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Cell Fusion by Canine Distemper Virus-Infected Cells

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AV3 cells (continuous human amnion) infected with the Onderstepoort strain of canine distemper virus produced cell fusion within 2 to 5 hr when added to AV3 cell monolayers. An apparent requirement for intact, infected cells was demonstrated by showing that (i) frozen-and-thawed infected cells failed to induce fusion, (ii) infected cells frozen in the presence of glycerol retained their ability to induce fusion, (iii) infected cells subjected to swelling in hypotonic buffer and homogenization lost their ability to fuse cells, and (iv) semipurified and concentrated virus preparations with infectivity titers as high as $10^{7.5}$ mean tissue culture doses per ml failed to induce fusion within 5 hr. Preparations of intact, infected cells had a mean $\log_{10}$ ratio of infectivity to fusion activity of 3.6. Treatment with beta-propiolactone rendered the active preparations free from detectable infectivity while they retained their ability to cause cell fusion. Cycloheximide did not block the formation of syncytia in assay cells. This type of cell fusion was neutralized by canine distemper virus immune antisera, and measles virus immune sera showed a slight degree of cross-neutralization. Other cell lines, HEp-2, MA 139 (embryonic ferret lung), MA 104 (embryonic rhesus monkey kidney), and Vero (African green monkey kidney) were also susceptible.

The formation of multinucleated giant cells (syncytia) has been shown to accompany the replication of canine distemper virus (CDV) in a variety of cell cultures (3–5, 21). Other viruses such as measles (6, 23), Sendai (18), simian virus 5 (9), and Newcastle disease virus (1, 2, 12–14) which can induce syncytia as the result of multiplication have been shown to cause early cell fusion (1–3 hr). Early cell fusion resulted after exposure to high titers of active or inactivated virus.

This study was undertaken to determine whether early cell fusion occurs with CDV. CDV preparations ($10^{7.5}$ mean tissue culture doses [TCD_{50}] per ml) failed to induce early cell fusion. However, cell fusion did occur within 2 to 5 hr when intact, infected cells were employed.

MATERIALS AND METHODS

Cell cultures. A continuous line of human amnion cells (AV3) was employed in most of these studies. Tube cultures of HEp-2, MA 104 (embryonic rhesus monkey kidney), MA 139 (embryonic ferret lung), and Vero cells (African green monkey kidney) were obtained commercially (Microbiological Associates, Bethesda, Md.). The AV3 cells were washed with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) and dispersed with CMF-PBS containing 0.02% ethylenediaminetetraacetic acid. The cells were suspended in growth media (minimal essential medium [MEM] plus 10% newborn calf serum) (350,000 cells per ml), 1 ml was plated per tube, and 25 ml was dispensed per 16-oz (ca. 0.48-liter) prescription bottle. These were incubated at 37 C, and the monolayers were ready to use in 2 to 3 days.

Virus. The Onderstepoort strain of CDV was employed (3–5). Sixteen-ounce (ca. 0.48-liter) prescription bottles of AV3 cells were inoculated with CDV, the virus was allowed to adsorb at 37 C for 2 hr, and then 22 ml of MEM-5% newborn agamma calf serum, *p*H 8.3, was added. The virus was harvested at 36 hr by freezing and thawing, at which time almost all of the cells were included in syncytia. Intact virus-infected cells for demonstration of cell fusion were prepared in the same manner but were harvested by scraping and pipetting because frozen-and-thawed infected cells did not cause cell fusion within 2 to 5 hr. Infectivity titers in AV3 cells ranged from $10^{5.5}$ to $10^{7.5}$ TCD_{50}/ml.

Fusion assay. Cell fusion was demonstrated by inoculating tubes of AV3 cells with 0.25 ml of serial twofold dilutions of intact virus-infected cells and incubating them at 37 C for 5 hr (6). The diluent was MEM-5% newborn agamma calf serum, *p*H 8.3. The cells were then fixed with 5% acetic acid in 70% ethanol and stained with hematoxylin and eosin. The end point of the titration was considered to be the dilution that yielded 10 or more syncytia containing 10 or more nuclei.

BPL treatment. Intact virus-infected cells were treated with beta-propiolactone (BPL; "Betaprone," Fellows-Testagar, Oak Park, Mich.) (22). The procedure described by Neff and Enders (17) was employed, with the exception that we did not carry out the final overnight incubation at 4 C. Final concentration of BPL was 0.1%.

