Antibody-Mediated Neutralization of Virus Is Abrogated by Mycoplasma

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The ability of a mouse mammary tumor cell line to abrogate antibody neutralization of vesicular stomatitis virus was shown to be due to the presence of mycoplasma. The mycoplasma was isolated from the cell line and typed as *Mycoplasma orale*. Colonies of this mycoplasma were used to deliberately infect cell cultures which then gained the capacity to reactivate antibody-neutralized virus. The extent of the reactivation depended on the source of neutralizing antiserum. Other species of mycoplasma were tested and were found to reactivate neutralized virus, indicating that this may be a general phenomenon of mycoplasma contamination.

During studies on the preparation and characterization of murine mammary tumor virus pseudotypes of vesicular stomatitis virus (VSV), the presence of an infectious agent which could reactivate antibody-neutralized VSV was detected (15). The cell line in which this phenomenon was originally demonstrated was the mouse mammary tumor cell line GR (11). Preliminary attempts to determine the nature of this agent were unsuccessful. In this paper we report further investigations into this phenomenon and identify the agent responsible for reactivating neutralized VSV as a mycoplasma.

MATERIALS AND METHODS

Cells and viruses. The mouse mammary tumor cell line GR, the normal mammary gland cell line NMG, and the mink lung cell line Mv1Lu were maintained as previously described (15). Early-passage duck embryo fibroblasts were grown in Dulbecco modified Eagle medium containing 10% tryptose phosphate broth, 1% fetal calf serum, and 1% chick serum.

VSV ts045, a group V temperature-sensitive mutant of the Indiana serotype (4), and wild-type VSV of New Jersey serotype were propagated as previously described (13). Chandipura virus was kindly supplied by F. Brown, Animal Virus Research Institute, Pirbright, England.

Mycoplasma. The mycoplasma strains *Mycoplasma argini* and *M. hyorhynchus* were obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London, U.K.

Antisera. Sheep anti-VSV immunoglobulin was obtained and prepared as previously described (15). Sheep anti-Chandipura immunoglobulin fraction was generously supplied by J. Zavada, Institute of Virology, Bratislava, Czechoslovakia. The guinea pig antisera against VSV Indiana serotype, VSV New Jersey serotype, and Chandipura virus were all generously supplied by F. Brown. Mouse monoclonal antibodies

to VSV G protein were prepared by P. Napiorkowski and R. Weiss (unpublished data).

Neutralization plaque assays. Approximately 10^6 plaque-forming units (PFU) of VSV was incubated for 2 h with antiserum at the dilutions indicated in Results, in a volume of 0.3 ml. The virus was diluted in a 10-fold series in phosphate-buffered saline containing 1% fetal calf serum. Portions of 0.5 ml were inoculated, in duplicate, onto confluent sheets of assay cells in six-well culture trays (Costar) and left for 1 h at ambient temperature to facilitate virus adsorption. The inoculum was removed, and the cells were washed with buffer and overlaid with 1.2% agar in Dulbecco modified Eagle minimal essential medium containing 5% fetal calf serum and 100 U of penicillin per ml and 100 μ g of streptomycin per ml. After 2 or 3 days at 32°C, the cells were stained with neutral red and the plaques were counted.

Mycoplasma detection and isolation. The cells to be tested were grown on cover slips and stained with aceto-orcein, as described by Fogh and Fogh (5). [3 H]thymidine autoradiography was a modification of the procedure described by Nardone et al. (10). Cells were grown in 50-mm culture dishes and labeled when subconfluent with 1 μ Ci of [3 H]thymidine per ml containing 0.8 μ M unlabeled thymidine in normal growth medium for 18 to 20 h. The cells were then washed twice with tris(hydroxymethyl)aminomethane-buffered saline and then twice with cold 5% trichloroacetic acid for 3 min and twice with absolute alcohol. The cell monolayers were overlaid with stripping film (Kodak AR10) and exposed for 72 h.

Isolation of mycoplasma was attempted by directly growing cell culture fluid samples in a specifically prepared nutrient agar as described by Lemcke (9), or by a modification of the procedure outlined by House and Waddell (7). In this procedure, the inoculum was stabbed into dishes containing confluent BHK-21/C13 cells as a feeder layer overlaid with agar containing Dulbecco modified Eagle medium and 10% calf serum, and further overlaid with Hers mycoplasma growth medium. The cultures were incubated at 37°C in an

atmosphere of 5% carbon dioxide in nitrogen and observed daily for the presence of mycoplasma colonies.

