BIKEN'S JOURNAL 1, 103-110 (1958)

The Fusion of Ehrlich's Tumor Cells caused by HVJ Virus*1 *in vitro*2

YOSHIO OKADA

Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University

(Received for publication, November 25, 1958)

SUMMARY

In the Ehrlich's tumor cell-HVJ system, fused cells are easily formed *in vitro* following conditions are fulfilled; 1) Concentrated viable tumor cells are used. 2) Incubation is made with mild shaking at 37°C. 3) A sufficient hemolytic activity of virus is used capable of causing a striking change or changes in the cell membrane. 4) An excess of virus over the maximal adsorption capacity of the cells is used.

The fusion phenomenon of the tumor cells seems to be a compensation for the injury of the cell membrane caused by the highly hemolytic virus.

INTRODUCTION

Recently the fusion of cells caused by viral action has been described in several reports. Fusion phenomena are classified into two categories according to their development. i) The cell fusion results from the direct action of virus on the cell membrane without any multiplication of virus. ii) The cell fusion appears in the later period of virus multiplication, i.e. a phenomenon caused by virus multiplication: e.g. giant cells in a measles virus-HeLa cell system. The fusion phenomenon treated in this paper belongs to the former case.

The author has described the appearance of many large fused cells within one hour after a large dose inoculation of HVJ into the abdominal cavity of mice implanted with Ehrlich's tumor cells. This effect of causing the fusion of cells seems to be proportional to the hemolytic activity of the virus as described in the preceding report (Okada *et al.*, 1957). These findings lead the author to a working hypothesis that the cell fusion phenomenon by HVJ is closely related to specific systematic, chemical and physical changes in the cell membrane similar to the hemolytic reaction of the red blood cell-HVJ system. However the fusion phenomenon which developes at a later period of the virus growth cycle is considered as a modified type of cytolysis. Though in such a case an autocatalytic processes may be involved in the final steps of the reaction, it is probable that there is reduction in some components of cell membrane. From the above mentioned view point the fusion phenomena are not essentially difference in nature.

This paper describes the results of experiments on the giant cell formation and the cytolytic action of HVJ virus *in vitro*.

---

*1 Synonym: Sendai virus, *Myxovirus parainfluenzae I.*

2 This paper was given at the annual meeting of the Society of Japanese Virologists in 1957.

Materials and Methods

Ehrlich's tumor cells: The ascites were withdrawn from mice 7 days after inoculation with 10^6 Ehrlich's tumor cells. Ascites and cells were separated by centrifugation. After washing three times with Hanks' solution to remove contaminating red blood cells the separated cells at suitable concentrations were suspended in the media shown in Table 1.

Table 1. Suspending media

Mouse ascites	clarified by centrifugation at 2,500 r.p.m. for 10 minutes,	2 volumes
Hanks' solution*	without glucose	6 volumes
Heparin solution	0.1% in saline	2 volumes

Virus: The chorio-allantoic fluid infected with HVJ, Z strain, was diluted to 10^{-6} with broth and 0.2 ml. aliquots of this diluted inoculum were injected allantoically into 10 day-old embryonated eggs. After 72 hour incubation at 35.5°C the culture was harvested and centrifuged at 3.000 r.p.m. for 30 minutes and the virus in the supernatant fluid was spun down at 20,000 r.p.m. for 30 minutes in a Spinco L ultracentrifuge. The resulting pellet was resuspended in Hanks' solution. The concentrated virus suspension was 128,000 HAU./ml.. After freezing and thawing 8 times the hemolytic activity of this suspension was enhanced about 10 times without any change in hemagglutination titer. The sample was diluted with Hanks' solution to the desired concentration.

Fusion experiments in vitro and the index of cell fusion: One half ml. of virus suspension was added to 0.5 ml. of tumor cell suspension in a 20 ml. volume vaccine vial and incubated in a water bath for 1 hour at 37°C with gentle shaking. The fusion reaction was completed within 1 hour and did not proceed further under these conditions.

When an adequate concentration of tumor cells was used, a low concentration of virus produced small fused cells while a concentrated virus suspension resulted in large fused cells.

The ratio between fused cells and free cells was low in the former case and was higher in the latter. The fusion rate was represented as follows. One drop of each sample was poured into a hemocytometer. Then photomicrographs of 5 microscopic fields were taken at random with a phase contrast microscope at low magnification. The negatives were projected at a total magnification of 1000 and the largest 10 cells were traced on graph paper and their mean crose sections relative to the normal cells was expressed as the cell fusion index. This representation was used for convenience although it shows only one aspect of the fusion phenomenon quantitatively.

