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LR1: A candidate RNA virus of *Leishmania*

(*Leishmania braziliensis guyanensis*/single-stranded RNA/viral particle/electron microscopy)

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ABSTRACT **Although viruses are important biological agents and useful molecular tools, little is known about the viruses of parasites. We report here the discovery of a candidate for an RNA virus in a kinetoplastid parasite. This potential virus, which we term LR1, is present in the promastigote form of the human pathogen *Leishmania braziliensis guyanensis* CUMC1-1A but not in 11 other stocks of *Leishmania* that were examined nor in *Trypanosoma brucei*. The candidate viral RNA has a size of ≈6000 nucleotides, is single-stranded, and is largely, if not exclusively, located in the cytoplasm. No homologous LR1 sequences are detected in genomic DNA. The candidate viral RNA is associated with a spherical particle 32 nm in diameter that has a sedimentation coefficient of ≈130 S. There is as yet no evident effect of this potential virus on parasite physiology or the disease caused by the parasite.**

The presence of viruses in parasitic protozoa may be relevant to the diseases caused by these organisms and may be useful for molecular biological studies. DNA viruses have been reported in *Amoeba* (1) and virus-like particles have been observed in several *Plasmodium* species (1), in *Nagleria* (1), in *Endotrypanum* (2), in the cytoplasm of *Leishmania hertigi* (3, 4), and in the flagellum of *Trypanosoma melophagium* (5). Double-stranded RNA viruses have been found in *Giardia* (6) and *Trichomonas* (7). In addition, circular DNAs have been detected in *Leishmania* (8).

We report here the discovery and preliminary characterization of a multicopy RNA in the cytoplasm of *Leishmania braziliensis guyanensis* that is associated with a spherical particle 32 nm in diameter. This may be an RNA virus, to the best of our knowledge, the first virus found in a kinetoplastid parasite.

MATERIALS AND METHODS

Organisms. The *Leishmania* stocks examined in this study are shown in Table 1. They were grown as the promastigote forms at 28°C as described (9).

Nucleic Acid Isolation. Total cellular RNA was prepared by the urea/phenol/cesium chloride method (10). Genomic DNA was prepared as described in Milhausen *et al.* (11). Gel purified LR1 RNA was prepared from 1.35×10^{10} *L. braziliensis guyanensis* CUMC1-1A cells by lysis in 5 ml of 1% $NaDodSO_4$/proteinase K (1 mg/ml)/25 mM EDTA for 1 hr at 50°C. Chromosomal DNA was removed by potassium acetate precipitation and the supernatant was precipitated with 1-propanol, resuspended, and electrophoresed in a 0.7% agarose gel in TBE (89 mM Tris borate, pH 8.3/2 mM EDTA). The 6000-nucleotide band was excised from the gel, reelectrophoresed, electroeluted, and ethanol-precipitated.

Electrophoresis and Hybridizations. Pulse-field gel electrophoresis was performed as described (9). RNA was electrophoresed either in 1.2% agarose/2.2 M formaldehyde gels as described (10) or in native 0.6% agarose gels in TBE at 1.8 V/cm for 12–16 hr. After electrophoresis RNA was treated with 50 mM NaOH for 30 min and transferred to a Nytran membrane (Schleicher & Schuell) (10). Hybridization to the LR1 cDNA riboprobe was carried out in 50% (vol/vol) formamide/5× SSPE (1× SSPE = 0.18 M NaCl/10 mM NaH_2PO_4, pH 7.0/0.1 mM EDTA)/1× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumen)/denatured salmon sperm DNA (100 μg/ml)/1% Sarkosyl for 20 hr at 65°C. Hybridization to nick-translated LR1 cDNA was carried out at 42°C. Hybridizations were washed with 1× SSPE/0.1% $NaDodSO_4$ at 65°C for 1 hr and with 0.1× SSPE/0.1% $NaDodSO_4$ at 65°C for 1 hr.

Preparation of LR1 cDNA and Riboprobe. Double-stranded cDNA was prepared by a modification of the RNase H method (12). Gel-purified LR1 RNA (25 μl) was boiled with 10 μl of random hexamers (Pharmacia, 0.3 A_{260} unit/μl) in the absence of Mg^{2+}, cooled, and incubated with Moloney murine leukemia virus reverse transcriptase at 20 units/ml (Bethesda Research Laboratories) in the appropriate buffer (13) in a volume of 80 μl for 1 hr at 37°C. The second-strand synthesis reaction mixture was incubated for 1 hr at 14°C followed by 1 hr at room temperature. Ends were filled in with the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) and *Bam*HI linkers were added. The cDNA was ligated into Bluescribe vector (Stratagene) and transformed into DH5α cells (Bethesda Research Laboratories), and LR1 cDNAs were identified by colony hybridizations (14) to the radiolabeled first-strand synthesis product.

