Giardia virus (GLV) is a small nonenveloped double-stranded (ds) RNA virus that belongs to the genus of Giardiavirus in the family of Toittiviridae (22). GLV infects many strains and isolates of a binucleate flagellated parasite, Giardia, that causes intestinal infections in a very broad range of mammalian hosts. The 6.1-kb dsRNA genome of GLV encodes a 100-kDa capsid protein and a 190-kDa gag-pol-like fusion protein by a translation frameshift (23). The viral genome has a single-stranded RNA intermediate, representing a full-length copy of the positive strand of the dsRNA (3), as demonstrated by successful transfection of uninfected Giardia cells with these RNA species via electroporation (2). Mature virus particles are released into the culture medium of infected cells (20), and GLV is the only infectious virus among all the known small dsRNA viruses isolated from protozoa or fungi (22). The infection of Giardia cells with this virus causes no obvious effects, such as cell lysis or syncytium or inclusion body formation, which would enable visual detection of virus infection. Nevertheless, because of the high number of virus particles produced in the infected cells, this infection can be easily monitored by the presence of viral dsRNA in the infected cells by agarose gel electrophoresis or by Northern (RNA) blot analysis after extracting RNA from the infected cells (2). GLV infection can be inhibited by the inhibitors of endocytosis such as chloroquine and N-ethylmaleimide, demonstrating that the virus probably enters G. lamblia cells via endocytosis (16). Antisera raised in mice against the purified GLV were shown to abolish the viral infectivity (19). Some strains of Giardia, originally free of GLV, however, found to be resistant to the viral infection at an extremely high multiplicity of infection (MOI) of 10⁶ particles per cell (9). The GLV susceptible strains, on the other hand, could be infected at an MOI of 10 particles per cell (9). These data suggest that specific receptors for the virus may be present on the surface of GLV-sensitive G. lamblia cells but missing from the resistant cells as demonstrated in the case of several mammalian viruses (6, 7). Alternatively, the resistant strains may lack a certain cellular factor(s) important for replication of the virus (24).

In this report we demonstrate that two GLV-resistant strains of G. lamblia could become infected with the virus via viral RNA transfection. The presence of the viral dsRNA genome as well as the expression of viral antigen were detectable in the transfected cells resulting in the productive cycle of virus replication. We also demonstrated by indirect immunofluorescence that GLV was bound to the surface of virus-susceptible cells but not to the resistant cells after the virus-cell mixtures were treated with a cross-linking reagent. These observations suggest the presence of a specific virus receptor(s) on the surface of virus-susceptible G. lamblia cells mediating the viral infection. The same receptors appear to be absent from the surface of resistant cells.

MATERIALS AND METHODS

**Materials.** All chemicals used in this study were of reagent grade. Disuccinimidyl suberate (DSS) was purchased from Pierce (Rockford, Ill.). Fluorescein isothiocyanate-goat anti-rabbit immunoglobulin G (IgG) was obtained from Zymed (South San Francisco, Calif.).

**Cell cultures and preparation of RNA.** G. lamblia WB, clone 1, was obtained from R. L. Berens, University of Colorado. It is GLV-free and susceptible to the viral infection (20). G. lamblia Ac (NIH 1083-1) and JH (NIH 1182) were generous gifts from T. E. Nash of the National Institutes of Health. These strains do not contain GLV and are resistant to its infection (9). The culturing conditions have been described previously (20). Total RNA from GLV-infected WB cells was prepared by the hot-phenol extraction method (20).

**Two other species of anaerobic flagellated protozoan para-
sites, Trichomonas foetus KV, and Trichomonas vaginalis ATCC 30001, were maintained and cultivated as described previously (21).

Virus purification and preparation of antisera. GLV was purified as described previously (10), and the antisera against intact purified virus were prepared in rabbits by standard protocols (CalTag, Healdsburg, Calif.). Titers of the antisera used for immunofluorescence were beyond a $5 \times 10^4$-fold dilution as monitored by enzyme-linked immunosorbent assay. The number of virus particles was estimated on the basis of viral RNA content, assuming one dsRNA molecule per particle (20). The antisera stained specifically the 100-kDa capsid protein and the 190-kDa fusion protein but did not recognize the dsRNA (22).