Determination of hemolysis: Chicken red blood cells were washed 4 times with phosphate buffered saline at pH 7.2 and suspended in the same medium. An equal amount of the red blood cell suspension and the virus sample were mixed together and incubated in a water bath. After one hour's incubation at 37°C

*Hanks' solution without glucose was used throughout.

the samples were centrifuged at 1,500 r.p.m. for 5 minutes and the optical density of the supernatant was determined at 540 mμ. in a Coleman Junior spectrophotometer. The turbidity of the virus suspensions were subtracted from the optical density of the supernatant and the resulting values represented the amount of hemoglobin liberated.

Manometric determination: A 1.8 ml. aliquot of the tumor cell suspension (1.5×10^7/ml.), 120,000 HAU./ml. 0.2 ml. of virus, and 0.2 ml. of 10% KOH were placed in the main chamber, side arm, and center well, respectively of a working manometer. After 15 minutes shaking at 37°C, the virus in the side arm was tipped into the main chamber and manometer readings were taken for the next 60 minutes at 10 minutes intervals.

EXPERIMENTALS

Production of fused cells by incubation with shaking: As reported in the previous paper, while fused cells appear consistently in *in vivo* experiments, difficulties are encountered in forming them in *in vitro* experiments.

When the cell-virus mixture is incubated without shakings cytolysis chiefly occurs and no fused cells are formed. When concentrated HVJ which was freeze-thawed to increase the hemolytic activity was added to the tumor cells in a small test tube and incubated without shaking at 37°C, deformation and rupture of the cytoplasm was observed in almost all cells within 15 minutes and as a result cytolysis corresponding to hemolysis in a red blood cell-HVJ system was observed. (Fig. 1A) However when the cultures were shaken many giant fused cells were formed. (Fig. 1B)

As presented in Fig. 2 the electronmicrographs of these cells showed a rough cell membrane and destruction and vacuolization of the cytoplasm in lysed cells but no abnormality in the cytoplasm of the fused cells was observed except for an increase in numbers of microvirii.

Since the fusion phenomenon occurred even under anaerobic conditions with shaking and DNP had no effect on giant cell formation at a concentration lower than the concentration causing cell degeneration, the effect of shaking is not that of simple aeration of the reaction mixture. From the author's impression it seems that the tumor cell membranes which were strongly effected by the viral action must receive some hearling effect from mechanical shaking to complete fusion of the cell membranes. If they are not shaken the injured cells are not able to keep their live state as they lack functionate-limiting membranes.

Endogeneons O_2 uptake by fused cells: Within 30 minutes of the addition of the virus more than 80% of the tumor cells were fused into giant cells. As shown in Fig. 3, O_2 uptake by the cells through this fusion reaction and that of completed fused cells was nearly equal to that of normal cells. In the cells lysed by freezing and thawing once, O_2 uptake was much reduced.

Relationship between the cell fusion index, the concentration of cells and the virus concentration: Fig. 4 illustrates the changes of fusion index at various concentration of cells and virus. At a high cell concentration (1×10^7/ml.) the mean size of the fused cells is proportional to the virus concentration and at a low cell concentra-

