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Viral Spread in the Presence of Neutralizing Antibody: Mechanisms of Persistence in Foamy Virus Infection

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Several viruses were categorized on the basis of their ability to spread from cell to contiguous cell and form plaques in the presence of antiviral antibody. Herpes simplex virus, cytomegalovirus, and vaccinia, measles, and foamy viruses were able to spread in the presence of neutralizing antibody, whereas coxsackievirus, encephalomyocarditis virus, vesicular stomatitis virus, mumps virus, and simian virus 5 failed to spread. A detailed study of one of these virus groups (simian foamy viruses) suggested that the ability of these viruses to spread from cell to cell in the presence of antiviral antibody, the failure of antiviral antibody and complement to lyse infected cells, and the poor induction and relative resistance of these viruses to the antiviral action of interferon contribute to the persistent nature of this infection.

The relative importance of the specific immunological mechanism used by the host to protect itself against a viral infection depends, at least in part, upon the route by which the virus spreads from one cell to another. Virus can spread by one or more of three different routes: extracellularly, from infected cells to nearby or distant uninfected cells (type 1 spread); directly from infected to contiguous uninfected cells as a result of cell fusion or viral budding (type 2 spread); or from parent to progeny cells during cell division (type 3 spread) (11). In general, it appears that neutralizing antibody can stop type 1 but not type 2 or 3 spread. Very little, however, is as yet known about the mode of spread of most viruses. The present investigation was initiated to determine the mode of spread of several common viruses and to study in greater depth the factors responsible for the persistence of one of these viruses (foamy virus) in the presence of neutralizing antibody.

MATERIALS AND METHODS

Viruses and cell cultures. Simian foamy virus (SFV) types 1 and 2 were obtained from the National Cancer Institute Reagent Branch, and SFV types 6 and 7 were isolated from chimpanzee tissues (6). The foamy viruses, herpes simplex virus (HSV) type 1, vesicular stomatitis virus (VSV), simian virus 5 and vaccinia viruses were propagated in primary or first subcultured rabbit kidney cells (PRK). Coxsackievirus B1, encephalomyocarditis virus, mumps virus, and measles virus were propagated in Vero cells. Human cytomegalovirus (CMV) (AD169) and murine CMV were propagated in WI-38 and primary mouse embryo cells, respectively. Cell cultures were maintained with Eagle minimal essential medium supplemented with penicillin (100 U/ml), streptomycin (100 × 10^6 μg/ml) and 2 to 10% fetal bovine serum inactivated at 56°C for 30 min. Newcastle disease virus (NDV) was propagated in the allantoic fluid of embryonating hen eggs.

Formation of viral plaques in the presence of antibody. Standard agar overlay plaque techniques were used with coxsackie, mumps, SV5, vaccinia, CMV (murine), and measles viruses, whereas methylcellulose overlay techniques were used with EMC, VSV, CMV (human), and HSV. Antibody was prepared in rabbits, and the 50% neutralization titer was determined. In a typical experiment, virus was allowed to adsorb to the monolayers for 2 h, the plates were washed, and the appropriate overlay media and antibody concentrations were added. The viral plaques were counted 2 to 8 days later, depending upon the virus studied.

Assay for foamy viruses. The PRK cells were treated with a mixture of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) and resuspended in maintenance medium at a concentration of 1 × 10^5 to 2 × 10^5 cells/ml. The cells in a 1-ml volume were seeded onto 35-mm tissue culture dishes (Falcon plastic) and incubated at 36°C in a 5% CO_2 atmosphere. After 24 h, the cells were infected with 10-fold serial dilutions of foamy virus in a 0.5-ml volume and incubated for 90 min at 36°C on a rocker. The inocula were then aspirated, and the antibody overlay medium consisting of specific antiviral rabbit sera and maintenance media was added (1 to 2% rabbit anti-foamy virus serum containing approximately 25 neutralization units and 5 to 8% fetal bovine sera). Antibody overlay medium can be added within the first 24 h after viral incubation. The cell cultures were refed after 3 to 4 days of

incubation. After 8 to 10 days of incubation, medium was aspirated and the cells were fixed with 95% ethanol, stained with 20% Giemsa, washed in tap water, and allowed to air dry. Plaques were read on a plaque counter. The size and number of plaques increased until day 8; after this time there was little change in the number or size of plaques.