Electroporation. Total RNA was harvested from the early-log-phase cultures of GLV-infected G. lamblia WB trophozoites and electroporated into the virus-resistant strains of G. lamblia as described earlier (2) with minor modifications. Briefly, the cells were grown to confluence, harvested by centrifugation, and resuspended to $10^7$ cells per ml with phosphate-buffered saline (PBS). Total cellular RNA (final concentration, 500 mg/ml) extracted from the GLV-infected WB cells was incubated with 0.5 ml of the cell suspension on ice for 10 min and electroporated in a 0.2-cm cuvette with a Gene Pulser apparatus (BioRad). The settings for introducing RNA into G. lamblia WB trophozoites were at 2.5 kV, 25 $\mu$F, 400 ohm, and a 0.7-s time constant. After three or four pulses, the cells were incubated on ice for an additional 15 min, diluted into 15 ml of fresh culture medium, grown to confluence, and subjected to serial passages (assuming a 1:15 dilution for each passage). Total RNA was extracted from the cells and examined on agarose gel electrophoresis for GLV dsRNA. Each RNA sample applied to the gel was derived from $1.5 \times 10^7$ G. lamblia cells. After staining with ethidium bromide and photographing of the gels, the photos were scanned with an LKB Laser Densitometer (model 2202; Ultroscan) for a comparative estimation of the quantities of GLV dsRNA in each sample. For an estimation of the quantity of GLV dsRNA in the supernatant fraction of G. lamblia culture medium, the latter was filtered through a 0.22- $\mu$m Millipore filter. The filtrate (12 ml) was centrifuged at 65,000 rpm for 45 min with a TLA 100.1 rotor in a Beckman TL-100 ultracentrifuge to sediment the viral particles, which were then examined for dsRNA and its GLV dsRNA content estimated as previously described.

Immunofluorescence microscopy. G. lamblia cells were adhered onto microscope slides, fixed in 2.5% paraformaldehyde-PBS, treated with 1% BSA in PBS containing 0.2% Nonidet P-40 (NP40) for 30 min at room temperature and then permeabilized with the same solution containing 1% NP40. Immunocytochemistry was performed as follows. The cells were reacted with the IgG purified from rabbit anti-GLV antiserum or preimmune serum diluted 200- to 500-fold in PBS containing 0.2% NP40 for 40 min at room temperature and then with fluorescein-conjugated goat anti-rabbit IgG. Fluorescence staining was photographed with an Nikon Epifluorescence microscope.

Crosslinking of GLV to the cellular membrane. G. lamblia WB, Ac, and JH cells were each incubated with GLV at an MOI of $10^3$ viral particles per cell at 4°C in PBS for 30 min. DSS was added to a final concentration of 2.5 mM, and the mixtures were incubated for an additional 30 min at room temperature. The reaction was then quenched by adding Tris-HCl (pH 7.4) to a final concentration of 100 mM, and the cells were pelleted by centrifugation at 1,000 x g for 5 min. The cells were then resuspended in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, washed twice, adhered onto microscope slides, and subjected to indirect immunofluorescence staining as described above.

Viral infection assays. The infection assays were carried out as previously described (19). The culture medium of transfected cultures was tested for the presence of infectious GLV as follows. Cells of uninfected, virus-susceptible G. lamblia WB (4 x $10^8$) were mixed with 14 ml of the Millipore-filtered (0.2-$\mu$m pore size), undiluted culture supernatant. The cells were allowed to grow to confluence, and the presence of dsRNA in the cells was examined by agarose gel electrophoresis.

Metabolic labeling and protein analysis. Metabolic labeling of the proteins of G. lamblia with C$^{14}$S-cysteine (20 mM, 5 Ci/mmol) was carried out as described previously (1). The Triton X-114 detergent phase extracts of trophozoites were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 15% polyacrylamide) under nonreducing conditions as described previously (13).

