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Title: Relationship of Oxygen Tension to Viral Disease

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LABORATORY DIAGNOSIS OF LYMPHOCYTIC CHORIOMENINGITIS

As indicated in the initial application, the first goal of this project was to develop a tissue culture system which would be useful for isolation and quantitation of LCM virus. Using a number of different types of cell cultures, culture conditions were manipulated in attempts to induce a direct cytopathic effect by the virus which might be used as an index of infection. These attempts have been based on reports of other investigators regarding similar effects with the same or different viruses as well as leads obtained in our own work. Results obtained to date are categorized below for the most part according to the type of cell used.

1. Chick embryo cells

Based on the report of Benson and Hotchin (Proc. Soc. Exp. Biol. Med. 103: 623, 1960) that chick embryo cells were destroyed by infection with LCM virus, considerable work has been done with this system. However, a later report of Hotchin indicated this effect occurred only with one strain of LCM virus which he subsequently lost. In general, we have not found this system useful for assay of LCM virus either directly or by interference with other viruses.

Since Benson and Hotchin had used chick embryo extract in their work, we have prepared and tested 16 batches of extract for their ability to enhance CPE in chick embryo cultures infected with LCM virus. Of these, two batches have shown some activity. When observed, CPE developed around the ninth day after infection. A commercial lot of extract was also inactive. Extract from 9, 11, and 13 day embryos were tried and none were active. Thus, while there appeared to be some enhancing effect of the extract on development of CPE this could not be related to the method of preparation. Due to the low frequency of batches which were active, this line of work has been dropped.

Inasmuch as the appearance of CPE in cells infected with LCM virus is apparently a marginal phenomenon, numerous changes in culture conditions were made to enhance this effect. Embryos were taken at 9, 10, and 11 days of incubation as a source of cells for cultures. In the series of tests done, CPE did not develop in any. Components of growth and maintenance media were changed for similar reasons. Various combinations of Tris and bicarbonate concentrations have been shown to be of importance (Am. J. Hyg. 73: 36, 1961). Concentrations of calf serum of 20, 10, 5, and 0% in both growth and maintenance medium were tried without effect. Also, concentrations of chick embryo extract of 20, 10, 5, and 0% and in various combinations with the above were used without effect. We have noted repeatedly that cultures of chick embryo cells cultivated in the presence of extract have in the population heart cells which are actively contractile. Infection of the cultures with LCM virus had no effect on the contractions of these cells. Modifications were made in method of trypsinizing tissues for cultivation using minced tissue or tissue forced through a screen both with and without methyl cellulose as a stabilizing agent also without effect. Incubation of infected cells at 41 C, 37 C, or at room temperature was without effect. This was suggested by the work of Tyrrell (Lancet 1: 239, 1960). Likewise rolled tubes had no advantage over stationary tubes as suggested by Holper (J. Inf. Dis. 107: 395, 1960). Effect of changing the medium at the time of infection showed if anything that CPE was more likely to occur in infected cells if the medium was not changed. Furthermore, a difference in pH change between infected and non-infected cells has been noted. Attempts to enhance this pH change as well as the effect of medium change has not provided sufficiently consistent differentiation between infected and non-infected cultures. Omission of glutamine from the maintenance medium has not been beneficial either with or without a medium

change (Proc. Soc. Exp. Biol. Med. 124: 95, 1967). Passage of LCM virus in either chick embryo or HeLa cells has not resulted in any change in ability of the resulting virus population to induce CPE.

As a method for demonstrating LCM virus, we have investigated the ability of this virus to interfere with the CPE induced by other viruses. Infection of chick embryo cells with the more virulent WE strain of LCM virus has not protected the cells from infection with Newcastle disease virus. Neither the WE strain nor the less virulent Armstrong strain of LCM virus protected these cells from infection with vaccinia virus. Furthermore, cells infected with either strain of LCM virus and challenged with vaccinia virus on varying days later produced equal quantities of vaccinia virus as cells not infected with LCM virus. This was true with both primary and secondary chick embryo cultures.

