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Difference in Capacities for Virion-to-Virion Fusion of Young and Aged HVJ (Sendai Virus): A Model of Membrane Fusion

Jeman Kim and Yoshio Okada
Institute for Molecular and Cellular Biology, Osaka University, Osaka, 565, Japan

Summary. Young and aged HVJ virions differ structurally and morphologically due to changes that occur during aging in vitro or in ovo. Young virions soon after their budding off are rod-shaped, rigid and relatively uniform in size, whereas virions that have aged in vitro after their formation are round, nonrigid and variable in size. These changes during aging seem to be due to the variation of M protein, a "skeletal" protein that is associated with both the envelope membrane proteins and nucleocapsid strands in the virions. The capacities for virion-to-virion fusion of young and aged virions were compared to clarify the relation between the membrane fusion and membrane-associating skeletal proteins. On treatment with polyethylene glycol (PEG), aged virions readily fused, forming large virion vesicles, but young virions were resistant to fusion. Further, aged virions fused even on incubation at 37°C without the fusogen. Thus the capacity for virion-to-virion fusion evidently increases during aging of virions. This result suggests that skeletal proteins associating with the biological membrane are important for preventing membrane fusion, and that virion-to-virion fusion is a good model system for use in studies on the mechanism of membrane fusion.

Key Words Sendai virus · virion-to-virion fusion · membrane-associating protein · membrane fusion · virus aging

Introduction

Membrane fusion is an important biological event that is observed in various biological phenomena. There have been many studies on the mechanism of membrane fusion, but still little is known about the molecular mechanisms involved, or the factors that control it, because of the complexity of the membrane structure and its dynamics. We have been studying events in cell fusion with HVJ (Sendai virus) to clarify the fusion mechanisms (Kim & Okada, 1980, 1981, 1982). When cell fusion is induced by the virus, the cell membrane transiently becomes a labile state; the membrane loses its function as an ion barrier, the cytoskeletal structure is destroyed, and integral proteins of the cell membrane become very mobile in the lipid plane. With progress of fusion reaction, these alterations are reversed. Similar phenomena were seen during cell fusion induced by polyethylene glycol (PEG) (Robinson et al., 1979). These findings suggest that the interaction of membrane proteins with associated skeletal proteins that may fix the integral proteins is transiently disrupted during membrane fusion and that the skeletal proteins are involved in supporting the membrane structure. In this work, we tried to examine the relation of membrane-associated skeletal proteins with membrane fusion, using HVJ envelope membranes.

HVJ is an enveloped virus of the paramyxovirus family. Of the proteins in this virus, only one (M protein) could be a candidate of viral skeletal protein and two could be membrane proteins (Chen, Compans & Choppin, 1971; Mountcastle, Compans & Choppin, 1971; Homma & Ohuchi, 1973). So the envelope membrane of this virus is one of the simplest biological membranes known. Moreover, this virus can be obtained in larger quantity than other animal viruses, and it has no energy-producing or protein-supplying system. It is thus a convenient model for use in studies on the mechanism of membrane fusion.

Another advantage of use of HVJ is that the virions show unique structural changes after their formation (Kim et al., 1979; Bächi, 1980). Usually HVJ is propagated in the chorioallantoic cavity of 10-day-old embryonated eggs for three days after infection, and then the chorioallantoic fluid containing HVJ virions is chilled for one day and harvested. This chorioallantoic fluid is then stocked in a cold room at 4°C, and the virions are purified by repeated ultracentrifugation. Therefore, it takes at least more than a week to purify the virions after their formation by budding from the host cell, and consequently aging of the virions occurs. As reported previously, most virions prepared in this way are round and variable in size and contain randomly coiled nucleocapsid strands (Kim et al.,

1979). These virions are not rigid, and so their shape is affected by physical forces. Further, large intramembrane particles can be detected in them on the plane of freeze-fracture. However, when the virus is harvested 24 hr after infection and purified within a few days, we can obtain relatively young virions soon after budding off. Most of these virions are rod-shaped, relatively small and uniform in size, and contain nucleocapsid strands that are regularly folded along the long axis and located just under the envelope membrane (Kim et al., 1979). Virions harvested in this way are rigid and resistant to external physical force. Moreover, no intramembrane particles are observed on freeze-fracturing. Namely, HVJ virions change structurally with age as follows: (1) they become variable in size, (2) nucleocapsid strands dissociate from the envelope membrane, (3) spike protein molecules associate with each other in the lipid bilayer. These changes seem to be due to a variation of M protein which may have less interaction with the envelope membrane and nucleocapsid strands with age. Thus, to obtain a clue to the relation between membrane fusion and the skeletal proteins associated with the biological membrane, we compared the virion-to-virion fusion capacities of young and aged virions.