Fluorescent microscopy and ^{125}I-labeled antibody. The basic procedures for fluorescent antibody and ^{125}I-labeled antibody techniques have been described (5). New Zealand white rabbits were immunized by repeated intravenous inoculations with partially purified SFV-1 or SFV-7 virus pools (6). Virus-immune serum and sera from unimmunized animals was precipitated with ammonium sulfate. Immunoglobulin G (IgG) fractions were separated on a Sephadex G-200 column and labeled with ^{125}I or fluorescein isothiocyanate.

Cells grown on cover slips were infected with SFV-7 at an input multiplicity of 3 plaque-forming units/cell. After an adsorption period of 2 h at 37°C, the inoculum was removed, the monolayers were washed, fresh culture medium was added, and the cells were placed at 37°C. At various times after infection, cover slips were removed and washed with phosphate-buffered saline, and half of the cover slips were fixed with acetone for 5 min at room temperature. Fixed cells were used to detect cytoplasmic and nuclear antigen, and unfixed cells were used to detect membrane antigens. Cells were incubated with either ^{125}I-labeled or fluorescein-labeled antiviral IgG. The cover slips were washed, and the amount of ^{125}I-labeled antiviral IgG that bound to the cells was determined. Cover slips were mounted on slides and observed through a Zeiss fluorescent microscope with a UG-IP exciter filter system. The approximate percentage of cells showing specific fluorescence and localization of the fluorescence was then determined.

Attempts to detect capping of viral antigens on the cell surface were carried out at various intervals after infection. Monolayers were treated with EDTA and 0.2-ml volumes of cell suspensions were mixed with fluorescein isothiocyanate-labeled antiviral IgG. The mixtures were incubated for 20 min at 37°C, washed, resuspended in culture media, and placed at 37°C. After 20, 40, 60, and 120 min of incubation, the cells were removed and observed with the fluorescent microscope.

Complement-mediated cytotoxicity. For complement-mediated cell lysis studies, PRK cells seeded in petri plates, but without cover slips, were infected with SFV-7 or HSV, as described above. After 5, 24, 48, and 72 h of incubation, the cells were washed with phosphate-buffered saline, and 1 ml of rabbit anti-SFV serum or anti-HSV serum was added. Each serum had a neutralizing antibody titer of 1:2,000. Controls consisted of uninfected cells incubated with immune sera and infected cells incubated with nonimmune sera. The sera were removed after 1 h of incubation at 35°C in 5% CO_2, and fresh or heat-inactivated complement (guinea pig and rabbit) was added. After an additional 1 h of incubation, cells were washed and removed with a trypsin-EDTA mixture, and the viable cell count was determined with trypan blue (0.05%) (4). These experiments also were performed adding the serum and complement simultaneously (2).

Interferon assay. Interferon activity was assayed by reduction of VSV plaque formation on PRK cells. The titer of interferon activity of a given sample is expressed as the reciprocal of the highest dilution that reduced VSV plaques by 50%. Standard rabbit serum interferon containing 2×10^4 IU/ml was obtained from the National Institute of Allergy and Infectious Diseases and contained the same number of interferon units in our assay system.

RESULTS

Viral spread in the presence of neutralizing antibody. The effectiveness of antiviral antibody in preventing the development of viral plaques when added after the viral absorption period is shown in Table 1. The development of plaques by coxsackie B_1 virus, encephalomyocarditis, VSV, mumps virus, and SV5 was completely inhibited by antibody. In contrast, antibody did not inhibit plaque formation by vaccinia, HSV-1, and human and murine CMV. Since an absorption period of only 2 h was used with human CMV-infected cultures, the decreased number of plaques may have been due to neutralization of partially absorbed virus. Similarly, low concentrations of neutralizing antibody did not inhibit the formation of plaques by measles virus, SFV-1 and SFV-7; high concentrations of neutralizing antibody, however, did inhibit the formation of plaques by measles and partially inhibited the formation of plaques by SFV. SV5 plaque formation was tested only at relatively high concentrations of antibody (100 U). The possibility exists that plaque formation would not be inhibited by lower concentrations of antibody. In other experiments (data not shown) in which coxsackie, measles, SV5, and SFV plaque formation was suppressed by antibody, the reappearance of virus was studied after the removal of antibody. No virus could be recovered from coxsackievirus-infected cultures, but virus could be recovered from cultures that had been infected with measles, SV5, and SFV, indicating that antibody had not cured the latter cultures. The foamy virus group then was selected for further in depth analysis.

