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Persistent Reovirus Infection of CHO Cells Resulting in Virus Resistance

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We obtained a persistently infected line of Chinese hamster ovary cells by selection for resistance to reovirus infection. The cells were persistently infected by a population of viruses that were (i) cytopathic for parental chinese hamster ovary cells and (ii) similar to wild-type reovirus in molecular characteristics. The growth rate, plating efficiency, and morphology of the cells were altered. A large majority of the cells in the population were infected. There was no detectable interferon present in the medium. The cells were relatively resistant to a wide range of viruses.

Cells can acquire resistance to virus infection by a number of mechanisms. The least understood of these are mechanisms that do not involve extracellular factors such as interferon and humoral antibodies (15, 24). To study mechanisms of intracellular resistance we have selected Chinese hamster ovary (CHO) cells that are resistant to reovirus. In this communication, we describe one such cell line (VRR) that is characterized by the constant presence of infectious reovirus in the media. Such persistent infections are well known in virology, primarily with viruses that are weakly cytopathic such as certain enveloped viruses (2, 5, 14, 17, 19, 22-24). The molecular nature of such infections is poorly understood.

We believe that this cell line is a unique manifestation of a reovirus infection and that the further study of such an infection with a virus whose molecular biology is extremely well characterized (11) should yield important information regarding the general phenomena of persistence.

MATERIALS AND METHODS

Cells. The parental cell line was CHO-Kl (CCL 61) obtained from the American Type Culture Collection and periodically cloned (10). The cells are routinely maintained in Ham F-12 medium (6) buffered with 25 mM *N*-2-hydroxymethylpiperazine-*N'*2-ethanesulfonic acid (with a consequent reduction in NaH_2CO_3 to maintain isotonicity) (Grand Island Biological Co.). This medium was supplemented with 10% fetal calf serum (Microbiological Associates). The cells are grown at 37 C in Forma water-jacketed incubators. Both CHO and VRR cells were periodically assayed for mycoplasma contamination by L. Hayflick.

Cloning and growth experiments are performed in 50-ml flasks (Falcon no. 1013). Colonies are stained with 0.1% crystal violet in 10% Formalin. Cloning efficiency is routinely 50 to 70% with parental CHO cells.

Virus. Reovirus type III (obtained originally from W. Joklik) was used for the selection and experimentation described. Virus is routinely assayed on CHO cells by serial dilutions of virus (over 10^5 CHO cells/well, plated the previous day on multiwell [Falcon no. 3008] plates). After 3 days, the cells were stained, with small plaques usually visible. It appears that no overlay is necessary for the development of these plaques. The assay for VRR virus is identical and results in plaque sizes identical to those with stock reovirus. Infectious center assays of VRR cells are similar, except the VRR cells must be added simultaneously with the CHO cells. Vesicular stomatitis virus and Newcastle disease virus were gifts of Judith O'Malley, and encephalomyocarditis (EMC) virus was given by Roland Rueckert.

Scanning electron microscopy. Microscopy was performed by F. S. Fay of the Department of Physiology, University of Massachusetts School of Medicine. Standard techniques of fixation, dehydration, and coating were used (3). Pictures were obtained with an Etek autoscan.

Transmission electron microscopy. Media from the infection of CHO cells with the VRR virus were centrifuged at 105,000 × *g* for 1 h. The pellet was resuspended and negatively stained with 2% phosphotungstic acid on carbon- and Parlodion-coated 400-mesh grids. The grids were examined with a Hitachi 11A electron microscope.

Immunofluorescence. All antisera used for immunofluorescence and neutralization experiments were prepared by injecting rabbits in multiple intradermal sites with approximately 0.5 mg of purified reovirus emulsified with Freund adjuvant. Booster injections consisted of 0.1 mg of purified virus injected

intravenously. The antisera obtained neutralized reovirus (but not vesicular stomatitis virus or EMC) at a dilution of $1:10^4$. This antiserum was absorbed with 10^7 disrupted CHO cells/0.5 ml before use, and CHO cell debris was removed. For immunofluorescence, cells were grown on slides containing wells removable for staining (Lab-Tek tissue culture chambers). The cells were fixed for 5 min in methanol, washed, and then incubated 30 min at 37 C with various dilutions of antiserum. They were then washed with phosphate-buffered saline. Then various dilutions of fluorescein-coupled goat anti-rabbit immunoglobulin (Hyland Laboratory) were added for a 30-min, 37 C incubation and again washed with phosphate-buffered saline. We selected the combined concentrations that gave the highest concentration of antiserum with the least background fluorescence. Reovirus-infected cells were used as a positive control. The results shown were obtained with a dilution of reovirus antiserum of 1:40 and a dilution of goat anti-rabbit immunoglobulin G antiserum of 1:20. The slides were examined and photographed with a Leitz Ortholux II fluorescent microscope with transmitted fluorescent light and a K510 suppression filter.

Acrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis of proteins was performed by the method of Laemmli and Favre (12) with a Hoefer slab gel apparatus with 7.5%, 10-cm-long gel.

SDS-urea acrylamide gels of reovirus RNA were run as described by Schuerch and Joklik (20). The RNA was prepared from particles purified on $CsCl_2$ equilibrium density gradients (see below). The virus was heated to 80 C in 0.2 M NaCl with 0.5% SDS for 5 min to release the RNA from the virion.

Iodination. Protein was iodinated essentially by the method of Hubbard and Cohn (8), with modifications introduced by J. Tweto and D. Doyle (personal communication).