Materials and Methods

Preparation of HVJ Virions

Young and aged virions were prepared as reported previously (Kim et al., 1979). Briefly, 10-day-old embryonic eggs infected with HVJ were incubated for one day ('early harvest' for young virions) or three days ('late harvest' for old, aged virions) at 37°C, and then the eggs were chilled overnight and the chorioallantoic fluid was harvested. The harvested chorioallantoic fluid was kept at 4°C until use. The virions were purified from the chorioallantoic fluid by repeated ultracentrifugation as reported previously (Kim et al., 1979). Aged virions were aged for more than three weeks in the chorioallantoic fluid, while young virions were used within two to three days after harvest.

Fusion of Virions

Young and aged HVJ virions purified by differential ultracentrifugation were suspended at 20,000 to 30,000 HAU/ml in BSS (balanced salt solution buffer, pH 7.4) containing 22.5% PEG 4,000 (Nakarai Chemical Co., Ltd., Kyoto, Japan) and centrifuged at 20,000 rpm (Beckman rotor #30) for 30 min at 4°C. The resulting pellet was overlayed with 1 ml of 45% PEG solution and incubated for 30 min at 37°C with gentle shaking. After the incubation, PEG was removed by ultracentrifugation and the pellet was washed twice with PBS (phosphate-buffered saline) by centrifugation. In studies on fusion without PEG the suspension of aged virions in BSS buffer was centrifuged at 18,000 rpm (Sorvall ss-34 rotor) for 45 min and the resulting pellet of virions was incubated in BSS for 60 min at 37°C. When necessary, calcium chloride was added in the reaction medium at a concentration of 1 mM.

Electron Microscopy

The pellet of virions was fixed with 2% glutaraldehyde and then 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4. The sample was then routinely dehydrated with ethanol and embedded with Epon resin. Ultrathin sections were observed after double staining with uranyl acetate and lead acetate solution (Kim et al., 1979). When necessary, part of the pellet was resuspended in PBS and negatively stained with 3% potassium tungstate.

Results

Fusion of Aged Virions Induced by PEG

Before comparing the fusion capacities of young and aged virions, we examined the fusion of HVJ virions under drastic conditions to determine whether their fusion actually occurred. For this, aged virions were treated with a high concentration of PEG for a long time; namely, the aged virions collected together as a pellet by ultracentrifugation were incubated with 45% PEG at 37°C for 30 min.

When the resulting virions were examined by negative staining, many very large virions were seen that were much bigger than any detected in preparations before treatment with PEG, even though preparations obtained by late harvest consist of virions of various sizes. The presence of these large virions suggested that the HVJ virions had fused together. Then thin sections of the preparation were examined. Figure 1 is a section of aged virions treated with PEG. Many large vesicles are seen that appear to be fused virions. These large vesicles were about 1 μm in diameter and constituted about 30 to 50% of the total number of virions. These large vesicles had spikes on their membrane and contained nucleocapsid strands. These results suggested that the large vesicles were virion vesicles formed by fusion of virions. Most of these virion vesicles were unilamellar.

At higher magnification, the membrane of these large virion vesicles was formed to have a typical lipid bilayer structure, but with fewer spikes than on the membrane of unfused virions (Fig. 2). In many cases, the spikes had a patchy distribution interspersed with regions of membrane with no spike. It is noteworthy that viral spikes were often arrayed on both sides of the membrane (arrowheads in Fig. 2), suggesting that the lipid bilayer structure of the