RESULTS

Transfection of GLV-resistant G. lamblia with the RNA from GLV-infected cells. In the present study, one GLV-susceptible (WB) and two GLV-resistant (Ac and JH) strains of G. lamblia were used (9). None of these three strains harbored GLV originally (Fig. 1A). Cells of the susceptible and resistant strains were each infected with the purified GLV at an MOI of $10^3$. It is known that an infection at an MOI of $10^3$ does not reduce the growth rate of the virus-susceptible parasite significantly (10). The percentage of adherent and nonadherent cells in the infected cultures, which reflects the viability of G. lamblia, is similar to that in the uninfected cultures (10). After infection, the GLV-treated cultures were allowed to grow to confluence and subjected to serial passages. Total RNA samples extracted from the cells were subsequently analyzed by agarose gel electrophoresis. No viral dsRNA was detected in the virus-treated Ac and JH cells after five subsequent passages, confirming that they are resistant to GLV infection (9), whereas in the GLV-susceptible WB cells the viral RNA was readily detectable at 24 h postinfection (Fig. 1B).

Electroporation experiments were carried out in parallel.
After electroporating each of the G. lamblia WB, Ac, and JH strains with the total RNA isolated from the GLV-infected G. lamblia WB cells, the viral dsRNA was detectable in all three recipients after the third serial passage and persisted in all of them up to the tenth passage (Fig. 1C). At the third and the tenth passage, RNA samples, each derived from $1.5 \times 10^9$ cells, were analyzed in agarose gel electrophoresis and densitometry tracing. The results indicated that the ratios of intensities of ethidium bromide-stained GLV dsRNA band among WB, Ac, and JH samples were 1:1.04:1.08 at the third passage and 1:1.30:1.36 at the tenth passage. Thus, assuming that an equal number of RNA molecules entered each GLV-sensitive and GLV-resistant G. lamblia cell during the initial electroporation, the efficiency of GLV replication in the GLV-resistant cells is equivalent to, if not higher than, that in the GLV-sensitive cells.

After nine passages, RNAs were extracted from the pellets of 12 ml of filtrate from the transfected G. lamblia culture medium and analyzed for GLV dsRNA as described before. The ratios among the WB, Ac, and JH samples were estimated to be 1:0.90:0.97, suggesting that the three transfected G. lamblia strains released about the same number of GLV into the culture medium on the ninth passage. It again suggests that the efficiencies of GLV replication in the virus-sensitive and resistant cells are about the same. In the controls, where the same amount of viral RNA was added without electroporation, no dsRNA of GLV was detected in any of the treated cells after 10 passages (data not shown). These results demonstrate that the two GLV-resistant G. lamblia strains can be infected by GLV with the same efficiency as in the susceptible strain via introduction of GLV RNA directly into the cells.

Expression of the viral proteins in the cells transfected with viral RNA. Indirect immunofluorescence studies were performed on the GLV-infected and the RNA-transfected cells to monitor the expression of viral protein. An IgG fraction purified from anti-GLV rabbit antiserum was used as the primary antibody, followed by incubation with goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody. Bright punctate and positively fluorescent granules were found in the GLV-infected G. lamblia WB cells at 24 h postinfection (Fig. 2A). The fluorescent granules were present mainly in the cytoplasm, although, some of them could also be seen attached to the cell surface. The intensity of the stainings varied from cell to cell, from diffused to intense and punctate. The variation of immunostaining could be due to varying phases of virus development in different cells. The same antiserum revealed no positive signal in the resistant Ac and JH cells following an incubation with GLV at an MOI of $10^9$ (Fig. 2A). Preimmune serum did not generate any fluorescent signals from any of the tested cell samples (data not shown).

Among the RNA-transfected cells, however, positive fluorescent signals were detected in all three G. lamblia strains (Fig. 2B). This result indicates that viral protein is expressed in the cytoplasm of all the RNA-transfected cells. The intensities of the fluorescence in the resistant and susceptible cells were similar, suggesting that all three strains were similarly infected by GLV via viral RNA transfection. However, there are large granular fluorescent patches found around the surface of WB cells that are not found around the virus-resistant cells. This difference may indicate binding of progeny GLV to the membrane of the WB cells (see below) but not to the surfaces of Ac and JH cells. The cells incubated with the RNA without electroporation revealed no positive signal at all (data not shown).

