RESTRICTION OF HERPES SIMPLEX VIRUS
BY MACROPHAGES

AN ANALYSIS OF THE CELL-VIRUS INTERACTION

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Macrophages are generally recognized as important elements in determining the outcome of viral infection (cf. 12,5). Thus, in his review of viral pathogenesis, Mims states: "Macrophages are literally in a position to control the susceptibility of animals to virus infection since they monitor the main body compartments and may control the entry of viruses to target organs like the liver" (12).

Herpes simplex virus (HSV), infection of mice is a model system in which the role of macrophages has been studied in relative detail. Some years ago, Johnson (8) showed conclusively that the difference in susceptibility of different age groups to extracranial inoculation of herpes virus correlated with a difference in the ability of macrophages to disseminate infection. Specifically, infected macrophages derived from infant mice passed the infection to other cells while those from mature animals did not. In extensions of Johnson's work, Zisman et al. (23) and Hirsch et al. (7) were able to increase susceptibility to herpetic hepatitis in adult mice by injection of silica particles or anti-macrophage serum. In addition, infant mice could be protected against disease by adoptive transfer of macrophages derived from syngeneic adults.

These studies provide ample evidence that macrophages play a crucial role in the pathogenesis of herpetic infection. However, the mechanism by which virus replication is restricted in adult macrophages has not been systematically investigated. In addition to extending knowledge concerning the pathogenesis of herpetic disease, a definition of this process would contribute an understanding of macrophage function in viral disease generally. The present report characterizes this cell-virus system by defining viral-specified macromolecular syntheses in macrophages.

Materials and Methods

Media and Physical Conditions.—All cells were propagated as monolayers on glass in Eagle's minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS). Incubation was at 37°C in an atmosphere of 5% CO₂ in air.

1 Abbreviations used in this paper: FCS, fetal calf serum; HSV, Herpes simplex virus; MEF, mouse embryo fibroblasts; MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline; PFU, plaque-forming units; SDS, sodium dodecyl sulfate, SSC, standard saline citrate.
RK13 Cells.—RK13 rabbit kidney cells were used for propagation of all virus stocks and as nonrestrictive control cells for comparative studies with macrophages. The source and methods for propagation of this cell line have been described (22).

Macrophages.—Unstimulated, 8-wk old BRVS Swiss-Webster mice were used as donors of peritoneal macrophages. Macrophages were obtained from the peritoneal cavity using standard methods (2) and propagated as monolayers on glass. In addition to MEM and 5% FCS, in some experiments 1% normal rabbit serum was added since we have found that macrophages adhere to glass better when this is used. Macrophages were maintained in vitro for 48 hr before use.

Mouse Embryo Fibroblasts.—BRVS mouse embryos processed at the 19th day of gestation were the source of fibroblasts. Cells were prepared using methods developed for chick embryo fibroblasts (19).

Virus.—The H4 strain of HSV was kindly supplied by Dr. Albert Kaplan, Albert Einstein Medical Center, Philadelphia, Pa. Virus stocks were prepared in RK13 cells propagated in roller bottles (17) and contained 2 × 10⁶ to 1 × 10⁷ plaque-forming units (PFU)/ml. Titrations were performed on RK13 cell monolayers in 2 oz French square bottles by adsorbing 0.2 ml of the virus dilution at 37°C for 1 hr. Monolayers were then overlayed with MEM supplemented with 1% FCS and 0.3% pooled human gamma globulin. This overlay medium permits direct cell-to-cell spread of virus with consequent plaque development, but prevents formation of secondary plaques (6). After 48 hr of incubation at 37°C, the overlay medium was poured off, the monolayer stained with crystal violet, and the plaques enumerated.

Reagents and Chemicals.—(Methyl-3H) thymidine (10 Ci/mmole), (methyl-14C) thymidine (30-50 mCi/mmole), a4C-reconstituted algal protein hydrolysate, and 3H-reconstituted algal protein hydrolysate were purchased from Schwarz BioResearch, Inc., Van Nuys, Calif. All sera were obtained from Microbiological Associates Inc., Bethesda, Md. Sheep anti-rabbit gamma globulin conjugated with fluorescein isothiocyanate was purchased from Sylvania, Millburn, N. J.