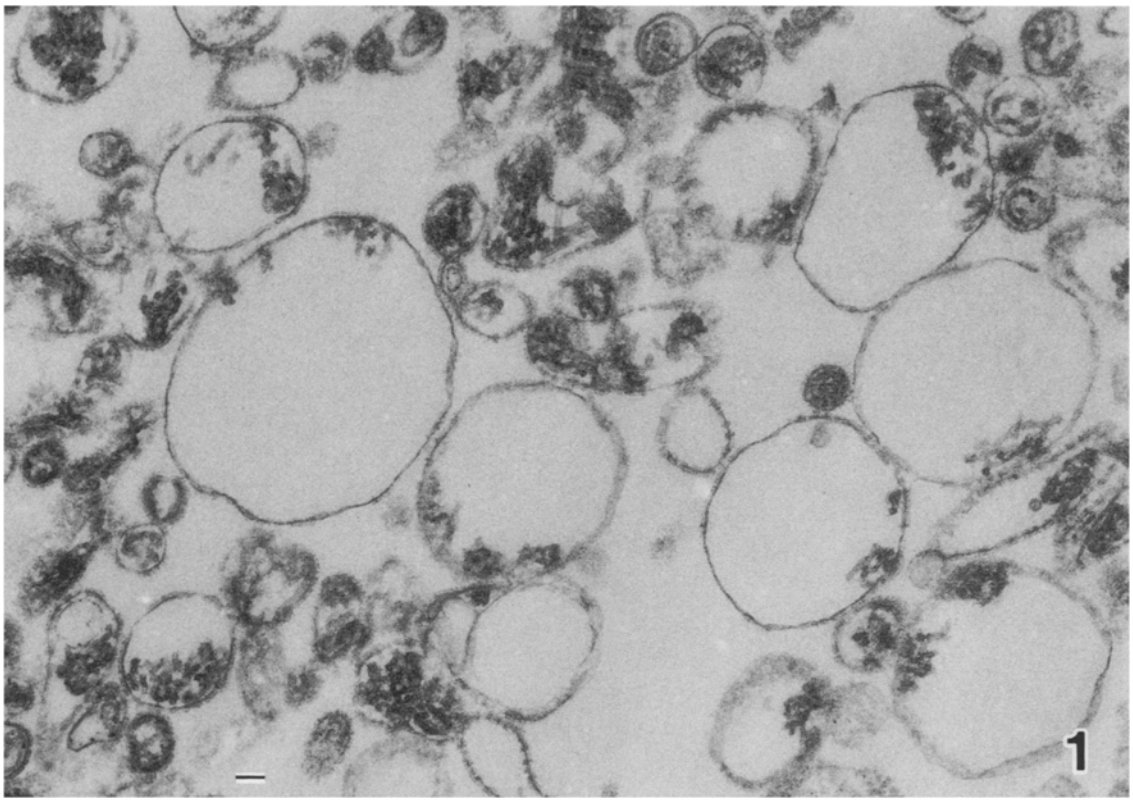

Fig. 1. Section of aged HVJ virions treated with PEG. HVJ virions obtained by late harvest that were aged in ovo and in vitro were precipitated by ultracentrifugation and incubated with 45% PEG at 37°C for 30 min. Many large virion vesicles formed by fusion of virions are seen among unfused virions. In the large fused virions, nucleocapsid strands are present associated with the membrane in limited regions. Many nucleocapsid strands have become amorphous and electron dense on treatment with PEG. 40,000×; bar, 0.1 μm

viral envelope was broken down by treatment with the fusogen. Another noteworthy point was that nucleocapsid strands in both fused and unfused virions were condensed, and often appeared amorphous. Consequently, most of the nucleocapsid strands in large virion vesicles were detached from the membrane, forming closely packed clusters and were characteristically located at one side of the vesicles still partially associated with limited areas of the inner surface of the membrane. In such cases, spikes were often detected on the surface of the membrane above the regions to which the nucleocapsid strands were still bound on the inner surface. Further, the spikes in this area were seen only on the outer side of the membrane (arrows in Fig. 2). After treatment with PEG, unfused virions appeared smaller than before treatment and irrespective of virion fusion, the nucleocapsid strands became more electron dense (*compare* Figs. 1 and 2 with Fig. 7). These changes may be due to loss of some components from the virions on PEG treatment.

Fusion of Young Virions with PEG

As shown in Fig. 3, the young virions are mostly rod-shaped and contain regularly folded nucleocapsid strands. Many connecting structures between the envelope membrane and nucleocapsid strands or between nucleocapsid strands are observed in these virions at higher magnification (inset of Fig. 3). These connecting structures disappear during aging of virions. Thus, the structure of young virions appears rigid, unlike that of aged virions. We next examined whether these structural differences between young and aged virions affected virion-to-virion fusion. The young virions were prepared from early harvest virions and treated with PEG in the same way as aged virions.

In contrast to aged virions, the young virions showed strong resistance to virion-to-virion fusion with PEG. Figure 4 shows a section of these virions after treatment with PEG. Most virions did not fuse. Moreover, this treatment did not cause any obvious changes in morphology; many virions were

