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Persistent Infection of Some Standard Cell Lines by Lymphocytic Choriomeningitis Virus: Transmission of Infection by an Intracellular Agent

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Cell-free cytoplasmic extracts of the Syrian hamster cell lines C13/SV28 and BHK-21F were immunogenic in Syrian hamsters. The resulting antisera cross-reacted completely with antisera against lymphocytic choriomeningitis virus (LCMV) in an immunoradiometric assay employing BHK-21F antigen. Several other Syrian hamster cell lines not previously known to be infected with LCMV were also strongly positive when assayed for viral antigens. Also, several mouse sera and antisera raised in Syrian hamsters against cells transformed by papovaviruses had high titers of anti-LCMV activity. No cytopathic effect was evident in any of the persistently infected cell lines. Culture media from these cells were not infectious and showed no evidence of defective interfering particles. However, cell-free extracts of all the persistently infected cells contained material capable of transmitting the persistent infection to uninfected cells of Syrian hamsters, rats, mice, green monkeys, and humans. The onset of infection is much slower than when LCMV virions are used. When 2×10^6 uninfected BHK cells were treated with an extract from 100 persistently infected cells, the new infection was apparent within about 12 days. When an extract from 10^6 cells was used, the new infection was apparent within about 5 days, but not sooner. The intracellular infectious material was sensitive to treatment with deoxycholate, Nonidet P-40, or ether but resistant to treatment with RNase or trypsin. It was also large (5,000S) and heterodisperse on sucrose gradients. The infectious material was probably contained in large lipid vesicles and their integrity was probably essential for infection. When a few persistently infected cells were cocultivated with many uninfected cells, a few discrete colonies positive for LCMV antigens were observed after about 5 days. Since the culture media were not infectious, the infection probably spread by cell-cell contact. Several different experiments indicated that interferon did not play a major role in mediating persistence in this case. Persistent infections by LCMV can be maintained without expression of extracellular virus particles and without appearance of large amounts of viral antigens on the cell surface. Cell-cell contact could still allow transmission of intracellular infectious material. In an animal, these properties could circumvent immune surveillance.

Mirault et al. (19) reported that a nucleoprotein antigen of unknown origin ("X antigen") is associated with ribosomes of C13/SV28, a line of simian virus 40 (SV40)-transformed BHK cells. X is not an SV40 antigen since sera from hamsters with C13/SV28 tumors do not react with other SV40-transformed cell lines and since BHK-21F cells (9), which are not transformed by SV40, contain an antigen that cross-reacts with X completely. Preparations of ribosomes from C13/SV28 contain large amounts of X antigen. These were used to induce potent and specific antisera in hamsters, and a sensitive, specific immunoradiometric assay was developed (19). We found that X antigen is derived from lymphocytic choriomeningitis virus (LCMV), a well-known arenavirus (22) that commonly infects populations of mice (31) and hamsters (16). Using the immunoradiometric assay to follow the appearance of LCMV anti-

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gens in uninfected cells exposed either to culture media or to cell-free extracts from infected cells, we found that all of the infectious material is intracellular. We discuss how infections by an intracellular agent might be initiated and propagated in cultures of cells, and we consider the relevance of the work in cell culture to persistent infections in animals.

MATERIALS AND METHODS

Cells and antisera. Cells were grown in a CO_2 incubator in Eagle medium as modified by Dulbecco (DMEM) with 10% calf or fetal calf serum (Irvine Scientific, Irvine, Calif.). Sources of the cells are given in Tables 2 and 3. Cytoplasmic antigen was prepared from BHK-21F or C13/SV28 cells essentially as described by Mirault et al. (19) but with more extensive purification. The crude ribosomal pellet (2,500 absorbance units at 260 nm), dialyzed into a buffer containing 10 mM triethanolamine, pH 7.4, 10 mM EDTA, and 10 mM NaCl for 8 h at 4°C to dissociate the polysomes, was sedimented in four 30-ml linear 20 to 35% sucrose gradients in the same buffer with 5-ml cushions of 2 M sucrose, using an SW27 rotor at 20,000 rpm for 16 h at 4°C. Almost all the antigen was within the bottom 5 ml, which had about 2% of the total absorbance at 260 nm. These fractions were pooled and stored at −20°C. Male Syrian hamsters were injected in a hind leg with about 0.1 ml of antigen mixed with an equal volume of complete Freund adjuvant. After 4 weeks, 0.1 ml of antigen mixed with 0.1 ml of incomplete adjuvant was injected four times at weekly intervals. After 1 week more, individual sera were assayed and two pools with high and low potency were prepared. Immunoglobulin G (IgG) was prepared by precipitation with ammonium sulfate at 40% saturation, followed by chromatography on DEAE- and carboxymethyl-celluloses in a buffer containing 10 mM sodium phosphate, pH 7.2, and 15 mM NaCl. A pool of the peak fractions, 0.7 mg/ml in 20 mM potassium phosphate buffer, pH 7.1, was stored at −20°C or at 4°C in the presence of 0.05% sodium azide. Using the immunoradiometric assay with excess antigen (see below), we found about 10% of the total IgG in the high potency pool and 2% of the IgG in the low potency pool to be directed against LCMV antigens.

Immunoradiometric assays. For immunoradiometric assays, the method of Miles et al. (18) was used, with modifications. Iodinated IgG prepared with Iodogen (Pierce Chemical Co., Rockford, Ill.) according to the method of Markwell and Fox (17) was diluted with buffer A (2.06 g of sodium barbital, about 0.4 ml of 12 M HCl to give pH 7.5, 29 g of NaCl, 4 ml of 5% sodium azide, 1 g of bovine serum albumin, 1 ml of calf serum per liter) and stored at 4°C for up to 3 months. The only loss of potency was due to radioactive decay. For use, this stock solution was diluted periodically with unlabeled IgG in buffer A to final concentrations of 5×10^5 cpm/ml and 140 ng of IgG per ml. Sterile polystyrene tissue culture tubes (Falcon no. 2052, 12 by 75 mm, clear, without caps) were coated by incubating them for 24 h at room temperature with 0.5 ml of unlabeled IgG, 7 μg/ml in 0.2 M $NaHCO_3$. The same solution was reused immediately to coat a total of four sets of tubes. After the IgG was removed, 0.6 ml of buffer A was added to each tube, and the tubes were stored at −20°C. They remain useful for about 6 weeks.

