Studies on Lymphocystis Tumor Cells of Fish

II. Granular Structures of the Inclusion Substance as Stages of the Developmental Cycle of the Lymphocystis Virus

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The lymphocystis tumors of fish consist of round, uninucleate cells of gigantic size which in the perch Stizostedion can reach 1,200 μ in diameter, and in the flounder Pleuronectes almost 2,000 μ. They are not parasitic protozoa as Woodcock supposed, who described them in 1904 as Lymphocystis johnstonei Woodc. (15), but are actually fibroblasts of the fish which have undergone an enormous hypertrophy (9, 10, 11). The cytoplasm of these "lymphocystis cells" contains basophilic inclusion bodies which can reach very large dimensions but start their development as tiny corpuscles. This observation, together with the fact that the lymphocystis disease proved to be very infectious, clearly indicated that the disease is produced by a virus which is located in the hypertrophying cells.

Recently specific granules were demonstrated in the outer layers of the inclusion bodies (14). The granules are stained dark brown when exposed to an osmic acid solution for several hours or longer. Because these components of the inclusions were small elements of rather uniform size, which increase considerably in number during the growth of the lymphocystis cells, it was concluded that they probably represented the transmission stage of the virus, to be compared with the elementary bodies of macroviruses (e.g., the viruses of vaccinia, fowl-pox, psittacosis), which are likewise visible under the light microscope. Any suspicion that the "osmiophilic granules" might be artifacts, produced by osmium precipitations, was dispelled by their demonstration as refractive structures in areas unstained by the osmium, and further by the fact that similar granules were seen in fresh preparations when the cytoplasm was made transparent by adding drops of dilute acetic acid.

Because the osmiophilic granules could not be demonstrated in the early period of the development of the inclusion bodies, the possibility was suggested that they were formed at intermediate stages, as differentiation products of the inclusion substance. However, details of such a process were not known. It was a problem whether the granules appear abruptly or whether certain microstructures in the inclusion substance are precursors to initiate the process. The present paper attempts to answer these questions and thus to contribute to the knowledge of the developmental cycle of the lymphocystis virus by a study of finer structures of the inclusion substance in stages preceding the appearance of the osmiophilic granules, and by a detailed investigation of the following stage in which osmiophilic granules have just made their appearance.

MATERIALS AND METHODS

For this morphological study lymphocystis tumor material was used from three fish species, the American perch Stizostedion vitreum and two European fish from the Baltic Sea: the perch Acerina cornua and the flounder Pleuronectes flesus. The material consists partly of collected fish specimens bearing spontaneous lymphocystis tumors, and partly of fish with experimental infection. Various methods of fixation and staining were applied (see legends of figures). The methods of staining with osmium have been described in detail (14).

RESULTS

Figures 1–5 show successive stages of the growth of inclusion bodies studied in young Pleuronectes lymphocystis cells of 40–200 μ in diameter which were obtained by experimental infection. In addition to the increase in size, the appearance of vacuoles and the formation of large inner spaces can be observed. When the superficial layer ruptures, as
shown in Figure 2 at the points marked by arrows, indentations are formed which may be followed later by fenestrations when the inclusion body further expands into the cytoplasm. The basophilic inclusion substance, in which the vacuoles are imbedded, could not be resolved into finer structures in the preparations shown in Figures 1 and 2, which are stained with hematoxylin after fixation in acetified alcohol. In the inclusion (Fig. 3) which was fixed in Meves' fluid, a number of highly refractive granules can be seen stained intensely with safranin. They are loosely scattered among the vacuoles. Their number is variable, and in many inclusions they are not present at all. In several inclusion bodies of the same material, concretions with a diameter of up to 1.5 μ were observed which likewise stain with safranin and have a high index of refraction. Under these circumstances, it seems to be quite possible that these granules have only the significance of small concrements.

The Sjövall method, by which in more advanced stages the osmiophilic granules can be demonstrated, makes the inclusion substance often appear as a rather homogeneous mass lightly stained by the osmium (Fig. 4). However, a continued study of such Sjövall preparations has shown that, in addition to the “clear type” of inclusion bodies, another type also occurs in which the inclusion substance shows a brown color as the result of the osmium staining. In thin layers of such “brown inclusions” a mosaic of dull brown granules could be discerned by use of oil immersion lenses of high aperture, as shown in (a), (b), (c), and (d) of Figure 5. The granules are imbedded in a lightly stained, delicate intergranular substance. These granular components of the inclusion substance are, in comparison with the osmiophilic granules, characterized by a less intensive staining reaction with osmic acid and a lower index of refraction.