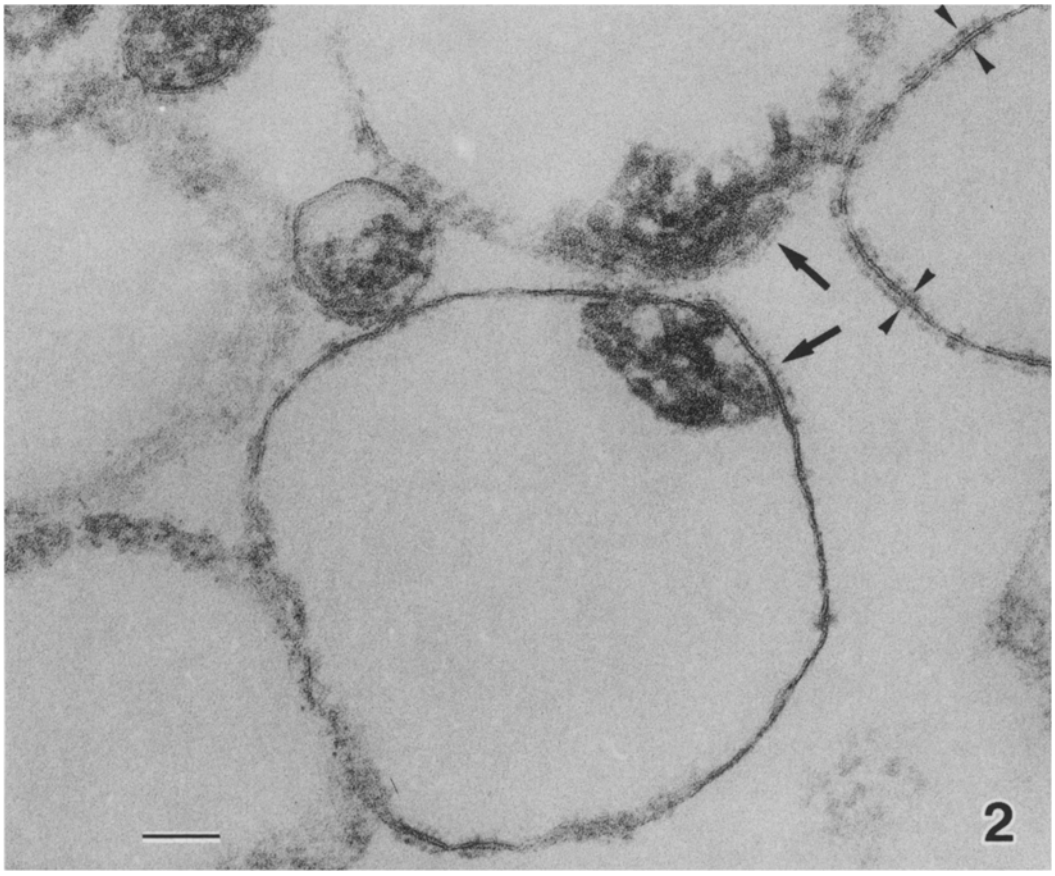

Fig. 2. Higher magnification of aged HVJ virions treated with PEG. The membrane of the large virion vesicles shows a typical lipid bilayer structure and spikes are detected on the membrane. Spikes on the large virion vesicles are generally sparse and localized in patches. Occasionally spikes are arrayed on both sides of the membrane (arrowheads). The nucleocapsid strands form clusters and are located on one side of the vesicles, partially associated with the restricted region of the membrane in which the spikes are present only on the outer surface (arrows). 100,000×; bar, 0.1 μm

still rod-shaped and retained the typical morphology of young virions after PEG treatment. But many of these nucleocapsid strands became amorphous, although they did not become compactly condensed like those of aged virions; namely, they mainly remained largely associated with the inner surface of the membrane. Moreover, the spike array of the young virions was not changed. However, a few larger virion vesicles were seen that seemed to have been formed by the viral fusion (inset of Fig. 4). In these larger virions, the nucleocapsid strands were clustered at one side of the vesicles as in the vesicles of aged virions. These larger virion vesicles could also be seen by negative staining (*data not shown*). Thus, a few virions in the early harvest, possibly relatively aged virions, seemed to fuse. The frequency of these virions was, however, much lower than that of large virion vesicles in the late harvest preparation, and these fused vesicles were smaller than those formed from aged virions. Thus treatment with PEG had clearly different effects on the virions in the early harvest and late harvest preparations (*compare* Fig. 1 with Fig. 4).

Fusion of Aged Virions Without Fusogen

The results with PEG, described above, show that envelope membranes of aged virions are readily fusible, whereas those of young virions are strongly resistant to fusion. This seems to reflect the difference in rigidity of young and aged virions. To confirm this, we tried to fuse aged virions without a fusogen. For this we used virions that had been aged for a longer time; namely, we purified virions from chorioallantoic fluid that had been stored for