Electron Microscopy.—For macrophages infected synchronously, cells were scraped from the glass at various times after infection, centrifuged into a pellet, and processed for electron microscopy using the techniques of Morgan et al. (13). Macrophages to be used for infection at low multiplicities were grown on carbon-coated glass slides. After polykaryocytes had formed, the cells were processed on the slides using methods which we have published (3).

Radioautography.—Macrophages grown on cover slips were synchronously infected with HSV. After adsorption, 2.5 μCi/ml 3H-thymidine was added to infected and uninfected cells. 5–6 hr postinfection all cells were washed with PBS, fixed in absolute methanol at 4°C, and further prepared for observation as described by Rogers (16). The exposure period was 7 days.

Immunofluorescence Procedures.—Herpes simplex antiserum was produced in New Zealand white rabbits by injection of maximally infected RK13 cells in Freund's complete adjuvant (8 biweekly injections over a 4 wk period, 2 × 10⁶ PFU per injection). The serum pool obtained possessed a neutralization constant of 129 at 37°C. Samples of both pre- and postimmunization sera were absorbed with mouse liver powder before use in immunofluorescent studies. For the immunofluorescent studies, macrophages grown on cover slips were synchronously infected with virus. At 6 hr postinfection, infected and uninfected cultures were fixed in acetone overnight at −20°C. Using standard methods (see reference 10) the indirect immunofluorescence technique was employed utilizing pre- and postimmunization sera and fluorescein-labeled goat and anti-rabbit gamma globulin. Stained slides were observed with a Zeiss fluorescence microscope.

Assay of DNA on CsCl Gradients.—Cellular and viral DNA were assayed by first labeling infected and noninfected cells with 3H- or 14C-thymidine (10 μCi or 2 μCi/ml respectively). After the appropriate labeling times, cells were washed once with standard saline citrate
(SSC), incubated at 37°C for 1/2 hour in 3% sodium dodecyl sulfate (SDS) in SSC, and
dialyzed overnight against 0.2% SDS in SSC. They were then treated for 30 min at 37°C
with 100 μg/ml of self-digested pronase. Suitable amounts of 14C- and 3H-labeled samples
were mixed and added to sufficient CsCl and distilled water to give 4.5 ml of a mixture with
a density of 1.70 g/cc. Samples were then centrifuged in a Spinco SW-50 rotor at 35,000 rpm for
40-44 hr at 25°C. Samples (about 100 per tube) were collected by drops from the bottom
of the tube. Refractive index measurements for density determinations were made on se-
lected samples using a Zeiss Abbé refractometer. Radioactive DNA was precipitated and
washed on 0.45 μl millipore filters with 5% cold trichloroacetic acid (TCA) in the presence of
100 μg of BSA as carrier. A Mark I liquid scintillation counter (Nuclear-Chicago Corp.,
Des Plaines, Ill.) adjusted for double label counting was used to monitor radioactive dis-
integrations.

Assay of Radioactive Proteins on Polyacrylamide Gels.—Infected
cells were labeled for appropriate times with 3H- or 14C-algal hydrolysate (25 μCi and 2 μCi/ml respectively) in
MEM with 1/5 the usual concentration of amino acids and 2% dialyzed FCS. They were
then scraped from the glass and pelleted. Pellets were treated with SDS, 2-mercaptoethanol,
and urea and were electrophoresed on polyacrylamide gels. Gels were fractionated and the
radioactivity determined. In most cases, appropriate 14C- and 3H-labeled samples were run
on the same gel. We have described all these procedures in detail (22).

RESULTS

Capacity of Macrophages to Replicate HSV.—

RK13 cells and mouse macrophages were infected with virus at multiplicities sufficient to
infect all RK13 cells. Then, unadsorbed inocula were removed by three washes with MEM,
serum was readded, and the cultures were incubated. At 5 hr intervals, duplicate cultures of
both RK13 cells and macrophages were removed and frozen at -70°C. When cultures for all
time intervals had been collected, frozen, and thawed one additional time, they were titrated
on RK13 monolayers.