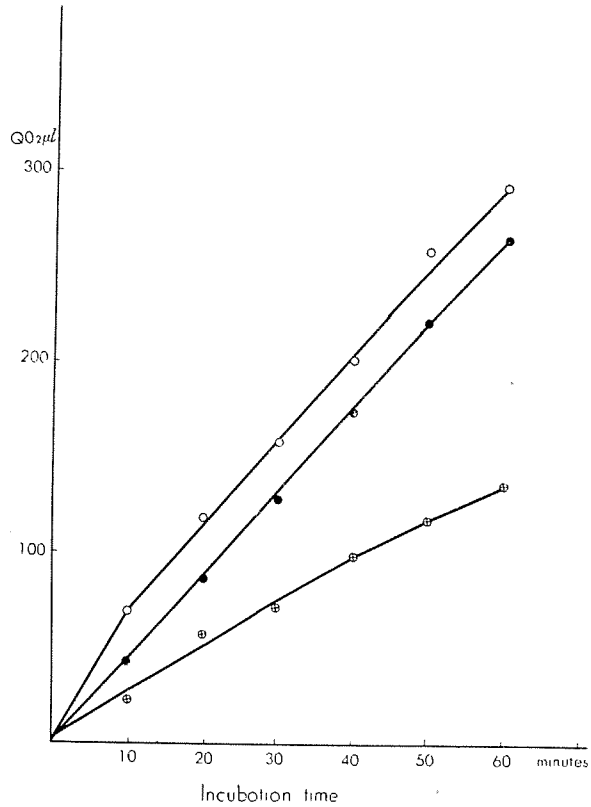

Fig. 3 O_2 uptake of Ehrlich's tumor cells fused by HVJ. The cell number at the beginning of the experiment: 3.0×10^7 ETC
○——○ Fused giant cells (in shaking culture)
●——● Normal cells (control)
⊕——⊕ Cells lysed by freezing-thawing once (without virus)

tion (2.5×10^6/ml. and 1.25×10^6/ml.) the size is independent of the virus concentration though it is a little larger than that of the control. The mononuclear cells remaining in the reaction mixture also increase in size when virus adsorption occurs and some show cytolysis. The higher the virus concentration, the more cytolysed cells appear.

At an intermediate concentration of cells (5×10^6/ml.) the maximum point for the mean size was found at 8192 HAU. virus titer and when a large amount of virus was used at this cell concentration the mean size decreased. Tentatively it is concluded that when a sufficient amount of virus was added to tumor cells at low concentration cytolysis was dominant and fusion occurred only at a high cell concentration.

Relation between the cell fusion index and the hemolytic activity: Figure 5 shows the relationships between the hemolytic activity of the virus and the cell fusion index when a constant amount of the cells (1×10^7/ml.) was used. The whole cell

A

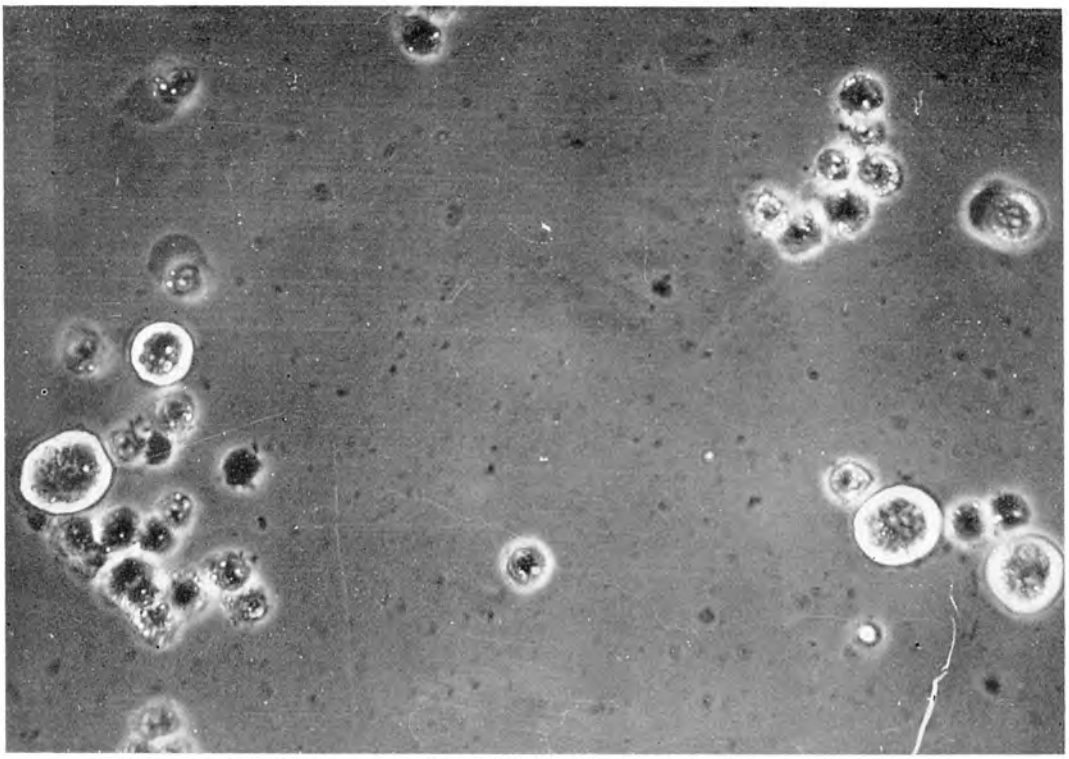

Fig. 1A. Ehrlich's tumor cells mixed with HVJ *in vitro* showing cytolysis. (Stationary culture)