Production of LCM virus by chick cells has been demonstrated for as long as 52 days after infection with no CPE. Virus production was maximum at 4.5 logs on the 11 day after infection, declined to 3.6 to 4.0 logs by the 13 day, and continued to be produced at this same level throughout the 52 day period.

Incubation of infected cells in an atmosphere of 95% nitrogen, 5% carbondioxide or in an atmosphere of 95% oxygen, 5% carbon dioxide had no effect on the cells when compared to conventional cultures (Proc. Soc. Exp. Biol. Med. 117: 567, 1964).

A report by Lukert (Avian Diseases 9: 308, 1965) suggested that cultures from specific organs of the chick embryo might be more susceptible than cultures grown from the whole embryo. Using cultures of lung or kidney from 16 day embryos prepared by Lukert's method, we have not found them to offer any advantage over cultures prepared from whole embryos.

In an attempt to change the metabolism of infected cells we have used pyruvate at 0, 1, 2, 5, and 10 millimolar concentrations in the maintenance medium without effect. Likewise, varying the calcium content from 0 to 10 times the usual amount was not useful (Virology 16: 122, 1962). Thus, our overall experience with chick embryo cells has not been very productive.

2. Rabbit embryo cells

Due to their unique susceptibility to herpes virus, rabbit embryos were taken at 20 days gestation and used as trypsinized monolayers. Although we do not have quantitative results, primary, secondary, and tertiary cultures infected with either the Armstrong or WE strains of LCM virus in the presence of 5 or 10% chick embryo extract and incubated at room temperature, 30 C or 37 C either stationary or rolled did not exhibit CPE during the 20 day observation period. Also, addition of endotoxin at 10 micrograms per ml did not change susceptibility of these cells (Bacterial Endotoxins, by Landy and Braun, 1965, p. 448).

3. Mouse embryo cells

Mouse embryo cell monolayers with tris or bicarbonate buffer at pH 6.9 of 7.5 and maintained in 5 or 10% chick embryo extract, 5 or 10% ascitic fluid, or 5 or 10% amniotic fluid after infection with either strain of LCM virus exhibited no CPE.

4. Monkey kidney cells

Investigators reporting that monkey kidney cells are susceptible to LCM virus have apparently not utilized chick embryo extract in the test system. Based on our original premise that embryo extract would enhance CPE, we have devoted considerable time to this system. On testing the same 16 lots of extract in addition to a commercial lot we have found the same two lots which were active in chick embryo cells also active to a limited extent

in monkey kidney cells. One of these batches when used at a 10% concentration in the maintenance medium resulted in CPE on two occasions. Again there appears to be some limited effect of added extract. However, due to the generally poor response of most batches, this approach has been discontinued. Various combinations of bovine embryo extract, ascitic fluid, and gamma globulin-free serum with extract have appeared promising at times but we have been unable to repeat the results. Use of 5, 10, 15, and 20% chick embryo extract with tris or bicarbonate buffers at pH 7.4 or 8.0 resulted in no CPE in the 10 day observation period. Plaque experiments using 0.5% extract were unsuccessful.

Barlow (Bacterial Endotoxins, by Landy and Braun, 1965, p. 448) reported that mice infected with LCM virus were more susceptible to bacterial endotoxins than normal mice. Based on this information we added 5 to 50 micrograms of endotoxin to cultures one to five days before infection with LCM virus with no apparent effect by 16 days after infection.

From the reported effects of phytohemagglutinin and cortisone on the CPE and yield of some viruses, we have investigated the effect of these compounds in this system. Cells were planted in medium containing 10% calf serum with 1% phytohemagglutinin, 10% chick embryo extract, 2.5 micrograms per ml cortisone or calf serum only and infected when confluent. No CPE resulted in any cultures.

5. Mouse peritoneal macrophages

Both the Armstrong and WE strains of LCM virus were replicated in cultures of both adult and suckling mouse peritoneal macrophages. However, a cytopathic effect (CPE) was never observed in these cultures infected with LCM virus. Addition of phytohemagglutinin (PHA) to cultures of mouse macrophages resulted in a delay of maximum virus yields in both adult and

suckling mouse macrophages. Addition of cortisone had no effect on the yield of LCM virus in macrophage culture. Serial passage of LCM virus did not increase its virulence for mice or its cytopathogenic effect for mouse macrophage cultures.

