A comparison of biochemical and biological properties of standard and defective lymphocytic choriomeningitis virus

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Lymphocytic choriomeningitis (LCM) virus infection of the mouse is the best-studied model of persistent viral infection. In cell culture, persistent LCM virus infections are associated with the production of large quantities of defective interfering (DI) LCM virus. These defective interfering particles cannot replicate by themselves yet can interfere with the replication of the standard virus and prevent the cytolytic effect caused by the standard virus. It is important to determine the mechanism of interference and to establish whether the DI virus plays a role in vivo. Biological and biochemical properties of the standard and DI virus particles and also virus enzymes are compared. Antigenic analyses reveal that cells releasing only DI virus particles have less cell surface expression of viral antigens than cells releasing the standard virus. In the animal model, the DI virus is shown to have a protective effect against the pathogenesis of the LCM virus disease both in the mouse and in the rat.

Homologous interference with lymphocytic choriomeningitis (LCM) virus in cell culture has been known for several years (9) and has been shown to be due to the presence of defective interfering (DI) LCM virus, which is generated rapidly after standard (S) virus infection (32, 33, 34). DI LCM virus can be obtained relatively free from S virus contamination from the culture fluid of cells harbouring persistent LCM virus infection (10, 26) for over 50 cell generations. This DI virus interferes with arenavirus synthesis but not with the synthesis of heterologous viruses (10, 32). It is defective because its interfering capacity and antigenicity cannot be passed through cell culture in the absence of added S virus (31, 32). The purpose of this paper is to present a brief review of previously published and current data (soon to be published elsewhere) on the biochemical and biological properties of S and DI LCM virus and virus-infected cells.

METHODS

Virus assay, growth, and purification

S LCM virus (Armstrong strain) was quantitated by the BHK cell agarose suspension plaque assay (24), while DI virus was assayed by inhibition of S LCM virus infective centre formation (32). Unless otherwise stated, S and DI LCM virus were propagated in acutely infected and persistently infected (26) BHK 21/13S cells, respectively. Virus was purified by methanol precipitation followed by centrifugation through two discontinuous and one continuous 20–50 g/100 g sucrose gradients (23; Welsh and Oldstone, unpublished observations). The S LCM virus preparation used for the protein synthesis, polymerase, and infectious nucleic acid assays contained 2×10⁶ PFU/ml and an estimated 10¹² particles/ml. The DI virus preparation for the polymerase study represented a 150-fold concentration from the culture fluid.

RNA analysis

Both acutely and persistently infected BHK cells were labelled with uridine-5,6-³H (40–50 Ci/mmol, New England Nuclear) at a concentration of
33 \mu Ci/ml and incubated for 6 to 24 h after labelling. Alternatively, cultures were treated with actinomycin D (AD) at a concentration of 0.05 \mu g/ml for 2–3 h before addition of the label. The labelled virus in the culture fluid was purified by centrifuging the fluid on a 20–50 g/100 g sucrose discontinuous gradient and recentrifuging the virus in the interphase to equilibrium on a continuous 20–50 g/100 g sucrose gradient. RNA from purified virions was extracted with hot phenol (25) in the presence of sodium dodecyl sulfate (10 g/litre), 8-hydroxyquinoline (1 g/litre), sodium acetate buffer (0.01 mol/litre, pH 5.2), and Baycovan (to inhibit nuclease activity). The RNA was analysed on 5–30 g/100 g sucrose gradients in a Beckman SW60 rotor, and 100-\mu l fractions were collected onto DEAE filters, washed, and counted.

Infectious nucleic acid and nucleocapsid assay

Infectious viral nucleic acids and nucleocapsids were assayed by the DEAE method described by Pagano (16). Nucleic acids were prepared by phenol extraction, and nucleocapsid preparations were made by treating virions with NP-40. BHK and L-929 cells were used as targets, and the transfected cells were examined by infectivity and immunofluorescence assays.