Antibiotic treatment of cells. The cells were treated with a mixture of antibiotics effective against mycoplasma: kanamycin, neomycin, and gentamycin, all at 100 µg/ml, and Tylocine with chlortetracycline, both at 50 µg/ml. The cells were treated for 8 to 10 days in a tissue culture flask completely filled with medium containing all the antibiotics. The medium was changed every 2 to 3 days, and on transfer the cells were plated in 90-mm dishes and maintained in normal growth medium for a further 3 to 4 days before being tested for their ability to reactivate neutralized VSV.

RESULTS

Properties of the reactivation agent. Previous studies (15) demonstrated a filterable infectious agent present in a mouse mammary tumor cell line (GR) which exhibited the capacity to reactivate antibody-neutralized VSV. This agent has been shown to infect a variety of cell types by its ability to impart to the recipient cells the capacity to reactivate antibody-neutralized VSV. Infected cell lines were passaged once or twice before being used in a standard VSV neutralization plaque assay to determine the extent of reactivation. The uninfected cell line served as a control for the neutralization of the VSV (Table 1). These experiments showed that mink, mouse, and avian cells could be used to assay VSV, giving titers of about 10^9 PFU of virus per ml. After treatment with sheep anti-VSV immunoglobulins, the VSV was completely neutralized when assayed on uninfected cells. However, when the assay cells were exposed to the tissue culture medium originating from the GR cell line and subsequently used in the plaque assay, the VSV was no longer completely neutralized, but demonstrated a residual titer of 10^4 to 10^6 PFU/ml. The titer of the non-neutralized VSV was not significantly different when assayed on infected or uninfected cells. The cells which showed the greatest capacity to reactivate neutralized VSV were the infected mink lung cells (mink/GR cells).

The VSV used for these studies was a temperature-sensitive mutant in complementation group V, which corresponds to the G protein (glycoprotein) of the virus. It is possible to mimic the antibody neutralization of VSV by heat inactivating (45°C for 1 h) this mutant (2, 14), since the glycoprotein is extremely heat sensitive. When the virus particles were inactivated by heating, no increase in the fraction surviving heat treatment was observed when the assays were performed on mink or mink/GR cells (data not shown), thus indicating that the agent is

restricted to reactivating antibody-neutralized virus.

The ability of the agent to reactivate antibody-neutralized VSV depended to some extent on the source of antibody used in the neutralization (Table 2). VSV_{Indiana} serotype neutralized with a sheep immunoglobulin fraction showed a dramatic difference in titer depending on the

TABLE 1. Infection of cell lines with an agent which imparts the capacity to reactivate antibody-neutralized VSV

Cells	Infected ^a	Titer of VSV (log ₁₀ PFU/ml)	
		Before neutralization	After neutralization ^b
Mink lung	—	9.3	<1.0
	+	9.4	6.8
NMG	—	9.9	<1.0
	+	9.1	5.7
Duck embryo	—	8.4	<1.0
	+	8.5	4.7
GR	—	8.4	4.4

^a Infection was established by exposing the cells to membrane-filtered (Millipore; 0.45-µm pore size) GR cell tissue culture fluid for 1 to 2 days. The cells were used in the experiments after 2 to 3 cell passages following infection.

^b Neutralization of the VSV (ts045) stock was performed as described in the text, using 0.1 ml of virus in a 0.3-ml neutralization reaction mixture.

TABLE 2. Dependence of extent of virus reactivation on neutralizing antiserum

Virus ^a	Antiserum	Dilution	Titer of neutralized virus (log ₁₀ PFU/ml) assayed on:	
			Mink	Mink/GR
VSV _{IND}	Sheep ^b	1/30	<1.0	6.8
VSV _{IND}	Guinea pig	1/30	1.7	2.0
VSV _{IND}	Mouse ^c IgG1	1/100	4.4	4.6
VSV _{IND}	Mouse ^c IgG2	1/100	3.4	6.5
VSV _{NJ}	Guinea pig	1/30	3.0	4.2
Chandipura	Sheep ^b	1/10	2.0	6.1
Chandipura	Guinea pig	1/10	1.4	2.7

^a VSV_{IND}, VSV, Indiana serotype; VSV_{NJ}, VSV, New Jersey serotype.

^b Sheep anti-VSV was used as an IgG fraction derived by ammonium sulfate precipitation as described in Zavada et al. (15).