As shown in Fig. 1, the one-step growth cycle for H4 HSV in RK13 cells is
the same as that described by Kaplan (9), with an eclipse period of about 5 hr
and a rise period of 15 hr. In macrophages, on the other hand, there is no rise
after 5 hr and the amount of infectious virus falls steadily to a level beyond de-
tection at 25 hr. This level corresponds to about 2 × 10^-4 PFU/macrophage.
The slight “plateau” observed at 15 hr probably represents replication of a
small amount of virus. This possibility will be treated in detail in a later section.
This result represents lack of virus production and is not due to the pres-
ence of materials in macrophage lysates which interfere with the assay in RK13
cells, since significant amounts of such substances could not be detected (Stevens,
J. G., and M. L. Cook, unpublished observations). Finally, it is important to
note that all cells were killed and released from the glass in both cell types. Ac-
cording to these results, HSV appears to undergo an abortive infection in mouse
macrophages.

Specificity of Restriction.—It is possible that the ability to restrict HSV is not
peculiar to macrophages but is a general property of mouse cells. Therefore, the
capacity of other mouse cell types to replicate virus in vivo and in vitro was
investigated.
In in vivo experiments, virus was inoculated intracerebrally or intradermally. In intracerebral experiments, $2 \times 10^6$ PFU were inoculated per 4 wk old mouse and, at 5 hr intervals, four mice were sacrificed, the brains pooled and assayed for virus. The results of a typical experiment are given in Fig. 2.

As can be seen, the titer of virus increased steadily between 2 and 25 hr showing that the virus replicates in the brain. Mice inoculated in this fashion usually begin to die about 24 hr after inoculation.

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**Fig. 1.** Growth cycle of herpes simplex virus in RK13 cells (○—○) and in mouse macrophages (■—■). The triangle at 25 hr on the curve drawn for macrophages represents the level of sensitivity for the assay.
For the intradermal experiments, $5 \times 10^5$ PFU were inoculated intradermally into both hind footpads of 28-day old mice using the method described by Olitsky and Schlesinger (15). The results, also shown in Fig. 2, indicate that the virus does replicate following intradermal inoculation and reaches a peak titer at 2 days after inoculation.

Finally, the results of an in vitro experiment in which mouse embryo fibroblasts were infected under the conditions described for Fig. 1 are presented as the third curve in Fig. 2. It is clear that HSV also replicates in mouse embryo fibroblasts. Since HSV replicates in other mouse cells both in vivo and in vitro, we conclude that the restriction in mouse macrophages is specific to these cells. **Adsorption of HSV to Mouse Macrophages.**—Restriction of virus replication can be manifested at many stages of the replication cycle. Adsorption is the obvious first step.
To assess the capacity of macrophages to adsorb virus, the kinetics of viral adsorption to RK13 cells and macrophages was determined. The methods used have been described in detail elsewhere (21). In brief, stock virus containing about 20,000 PFU/0.2 ml was plated onto monolayers of about 10^6 cells for adsorption at 37°C. At various times between 0 and 60 min, infected monolayers were removed from the incubator and the inoculum diluted to 20 ml to stop adsorption. Samples were then assayed on RK13 cells, curves were constructed, and an adsorption constant was derived. The results are presented in Table I.

Macrophages adsorb virus at a rate about one-half that of RK13 cells. Using this data it can be shown that all macrophages in the experiment presented in Fig. 1 adsorbed at least one RK13 PFU. Thus, lack of adsorption cannot be the reason for virus restriction in macrophages.

**Synthesis of Virus-Specific Macromolecules in Macrophages.**—Events following adsorption include uncoating and expression of the viral genome. Genetic expression can be studied by searching for nascent, viral-specified nucleic acids and proteins in cells.

| Experiment | RK13 cells | Macrophages |
|------------|------------|-------------|
| 1          | 2.4*       | 1.3         |
| 2          | 3.3        | 1.7         |

* Expressed as number × 10^-9 ml/min.

**Deoxyribonucleic Acid Synthesis.**—HSV DNA possesses a relatively high buoyant density and can be readily separated from mammalian cell DNA after equilibrium sedimentation in CsCl gradients (18). Thus, if virus-specific DNA is made in macrophages, one should be able to find it by coupling this technique to radioactive labeling.

To label DNA, infected and uninfected macrophages or RK13 cells were labeled with 3H-thymidine (10 μCi/ml) or 14C-thymidine (2 μCi/ml). Infected cells were labeled between 0 and 10 hr (macrophages) or 0 and 12 hr (RK13 cells), while uninfected cells were labeled for 2–3 days. At the conclusion of the labeling period, DNA was extracted and CsCl gradients were run. The results of several experiments are presented as pooled data in Fig. 3.

