# STUDIES ON THE MECHANISM OF ACTION OF RILEY VIRUS

## III. Replication of Riley's Plasma Enzyme-Elevating Virus in Vitro

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(Received for publication, June 28, 1965)

The first report of replication of Riley's plasma enzyme-elevating virus (Riley virus) in tissue culture was by Yaffe (1), 2 years after the discovery of the virus in 1960 (2). Yaffe found that Riley virus multiplied in primary mouse embryo cultures until the death of the cultures through overcrowding (3 to 4 weeks), without producing cytopathic or other detectable changes, and could be serially passaged indefinitely by transference of supernatant fluid at weekly intervals to fresh primary cultures. However, when an infected culture was serially trypsinized and replated, infective Riley virus was lost after the 2nd passage, and the passaged cultures were insusceptible to a 2nd infection. Similarly, passaged uninfected cultures also became insusceptible to infection. Subsequently, preliminary experiments were reported by Plagemann *et al.* (3) which suggested that replication of Riley virus occurred in cultures of mouse embryo, spleen, lung, and kidney. No detailed account of this work has been published. Georgii *et al.* (4, 5) failed to propagate Riley virus in primary mouse embryo cultures for longer than 14 days, and concluded that Riley virus merely survived without replicating in such cultures. Their results were in marked contrast to those of Yaffe (1). As they explained, two possible reasons for this might be considered: differences of method, or the existence of various plasma enzyme–elevating viruses differing in their biological properties. Anderson *et al.* (6) have recently reported propagation of Riley virus in primary mouse embryo cultures for as long as 88 days.

In this laboratory evidence has been obtained of true replication of Riley virus in primary mouse embryo cultures, but only for 8 to 12 days. Yaffe's observation that replated cultures would not support replication was confirmed. Similar temporary growth occurred in primary mouse embryo liver, and adult spleen, but not in Hela, Rhesus monkey kidney, or rat peritoneal macrophage cultures.

An hypothesis which would explain the limitation of growth of the virus in

mouse tissue cultures is that multiplication takes place exclusively in a type, or types, of cells which have only a limited life under the cultural condition used. In order to test this hypothesis cultures of various types of mouse tissue have been infected with Riley virus. An account of preliminary experiments has been published (7).

Researches in this and other laboratories have suggested that Riley virus affects the phagocytic activity of cells of the reticuloendothelial system (RES) (8 to 11). Recent results in this field are reported in accompanying papers (12, 13). For this reason a study was made of the growth of Riley virus in cultures of mouse macrophages. It has been shown that Riley virus will proliferate in cultures of mouse macrophages as long as the cultures are kept alive, and that the virus can be maintained by serial twice weekly passage to fresh macrophage cultures for an indefinite period without loss of titre.

In the present paper these experiments are fully described and further results reported.

## *Materials and Methods*

*Animals.*—The mice used were albinos of the randomly bred Parkes or ICI/SPF strains, or inbred BALB/c strain.

*Mouse Embryo cultures.*—These were prepared from 16- to 19-day-old embryos. There was no apparent difference between the strains used in the response of cultures to Riley virus infection. Embryos were minced, and trypsinized in 0.25 per cent trypsin (Difco 1:250), pH 7.6 at 36.5°C for 1½ hours. Cell suspensions were spun at 2000 RPM for 15 minutes, and the cells were resuspended in Eagle's basal medium (1959) supplemented with 5 to 10 per cent lamb serum and antibiotics (growth medium). Approximately $4.8 \times 10^6$ cells/5 ml were placed in 50 ml medicine bottles, and incubated at 36.5°C.

*Mouse Embryo Liver and Adult Mouse Spleen Cultures.*—These were prepared in the same manner as mouse embryo cultures.