VRR virus purification. The media from VRR cells was removed and centrifuged at 15,000 × *g* in a Sorvall RC-2B (SS-34 rotor) to remove cell debris. The supernatant was then centrifuged at 19,000 rpm in a Beckman L-5 ultracentrifuge (19 rotor) for 5 h. The pellet was suspended in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.5) and layered on a $CsCl_2$ gradient (1.325 to 1.430 g/cm^3). The gradient was centrifuged for 16 h at 30,000 rpm (SW50 rotor). The virus band was removed by horizontal puncture of the tube, diluted, and centrifuged at 40,000 rpm (40 rotor) for 2 h. The pellet was resuspended in 1× SSC for iodination and RNA extraction.

Isotopes. ^{125}I was purchased from New England Nuclear (NEZ-033L).

RESULTS

Selection. We selected the cell line described below (VRR) by infecting 10^7 logarithmically growing CHO cells with reovirus type III at a multiplicity of infection of 0.01. Within 2 days after infection at 37 C, widespread cytopathic effects were observed, and by day 3, there were no apparent viable cells remaining. The flask was further incubated at 37 C for 3 weeks. At this time, three distinct colonies of cells existed. The studies reported below were done on one of these colonies that was grown up, subcloned, and then maintained in continuous culture for over 6 months.

Growth characteristics. We first examined the growth rate of the cells and found that they doubled at approximately half the rate of our original CHO population (24 versus 14 h). This was determined by counting the number of cells in large numbers of colonies after sparse plating. The average-size colony was determined on 3 successive days, and the doubling time was determined from this data. Similar results were obtained by trypsinizing cell populations and counting the detached cells in an electronic particle counter.

The cloning efficiency of these cells was found to be about 2 to 5% that of the parent line. This efficiency was not changed by increasing serum concentration from 10 to 20%.

Morphology. The VRR cells exhibit an altered morphology as is shown in Fig. 1. With phase contrast microscopy it can be seen that these cells form relatively diffuse colonies, in contrast to the tightly packed colonies of the parent line. It also appears that there are a number of necrotic cells in the field shown, whereas these are not seen in the parent line. Scanning electron microscopy examination of the cells (Fig. 2) reveals a similar difference showing less apparent adherence of the cells and less cell-cell contact. The microvilli on the cell surface do not seem to be different in the two cell types.

Characteristics of the persistent infection. The fact that there appeared to be necrotic cells in the VRR population led us to believe that virus was present. To investigate this possibility, we performed the experiment shown in Fig. 3. Parental CHO cells were put in the wells of a Falcon multiwell plate at a concentration of 10^5 cells/well. The indicated number of thoroughly washed VRR cells was then added to rows A and B. The medium from 10^6 VRR cells, after 2 days of growth, was added to rows C and D in the indicated dilutions after intact cells had been removed from this supernatant by low-speed centrifugation. Rabbit antiserum prepared against purified reovirus type III was added to rows B and D. After 3 days at 37 C the wells were stained.

The results indicated that (i) both VRR cells and the media from VRR cells were capable of killing parental CHO cells, (ii) the media contained about 10^7 IU/ml, and (iii) reovirus antisera prevented the cytopathic effects of both

