Arenavirus Defective Interfering Particles Mask the Cell-Killing Potential of Standard Virus

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SUMMARY

Lymphocytic choriomeningitis virus (LCM) and Pichinde virus grew readily and produced cytopathology in MDCK and PK-15 cells. It is known that in these cell lines, the synthesis or function of defective interfering (DI) virus particles is restricted. Survival curves of single MDCK cells infected with low multiplicities of LCM showed one-particle-to-kill kinetics. At high multiplicities of infection, there was a maximum degree of cell-killing, or even a reduction in the amount of cell-killing, depending on how much DI virus was present in a particular standard virus stock. DI LCM virus could completely prevent standard virus from producing c.p.e. in MDCK monolayers with one-particle-to-protect kinetics. It could still prevent killing of the cells when added within a short time after infection with standard virus, but was able to interfere with synthesis of standard virus when added even later. On passage of LCM or Pichinde virus without dilution in MDCK cells, there was no homologous auto-interference. Furthermore, there was only slight interference with the synthesis of standard virus when these cells were pre-treated with DI virus.

INTRODUCTION

The terminal event in many well studied virus-animal interactions is the rapid irreversible loss of bodily function caused by cell death. At the other extreme, viruses like lymphocytic choriomeningitis virus (LCM) multiply in congenitally infected mice without apparent adverse effect on their cells. This absence of virus-induced cytolytic activity, combined with an immune response that does not limit spread of the infection, results in a life-long persistent infection. Strikingly constant and relatively low levels of virus are found in the various organs of such mice throughout their entire life-span. This regulation of virus synthesis appears to be mediated neither by interferon (Traub, 1961; Wagner & Snyder, 1962; Volkert, Larsen & Pfau, 1964) nor by the competence of the immune system (Hoffsten & Dixon, 1973; Bro-Jørgensen & Volkert, 1974).

It has long been known that LCM-infected tissue cultures mimic the in vivo non-cytolytic response (Hotchin & Cinitis, 1958). Only recently has it become apparent that the synthesis of LCM and other arenaviruses (Pfau et al. 1974) in these cells is controlled by defective interfering (DI) virus (Welsh & Pfau, 1972; Welsh, O'Connell & Pfau, 1972; Staneck & Pfau, 1974; Dutko, Wright & Pfau, 1976; Help, Leon & Coto, 1976). The latter viruses arise very quickly after cells are infected with standard (plaque-forming) virus (Welsh & Pfau, 1972)

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and are synthesized almost exclusively in long-term persistently infected cultures (Staneck et al. 1972; Dutko et al. 1976; Help et al. 1976). They have thus far been found to conform (Dutko et al. 1976) to the general criterion that these particles are a special class of deletion mutants (Huang & Baltimore, 1977).

This communication presents data indicating that standard arenaviruses readily kill cells, and that it is only through DI particle intervention that cells are spared. These cytopathic effects are shown in tissue culture lines which prevent or delay genesis of DI particles, while allowing their expression if exogenously added.

**METHODS**

**Cells.** MDCK (Madin-Darby canine kidney) cells from the Sloan-Kettering Institute, N.Y., were routinely used (Krug, 1971). Similar cells from the University of California at San Diego (Perrault & Holland, 1972) and the American Type Culture Collection (Repository Designation CCL 34) Rockville, Md. were found to respond to virus infection in an identical manner. These cells, periodically checked for mycoplasma (Zgorniak-Nowosielska et al., 1967), were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% heat-inactivated foetal bovine serum. Cells were monodispersed with a 0.0625% (1:300) trypsin solution (in phosphate buffered saline) containing 0.0125% of the disodium salt of [ethylenedinitrilo] tetra acetic acid (EDTA). PK-15 (porcine kidney) cells, ATCC-CCL33, were treated similarly to MDCK cells except that the growth medium was also supplemented with Eagle's non-essential amino acids. BHK21/13S cells, originally from the Wistar Institute, were carried in suspension or as monolayer cultures by established procedures (Pulkkinen & Pfau, 1970). L-929 cells were grown in MEM plus 10% calf serum and dispersed with a 0.25% trypsin solution (without EDTA).