*Adult Mouse Peritoneal Exudate Cultures.*—2 ml of Dulbecco's phosphate-buffered saline (P.B.S.) without Ca or Mg ions, containing 1 unit of heparin per ml, were injected intraperitoneally into adult mice, which were killed immediately afterwards. The abdomen was massaged and the exudate pipetted off. Approximately $3 \times 10^6$ cells were obtained from each mouse, seeded into 50 ml bottles in growth medium and incubated at 36.5°C. 30 minutes later the supernatants were decanted to remove suspended cells, mainly lymphocytes, and replaced by fresh growth medium. It was not possible to maintain peritoneal cultures (macrophage cultures) in a healthy state for longer than 2 to 3 weeks.

*Hela Cells and Rhesus Monkey Kidney Cells.*—Cells were kindly supplied by the Bacteriology Department of the London Hospital, and were cultivated as described for mouse embryo cultures.

*Adult Rat Peritoneal Cultures.*—Prepared and cultured in the same way as mouse macrophage cultures.

*Riley Virus.*—The preparation used was the same as that described previously (12). The dose which infected 50 per cent of the mice ($ID_{50}$) was calculated by the method of Thompson (14) and expressed on the basis of 1.0 ml of material. The stock virus preparation had an infectivity titre of $10^{7.0} ID_{50}$/ml for mice and was stored at −20°C.

*Infection of Cultures.*—1 ml of thawed virus was diluted with 3 ml of maintenance medium

(Eagle's basal medium plus 1 per cent lamb serum) or growth medium in the case of macrophage cultures, and added to the cultures after removal of supernatant fluids. After 1 hour, the cultures were washed with Hanks' balanced salt solution (B.S.S.) or P.B.S. 4 times every 15 minutes for the following hour, to remove as much unadsorbed virus as possible.

*Titration of Riley Virus.*—Samples to be tested for infectivity were serially diluted 10-fold in B.S.S., and 1 ml per mouse of each dilution was inoculated intraperitoneally into 2 mice. Mice were bled retro-orbitally 72 hours later. The blood was diluted $\frac{1}{12}$ in heparinized P.B.S., and spun at 3000 RPM for 15 minutes to sediment the cells. The plasma was removed and lactate dehydrogenase (LDH) activity was determined colorimetrically by the plate technique described in an accompanying paper (13).

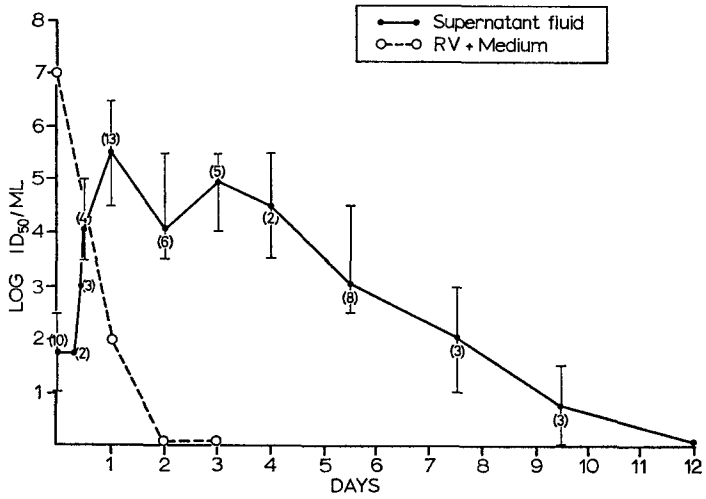


FIG. 1. Riley virus (RV) titre of the supernatant fluids of primary mouse embryo cultures, infected 24 hours after preparation, and of control suspension of RV in medium alone.

## RESULTS

*The Response of Mouse Embryo Cultures to Riley Virus Infection.*—Supernatant fluids of Riley virus–infected primary mouse embryo cultures were titrated for infective virus, and the results are shown graphically in Fig. 1. After the infection and washing periods less than $10^{2 \cdot 0} ID_{50}$/ml was detectable in the supernatant fluids. The titre began to rise between the 7th and 9th hours after infection of cultures. The peak titre was reached between 16 and 24 hours, and remained approximately constant for 2 to 4 days, after which there was a slow decline. By the 12th day the supernatants were noninfective. Each point on the graph represents the mean of the number of experiments shown in brackets, and the range of titres recorded at each point is indicated. The thermal inactivation of Riley virus in medium only is also shown. There was no apparent cytopathic effect, and no elevation of LDH activity in the supernatant fluids.