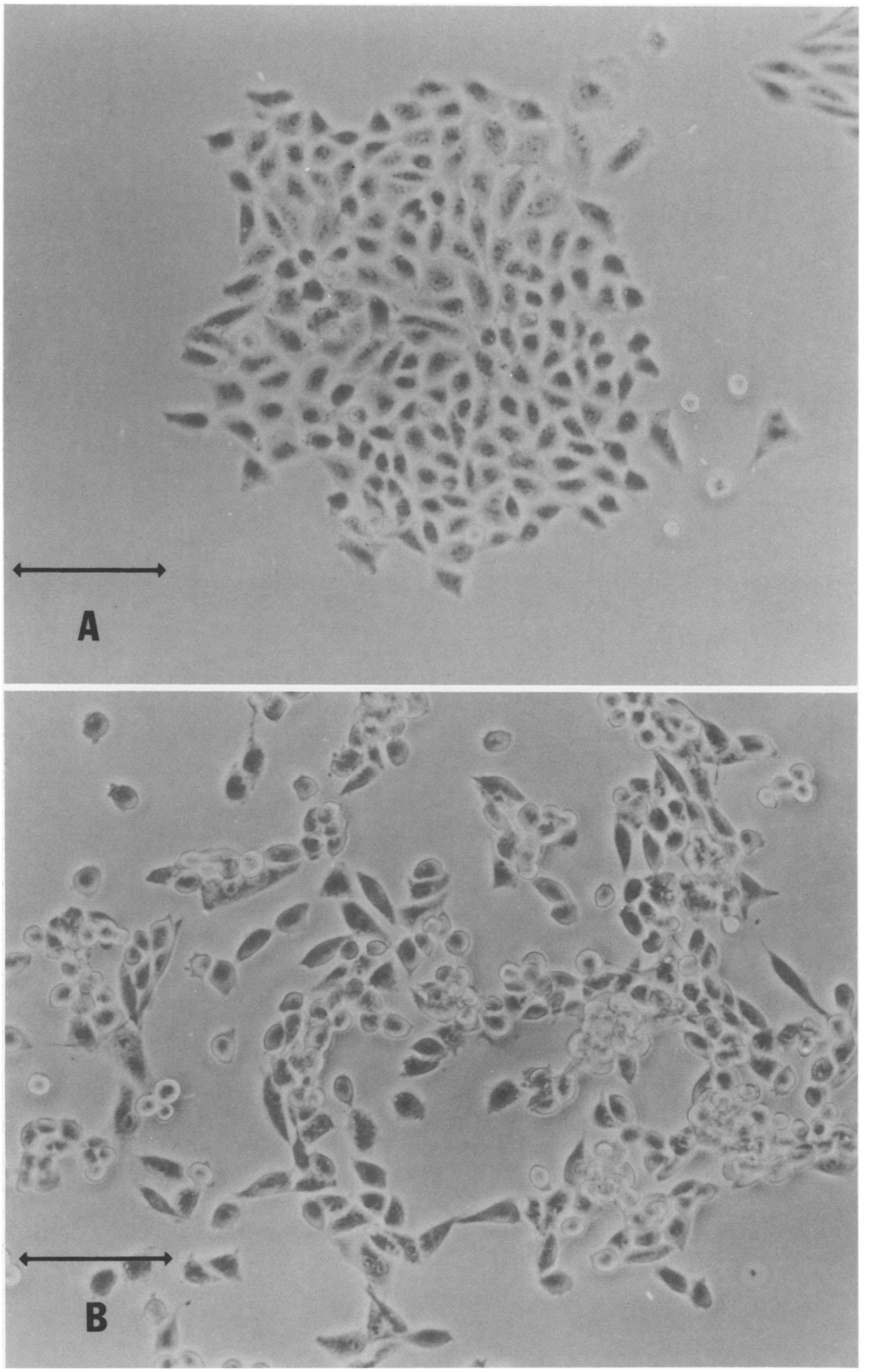


FIG. 1. *Phase contrast microscopy. (A) A discrete colony of parental CHO cells seen by inverted phase microscopy. (B) A colony of VRR cells is seen. The bar represents 50 μm.*

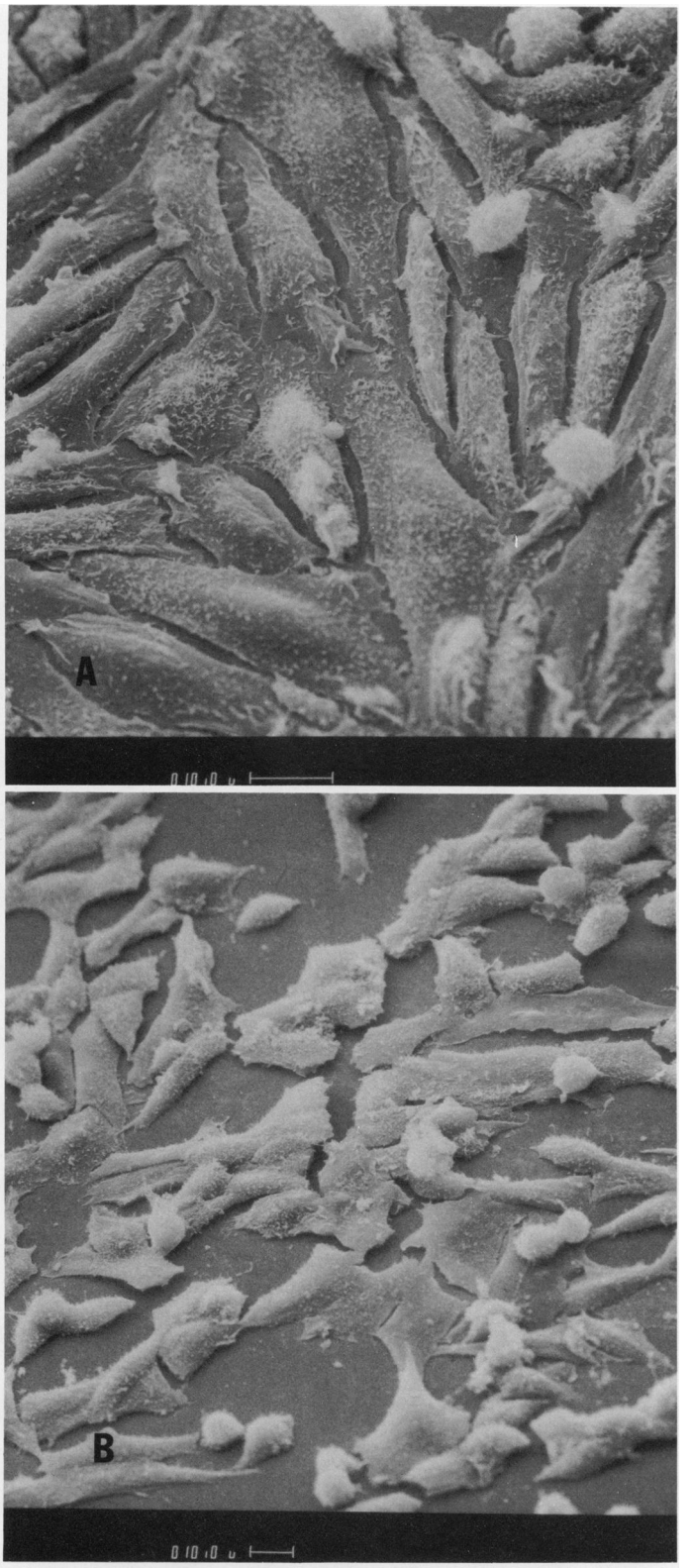


FIG. 2. *Scanning electron microscopy. (A) Parental CHO cells and (B) VRR cells are visualized by scanning electron microscopy. The bar represents 10 μm.*

