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# Identification of Galactose as the Immunodominant Sugar of Leishmanial Excreted Factor and Subsequent Labeling with Galactose Oxidase and Sodium Boro[$^3$H]hydride

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Inhibition by low-molecular-weight sugars of precipitin line formation between a polysaccharide (EF) excreted by *Leishmania tropica* subsp. *major*, *Leishmania enriettii*, and rabbit antileishmanial antibodies on double gel diffusion plates revealed that galactose residues, possibly as components of lactosyl groups, were the critical immunodominant sugars mediating antibody recognition of EF. The galactose residues of the EF of *L. tropica* subsp. *major* were specifically labeled with tritium via galactose oxidase and sodium boro[$^3$H]hydride. The radioactive EF had an apparent molecular weight of about 85,000 on sodium dodecyl sulfate-polyacrylamide gels and was precipitated by antileishmanial antibodies as well as *Ricinus communis* lectins I and II (galactose specific). Lectins specific for glucose-mannose residues, fucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine did not precipitate the labeled EF. Treatment of [$^3$H]EF with proteolytic (trypsin, papain, protease) or glycosidic (α-amylase, β-galactosidase) enzymes had no effect on either the electrophoretic pattern of the material or on its recognition by antileishmanial antibodies or *R. communis* lectin. This resistance to enzyme activity suggests that EF may be a useful marker for the presence of the parasite in vivo if it can be detected in minute quantities.

Simple sugars interfere with antigen-antibody reactions if they have structures similar to sites on the antigen that are recognized by the antibody (9). For example, agglutination of T antigen-activated erythrocytes by anti-T antigen antibodies from guinea pigs recovering from *Leishmania enriettii* infections was inhibited by both galactose and lactose. The reaction was also inhibited by an *L. enriettii* surface glycoprotein, and it was concluded that immunodominant galactosyl residues in the glycoprotein were responsible (16).

*L. enriettii* and other leishmanial species are classified by serotypes that are distinguished by the specific precipitation reaction between rabbit antisera raised against whole parasites and a polysaccharide (excreted factor, EF) excreted by the growing parasite (4, 17, 20, 21). EF contains glucose, galactose, mannose, and amino sugars, and oxidation of EF with sodium periodate completely abrogates the precipitation reaction (20, 21). On the other hand, proteolytic enzymes, heating, and acidic or basic conditions have no effect on antibody recognition of EF (4). It was therefore of interest to determine which of the sugars are immunodominant in this system and to capitalize on their stability for the radioactive labeling of EF.

Our results show that galactose residues, possibly as components of lactosyl groups, were the critical immunodominant sugars. Furthermore, these galactosyl groups may be specifically labeled by treatment with galactose oxidase and sodium boro[$^3$H]hydride. The radioactive EF had an apparent molecular weight of about 85,000, and treatment with proteolytic or glycosidic enzymes had no effect on its structural or immunological characteristics.

## MATERIALS AND METHODS

**Strains.** Standard leishmanial isolates of serotypes $A_1$ (*Leishmania tropica* subsp. *major*, LRC-L 137) and $B_3$ (*L. enriettii*, LRC-L 144) were obtained from the World Health Organization Leishmania Reference Collection, Jerusalem.

**Antisera.** Antibodies against living promastigotes were prepared by six intravenous injections into rabbits (17). Antirabbit serum (goat) was a commercial preparation obtained from Miles-Yeda, Rehovot, Israel.

**Lectins.** Lectins used in these studies were obtained from either Sigma Chemical Co., St. Louis, Mo., or Miles-Yeda.

**EF.** EF was prepared by a modification of the method of Slutzky et al. (20). After boiling and dialysis, the media was extracted three times at 68°C with 90% phenol. The aqueous phases were pooled, dia-

lyzed against distilled water, and then chromatographed on a column of Sephadex G-50 equilibrated with 0.02 M ammonium bicarbonate. The EF, which elutes in the void volume, was indistinguishable from the protein-free EF described previously (20).