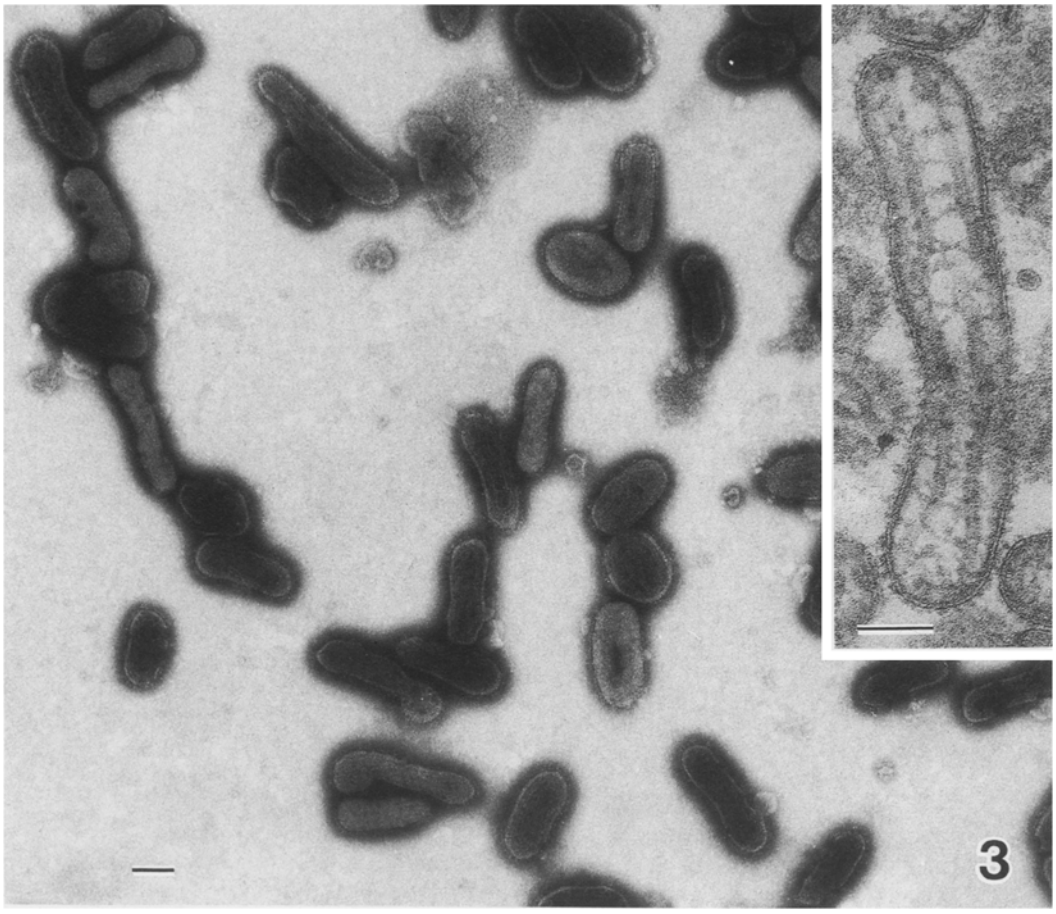

Fig. 3. Negative staining of young virions obtained by early harvest after fixation with 1% glutaraldehyde. Most virions are similar in size and rod-shaped. The nucleocapsid strands are regularly folded along the long axis. Inset: thin section of a young virion showing structural connection of substances between nucleocapsid strands or nucleocapsid strands and the envelope membrane. 48,000×; bar, 0.1 μm. Inset, 100,000×; bar, 0.1 μm

about 5 weeks at 4°C after harvest and finally pelleted by ultracentrifugation, and incubated the pellet at 37°C for 60 min in BSS buffer. Results are shown in Figs. 5 and 6. After negative staining, many very large virions were seen (Fig. 5), and some were giant virions covered with many spikes. Very large virions were also seen among the virions in thin sections (Fig. 6), although at lower frequency than after treatment with PEG. Moreover, unlike in preparations treated with PEG, intermediate steps of virion-to-virion fusion were frequent in preparations of aged virions incubated at 37°C (arrow in Fig. 6). In addition to the intermediate step (arrow), contact regions of two apposing viral envelope membranes (arrowheads) are also detectable. Figure 7 is a section of control aged virions from the same lot as those in Figs. 5 and 6 (incubated at 0°C). A clear contrast can be seen (*compare* Fig. 6 with Fig. 7). The sample incubated at 0°C has the typical appearance of late harvest virions; the virions vary in size, but these are not very large virions. HVJ virions are generally thought to be 150 to 600 nm in diameter (Hosaka, Nishi & Fukai, 1961). However, most of the virions harvested from embryonated eggs are 200 to 350 nm in diameter and few are more than 500 nm in diameter. On the other hand, as shown in Fig. 6, many large virions were seen in the sample incubated at 37°C; some of them were more than 1 μm in diameter and intermediate steps of fusion were also detected. These findings indicate that aged virions fuse with each other at 37°C. This fusion occurred irrespective of the presence of calcium ion in the buffer (*data not shown*).

The large virion vesicles differed morphologically from those formed on treatment with PEG. Virions incubated without the fusogen were with

