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Pathological Changes Induced by Herpes Simplex Virus in the Chorioallantoic Membranes of Hatching Eggs

SHIRO NII AND JUNTARO KAMAHORA

Department of Pathology, The Research Institute for Microbial Diseases, Osaka University, Osaka
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SUMMARY

Histopathological changes in the chorioallantoic membranes of hatching eggs induced by three variants of the Miyama strain of herpes simplex virus, which had been differentiated by their cytopathic effects on FL cell monolayers, were studied. Virus +GC induced the formation of syncytia in chorioallantoic membranes, just as in monolayer cells *in vitro*. The other two variants, *i. e.* -GCr and -GCf, did not cause formation of syncytia but cytopathic change in individual cells in the membrane was observed. The morphological changes in the membranes induced by the latter two variants could not be distinguished from each other, although they induced different types of cytopathic change *in vitro*.

INTRODUCTION

Preceding papers described three substrains of the Miyama strain of herpes simplex virus, which were distinguishable from each other by their cytopathic effcts on FL monolayers and these variants were designated as +GC, −GCr and −GCf respectively (Nii, 1961a; Nii *et al.*, 1961b).

The +GC variant caused syncytium formation not only in FL cells but also in many other established cell lines, *e. g.* L, HeLa, Hep-2 and PS cells (Nii *et al.*, unpublished).

The phenomenon of giant cell formation has been recognized *in vitro* as well as *in vivo* during infection by many other viruses. Among these, measles virus is well known to cause characteristic giant cell formation *in vivo*, named Warthin-Finkeldey giant cells, and this is an important pathological change in animals infected with measles. Similar giant cells have been also observed in monolayer cell cultures *in vitro*. (Taniguchi *et al.*, 1954; Enders *et al.*, 1954)

It is interesting to see whether the variants of herpes simplex virus differentiated by their cytopathic effects *in vitro* cause different characteristic changes in the tissues of infected animals.

This report describes studies on the effects of three variants of the virus in chorioallantoic membranes, these membranes being composed of more complex cellular populations than monolayer cells *in vitro*.

MATERIALS AND METHODS

1. *Virus*

Three substrains of the Miyama strain of herpes simplex virus were used (Nii, 1961a). Viral samples for experiments were prepared from culture fluids of FL cells infected with each variant. They were used after centrifugation at 2,500 rpm for 15 minutes. Their viral titers were approximately 10^8 $TCID_{50}$ per ml.

2. *Eggs*

Twelve day old developing eggs were used.

3. *Experimental procedures*

A series of eggs were inoculated with 0.05 ml of viral materials by the chorioallanotic route and incubated at 35°C. At various intervals after virus inoculation three to five eggs were removed for examinations.

The paraffin seal over the hole in the shell was removed and the shell was broken away to the level of the fallen chorioallantois. At a certain distance beyond the level of the membrane the shell was cut away with scissors and then a ring segment of the shell with the membrane was obtained. The membrane was pulled off from the shell and immersed in Hanks' balanced salt solution in a Petri dish. It was washed with two or three changes of this solution and after the last wash the rest of the membrane outside the inoculated area was cut away with scissors and the part of the membrane corresponding to the inoculated area was fixed in Bouin's fixative. Histological sectioning and staining with hematoxylin and eosin of the membrane were performed by routine methods.

4. *Control membranes were obtained as follows.*

a) 0.05 ml of L-E solution (Earle's saline containing 0.5 per cent Lactalbumin hydrolysate) was inoculated into two or three eggs and the extent of non-specific lesions of the chorioallantoic membrane was observed histologically.

b) Chorioallantoic membrane was merely separated from the shell membrane by production of an artificial air space and was not inoculated.

After incubation for the same periods as the eggs inoculated with viral samples, the above membranes (a and b) were harvested to make histological preparations.

c) The chorioallantoic membrane in normal contact with the shell membrane between the 12th day and the 14th day of incubation, was observed histologically.