LR1 5-30 cDNA riboprobe was prepared with T7 RNA polymerase. cDNA plasmid (1 μg, linearized with *Sal* I) was transcribed under conditions recommended by Stratagene for high-specific-activity transcripts.

Subcellular Fractionation. *L. braziliensis guyanensis* CUMC1-1A cells were lysed with Triton X-100 by a procedure modified from that described in Shapiro and Young (15). Cells (2×10^9 per ml) were lysed in lysis buffer containing 0.25 M sucrose/0.25% Triton X-100 without heparin or cycloheximide. Nuclei were separated from cytoplasm by centrifugation at $2000 \times g$ for 10 min and resuspended in storage buffer [50% (vol/vol) glycerol/20 mM Tris·HCl, pH 8.0/75 mM NaCl/0.85 mM dithiothreitol/1 mM EDTA] at 2×10^6 nuclei per μl.

The cytoplasm was further fractionated by centrifugation. Cytoplasm (0.5 ml) was centrifuged through a 14-ml linear 15–30% (wt/vol) sucrose gradient in 1× SSPE for 2.5 hr in a Beckman SW 40 rotor at 40,000 rpm and 1-ml fractions were collected. The sedimentation coefficients were determined from the average density of the gradient fraction by the method of McEwen (19).

Electron Microscopy. Sucrose gradient fractions were concentrated 5-fold by dialysis against dry Sephadex G-200

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Table 1. *Leishmania* isolates tested for LR1 RNA

Species of *Leishmania*	World Health Organization designation	RNA
L. braziliensis guyanensis	MHOM/SR/81/CUMC1-1A	LR1
L. braziliensis guyanensis	MHOM/BR/75/M4147*	—
L. braziliensis braziliensis	MHOM/PE/83/CUMC3	LR2
L. braziliensis braziliensis	MHOM/BR/75/M2903	—
L. braziliensis braziliensis	MHOM/BR/00/LTB300*	—
L. braziliensis braziliensis	MHOM/BR/75/M2904	—
L. braziliensis panamensis	MHOM/PA/71/LS94*	—
L. braziliensis panamensis	MHOM/BZ/00/470	—
L. donovani chagasi	MHOM/BR/82/BA-3	—
L. mexicana amazonensis	MHOM/BR/76/Josepha	—
L. mexicana amazonensis	MHOM/BR/80/Maria	—
L. mexicana mexicana	MHOM/BZ/82/BEL21*	—
L. major	MHOM/IL/67/Jericho II	—

L. major was provided by Steven Reed (Seattle Biomedical Research Institute). *L. braziliensis braziliensis* M2904 was provided by J.K. Other strains were described (9).
*World Health Organization reference strains.

(Pharmacia), deposited on parlodion-coated grids, negatively stained with 50 μl of 0.3% uranyl acetate and 0.5 μl of 0.015% octadecanol in hexanes (16), and visualized with a Philips model EM-300 electron microscope.

RESULTS

We examined the promastigote forms of 12 stocks of New World and one stock of Old World *Leishmania* and found two that contain an abundant 6000-nucleotide nucleic acid (Fig. 1 and Table 1), based upon ethidium bromide staining of cellular nucleic acids resolved by agarose gel electrophoresis. This nucleic acid was absent from *Trypanosoma brucei* and *Trypanosoma cruzi* (data not shown). The high fluorescent intensity of this nucleic acid in *L. braziliensis guyanensis* CUMC1-1A relative to its size and the fluorescence of the chromosomal DNA in ethidium bromide-stained gels indicates that it has a high copy number, estimated to be >500 copies per cell, based upon its relative fluorescence compared to that of the rRNAs. It is degraded by RNase A and alkali but not by DNase (Fig. 2*A*) showing that it is RNA. It was designated LR1 and examined in greater detail. LR1 is retained after passage of *L. braziliensis guyanensis* CUMC1-1A through the sandfly (data not shown). A similar-sized nucleic acid, designated LR2, was found in *L. braziliensis braziliensis* CUMC3 but since it does not cross-hybridize to an LR1 cDNA (see below) its relationship to LR1 is unknown.