**Sugar inhibition assay.** The effect of sugars on the EF-antibody precipitation reaction was determined by adapting the standard double gel diffusion method (Ouchterlony; 17). Increasing dilutions of EF solutions from 2.0 to 0.0625 mg/ml were tested against full-strength antiserum on agar plates. In addition to 1.1% Noble agar, 0.6% NaCl, and 0.02% $NaN_3$, each plate contained a concentration of the sugar to be tested (see Table 1). All plates were maintained at room temperature and read every 24 h for 3 days. Results were expressed qualitatively: inhibition was rated as significant when precipitin lines failed to develop at EF concentrations up to twice the concentration giving a precipitin line on control plates. Inhibition of precipitin line formation at EF concentrations lower than this was rated as partial inhibition (see Table 1). All inhibition assays were carried out in triplicate, and little variation was found among the test plates.

**Labeling of EF.** Samples of EF (5 mg dry weight containing 3 mg of hexose as determined by the method of Dubois et al. [3]) were labeled by treatment with galactose oxidase (20 U/ml; Sigma) for 25 h at 37°C in phosphate-buffered saline, pH 7.2, succeeded by reduction with sodium boro[$^3$H]hydride (30.8 Ci/mol; New England Nuclear Corp., Boston, Mass.) for 10 min. Labeled EF was recovered by precipitation with *R. communis* lectin II (molecular weight, 120,000; Miles-Yeda), solubilization in 0.2 M galactose, and chromatography on a small agarose column. Control samples were incubated with sodium boro[$^3$H]hydride without prior treatment with galactose oxidase. The amount of lectin protein in precipitates was determined by the method of Lowry et al. (12).

**Enzyme treatments.** Samples of labeled EF were incubated with the following enzymes (Sigma) for either 2 h at 37°C or 24 h at room temperature. All incubations were carried out in phosphate-buffered saline, pH 7.2. The enzymes tested were trypsin (Type III from bovine pancreas; 10,000 to 13,000 U per mg of protein; tested at 2 mg/ml), papain (Type IV from *Papaya latex*; 10 to 15 U per mg of protein; tested at 2 mg/ml), protease (insoluble bound to carboxymethyl cellulose, from *Streptomyces griseus*; 60 to 80 U per g of solid; tested at 0.12 to 0.16 U/ml), α-amylase (Type VI A from porcine pancreas; 12 U per mg of solid; tested at 2 mg/ml), and β-galactosidase (Grade III crude from bovine liver; 0.15 U per mg of protein;

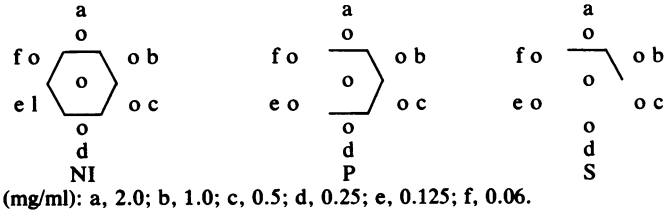
TABLE 1. Inhibition of EF-antibody precipitation by various sugars

| Sugar[a] | Inhibitory effect[b] (active concn) | |
|---|---|---|
| | $A_1$ serotype | $B_3$ serotype |
| Monosaccharides | | |
| Glucose | NI | S (500 mM) |
| Mannose | NI | S (250 mM) |
| Fucose | NI | NI |
| Arabinose | NI | NI |
| Galactose | S (125 mM) | S (125 mM) |
| α-Galactoside | ND | S (125 mM) |
| β-Galactoside | ND | S (125 mM) |
| Disaccharides | | |
| Sucrose (α-D-glucose-β-D-fructose) | NI | NI |
| Trehalose (α-D-glucose-α-D-glucose) | NI | NI |
| Lactose (β-D-galactose-D-glucose) | P (60 mM), S (250 mM) | P (60 mM), S (125 mM) |
| Trisaccharides | | |
| Raffinose (α-D-galactose-sucrose) | NI | NI |
| Polysaccharides | | |
| Arabinogalactan | NI | NI |

[a] All sugars were from Sigma except glucose (Mallinckrodt) and trehalose (Nutritional Biochemicals).