B

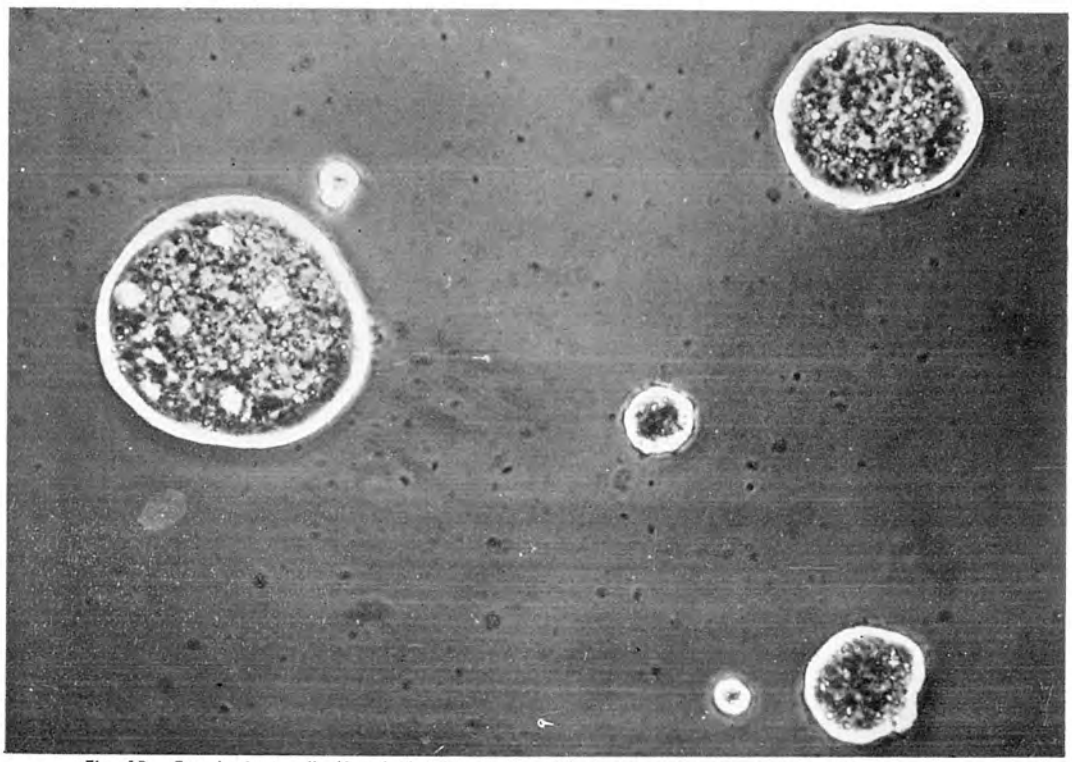

Fig. 1B. Fused giant cells (fused Ehrlich's tumor cells) produced by HVJ *in vitro*. (Shaking culture)

(OKADA)

A

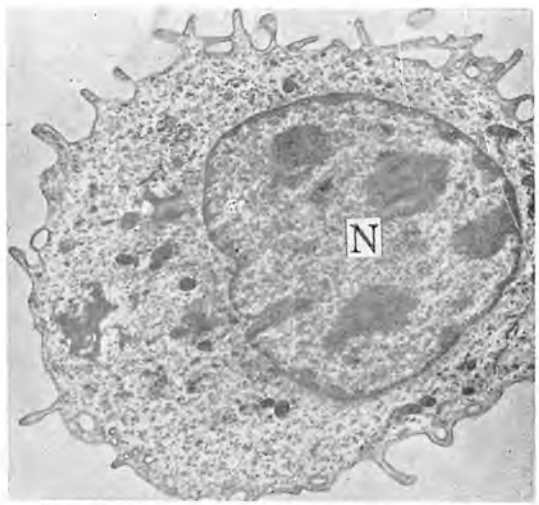

Fig. 2A. Electronmicrographs of Ehrlich's tumor cellssh owing the effects of HVJ *in vitro*. Normal cell. N : Nucleus