For an assay, coated tubes were thawed to room temperature, buffer was removed by aspiration, and the tubes were rinsed once with 0.6 ml of buffer A; 0.4 ml of buffer B (buffer A supplemented with 25 ml of RNase A solution per liter, 1 mg/ml in 20 mM sodium acetate, heated to 100°C for 5 min) was added, followed by up to 50 μl of antigen. To prepare the antigen, a frozen cell pellet from one 100-mm tissue culture plate was mixed with 1.0 ml of cold extraction buffer (20 mM Tris-hydrochloride, pH 7.4, 20 mM EDTA, 80 mM NaCl, 10% glycerol) and sonicated for about 20 s with a probe at low setting. The set of tubes was covered with foil and incubated at 37°C for about 4 h. The solution of antigen was removed by aspiration, the tubes were rinsed once with buffer A, and 0.5 ml of radioactive antibody stock solution, diluted with buffer A to give about 2×10^5 cpm per tube, was added. After 16 h (overnight) at room temperature, the antibody was removed, the tubes were rinsed twice with 0.6 ml of buffer A, and the amount of bound radioactivity was determined.

The assays were run under conditions of moderate antibody excess. To determine these conditions, tubes were saturated with excess antigen and the amount of undiluted radioactive antibody stock solution that was able to bind under these conditions was determined. For the IgG used here, 2 to 10% of the labeled IgG bound. Unlabeled IgG was then added to some of the radioactive stock until only about 30% of the maximum (0.6 to 3% of the total) bound to saturating antigen. Next, a standard curve was constructed by using this dilution of the IgG. With IgG that was 10% specific, the assay was linear in antigen until about 1% of the input radioactivity was bound, and about 0.06% was bound with no antigen present. This background was very constant and individual assays were quite reproducible. Routinely, several dilutions of standard antigen were included in each set of assays for calibration. Specificity and sensitivity are illustrated by the following examples. An extract from 10^4 BHK-21F, C13/SV28, or newly-infected BHK cells gave a half-maximal response 5 to 20 times the background. An extract from 10^3 infected cells gave a significant and reproducible response over background. An extract from 10^5 uninfected cells gave no detectable increase over the background obtained without antigen.

For competition immunoradiometric assays, antibody-coated tubes were incubated with a large excess of standard antigen in buffer B and washed as usual. Unlabeled test serum, up to 20 μl in 0.5 ml of buffer A, was added for 7 h at room temperature, and 50 μl of labeled anti-X IgG (about 2×10^5 cpm) was added directly to the same solution for 16 h more. The tubes were then washed and counted as usual. Standard dilutions or unlabeled anti-X IgG as the competing serum were included in each set of assays.

To assay for intracellular infectivity, cell-free extracts (prepared from BHK-21F cells unless stated otherwise) were made from three or four nearly confluent 100-mm plates. The cells were washed twice on the plates with ice-cold TS buffer (130 mM NaCl, 5 mM KCl, 0.6 mM sodium phosphate, 25 mM Tris-hydrochloride, pH 7.4, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$), scraped into 2 ml of TS buffer with a rubber

policeman, and centrifuged to yield 0.05 to 0.1 ml of packed cells per plate. The cells were suspended in 0.4 ml per plate of hypotonic buffer (10 mM triethanolamine, pH 7.4, 5 mM $MgCl_2$, 2 mM $CaCl_2$) and homogenized with 40 strokes of a tight-fitting (A type) Dounce homogenizer on ice until virtually all the cells were disrupted, as assayed by trypan blue staining. The mixture was frozen quickly on dry ice and thawed twice to lyse any whole cells remaining, and dilutions were made immediately with TS buffer containing 2% fetal bovine serum. Culture media to be tested for infectivity were centrifuged at 10,000 × *g* for 15 min at 4°C and carefully separated from the pellet before use. Virus was separated from clarified medium by centrifugation at 145,000 × *g* for 40 min at 4°C, resuspended in a buffer containing 10 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA, and sonicated as described by Welsh and Buchmeier (33). Cells for infection (BHK cells unless stated otherwise) were plated at one-fifth confluency in T flasks (25 cm^2) 4 to 16 h before use. At the time of infection, medium was removed, and 1 ml of cell-free extract was added (range, 1/2 to 1/128,000 of a plate equivalent per flask) and the flasks were capped. After 1 h at room temperature, supernatant fluid was removed from the cells, fresh medium was added, and the flask was flushed with CO_2 and replaced in the incubator. The cells were subcultured into 100-mm plates 3 days after infection and at 3- or 4-day intervals thereafter, using about 20% of the cells for subculture and reserving the remainder at −20°C for subsequent immunoradiometric assay.

Plaque assay. L-929 cells were used as described by Popescu and Lehmann-Grube (23), with some modifications. Cells were plated in 3 ml of DMEM–5% fetal calf serum at a density of 3×10^5 per 35-mm tissue culture dish 24 h before infection with 0.2 ml of virus and diluted in phosphate-buffered saline plus 1% fetal calf serum. After 30 min at 37°C the inoculum was removed and the cells were overlaid with 2 ml of 0.4% agarose in DMEM–5% fetal calf serum and incubated at 37°C. After 4 days, 1.5 ml of medium without agarose was added. After 5 days, the cells were fixed for 2 h with 1.5 ml of 10% formaldehyde and then stained.

Assay for DI particles. The assay for defective interfering (DI) particles depends on the ability of the particles to protect L-929 cells against superinfection with standard LCMV (24). Cells were plated at a density of 4×10^4 per 35-mm tissue culture dish 24 h before assay. Challenge virus (LCMV strain WE, about 5×10^4 PFU in 0.1 ml of phosphate-buffered saline plus 1% fetal calf serum) and 0.1 ml of serially diluted test materials were adsorbed to the cells at 37°C for 30 min. The cells were then overlaid and incubated as described above. The number of interference focus-forming units added with the challenge virus (about three per dish) was subtracted in calculating the titer.

Immunofluorescence assays. Cells grown on cover slips were washed with buffer and fixed with acetone. After incubation for 30 min with a 1:20 dilution of hamster anti-LCMV serum, the cover slips were rinsed and the procedure was repeated with a 1:30 dilution of anti-hamster IgG serum labeled with fluorescein isothiocyanate (Miles-Yeda Ltd., Rehovot, Israel).

Biohazards. The L-929 (LCMV) line is persistently infected with the Armstrong strain of LCMV, which has been studied for many years with no evidence of human disease (M. Buchmeier, personal communication). However, the strains of LCMV present in the other persistently infected cells we used are not known. Since LCMV is infectious in humans and since we have been able to infect human cells persistently with extracts of infected hamster cells, we worked under P2 containment. It is possible that reassortment of genome fragments of two LCMV strains could lead to a more pathogenic virus. Therefore, interference assays were done only under an overlay of agarose, and the cells were treated with formaldehyde before plaques were counted.