Polymerase assays

The assays for virion RNA-dependent RNA polymerase and for cytoplasmic polymerase -RNA complexes were those described by Villarreal & Holland (28). At times the assay mixture was modified with various permutations of different concentrations of the following substances: NAD, Mn++, Mg++, S-adenosyl methionine, trypsin, chymotrypsin, and cytoplasmic extracts. The reverse transcriptase assay is described by Welsh et al. (37).

Protein synthesis assay

Virions were disrupted with NP-40 and assayed for protein synthetic activity, employing a combination of \(^3\)H-amino acyl transfer RNA's or \(^3\)H-phenylalanyl tRNA, with and without the addition of an exogenous polyuridylic acid template. The assays were those described by Moldave & Skogerson (13) and were run in the presence of rat liver and/or BHK cell supernatants after centrifugation at 100 000 g. In addition to the standard assay components, various concentrations of adenosine triphosphate, additional magnesium chloride, and peptide elongation factors EF-1 and EF-2 (Moldave et al., 12) were sometimes added to the assay mix. Ribosomes isolated from uninfected BHK cells served as positive controls.

Antibody to LCM virus

The antibody to LCM virus was a purified IgG fraction from guinea-pigs. The antibody was labelled with \(^{125}\)I by the method of McConahey & Dixon (11).

RESULTS AND DISCUSSION

Purification and RNA analysis

When centrifuged to equilibrium in sucrose, S LCM virus infectivity and radioactivity banded at about 1.17 g/cm\(^3\), in general agreement with the results of other research workers (3, 17). DI LCM virus was more heterogeneous, with peak radioactivity and interference activity banding at a slightly lower density (1.15–1.17) than for S LCM virus. Such a decrease in density is a common feature of DI particles (29). No radioactive peak at the expected virus density was observed when S LCM virus was propagated in the presence of \(^3\)H-thymidine, a DNA precursor, indicating that no significant quantities of DNA were located within the LCM virion. Pedersen (17, 18, 19) has reported LCM virus RNA species of 31S, 28S, 23S, and 18S, as well as some small molecular weight species. Since LCM virus contains ribosomes (6) and since virions purified from cells treated with AD, an inhibitor of DNA-directed RNA synthesis, contained only the 31S and 23S species, Pedersen concluded that the 28S and 18S species were ribosomal. Our studies have confirmed his work and indicate that S LCM virus propagated in the absence of AD can be resolved by gradients into 28S, 23S, and 18S RNA species. Propagation in the presence of AD eliminates the ribosomal RNA species and reveals a 31S peak that is considerably smaller than the 23S peak. The relative peak sizes remained constant with virus harvested 24 h, 48 h, or 72 h after infection. DI particles in most (8) but not all (2) virus systems contain less genetic material than S virus. The incomplete genome is presumably responsible for the defectiveness and for the packaging of lower density particles. Consistent with the concept of reduced nucleic acid (target) size was the fact that interference produced by DI LCM virus was considerably more resistant to neutral red and ultraviolet light inactivation than was the infectivity produced by S LCM virus (32). Our preliminary data, however, clearly demonstrate the presence of
both 31S and 23S as well as ribosomal RNA species in DI LCM virus. The reason for the defectiveness is therefore unclear, but it may become apparent following further qualitative and quantitative resolution of viral RNA species. The defectiveness could possibly be explained if there were more than one class of LCM virus 23S RNA species.

Because it is unclear whether LCM virus is a positive strand RNA virus, in which the RNA is a message and therefore infectious, or a negative strand virus, in which the RNA is complementary to the message (1), we attempted to infect cells with the nucleic acid extracted from purified S LCM virus. Under conditions where mengo virus RNA was successfully transfected, no infectivity was obtained from cultures transfected with nucleic acid from LCM virions. Similar negative results were obtained with nucleic acids extracted from acutely infected L-929 and BHK cells. In the light of recent evidence indicating that some non-oncogenic RNA viruses may become incorporated in cell DNA during conditions of persistent infection (35, 36), we attempted to transfect the nucleic acids from L-929 and BHK cells persistently infected with LCM virus. The target cells failed to produce PFU or antigens of LCM virus detectable by immunofluorescence. Though it appears that LCM virus nucleic acid is not infectious, one should consider that there are several species of LCM virus RNA and that the probability of transfecting cells with all the viral RNA species necessary for infection may be quite low. This interpretation is consistent with the negative results we have obtained in trying to infect cells with NP-40-disrupted LCM virus (nucleocapsid) preparations.

