Further Studies on the Replication of the Lactate Dehydrogenase-Elevating Virus (LDH Virus) in Mouse Peritoneal Macrophage Cultures (34574)

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(Introduced by Jørgen Fogh)
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Evidence suggesting that macrophages are the site of replication of the lactate dehydrogenase-elevating virus (LDH virus) comes mainly from tissue culture (1–5) and electron microscopic studies (1, 6, 7). Peritoneal macrophages are of particular interest in that they have shown to support LDH virus replication *in vitro* to high levels (4) comparable to those reached in the infected mouse during the first few days of infection (8). However, the level of virus infectivity in macrophage cultures was shown to fall after 3 days and to be immeasurable by 3 weeks, and such cultures could not be reinfected with LDH virus (4). The virus titer also declines in the infected mouse after about 3 days but a stable level of viremia is established and retained throughout life (8). In view of their response to LDH virus inoculation, peritoneal macrophage cultures were the subject of further studies to provide more data on their relationship with the LDH virus.

Materials and Methods. Animals. Mice, 4–6 weeks old, of randomly bred Parkes strain were used as a source of peritoneal macrophages and for LDH virus infectivity titrations. Swiss ICR/Ha mice were used during interferon studies.

Media. In most experiments growth medium (GM) consisted of Eagle's basal medium supplemented with 10 % calf serum. For interferon experiments, medium 512 (9) supplemented with 10 % fetal calf serum was used.

Peritoneal macrophage cultures. Their preparation was described previously (3). Each culture was prepared from an individual mouse without prior stimulation with irritants. Five milliliters of GM with approximately 1.5×10^6 macrophages were seeded into 50-ml bottles. For routine purposes, cultures were thoroughly washed with phosphate-buffered saline (PBS) 30 min after cultivation to remove floating debris and cells, particularly lymphocytes.

Peritoneal lymphocyte cultures. The entire peritoneal cell population was seeded into 50-ml bottles and left to settle for 15 min at room temperature, after which the bottles were gently agitated, and the culture medium was poured into clean bottles. The cells were again allowed to settle for 15 min and the procedure was repeated.

Up to six transfers were made before the lymphocyte population was about 99 % pure. The final cell suspension was centrifuged at 500 rpm for 5 min and resuspended at a concentration of 300,000 cells/ml. Five milliliters of this were seeded into 50-ml culture bottles.

Viruses. LDH virus. Stock virus was prepared from infected mouse plasma as described by Mahy *et al.* (10) and stored at −20°. Infectivity titers were calculated by the method of Thompson (11) and expressed as the dose which infected 50 % of the mice (ID_{50}/ml). Two mice each were injected intraperitoneally with 1 ml of 10-fold serial dilutions. The stock virus had a titer of $10^{7.0}$ ID_{50}/ml and was diluted 1/10 in GM for all experiments. Unless otherwise stated, a standard dose of $10^{6.0}$ ID_{50}/ml was inoculated into cultures.

Vaccinia virus. This was obtained by growing freeze-dried chick embryo-passaged

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smallpox vaccine in mouse embryo subcultures. After two passages, cells were homogenized and debris was deposited by centrifugation. The supernatant fluid was stored at —20° in 1-ml aliquots containing approximately 10^5 plaque-forming units (pfu)/ml on mouse embryo subcultures.

Vesicular stomatitis virus. Stock virus was prepared by passage of virus in mouse embryo cultures. One-milliliter aliquots contained approximately 10^4 tissue culture doses (TCD), which produced gross cytopathic effects in unprotected mouse embryo cultures within 24 hr.

Assay for interferon or viral interference. Two methods were used: The first method used was described by Lindemann and Gifford (12). Tissue culture fluids from LDH virus-infected and uninfected control cultures were diluted ½ in serum-free culture medium and mixed with approximately 100 pfu of vaccinia virus. Three milliliters were added to 5-cm plastic dishes containing monolayers of subcultured mouse embryo cells and left for 3 days. Sheets were stained with 0.1 % crystal violet for 2 min, washed, and scored for plaques. The second method, described elsewhere (14), assayed the protective effect of culture fluids from LDH virus-infected and uninfected cultures in mouse embryo subcultures when the latter were challenged with 10^4 TCD of vesicular stomatitis virus. No attempt was made to purify the culture fluids or to remove LDH virus, except where specified.

Actinomycin D. The antimetabolite (LYO Meractinomycin, Merck, Sharp and Dohme, Research Laboratories, West Point, Pennsylvania) was used at a subtoxic concentration of 0.01 μg/ml of culture medium.