Generation of infectious GLV from viral RNA-transfected cells. After the detection of viral protein in the transfected resistant cells, we tested whether virus was formed in these cells and released into the culture medium. The culture medium from each of the three electroporated and serially passaged cells was filtered through 0.22-µm-pore-size Millipore filters, and the filtrates were used to infect the WB cells (20). As shown in Fig. 3, all three culture medium supernatants were found capable of infecting the WB cells to regenerate the GLV dsRNA in the cells. Thus, packaged and infectious GLV particles were apparently produced by all three electroporated strains and released into the culture supernatants.

Electroporation of T. foetus and T. vaginalis cells with the RNA from GLV-infected G. lamblia. The in vitro cultures of T. foetus and T. vaginalis were electroporated with the total RNA from GLV-infected G. lamblia WB cells under the same experimental conditions as described for G. lamblia WB (2) to determine whether the two related protozoa, known to be resistant to GLV infection (18), are also competent in supporting the replication and maturation of GLV after transfection with the viral RNA. T. foetus or T. vaginalis cells were collected at the early log phase of growth, washed, and electroporated with the RNA extracted from GLV-infected G. lamblia WB cells. The electroporated cells were kept up to the sixth passage. No GLV dsRNA was detectable in their nucleic acid extract and no infectious GLV particles could be recovered from these serially passaged cultures (data not shown).

Cross-linking of GLV to the surface of G. lamblia WB cells. The successful viral RNA transfection of GLV-resistant G. lamblia cells indicated that resistance of the two G. lamblia strains Ac and JH to GLV infection is not due to the lack of any necessary cellular factors for the viral replication but rather to the lack of GLV-specific receptor(s) on the membrane surface of GLV-resistant G. lamblia cells. This putative receptor(s) is expected to be present on the surface of the virus-susceptible cells, responsible for the attachment and internalization of the GLV, but missing from the resistant cells. We tested this hypothesis by trying to cross-link GLV to the surface of G. lamblia cells by DSS after incubating the cells with virus at 4°C, which is the temperature allowing mainly the attachment and not the internalization of the virus (16). We then monitored the association between the cell and the virus by indirect immunofluorescence. DSS is a bifunctional cross-linker that has been successfully applied to cross-link various different ligands to their receptors (15, 17, 25). After exposing the cells to GLV at 4°C for 30 min, the mixture of the cells, viruses, and DSS was incubated at room temperature for another 30 min to facilitate the cross-linking reaction before the immunofluorescence stainings. As shown in Fig. 4, the immunofluorescence stain revealed a punctate fluorescence on the membrane surface of the WB cells. Very little, shallow and weak fluorescence can be seen inside the cells (Fig. 4), suggesting that DSS has indeed successfully cross-linked GLV to the surface of WB cells at a noninvasive temperature. The subsequent 30-min incubation at room temperature may have allowed some limited viral invasion, as a similarly weak and shallow fluorescence could also be seen in few cells incubated for the same time periods with GLV without adding the cross-linker (data not shown), though the strong punctate fluorescence on the surface seen with DSS (Fig. 4) was missing. Under identical experimental conditions, neither the Ac strain nor the JH strain demonstrated any sign of fluorescent stainings, thus once again supporting the hypothesis that the virus receptor(s) may be missing from the surface of GLV-resistant G. lamblia cells (data not shown).

Surface protein profile analysis of GLV-susceptible and -resistant G. lamblia cells. It is known that cysteine-rich proteins are abundant on the membrane surfaces of G. lamblia
GLV-RESISTANT G. LAMBLIA LACKS VIRUS RECEPTOR

FIG. 2. Detection of GLV antigen in G. lamblia trophozoites by indirect immunofluorescence with rabbit anti-GLV IgG and fluorescein-conjugated goat anti-rabbit IgG. (A) GLV-infected G. lamblia cells at 24 h postinfection for WB and 5 passages for Ac and JH. (B) GLV RNA-transfected G. lamblia WB, Ac, and JH cells after 9 passages.

which can be readily identified by metabolic labeling of the trophozoites with [35S]cysteine and extracted with Triton X-114 (4). To compare the cell membrane surface protein profiles among the GLV-resistant and -susceptible G. lamblia in the present investigations, the cells were metabolically labeled with [35S]cysteine. The labeled proteins solubilized in Triton X-114 were analyzed by SDS-PAGE and autoradiography (13). The results revealed differences between the susceptible and the resistant cells. The resistant cells lack a major 170-kDa cysteine-rich protein, known to be present on the surface of the virus-susceptible WB cells (12) (Fig. 5). On the other hand, a major 105-kDa protein band present in the Ac and JH Triton X-114 extracts is missing from the extract of virus-susceptible WB cells (Fig. 5).