Alignment of peaks presented in separate panels was achieved by use of appropriately paired combinations of DNA labeled with either 14C-thymidine or 3H-thymidine in the same gradient. From the results it can be concluded that DNA with a density characteristic for H1 HSV is induced in mouse macrophages. If the virus DNA is assigned a density of 1.727 g/cc (18) then the RK13 cell DNA bands at an apparent density of 1.720 g/cc, and the Macrophage DNA...
at 1.712 g/cc. These densities can only be considered to be approximate since densities were not determined on samples used for radioactive measurement.

The number of cells induced to synthesize DNA was determined. Here, advantage was taken of the fact that very few peritoneal macrophages synthesize DNA in culture (1).

![Diagram](image)

**Fig. 3.** CsCl density gradient profiles of $^3$H- or $^4$C-thymidine-labeled DNA in herpes simplex infected and uninfected mouse macrophages and RK13 cells.
Infected and noninfected cells were labeled with $^3$H-thymidine from 0 to 6 hr after infection and radioautographs were prepared. The results of one such experiment are presented in Fig. 4.

It is clear from Fig. 4 b that DNA synthesis is induced in the majority of infected cells. In two such experiments, of 1000 nuclei scored per group, the number of labeled nuclei went from 1% in uninfected cells to 55 and 85% respectively in infected cells. It is probable that the other infected cells would have incorporated label if the incubation were continued beyond 6 hr. Because of technical difficulties we did not determine the kinetics of labeling or the absolute amounts of viral DNA made in macrophages.

From these experiments, if the unprecedented possibility that HSV induces
cellular DNA synthesis is discounted, it can be concluded that DNA with a density of herpes simplex DNA is induced in a majority of infected macrophages.

Protein Synthesis.—At present, polyacrylamide gel electrophoresis utilizing SDS is probably the best method for resolution and subsequent identification of viral-induced proteins from infected cells (11). Using this method, Spear and Roizman have identified at least 25 herpes virus-specific proteins after productive infection (20). In similar experiments, we have found that if radioactive amino acids are added in the period between 6 and 12 hr after productive infection of RK13 cells, all virus-specific proteins which can be identified at any other time period are labeled (Stevens, J. G., and M. L. Cook, unpublished observations).

To determine whether virus-specific proteins were induced in macrophages, synchronously infected cells were labeled with 3H-amino acids between the period 6 and 12 hr after infection. Then cells were solubilized with SDS, urea, and 2-mercaptoethanol and coelectrophoresed with marker proteins prepared from infected RK13 cells labeled with 14C-amino acids during the same time interval.

![Graph showing electropherogram of radioactive proteins induced by herpes simplex virus in RK13 cells and mouse macrophages. Cells were labeled between 6 and 12 hr after infection. 14C-amino acids were used in RK13 cells (broken line) and 3H-amino acids in macrophages (solid line).]
The results, shown in Fig. 5, permit the tentative conclusion that, qualitatively at least, the major peptides present in RK13 cells during productive infection are present in the restrictive macrophages. In this electropherogram, the discrete peak which appears at fraction number 37 in the RK13 sample is a shoulder on the preceding peak in the sample prepared from macrophages.

However, it has been resolved in other experiments. In addition, the minor peaks after fraction 75 in RK13 cells are not always present. Two qualifications concerning the method must be mentioned. First, all proteins in this complex system are most certainly not resolved even by this method. Secondly, identity is based only upon molecular weight. Therefore, although it seems likely, we cannot unequivocally conclude from these data that all virus-specified proteins are induced in macrophages.
The proportion of macrophages producing virus-specific proteins was assessed by methods involving fluorescent antibody and electron microscopy. In the experiments involving fluorescent antibody, synchronously infected macrophages were incubated for 6 hr, and then processed for indirect fluorescent antibody staining. The results of a typical experiment are shown in Fig. 6. It is clear that antigen which specifically reacts with immune serum is present in all macrophages in infected cultures (Fig. 6a). Controls in which uninfected cells were treated with HSV-immune serum (Fig. 6b) or in which infected cells were treated with preimmune serum (Fig. 6c) are presented for comparison.