B

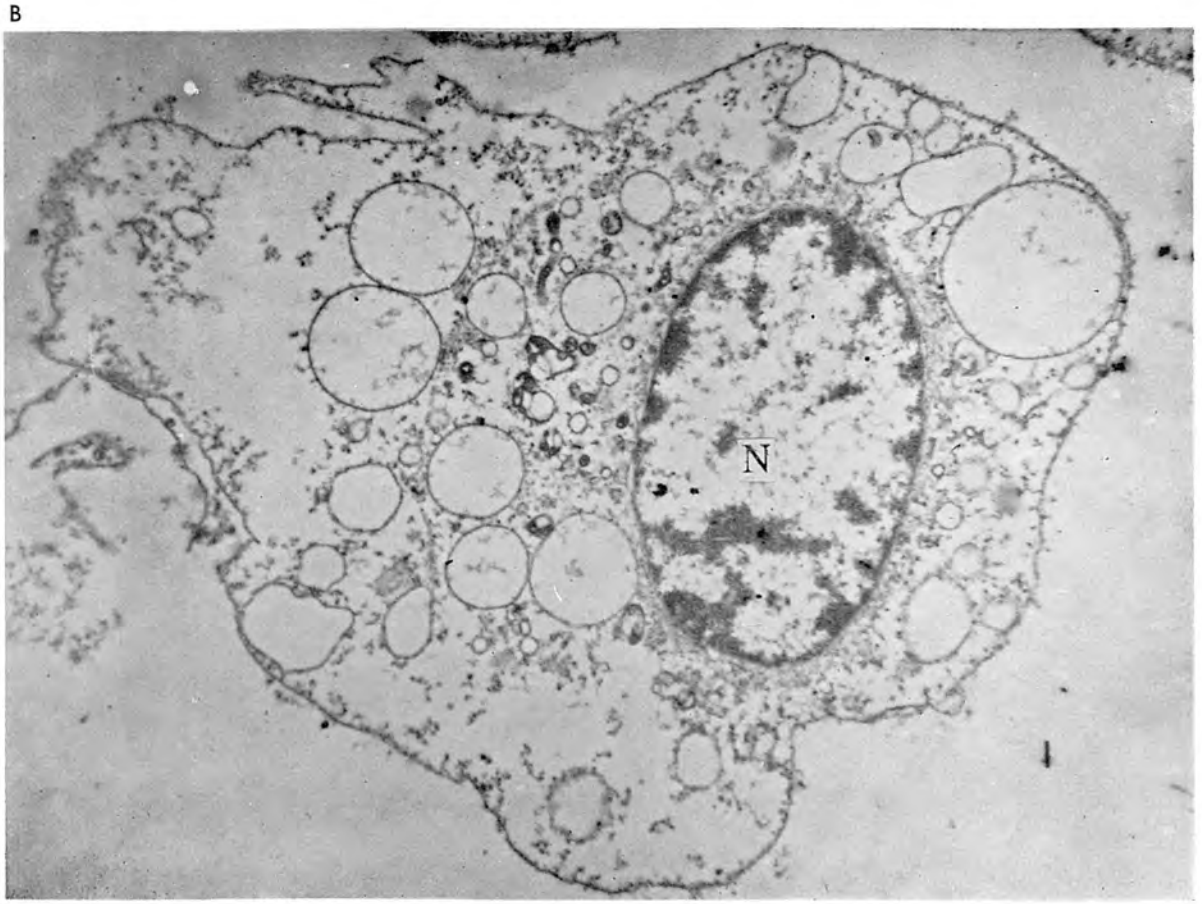

Fig. 2B. Electronmicrographs of Ehlicher's tumor cells shownig the effects of HVJ *in vitro*.
Cell from stationary culture showing heavy damages in cytoplasm and cell membrane.

(OKADA)