RESULTS

X virus. Two preparations of standard LCMV antigens (7015/20, Fortner strain in 3T3 cells, and 7022/30, CA1371 strain in BHK-21 cells) obtained from Michael Collins, Microbiological Associates, Bethesda, Md., gave the same response as X antigen from BHK-21F cells when saturating levels of antigen were used in the immunoradiometric assay. Another antigen from the same source (7015/2, Fortner strain, hamster tumor) and extracts of L-929 mouse cells persistently infected with LCMV in vitro each gave about half the response of X antigen at saturation. Furthermore, all anti-LCMV sera tested cross-reacted with anti-21F serum completely at comparable titers (Table 1). However, an antiserum against the related arenavirus Pichinde did not cross-react at all, in agreement with previous results for LCMV virions (3). Unlabeled anti-21F serum also competed completely when a competition assay was done with bound unlabeled anti-LCMV (3), LCMV antigen from L-929(LCMV) cells, and labeled anti-LCMV. Thus, anti-LCMV and anti-21F sera are directed against very similar or identical determinants.

LCMV in other cell lines and anti-LCMV in other antiviral sera. The results of assays for LCMV antigens in several cell lines are shown in Table 2. Of the positive lines, only L-929(LCMV) had been infected intentionally. It seems likely that most of the LCMV-positive transformed cells were infected during passage in infected hamsters, as described previously by Lewis et al. (16). The source of LCMV in BHK-21F is not known.

Reference antisera against 16 viruses known to cause inapparent or persistent infections in mice were tested by competition for cross-reactivity with anti-X serum, and anti-X serum was tested directly for activity against the same viruses (mouse adenovirus, polyoma virus, Kilham rat virus, minute virus of mice, Toolan virus, rotavirus, mouse hepatitis virus, pneumonia virus of mice, Sendai virus, parainfluenza virus type 3, simian virus 5, Theiler's GDVII virus, reovirus type 3, lactic dehydrogenase

TABLE 1. Cross-reactivity of pooled anti-X sera with anti-LCMV and anti-Pichinde sera[a]

Serum	Source	Amt of serum for half-maximal inhibition (μl)	Amt of labeled anti-X IgG competed at saturation (%)
Anti-X (anti-21F)	Syrian hamsters injected with partially purified C13/SV28 or BHK-21F antigen	0.03–0.10 (various pools)	100
Anti-LCMV	Guinea pig anti-Armstrong strain (M. Buchmeier)	0.03	100
Anti-LCMV 17011	Mouse anti-CA 1371 strain (M. Collins)	0.3	88[b]
Anti-LCMV 17005	Guinea pig anti-Fortner strain (M. Collins)	0.1	100
Anti-LCMV	Rabbit anti-WE strain (F. Lehmann-Grube)	0.01	100
Anti-Pichinde	Guinea pig anti-AN 3739 strain (M. Buchmeier)	No inhibition at 3 μl	0

[a] Various amounts of unlabled test serum were incubated with assay tubes saturated with BHK-21F antigen before ^{125}I-labeled anti-21F IgG was added.

[b] Not carried to saturation; 3 μl of serum was used.

virus, mouse mammary tumor virus, and LCMV). Only anti-LCMV and LCMV were positive in these assays. However, several sera raised against SV40 or polyoma in hamsters did cross-react with anti-LCMV, with titers from 100 to 1% of a standard anti-C13/SV28 serum. In some cases it is clear that the transformed cells used to generate tumors were infected with LCMV to begin with (see Table 2), but hamsters carrying tumors induced by HY1A, TSV_5Cl_2, H65, J1, or several other transformed cell lines which showed little or no evidence of LCMV antigens also produced anti-LCMV antibodies. Since prebleed sera were not obtained, it was impossible to determine whether these hamsters had been infected with LCMV before induction of the tumors.

Absence of extracellular infectious virus and DI particles. Persistent infections of cell lines with LCMV are easily established and have been well described before (13, 15, 28, 29). Generally, carrier cell lines produce infectious virus in early passages, but the amount of infectious virus decreases, and more DI particles are found in later passages. We attempted to find LCMV antigen, DI particles, and standard virus in culture media from BHK-21F cells or from BHK-21L cells infected with extracts of BHK-21F cells. After 100-fold concentration of the media, low concentrations of antigen were found, equivalent to the antigen in a preparation of standard virus containing 2×10^4 PFU/ml. This amount of antigen represented less than 0.1% of that present in the cells themselves and could easily have been derived from a small number of disrupted cells. When culture media from BHK-21F cells was tested for infectious LCMV virus in a plaque assay, none was found (less than 2.5 PFU/ml). The immunoradiometric infectivity assay also showed that culture medium from BHK-21F cells was negative (less than 0.1% of the total amount of infectivity present in the cells). Therefore, it is unlikely that an LCMV variant which does not produce plaques (10) was present.

DI particles were determined by the assay of Popescu et al. (24), in which such a DI particle gives rise to a colony of cells protected against standard virus. We could readily demonstrate that DI particles were present in medium from cells infected with standard LCMV at high multiplicity of infection. However, no interfering particles (less than 5 interference focus-forming units per ml) were found in media from BHK-21F cells or from BHK-21L cells infected with an extract of BHK-21F cells. One possible explanation for the apparent lack of DI particles might be that the strain of LCMV present in BHK-21F cells is so unrelated to the WE strain that it does not interfere. This seems unlikely since Welsh and Pfau (35) have found that strains CA1371, M7, UBC, and WE all show cross-interference. Staneck et al. (28) have made similar observations and have also shown that LCMV interferes with Tacaribe, Amapari, and, to a lesser extent, Parana viruses. We found that LCMV strain WE did not plaque on persistently infected BHK-21L cells, whereas it did give the same number of plaques on uninfected BHK-21L cells as on L cells. Vesicular stomatitis virus and Sindbis virus plaqued equally well on all these cell lines (data not shown).