Morphologic studies of the infectious process in individual cells were performed on macrophages maintained for 5 hr after infection. Morphologic features characteristic of herpes simplex infection are evident in Fig. 7. The particles most commonly seen in this and later time periods are capsids lacking
a dense central core. Most of them do, however, possess a central, diffuse "ring" with relatively low density. A very few morphologically complete particles with membranes were seen extracellularly and in the cytoplasm of a few cells. Quantitative determinations indicated that of 58 nuclei examined, 39 (67%) contained herpes virus capsids, thus corroborating the fluorescent antibody studies in which all cells demonstrated specific fluorescence. Taken together, these experiments show unequivocally that herpes simplex virus induces major virus-specific proteins in the majority of macrophages.

The kinetics of synthesis and amounts of protein induced in macrophages was compared to RK13 cells. Cells were pulsed with 3H-amino acids for 3-hr intervals between 0 and 24 hr after infection, and acid-insoluble radioactivity was used as a measure of protein synthesis. As shown in Fig. 8, macrophages produce considerably less protein per cell at all time intervals than do RK13 cells, the kinetics of protein synthesis is different, and macrophages appear to be functionally dead by 9 hr after infection since incorporation falls to background levels by this time.

From the results presented to this point, we conclude that HSV infection of mouse macrophages results in an abortive infection in which virus-specific DNA and proteins, and subviral particles, are induced in the majority of infected macrophages. The following experiments represent further attempts to characterize the system and define the reason(s) for this restriction.
Evidence for Replication of a Small Amount of Virus.—The data presented in Fig. 1 and the electron micrographs of infected macrophages suggested that a small amount of infectious virus was replicated in macrophages. To investigate this more completely, macrophages were infected at low multiplicities to reduce background levels of input virus, and were held for 3 days. Supernatants from these monolayers were assayed for the presence of virus and the cells were stained and observed for the presence of polykaryocytes. It was found (Figs. 9 and 10) that polykaryocytes did develop and that nuclei in these polykaryocytes possessed capsids containing a central dense core. In addition, a very few morphologically complete particles were seen in the cytoplasm and in extracellular spaces. When supernatants from these cells were assayed for the presence of virus, the titers increased from below the level of detection immediately after adsorption and washing to 40 PFU/ml at 3 days after infection. Thus, it
Fig. 10. Electron micrograph of a polykaryocyte induced by herpes simplex virus in mouse macrophages. Some of the capsids with central dense cores are noted by small arrows. One virion is seen at the top (large arrow). $\times$ 24,000.
appears that a few infectious virions are produced by some cells. It should be noted in passing that the efficiency of polykaryocyte induction in macrophages was 1/200–1/1500 that in RK13 cells, indicating again that the majority of macrophages undergo an abortive infection.

**Figs. 11 a and b.** Electron micrographs of nuclei from herpes simplex virus-infected mouse macrophages (a) and RK13 cells (b). The capsids seen in the macrophage nucleus contain only cores with low density while most of those in the RK13 nucleus have central dense cores. An arrow designates one exception. X 55,000.

**Comparative Electron Microscopy.**—As a final characterization, virus production in macrophages was compared to that in RK13 cells by electron microscopy. Cells were synchronously infected, held for 15 hr, and processed for electron microscopy. In nuclei (Fig. 11), the most conspicuous difference between infections in the two cell types is the relative paucity of capsids with central dense cores in macrophages (Fig. 11 a). The internal structure in macrophages consisted of a ring with comparatively low density. This impression is
supported by quantitative evidence. In RK₁₃ cells, of 34 nuclei containing capsids, 32 (94%) possessed capsids with dense cores. In macrophages only 9 of 39 (23%) contained such structures. Very few enveloped capsids were found in macrophages.