Results. LDH virus in peritoneal macrophages. It was shown previously (4) that when the medium was replaced at twice-weekly intervals, virus infectivity was lost from cultures within 21 days. Recently, Du Buy and Johnson (2) reported that more frequent changes resulted in prolonged infectivity in their cultures. In view of this, the following experiments are reported. Twenty-four hours after their preparation, cultures

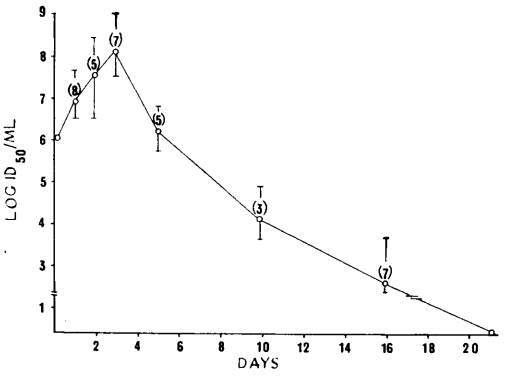

FIG. 1. Replication of LDH virus in 1-day-old peritoneal macrophage cultures. Each point represents the mean of a number of experiments shown in brackets.

were inoculated with a standard dose of virus, $10^{6.0}$ ID_{50}/ml of medium. The culture medium was completely replaced daily for the first 72 hr and thereafter every 48 hr and titrated for LDH-virus infectivity. The results of a number of experiments are shown in Fig. 1. Three points only are shown after Day 5. It is seen that after frequent changes of medium, infectivity was not prolonged beyond 21 days. The cells at this stage were viable as shown by the dye-exclusion test with trypan blue.

Loss of virus infectivity by peritoneal macrophage cultures. Since the response to infection of macrophage cultures was fairly consistent and reproducible, the above system was used to study the possible involvement of a number of factors which might affect LDH-virus replication *in vitro*.

(a) *The role of peritoneal lymphocytes in the replicative process.* These invariably contaminate peritoneal macrophage cultures for the first 2 or 3 days. They do not survive for long, however, and do not adhere strongly to glass; thus it was of interest also to see if their loss from cultures related to the decline in virus titer seen after 3 days. Lymphocyte cultures were prepared as described in Methods. Cells from the second, third, and sixth stages of purification were used, and their response to virus inoculation was compared with that of peritoneal macrophages, which were obtained from the original peritoneal exudate population after the lymphocytes

TABLE I. Relationship Between Lymphocyte and Macrophage Content of Cultures and LDH Virus Replication.

Culture	% Lymphocytes	% Macrophages	LDH virus titer[a] (log ID_{50}/ml)
Lymphocyte	100	0	<0.5
Lymphocyte	90	10	1.5
Lymphocyte	70	30	3.5
Macrophage	0	100	7.5
Mixed	50	50	7.5

[a] Twenty-four hours after inoculation of cultures.

had been removed. To show that there was no synergistic effect on replication in the presence of both cell populations, whole peritoneal exudates were also seeded into culture bottles. All cultures were inoculated with a standard dose of virus 2 hr after their preparation. Twenty-four hours later culture fluids were titrated for virus infectivity. Table I shows the response of the three types of cultures. The level of infectivity at 24 hr apparently related to the varying macrophage content of the lymphocyte cultures, and relatively pure lymphocyte cultures were not infective at all at this time.

To show conclusively that lymphocytes were not required for the replicative process, macrophage cultures were prepared from the peritoneal exudates of mice which had received 600 R whole body X-irradiation 3 hr previously (this level of irradiation significantly reduced the number of circulating lymphocytes, and peritoneal lymphocytes from the mice did not survive long *in vitro*). Control cultures were prepared from normal unirradiated mice. Both sets of cultures were thoroughly washed with PBS 30 min after preparation. At this stage none of the cultures contained more than 2 % lymphocytes. Twenty-four hours later lymphocytes could not be detected in cultures prepared from irradiated mice, and after three further washes lymphocytes could not be detected in cultures from unirradiated mice. All cultures were inoculated with a standard dose of virus and sampled at intervals (Table II). It is seen that cultures from both normal and irradiated mice supported replication during the short period of observation. From these and the foregoing data, it is evident that loss of lymphocytes did not affect the level of virus replication.

(b) *Interferon production* in vitro. Although standard techniques have not detected interferon production by LDH-virus *in vitro* (1), it was a possibility that immeasurable quantities of interferon were produced at a level which might exert an antiviral effect. If this were blocked, the replication of LDH virus might be enhanced or prolonged. To block interferon production, or any DNA-dependent synthesis of protein inhibitors, peritoneal macrophage cultures were treated with the antimetabolite, actinomycin D.

Macrophage cultures were divided into two groups. The first was inoculated with a standard dose of LDH virus, and the medium was tested at intervals for interference potential by inhibition of vaccinia virus plaque formation or protection against vesicular stomatitis virus. No attempt was made to purify the samples, though any plaque inhibition or protection against cytopathic affects would have necessitated this. As a positive interferon control, plasma from mice infected with LDH virus 20 hr previously was treated with 3 *N* HCl for 6 days and ultracentrifuged to yield a preparation with the properties of interferon (14). In the second group, virus was inoculated either at the same time as actinomycin (0.01 μg/ml of culture fluid) or 4 hr and 24 hr after addition of the antimetabolite. Culture fluids were sampled at intervals and titrated for virus infectivity.