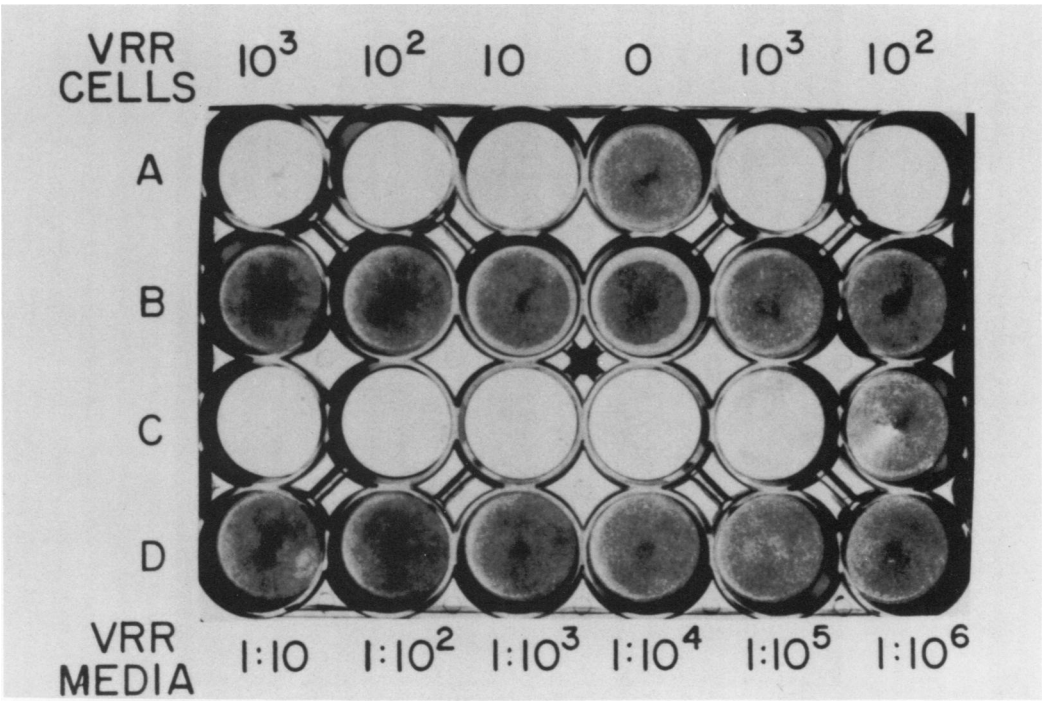


FIG. 3. *Titration of VRR cells and VRR media. All wells were seeded with 10^5 CHO cells/well. Rows A and B received the indicated numbers of throughly washed VRR cells at the same time. Rows C and D received the indicated dilutions of cell-free media from 10^6 VRR cells grown for 2 days in 5 ml of media. Reovirus antiserum was added at a dilution of $1:10^2$ to rows B and D.*

media and cells. Diffuse colonies typical of VRR cells could be seen in wells where 10^3 VRR were added to the CHO cells. The growth of VRR cells after killing of CHO by the VRR was a usual result. The number of VRR colonies was dependent on the number of VRR cells added. This result indicated that the cells carried infectious reovirus that was certainly normal in that it was highly infectious for parental CHO cells. To test the nature of the virus further, we examined the virus produced after infection of CHO cells by the media of VRR cells (infection was done to amplify the amount of virus present). Figure 4 shows an electron micrograph of particles isolated by centrifugation of cell-free media after infection. No such particles were seen in control media. These particles have a morphology apparently identical to that of normal reovirus. When such virus particles were extensively iodinated with ^{125}I and compared with similarly iodinated standard reovirus proteins on SDS-acrylamide gels, the pattern shown in Fig. 5 was obtained. There is no evident difference between the two patterns. These two figures show that the VRR cells generate an infectivity that produces reovirus virions with a normal morphology and protein composition.

There are two basic explanations of the presence of virus in the media of VRR cells. One is that an occasional cell produces large amounts of virus or that most cells are producing virus. We investigated this problem in two ways. The first was to clone thoroughly washed VRR cells. This was done in such a way as to produce approximately one cell per chamber, and any chamber containing more than one attached cell was not used. This method considerably reduced the possibility of cross-infectivity of cells while the clones were being grown. These clonal isolates were then tested for infectivity. We found all to be infectious. Two of the clones had considerably lower titers than the others, and we are presently investigating this result further. The second approach was to examine the cells with an indirect immunofluorescence assay. We used an initial treatment with rabbit antiserum prepared against purified reovirus type III and absorbed with disrupted CHO cells. This procedure was followed by treatment with goat anti-rabbit immunoglobulin G conjugated with fluorescein. Figure 6