RESULTS

1. *Histological appearance of control membranes*

In the chorioallantoic membrane in normal contact with the shell membrane between the 12th day and the 14th day of incubation, ectodermal and entodermal layers appeared as single layers of flattened cells.

The membrane, which was separated from the shell membrane by an air space and was not inoculated, occasionally showed non-specific lesions, the so called "traumatic ulcers" (Beveridge *et al.*, 1946). The ectodermal layer was slightly thickened.

In membranes inoculated with L-E solution, thickening of the ectodermal

layer was occasionally seen and oedema of the mesodermal layer was sometimes observable. A slight cellular proliferation and accumulation could be detected beneath the ectodermal layer (Fig. 1).

2. *Pathological changes induced by +GC virus*

The eggs inoculated with +GC virus were taken out from the incubator 16, 20, 24 and 40 hours after virus inoculation. In the membranes obtained 16 hours after infection, giant cells containing intranuclear inclusion bodies were found at the ectodermal layer and the mesodermal layer was moderately affected.

In preparations derived from membrances 20 and 24 hours after infection, the specific change induced by +GC virus *in vitro* was also seen in the ectodermal layer, although it was not so easily detected as *in vitro*, and necrosis of proliferating epithelium and marked accumulation of inflammatory cells in the mesoderm were observed. These pathological changes appeared to vary more or less in serverity in different membranes and in different microscopic loci in a single membrane. The membrane 40 hours after infection showed more extensive syncytial formation as shown in Figs. 2 and 3 and many intranuclear inclusion bodies were readily seen in the syncytia.

3. *Pathological changes induced by —GCr virus*

Eggs were taken out from the incubator 20, 40 and 48 hours after inoculation with —GCr virus. In no histological preparations could giant cells be detected but cytopathic changes of individual cells were clearly seen (Fig. 4, Fig. 5). Other inflammatory changes of the mesodermal layer were clearly observed, as shown in the membranes inoculated with +GC virus.

4. *Pathological changes induced by —GCf virus*

Infected membranes were obtained 28 and 52 hours after virus inoculation. Histological examination revealed cytopathic changes of individual cells but giant cells were not found (Fig. 6). However, the cytopathic changes in these membranes were the same as those induced by —GCr virus, although the cytopathic changes *in vitro* caused by the two substrains differed.

DISCUSSION

In virus infected animals, giant cells have sometimes been recognized as an important pathological change while in monolayer cell cultures *in vitro* cytopathic changes are thought to be characteristic of the infecting virus.

The variants of herpes simplex virus were differentiated by some workers by the cytopathic effects they caused in monolayer cultures and it was found that the morphological changes induced by them were controlled genetically (Nii *et al.*, 1962). However, the biological significance of these variants in the infection of

human beings has not been clarified, nor has it been shown whether they induce the same effects in animal tissues as *in vitro*.

To study the above problems chorioallantoic membranes of hatching eggs were used. Histologically, these were composed of three layers; the ectodermal, mesodermal and entodermal layers. The histological structure of the membranes as well as the biological interactions of these tissue cells is supposed to be rather similar to those of animal tissues. Cytopathic effects of three variants of herpes simplex virus on the membranes were tested and it was found that the +GC variant induced syncytial formation in the membranes, while cytopathic changes in individual cells occurred with the −GCr and −GCf variants. Therefore, it is supposed that the giant cell forming variant has the property of inducing giant cells in herpetic lesions in human beings although it may be difficult to prove this occurs in human tissues.

The histological changes in the membranes induced by −GCr and −GCf could not be distinguished, although the changes caused by each differed in monolayers *in vitro*. The first reason for this is that the difference between the changes characteristic of the two variants, (*i.e.* cell rounding induced by −GCr virus, and cell agglutination and fusiform development of cells induced by −GCf virus) is rather more delicate than the distinct difference between the changes induced by +GC virus (*i. e.* syncytial formation) and by either of the former two variants. The second reason is the impossibility of exact and repeated observations of cytopathic changes in the membranes in paraffin sealed eggs. The third reason is the difference in the interactions or connections of cells in the *in situ* ectodermal cells and the *in vitro* cells. In the former cells neighbouring cells are combined in a more regular and stronger manner to maintain the organization of the living animal.