Intracellular infectivity. Cell-free extracts of C13/SV28, BHK-21F(LCMV), and BHK-21L(LCMV) cells contained infectious material when tested on BHK-21L cells. Representative time courses for infections by different amounts of extracts from BHK-21F(LCMV) cells are

TABLE 2. LCMV antigens in cells[a]

Cells	Source	Comments
Positive		
C13/SV28 (Syrian hamster)	Wiblin and MacPherson (36)	
BHK-21F (Syrian hamster)	K. Holmes (3)	
L-929 (LCMV) (mouse)	M. Buchmeier (33)	Intentionally infected with LCMV (Armstrong)
Flow SV (Syrian hamster)	Flow Laboratories	Anti-Flow SV serum is positive
HT 2, 3, 5, and 9 (Syrian hamster)	Polyoma-transformed lines, Imperial Cancer Research Fund, London	Anti-HT2 and anti-HT9 sera are positive
HYIA (mouse-Syrian hamster hybrid)	Imperial Cancer Research Fund	Very low level of antigen by immunofluorescence
Negative		
BHK-spinner (Syrian hamster)	BHK-21/13 cells from E. Penhoet, University of California, Berkeley	
BHK-21L (Syrian hamster)	Cell line carried in Utrecht	
GD 36A, B; GD 248A, B (Syrian hamster)	SV40-induced lymphosarcomas from G. T. Diamondopoulis, Harvard University	
H65 (Syrian hamster)	Imperial Cancer Research Fund	Anti-H65 serum is weakly positive
TSV_5Cl_2 (Syrian hamster)	Tilz et al. (30)	Anti TSV_5Cl_2 sera are positive
J1, AP23, PY3T3, H31/11, 3T3/K, C13/11, 3T3 (mouse and Syrian hamster)	Various polyoma-transformed mouse and hamster lines, from Imperial Cancer Research Fund	Anti-J1 serum is positive

[a] Extracts of the cells were tested by the immunoradiometric assay, with anti-21F serum. With the exception of HYIA (see comments), all positive cells gave a strong response, roughly equal to that of BHK-21F. All cells listed in Table 4.

shown in Table 3. The results of other experiments (not shown) were quite similar. As the amount of extract was decreased, the time needed before antigen appeared in the newly infected cells increased, but the amount of antigen finally present was independent of the amount of extract used, indicating that an infection initiated in a small fraction of the BHK-21L cells can eventually spread throughout the culture. Sometimes extract derived from as few as 100 BHK-21F(LCMV) cells was infectious. No antigen could be detected in newly infected BHK cells until about 5 days after infection, even when an extract derived from 10^6 cells was used to infect 2×10^6 cells. In contrast, in infections with standard LCMV, new virus is produced in less than 1 day (22). Newly infected BHK cells did not show a significant cytopathic effect, in agreement with observations of others for persistent infections by LCMV (28, 29).

All cell lines with a high level of LCMV antigen (Table 2) had intracellular infectious material. Furthermore, BHK-spinner, MA-134, or WI38 cells infected by extracts of BHK-21F or C13/SV28 cells (see below) also contained infectious material capable of infecting hamster cells in a second cycle. The L-929(LCMV) cells infected with LCMV intentionally by Welsh and Buchmeier (33) have both intracellular infectivity and extracellular DI particles (data not shown).

About 10^4 BHK-21F(LCMV) cells or about 5×10^4 PFU of a preparation of LCMV (treated

TABLE 3. Rate of appearance of LCMV antigen in BHK-21L cells infected with cell-free extracts of BHK-21F(LCMV) cells[a]

No. of BHK-21F(LCMV) cells used for extract to infect 2×10^6 BHK-21L cells	% Final amount of LCMV antigen after:		
	6 days	9 days	12 days
400,000	36	100	100
100,000	28	100	100
25,000	28	100	100
6,000	6	90	100
1,500	0	56	100
400	0	45	100
100	0	3	13
25	0	1	0

[a] Quantitative immunoradiometric assays were performed, and the final amounts of antigen found were similar to the levels of BHK-21F(LCMV) cells in all cases.

TABLE 4. Infectivity of a cell-free extract from BHK-21F cells toward uninfected cells

Cells	Source	Day postinfection when antigen was first detected	Day postinfection when last negative test for antigen was performed
Infected			
BHK-spinner (Syrian hamster)	E. Penhoet, University of California, Berkeley	6	
BHK-21L (Syrian hamster)	Cell line carried in Utrecht	6	
Kidney fibroblasts F2408 (rat)	G. Di Mayorca, New Jersey Medical College, Newark	7	
BK11 embryo fibroblasts (mouse)	H. Kaplan, Stanford University	14	
MA-134 kidney (green monkey)	Microbiological Associates	13	
CV-1 kidney (green monkey)	Jensen et al. (11)	10	
WI38 fibroblasts (human)	Hayflick (7)	24	
TSV_5Cl_2 (Syrian hamster)	Tilz et al. (30)	21	
Not infected			
HY1A (mouse-Syrian hamster hybrid)	See Table 2		21
Embryo fibroblasts (chicken)	Primary cells		21
Kidney (cow)	Microbiological Associates		29
Hepatoma (FAZA) (rat)	G. Ringold, Stanford University		41

with Triton X-100, gradient-purified and free of DI particles) gave half-maximal response in the immunoradiometric assay. In the virus preparation used, one PFU corresponded to about 350 physical particles as determined by electron microscopy. Therefore, each persistently infected cell contained enough viral antigen for about 1,750 virus particles. Since less than 10% of the intracellular antigen was associated with intracellular infectious material (see below), each cell contained fewer than 175 infectious virus particles.

The infectivity in a postnuclear supernatant solution from BHK-21F(LCMV) cells was abolished completely by any of the following treatments: 1% deoxycholate at 4°C for 30 min, 0.1% Nonidet P-40 (NP40) at 4°C for 30 min, extraction with ether at 25°C, or heating in a neutral buffer at 60°C for 30 min. Infectivity was not affected substantially by prolonged exposure to 15 mM EDTA at 4°C, by freezing and thawing once, by storage for 1 month at −20°C, by exposure to pancreatic RNase (10 μg/ml) at 25°C for 30 min, or by exposure to trypsin (1 mg/ml) at 4°C for 30 min. Standard LCMV virions were also resistant to the above treatments with RNase or trypsin.

Cells that can be infected. Cells susceptible or resistant to infection by cell-free extracts of BHK-21F or C13/SV28 are shown in Table 4. Most hamster lines could be infected readily, the only exceptions being HY1A (a hamster-mouse hybrid) and TSV_5Cl_2, which was highly resistant but eventually positive. Most mouse cells tested were resistant, but mouse embryo fibroblasts could be infected, although with difficulty. Infection of monkey and human cells was also extremely slow, but eventually positive. No infection of bovine kidney cells, chick embryo fibroblasts, or rat hepatoma cells was obtained in repeated attempts. In many of the above cases of absolute or relative resistance to infection by extracts of LCMV-infected hamster cells, it is well known that standard LCMV can readily infect the cells in question (14). However, we did not test their sensitivity to standard virus ourselves.