[b] Serial dilutions of EF were tested by double gel diffusion on agar plates containing 30 to 500 mM of the inhibitory sugar. Abbreviations: NI, No inhibitory effect at sugar concentrations of 500 mM or less; S, (significant inhibition) formation of precipitin line inhibited at twice the EF concentration giving a line on control plates; P, (partial inhibition) formation of precipitin line inhibited at lowest EF concentration giving a line on control plates; ND, not done.

Examples:




EF concentrations (mg/ml): a, 2.0; b, 1.0; c, 0.5; d, 0.25; e, 0.125; f, 0.06.

tested at 2 mg/ml). All enzymes solutions were prepared from newly opened bottles immediately before use.

**Electrophoresis.** Samples were run on disc gels containing 5.6% acrylamide and 0.1% sodium dodecyl sulfate (SDS; 21). After electrophoresis the gels were sliced into 2-mm sections, and the slices were incubated for 2 h at 50°C in Soluene (Packard Instrument Co., Rockville, Md.). Samples were assayed for radioactivity by counting in a toluene-2,5-diphenyloxazole–1,4-bis-(5-phenyloxazoly) benzene (PPO-POPOP) scintillation cocktail.

## RESULTS

**Inhibition of EF-antibody precipitation by sugars.** The qualitative assessment of the inhibitory activity of the various sugars on the formation of EF-antibody precipitin lines is shown in Table 1. Lactose and galactose were the most effective sugars. Lactose completely eliminated the reaction of the $A_1$ serotype at 250 mM and the $B_3$ reaction at 125 mM. Even concentrations as low as 60 mM were partially inhibitory for both serotypes. Galactose gave significant inhibition at concentrations above 125 mM. The two structural isomers 1-*O*-methyl-α-D-galactopyranoside and 1-*O*-methyl-β-D-galactopyranoside were equally as effective as galactose when tested with the $B_3$ serotype. The $B_3$ reaction was also significantly inhibited by mannose concentrations above 250 mM and by glucose at concentrations above 500 mM. However, these sugars did not inhibit the $A_1$ reaction. Fucose was not inhibitory at the concentrations tested.

As a control the effect of lactose on the formation of precipitin lines between rabbit serum albumin and goat antirabbit serum was tested. None of the lactose concentrations tested had any effect on this reaction.

**Labeling of EF.** Purified EF of *L. tropica* subsp. *major* was labeled with tritium by treatment with galactose oxidase and then with sodium boro[$^3$H]hydride. The specific activity of the labeled EF was $8.6 \times 10^6$ cpm per mg of hexose. An identical sample was exposed to the sodium boro[$^3$H]hydride without prior galactose oxidase treatment. The specific activity of this sample was $7.3 \times 10^5$ cpm per mg of hexose, indicating that more than 90% of the label was specifically incorporated into galactose or galactosamine residues. Treatment with galactose oxidase and incorporation of the label did not effect recognition of EF by antibody or by *R. communis* lectins I and II (galactose specific).

**Electrophoretic analysis of labeled EF.** A portion of the labeled EF (ca. $5 \times 10^4$ cpm, corresponding to 5.8 μg of hexose) was dissolved in SDS and analyzed by polyacrylamide gel electrophoresis (Fig. 1, solid line). A broad peak corresponding to approximately 85,000 daltons was found. The only other radioactive band

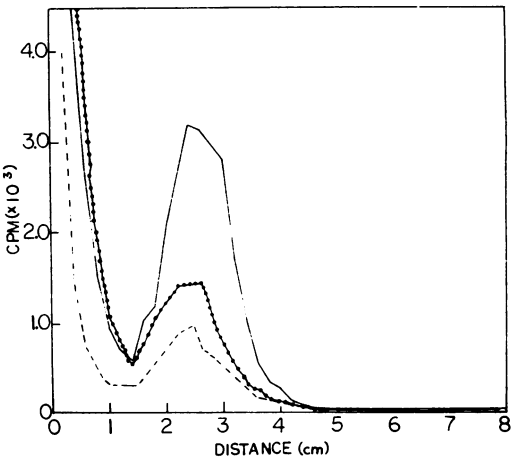



FIG. 1. Analysis of [$^3$H]EF by SDS-polyacrylamide gel electrophoresis. Samples of labeled EF (—), EF *R. communis* lectin II complex (----), or EF-antibody complex (-●-●-●-) were dissolved in 2% SDS and electrophoresed on gels containing 5.6% acrylamide and 0.1% SDS as described in the text. Slices of 2 mm thickness were assayed for radioactivity in a Packard Tricarb liquid scintillation counter. Approximate molecular weights were determined by electrophoresis of proteins of known molecular weight (20–22).

appearing on the gels was a highly labeled band at the very top of the gel (apparent molecular weight, >200,000).