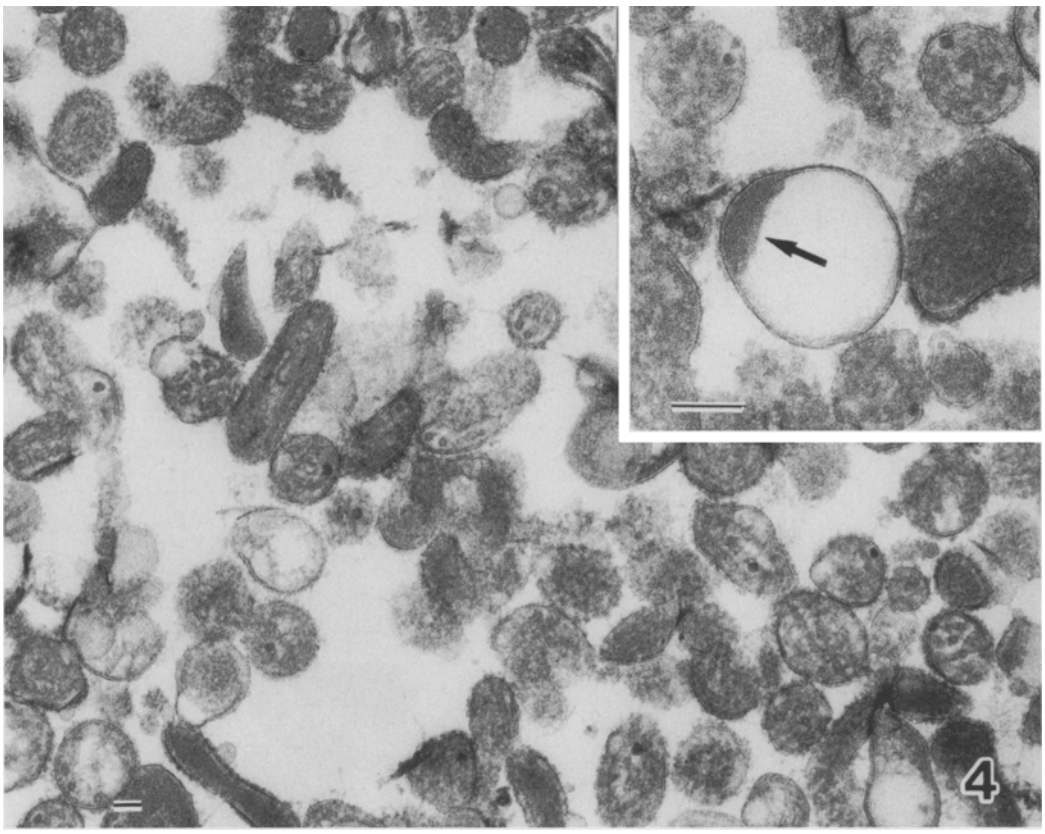

Fig. 4. Section of young HVJ virions treated with PEG. Young virions obtained by early harvest were treated with PEG under the same conditions as aged virions. Unlike aged virions, most of the virions are not fused and retain the rod shape and morphology of young virions. The nucleocapsid strands in many virions have become amorphous, but are not clustered and are still broadly associating with the envelope membrane. The nucleocapsid strands are clearly different from those in aged virions: Inset: A relatively large vesicle that seems to have been formed by virion-to-virion fusion. The amorphous nucleocapsid strand is located on one side of the vesicle (arrow) as in aged virions. Spikes are seen only on the outer surface in the region with which the amorphous nucleocapsid strand is still associated. 60,000×; bar, 0.1 μm. Inset, 100,000×; bar, 0.1 μm

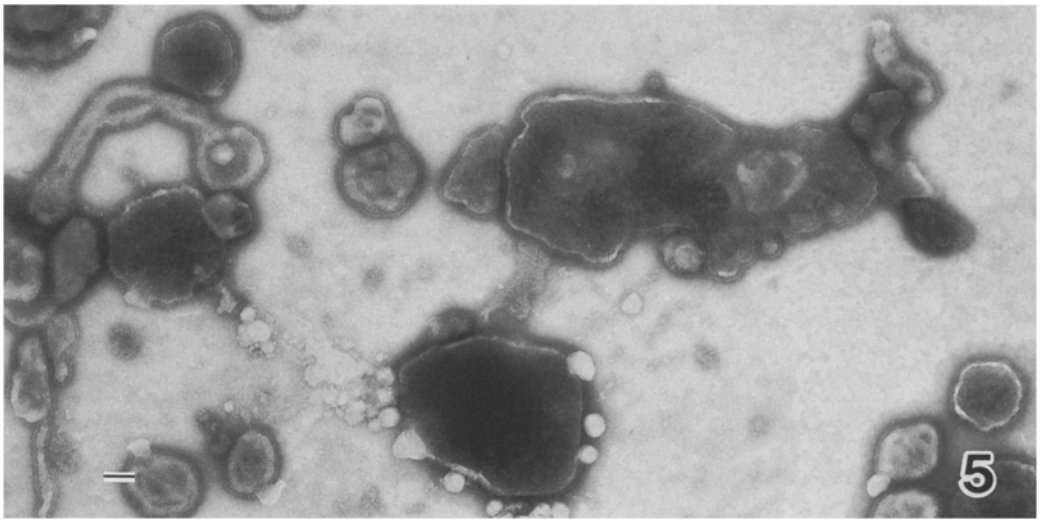

Fig. 5. Negative staining of aged virions incubated at 37°C for 60 min without PEG. Virions show the typical morphology of aged virions with various shapes. Very large virions are frequently seen among the virions of various sizes. Virions are covered with many spikes and the randomly coiled nucleocapsid strands are seen. 36,000×; bar, 0.1 μm