Intracellular infectious material separated from most of the intracellular antigen. In the experiment shown in Fig. 1A, a cell-free homogenate of BHK-21F(LCMV) cells was sedimented for 15 min at 35,000 × *g* in a 5 to 20% (wt/vol) sucrose gradient. It is clear that the bulk of the antigen remained near the top of the gradient, whereas the infectivity sedimented further. The major peak of infectivity in tube 9 had a sedimentation coefficient of at least 5,000S. The results of a parallel sedimentation in a 20 to 40% (wt/vol) sucrose gradient were similar except that more infectivity was present in the bottom fractions (data not shown). The material containing the bulk of the antigen could be converted to material with the density of nucleocapsids by treatment with a detergent (see below). The infectious material seemed to be present predominantly in large structures that differed from each other in size, density, or both. Attempts to remove nuclei from the whole-cell homogenates by sedimentation at low speed often caused most of the infectious material to be lost, proba-

bly because it cosedimented with the nuclei under these conditions. However, nuclei that had been pelleted through 1.6 M sucrose cushions in the experiment of Fig. 1A were devoid of infectivity. In another experiment (Fig. 1B), EDTA was included in a 20 to 40% sucrose gradient in an effort to free viral antigens from their association with polysomes as noted previously (1, 19). In this case, the major peak of infectivity in fractions 7 and 8 still had a sedimentation coefficient of at least 1,500S and was associated with very little antigen. Most of the antigen appeared at the extreme top of the gradient.

Density of intracellular LCMV antigens before and after treatment with NP40. An extract of BHK-21F(LCMV) cells was sedimented to equilibrium in a 20 to 50% (wt/vol) sucrose gradient in D_2O and assayed for LCMV antigens with hamster anti-LCMV serum (Fig. 2A). The material in the main peak (density, 1.22) was pooled, made 0.25% in NP40, and sedimented again to equilibrium in a 40 to 80% (wt/vol) sucrose gradient in D_2O in the presence of 0.25% NP40 (Fig. 2B). The major peak in the second gradient had a density of 1.31, about the same as that of LCMV nucleocapsid cores (22). The peaks in both gradients also contained LCMV RNA molecules bound to LCMV proteins (32). Analyses (Fig. 2C) were carried out with rabbit anti-LCMV serum after separation of the LCMV proteins by electrophoresis and transfer to diazotized paper (32). Note that the nucleocapsid protein was present throughout the lower part of the first gradient and was also present in the position of cores in the NP40 gradient. The peak of cores also contained a significant amount of a cellular protein of molecular weight 38,000 (32), showing its close association with LCMV nucleocapsids. The small peak of antigen at the top of the NP40 gradient was free of the nucleocapsid protein. It probably did contain other LCMV proteins, but these were not abundant enough to detect in the transfer experiment shown. Several additional LCMV proteins were detected in transfers carried out with whole cell extracts (32).

Intracellular virions. BHK-21F(LCMV) cells contained at least two distinct types of enveloped virus particles within intracytoplasmic vesicles (Fig. 3). Most of the particles (Fig. 3A) corresponded to the R-type virus already well described in BHK-21F cells (2). Similar particles were seen in control BHK cells free of LCMV. Each cell contained an average of about 800 R-type particles. Much more rarely, BHK-21F(LCMV) cells, but not control BHK cells, contained a different kind of virus particle within intracytoplasmic vesicles. We observed four such examples in 100 thin sections (Fig. 3B

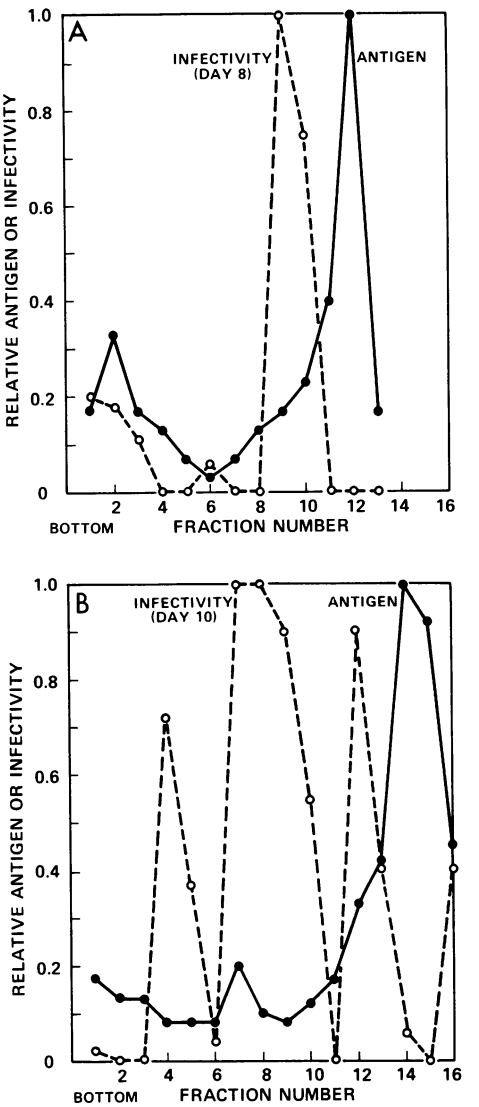

FIG. 1. Analysis of LCMV antigens and infectious material on sucrose gradients. Cells were broken by homogenization at 0°C in a hypotonic buffer containing 7 mM Mg^{2+} and Ca^{2+}. After isotonicity was restored, 1.5-ml portions of the entire homogenate were layered on top of sucrose gradients (8 ml) with 1.6 M sucrose cushions (3 ml) in polyallomer tubes for an SW41 rotor. The gradients were made in 10 mM triethanolamine hydrochloride buffer, pH 7.4, containing 0.08% gelatin and 0.15 M NaCl. (A) A 5 to 20% gradient in 7 mM $MgCl_2$, sedimentation for 15 min. (B) A 20 to 40% gradient in 15 mM EDTA, sedimentation for 60 min. After sedimentation at 14,000 rpm (35,000 × *g*) and 4°C for the times indicated, fractions collected by puncturing the tubes just above the sucrose cushions were assayed for LCMV antigens. Nuclei, which pelleted through the sucrose cushion, were assayed separately. All procedures were carried out under sterile conditions so that assays for infectivity could be carried out.