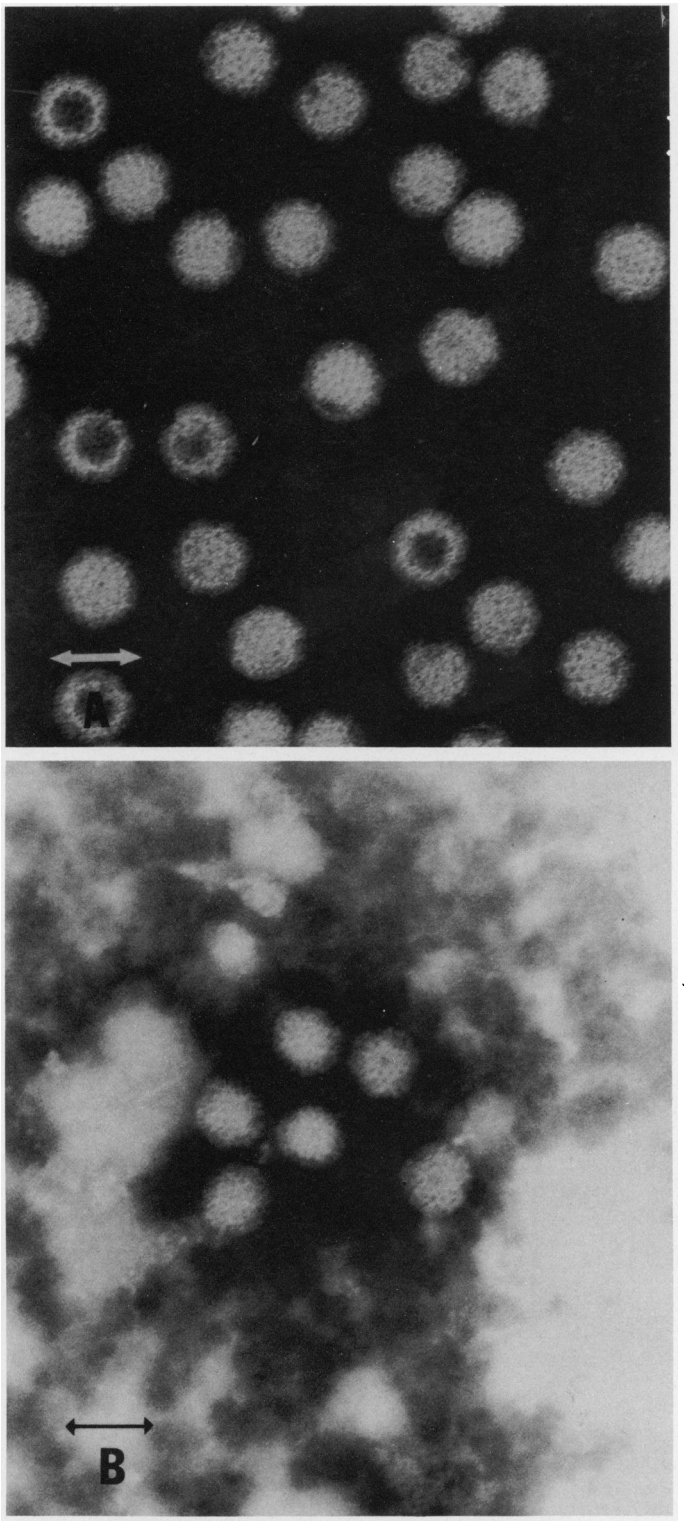


FIG. 4. *Electron microscopy. (A) Purified reovirus virions are shown by negative staining (see text). (B) Particles obtained by centrifugation of media after infection of parental CHO cells with VRR-conditioned media are shown. No such particles were visible with media from parental cells. The bar represents 0.1 μm.*

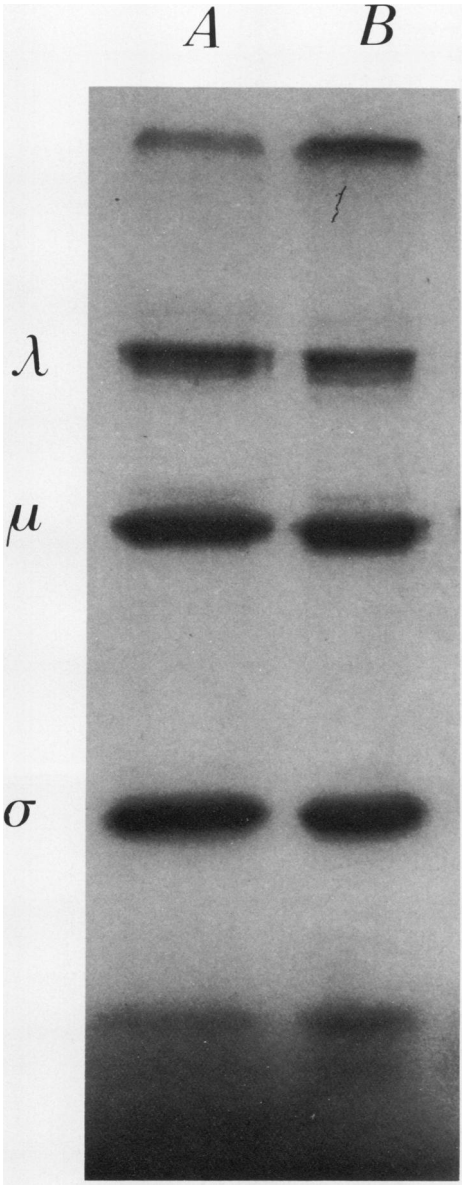


FIG. 5. *SDS-acrylamide gel electrophoresis. Gel electrophoresis of iodinated proteins from purified reovirus (A) and virus from VRR-conditioned media (B) was performed as described in the text. The gel slab was dried on filter paper under heat and vacuum and autoradiographed with Kodak no-screen X-ray film. The λ-, μ-, and σ-protein species are indicated. Migration was from top to bottom.*