The sensitivity of LR1 to RNase A was retained over a range of salt concentrations as high as 200 mM (Fig. 2*B*), indicating that the RNA is single-stranded. Its presence in RNA preparations that have been pelleted through cesium chloride reveal it has a density >1.7 g/cm^3, also indicating that the RNA is single- rather than double-stranded (17). A segment of LR1 RNA was cloned as a cDNA from the gel-purified 6000-nucleotide RNA and thus is probably from the viral genomic RNA rather than a transcript. The cDNA clone 5-30 (383 nucleotides long) hybridized to RNA from *L. braziliensis guyanensis* CUMC1-1A but not to RNA from *L.*

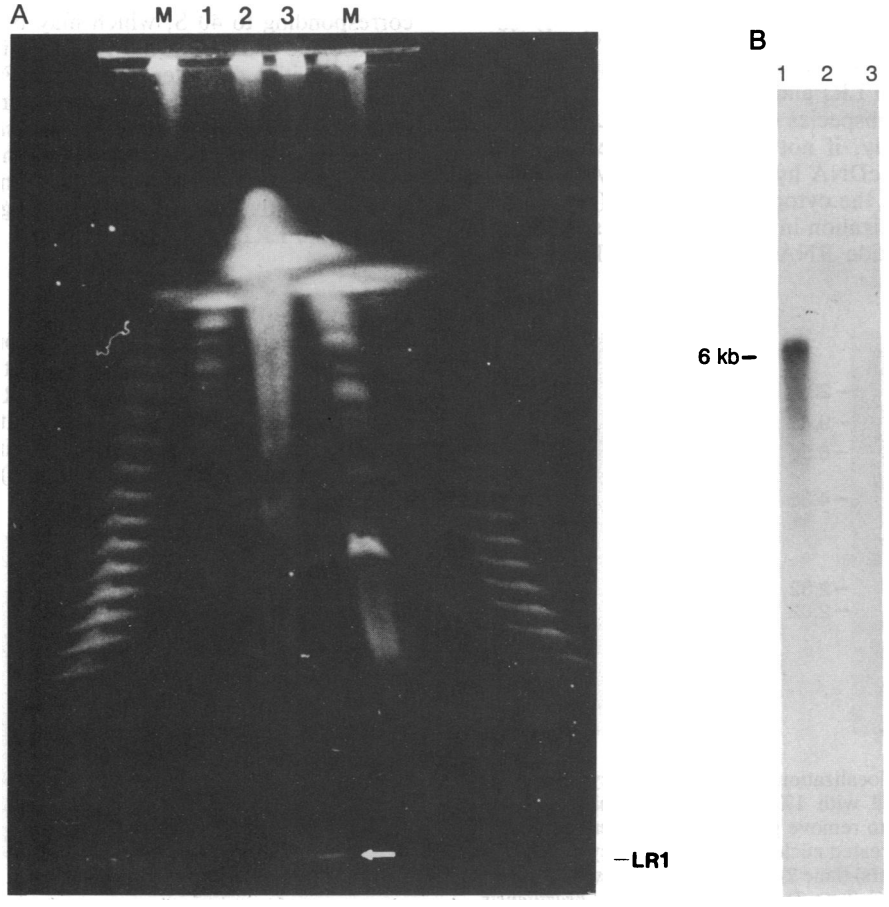

FIG. 1. LR1 viral RNA in *Leishmania*. (*A*) Ethidium bromide-stained pulse-field gel electropherogram showing the multicopy LR1. Lanes: M, ligated λ DNA markers; 1, *L. braziliensis guyanensis* M4147; 2, *L. braziliensis guyanensis* CUMC1-1A; 3, *L. braziliensis braziliensis* M2903. The LR1 band is indicated by the arrow. (*B*) Autoradiogram of a Northern blot showing hybridization of the nick-translated LR1 cDNA clone with total cellular RNA from *L. braziliensis guyanensis* CUMC1-1A (lane 1) but not *L. braziliensis braziliensis* M2903 (lane 2) or *L. major* (lane 3). kb, Kilobases.