A further study was made of the thermal inactivation of Riley virus in me-

dium alone at 36.5°C. Fig. 2 shows the rates of inactivation of various virus dilutions. $10^{6.0}$ID$_{50}$/ml and $10^{7.0}$ID$_{50}$/ml were inactivated within 48 hours while $10^{4.5}$ID$_{50}$/ml and less were inactivated within 24 hours.

The regular response of infected cultures clearly indicated virus replication, though it was apparent that as cultures aged the rate of virus replication decreased. The ability of cultures of different ages to support replication of Riley virus was examined in the following experiment.

Cultures were infected on the 1st, 8th, and 14th day after preparation. Unabsorbed virus was washed away after 1 hour. Supernatant fluids were sampled

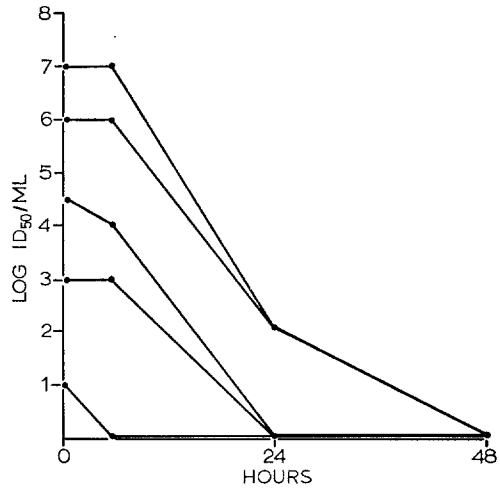


FIG. 2. Rate of thermal inactivation of Riley virus dilutions, incubated at 36.5°C.

periodically and tested for infectivity. The results are shown in Fig. 3. Cultures infected with Riley virus 1 day after preparation showed the response which has been described. Infection on the 8th and 14th days gave progressively less replication. Cultures became incapable of supporting replication between 8 and 14 days. All cultures remained apparently healthy for at least 2 weeks, by which time cell sheets were well developed. Superinfection of cultures from which infective Riley virus had disappeared showed no evidence of virus replication: $10^{7}$ID$_{50}$/ml of added virus became non-infective within 5 days.

*Replication of Riley Virus in Cultures of Various Mouse Tissues.*—

*Embryo liver:* The livers were excised from mouse embryos, and cultures were prepared from the livers and from the embryos minus livers separately (Fig. 4). Virus replication continued for a much longer period in the liver than in the embryo minus liver cultures, and the maximum titre reached was higher in the former.

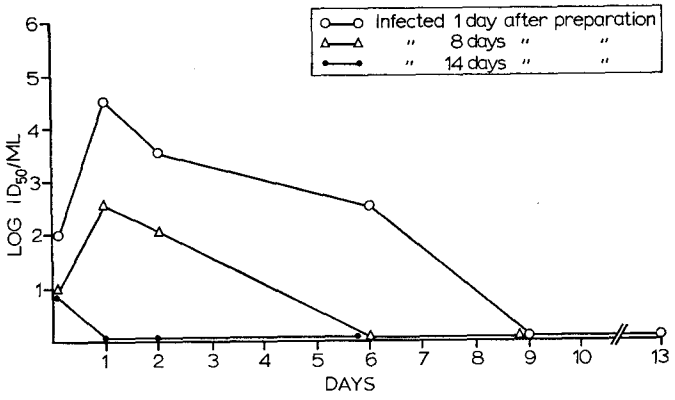


FIG. 3. Susceptibility of primary mouse embryo cultures to Riley virus infection. Titres of supernatant fluids of cultures infected on the 1st, 8th, and 14th day after preparation.