Effect of antiviral antibody on foamy virus spread. To evaluate the effect of different concentrations of antiviral antibody on the formation of plaques, PRK cells were inoculated with SFV-7 and then overlayed with antibody. Approximately the same number of plaques developed in the presence of 1 or 125 U of antibody

TABLE 1. *Viral spread in the presence of neutralizing antibody*[a]

Virus	Cell type	Neutralizing antibody (U)[b]	No. of plaques formed	Plaque formation inhibited
Coxsackie B_1	Vero	0	>300	
		10	0	Yes
Encephalomyocarditis	Vero	0	120	
		4	0	Yes
Vescular stomatitis (Indiana)		0	61	
	PRK	5	0	Yes
Mumps	Vero	0	>300	Yes
		20	0	Yes
Simian virus 5	PRK	0	182	
		120	0	Yes
Vaccinia	PRK	0	22	
		500	18	No
Herpes simplex (type 1)		0	28	
	PRK	640	29	No
Cytomegalovirus (AD169)	WI-38	0	310	
		256	159	No
Cytomegalovirus (murine)	ME	0	73	
		15	62	No
Measles (Edmonston)	Vero	0	74	
		25	76	No
		100	0	Yes
Simian foamy virus (type 1)	PRK	0	TMTC[c]	
		4	35	No
		64	4	Partial
Simian foamy virus (type 7)	PRK	0	TMTC	
		1	27	
		128	21	No
		256	10	Partial

[a] Different viruses were allowed to adsorb on cell monolayers for 2 h. The plates were washed, and the appropriate overlay medium (agar or methylcellulose) was added either alone or mixed with antiviral antibody. Plaques were counted 2 to 8 days later, depending upon the virus studied.

[b] Antibody in overlay medium is expressed in terms of units of neutralizing antibody; 1 unit is equivalent to the concentration of antibody that neutralized 50% of the viral plaques.

[c] TMTC, Too many to count. For the SFV-infected cultures, the overlay medium that contained no antibody was just maintenance medium (rather than agar on methylcellulose). In the presence of medium alone, the viral spread was so extensive that discrete plaques could not be counted.

(Table 2). If antibody was not added to the media, viral spread occurred and discrete plaques were not discernible. When high concentrations of antibody were used (250 or 500 neutralization units), plaque size decreased by approximately 50% and the number of plaques was substantially reduced. Similar results were obtained when SFV-1 was used.

Plaque assay. The plaques formed in SFV-infected PRK cells consist of foci of numerous nuclei and occasionally some vacuolation. Other experiments (not shown) demonstrated a linear relationship between the relative concentration of the virus inoculated and the number of plaques formed. A comparison of the virus infectivity titers obtained by the plaque assay with the standard tissue culture infectious dose assay showed that the infectivity titers calculated by the two methods were similar.

Other experiments performed to elucidate the optimal conditions for the plaque assay revealed that a 3-h adsorption period was optimal. Moreover, the number of plaques that

TABLE 2. *SFV-7 plaque production in the presence of varying concentrations of neutralizing antibody*[a]

Neutralizing antibody (U)	No. of plaques
None	Confluent, cytopathic effect
0.5	83
1	54
2	56
4	52
8	50
16	53
32	57
64	41
125	46
250	21
500	7

[a] The antiserum overlay medium was prepared in maintenance medium supplemented with anti-SFV-7 rabbit serum and with normal rabbit serum so that all of the dishes were incubated with medium containing 20% serum. After 10 days of incubation, the plaques were read and the average plaque number of four dishes was recorded.

formed and the time required for plaque development varied with the concentration of cells in the monolayer (Table 3). Maximal size and number of plaques were obtained when 1×10^5 to 3×10^5 cells were seeded onto dishes. If higher concentrations of cells were used (5×10^5 to 1×10^6), the development of cytopathic effect was delayed by 2 days, and the size and number of plaques were decreased. The development of maximal numbers of plaques in the presence of relatively low numbers of cells (i.e., noncontact inhibited) may simply reflect the fact that cell division is required for foamy virus replication (14).