**Viruses.** The strains of LCM virus used in these studies were UBC, Traub and WCP. The origin of these L cell-grown stocks has been described (Pulkkinen & Pfau, 1970). LCM-UBC DI virus was concentrated 20-fold by differential centrifugation (Welsh et al. 1972) of medium from persistently infected L cells which had ceased to produce detectable standard virus particles (Staneck et al. 1972). Pichinde standard and DI virus (concentrated as above) were those used in previous studies (Logan et al. 1975; Dutko et al. 1976). All standard virus stocks were grown at low m.o.i. (usually 0.1) and harvested 27 h after infection.

**Standard virus cell-killing: evaluation by monolayer and cloning assays.** LCM or Pichinde virus cell-killing ability was determined in MDCK or L cell monolayers using essentially the procedure of Marcus & Sekellick (1974). Medium was decanted from confluent monolayers (in Falcon 3013 flasks seeded with 10⁶ cells 48 h before use) and replaced with 1 ml of either virus at the appropriate m.o.i. All such inocula contained 50 µg DEAE-dextran. After 1.5 h at 37 °C the inocula were removed and the monolayers washed three times with 10 ml of medium. These cell monolayers were then photographed (after medium replacement and incubation at 37 °C) at various intervals or immediately dispersed with the appropriate trypsin solution and counted in a haemocytometer. Tenfold dilutions of the cells were added to plastic tissue culture plates (Falcon no. 3002) with a final vol. of 5 ml of the original type of medium. After incubation for 8 to 10 days at 37 °C in a 5% CO₂ atmosphere, the cell colonies (the numbers of which were always proportional to the cell dilution factor) were fixed in methanol-acetic acid (3:1), stained with a standard Giemsa solution, and counted. In all flasks containing LCM-infected cells the medium during the 8- to 10-day incubation period contained neutralizing antibodies in the form of heat-inactivated (56 °C for 30 min), filtered human ascites fluid (Webb et al. 1975). This was necessary to prevent spread of virus
 Arenavirus defective interfering particles

from productively infected cells to colonies arising from cells that had escaped infection on
the original intact monolayer. The efficiency of plating (e.o.p.) was determined by dividing
the colony number/flask by the total number of cells added/flask. The e.o.p. during an eight
month period of experimentation varied from 50 to 80% but was constant in individual
experiments and unaffected by either the dextran used in the infection procedure or the
antiserum in the cloning medium. Cell-killing was determined by comparing the colony
counts from virus-infected flasks to those from mock-infected flasks.

Plaque assays. Infectious Pichinde or LCM virus (samples frozen at -60 °C until use)
was measured either by a BHK-agarose suspension assay (Logan et al. 1975) or an MDCK
monolayer assay (Krug, 1971). With the latter assay, LCM plaques were counted without
the need of counterstain on the fourth day after infection, whereas at that time Pichinde
plaques were stained with a 1:10000 dilution of neutral red and counted one day later.

DI virus intervention with standard virus function. BHK or MDCK monolayers, as prepared
for a standard virus cell-killing assay, were infected with 1 ml of an LCM or Pichinde DI
virus preparation. After 1.5 h at 37 °C the inoculum was removed, the monolayers were
repeatedly washed and then challenged with standard virus (as above) at a m.o.i. of 3.
DEAE-dextran (50 μg/ml) was added only once to the cultures in the DI virus preparations
or in the mock-infecting solution. The monolayers were then re-fed or dispersed with
trypsin-EDTA for cloning purposes. The supernatant content of standard virus from intact
BHK and MDCK monolayers was determined 21 and 18 h after challenge infection,
respectively.

RESULTS

Virus growth and cytopathic effects in various cell lines

BHK, MDCK and PK-15 cell monolayers were infected with LCM or Pichinde virus at
a m.o.i. of 3 to 5. During a 72 h observation period, the arenavirus-infected BHK cells
appeared no different from the mock-infected cells. However, with MDCK cells and unlike
the mock-infected cell controls (Fig. 1a), small clusters of rounded cells were first noted
14 h after infection with either virus. Within 3 to 4 h after the appearance of clusters in the
LCM-infected monolayers, an abnormal number of cells were noted in the surrounding
medium (Fig. 1b). At this time patches of large cells were apparent – presumably filling the
space left by the now-floating cells. By 38 h after infection (Fig. 1c) so many cells had lifted
off the monolayer that only a few islands of intact cells could be seen. Higher input m.o.i.
decreased the interval between infection and first appearance of c.p.e. The extent of the
cytopathic effect was much more limited with Pichinde virus and never exceeded that seen
with LCM at 18 h after infection. A weak c.p.e. was also evident in PK-15 cells 48 h after
LCM infection.