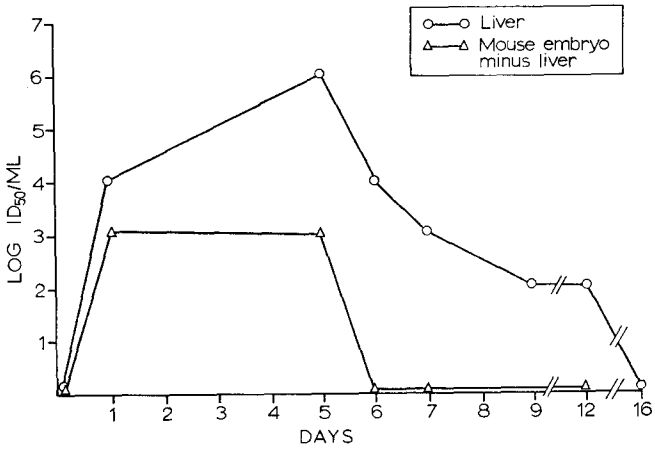


FIG. 4. Riley virus titres of the supernatant fluids of mouse embryo liver and embryo minus liver cultures.

*Adult mouse spleen:* There was no significant difference between the virus titres of Riley virus in the supernatant fluids of adult mouse spleen and whole mouse embryo cultures, but in the former the virus persisted longer, namely for at least 14 days.

*Adult mouse macrophages:* In view of the possible relationship between the RES and the level of viraemia (13), a study was made of the proliferation of Riley virus in macrophage cultures. Cultures of mouse peritoneal macrophages were infected with the virus either on the 1st or on the 8th day of cultivation. The virus titres of the supernatant fluids are shown in Fig. 5. In the cultures infected on the 1st day, a maximum titre was reached 5 days later. Furthermore,

infective virus could be demonstrated in the supernatant for at least 21 days, that is as long as the cultures could be maintained in good condition. Cultures infected 8 days after preparation showed almost as good replication. In general, macrophage cultures yielded higher titres than other types of cultures, though usually the peak titres were not reached until 24 hours after infection of cultures.

*Serial Passage of Riley Virus.*—Proof of viral replication *in vitro* requires that a virus be passed serially for an indefinite period without loss of infectivity. As has been shown the infective titre of the supernatant fluids reached a peak at

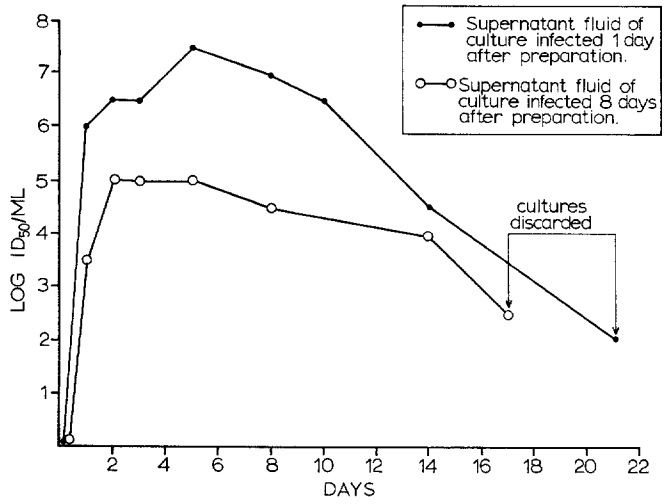


FIG. 5. Riley virus titres of the supernatant fluids of mouse macrophage cultures, infected on the 1st and 8th day after preparation.

about 1 day after infection in the case of mouse embryo cultures, and 1 to 5 days in the case of macrophage cultures.