Antisera. Hyperimmune rabbit anti-CDV and anti-measles virus antisera were prepared by intravenous inoculation of CDV (Onderstepoort) grown on the chorioallantoic membrane of chicken embryos or measles virus (Edmonston) grown in monkey kidney cell culture. All sera were inactivated at 56 C for 1 hr before use.

RESULTS

Demonstration of cell fusion. Tubes of AV3 cells were inoculated with intact virus-infected cells and incubated at 37 C. Small syncytia containing 10 to 20 nuclei were observed within 2 hr (Table 1). After 3 hr, the syncytia had increased in size (20 to 30 nuclei), and 50% of the cells were contained in syncytia. After 5 hr, the syncytia contained 50 to 70 nuclei and included 70 to 100% of the cells. No effect on the nuclei was observed, and they were dispersed throughout the syncytia.

Quantitation of cell fusion. Duplicate tubes of AV3 cells were inoculated with three separate series of dilutions prepared from a single, intact virus-infected cell preparation. Focal syncytia were observed at limiting dilutions. The number of syncytia containing 10 or more nuclei per tube was counted (Table 2). The number of syncytia was proportional to the dilution in the range between 1:64 through 1:256 (18–70 syncytia). Several methods of determining end points were evaluated. These were based upon either the percentage of cells involved in syncytia or the count of syncytia containing various numbers of nuclei. The dilution which caused the formation of 10 or more syncytia per tube was taken as the end point because this could be determined quickly and gave consistent results. The reciprocal of the limiting dilution gave the number of early cell fusion units per 0.25 ml. Syncytia containing less than 10 nuclei were not counted because uninoculated cells frequently contained syncytia with 3 to 5 and, rarely, up to 10 nuclei.

TABLE 1. *Effect of time of incubation on the development of syncytia*[a]

Time	Cells involved in syncytia (%)	No. of nuclei/ syncytium
30 Min	0	
1 Hr	0	
2 Hr	1	10–20
3 Hr	50–60	20–30
4 Hr	75–100	30–50
5 Hr	75–100	50–70

[a] AV3 cells were infected with CDV and harvested by scraping at 36 hr. The infectivity titer of this preparation was $10^{6.8}$ TCD_{50}/ml. A 0.25-ml amount of undiluted infected cells was inoculated into each replicate tube culture of AV3 cells, and these were incubated at 37 C. At the times shown, duplicate tubes were fixed and stained. The percentage of cells involved in syncytia (10 or more nuclei) and the number of nuclei per syncytium were estimated.

TABLE 2. *Quantitation of cell fusion by CDV-infected cells*[a]

Dilution	No. of syncytia per tube[b]	Mean	Range
1:64	73, 74; 68, 82; 64, 62	70.5	62–82
1:128	33, 36; 40, 44; 34, 32	36.5	32–44
1:256	17, 18; 16, 20; 17, 18	17.7	16–20
1:512	7, 6; 6, 4; 5, 8	6.0	4–8
1:1,024	3, 5; 6, 4; 3, 3	4.0	3–6

[a] Duplicate tubes of AV3 cells were inoculated with each of three separate series of dilutions prepared from a single intact virus-infected cell preparation. These cells were harvested by scraping at 36 hr postinfection. The assay cell cultures were incubated at 37 C for 5 hr, fixed, stained, and counted.

[b] Number of syncytia containing 10 or more nuclei.

Titrations were performed on six different intact virus-infected cell preparations and compared with their infectivity titers (Table 3). Infectivity titers ranged from $10^{5.5}$ to $10^{6.3}$ TCD_{50}/ml, whereas fusion titers ranged from 32 to 1,024/ml. The $\log_{10}$ ratio of infectivity to cell fusion titers ranged from 2.5 to 4.3 with a mean of 3.6. When the same preparations were frozen and thawed, slightly higher infectivity titers were obtained (0.5 $\log_{10}$ or less increase). However, these frozen-and-thawed preparations failed to induce cell fusion within the 5-hr assay period. Virus semipurified and concentrated by differential centrifugation or ammonium sulfate precipitation also failed to induce fusion even though infectivity titers of $10^{7.5}$ TCD_{50}/ml were obtained.

Neutralization of cell fusion by immune sera. Twofold serial dilutions of preimmune, anti-CDV and anti-measles sera were made in MEM-5% newborn agamma calf serum, *p*H 8.3. Equal parts of sera and intact CDV-infected cell suspension diluted to contain 2.5 or 23 early cell fusion units were mixed and incubated at 25 C for 1 hr, and 0.25-ml fractions of the mixture were assayed for cell fusion (Table 4). The 50% neutralizing antibody titers obtained against 100 TCD_{50} of CDV are shown for comparison.