Viral replication or demonstration of CPE could not be detected in either adult or suckling mouse peritoneal macrophage cultures infected with either herpes simplex virus or vaccinia virus.

Newcastle disease virus (NDV) caused complete CPE within 5 to 6 days in both adult and suckling mouse peritoneal macrophage cultures. However, only small amounts of infectious virus could be recovered from these infected cultures. Serial passage of virus using either infected culture fluids or viable infected cells was unsuccessful. Ultraviolet irradiated NDV, which was non-infectious, still caused CPE in macrophage cultures. Thus, a cytotoxic effect was suspected.

Disruption of the NDV virus particle by ether treatment resulted in fractions consisting of hemagglutinin and nucleoprotein antigens. Following the inoculation of these individual fractions into mouse macrophage cultures, it was found that the toxic substance was associated with the nucleoprotein fraction, which was toxic for cultures of mouse peritoneal macrophages, was not toxic for chick embryo tissue culture cells, primary monkey kidney cells or baby hamster kidney cells (BHK-21, clone 13).

6. BHK-21, clone 13 cells

From our own work as well as from reports in the literature we have noted a number of similarities in the biological properties of rubella and LCM viruses. Due to the usefulness of the BHK-21, clone 13 cell line for the study of rubella virus, we have studied some of the basic aspects of LCM virus replication in this cell line.

For the purpose of estimating the quantity of intracellular virus, methods of extraction of LCM virus from infected BHK cells were compared. In this and other experiments, virus was quantitated by intracerebral injection of mice. Best yields of virus from infected cells was achieved after adding tryptose phosphate broth (TPB) to the washed culture followed by two cycles of freezing and thawing. Extraction of cell-associated virus with borate buffer at pH 9.0 as suggested by Hallauer and Kronauer (Arch. Ges. Virusforsch. 15: 433, 1965) or freezing and thawing the cells covered with borate buffer released approximately one-tenth the amount of virus recovered on TPB. However, based on studies on stability of virus a part of this difference has likely resulted from a decreased stability of infectious virus in the borate buffer.

In order to arrive at a better estimate of viral adsorption to BHK-21 cells, the thermal stability of crude LCM virus in cell culture fluids at 37 C was assayed. Based on these experiments, we estimate the half-life of the Armstrong strain to be on the order of 115 minutes, whereas the half-life of the WE strain was approximately 90 minutes. Compensating for thermal inactivation of virus, the adsorption rate of the two strains of virus to BHK-21 cells was similar. One hour after addition of the Armstrong strain virus to cell cultures, 78% of the virus had disappeared from the medium.

Of this amount, 47% had adsorbed to the cells and 31% had become inactivated. For the WE strain, 83% had disappeared within an hour of which 45% had adsorbed and 38% was lost due to thermal inactivation. During the second hour, most of the remaining virus was lost due to thermal inactivation with only a small proportion adsorbing to the cells.

Studies of virus replication in BHK-21 cells with addition of Armstrong strain virus at varying multiplicities are summarized below.

<u>Multiplicity of infection</u>	<u>Virus first detectable (hours after infection)</u>		<u>Extracellular virus titer after 70 hours (Log₁₀ LD₅₀ per 0.03 ml)</u>
	<u>Intracellular</u>	<u>Extracellular</u>	
1.0	12	13	5.2
0.1	12	15	5.1
0.01	16	18	2.4
0.001	19	19	1.6

After appearance of virus in infected cultures, the amount of virus recovered intracellularly is practically the same as the amount free in the culture fluids. This was found to be true with both strains of virus. In addition, peak production of both virus strains occurred after three to four days following which yields decreased.

7. Complement fixing antigen in BHK-21 cell cultures

Reference has been made in the literature to the appearance of complement fixing antigen appearing in cell cultures infected with LCM virus. However, cells other than BHK-21 were used. We have demonstrated that a complement fixing antigen is produced in BHK-21 cells, and that it occurs in a quantity and quality comparable to the antigen prepared from spleens of infected guinea pigs as routinely prepared by the National Communicable Disease Center. In our opinion, antigen prepared in BHK-21 cells would be less expensive and constitute less of a hazard than antigen prepared from guinea pig spleen.