1 ml volumes of stock Riley virus, diluted 1:3 in maintenance or growth medium, were added to mouse embryo and macrophage cultures respectively. Supernatants, diluted 1:3, were transferred to fresh cultures every 24 hours for mouse embryo cultures, and every 3 or 4 days, that is twice weekly, for macrophage cultures. At each transference the supernatants were titrated for infectivity.

After 3 to 5 transfers infective Riley virus had disappeared from the supernatants of mouse embryo cultures. An attempt was made to pass Riley virus twice weekly in mouse embryo cultures, but in all cases the virus disappeared from culture supernatants about the 3rd or 4th passage.

In macrophage cultures the virus could be maintained indefinitely at a fairly

steady titre (Fig. 6). The range of titres during passages was from $10^{4.5} \pm {}^{1.0}ID_{50}$/ml to $10^{8.0} \pm {}^{0.5}ID_{50}$/ml. The theoretical drop in supernatant titre, diluted 1:3 at each transference, assuming no inactivation, and the rate of thermal inactivation of Riley virus in the absence of cells, are also shown. Infective supernatants were transferred 23 times over a period of 80 days, during which the original inoculum was theoretically diluted to $10^{-14}$.

*Cultures from Other Species.*—Riley virus was added to cultures of Hela cells, Rhesus monkey kidney cells, and rat peritoneal macrophages. There was no detectable replication, as judged by the infective virus titre of supernatant fluids.

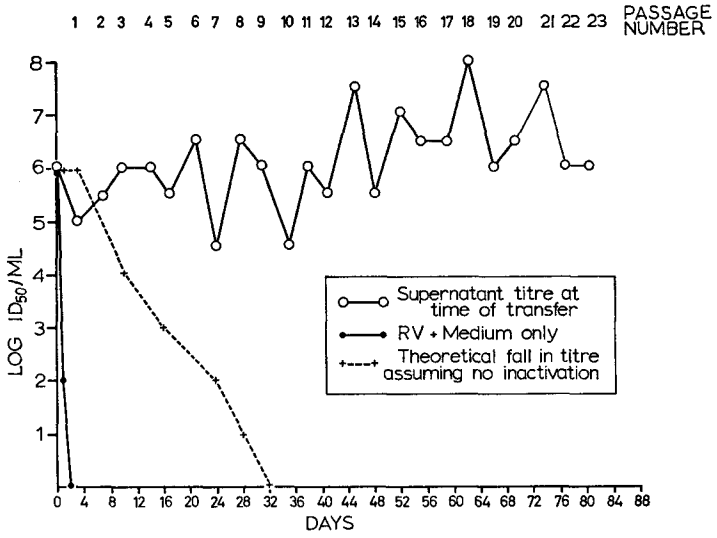


FIG. 6. Twice-weekly passage of supernatant fluids to fresh macrophage cultures.

## DISCUSSION

While it is beyond doubt that Riley virus replicates in mouse tissue cultures, certain aspects are far from clear. The inability of mouse embryo cultures, in our hands, to support replication for longer than 8 to 12 days contrasts with the prolonged propagation reported by Yaffe (1) and by Anderson *et al.* (6), but agrees substantially with the results of Georgii *et al.* (4, 5). However, all available evidence supports the view that replication occurs in mouse embryo cultures, if only for a limited period. We have found that as cultures age they lose their susceptibility to Riley virus infection, and as infected cultures age they produce fewer infective units. The reasons for this and for the failure to propagate the virus for longer periods are not yet clear. Perhaps a susceptible cell is involved, a cell which gradually disappears or changes physiologically during

cultivation. Failure to pass the virus through more than three or four successive fresh embryo cultures suggests that other factors are involved.

The ability of the cells of the RES to phagocytose virus particles is well documented (15). Some viruses will replicate in macrophage cultures, for example mouse hepatitis virus (16, 17), while others are phagocytosed without subsequently replicating, for example vaccinia virus (18, 19). It is not possible to say at this stage what happens to Riley virus or the cells at the time of infection and during replication. We, as others (1, 3, 6), could find no visible cell damage.