Figs. 12 a and b. Electron micrographs of extracellular herpes simplex viruses in infected RK₁₃ (a) and mouse macrophage (b) cell cultures. × 74,000.

| Table II |
| Inactivation of Herpes Simplex Virus by Culture Media or Supernatants from Infected Macrophages |
| Suspending medium | Exp. 1 | Exp. 2 |
|-------------------|-------|-------|
| Culture medium    | 48*   | 50    |
| Infected macrophage supernatant | 10 | 16 |

* Number of PFU surviving treatment for 1/2 hr at 37°C.
In the cytoplasm and in extracellular spaces, morphologically complete virions are abundant in RK13 cells (Fig. 12 a), but are extremely rare in macrophages. Those that are present appear to be degraded (Fig. 12 b). That such degradation does occur is proved by experiments in which supernatants from macrophages infected for 24 hr were incubated with known amounts of virus at 37°C for 30 min before plating on RK13 cells. As shown in Table II, the amount of infectious virus surviving this treatment was three to five times less than that surviving in culture medium only.

**DISCUSSION**

The results presented here confirm and extend the work of others by showing unequivocally that the herpes simplex genome is actively expressed under restrictive conditions in mouse macrophages. This expression is manifested by the production of viral DNA and proteins and, subsequently, by the death of the macrophage. Although some reservations must be made concerning induction of the entire spectrum of proteins in each macrophage, our tentative conclusion is that all viral components are made in all cells and that a very few infectious virions are produced by some cells. Thus, for practical purposes, the infection can be considered to be an abortive one in which all the viral components are made but not efficiently assembled. Assembly could be influenced if amounts of all or selected components are limiting or if the components are structurally faulty.

From the ultrastructural studies, two deviations from normal viral morphogenesis were noted in macrophages. First, there was a paucity of central dense cores in capsids and, secondly, very few capsids were subsequently enveloped. The numerous capsids with cores of low density seen (Fig. 11 a) probably contain no DNA. Evidence to support this hypothesis comes from two sources. First, dense cores are susceptible to DNAase (4). Secondly, capsids seen in permissive cells when virus is grown in the presence of DNA analogues possess structures morphologically identical to those seen in macrophages. Thus, RK13 cells infected in the presence of hydroxyurea or cytosine arabinoside under conditions in which DNA synthesis is inhibited by 90%, exhibit almost exclusively capsids with cores of low density. Macrophages are insusceptible to cytosine arabinoside, but the few capsids which are produced in the presence of hydroxyurea possess cores of low density (Stevens, J. G., and M. L. Cook, unpublished observations). From these considerations, it seems that the primary block to replication may reside somewhere in the pathway of viral DNA synthesis or processing. Thus, although DNA synthesis is induced in macrophages and the DNA possesses a buoyant density which is characteristic of herpes simplex DNA, it is possible that insufficient amounts are made, that it is structurally defective, or that some process related to its association with capsids does not proceed efficiently. Since herpes simplex capsids lacking central dense cores are
“enveloped” inefficiently (14), the paucity of enveloped capsids observed in macrophages might represent a later manifestation of abnormal DNA metabolism and not indicate a primary lesion in the process of envelopment.

In addition to restrictive events concerned with assembly, most of the virions that are assembled in macrophages are destroyed by some cellular product(s) (Table II). Released lysosomal hydrolases which are abundant in macrophages are the most likely candidates for this effect.

In conclusion, we show that the interaction between HSV and mouse macrophages is a complex one in which virus-specific macromolecules, but very few virions, are produced. As a consequence of the active expression of viral genes, both macrophage and virus are destroyed. It is obvious that this type of interaction could effectively influence the course of herpetic disease in vivo.

SUMMARY

Macrophages restrict herpes simplex virus replication and can prevent the development of herpetic disease in mice. In an attempt to define the nature of this restriction, an analysis of virus-specified macromolecular syntheses in infected macrophages was undertaken. The significant results were the following: All cells were killed, but the infection was considered to be abortive since the level of infectious virus in macrophage cultures dropped steadily to a level beyond detection by 25 hr after infection. This restriction appeared to be specific for macrophages; the virus replicated efficiently in other mouse cells. DNA with a density characteristic for herpes simplex virus DNA was extracted from infected cultures, and the proportion of macrophages synthesizing DNA increased from less than 1% to greater than 50% by 6 hr after infection. Studies employing polyacrylamide gel electrophoresis indicated that the major viral-specific proteins were induced in macrophage cultures. In addition, all cells showing cytopathic changes characteristic of herpes virus infection also contained viral antigens which could be detected by fluorescent antibody techniques and, by 15 hr after infection, most contained nascent capsids lacking central dense cores. It is suggested that an error in DNA metabolism may be the primary cause of restriction.

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