A second portion of radioactive EF ($5 \times 10^4$ cpm, corresponding to 5.8 μg of hexose) was added as a tracer to a solution of unlabeled EF (5 mg/ml). *R. communis* lectin II (molecular weight, 120,000) was added to final concentration of 0.5 mg/ml; and the resulting precipitate, which contained virtually all of the radioactivity, was washed three times in cold water, dissolved in SDS, and electrophoresed (Fig. 1, broken line). A third portion of labeled EF ($10^5$ cpm, corresponding to 11.6 μg of hexose) was added to 5 ml of unlabeled used growth medium (crude EF). Homologous rabbit anti-*L. tropica* serum (0.25 ml) was added, and the sample was incubated at 37°C for 1 h at 4°C overnight. The insoluble immune complex, which contained about $5 \times 10^4$ or nearly 50% of the counts per minute, was washed three times in cold water and then dissolved in SDS. A sample containing $2 \times 10^4$ cpm was then electrophoresed (Fig. 1, dotted line). The electrophoretic profiles (Fig. 1) indicate that the labeled EF component of a molecular weight of about 85,000 as well as a high molecular weight fraction were recognized by both lectin and antibodies. All electrophoretic runs were performed in triplicate, and Fig. 1 shows representative mobility profiles.

The radioactive EF was treated with proteo-

lytic and glycosidic enzymes (as previously described) for periods of 2 to 24 h. None of the enzymes tested caused any changes in the electrophoretic profile of the EF or in its recognition by either *R. communis* II lectin or anti-*L. tropica* serum. These results are in accord with those of El-On et al. (4) who have found that EF-antibody recognition was not effected by pronase, trypsin, neuraminidase, or hyaluronidase.

**Precipitation of EF by lectins.** Use of labeled EF enabled the determination of the affinity for EF of several lectins of different sugar specificities. Of the lectins tested, concanavalin A (glucose-mannose specific), wheat germ lectin (*N*-acetylglucosamine specific), soybean (*N*-acetylgalactosamine specific), lotus (L-fucose specific), pea (glucose-mannose specific) and *Dolichos biflorus* (*N*-acetyl-galactosamine specific) did not precipitate with the labeled EF. On the other hand, *R. communis* lectins I and II (specific for galactose) recognized and precipitated the radioactive EF. This recognition was inhibited by the inclusion of galactose in the reaction mixture, and the lectin-EF complex could be solubilized by the addition of galactose.

In a typical experiment with *R. communis* lectin II, 600 μg of labeled EF (specific activity, $5.5 \times 10^4$ cpm per mg of EF) was precipitated with 500 μg of lectin. The pellet contained 81.2% of the labeled material or 487.5 μg of EF. Analysis by the Lowry method showed that this EF was complexed with 470 μg of lectin protein. The molecular weight of the lectin is 120,000 and that of the EF is 85,000 (Fig. 1). Therefore, the molar ratio of EF bound to the lectin is 1.5. However, if from the discussion which follows, we assign 18,000 as the molecular weight of EF, then the ratio would be 7.1.

## DISCUSSION

The results of the sugar inhibition test indicate that D-galactose residues form part of the site on EF recognized by rabbit antileishmanial antibodies. Lactose was equally active in inhibiting the precipitation reaction of both of the serotypes tested, whereas the α-methyl and β-methyl galactosides were not more effective than galactose alone, perhaps suggesting that the binding site on the antibody has greater affinity for a di- than for a monosaccharide. However, raffinose, a trisaccharide with α-D-galactose as the first sugar, was not inhibitory. When the galactose was masked by other sugars, as in the case of arabinogalactan, it was completely without inhibitory activity (nonetheless, arabinogalactan is readily precipitated by *R. communis* II lectin).