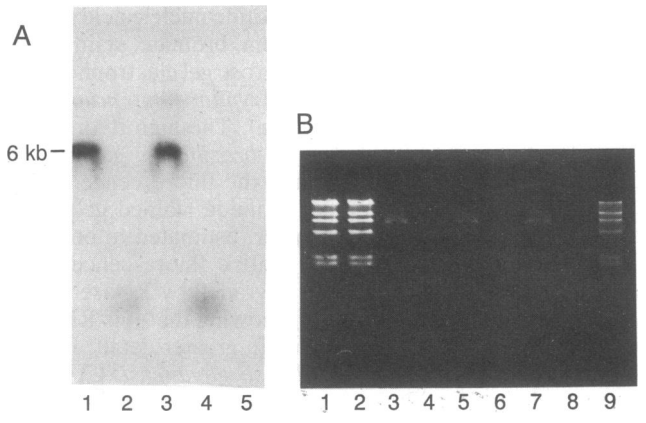

FIG. 2. LR1 contains single-stranded RNA. (*A*) LR1 was degraded by RNase A and NaOH but was not degraded by DNase. Gel-purified LR1 RNA was treated for 10 min at 37°C with buffer alone (lane 1) or with RNase A at 10 μg/ml in low-salt buffer (20 mM Tris·HCl, pH 7.5/3 mM $MgCl_2$) (lane 2), RNase-free DNase I (Pharmacia) at 47 units/ml (lane 3), RNase A plus DNase I (lane 4), or 100 mM NaOH (lane 5). All samples were adjusted to the same salt concentration and electrophoresed in a 0.7% agarose gel, transferred to a Nytran membrane, and hybridized to the nick-translated LR1 cDNA. kb, Kilobases. (*B*) RNase sensitivity is unaffected by salt concentration. Gel purified LR1 RNA was treated with RNase A as described for *A* except that the NaCl concentration was varied. Lanes: 1, 2, and 9, DNA size markers [without RNase (lane 1), with RNase (lane 2), and at lower concentration without RNase (lane 9), respectively]; 3, no NaCl or RNase A; 4, no NaCl but with RNase A; 5, 100 mM NaCl; 6, 100 mM NaCl plus RNase A; 7, 200 mM NaCl; 8, 200 mM NaCl plus RNase A.

braziliensis braziliensis M2903 or *L. major* Jericho II (Fig. 1*B*) nor to *T. brucei* RNA (data not shown). Thus, the 5-30 cDNA originates from LR1 and has a restricted distribution among species and subspecies of kinetoplastids.

LR1 RNA is largely, if not exclusively, located in the cytoplasm. The 5-30 cDNA hybridizes strongly to a 6000-nucleotide RNA from the cytoplasmic fraction (Fig. 3, lane 2). A smear of hybridization in the region corresponding to 2000- to 2500-nucleotide RNAs is also seen. There is no hybridization to RNA from the nuclear fraction in the experiment shown (Fig. 3, lane 1). In some experiments, however, a slight amount of hybridization to a 6000-nucleotide nuclear RNA was detected, which was probably due to cytoplasmic contamination since the nuclear fractions were not washed. Identical results are obtained whether the cells are ruptured by Triton-X100 or by silica carbide grinding (18). Genomic DNA was only barely detectable in the cytoplasmic fraction based upon ethidium bromide staining (data not shown), suggesting only slight contamination of this fraction with nuclear components. Since the nuclear fraction also contains membrane components, these experiments indicate that little, if any, LR1 is associated with the cell membrane. The 5-30 cDNA did not hybridize to Southern blots of genomic DNA from *L. braziliensis guyanensis* CUMC1-1A (data not shown). These experiments incorporated sensitivity controls detecting less than one copy per genome equivalent of the cDNA sequence. Thus, there is no genomic DNA copy of LR1 indicating that it is not a retrovirus.