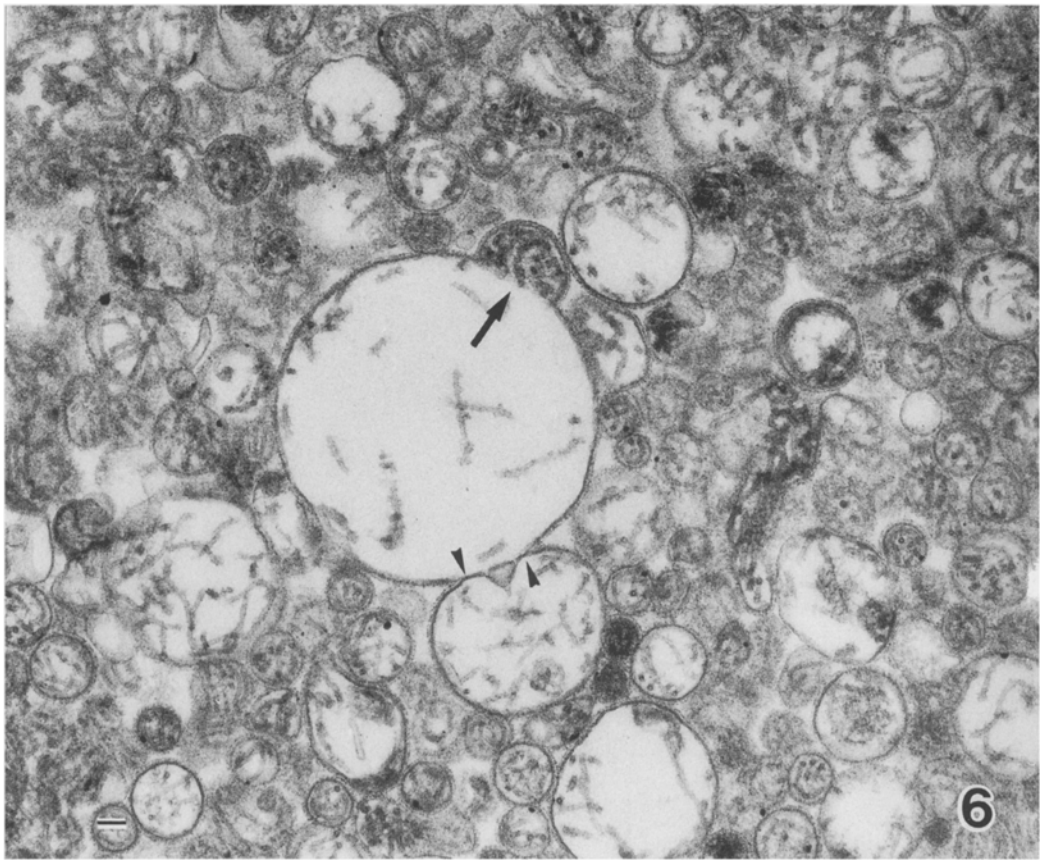

Fig. 6. Section of aged virions incubated at 37°C for 60 min without PEG. The sample is from the same lot as for Figs. 1 and 5. Very large virions are seen. An intermediate step of viral fusion is seen (arrow). The fused virion is covered with many spikes, unlike the virion vesicle, formed by PEG. In addition to the intermediate step of fusion (arrow), contact regions of two apposing viral envelope membranes where fusion will occur (arrowheads) are also seen. 36,000×; bar, 0.1 μm

many spikes like the natural virions, and no spikes were detected on the inner surface of the membrane. Moreover, nucleocapsid strands were not clustered, but distributed randomly as in intact aged virion. Thus the large virion vesicles were morphologically similar to natural virions.

Discussion

From the results in this paper, it is clear that aged virions tended to fuse, whereas young virions were strongly resistant to fusion. These findings suggest that structural changes during aging of the HVJ virions are closely related to the capacity for virion-to-virion fusion. Thus one reason why virions obtained by late harvest contain virions of variable size including large virions may be because virion-to-virion fusion occurs in aged virions.

We think this difference in the fusion capacity may be related to change in the interaction of M protein with the envelope membrane. M protein, which is present inside the virion, is associated with both nucleocapsid strands and the membrane proteins that form spikes on the envelope (Yoshida, et al., 1976, 1979). So it may be considered as a kind of 'viroskeleton,' like the cytoskeleton in the cell. Binding of nucleocapsid strands to the inner surface of the envelope membrane resulting in regular folding along the axis may be mediated by M protein. Thus M protein seems to be important in maintenance of the characteristic rigid structure of young virions. On the other hand, with virion aging, the regular folding of nucleocapsid strands is lost, and the virions become nonrigid and round. Further, spike proteins of the envelope membrane become more mobile in the plane of the lipid bilayer (Kim et al., 1979). These seem to be associated with some variation of M protein occurring during aging for unknown reason. On treatment with PEG, most nucleocapsid strands of aged virions become detached from the envelope membrane, whereas those of young virions remain associated with the membrane. These observations also suggest some variation in M protein which made the association less.