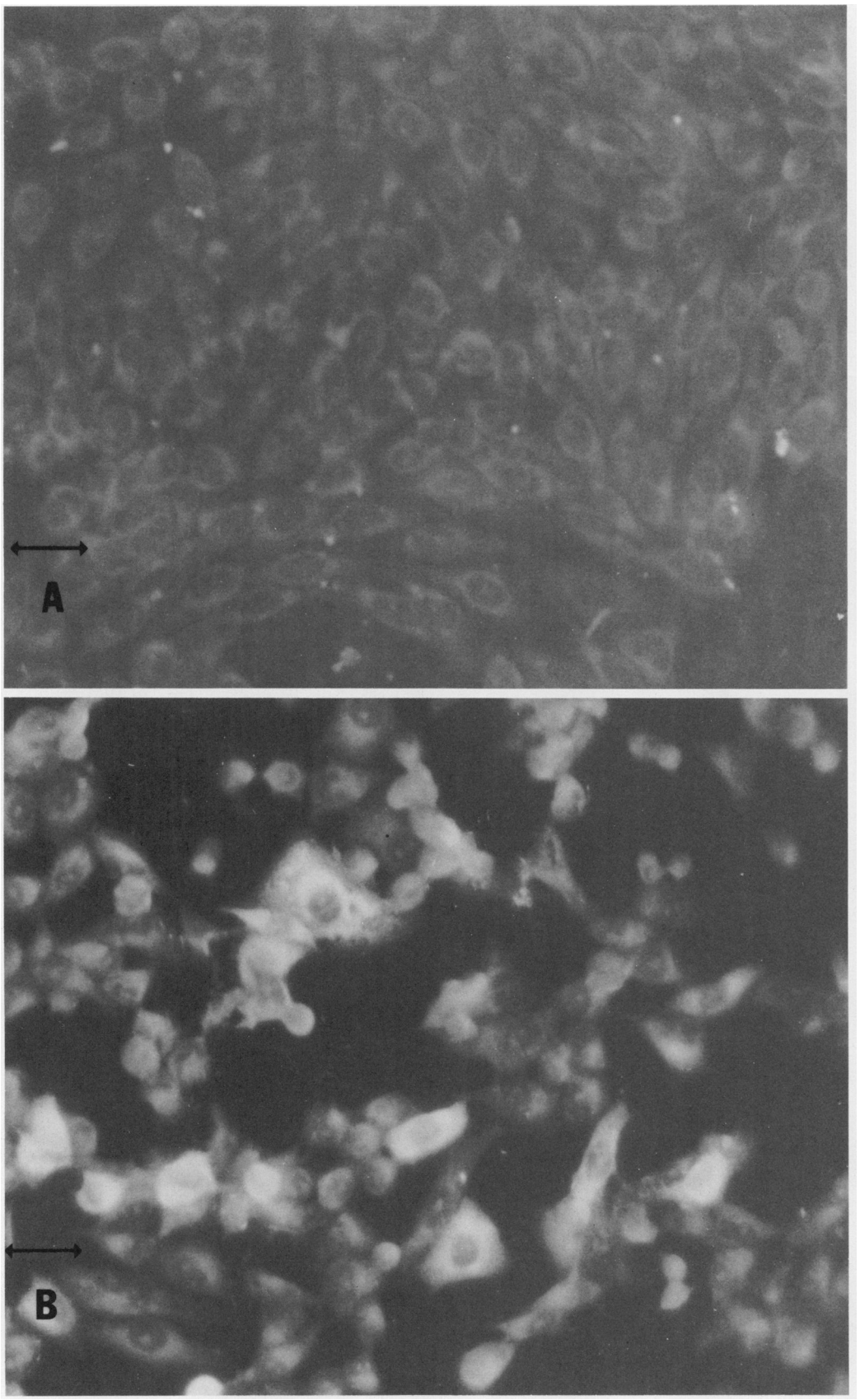
shows that most of the methanol-fixed VRR cells contain cytoplasmic fluorescent material, but that the amount seems to vary widely from cell to cell.

These two results and Fig. 3 indicate that most if not all of the VRR cells contain at least certain virus antigens and most likely are capable of releasing complete infectious virions. The immunofluorescence result further indicates the virus antigens are intracellular.

The fact that these VRR cells grew in a medium containing high concentrations of infectious cytopathic virus and contained viral antigens in their cytoplasm is indeed unusual, and we performed a number of experiments in an attempt to account for it.

One possibility was that the cells were producing interferon to generate a balance between viral synthesis and inhibition (23). We tested this possibility by adding cell-free media from 10^6 VRR cells grown 2 days in 5 ml (containing 10^6 IU of reovirus per ml) to 10^5 CHO cells at a 1:5 dilution. Killing by the VRR infectious particle was prevented by the addition of reovirus antiserum (1:10^2 dilution). One day after the addition of VRR media and antiserum, dilutions of EMC virus were added to the cells. With VRR media, EMC virus dilutions of 1:10^3 and 1:10^4 produced 73 plaques and 1 plaque, respectively. Without VRR media, EMC virus dilutions of 1:10^3 and 1:10^4 yielded 86 plaques and 3 plaques, respectively. (Dilutions of 1:10 and 1:10^2 produced confluent or nearly confluent lysis.) Therefore, it is clear that no protection of the cells resulted from the addition of the VRR-conditioned media. If VRR cells were producing interferon, one would have expected considerable protection from EMC infection.