C

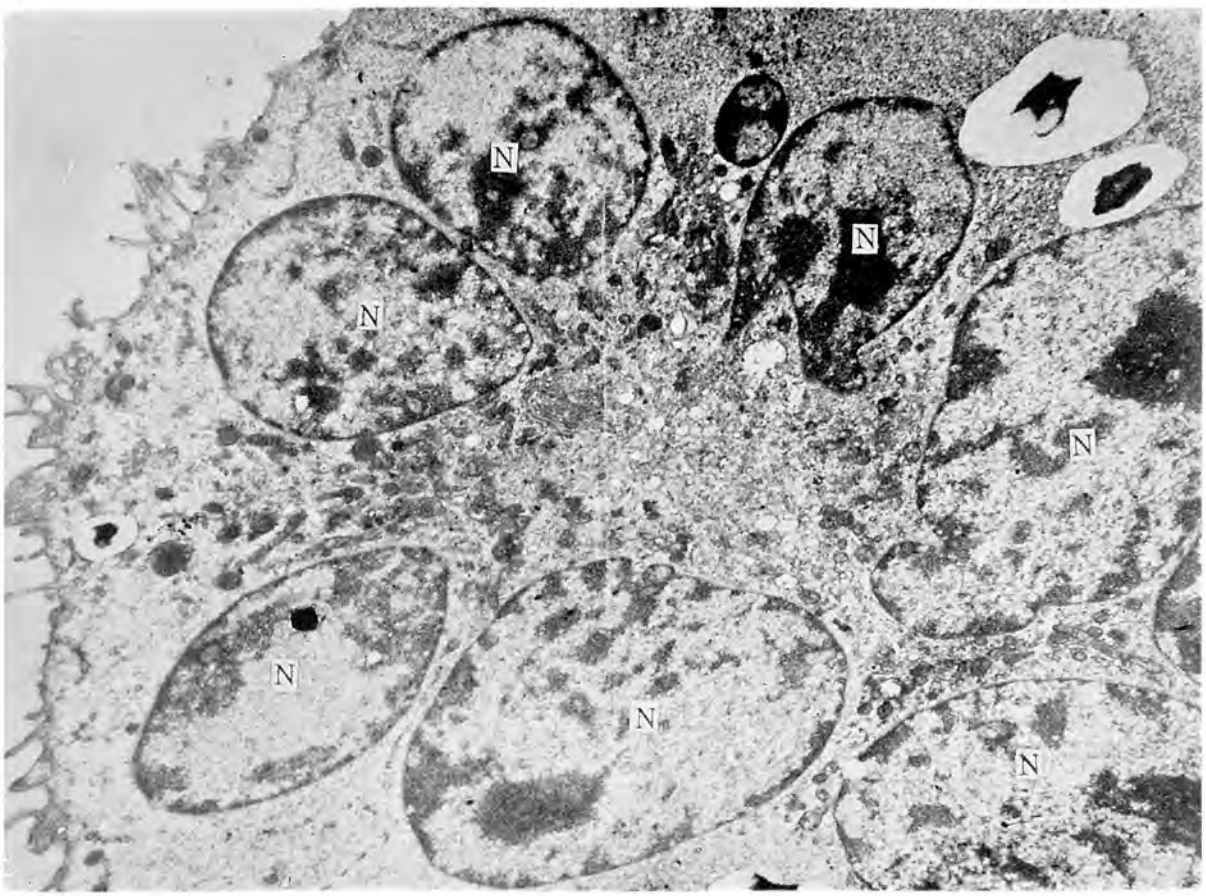

Fig. 2C. Electronmicrographs of Ehrlich's tumor cells showing the effects of HVJ *in vitro*.
A part of fused giant cell from shaking culture.

(OKADA)

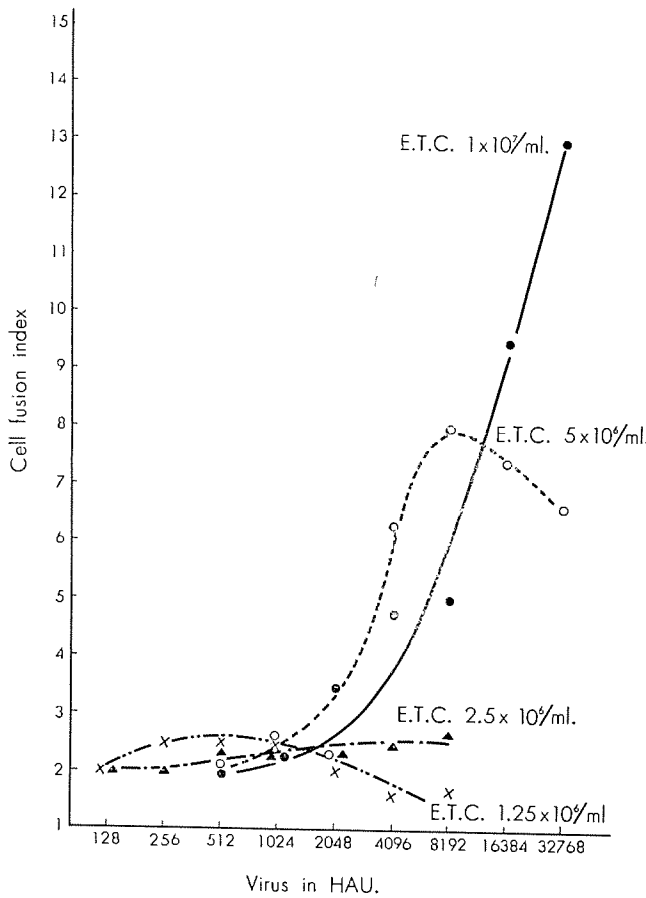

Fig. 4. Relationship between the cell fusion index, cell concentration and virus concentration.

number in the reaction mixture, that is the sum of the number of fused cells and non-fused cells, is inversely proportional to cell fusion index. This means that these are conversion of nonfused cells into fused cells.

