirin-N on amebic phagocytosis *in vitro* (shown here) and of antibodies to the Gal/GalNAc lectin (32, 39, 43) and to PPG (6, 33, 35, 40, 48) on amebic virulence *in vivo* are likely not simply based upon clearing the relevant proteins from the parasite surface. Instead, the effects of cyanovirin-N and of antibodies to the Gal/GalNAc lectin or to PPG are likely also mediated by perturbation of the cytoskeleton during capping (3, 16, 18, 19, 50) and/or by signals transduced by various receptors (Gal/GalNAc lectin and/or receptor kinases) (8). Conversely, it does not appear that E. histolytica trophozoites escape anti-Gal/GalNAc lectin or anti-PPG antibodies by capping and removing antigens from their surfaces as both the Gal/GalNAc and PPG are rapidly replenished from large intracellular pools.

While there was no surprise that the E. histolytica Gal/GalNAc lectin has occupied N-glycan sites (32), it was not possible in advance to predict that glycoproteins recognized by anti-PPG antibodies are also capped by cyanovirin-N (6, 33, 35, 40, 48). The latter result suggests that some E. histolytica glycoproteins contain both N-glycans and O-phosphodiester-linked glycans.

**E. histolytica glycoproteins include well-characterized virulence factors, as well as numerous unique proteins that may be novel vaccine candidates.** ConA affinity chromatography enabled the identification of >100 E. histolytica secreted and membrane proteins by mass spectrometry. The gel-free mass spectrometric methods used here are easier than cutting proteins from two-dimensional protein gels and result in relatively fewer cytosolic proteins identified than methods in which membranes or lysosomes are isolated (12, 26, 37, 51, 52, 55, 56). However, these other mass spectrometric studies reveal differences between virulent and avirulent strains of *Entamoeba* and demonstrate accessory proteins (e.g., Rabs) involved in vesicle sorting, endocytosis, and protein secretion.

Gal/GalNAc lectins are among the most abundant E. histolytica glycoproteins identified here, consistent with their prior identification by monoclonal antibodies and their importance in amebic pathogenesis (32, 39, 43). Dozens of unique and abundant E. histolytica glycoproteins identified here by mass spectrometry include new vaccine candidates (type 1 membrane proteins and GPI-anchored proteins) and/or new proteins involved in pathogenesis (secreted proteins). Of course, vaccine candidates and proteins involved in pathogenesis may be overlapping (e.g., the Gal/GalNAc lectin) (39). Recombinant versions of these unique E. histolytica glycoproteins, many of which are relatively small and not too Cys rich, might be used to vaccinate animal models and so add to the relatively short list of amebic vaccine candidates (Gal/GalNAc lectins, serine-rich E. histolytica protein [SREHP], and the 29-kDa protein) (9, 32, 39, 46, 47). Knockdown or knockout methods might be used to test the roles of these proteins in amebic virulence (27, 34).

These results suggest the possibility that E. histolytica N-glycans may be a new target for antiamoebic reagents. Unprocessed Manα1,GlcNAc2, by far the most abundant E. histolytica N-glycan, is recognized by the antiretroviral lectin cyanovirin-N that has been overexpressed in *Lactobacillus* (1, 25, 28, 31, 41, 46). Cyanovirin-N-expressing lactobacilli (“yogurt plus”) might be introduced into the gastrointestinal tract, where the bacteria may have an antiamoebic effect. Other bacterial lectins that target high-mannose N-glycans of HIV (e.g., griffithsin and banana lectin [BanLec]) may have even greater efficacy than cyanovirin-N versus E. histolytica (38, 49). Conversely, it may be possible to vaccinate against amebic infection...
with high-mannose N-glycans present on *Saccharomyces* mutants (14, 30).

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