TABLE II. LDH Virus Replication in Peritoneal Macrophage Removed from X-Irradiated Mice.[a]

Cultures	LDH virus titer (log ID_{50}/ml)[b] 24 hr	48 hr
Nonirradiated cells	7.0	8.0
X-irradiated cells	6.5	7.0

[a] Parkes mice received 660 R whole body irradiation 3 hr before removal of peritoneal macrophages.

[b] Cultures inoculated with 10^6 ID_{50}/ml.

TABLE III. Effect of Actinomycin D on LDH Virus Replication in Peritoneal Macrophages.[a]

Actinomycin D administered	LDH virus titer[b] (log ID_{50}/ml)		
	Days		
	1	3	5
24 hr before virus	7.0	8.5	6.0
4 hr before virus	6.5	7.5	6.5
Same time as virus	7.0	8.0	5.5
Control (not treated)	6.5	8.0	6.0

[a] Actinomycin D (0.01 μg/ml culture fluid).
[b] Culture inoculated with 10^6 ID_{50}/ml.

In the first group there was no evidence of interferon production, or of interference by LDH virus when compared with the positive interferon control plasma, which produced approximately 95 % plaque reduction at a 1/10 dilution, and complete protection against vesicular stomatitis virus. In the second group (Table III) there was no significant difference in the replication of LDH virus in treated and control cultures. It is concluded, therefore, that interferon or protein inhibitors were not produced after LDH virus infection of peritoneal macrophage cultures. The failure to demonstrate inhibition of LDH virus replication by Actinomycin D would indicate that LDH virus, which is reported to be an RNA virus (15), is not dependent on DNA-directed RNA synthesis.

To show that the dose of Actinomycin D was potentially effective in inhibiting DNA-directed protein synthesis the same dose was added to mouse embryo subcultures 4 hr before challenge with 100 pfu vaccinia virus. Three days later the plaques were counted. There was a 50 % reduction compared with the controls ($p < 0.01$).

Inhibition of LDH virus replication in macrophage cultures. As reported previously (14) interferon stimulated in mice after injection of LDH virus significantly reduced the level of replication in primary mouse embryo cultures. To show that interferon could also inhibit LDH virus growth in peritoneal macrophages, cultures were incubated for 24 hr with a 1/5 dilution of interferon, prepared from LDH virus-infected mouse serum and partially purified as described previously (14). Control cultures were incubated with normal mouse serum treated similarly. A standard dose of virus was then inoculated into the cultures, which were incubated for a further 1 hr and then washed twice with GM. Finally 5 ml of GM were pipetted into each culture bottle. At intervals the level of virus infectivity in the culture fluids was titrated. Table IV shows that over a period of 6 days there was a significant inhibition of LDH virus replication in macrophage cultures treated with the interferon preparation.

LDH virus dose response of peritoneal macrophage cultures. It has been shown that inoculation of cultures with multiplicities of virus greater than 1, did not increase the yield of virus (13), though peak virus titers were usually reached more quickly. Similar findings were reported by Du Buy and Johnson (2). To demonstrate the sensitivity of macrophages to various multiplicities of virus below 1, cultures were inoculated with 5 ml of varying dilutions of stock virus, from 10^{-1} to 10^{-5}. At the same time two mice were injected with 1 ml of each dilution. At intervals the culture medium was titrated for infectivity. Figure 2 indicates that with decreasing dose of virus cultures took longer to reach peak titers, and that at $10^{2.0}$ ID_{50}/ml and less, cultures lost infectivity within 2 days. Mice, however, were infected by 10^1 and 10^2 ID_{50}/ml. Thus under these conditions the dose required to infect macrophage cultures was as least 100 times greater than that required to infect the intact mouse.

TABLE IV. Inhibition of LDH Virus Replication in Peritoneal Macrophage Cultures Pretreated with Interferon.[a]

Cultures	LDH virus titer[b] (log ID_{50}/ml)		
	Days		
	2	3	6
Interferon-treated	1.5	2.0	1.5
Control serum-treated	7.5	7.5	5.5

[a] Twenty per cent dilution of partially purified LDH virus-infected serum.
[b] Cultures inoculated with 10^6 ID_{50}/ml.