Viral enzymatic activity

(a) RNA-dependent RNA polymerase. The apparent absence of infectious LCM virus RNA and a report of an RNA-dependent RNA polymerase associated with Pichinde virus, which is related to LCM virus (4), suggested that LCM virus might be a negative-strand virus that carries a polymerase into the infected cell in order to initiate transcription. Purified S LCM virus preparations were therefore examined for RNA polymerase activity. Using viral concentrations and assay conditions (see Methods) that reveal enzyme activity in other RNA viruses, we could detect no activity associated with LCM virions. Enzymatically active RNA-RNA polymerase complexes sedimenting at about 200S were, however, isolated from the cytoplasms of BHK cells acutely infected with LCM virus. Polymerase activity detectable in cytoplasmic extracts but not with purified virions has also been shown with rabies, measles, and mumps viruses (28, and unpublished observations). Thus far we have not been able to detect polymerase complexes in BHK cells persistently infected with LCM virus, but this may be related to the observation that persistently infected cells in other virus systems contain lower polymerase levels than acutely infected cultures (28). The data of Carter et al. (4) on Pichinde virus polymerase may be open to question, as (1) the specific activity of their enzyme was very low and (2) a requirement for nucleotides other than UTP was not shown. A poly-U polymerase activity associated with cytoplasmic extracts and ribosomes from uninfected BHK cells (28) may account for that enzyme activity. This could be resolved by determining whether labelled nucleotides other than UTP can be utilized by the Pichinde virus enzyme. GTP is utilized by the LCM virus cytoplasmic polymerase.

(b) Reverse transcriptase (RNA-dependent DNA polymerase). Reverse transcriptase activity associated with Newcastle disease virus particles purified from persistently infected cells has been reported (7). To determine whether LCM virus might also acquire this modification, S and DI virions purified from BHK cells were examined for reverse transcriptase activity and found to be negative.

(c) Protein synthesis. The presence of ribosomes within the LCM virion suggests that disrupted virions could be capable of protein synthesis. Under the conditions tested, however, no protein synthetic activity was detected in the LCM virus preparations. The ribosome number, estimated from the number of ribosomes/virion as seen by electron microscopy, should have exceeded the number required to detect activity, and control BHK cell ribosomes were highly active. Thus, either the ribosomes within the LCM virion were inactive, or the several conditions selected for demonstrating their activity were inappropriate.

Antigenic analysis of S and DI LCM virus

Purified S and DI virions both fix guinea-pig complement after incubation with guinea-pig antibody to LCM virus. Human antibody neutralizes the infectivity of S LCM virus and the interfering capacity of DI LCM virus (32, 34). Injection of mice with DI LCM virus elicits the production of complement-fixing antibody (22) and immunizes the mice against S LCM virus infection (10, 32). Welsh et al. (32)
expressed reservations, suggesting that the immunization might be due to a silent immunizing infection produced by undetectable S LCM virus contamination in the DI LCM virus preparation. This question was resolved by immunizing mice with dilutions of DI virus preparations and with DI virus preparations from which S LCM virus could not be rescued (15, 22). Several investigators have reported that the cytoplasms from cells acutely or persistently infected with LCM virus stains equally well with LCM virus fluorescent antibody (5, 10, 26), but persistently infected cells display reduced cell surface staining (5). Using 125I-labelled antibody to LCM virus we have confirmed this cell surface observation, showing that there was 9 times more antibody binding to acutely infected murine neuroblastoma cells than to persistently infected cells. Persistently infected cells release less complement-fixing antigen and 10–50-fold fewer radioactive counts from RNA-labelled virus than do acutely infected cells. This reduced surface antigenicity may be important in the in vivo persistent infection by reducing the cell susceptibility to immunological attack. Purified S and DI LCM virions can each block antibody binding to the surfaces of both acutely and persistently infected cells. DI viruses in other systems contain most if not all S virus structural proteins (8). Our results are consistent with these findings, as no differences in proteins between S and DI LCM virus have been demonstrated by antigenic analysis.