Demonstration of cell fusion with BPL-treated

TABLE 3. *Ratio of CDV infectivity titers to cell fusion titers*

CDV prepn	TCD_{50}/ml (log_{10})	Cell fusion units/ml (log_{10})	Log ratio: TCD_{50}/cell fusion
AV3-7	6.3	2.1	4.2
AV3-10	5.8	1.5	4.3
AV3-12	5.5	3.0	2.5
AV3-13	6.3	3.0	3.3
AV3-14	5.8	2.7	3.1
AV3-14a	5.8	1.5	4.3

TABLE 4. *Neutralization of cell fusion by CDV-infected cells*

Serum	50% Syncytia reduction titers[a]		50% Neutralization titers
	2.5 Cell fusion units	23 Cell fusion units	100 TCD_{50}
Preimmune	<1	<1	<1
Anti-CDV	464	312	44.7
Anti-measles	6	3	5.6

[a] Reciprocal of the serum dilution required to reduce the number of syncytia by 50%.

virus-infected cells. Intact virus-infected cells treated with BPL retained their ability to cause cell fusion. BPL caused an eightfold decrease in the number of cell fusion units (from 64 to 8), and a reduction in the number of nuclei per syncytium from approximately 50 to 30. The infectivity titer was reduced from $10^{5.8}$ TCD_{50}/ml to less than 1.

Requirement of intact cells for demonstration of cell fusion. The ability to cause cell fusion appeared to be associated with intact virus-infected cells. After centrifugation at 250 × *g* for 10 min, cell fusion activity was found in the pellet only. Cell fusion induced by Sendai virus was labile at either 4 or −70 C (8, 17, 19). To determine whether cell fusion activity was labile at low temperature (−70 C), CDV-infected cells were frozen in the presence of glycerol. The number of cell fusion units increased twofold from 128 to 256. This result indicated that exposure to low temperature per se was not the reason for the loss of cell fusion activity. Since freezing and thawing causes disruption of cells, the intact virus-infected cells were subjected to another means of disruption (20). An intact virus-infected cell suspension was centrifuged at 250 × *g* for 10 min, and the cell pellet was suspended in hypotonic buffer. The cells were allowed to swell for 5 min and were transferred to a Dounce homogenizer, and 5 to 10 strokes were applied to rupture the cells. The preparation was returned to isotonicity by addition of concentrated MEM. The number of cell fusion units decreased from 8 to less than 1, but the number of TCD_{50}/ml increased from $10^{5.8}$ to $10^{6.3}$. Since intact virus-infected cells lost their ability to cause cell fusion when treated in this manner, it seems likely that disruption of cells after freezing and thawing is also responsible for loss of activity.

The effect of cycloheximide on fusion induced by intact CDV-infected cells. Early cell fusion or fusion from without (FFWO) by Newcastle disease virus did not require protein synthesis, as shown by experiments with cycloheximide (1, 2). Intact CDV-infected cells were harvested at 36 hr postinfection and diluted 1:8 in MEM-5% calf serum with or without added cycloheximide. The intact infected cells were added to duplicate tubes of AV3 assay cells (0.5 ml/tube). The final concentrations of cycloheximide were 2 and 10 μg/ml. The assay tubes were incubated for 5 hr at 37 C, fixed, and stained, and the syncytia were counted. There were a total of 74 syncytia in the control tubes (no cycloheximide); in the presence of 2 μg and 10 μg of cycloheximide per ml there were 60 and 63 syncytia, respectively. Other experiments showed that cycloheximide in concentrations of 1 μg/ml and 10 μg/ml inhibited infectious CDV production in AV3 cells by 97 to 99.9% and 99.99%, respectively. These results show that cycloheximide in concentrations that markedly inhibit infectious virus production does not prevent the cell fusion induced by CDV-infected cells.

Susceptibility of other cell lines to cell fusion. Intact CDV-infected AV3 cells also caused cell fusion in HEp-2 cells to approximately the same titer as in AV3 cells. MA 139 ferret lung fibroblasts were also sensitive: approximately two-thirds of the syncytia contained 20 to 30 nuclei, and the others contained 50 to 70. The nuclei were often arranged in a ring-like fashion near the center of the syncytium. MA 104 (embryonic rhesus monkey kidney) and Vero cells were also sensitive. The resulting syncytia were consistent in size but were smaller (10–30 nuclei) than the ones formed in AV3 cells. The MA 104 cells gave a 16-fold lower cell fusion titer than the AV3 cells (256 versus 16) and the Vero cells gave a twofold lower titer (64 versus 32). Differences in cell fusion titers and appearance of syncytia might have been due to differences in the age or condition of the cells.