Growth curves of these viruses were determined in the three cell lines. Monolayers in
75 cm² plastic flasks that had just reached confluency were exposed to a 5 ml inoculum at
an input m.o.i. of 3 (LCM) or 5 (Pichinde). After the adsorption period the virus was removed
by repeated washing, and the growth medium (Eagle’s MEM) was replaced to the original
volume (30 ml). Virus titres were highest (Fig. 2) in the BHK cells which developed no c.p.e.
after infection with Pichinde virus (or with LCM virus, data not shown). Yields in MDCK
cells were at least one log₁₀ unit lower and were maximal shortly after the initial detection of
c.p.e. Incomplete data were obtained on the growth of LCM virus in PK-15 cells, but the
yields appeared somewhat better than those from the MDCK system (Fig. 2).
Survival of single MDCK cells as a function of m.o.i.

Cell-killing was shown to be the cause of the c.p.e. observed in MDCK cultures. Fig. 3 illustrates the survival data after exposing MDCK monolayers to various concentrations of the UBC strain of LCM virus. The linear nature of the curve at relatively low virus concentrations demonstrates that the inability of a cell to form a clone, i.e. a killed cell, is the result of infection with an individual virus rather than the cumulative action of a number
of viruses. Prevention of cloning was not simply due to failure of infected cells to attach to a surface, since this event was observed to proceed normally with trypsin-dispersed cells taken from monolayers up to 6 h after infection. From the data in Fig. 3, the virus concentration which kills 63% of the cell population can be determined. Based on the Poisson distribution this concentration of virus represents, on average, one cell-killing particle attached per cell. Knowing the number of cells in the monolayer at the time of infection and the input standard virus titre (identical e.o.p. in either BHK or MDCK cells), the m.o.i. (in terms of p.f.u.) at the 63% kill point can be determined. This was 0.69, or 1 p.f.u./1.45 cell-killing particles. Similar cell-killing experiments with Pichinde virus have not been attempted because we could not obtain or produce potent neutralizing antibody. However, when undiluted Pichinde virus was used to infect MDCK monolayers, the cell-killing measured in the absence of antibody was 44% (use of antisera was necessary only with diluted LCM virus).
Cell-killing particle multiplicity

Fig. 3. Survival curve of MDCK cells exposed to various concentrations of LCM-UBC virus. The upper abscissa represents the cell-killing particle multiplicity calculated from the 37% cell survival level. Undiluted virus stock was assigned a relative concentration of 20 (lower abscissa) and corresponded to an input m.o.i. of 5.5. △, Experiment 1; □, experiment 2. Theoretical hit kinetics: ——, single; ——, double.

Susceptibility of MDCK sub-lines to cell-killing by standard virus

In numerous experiments similar to that presented in Fig. 3, no more than 80% cell-killing was observed. Since similar experiments with L cells, instead of MDCK cells, failed to reveal any cell-killing, it was considered that there might be a sub-population of MDCK cells that was resistant to the cell-killing potential of LCM virus. In fact, two sub-lines of MDCK cells are known to exist (J. Robb, 1976, personal communication), one epithelial and the other fibroblastic and constituting less than 2% of the cell population in an uncloned culture. Seven clones of each type of cell, easily distinguished by their compact or spreading/swirling appearance, were isolated in our laboratory. However, both types were equally susceptible to killing.
Prevention of standard virus cell-killing by DI virus