Studies have been made on the time and amount of antigen produced as well as methodology for extracting cell-associated antigen. The antigen appeared in the culture fluids on the third day after infection and reached a maximum titer of 1:8 on the fifth day. Extraction of antigen from infected cells with borate buffer at pH 9.0 both with and without freezing

the cell sheet was less effective in recovering antigen than freezing with tryptose phosphate broth (TPB). Using two freeze thaw cycles with TPB, antigen was recovered with titers of 1:16 on days 3, 4, and 5. Furthermore, volumes of TPB equal to the original volume of medium were used. By reducing this volume, it should be possible to produce antigen of higher potency. Stability of the antigen in borate buffer appears to be less than in TPB which may account for part of this effect.

Using the appearance of complement fixing antigen as an index of infection of BHK-21 cells, we have compared infectivity of both strains of LCM virus in cell cultures with infectivity by intracerebral injection of mice. Based on these results, the cell culture procedure seems to be practical, but titers by this method were approximately one log lower than determined by injection of mice.

Previous reports concerning the complement fixing antigen in spleens of infected guinea pigs indicated the antigen was separate from the virus itself. By ultracentrifugation we have been able to sediment most of the virus while most of the antigen activity remained in the supernate. By fluorocarbon extraction, the antigen was removed with little effect on the level of infectious virus.

We have also compared complement fixing antibody titers in a number of sera using both BHK-21 cell culture antigen and NCDC guinea pig spleen antigen. Sera of guinea pig, mouse, and human origin, both normal and immune, were used. Sera which were non-reactive with one antigen were non-reactive with both. Furthermore, titers determined with the cell culture antigen were as high or higher than titers for the same sera determined with the NCDC antigen. Based on these results, we feel the cell culture antigen is at least as sensitive and specific as the NCDC antigen.

8. Development of CPE in BHK cells

In our early experiments with BHK cell cultures we found the cultures to be contaminated with Mycoplasma orale type 1. These cultures also infected with LCM virus did develop CPE in approximately 9 days. However, using non-contaminated cultures, LCM virus has consistently failed to develop CPE. Inasmuch as this Mycoplasma produces an arginase, attempts were made to reproduce the effect in non-contaminated cultures by omitting arginine from the medium, adding ammonia, urea, and semicarbazide in the presence and absence of arginine in neutral, high, and low pH maintenance medium all without success. Addition of vitamin A which reduces the stability of lysosomes had some activity, but was not sufficiently reproducible. Addition of DEAE dextran at non-toxic levels also was without effect.

9. Development of CPE in VERO cells

Work along the same lines was recently begun using VERO cell cultures. By lowering the pH of the medium and adding ammonium chloride to the medium, definite CPE developed in cultures infected with LCM virus on one occasion. This is currently being repeated.

10. Effect of serum on LCM virus in BHK cells

Inasmuch as the species of serum used for cultivation and maintenance of cell cultures has sometimes influenced the outcome of viral infection, we have compared cell growth and virus yield in BHK cells cultivated in and maintained in fetal bovine, calf, bovine, and horse serum. Fetal bovine serum (FBS) has been used routinely for culturing and maintaining BHK cultures, and was used as a reference system. On first passage in serum other than FBS, the population doubling time was prolonged. However, by the third passage in any serum, the doubling time was the same in all cultures.

LCM virus yield was consistently higher in cells grown in and maintained in FCS than in cells grown in other serums. Determination of virus yield per unit volume of culture fluid was considerably more variable than the virus yield per cell. Therefore, virus yields per cell were determined for this portion of the work. The type of serum used in culture medium had no effect on the percent of cells in a culture which were infected. No more than 60% of the cells were found to be infected at any time. Actinomycin D at concentrations of 5 and 10 ng/ml of culture medium stimulated production of LCM virus. However, higher concentrations of actinomycin D were inhibitory. The type of serum used in the culture medium had little influence on this effect. Using FBS in the medium, infection of BHK cells with the same dose of LCM virus cultivated either in mouse brain or BHK cell cultures resulted in similar yields of virus. These findings were similar using both the Armstrong and the WE strains of LCM virus.