DISCUSSION

We have clearly demonstrated that (i) two GLV-resistant G. lamblia strains can be readily infected by the virus via viral RNA transfection and (ii) GLV can be cross-linked to the surface of virus-susceptible G. lamblia WB cells but not to the virus-resistant cells. Therefore, (i) GLV-specific receptor(s) must be present on the surface of GLV-susceptible G. lamblia WB cells but missing from the two GLV-resistant strains and (ii) this lack of virus receptor(s) may have contributed to the mechanism of GLV resistance in the two G. lamblia strains. This phenomenon has found its close parallel in the case of poliovirus (5, 8, 14).

On the other hand, two very closely related parasitic protozoa, T. foetus and T. vaginalis, have been shown to be GLV resistant in both the virus infection and the viral RNA transfection tests. Apparently, neither T. foetus nor T. vaginalis can provide a suitable environment for GLV replication, whereas G. lamblia Ac and JH do. This discrepancy is particularly interesting in the case of T. vaginalis because this protozoan is known to have its own specific dsRNA virus, the T. vaginalis virus (21, 22). However, since successful examples of RNA transfection of T. foetus or T. vaginalis have yet to be estab-
established, the present negative results must be viewed with some caution.

Although the mechanism of GLV invasion of *G. lamblia* is not at all understood other than the possible involvement of a specific receptor(s), GLV infection was found sensitive to the lysosomotropic agents chloroquine and ammonium chloride (16). Less than 2% of the virus was recovered from the cells treated with chloroquine or ammonium chloride. Considering these results and our immunostaining data, we believe that GLV invasion is most likely mediated by a receptor-directed endocytosis in *G. lamblia*.

The virus-susceptible and -resistant *G. lamblia* strains used for the present studies belong to two different groups of *G. lamblia* classified by their major immunogenic surface antigens (11, 12). The GLV-susceptible strain WB is a representative of group I and is known to possess a cysteine-rich surface antigen of 170 kDa (12), while the virus-resistant strains Ac and JH belong to group II, which does not have this particular surface antigen. Our preliminary studies indicated also the presence of a cysteine-labeled, Triton X-114-soluble 170-kDa protein in the WB strain and not in the Ac and JH strains. Conversely, a 105-kDa cysteine-rich, Triton X-114-soluble protein in the Ac and JH strains is missing from the WB strain. It is thus highly likely that the 170-kDa protein in our hands is the 170-kDa surface antigen of group I *G. lamblia*. There have been six *G. lamblia* strains found belonging to group I (11). Among them, WB and Isr are GLV negative but susceptible to GLV infection (9). LT, Be2, CAT, and RS are already infected with GLV (9). Presumably, they were sensitive to GLV infection before becoming infected. Among the nine strains or isolates of *G. lamblia* found resistant to GLV infection in our previous studies (9), Ac, JH, and N belong to group II whereas CM is a member of group III, which has also a profile of surface antigens distinct from that of group I (11). The rest of the GLV-resistant *G. lamblia* strains have yet to have their surface antigen profiles analyzed.

All of the above information points to a possible close relationship between the 170-kDa surface antigen and the GLV receptor on the cellular membrane of *G. lamblia*. However, extreme caution must be paid to this hypothesis, because the 170-kDa antigen is known to undergo frequent variations during in vitro cultivation of group I *G. lamblia* (13), whereas the susceptibility of *G. lamblia* WB toward GLV infection never changes. Fortunately, we have now cloned the full-length cDNA of the GLV genome (23). It should not be too difficult to express the viral capsid protein in an artificial system and use it in affinity column chromatography to test for possible specific binding to the 170-kDa protein. Alternatively, it could be useful for purifying the genuine GLV receptor, with the membrane proteins from GLV-resistant *G. lamblia* serving as the negative control.
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