Time of appearance of virus-induced cell surface antigens. To determine if the foamy viruses induced antigens on the surface of infected cells, PRK cells were infected with SFV-7 at an input multiplicity of 3 plaque-forming units/cell. After incubation for 5, 24, 48, and 72 h, the presence of specific intracellular and membrane fluorescence was determined (Table 4). Intracytoplasmic and nuclear fluorescence was noted in nearly all of the cells at 24 to 72 h. The intensity of nuclear fluorescence decreased slightly after 24 h, whereas brilliant cytoplasmic fluorescence was still present at 72 h. The intensity of the membrane fluorescence was maximal at 24 to 48 h but had decreased by 72 h. Foamy virus antigens, present on infected cell membranes, were dispersed over the whole cell membrane but appeared patchy in comparison with antigens induced by HSV-1 or vaccinia virus that served as controls. Attempts to cap foamy virus antigens with anti-foamy virus antibody were unsuccessful. Growth curve studies showed that the highest virus yield was obtained at 48 h after infection for both cell-associated virus and virus present in the extracellular culture fluid.

The use of the ^{125}I-labeled antiviral antibody-binding technique to detect viral antigens on the surface of SFV-infected cells yielded results similar to those obtained by membrane immunofluorescence. Unfixed SFV-1- or SFV-7-infected PRK cells bound two to three times more ^{125}I-labeled antiviral antibody (neutralizing antibody titer 1:2,000) than did uninfected controls. In contrast, unfixed HSV-infected PRK cells and measles virus-infected Vero cells bound 20 to 10 times more ^{125}I-labeled antiviral antibody (neutralizing antibody titer 1:2,000), respectively, than did control cells (data not shown).

Failure of anti-foamy virus antibody and complement to lyse infected cells. Previous studies showed that cells infected with HSV, vaccinia, influenza, NDV, and measles could be destroyed by antiviral antibody and complement (5, 9). However, at least in the case of HSV-1, the virus is able to spread to uninfected contiguous cells prior to immune lysis. To test whether foamy virus-infected cells could be destroyed by immune lysis, monolayers infected with SFV-1, SFV-7, or HSV-1 were incubated with antiviral antibody or antiviral antibody and complement. Table 5 shows that 92% of the HSV-infected cells were destroyed by anti-HSV antibody and complement. In contrast, the SFV-infected cells were not destroyed by anti-SFV-antibody in the presence of either guinea pig or rabbit complement.

TABLE 3. *Relationship between cell concentration and plaque formation with SFV-7*

Cell concn[a]	Virus-induced cytopathic effect[b]			No. of plaques[d]
	4[c]	8	10	
1×10^6	−	−	+	3
5×10^5	−	−	+	8
4×10^5	−	+	+	9
3×10^5	−	+	+	14
2×10^5	−	+	+	15
1×10^5	−	+	+	12
5×10^4	−	+	+	7

[a] Varying PRK cell concentrations were seeded onto 35-mm plastic petri dishes. After overnight incubation, the cells were infected with SFV-7 and maintained with anti-SFV-7 antibody overlay medium containing approximately 20 neutralizing units.

[b] The cells were observed microscopically for the presence (+) or absence (−) of virus-induced cytopathic effect.

[c] Days after infection.

[d] Number of plaques (average of four plates) observed 12 days after incubation.