We also considered that the VRR phenotype might be due to defective interfering (DI) virus (7). Such forms are known to occur in reovirus and are missing either the L1 and/or L3 segments of the double-stranded RNA found in these viruses (11, 16). To investigate this possibility, we collected approximately 1 liter of medium conditioned by growth of VRR cells. The medium was freed of cells by low-speed centrifugation, and the virus was concentrated by high-speed centrifugation. The pellet from the high-speed centrifugation was then purified by $CsCl_2$ equilibrium density centrifugation. By this technique we generally obtained one band with a density of about 1.36 g/cm^3, identical to that produced by our standard reovirus. Occasionally, we observed a minor, lighter band. We have analyzed the RNA content of the band at 1.36 by SDS-urea acrylamide gel electrophoresis. The result of this electrophoresis is shown in Fig. 7. It is apparent that all 10 of the reovirus double-stranded RNA molecules are present in the virus from the VRR cells. It is also apparent that the double-stranded RNA molecules are present in the VRR virus in approximately


A
B

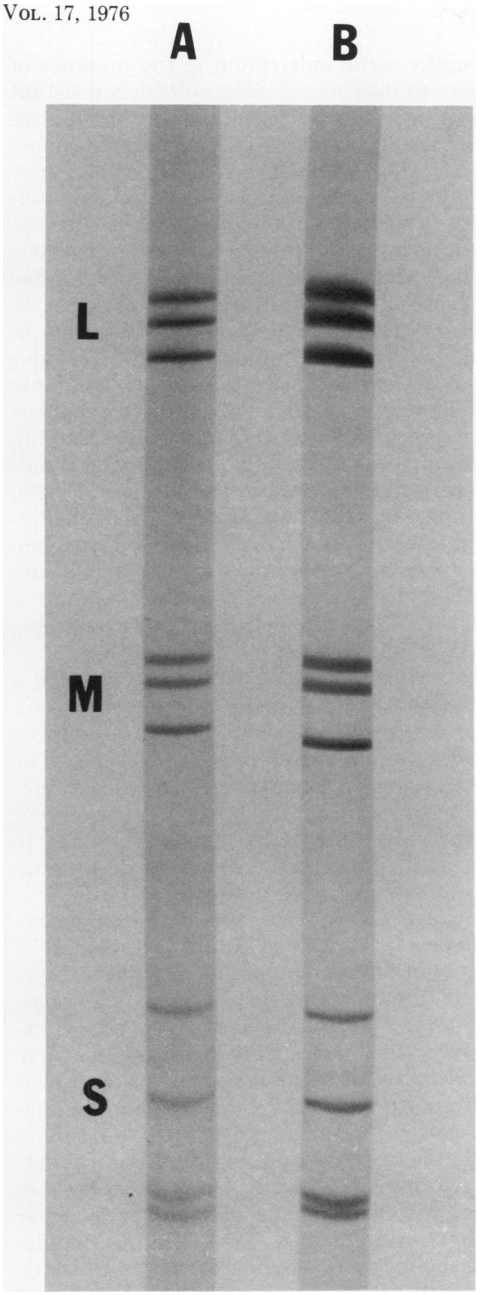


FIG. 7. *SDS-urea acrylamide gel electrophoresis. Gel electrophoresis was performed by the technique of Schuerch and Joklik (20) on RNA extracted from purified reovirus (A) and the 1.36-g/cm³ band from VRR-conditioned media (B). The RNA was extracted as described in the text. The tube gels were stained with 0.1% Stain-all (Eastman) in 50% formamide. The L, M, and S RNA species are indicated. Migration was from top to bottom.*

equimolar amounts. This precludes the possibility that large amounts of DI virus are present in the band or that there are major differences in the size of the RNA molecules. When known DIs are present in virus stocks, they usually represent approximately half the virus population (21). Differences of such magnitude would be obvious on the gels shown in Fig. 7. Further, we have analyzed the RNA from the lighter band when it does occur and found 10 pieces of RNA also. We believe that the second band most likely represents an artifact related to the time the virus remains in the media after its production. We think this is true (i) because there are as yet no known reovirus defectives that have a sufficient density difference to allow resolution from normal reovirus on $CsCl_2$ gradients without the production of cores, and (ii) we obtain more upper band when the virus is allowed to exist longer at 37 C.

Resistance characteristics of the VRR cells. To further investigate the nature of the VRR cells, we investigated their resistance to virus infection. In these experiments, resistance was measured by the ability of the cells to form colonies of 100 cells or more in the presence of various titers of virus. VRR cells and CHO cells were plated such that control flasks produced approximately 200 colonies. The day after plating the challenge virus was added at the indicated dilution. Table 1 demonstrates that the cells are resistant to a wide spectrum of viruses including both enveloped and unenveloped virions, containing plus- or minus-stranded nucleic acid. We have also found that cells are only relatively resistant. If one increases the multiplicity of infection to 10 or more with EMC and reovirus, the cells do not form colonies. The fact that the cells are resistant to a wide range of viruses suggests that we are observing an interference similar to the intrinsic interference described by Marcus and co-workers (9).

We also considered the possibility that we are observing a genetic change in the cell population that allows the presence of infectious reovirus with continuing cell growth. We attempted to test this idea by growing cells in the presence of anti-reovirus antiserum. After a period of 2 months of continuous cell passage in the presence of antiserum, we finally obtained a population of cells that were free of infectious virus and virus antigen by immunofluorescence. These cells, however, proved to have normal susceptibility to reovirus infection. Two simple explanations are available for this result. The first is

FIG. 6. *Immunofluorescence. Immunofluorescence analysis of the VRR cells was performed as described in the text. (A) Parental CHO cells. (B) VRR cells. The two experiments were performed simultaneously with the same reagents. The bar represents 10 μm.*

TABLE 1. *Resistance characteristics*[a]

Virus dilution	Resistance (%)	
	VRR	CHO
Vesicular stomatitis virus		
10^{-1}	128	0
10^{-2}	101	0
10^{-3}	87	105
10^{-4}	114	112
10^{-5}	122	82
Newcastle disease virus		
10^{-1}	50	1
10^{-2}	66	0
10^{-3}	103	0
10^{-4}	155	0
10^{-5}	117	73
EMC virus		
10^{-1}	108	0
10^{-2}	132	0
10^{-3}	110	0
10^{-4}	96	0
10^{-5}	117	101
Reovirus		
10^{-1}	125	0
10^{-2}	82	0
10^{-3}	115	0
10^{-4}	91	86
10^{-5}	97	99