**Biological properties of DI LCM virus**

(a) **Interference in vitro.** It is now well documented that DI LCM virus inhibits S LCM virus synthesis, infective centre formation, and cytoplastic effect (15, 21, 26, 32, 33, 34), and the cytoplastic interference may be important for the maintenance of persistent in vitro and in vivo infections. The interference can be extended to other arenaviruses (26, 32) but not to unrelated heterologous viruses. DI LCM virus itself has no detectable cytoplastic effect, although neuroblastoma cell dysfunction in terms of reduced levels of neurotransmitter enzymes has been associated with persistent DI LCM virus infection (15). Owing to the rapid generation of DI virus during acute infection, it is difficult to determine whether S LCM virus alone has interfering potential.

(b) **Interference in vivo.** There is suggestive evidence for the generation of DI virus in mice because of demonstrations of interference in vivo. Cells taken from persistently infected mice are resistant to superinfection with another LCM virus strain (27), and mice persistently infected with a small LCM virus plaque variant (strain CA1371) do not produce detectable levels of challenge virus when superinfected with faster growing and more virulent large plaque variants (strains WE and UBC) (30). In our hands, viral isolates from chronically infected mice display autointerference at high concentrations on BHK plaque assay plates, whereas isolates from acutely infected mice (samples taken 3 days after infection) display no such interference. Although previous attempts to demonstrate therapeutic and non-immunizing prophylactic effects for DI LCM virus in vivo have failed (32), our recent experiments indicate that DI LCM virus may have prophylactic value. In order to assure co-infection of the large number of cells in an animal with both virion types, the DI virus was aggregated to S LCM virus by pelleting in an ultracentrifuge (20, 30). Under these conditions C3H/HeJ mice were spared from the normally lethal infection observed with aggregated S LCM virus alone, and young Lewis rats were protected from the expected destruction of the cerebellum (14) normally produced by S LCM virus.

(c) **Defectiveness.** DI LCM virus is capable of being synthesized in the presence of large quantities of S LCM virus during acute infection (34) and continues to be synthesized in persistently infected cultures where S LCM virus cannot be easily detected. These persistently infected cultures are not devoid of S LCM virus genetic information, however, as S LCM virus can be rescued from them under the appropriate conditions (26). Although S LCM virus is rescued with ease in some cultures by passing persistently infected cell culture fluid in uninfected cells, it is not easily rescued in other cultures. Those cultures can be more easily studied to determine whether the interfering virus is indeed defective. Cells exposed to such culture fluids produce no detectable LCM virus antigens and release no detectable interfering component, indicating that the DI virus is truly defective and cannot be synthesized without S LCM virus helper.

**CONCLUSIONS**

Comparisons of the biological and biochemical properties of S and DI LCM virus and virus-infected cells are presented in Tables 1 and 2. At this moment it is uncertain why DI LCM virus is defective, and further resolution of viral RNA and protein species
BIOCHEMICAL AND BIOLOGICAL PROPERTIES OF LCM VIRUS

Table 1. Properties of lymphocytic choriomeningitis virus virions

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Defective</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/cm³)</td>
<td>1.17</td>
<td>1.15-1.17</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Protein synthesis activity</td>
<td>–</td>
<td>no data</td>
</tr>
<tr>
<td>RNA (cellular) species</td>
<td>28S, 18S</td>
<td>28S, 18S</td>
</tr>
<tr>
<td>RNA (viral) species</td>
<td>31S, 23S</td>
<td>31S a, 23S</td>
</tr>
<tr>
<td>DNA</td>
<td>–</td>
<td>no data</td>
</tr>
<tr>
<td>Infectious nucleic acid</td>
<td>–</td>
<td>no data</td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>–</td>
<td>no data</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Infectious nucleocapsid</td>
<td>–</td>
<td>no data</td>
</tr>
<tr>
<td>Ultraviolet light sensitivity</td>
<td>+ (high)</td>
<td>+ (low)</td>
</tr>
<tr>
<td>Neutral red sensitivity</td>
<td>+ (high)</td>
<td>+ (low)</td>
</tr>
<tr>
<td>Cell surface antigen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Complement-fixing antigen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fluorescent antigen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Immunizing antigen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neutralizing antigen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cytolytic potential</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Interfering potential</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Replicating potential</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