The phenomenon of the appearance of giant cells in lesions of herpes infected human beings has already been reported (Scott, 1952). The data described in this paper strongly suggest that this pathological change is induced by +GC virus contained in viral populations carried by human beings although other pathogenic changes such as amitotic nuclear division (Reissig *et al.*, 1960; Nii *et al.*, 1963) must also be considered in actual cases. However, a lower ratio of +GC virus is carried in man in viral populations, as shown by the fact that a large number of freshly isolated virus particles reveal −GCr type cytopathic changes *in vitro* (Scott *et al.*, 1959; Nii *et al.*, unpublished data).

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EXPLANATION OF FIGURES

Fig. 1. In the control chorioallantoic membrane inoculated only with L-E solution, slight thickening of the ectodermal layer and slight cell proliferation and accumulation are shown.

Fig. 2. The membrane 40 hours after inoculation with +GC virus.
Syncytial formation in the ectodermal layer and marked accumulation of inflammatory cells in the mesodermal layer just beneath the ectoderm are shown.

Fig. 3. Magnified portion of Fig. 2.
A syncytium containing numerous nuclei with intranuclear inclusion bodies is shown.

Fig. 4. The membrane 40 hours after inoculation with -GCr virus.
Cytopathic changes of single cells in the ectodermal layer but no syncytial formation are seen.
Cellular proliferation in the mesodermal layer just below the ectoderm is shown.

Fig. 5. Magnified portion of Fig. 4.
Single cells showing cytopathic rounding are seen in the ectodermal layer and some nuclei contain homogeneous intranuclear inclusions.

Fig. 6. The membrane 28 hours after inoculation with -GCf virus.
Cytopathic changes of single cells but no giant cells are seen.