TABLE 4. *SFV-7 infection of PRK cells*[a]

Properties	Infection of PRK cells			
	5[b]	24	48	72
Cytopathic effect	0	15%	60–70%	90%
Immunofluorescence				
Nuclear				
Intensity	±	++++[c]	+++	++
Description		Throughout nucleus and membrane	Throughout entire nucleus	Throughout nucleus
Cytoplasmic				
Intensity	±	++++	+++	+++
Description		Throughout cytoplasm	Intensity greatest in perinuclear region	Intensity greatest in perinuclear region
Membrane				
Intensity	–	++++	+++	++
Description		Patchy, widely dispersed	Patchy	Patchy
% Cells showing specific fluorescence	5	100	100	100
Infectivity titer (PFU/ml)				
Cell associated	7.4×10^2	1.3×10^4	1.0×10^5	2.8×10^4
Supernatant	4.4×10^2	6.0×10^3	1.2×10^5	4.6×10^4
Complement-mediated cytolysis		Neg	Neg	Neg
Capping with antibody		Neg	Neg	Neg

[a] PRK cells grown on cover slips in 35-mm petri dishes were infected with SFV-7 with an input multiplicity of 3 plaque-forming units (PFU)/cell. The infected cells were sequentially followed by light microscopy, immunofluorescence, infectivity, complement-mediated lysis, and capping. Neg, Negligible.

[b] Hours after infection.

[c] Intensity of immunofluorescence is graded from + to ++++.

TABLE 5. *Immunological destruction of virus-infected cells by specific antiviral antibody and complement*

Virus	Hours after infection	% Cells lysed[a]		
		1,000[b]	200	None
SFV-1	24	2	NT[c]	0
SFV-7	20	2	0	0
	24	2	0	1
	48	1	NT	0
	72	0	NT	0
HSV-1	24	92	NT	0

[a] Specific lysis is calculated by subtracting the percentage of cells lysed by antibody and heat-inactivated complement from the percentage of cells lysed by antibody and fresh complement.

[b] Neutralizing antibody titer.

[c] NT, Not tested.

Role of interferon. Studies were performed to determine whether the foamy viruses induced interferon. PRK cells were infected with SFV-1, SFV-2, SFV-6, and SFV-7. Foamy virus cytopathic effect was first noted at 2 days and progressed to involve 80 to 100% of the cells by day 7. At various times after infection (1, 2, 3, 4, and 7 days), the supernatant fluids were assayed for interferon activity. Interferon activity was never detected in supernatant fluids tested at a 1:5 dilution. In contrast, supernatant fluids from NDV-infected cultures contained more than 8,000 U of interferon as early as 24 h after infection.

In another experiment, SFV, HSV, and NDV were ultraviolet inactivated and added to PRK cell cultures. Supernatant fluids were removed after 24 h and assayed for interferon activity. Interferon activity was found in NDV-treated cells (320 U) but was not found in cultures treated with HSV or SFV.

In an attempt to use a more sensitive assay to detect interferon activity, ultraviolet-inactivated viruses were added to PRK monolayers, and after 24 h the medium was removed and the monolayers were infected with 25 plaque-forming units of VSV. The PRK cells, treated with

ultraviolet-inactivated NDV or HSV-1, were protected when challenged with VSV (Table 6). However, PRK cells treated with ultraviolet-inactivated SFV were not protected when challenged with VSV.

To determine if foamy viruses were sensitive to the antiviral action of interferon, PRK cells were treated with dilutions of interferon for 24 h and then challenged with VSV, HSV-1, SFV-1, SFV-2, and SFV-7. One unit of interferon inhibited more than 50% of VSV plaques (Table 7). In contrast, 256 U of interferon did not significantly inhibit the number, size, or time of appearance of SFV plaques.

DISCUSSION

The present study expands the list of viruses that spread by the type 1 (i.e., coxsackie, encephalomyocarditis, VSV, mumps, and SV5) and type 2 (i.e., HSV, CMV, vaccinia, measles, and SFV) routes. It should be emphasized, however, that certain viruses spread by more than one route, and analysis of the route of viral spread is particularly important in understanding the efficacy of the different immunological defense mechanisms that operate in vivo. In this connection, it is known that a number of viruses can produce a persistent infection in the presence of high concentrations of antiviral antibody. The foamy viruses have now been added to this group (7, 15).

The demonstration here that foamy viruses can spread in vitro in the presence of concentrations of antiviral antibody sufficient to neutralize extracellular virus made it possible to develop a simple plaque assay. Although very high concentrations of neutralizing antibody (250 to 500 neutralization units) moderately inhibit plaque formation, the cultures were not free of virus or "cured." A number of factors might be responsible for this partial inhibition of viral spread by very high concentrations of antibody. Transient exposure of virus to serum may take place during the egress or budding of the virus into adjacent cells, and in the presence of high concentrations of antiviral antibody this might result in neutralization. High concentration of antiviral antibody attached to the surface of infected cells also might block viral-induced cell fusion and thereby prevent cell-to-cell spread. Alternatively, high concentrations of antiviral antibody might affect the expression or synthesis of viral antigens (3).