^c Two monoclonal antibodies to VSV G protein were used; one was shown to be IgG1 and the other IgG2 by immunodiffusion using specific antisera to the mouse immunoglobulin clones (Napiorkowski and Weiss, unpublished observations).

assay cell (infected or uninfected), whereas the same virus stock showed no significant difference when neutralized by the guinea pig anti-VSV_{Indiana} serotype serum. The latter serum was not as effective in neutralization as the sheep immunoglobulin since a low, residual, non-neutralization fraction was found when assays were performed on both infected and uninfected mink cells. However, the difference did not entirely reside in the animal species from which the antiserum was obtained since guinea pig antisera to other rhabdoviruses were reactivated, albeit to a less dramatic extent.

Two neutralizing mouse monoclonal antibodies to VSV G protein were tested; one, of immunoglobulin class IgG1, did not allow reactivation of the virus, whereas the other, of class IgG2, did. The non-neutralizing fraction found for both immunoglobulins when assays were performed on control mink cells was probably the result of mutations affecting the protein domain interacting with the antibody, since increasing the concentration of IgG did not decrease the size of this resistant fraction. The basis for the differential capacity of these antibodies to allow reactivation of neutralized VSV was not apparent from these experiments and could reside with the specificity, type, or class of antibody, or nature of the antigen.

Further experiments were designed in order to characterize the agent responsible for the reactivation. The culture fluid from the mink/GR cells was subject to filtration procedures which showed that the agent was able to pass through membrane filters (Millipore Corp.) of pore size 0.45 μm and 0.22 μm but not 0.1 μm . The agent was inactivated by shaking the culture fluid with diethyl ether. These properties indicated that the agent was relatively large ($>0.1 \mu\text{m}$) and possessed a lipid membrane. In a dilution experiment, the titer of the agent in the mink/GR cells was estimated to be between 10^6 and 10^7 infectious units/ml (1 to 10 infectious units/cell). The filtered tissue culture fluid was assayed by infecting fresh mink cells and passaging the cells two or three times to allow the titer to reach optimal levels before being assayed. Uninfected mink cells were assayed in parallel as control cells (data not shown). The results from these experiments showed that the agent was present at a relatively high concentration and was able to proliferate to a level capable of reactivating neutralized virus within a few days. Consequently, the effect of the agent on the growth of the mink lung cells was determined. Infected and uninfected mink cells were plated at 2×10^5 cells per 90-mm dish, and the medium was changed every 2 or 3 days. Growth was

monitored by counting the cells at intervals from replicate dishes (Fig. 1). The rate of cell proliferation was slightly different, with the infected cells showing a marginally slower growth rate, although no gross difference in cell morphology or general condition was observed. However, the agent was cytopathic for chick cells and significantly decreased the rate of proliferation of duck cells.

Detection and isolation of a mycoplasma. Few known infectious organisms are capable of growing to high titer in cells without causing any obvious cytopathic effect. A likely candidate would be a mycoplasma, and so experiments were designed to test such a notion. Two methods for detecting mycoplasma were used: (i) staining cell cultures with aceto-orcein, and (ii) [^3H]thymidine autoradiography. Mink, mink/GR, and GR cells were grown on cover slips and stained with aceto-orcein, which shows myco-

FIG. 1. Growth curves for mink (●) and mink/GR (○) cells. Cultures were seeded at 2×10^5 cells per 90-mm petri dish. At the times indicated, duplicate cultures for each cell type were trypsinized and the number of cells per plate was determined.

plasma as dark-staining granules on the cell periphery. Mycoplasma were detected in the mink/GR cells but not the mink or GR cells by this procedure. The [^3H]thymidine autoradiography method gave a similar positive result for the mink/GR cells, which showed labeling of the cell cytoplasm with a concentration of label on the cell periphery, characteristic of mycoplasma infection. Both the uninfected mink and the GR cells demonstrated nuclear labeling only, indicating a negative result.

Treatment of GR and mink/GR cells with a potent antibiotic mixture, as described in Materials and Methods, was effective in eliminating the reactivation capacity for several cell passages. Concomitant with this change, the [^3H]thymidine autoradiography of the mink/GR cells ceased to show evidence for mycoplasma. However, after several months of culture in the absence of the antibiotic cocktail, the mycoplasma was detectable in the GR cells by growth in agar over feeder layers but not by staining with aceto-orcein, suggesting incomplete eradication.

The infected mink cells are the most active cells so far tested in their ability to reactivate neutralized VSV. However, the original source of the agent was the GR cell line, and therefore isolation of the agent from this cell line and the mink/GR cells was considered necessary to determine unambiguously the nature of the reactivation agent. To isolate a putative mycoplasma from these cell lines, we tried to grow the agent on nutrient agar with and without the presence of a cell feeder layer. A culture of mycoplasma was obtained from the GR and mink/GR culture fluid inoculum, using nutrient agar containing a cell feeder layer; nutrient agar alone did not support growth. Samples of the mycoplasma colonies and the mink/GR cell line were sent to the Mycoplasma Reference Laboratory, where in each case the mycoplasma isolate was typed by immunofluorescence as *M. orale*.