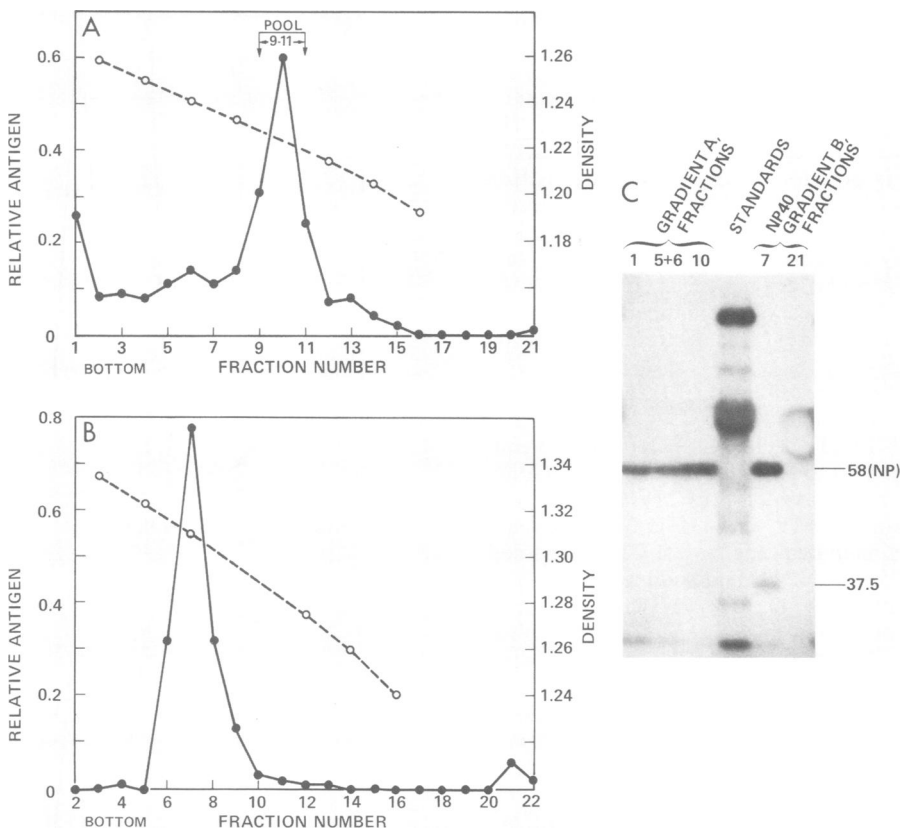

FIG. 2. Equilibrium density centrifugation of viral antigens from BHK-21F(LCMV) cells. (A) A cell-free homogenate (1.0 ml) was layered onto a 20 to 50% (wt/vol) sucrose gradient in D_2O, in 20 mM triethanolamine hydrochloride buffer, pH 7.4–20 mM EDTA–20 mM NaCl and was centrifuged for 18 h at 4°C in an SW50.1 rotor at 50,000 rpm. Portions of each fraction were assayed for LCMV antigens, using hamster anti-LCMV serum. (B) Fractions 9 to 11 from two similar gradients were pooled, NP40 was added to 0.25%, and the sample was layered on top of a 40 to 80% (wt/vol) sucrose gradient in D_2O, in the same buffer but with 0.25% NP40 added. Centrifugation was carried out for 18 h at 4°C in an SW50.1 rotor at 50,000 rpm, and portions of each fraction were assayed. (C) Portions of individual fractions (20 μl) were analyzed by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel and transfer to diazotized paper (32). Rabbit anti-LCMV serum was used to identify the specific proteins.

through E), corresponding to about 10 virus particles per cell. These intracellular particles were somewhat similar to extracellular LCMV virions (Fig. 3F). They were about 50 nm in diameter, compared to 50 to 300 nm for the virions, which are pleiomorphic (22). The example shown in Fig. 3F is about 150 nm in diameter. In the absence of more direct evidence that these cytoplasmic virus particles react with anti-LCMV serum, we cannot be certain of their identity. Such experiments would be prohibitively difficult to perform when there are so few particles per cell.

Spread of LCMV through newly infected cells. We used immunofluorescence to investigate the possibility that the virus could spread by cell-cell contact (Fig. 4). Foci of BHK-21L cells containing LCMV antigens were observed after infection with an extract of BHK-21F(LCMV) cells. The appearance of antigen, monitored by the immunoradiometric assay and by fluorescence, is shown in Fig. 4A. The cells were subcultured on the day of assay and 2, 6, or 12 days after infection. The culture was negative 5 days after infection except for one fluorescent colony, a part of which is shown in Fig. 4B. These data suggest that the infection did spread from cell to cell until all cells finally became positive. Periodic subculturing of infected cultures might enhance this process by dispersing the positive cells. To examine this possibility further, about 10^6 uninfected BHK-21L cells were plated together with about 100 intact BHK-21F(LCMV) or BHK-21L(LCMV) cells. When the cultures were assayed for fluorescent colonies on day 5, as described above, none were