Since neutralizing antibody does not stop the spread of foamy viruses, other immunological mechanisms such as antibody-dependent, complement-mediated lysis might be required to halt the infection. Although viral antigens were induced on the surface of foamy-infected cells, complement-mediated lysis was not demonstrated. There are several explanations. First, the cell type infected might be resistant to lysis by complement. This is unlikely, since the same cells infected with HSV could be lysed. Second, the density of viral antigens on the surface of infected cells might be a factor. If there are few antigenic sites or if these sites are widely separated, complement-mediated cell

TABLE 6. *VSV plaque reduction in PRK cells treated with ultraviolet-inactivated virus*[a]

Interferon inducer	VSV plaques
Maintenance medium	24
PRK cell control	20
NDV	0
HSV-1	0
SFV-1	18
SFV-2	24
SFV-6	16
SFV-7	23

[a] PRK cell monolayers in 35-mm petri dishes were incubated with ultraviolet-inactivated viruses for 24 h prior to challenge with 25 plaque-forming units (PFU) of VSV. The number of plaques that developed was determined. The viral infectivity titers prior to inactivation by ultraviolet light were: HSV-1 (4×10^6 PFU/ml), SFV-1 (3.2×10^6 tissue culture infectious doses [$TCID_{50}$]/ml), SFV-2 (1.6×10^7 $TCID_{50}$/ml), SFV-6 (1.3×10^5 $TCID_{50}$/ml), and SFV-7 (3.2×10^5 $TCID_{50}$/ml).

TABLE 7. *Sensitivity of foamy viruses to antiviral action of interferon*[a]

Virus	Interferon (U)	No. of plaques
VSV	256	0
	2	10
	1	14
	0.5	34
	0	32
HSV-1	256	3
	0	44
SFV-1	256	17
	128	21
	0	21
SFV-2	256	36
	128	36
	0	38
SFV-7	256	58
	128	60
	0	57

[a] PRK cell monolayers were inoculated with different concentrations of interferon for 24 h. The interferon was removed, and the cells were challenged with 25 to 50 plaque-forming units of VSV, HSV-1, SFV-1, SFV-2, or SFV-7. The average plaque number of four plates was recorded.

destruction may not occur (1, 10). Our studies with fluorescein isothiocyanate-labeled and ^{125}I-labeled antibody showed that the concentration of viral antigens on the surface of foamy virus-infected cells was, in fact, low. Third, antiviral antibody might induce fluctuations in the density of viral antigens by capping (8). However, we were unable to demonstrate capping. Fourth, the concentration and nature of the antiviral antibody in a particular serum may vary, i.e., the ratio of complement-fixing to noncomplement-fixing antibody or the ratio of cytotoxic to noncytotoxic antibody (1).

Whether antibody-dependent, cell-mediated cytolysis or cytotoxic T lymphocytes aid in stopping the spread of foamy virus has not yet been investigated. In fact, it is not known whether foamy viruses induce a cell-mediated immune response. Recent studies have shown that cellular immunity to viral infections may, in part, be mediated through immunologically induced interferon (13). Our studies, however, indicate that foamy viruses are relatively resistant to the antiviral action of standard interferon and are poor interferon inducers. Whether the antiviral action of immune-induced interferon would affect foamy virus replication more than standard interferon remains to be determined. The fact remains, however, that these viruses produce a persistent infection in vivo.

It is becoming increasingly apparent that a variety of viruses can produce persistent infections in the presence of immunity. More than a dozen factors that might contribute to viral persistence in the presence of immunity have been enumerated (12). In the case of foamy viruses, our studies suggest that the capacity of these viruses to spread from cell to contiguous cell in the presence of antibody, the patchy and widely dispersed expression of viral antigens on the cell surface, and the poor induction and response to interferon may be several of the important factors contributing to persistence.

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