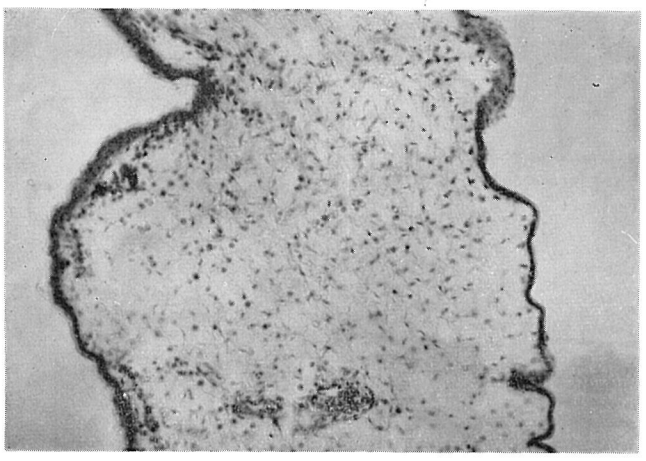

Fig. 1

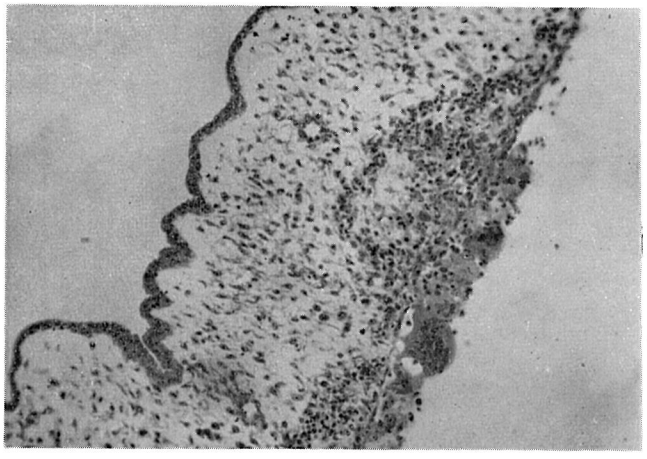

Fig. 2

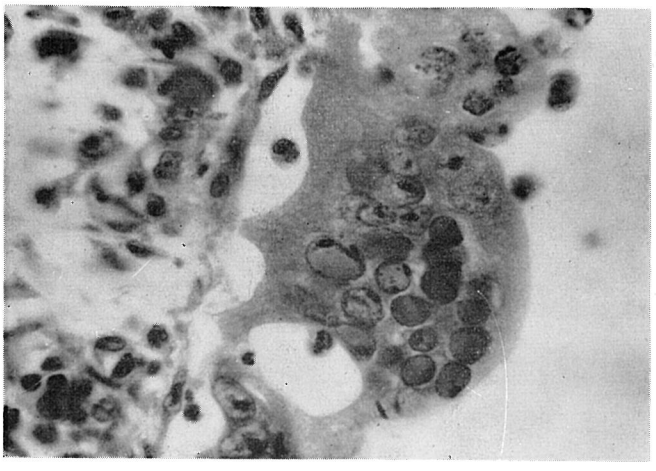

Fig. 3

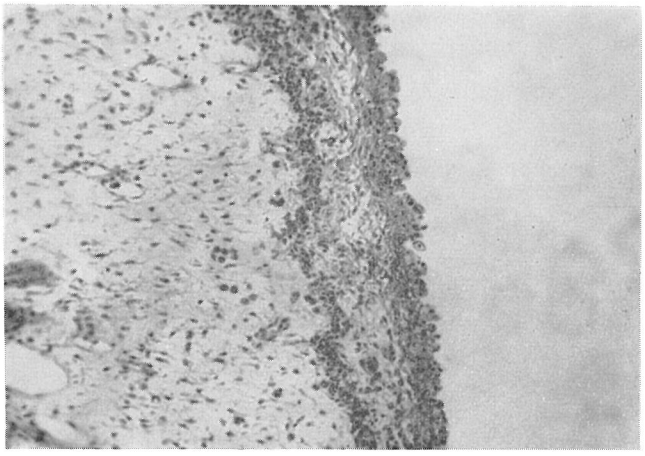

Fig. 4

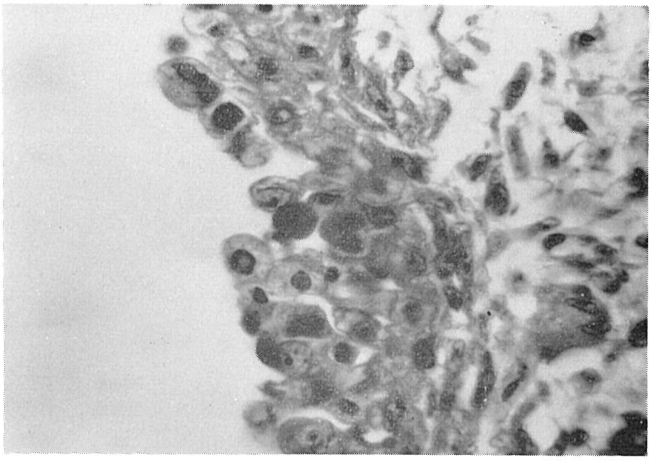

Fig. 5

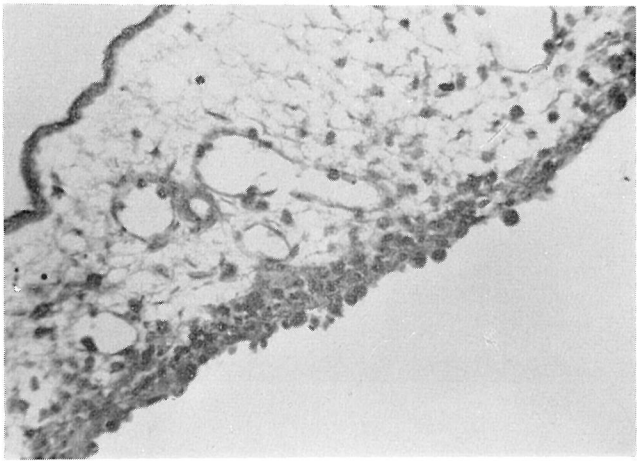

Fig. 6