The serial passage of Riley virus in macrophage cultures provides further evidence of replication. The period of transfer of infective supernatants, twice weekly, was chosen to correspond to the usual time taken to reach peak titres, but as Fig. 5 shows, high titres are maintained between the 1st and the 10th days or later, and it now seems probable that transfer every 24 hours, or every 10 days, would also result in successful passage. There is evidence (Evans, unpublished observations) that infection of cultures with doses of $10^4 ID_{50}$/culture will produce as great a yield of virus as $10^7 ID_{50}$/culture. Above a certain minimum, the infecting dose of virus does not seem to affect the rate of virus production. The same appears to be true of mouse embryo cultures. High doses (*e.g.* $10^9 ID_{50}$) did not result in greater yields of virus than lower doses ($10^5 ID_{50}$).

The *in vivo* experiments by Rowson *et al.* (13) have indicated a relationship between the level of viraemia in long term Riley virus-infected mice and the activity of the RES. The fact that cultures of macrophages, spleen, and liver, provide a more favourable milieu than mouse embryo cultures suggests that elements of the RES are involved in the uptake and replication of the virus.

The state of the RES in the mouse embryo has not been adequately explored (20). An embryo culture contains small proportions of many cell types. If, for example, macrophages are mainly responsible for Riley virus replication, the degree of replication will depend on the number of macrophages in the culture at the time of infection, their ability to survive in the presence of an ever increasing number of other cells, and their capacity for recovery after repeated ingestion and digestion of dead cells and debris. Ultimately, after the rigours of competition in such heterogeneous embryo cultures, a mixture of fibroblastic and epithelial cells is all that can be seen (6). Unless macrophages form an integral part of the cell sheet, which is unlikely due to their inherent wandering habit, or survive in small number on the cell sheet surface, it is probable that they are actually absent from cultures of more than a week old. Others have shown that macrophages disappear from mixed cultures (21) and from serially passaged cultures (22). In our experience, and that of Yaffe (1), Riley virus does not replicate in subcultured mouse embryo cultures. Replication in macrophage cultures appears to be limited by death of the cultures. In a recent report Chang *et al.* (23) claim to have maintained macrophages in special medium for well over 100 days. It would be interesting to

study replication of Riley virus in macrophage cultures grown under their conditions.

The possibility that Riley virus may replicate in lymphocytes, which are present initially in both embryo and macrophage cultures, has been considered. The population of lymphocytes visible in the latter drops from approximately 50 per cent when explanted to nil after 2 to 3 days in culture, and it is therefore unlikely that they are responsible for more than a very small part of the replication observed. In any case, under our conditions, most of the lymphocytes are removed from cultures when the supernatant fluids are decanted during the first 30 minutes of cultivation.

It would be premature to assert that macrophages are the major host cells involved in the replication of Riley virus *in vivo*, but the fact that cultures of macrophages and of macrophage-rich tissues support more active replication than any others tested suggests that this may well be so.

## SUMMARY

A study was made of the replication of Riley virus in various tissue cultures. It was observed that Riley virus replicated in primary mouse embryo cultures for 8 to 12 days. As they aged primary cultures became less susceptible to Riley virus infection, and subcultured cells were not susceptible. Suspensions of virus, in the absence of cells, were inactivated at 36.5°C. Serial passage by transfer of supernatant fluids to fresh embryo cultures was not successful. Replication of the virus was more active in mouse embryo liver cultures than in the cultures of the embryo minus the livers.

In cultures of mouse macrophages, the supernatants remained infective throughout the life of the cultures (21 days). The virus was passed 23 times through fresh macrophage cultures over a period of 80 days, during which the original inoculum ($10^{6.0}$ $ID_{50}$/ml) was theoretically diluted to $10^{-14}$.

The possibility that the cells of the RES are involved in Riley virus replication is discussed.

The expenses for this research were partly defrayed out of a block grant from the British Empire Cancer Campaign.

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