To confirm that *M. orale* was the agent responsible for the reactivation of the antibody-neutralized virus, mink cells were infected directly with mycoplasma from the agar colonies and used in the plaque assay procedure. The infected cells gained the capacity to reactivate neutralized virus (Table 3). To determine whether this property is specific for *M. orale* or a general property of mycoplasma, other strains of mycoplasma were obtained and used to infect mink cells directly. These infected cell cultures were then used in the neutralization plaque assay (Table 3). The findings demonstrated that each of the three strains tested could reactivate neutralized virus, indicating that this property of mycoplasma is a general phenomenon.

TABLE 3. Ability of several mycoplasma strains to reactivate antibody-neutralized VSV

Mycoplasma	Titer ($\log_{10}$ PFU/ml) of VSV assayed on mycoplasma-infected mink cells	
	Before neutralization	After neutralization ^a
Uninfected	9.8	<1.0
<i>M. orale</i>	9.8	6.7
<i>M. argini</i>	9.4	3.7
<i>M. hyorhina</i>	9.4	4.7

^a VSV ts045, Indiana serotype, was neutralized with sheep IgG at a 1/30 dilution as described in the text.

DISCUSSION

The results demonstrate that several mycoplasma species are able to partially abrogate the effect of antiserum neutralization. Two aspects of this phenomenon are important; the first is to understand how these microorganisms affect this property, and the second is the practical importance for workers using antibody to neutralize virus in tissue culture systems.

The mechanism by which mycoplasma may reactivate virus is obscure; however, several possible mechanisms may be envisaged. The mycoplasma could excrete or have on its surface proteases or glycosidases which are capable of destroying the antigen-antibody complexes, and so release virus which may in turn become infectious. This would most likely occur on the cell membrane, where the components of the system come into close contact. We have been unable to reactivate neutralized virus by using tissue culture fluid from mycoplasma-infected cells, which is consistent with this idea (unpublished observations). The production of an excreted protease which degrades antibodies has been described for the protozoan *Tetrahymena pyriformis*, where it probably acts to avoid immobilization in the host (3). In some preliminary experiments, we have been unable, by two different approaches, to detect a protease associated with the mycoplasma. In one set of experiments, labeled IgG was incubated with a suspension of mycoplasma and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results showed no detectable degradation of the immunoglobulin heavy or light chains, indicating the absence of a specific protease (unpublished observations). This approach was successful in demonstrating the presence of a protease in *T. pyriformis* cultures (3). A specific protease or glycosidase may be effective by acting directly on the viral glycoprotein to release the bound antibody-antigen complex, without cleaving the immunoglobulin chains. Consequently, a second approach was to use a gen-

eral protease assay utilizing a milk protein agar overlay, previously described for the detection of plasminogen activator induced by Rous sarcoma virus transformation in cell cultures (1, 6). In these experiments, neither the infected nor the uninfected cell cultures exhibited detectable hydrolysis of the milk protein overlay.

An alternative mechanism of reactivation might reside in an ability of the mycoplasma to facilitate virus penetration. This seems unlikely because no effect of mycoplasma was noted when heat-inactivated virions of the thermolabile VSV *ts045* were substituted for antibody-neutralized VSV. The infectivity of heat-inactivated preparations can be reconstituted to some extent by fusing the virus into cells, using inactivated Sendai virus (12).

Thus, the capacity of mycoplasma to reactivate antibody-neutralized virus has been established, but the mechanism remains unknown. Some form of enzymatic degradative process to remove the antibody seems the most likely mechanism, although preliminary experiments aimed at demonstrating this process have been unsuccessful. The importance of these observations to our own research was of a practical nature, because the reactivation phenomenon obscured the demonstration of viral interference at the membrane level. The uses of a potent antibiotic mixture was successful, however, in temporarily removing the mycoplasma by all the criteria we used to demonstrate its presence, for a period of several cell passages. The difficulty in detecting the presence of mycoplasma in the GR cell line and the complete absence of cytopathic effects in mammalian cells suggest that the presence of this type of agent could be quite widespread in many tissue culture systems and might explain assay cell-dependent variations in apparent neutralizing titers of antisera (8). The reactivation of neutralized VSV by mycoplasma is a simple method of detecting such contamination which appears to be more sensitive than conventional methods.

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