DISCUSSION

Intact CDV-infected cells caused the formation of syncytia in monolayers of AV3 cells within 2 to 5 hr after inoculation. This compares with an eclipse period of 8 to 12 hr observed in chicken embryo cells (3) and HEp-2 or AV3 cells (*unpublished data*) after infection with the Onderstepoort strain of CDV. The time course of syncytia formation differs after the inoculation of intact infected cells from that seen in the initially infected cultures. AV3 cells inoculated with multiplicity of infection of 1 to 5 show the first signs of syncytia at 18 to 24 hr postinfection, and complete fusion does not occur before 36 to 48 hr. This compares with the initial appearance of fusion at 2 hr and a rapid progression to complete fusion by 4 to 5 hr after the inoculation of high concentrations of intact infected cells.

Human continuous heteroploid cells were sensitive, whereas continuous monkey cells were resistant to early cell fusion by measles virus (6). We have shown that cells of human, monkey, and ferret origin were all sensitive to cell fusion by CDV-infected cells. This result suggests that cell fusion by intact, infected cells may be a more efficient process than fusion by virions.

The demonstration that BPL-treated CDV-infected cells retained their ability to cause cell fusion within 5 hr in the absence of detectable infectious virus was further proof that fusion may occur in the absence of viral multiplication. High titers of CDV ($10^{7.5}$ TCD_{50}/ml) released from infected cells by freezing and thawing failed to induce early cell fusion, yet similar titers of measles virus preparations prepared by freezing and thawing infected cell cultures produced early cell fusion (6). Nevertheless, we cannot exclude the possibility that higher concentrations of CDV virions would show early cell fusion.

The fusion observed with intact CDV-infected cells may be due to the presence of virions maturing at the surface of infected cells. The infected cell would serve to localize the virions, allowing a concerted action on the assay cell membranes in contact with the infected cells. This can be envisioned as being similar to hemadsorption reactions where the release of free virions is not sufficient to demonstrate hemagglutination. This mechanism would explain the focal nature of the syncytia we observed at limiting dilutions in contrast to the nonfocal nature of the early cell fusion seen with measels virus preparations (6). It would also explain the apparent high efficiency of fusion as compared with early cell fusion systems employing virion preparations. A second possible explanation is that fusion is due to alterations in the cell membrane unrelated to the actual presence of virions. Such alterations have been described for several parainfluenza viruses and for measles virus (7, 11, 16). These surface alterations may be marked even in the absence of large numbers of budding virions (7, 9). During replication of CDV in the chorioallantoic membrane of the chicken embryo, many infected cells showed surface projections on the cytoplasmic membrane even though no budding virions were observed (15). Such altered areas of the cytoplasmic membrane may be responsible for the cell fusion we report with intact CDV-infected cells. Loss of activity after disruption of cells could then be explained by a fragmentation of altered segments of the cytoplasmic membrane and consequent lack of localization. A correlation has been reported between cell fusing capacity and the size of virion envelope fragments of Sendai virus, with the smallest fragments completely lacking cell fusing capacity (10). CDV-infected cells gained the capacity to fuse assay cells prior to 10 hr postinfection, whereas the latent period was 10 to 12 hr (Fisher and Bussell, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 240, 1972). When cycloheximide was added 2 or 10 hr postinfection, infectious virus production was inhibited 2 to 3 $\log_{10}$ units, whereas the capacity to fuse assay cells was not significantly reduced at 36 hr. These results favor the interpretation that fusion by CDV-infected cells is not due to surface virions but is due to some other alteration in the cytoplasmic membrane.

The experiment which showed that cycloheximide did not block fusion of assay cells suggests that there is no requirement for protein synthesis for this type of cell fusion. Bratt and Gallaher (1, 2) listed several criteria which they employed to define early (FFWO) and late (fusion from within, FFWI) cell fusion. A comparison of our data with these criteria reveal that fusion induced by CDV-infected cells more nearly resembles FFWO than FFWI. Thus, more fusion occurs with high concentrations of infected cells; infected cells rendered noninfectious by BPL still induce fusion; cycloheximide does not block fusion; and fusion occurs before the end of the latent period. The major and perhaps critical difference between the cell fusion observed with CDV-infected cells and FFWO is that it does not appear to be induced by virions.

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