Tumors of flounders caught in the fall showed the lymphocystis cells in the next period of their growth in which they reach 220–270 μ in diameter. In the tumors of one freshly fixed specimen a number of degenerated lymphocystis cells was seen. Furthermore, in many cells the inclusions could be noted in a peculiar process of swelling which gave them the appearance of fluffy bodies. Early stages of the swelling are seen in Figure 6, advanced stages in Figure 9. With such simple methods as fixation in alcohol and staining with hematoxylin, a granular composition of the inclusion substance could distinctly be seen in the fluffy bodies, even with an ordinary oil immersion lens in combination with a low power ocular. The inclusion body (a) of Figure 6 is shown in Figure 8 in high magnification. Between vacuoles a large number of intensely stained granules is seen, often arranged in tetrads (for instance, at the arrow) or dyads and short chains. In the group of very small inclusion bodies marked (b) in Figure 6, which is shown in Figure 7 in high magnification, again such a pattern of intensely stained granules can be seen imbedded in a delicate intergranular substance. The inclusion body (b1) shows one tetrad in the central area. In (b2) several tetrads and dyads are seen. Some of the granules appear somewhat elongated, as short rods. In advanced stages of the swelling, as shown in Figure 9 at (f), the granules appear in high magnification (Fig. 10) as more lightly stained structures. Again tetrads and dyads with granules in a tandem position are frequently observed. In an interpretation and evaluation of patterns of granules there is, of course, always some danger of overemphasizing a topographic relation of granules which may only by chance lie close together. The arrangement of the granules can be noted in the characteristic groups of tetrads so often, however, that one cannot consider such arrangements as merely incidental. Thus, the presented evidence indicates that the granules are units which multiply by fission.

Secondary application of osmic acid solution to the alcohol-fixed material of the swollen inclusion bodies resulted in an osmium staining of the granules which, in its rather low intensity, contrasted to the characteristic staining reaction of osmiophilic granules. Furthermore, the granules in such osmium preparations appeared as dull structures, whereas osmiophilic granules show a high index of refraction. Therefore, it seems very probable that the granules demonstrated in the swollen inclusions correspond to the granules observed in the brown inclusion bodies of the Sjövall preparations. Such findings in young Pleuronectes cells lead to the interpretation that the inclusion substance has a granular microstructure even in the early stages of development before the osmiophilic granules appear. The difficulty of demonstrating the “granules of the inclusion substance” in the ordinary preparations of young Pleuronectes lymphocystis cells may be chiefly caused by their dense arrangement. Under favorable fixation and staining conditions, resulting in the brown type of the inclusions in Sjövall preparations, the granules become discernible if inspected with an oil immersion lens of high aperture. In swollen inclusions they are distinctly visible and can easily be demonstrated by simple methods of fixation and staining and with ordinary oil immersion lenses.

The next step was to find out whether such granules of the inclusion substance were still recognizable during the period of appearance of the
"osmiophilic granules." For this task formalin-fixed material of Pleuronectes tumors showing a small number of osmiophilic granules in the surface layer of the inclusion bodies proved unsatisfactory. Only the rather homogeneous "clear type" of the inclusion substance was observed in the osmium preparations, as was shown in Figure 5 of the preceding paper (14). The stage of progressive appearance of the osmiophilic granules is in our collection much better represented by preparations from lymphocystis-diseased perchels belonging to the European species Acerina cernua and the American species Stizostedion vitreum. An experimentally infected young specimen of Stizostedion, which was kept in a laboratory aquarium for 15 weeks, proved especially valuable, since innumerable tumors developed. This material was freshly fixed by various methods, as the fish showed signs of weakness on the 105th day of the infection experiment. From this specimen some Sjövall preparations of relatively small cells were illustrated in Figures 6 and 7 of the preceding paper (14). These pictures did not show granules in the core of the inclusion network. The photomicrograph of a lymphocystis cell in Figure 6 (14) represents a typical picture of the clear type of the inclusion substance. A revised and continued study of the series of Sjövall preparations has, however, led to similar findings, as described in the osmium preparations of young Pleuronectes cells. In addition to the clear type of inclusions the brown type has again been frequently observed, showing quite distinctly, under favorable optic conditions, a granular composition of the inclusion substance.