The data from the lectin precipitation experiments are in accord with those of the sugar inhibition study. Only the two *R. communis* lectins, which are specific for galactose, precipitated EF. That the galactosyl moieties bound by the lectin might be components of lactosyl groups is quite reasonable since lactose is about four times more effective an inhibitor of *R. communis* lectin than is galactose (15). The failure of other lectins with galactose-galactosamine affinities to precipitate the [$^3$H]EF has not been explained, but we suspect that some structural characteristic of the lectins themselves prevent the precipitation.

It has previously been reported that EF may be precipitated by concanavalin A (2, 8) and wheat germ lectin (20). However, we were able to separate EF, which does not precipitate with these lectins, from contaminating polysaccharides, which do. Even after phenol-water extraction and Sephadex G-50 chromatography, EF samples contain small amounts of concanavalin A- and wheat germ lectin-precipitable contaminants that do not react with antileishmanial serum. These contaminants can be removed by passage of EF over affinity columns of concanavalin A (which removes both the concanavalin A-reactive component and one of the wheat germ lectin-reactive components) and wheat germ lectin (which removes the other wheat germ lectin-reactive component). All of the contaminants as well as EF are retained by *R. communis* affinity columns (G.M.S., unpublished data). Whether these contaminating polysaccharides derive from serum in the original growth medium or whether they are immnologically nonactive parasite products is not known.

The reaction of $B_3$-serotype EF with antibody was inhibited by several sugars that did not affect the $A_1$-EF-antibody reaction. We are not certain what this implies about the recognition site on the anti-$B_3$ antibody since the lectin-binding characteristics of this EF are exactly the same as those for $A_1$-EF.

EF has been readily labeled with tritium by the galactose oxidase sodium boro[$^3$H]hydride technique (6). Previous attempts to label EF with $^{125}$I by the chloromine T method or with tritium via acetic anhydride were not successful. The failure of these methods was probably due to the lack of tyrosine residues in EF in the first case (23) and perhaps to the lack or masking of free amino groups in the second.

Analysis of [$^3$H]EF by SDS-polyacrylamide gel electrophoresis demonstrated the presence of a component with a molecular weight about 85,000 (Fig. 1). This value is undoubtedly high, however, because of the well-known anamolous behavior of highly glycosylated molecules on SDS gels (18). It is also likely that the material migrating at this locus is an aggregated form of EF, which tends to self-aggregate in aqueous buffers even in the presence of SDS (20). This

tendency is reflected by the amount of labeled material electrophoresing at the very top of the gels in Fig. 1 (apparent molecular weight, >200,000). Several molecular weight values have been reported for EF (both protein-bound and free), with the lowest value being about 18,000 (4, 19, 20). If this figure is correct, then the current value of 85,000 would imply that from four to five EF subunits form the aggregate. Indeed, Semprevivo and Honigberg (19) have suggested that any molecular weight value assigned to EF may be due to artifacts of the isolation procedure and is probably not indicative of the native size of the molecule.

Oxidation of EF with sodium periodate completely eliminates the EF-antibody reaction (20). Less drastic treatment of the material with various glycosidic and proteolytic enzymes seems to have no effect on either the structure of EF or on its immunological properties. Thus EF may be a useful marker for the presence of the parasite in vivo if it can be detected in minute quantities.

The lactosyl moiety is ubiquitous in the repertoire of mammalian cell surface carbohydrates. What benefits can the leishmanial parasite derive from the production and release of a material rich in galactose or lactose residues? Glycoproteins with terminal galactose residues are quickly removed from circulation (1, 14) and some types of macrophages have been shown to possess specific surface receptors for galactose (10, 13). Since EF has been implicated as being a macrophage conditioning agent on the one hand (7) and a lymphocyte inhibitor on the other (11), it may be that the galactose-rich nature of EF facilitates its rapid uptake by the cells crucial to the survival of the parasite in the host (5).

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