As shown in Fig. 5, the cell number apparently decreases when the amount of the virus in the system is increased. The cell fusion index curve shows one inflexion point at the virus amount of 16,384 HAU.. On the left side of this point the resulted fused cells are all small, while on the right side the cells showing larger diameter are observed. This abrupt change in the cell fusion index curve means that the fused cell production by smaller amount of the virus are qualitatively different from that by larger amount (i.e. more than 16,384 HAU.) of the virus.

The direct relationships between cell fusion and the hemolytic activity of the virus was studied. The surface area of 3×10^7 red blood cells was considered equivalent to the surface area of 1×10^7 Ehrlich's tumor cells because it had been accepted that the Ehrlich's tumor cells have a surface area about three times

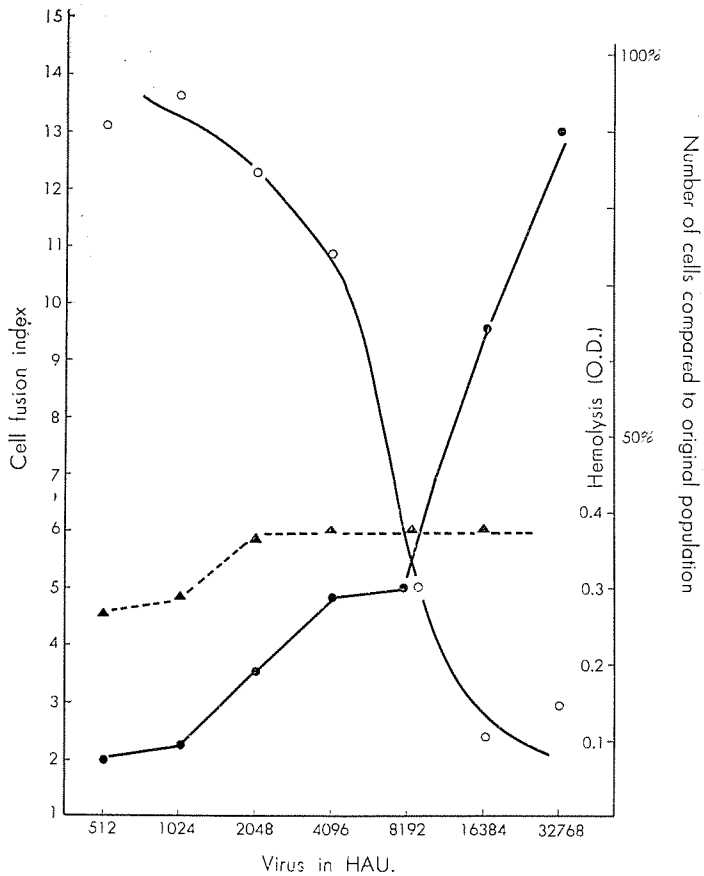

Fig. 5. Relationship between the cell fusion reaction and the hemolytic activity of HVJ.

○——○ Cell Number %　　●——● Cell fusion rate　　▲——▲ Hemolysis

that of fowl red cell and the receptors for the virus are distributed equally over both kinds of cells (Fukai, Okada and Suzuki, 1955).

When 3×10^7 red blood cells were mixed with amounts of virus from 64 to 33,000 HAU., the degree of hemolysis when less than 1024 HAU. of virus was used, was different from that with more than 2048 HAU. virus. In the former case the lysed red blood cells still held residual amounts of hemoglobin macroscopically (partial hemolysis) while in the latter case the pellet of lysed cells was white showing complete hemolysis.

In a similar experiment with Ehrlich's tumor cells it was found that for cell fusion at least 2048 HAU. of the virus was required for 1×10^7 cells. Therefore with red blood cells, 3×10^7 cells are completely hemolysed by 2048 HAU. of the virus.