In the early stage of the fusion reaction induced

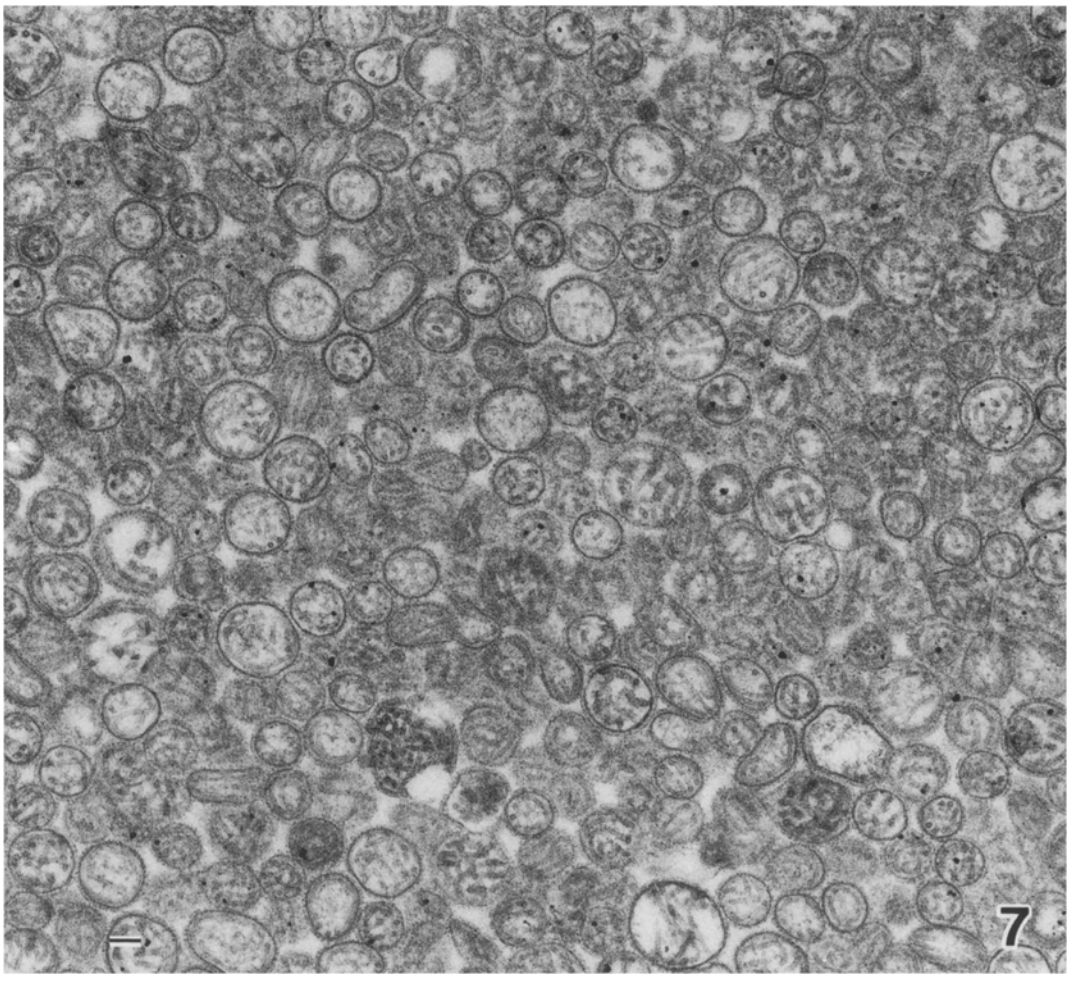

Fig. 7. Section of aged virions incubated at 0°C for 60 min as a control. The sample is from the same lot as for Figs. 1, 5 and 6. The virions show the typical morphology of late harvest virions, which consist of virions of various ages. No large virions are observed, unlike in samples incubated at 37°C (*compare* with Fig. 6). 36,000×; bar, 0.1 μm

by HVJ, the cytoskeletal network is disrupted and the membrane proteins become mobile (Kim & Okada, 1981). Similar changes during PEG treatment have been reported (Robinson et al., 1979). These changes suggest that interaction of cytoskeletal proteins with the cell membrane is lost during membrane fusion. These data and the present study strongly suggest that interaction of M protein as viroskeleton is disrupted in the aged virion. These findings also indicate that the skeletal proteins are important for preservation of the membrane structure. Dissociation of skeletal proteins supporting the biological membrane may be essential for membrane fusion. When the skeletal structure is disrupted, membrane proteins become freely mobile and so result in a condition in which membranes readily fuse with each other. In contrast, the cells in tissues of animals are separated by cell membranes, although they are in tight contact. In this case, the rigid cytoskeletal structure associated with the cell membrane may be considered to prevent membrane fusion, and maintain individual cell compartments.

On treatment with PEG, the assymmetrical distribution of spikes is lost in aged virions, but not in young virions. This difference may also suggest a possibility that some variation occurred in M protein which supports the envelope membrane. In this connection, the fact that spikes are only detected on the outer surface, but not the inner surface of the membrane when nucleocapsid strands are still bound to the inner surface of large virion vesicles seems noteworthy. Thus, we mainly discussed the fusibility of the envelope membrane with M protein as a skeletal protein. However, we have no direct

evidence on the variation of M protein. Other possibilities also remain.

Virion-to-virion fusion was also seen in aged virion without PEG, although at lower frequency. As is well known, the fusion activity of HVJ depends on two viral glycoproteins, HN and F (Homma & Ohuchi, 1973; Scheid & Choppin, 1974). To examine whether these proteins concern the virion-to-virion fusion, the virions were treated with anti-HVJ polyclonal antibody and then incubated at 37°C. As a result, large virion vesicles were also observed under this condition. This suggests that these two proteins are at least not directly associated with virion-to-virion fusion.

The large virions forming without PEG retain biological activities. So it should be possible to use these large virions as vectors of biologically active substances for injection into living cells. Experiments on this possibility are in progress.

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