11. LCM virus hemagglutinin

A hemagglutinin (HA) was found in rather low concentration in culture fluids of both ^{LCM virus infected} BHK and monkey kidney cell cultures but not in fluids of noninfected cultures. The HA has been recovered in maximum titers of 1:16 provided that bovine serum albumin was used in the culture fluid instead of serum. HA was also present within infected cells, but yields following extraction by freezing the cells have been low. The HA was most active when the test was incubated at 4 C, when 0.1 M calcium chloride was present in the diluent, and when one-day-old chicken red cells were used for testing. This reaction was inhibited by LCM immune guinea pig serum but not by normal guinea pig serum. Serum inhibitors were removed by treatment with manganese and heparin.

12. Inhibitor in tissues of mice infected with LCM virus

Early in our tissue culture studies we had attempted to develop a procedure for assay of LCM virus based on the ability of LCM virus to interfere with the development of CPE induced by another unrelated virus. Such a procedure has been used routinely in a number of laboratories for assay of rubella virus in monkey kidney cell cultures. However, it soon became apparent that this sort of procedure was not applicable for assay of LCM virus. Using brains of mice infected with the Armstrong strain of LCM virus, interference was induced. On the other hand, neither the WE strain LCM virus from mouse brain or cell cultures nor the Armstrong strain virus from cell cultures would induce such an interfering effect. It has evolved that infection of mice with the Armstrong strain LCM virus causes the mice to produce a substance which is inhibitory to heterologous viruses. Because the inhibiting substance was unique in many ways, we have continued our work to characterize its biological activity.

The inhibitor has been assayed in most instances by its ability to inhibit production of CPE in monkey kidney cells by poliovirus. This combination of agents has proved to be useful since, as we have shown, it was possible to assay each virus when both were present as a mixture. In such a mixture, LCM virus was assayed by intracerebral injection of mice and poliovirus was assayed in monkey kidney cell cultures. Poliovirus has not interfered with the infectivity of LCM virus in mice, nor has LCM virus even with inhibitor interfered with poliovirus assays in cell cultures when both were added simultaneously. However, when poliovirus was added after the Armstrong strain LCM virus mouse brain suspension, an inhibitory effect was apparent. Based on this observation, we have shown that the

effect was first observed when poliovirus was added three days after addition of mouse brain preparation. This effect was maximal when cells were challenged on the fifth day and declined thereafter. On comparison with control cultures infected with poliovirus alone, the inhibitor reduced both the CPE caused by poliovirus as well as the amount of poliovirus produced. Furthermore, we have shown the inhibitor has the same effect on the CPE and yield of vesicular stomatitis virus.

A number of other aspects of the inhibitor have been investigated and are summarized here. Using dilutions of mouse brain preparations, we have found that an equivalent of 100 LD₅₀ of LCM virus (0.1 ml of a 10^{-5.02} dilution) but not one-tenth this amount was sufficient to inhibit synthesis of poliovirus. Using similar procedures, the mouse brain material inhibited six serotypes of coxsackie virus, ECHO-11 virus, and influenza virus but not vaccinia or reovirus type 1 under the same test conditions. The ability of the inhibitor to suppress poliovirus in HeLa cells was similar to the effect observed in monkey kidney cells, but quantitatively less dramatic. One unusual feature of this inhibitor is that replacing the culture medium at the time of addition of the challenge virus eliminates the inhibitory effect. This effect is unlike that of classical interferons. Using vesicular stomatitis virus for challenge, the inhibitor was demonstrable in monkey kidney, HeLa, and mouse embryo cultures, but not in chick embryo, L-929, or BHK-21 cultures. After infection of mice with the Armstrong strain LCM virus, inhibitor was demonstrable in brain, kidney, spleen, and serum but not in liver. All of these materials contained infectious virus. In keeping with other characteristics of interferon, antiserum to LCM virus had no effect on inhibitor activity.