[a] A number of cells sufficient to generate 200 colonies was seeded on day 1. On day 2, the various viruses in the indicated dilutions were added. After 1 week, the colonies were stained and counted. The number of colonies of more than 100 cells on the control plates represents 100%.

that the VRR phenotype is due solely to virological phenomena. The second is that a genetic change occurs with a significantly high frequency of reversion. When the virus is removed, the revertant, in the absence of selection, overgrows the population. We have not yet resolved this problem although preliminary experiments suggest that mutagenesis increases the frequency of the appearance of resistant colonies (R. Taber and N. Wald, unpublished data).

DISCUSSION

The literature on persistent infection is vast indeed and dates from the earliest experiments with viruses grown on cultured cells (26). Walker et al. (25) have reviewed this early literature and provided certain criteria for classification. We know that in our system (i) additional antiviral factors are not necessary for maintenance, (ii) the cultures can be cured only by lengthy serial cultivation in the presence of antisera to reovirus, (iii) the culture is resistant to superinfection by other viruses, (iv) a large percentage of the cell population contains infectious virus, (v) infected cells do divide and grow into infected clones, and (vi) interfering factors cannot be detected in the media of the cultures. From this information, it seems that our system falls into what Walker described as a "regulated infection." The only aspect that does not fit Walker's criteria is that the culture can be cured by the addition of antisera for long periods of time. These phenomena are similar in varying degree to those in cultures produced from (i) fetuses with "rubella" syndrome (19), (ii) mumps virus in human conjunctiva cells (26), (iii) measles virus in HeLa cells (27), and (iv) rabies in rabbit endothelial cells (4). Persistent infections previously described with reovirus arose without massive killing of cells and exhibited both interferon production and a small percentage of reovirus antigen-containing cells (1, 13).

The results presented show that we have a cell line that is persistently infected with cytopathic reovirus. This cell line is also resistant to some degree to a wide range of virus types. The basic unresolved questions are (i) what alterations have occurred that allow a persistent infection of a normally cytopathic virus to occur, and (ii) are these alterations of a fundamentally cellular or viral nature?

We have shown that the virus population obtained is (i) capable of killing CHO cells at 37 C, (ii) contains an apparently normal complement of protein, and (iii) contains an apparently normal complement of RNA. These results suggest that we are not dealing with a large population of DI virus similar to those previously reported. Further evidence that a previously reported DI does not account for our results is the wide-ranging nature of the interference we observe. Known DI particles are, in general, highly specific in their ability to confer resistance (12). Further, this VRR virus population is perfectly cytopathic for parental CHO. The fact that we do, on occasion, obtain a second band on $CsCl_2$ gradients makes us somewhat less certain that we do not have a defective particle present. However, this lighter band contains all 10 pieces of RNA and all normal protein species; it also becomes more evident with continuing incubation of the virus-containing media at 37 C.

The immunofluorescence data suggests that infection can proceed to a stage where viral antigen is produced. It is therefore unlikely that the resistance of the cells is due to a surface

effect. A more likely explanation is that the resistance is due to an effect similar to the "intrinsic interference" described by Marcus and co-workers (9). This interference is characterized by a cellular state of refractoriness to heterologous virus. The induction of this refractoriness requires the expression of viral genes and apparently affects the replication of the challenge virus, nucleic acid.

The obvious difference between our system and intrinsic interferences is that in our system the challenge virus and the initial infecting virus are identical. Even if the mechanism of intrinsic interference were completely understood, it would not explain why the initial infection does not result in cell death.

The data presented in no way exclude the possibility that the VRR phenotype is due to a small population of defective virus different from those previously described. For example, it is well known that temperature-sensitive viruses often appear in persistent infections of other viruses (18). We can only state that the VRR virus is capable of killing wild-type CHO cells with high efficiency (<0.001% survivors) at 33, 37, and 39 C (R. Taber and V. Alexander, unpublished data). It is also possible the VRR phenotype is due to a defective virus population that predominates intracellularly rather than extracellularly.

An interesting characteristic of the VRR cells is that reovirus is not lethal to them. This is not surprising, as they were selected for this property. The fact that they produce virus cytopathic to parental cells while continuing to grow suggests that there is a cellular defect in the killing mechanism. One possibility may be a reduced production of nonvirion double-stranded RNA in the cells.

To investigate these questions more thoroughly, we are at present attempting to determine exactly what reovirus components are present in the VRR cells by molecular hybridization for viral RNA components and radioimmunoassay for viral protein components to see if a possible intracellular defective virus exists or to determine at what step the molecular events that result in cell killing are inhibited.

Another related aspect is how the virus genome is maintained in the cells. Two basic possibilities exist. The first, and more likely, is that the reovirus information is spread by constant infection of newly divided cells by mature virus from the media or from the parental cell by an intracellular mechanism. The other possibility is that the reovirus information is carried in some stable form in the cell genome. The evidence we have for the latter are experiments that suggest halogenated pyrimidines are capable of inducing the production of reovirus from certain cell lines that do not carry infectious reovirus (R. Taber, K. Manly, and V. Alexander, manuscript in preparation). We intend to investigate further this possibility as well, especially in light of recent work that describes the integration of DNA copies of RNA viruses other than reovirus (28).

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