* + = positive; – = negative.
* a Preliminary figure.

Table 2. Properties of cells infected with lymphocytic choriomeningitis virus

<table>
<thead>
<tr>
<th></th>
<th>Acute (standard)</th>
<th>Persistent (defective)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytopathic effect</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Infective centres</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cytoplasmic LCM virus antigens</td>
<td>+ (high)</td>
<td>+ (high)</td>
</tr>
<tr>
<td>Cell surface LCM virus antigens</td>
<td>+ (high)</td>
<td>+ (low)</td>
</tr>
<tr>
<td>Infectious nucleic acid</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>RNA-dependent RNA polymerase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Resistance to superinfection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>arenavirus challenge</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>heterologous virus challenge</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

+ = positive; – = negative.
See also footnote to Table 1.

may be necessary to answer this question. The role of DI LCM virus in the persistent murine infection is as yet unclear, but it is tempting to speculate that it moderates the severity of the disease by (1) reducing the cytopathic effect produced directly by S LCM virus, (2) decreasing levels of viral cell surface antigens and the susceptibility of those cells to immunological attack, and (3) reducing the levels of released virus and the resulting damage due to immune complex deposits.

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RÉSUMÉ

COMPARAISON DES PROPRIÉTÉS BIOCHIMIQUES ET BIOLOGIQUES DU VIRUS DE LA CHORIOMÉNINGITE LYMPHOCYTaire STANDARD ET DU VIRUS DÉFECTIF


La centrifugation à l’équilibre en gradient de densité de saccharose montre que la densité du virus DI (1,15 à 1,17) est légèrement inférieure à celle du virus standard (1,17). Les deux types de particules contiennent des ARN de 31S, 28S, 23S et 18S ; toutefois, des expériences avec l’actinomycide D ont montré que les espèces 28S et 18S étaient d’origine cellulaire plutôt que virale. Cette caractérisation de l’ARN du virus standard de la CML est en accord avec les travaux de Pedersen.

Dans les conditions de l’expérience, les acides nucléiques isolés des cellules infectées ou des virions purifiés n’ont pas été trouvés infectants. De plus, les méthodes employées n’ont montré aucune activité d’ARN-polymerase dépendante-ARN, d’ADN-polymerase ARN-
dépendante, ni d’activité de synthèse des protéines, dans les virions. On a toutefois pu mettre en évidence une activité d’ARN-polymérase ARN-dépendante dans des extraits cytoplasmiques de cellules BHK présentant une infection aiguë, mais non dans des extraits des cellules BHK atteintes d’une infection persistante ou des extraits de cellules témoins de la même lignée.

On a réussi à provoquer des infections persistantes à virus CML en cultures de cellules; ces dernières produisaient des quantités notables de virus DI mais on ne pouvait déceler de virus standard. Les cellules atteintes d’une infection aiguë adsorbaient 9 fois plus d’anticorps anti-virus de la CML que les cellules présentant une infection chronique; ces dernières sembleraient donc relativement résistantes à une attaque immunologique.

Il a été démontré que le virus DI de la CML avait un effet protecteur in vivo. En effet, des souris infectées avec un agrégat de virus standard et de virus DI ne présentaient pas la réaction létale suscitée par le virus standard seul, et chez de jeunes rats infectés avec le même agrégat on n’observait pas la détérioration du cervelet que provoque le virus standard seul. Le rôle possible du virus DI dans la choroïdéméningite lymphocytaire chronique de la souris est discuté.

REFERENCES