It was next considered that a cell co-infected with both standard and DI virus might be spared, so that the amount of DI virus in a stock determines its maximum cell-killing ability. This hypothesis rested on the following observation. When two identical L cell monolayers were infected at an m.o.i. of 0.1 and the extracellular fluids were harvested 27 and 48 h later, both harvests had identical p.f.u. titres ($2 \times 10^7$/ml), yet they were markedly different in cell-killing ability (Fig. 4). The early harvested stock, when undiluted, killed 75% of the MDCK cells, whereas the late harvested stock killed only 31%. Over about a 10-fold dilution range the cell-killing capacity of either virus stock actually increased, the maxima being 92 and 56% respectively. Since previous growth curve studies (Welsh & Pfau, 1972) had shown that the initial peak (and plateau) in standard virus synthesis indicates the beginning of a
Table 1. The effect of antiserum on the ability of LCM virus DI particles to inhibit cell-killing

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Cell-killing by challenge virus (%)</th>
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<tbody>
<tr>
<td>None (control)</td>
<td>84</td>
</tr>
<tr>
<td>DI virus</td>
<td>37</td>
</tr>
<tr>
<td>Antiserum + DI virus</td>
<td>73</td>
</tr>
<tr>
<td>Antiserum</td>
<td>83</td>
</tr>
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</table>

Fig. 5. MDCK monolayers were exposed to two undiluted DI virus stocks (assigned a relative concentration of 20) for 1.5 h prior to infection with standard virus. In experiment 1 (△), the challenge standard virus stock killed 77% of the cells at zero relative DI virus concentration. In experiment 2 (□), the 100% maximum cell-killing value was established by a virus stock that would kill 84% of the cells. The line drawn represents theoretical single-hit kinetics.
burst in DI virus synthesis, the results in Fig. 4 were interpreted to indicate that the ratio between DI and standard virus in a stock, as well as the m.o.i., determine the extent of cell-killing.

Since it is not yet possible to separate a mixture of standard and DI arenaviruses physically (Dutko \textit{et al.} 1976), the role of DI virus in the above observations was confirmed by infecting monolayers with DI virus stocks (containing little or no standard virus) harvested from persistently infected L cells (Welsh, 1971). They were then challenged with a homotypic standard virus stock that by itself would display maximum c.p.e. as well as cell-killing activity. At 38 h after infection, monolayers receiving standard virus alone exhibited extensive c.p.e. (Fig. 1c) while those pre-treated with DI virus (Fig. 1d) appeared similar to non-infected control cultures (Fig. 1a). Identical results were obtained by infecting MDCK cells with Pichinde DI virus (F. J. Dutko and C. J. Pfau, unpublished data) prior to challenge with standard homotypic virus stocks. That LCM DI particles were responsible for preventing standard virus induced c.p.e. rested on their ability to be sedimented only under the conditions used for standard virus (see Methods), and their susceptibility to LCM-specific immune serum. In the latter experiments DI particles were exposed for 30 min at 37 °C to a dilution of human ascites fluid (see Methods) which would inactivate 98 % of the p.f.u. activity of a standard virus stock under the same conditions. MDCK cells were infected with the DI virus alone, the DI virus-antibody mixture, or antibody alone, then washed and challenged with standard virus. The data in Table 1 indicate that almost all interference activity of the DI virus preparations is destroyed by antibody. Furthermore, a 2 to 3 log\textsubscript{10} reduction in the p.f.u. titre of standard LCM virus stocks by either heat (37 °C) or u.v. failed to generate any detectable particles with the interfering ability of the DI virus preparations. The stoichiometry of the DI particle inhibition of cell-killing was studied by exposing MDCK monolayers to varying dilutions of DI virus followed by standard virus challenge. The data in Fig. 5 confirm that this is a dose-dependent phenomenon and follow single-hit kinetics. We make this statement only for the DI virus obtained from persistently infected cells. The DI particles in the standard virus preparations may be equally effective, because the regression of cell-killing in these stocks at high concentrations (Fig. 4) appears to follow single-hit kinetics, but insufficient data are available to be conclusive.

In order to rule out that the observed protection against c.p.e. was not simply due to blocking cell receptor sites by DI virus, these particles were added to monolayers at specific intervals either before or after challenge with standard virus (Fig. 6). Although the cell protecting ability of DI virus quickly waned, it could be added as late as 3 h after standard virus infection with retention of about 15 % of its original potency. On the other hand, as measured by its ability to inhibit standard virus synthesis, DI virus activity at that time was still 75 % of maximum. Detection of the longer lasting effect of DI virus in the latter part of these experiments depended on early harvest of supernatant fluids (16 h p.i.) followed immediately by sedimentation of cell debris. When infected MDCK cells lyse, virus titres fall quickly, for reasons which are not yet established (Fig. 2). Since c.p.e. is delayed in DI-treated cultures, a late harvest control virus stock would appear closer in p.f.u. titre to a comparable DI-treated stock than if both were harvested earlier.