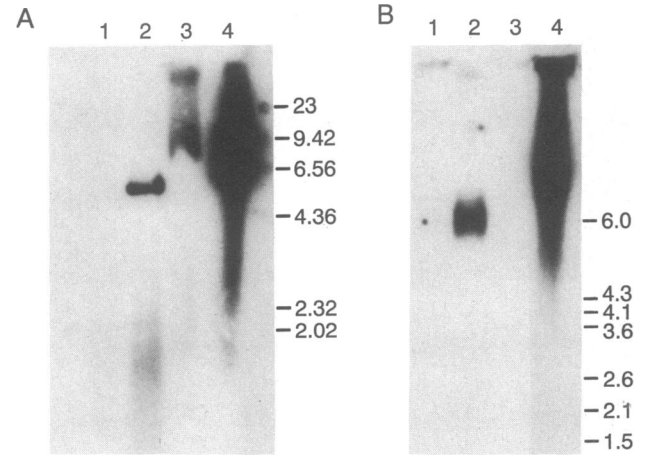

FIG. 3. Subcellular localization of LR1. Nuclei, cytoplasm, or intact cells were treated with 1% $NaDodSO_4$/proteinase K (20 μg/ml) for 1 hr at 65°C to remove protein. (*A*) Nondenaturing gel. Approximately 4×10^7 treated nuclei (lane 1), treated cytoplasm (20 μl, 3×10^7 cell-equivalents) (lane 2), untreated cytoplasm (20 μl, no $NaDodSO_4$/proteinase K) (lane 3), and 10^8 treated *L. braziliensis guyanensis* CUC1-1A cells (lane 4) were electrophoresed in a 0.6% agarose gel and hybridized to the LR1 cDNA riboprobe. (*B*) Denaturing gel. All fractions (lanes as in *A*) were electrophoresed in a formaldehyde-containing 1.2% agarose gel and hybridized to the LR1 cDNA riboprobe. Molecular size markers are in kilobases.

When the $NaDodSO_4$/proteinase K treatment of the cytoplasmic fraction was omitted, the 6000-nucleotide RNA band was absent but a smear of hybridization was observed extending from the well to the region of the gel containing RNA of ≈9000 nucleotides (Fig. 3, lane 3). This suggests that LR1 occurs in a complexed form, perhaps as a particle. This possibility was further examined by using sucrose density gradients. In cytoplasm resolved on a linear gradient, most 6000-nucleotide LR1 RNA is found in the fraction corresponding to a sedimentation coefficient of 130 S (Fig. 4). There is also a 6000-nucleotide LR1 RNA in the fraction corresponding to 40 S, which may be free RNA. Electron microscopic analysis of the 130S fraction revealed spherical particles with a diameter of ≈32 nm (Fig. 5). These particles were not present in comparable sucrose density gradient fractions from *L. braziliensis guyanensis* M4147, which lacks the LR1 RNA. The coincidence of the LR1 RNA and the 32-nm particles in the same fraction and their apparent absence in cells lacking LR1 RNA suggests that the particles contain the LR1 RNA.

DISCUSSION

We conclude that LR1 is probably a single-stranded RNA virus. Its distribution among only one or two of the stocks we examined, the size and morphology of the particle associated with the LR1 RNA, the size of the RNA, its maintenance during fly transmission, and its apparent cytoplasmic location are consistent with its being an RNA virus. Interestingly, the virus-like particles seen in *L. hertigi* (3) are also restricted to the cytoplasm.

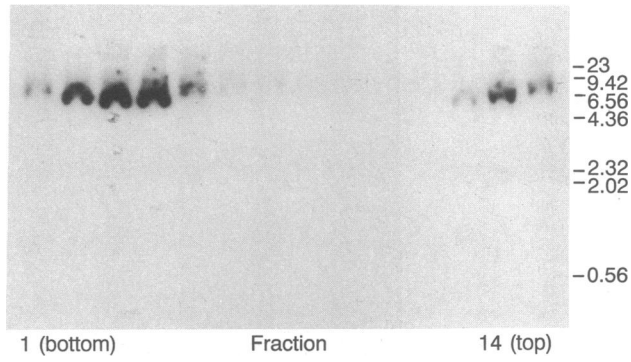

FIG. 4. Sucrose gradient analysis of LR1 particle. Aliquots (20 μl) of the gradient fractions (as indicated) were treated with 1% $NaDodSO_4$/proteinase K (20 μg/ml) for 1 hr at 65°C, electrophoresed on a nondenaturing 0.6% agarose gel, and hybridized to the LR1 cDNA riboprobe. Molecular size markers are in kilobases.