When a larger amount of the virus is given to the same number of red blood cells only 2048 HAU. are effective and the excess virus does not cause further hemolysis. However with Ehrlich's tumor cells, the threshold virus level for

fusion of 1×10^7 cell is 2048 HAU. and fusion increases with an increase in virus concentration above this threshold level. It also seems as if the injuries caused in Ehrlich's tumor cells during the fusion reaction may be greater than those in red blood cells during the hemolytic reactions.

In this experiment the amount of virus adsorbed onto the Ehrlich's tumor cells could scarcely be determined from the amount of unadsorbed virus because the adsorbed virus was much less than the total virus added. To determine the exact amount of virus adsorbed some new method may be necessary.

Determination of the adsorbed virus by elution was impossible because when once absorbed onto the cells, some part of the virus formed a strong combination and disappeared as incubation proceeds at 37°C (Okada and Suzuki, 1956).

DISCUSSION

The requirements for the completion of the cell fusion reaction in an HVJ-Ehrlich's tumor cell system *in vitro* are as follows:

1) Concentrated, viable tumor cells must be used.
2) The cell virus mixture must be incubated with mild shakings at 37° C.
3) A virus having sufficient hemolytic activity to effect the cell membranes greatly must be used.
4) An amount of virus in excess of the maximal adsorption capasity of the cells must be used.

When sufficient virus and cells are mixed at 37°C, the virus particles are adsorbed onto the cell surface and agglutination occurs. Then the cell membrane is strongly attacked by the action of the virus particles and, in the case of stationary culture cytolysis results. When the system is shaken, the cell aggregates form semipermeable barriers around them, recover the activities of the individual cells and fused cells result. From this view point the fusion reaction looks like a kind of repair of the damaged cell membrane. Ultra-thin section electronmicrographs of the cells during the fusion reaction supports this view.

While there is much work on the receptor destroying activity of the MNI group viruses, studies on the hemolytic activity of mumps, Newcastle disease virus and HVJ have not yet made. As described in this paper there is a definite relationship between the hemolytic activity of HVJ and its cell fusion activity on Ehrlich's tumor cells. Therefore it may be helpful to analyse the hemolytic reaction of this virus to understand what happens in the course of cell fusion.

Recently Mobery *et al.* (1958) showed a decrease of sphingomyelin in the membrane of fowl red blood cells hemolysed by mumps virus and Hosaka (1958) showed that the degree of hemolysis by HVJ is proportional to the decrease of sphingomyelin in the membrane of fowl red blood cells. It is well known that the hemolytic reaction is generally due to changes in phospholipid components in the membrane of red blood cells. Therefore a study of the relationship between the the destruction of sphingomyelin or some other components in the cell membrane and the cell fusion reaction in the HVJ-Ehrlich's tumor cell system is of interest for the analysis of the fusion reaction.

The biggest question is why the threshold level of the virus for cell fusion is

distinctly higher than the adsorption capacity of Ehrlich's tumor cells. The author is very much interested in analysing the meaning of this " excess " virus in the cell fusion reaction.

From the physical view point, great changes in the electric double layer of the cell membrane must be caused by viral action. The reason for the requirement for excess virus could be explained along these line.

The author wishes to thank Dr. Kazuo Doi for excellent electronmicrographs. Thanks are also due to Dr. Tetsuo Yoshishita for taking part in some of the experiments and Miss Kazuko Kawai for technical assistance.

REFERENCES

Fukai, K., Okada, Y. and Suzuki, T. (1955) The viral agglutination of Ehrlich's tumor cells *in vitro*. *Med. J. Osaka Univ.* **6**, 17–18.

Hosaka, Y. (1958) On the hemolytic action of HVJ. II. Decrease of sphingomyelin in red blood cells hemolysed by HVJ. *Biken's J.*, 1, 90–99.

Morbery, M. L., Marinetti, G. V., Writter, R. F. and Morgan H. F. (1958) Studies of the hemolysis of red blood cells by mumps virus. III. Alteration in lipoprotein of the red blood cell wall, *J. Exp. Med.*, **107**, 87–94.

Okada, Y., and Suzuki, T. (1956) Interaction between influenza virus and Ehrlich's tumor cells. II. Fate of the virus particles adsorbed onto Ehrlich's tumor cells. *Med. J. Osaka Univ.*, **6**, 987–994.

Okada, Y., Suzuki, T. and Hosaka, Y. (1957) Interaction between influenza virus and Ehrlich's tumor cells. III. Fusion phenomenon of Ehrlich's tumor cells by the action of HVJ Z strain. *Med. J. Osaka Univ.*, **7**, 709–717.