\textit{Cell type as a function of the level of homologous autointerference}

Since the above studies showed that DI virus could prevent the cell-killing ability as well as the synthesis of standard virus, it was of interest to compare the potency of the latter phenomenon with that observed in cells which are naturally resistant to cell-killing. Two approaches were used involving comparison of p.f.u. yields from BHK or MDCK cells to
Fig. 6. Effect of DI virus on the cloning ability and standard virus yields from lethally-infected MDCK cells. Experiment 1 (open symbols): the standard virus challenge stock by itself killed 73% of the cells and the maximum inhibition in standard virus yield by the DI virus stock was 92%. Experiment 2 (solid symbols): comparable figures were 81 and 99% respectively.

which known quantities of DI virus were added, or in which conditions were created to favour DI virus synthesis. The first type of experiment was to infect monolayers with varying concentrations of LCM-UBC DI virus and then challenge the cultures with a fixed concentration of a homotypic p.f.u. virus stock. Virus was harvested at the peak of the growth curve in each cell line and p.f.u. yields were plotted as a function of the relative concentration of DI virus used to pre-condition the cells. As can be seen in Fig. 7 the ability of DI virus to interfere with standard virus synthesis is markedly less in MDCK than in BHK cells. With the highest concentration of DI virus used, the p.f.u. titre from BHK cells was only 49% of the control titre, whereas under similar conditions the titre from MDCK cells was 27% of the control yield.

The second type of experiment was to determine the effect of high m.o.i. serial passages (in which the virus inoculum used was that obtained from the preceding infected cultures) on virus p.f.u. yields. One would predict that, if the cell type allows DI virus to replicate and interfere with standard virus synthesis, the procedure would lead to the enrichment of DI
Fig. 7. LCM DI virus interference with standard virus replication in BHK (○) and MDCK (●) cells. A concentrated stock of LCM DI virus (see Methods) was diluted 1:1 and assigned a relative concentration of 10. After a 1.5 h adsorption period, all DI virus-infected cultures were challenged with a fixed concentration of standard virus. The supernatant fluids were harvested at the initial peaks in virus synthesis (see Fig. 2).

Table 2. Variability of autointerference in different host cells

<table>
<thead>
<tr>
<th>Undiluted passage</th>
<th>Yields (p.f.u. x 10^-4)</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>LCM*</td>
<td>Pichinde*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MDCK</td>
<td>BHK</td>
<td>MDCK</td>
</tr>
<tr>
<td>1</td>
<td>13.5</td>
<td>3.6</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>16.9</td>
<td>0.04</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>9.2</td>
<td>1.2</td>
<td>3.9</td>
</tr>
<tr>
<td>4</td>
<td>19.0</td>
<td>22.8</td>
<td>—</td>
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</tbody>
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* Pichinde and LCM virus were used at an initial m.o.i. of 1 to 3 to infect both cell types.

virus at each successive passage with a concomitant lowering of standard virus yield (Perrault & Holland, 1972). To exclude the possibility that DI virus synthesized in one cell type was only effective in that type, the LCM virus used in these experiments was cloned and passed in MDCK cells while the Pichinde virus was repeatedly passed (at low m.o.i.) in BHK cells. As shown in Table 2 the yield of either virus was reduced by about 2 log_{10} units with only two to three passages in BHK cells, whereas there was little or no evidence of autointerference in the MDCK cell line.
DISCUSSION