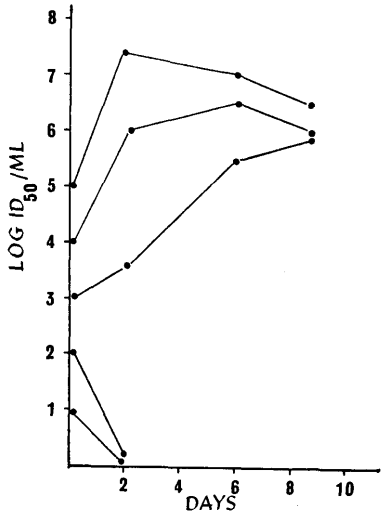

FIG. 2. Replication of LDH virus in 1-day-old peritoneal macrophage cultures after inoculation with various doses of virus (10^1–10^5 ID_{50}/ml). Each point on the abscissa indicates the dose of virus inoculated into each culture.

Discussion. The data from Fig. 1 demonstrate that within certain limits the response of peritoneal macrophage cultures to inoculation of a standard dose of LDH virus was consistently reproducible. That this response was not related to lymphocyte content was demonstrated and agrees with the *in vivo* results of Du Buy and Johnson (1) who showed that there was no change of virus titer in the blood of mice which had received whole body irradiation, when 99 % of the circulating lymphocytes were killed. Therefore, the disappearance of lymphocytes from macrophage cultures could not account for the decline of virus titer seen after 3 days, and neither could interferon production since no evidence for this could be found. Furthermore, the decline of virus titer below ID_{50} over the next 18 days could not be explained in terms of a sudden loss of susceptible or virus-producing cells, since it was shown previously that in nonsusceptible macrophage cultures the infectivity of the same dose of virus was lost within 6 days (4). In addition, when macrophage cultures were infected on various days after preparation, loss of susceptibility appeared to be a gradual process, not involving loss or death of cells as shown by trypan blue exclusion. Thus, cultures inoculated 8 days after preparation produced less virus than those inoculated on Day 1 (3), and 4-week-old cultures were completely insusceptible (4). Figure 2 would support the view that virus-producing cells persist for at least 10 days. However, the inability to infect cultures with doses less than 10^3 ID_{50}/ml suggests that only a small proportion of the macrophage population is susceptible to infection with the virus. In support of this is evidence from electron microscopic studies on cultures of peritoneal macrophages infected 3 days previously with a standard dose of virus (7). Although there were at least 10^8 ID_{50}/ml in the homogenates of the cell suspensions, when the cells were fixed and examined by electron microscopy very few cells in each section (less than 1 %) contained demonstrable virus particles, and these could not be positively identified as replicated, as opposed to phagocytosed virus. It appears, therefore, that there is only a small proportion of susceptible cells found in peritoneal macrophage cultures 1 day after seeding. These may be exhausted fairly rapidly by high multiplicities but more slowly by low multiplicities of virus, and this, together with the age-dependent susceptibility of macrophage cultures to virus infection, provides a possible explanation for the decline in the virus infectivity *in vitro*.

The apparent discrepancy between the above results and those of Du Buy and Johnson (1, 2), who reported that 12-day-old cultures supported LDH virus replication better than 3-day old, and that infectivity could be maintained for longer than 21 days, cannot be simply explained. However, these authors used macrophages from mice stimulated by tripticase soy broth, a procedure which increased the macrophage content of the peritoneal cavity by a mass migration of monocytes from the blood, as demonstrated by Van Furth and Cohn (16). It is possible that artificial stimulation affects the LDH virus susceptibility of cultures of such cell populations and might account for the difference in results.

Although the results from tissue culture

studies suggest that macrophages might be involved in the replicative process *in vivo* there is no direct evidence that the mouse reticuloendothelial system (RES) or any specific compartment of it, supports replication of the LDH virus. Electron microscopy has revealed virus particles in peritoneal (6), lymph node, and splenic macrophages (1) taken from infected mice, and Porter, Porter, and Deerhake (17) have recently demonstrated by immunofluorescence the presence *in vivo* of LDH virus antigen in spleen and liver macrophages. In addition, *in vivo* experiments have demonstrated that, after infection, a complex relationship is established between the virus, RES activity, and the level of certain enzymes, notably lactate dehydrogenase (8, 10, 15, 18). Direct evidence is now required to show whether the peritoneal macrophage is one of a number of sites for LDH virus replication *in vivo*.

Summary. The response of 1-day-old peritoneal macrophage cultures to inoculation with a standard dose of LDH virus, 10^6 ID_{50}/ml, was consistent in all experiments; peak virus infectivity up to 10^9 ID_{50}/ml was reached by 3 days followed by a decline to below the ID_{50} within 21 days. Cultures were not infected by doses of virus containing less than 10^3 ID_{50}/ml. Since lymphocytes were shown not to be involved in the replicative process, and interferon could not be detected in the supernatant fluids of infected cultures, it is suggested that the decline in virus infectivity might be due to the combined effect of the exhaustion of a small number of susceptible cells and an age-dependent decrease in susceptibility of macrophage cultures.

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