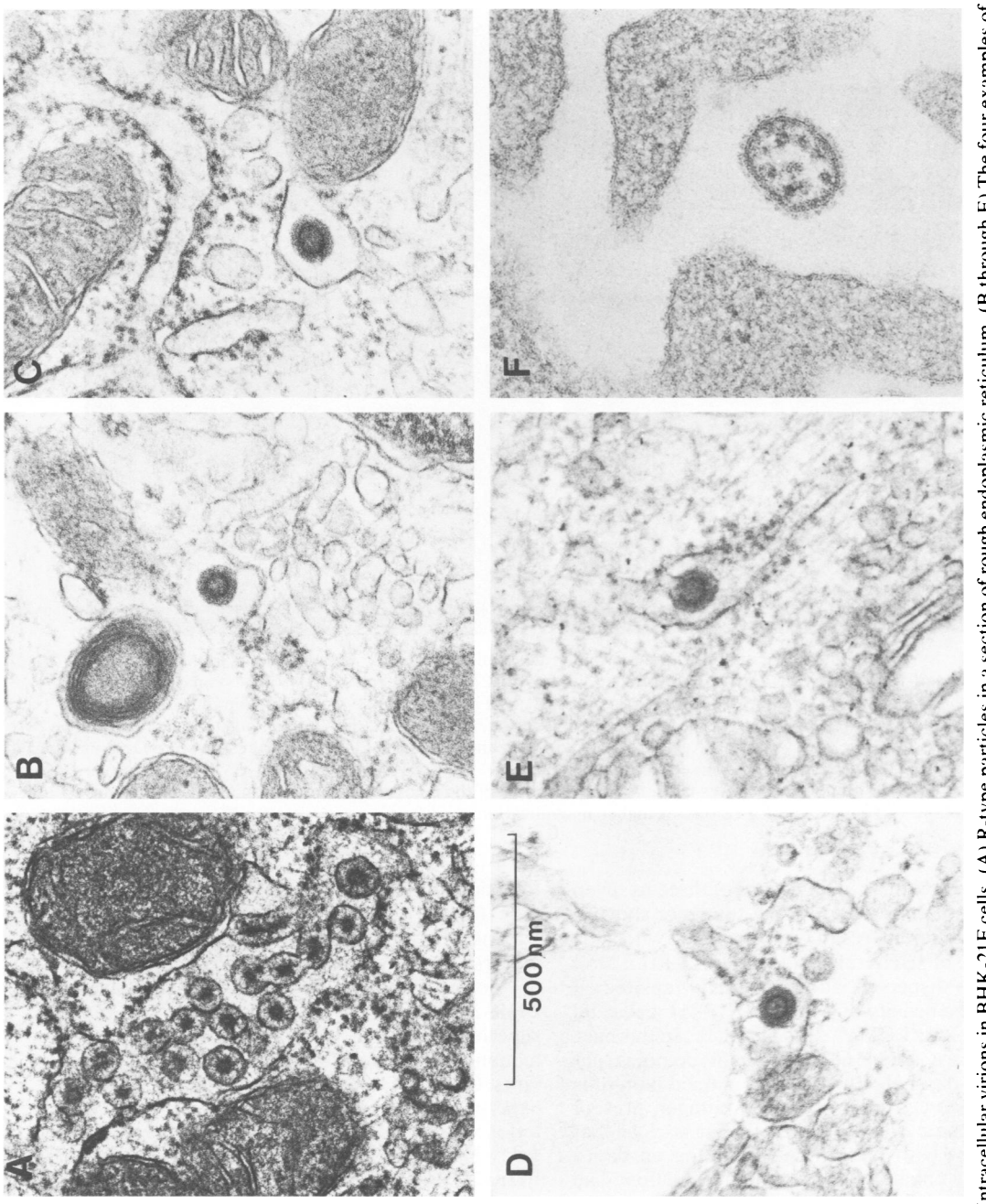

FIG. 3. Intracellular virions in BHK-21F cells. (A) R-type particles in a section of rough endoplasmic reticulum. (B through E) The four examples of different virus particles that were observed in 100 thin sections of BHK-21F(LCMV) cells. (F) An example of an extracellular LCMV virion. The micrograph shown was taken by Peter W. Lampert, University of California, San Diego, and we thank him and Michael J. Buchmeier for supplying a copy of the photograph, which has been published previously (4). The magnification is approximately 70,000, and the virion shown, which was formed in infected mouse L-929 cells, is about 150 nm in diameter.

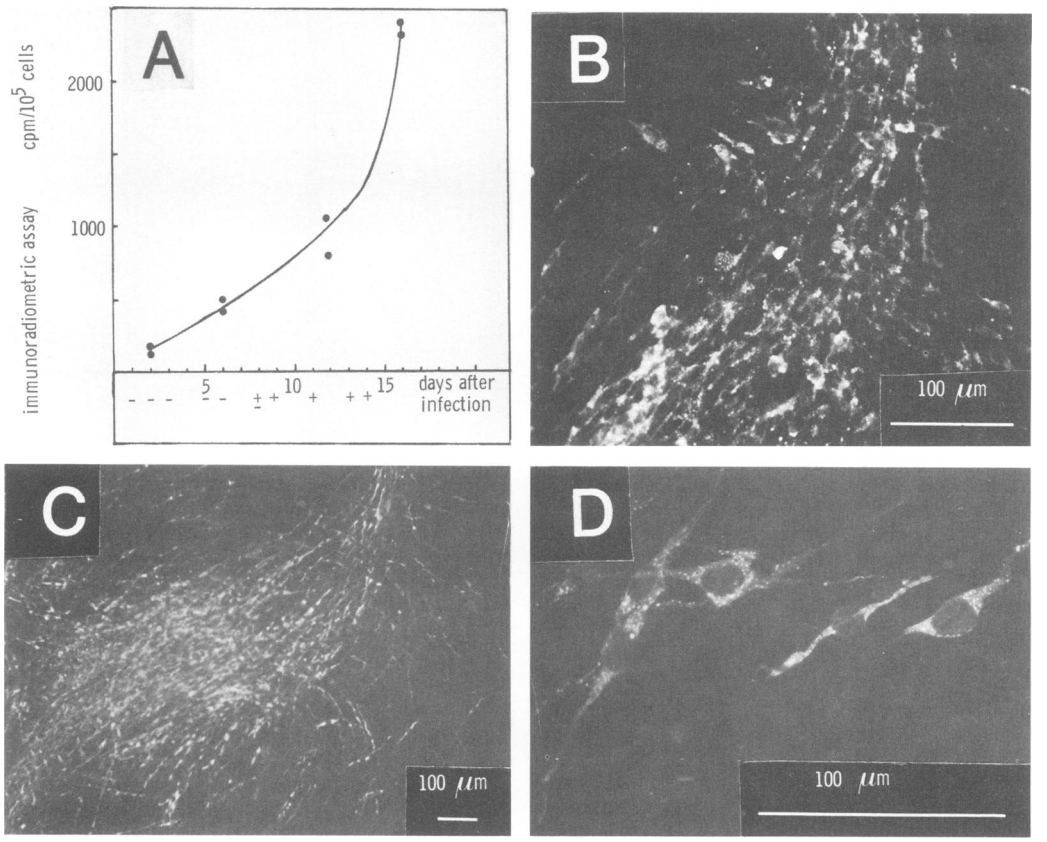

FIG. 4. Evidence for propagation of the infection by cell-cell contact. (A) Appearance of LCMV antigen in BHK-21L cells infected with a cell-free extract of BHK-F(LCMV) cells (ratio, 1:1). The symbols + and − indicate the presence and absence of LCMV antigens, measured by immunofluorescence in parallel cultures. (B) The single fluorescent colony that was apparent 5 days after infection. (C and D) Antigen-positive centers after infection with intact cells. Two days after 10^6 BHK-21L cells and 100 BHK-21L(LCMV) cells were mixed and plated together, the culture was dispersed with trypsin and 2×10^5 cells were replated. LCMV antigens were assayed by immunofluorescence 3 days later. (C) One example of the many fluorescent colonies observed 5 days after infection. (D) The edge of a colony at higher magnification.

observed and only small groups of three to seven fluorescent cells were present (data not shown). A very different result was obtained when the mixture of BHK-21L and BHK-21L(LCMV) cells was dispersed with trypsin and replated on day 2, at a density of 2×10^5 BHK-21L cells and 20 BHK-21L(LCMV) cells per plate. In this case there were about 60 fluorescent colonies per plate on day 5. Each colony contained hundreds of positive cells (Fig. 4C) with antigen present only in the cytoplasm (Fig. 4D). It is clear that treatment with trypsin and replating on day 2 markedly enhanced the spread of infection. The mechanism of this enhancement is not known.