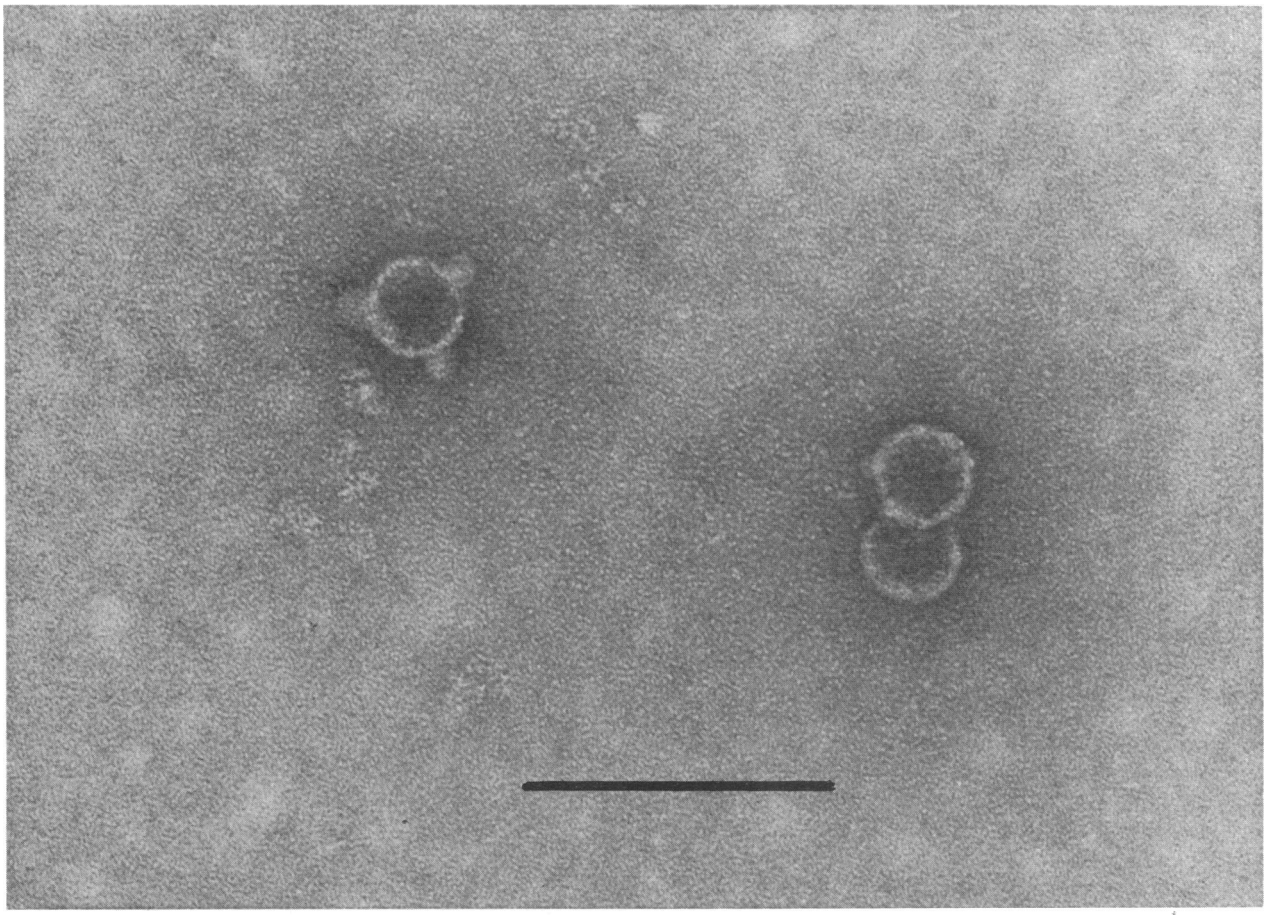

FIG. 5. Electron micrograph of particles from the 130S sucrose gradient fraction (fraction 3 in Fig. 4). (Bar = 100 nm.)

The effects of LR1 on the parasite are not obvious. The stock containing LR1 was originally isolated from a human cutaneous infection of a visitor to Surinam. The infection subsequently produced satellite lesions and symptoms of lymph node involvement but was cured by Pentostam treatment. Passage of the isolate through a hamster produced a mucocutaneous infection. There is as yet, however, no correlation between the presence of LR1 and any disease characteristic, host range, or growth characteristic of *L. braziliensis guyanensis*. The origin of LR1 is also unknown, although it is intriguing that the sandfly, the vector for *Leishmania*, is also a known vector of RNA viruses. The initial characterization presented here is insufficient to determine whether LR1 belongs to an existing class or constitutes an additional class of RNA virus. Perhaps of most significance, however, is the potential for LR1 to serve as a transformation vector for *Leishmania* and possibly other kinetoplastids. This would aid the molecular biological studies of these parasites immeasurably.

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