There is compelling evidence that LCM DI particles are generated rapidly after infection of most cell lines with standard LCM virus (Welsh & Pfau, 1972; Welsh et al. 1972; Pfau, 1977). Thus it was of interest to determine the characteristics of arenavirus synthesis in cell lines that had been found to restrict the replication or function of DI particles. Four cell lines were chosen for this study: MDCK, PK-15, MDBK, and Aedes albopictus, but only the first two of these could be conductively infected. Both displayed cytopathic effects when infected with any of three strains of LCM virus (UBC, WCP and Traub) or with Pichinde virus. Before these findings we were unaware of LCM-induced irreversible c.p.e. in a liquid culture medium that was not later traced to another agent (see Lehmann-Grube, 1971, for discussion and references). Repeated tests for PPLO in our virus stocks and cell lines were negative. The c.p.e. was clearly a specific virus-induced metabolic change since, unlike the effects of an adventitious virus or toxic substance, it could be readily prevented by exposing the cells to homotypic DI virus either before or after standard virus infection. We are aware, however, that arenaviruses can cause permanent damage to many cell types if the monolayers are under an agar overlay or in a nutritionally minimal liquid medium, or have been inoculated with virus taken directly from infected animals (Trapido & Sanmartin, 1971; Pfau, Welsh & Trowbridge, 1973; Popescu, Schaefer & Lehmann-Grube, 1976).

A preliminary survey of the arenaviruses indicated a spectrum of abilities to induce c.p.e. in MDCK cells. The most potent was the UBC strain of LCM, followed by the WCP and Traub strains and then by Pichinde virus. With almost identical LCM-UBC and Pichinde virus growth curves in MDCK cells (Fig. 2), the gradation in c.p.e. cannot be readily attributed to differences in primary virus-cell interactions (adsorption, penetration, uncoating). Although there are indirect indications (based on histopathology of persistently infected mice) that there may be intrinsic differences in the cell-killing ability of different strains of LCM virus (Volkert & Lundstedt, 1970), it could be argued that the amount of c.p.e. caused by a virus stock is inversely related to its DI particle content. On the other hand the Pichinde virus stocks, which killed only about half the number of cells killed by LCM-UBC, were early harvests from cultures infected at low m.o.i. - conditions known to produce virus with relatively low levels of DI particles (Welsh & Pfau, 1972; Dutko et al. 1976). If we assume that almost concomitant synthesis of DI and standard virus is the cause of the lack of c.p.e. in most cell lines, then a more likely explanation is that there is a direct relationship between the amount of c.p.e. caused by the standard virus and the extent of host cell modulation of the genesis or function of the DI virus. Prevention of function of DI virus in MDCK cells clearly appears untenable as a hypothesis since it has been established that deliberate addition of DI virus to these cells prevents c.p.e. Initiation of the cell-killing event probably occurs prior to genome replication, since delayed addition of DI particles to standard virus-infected cells has a markedly different effectiveness in interfering with these functions (Fig. 6). Alternatively, these results could be explained by proposing two types of DI virus - one preventing cell-killing, the other interfering with standard virus replication. Thus, at this time, it appears likely that, at least with certain arenaviruses, MDCK cells either prevent DI virus synthesis or delay its appearance beyond the point of interfering with the cell-killing programmed by standard virus.

The ratio between cell-killing particles and p.f.u. has been found to vary in different LCM-UBC virus stocks from 1:45:1 (Fig. 4) to 0:55:1. It appears reasonable to assume that the ratio is 1:1 and that cell-killing and p.f.u. activity reside in the same particle. LCM-strain CA1371 DI particle activity, as measured by the ability to inhibit infective centre
formation, has been shown to follow single-hit kinetics (Welsh et al. 1972). Since only one LCM-UBC DI particle is necessary to prevent cell-killing, it seems likely that the same DI particle may possess both these functions. Thus, in most cell lines synthesis of DI particles may act as a two-edged sword: on the one side interfering with standard virus synthesis and on the other preserving cell integrity without which even poorer virus yields would be obtained.

A number of observations has suggested the presence of DI particles in LCM-persistently infected mice (Welsh, 1971; Welsh et al. 1975). It has been shown recently that these particles are indeed synthesized in mice and are in greatest abundance during the neonatal period (Popescu et al. 1976; Popescu & Lehmann-Grube, 1977). Thus, the MDCK cell line may not only serve as an essential part of a tissue culture model for explaining the lack of cytopathology in rodents persistently infected with an arenavirus, but may also, because of its susceptibility to virus-induced suppression of cellular macromolecular synthesis, be the line of choice for studying the molecular events in arenavirus replication.

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