Role of interferon in persistent infection. Cells persistently infected with LCMV which produce extracellular DI particles are refractory to superinfection with standard LCMV but permissive for other viruses, indicating that the persistent infection is not mediated by interferon in this case (13, 33). It was important to determine whether this conclusion also applied to the type of persistent infection described here, in which no detectable virus particles are produced. BHK-21L(LCMV) cells were indeed resistant to superinfection with LCMV but not to superinfection with vesicular stomatitis virus or Sindbis virus (data not shown). In a different type of experiment, pretreating BHK-21L cells with interferon (5,000 U of mouse interferon per ml) for 1 day before infection with a cell-free extract of BHK-21F(LCMV) cells protected the cells only slightly but seemed to delay the onset of infection when large amounts of extract were used.

DISCUSSION

Persistent infection of cell lines by LCMV. A number of cell lines not intentionally inoculated

with LCMV are persistently infected with this virus, probably as a result of passage through infected hamsters or mice. It is not clear whether the persistent infection by LCMV has influenced experiments carried out with these cell lines in the past. BHK-21F cells have been used for many studies with paramyxoviruses because they are extremely sensitive to virus-induced cell fusion (9). It is not known whether the sensitivity to cell fusion is related to infection by LCMV. However, no artifacts were reported in the protein composition of Newcastle disease virus grown in BHK-21F (20). No infectious virus or DI particles were present in growth medium of the persistently infected cells we have studied. Essentially all the infectivity was cell associated, a property which has not been described before.

Intracellular antigens. The persistently infected BHK cells contained relatively small amounts of cytoplasmic LCMV antigen. Some of the antigen was present in ribonucleoprotein particles which also contained LCMV RNAs (32). It has been recognized previously that LCMV ribonucleoprotein particles are associated with ribosomes and polysomes (1, 19) and immunoprecipitates of these particles also contain rRNA (32). The apparent size of the particles was reduced somewhat in the presence of EDTA, which disrupts polysomes (Fig. 1B). The density of the particles was about 1.22 after dissociation from ribosomes with EDTA, a density greater than the values of 1.15 to 1.18 reported for LCMV virions (22). The density of the intracellular particles was increased to 1.31 in the presence of NP40 (Fig. 2B), the same as the density of nucleocapsid cores derived from virions in the presence of detergents (22). The density of the particles without NP40 and their sensitivity to EDTA suggest that they may have been associated with lipids but that they were not completely enveloped by a lipid bilayer, as are LCMV virions.

Intracellular infectious agent. Most of the intracellular nucleoprotein particles were not infectious and the infectious agent could be separated from them (Fig. 1A), especially after treatment with EDTA (Fig. 1B), which affected the size of the infectious agent very little (compare Fig. 1A and B). LCMV is a negative-strand RNA virus with a segmented genome (22), and the two different viral ribonucleoproteins must be present in the same cell to achieve infection. For this reason, the infectivity of the pool of ribonucleoprotein particles might be enhanced if their uptake were facilitated. We suggest tentatively that the large infectious agent we found may be intracytoplasmic vacuoles containing many enveloped LCMV nucleocapsids. Such structures could be formed if nucleocapsids were to bud through the membrane of an intracellular organelle such as the endoplasmic reticulum or the Golgi apparatus. The following observations are consistent with this possibility. (i) The intracellular infectious agent was very large and quite heterogeneous in size. (ii) The infectivity was sensitive to several agents which disrupt lipid bilayers. (iii) In contrast, the infectivity was insensitive to RNase and trypsin, suggesting that viral RNAs and proteins essential for infection may be protected by a lipid bilayer. (iv) A few particles somewhat similar in appearance to LCMV virions were observed within intracytoplasmic vacuoles in BHK-21F(LCMV) cells but not in uninfected controls (Fig. 3). Several attempts were made to block infections by extracts of BHK-21F(LCMV) by using antisera in conjunction with a second antibody or with fixed *Staphylococcus aureus* cells. Unfortunately, the experiments were inconclusive because substantial inhibition was obtained even with control antisera, probably due to the large size of the infectious material.

Infection by a large vesicle containing enveloped virions might be slow because fusion of the vesicle with a cell would release enveloped virions into the cytoplasm rather than nucleocapsids, as in an infection with standard virions. Removal of the lipid coat from these enveloped virions in the cytoplasm, presumably necessary to make the nucleocapsids available for active processes, might be slow. Loss of infectivity after treatment of cell extracts with detergents or lipid solvents does indicate that some entity involving a membrane is likely to be involved. Presumably the infectious agent is converted to noninfectious nucleocapsids by such treatment.

Persistent infections in vivo. A persistent infection will be maintained in an animal only if several conditions are met. Replication of the virus must be inhibited, the immune system must be circumvented, and there must be some way to propagate the infection. If replication were not inhibited, the animal would be killed. DI RNAs are likely to function as a major modulator of virus replication (25, 34). Accumulated mutations in viral proteins (25) may also help to limit replication. One way the persistent virus can avoid immune surveillance is to avoid forming extracellular virions. Another is to avoid placing large amounts of viral antigens on the cell surface—see discussions by Oldstone and Buchmeier (21) and Welsh and Oldstone (34). Both of these stratagems are used by the virus of BHK-21F(LCMV) cells. As discussed in detail by Holland et al. (8), the hypermutability of RNA viruses should give ample opportunity to generate many viral mutants defective in maturation, and strong selection of such mutants should occur in an immunocompetent animal.

There are examples of such mutants for vesicular stomatitis virus (12, 38), measles virus (6, 26), and Sindbis virus (27). Some step in the transport, processing, or assembly of viral membrane glycoproteins is impaired in these mutants. The failure of GP-C to appear on the surface of BHK-21F(LCMV) cells has been shown in an accompanying paper (32). It is possible that mutations in viral coat proteins may prevent them from appearing on the surfaces of some types of cells but may not prevent them from reaching the surfaces of other cell types. In such as case, the immune system of an infected animal would function only against those cells in which the antigen was displayed on the surface, with the surface-negative cells serving as a sanctuary for the virus, even in the presence of anti-viral antibody and natural killer cells.

As suggested by the data in this paper, cell-cell contact is a likely mechanism for propagating a persistent infection without exposing viral antigens to the immune system of the host. LCMV can achieve persistent infection of lymphoid cells (5), which could transmit the infection to other cell types throughout an animal. Lysis of infected cells with release of an intracellular infectious agent insensitive to antibody might also transmit the virus. Alternatively, membrane-bounded fragments of cells containing virus might be absorbed by other cells. Such fragments could be formed by a process termed apoptosis (37). The persistent virus might also be transmitted as a pseudotype, with its nucleocapsid coated by the envelope of some endogenous virus to which the animal is tolerant. We tried without success to produce LCMV pseudotypes from BHK-21L(LCMV) cells after superinfection with Newcastle disease virus (data not shown).

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