A section through a lymphocystis cell 200 μ in length, in which the inclusion network is of the brown type, can be noted (Fig. 11). The bars of the network have extended in the upper half of the cell body. The small inclusion area (a) is shown in high magnification in Figure 12. The two types of granules are here seen very clearly side by side. The number of osmiophilic granules is still relatively small. They are predominant in the right half of the picture, because here a more superficial plane is in focus. Granules of the inclusion substance, characterized by the lighter osmium staining and a lower index of refraction, are chiefly seen on the left side where a level close to the surface layer is in focus. Among the pale granules (i), some dark osmiophilic granules are scattered. It is obvious that the two types of granules are of the same size and that they also resemble one another in their arrangement. They appear rather frequently as dyads and often as tetrads, or arranged in short chains. Sometimes such a chain consists partly of granules of the inclusion substance and partly of osmiophilic granules. Some granules may be found which appear to be intermediate between the two types. The evidence of such Sjövall preparations strongly suggests that the osmiophilic granules originate as peripherally located granules of the inclusion substance which, by a process of transformation, develop a higher index of refraction and a stronger staining reaction with osmic acid. Concerning other structures in Figure 12 it may be pointed out that on the right side, between the groups of osmiophilic granules, a pale brown homogeneous lattice of inclusion substance can be seen which at (f) is replaced by clear capsules of a high index of refraction.

Figure 13 shows in a cell from the same tumor a more advanced stage of the development of the inclusion network which here has grown through a larger portion of the cytoplasm. The bar (b) of the brown inclusion network is, in Figure 14, demonstrated in high magnification. Whereas at this stage in the periphery a continuous layer of osmiophilic granules (o) has developed, the core of the bar shows a mosaic of the more lightly stained granules of the inclusion substance (i) which are inbedded into a delicate intergranular mass. In some of the bars, especially in the cortical zone of the cell, the granulated core is replaced by a homogeneous layer of brown inclusion substance or by a strongly refractive unstrained framework.

Concerning the occurrence of the clear type and the brown type inclusion substance in the Sjövall preparations of the tumors of this Stizostedion specimen, it can be stated that the clear type is seen chiefly in cortical cells, especially at spots where the covering epithelium is missing, so that here the fixative could exert its quickest and strongest action. In some larger cells the upper half of the cell body near such a surface showed clear inclusions, whereas the inclusion bodies in the opposite half of the cell were all of the brown type. Such observations suggest that the clear type of the inclusion bodies in Sjövall preparations is the result of a fixation phenomenon that reminds one somewhat of the so-called "Überfixation" (Raubits), which is known to occur frequently in the cortical layer of pieces of organs fixed with Fleming's fluid (cf. 5, Vol. I, p. 477; Vol. II, p. 343).

In the course of the further growth of the lymphocystis cells of perchels, which is accompanied by the rapid extension of their inclusion network, a continuous increase in number of the osmiophilic granules is evident. The demonstration of areas of granulated brown inclusion substance in more advanced stages is of interest in determining how this increase occurs. In the Sjövall preparations of the Stizostedion specimen, such areas were still
observed in cells of 350 μ in diameter. Corresponding results were obtained by the study of osmium preparations of Acerina lymphocystis cells. Here such areas have been observed in cells up to 400 μ in diameter. For Acerina this is a rather advanced stage of growth; in this species the tumor cells reach a maximal diameter of only about 700 μ, in contrast to those of Stizostedion which may grow to 1,200 μ. Concerning advanced stages of Pleuronectes cells, areas of granulated brown inclusion substance have been observed in osmium preparations of a tumor containing cells of about 900 μ in diameter.

These findings support the view that such areas may continue, during the growth period of the lymphocystis cells, to serve as germinative layers for the differentiation of osmiophilic granules, provided that in these zones divisions of stem granules balance their numerical reduction brought about by the process of transformation. In this connection, observations in Acerina are of interest in showing that sometimes even an excessive increase in the mass of granulated inclusion substance occurs in medium-sized cells which leads to swelling and finally to the bursting of inclusion bodies. In tumors of an Acerina specimen containing lymphocystis cells up to 320 μ in diameter, it was noted that portions of the inclusion network were subdivided into groups of rather small inclusion bodies which showed various stages of expansion. This is demonstrated (Fig. 15) in a preparation fixed with Meves' fluid and stained with crystal violet after Benda. In the inclusion bodies a basophilic framework of lamellae is reinforced by intensely stained filaments. The lamellae surround a more lightly stained basophilic mass, for which the noncommittal expression "ground substance" may be used. This mass shows a tendency to expand. The small inclusion bodies (a) still show the original condition. At (b) expanding inclusion bodies are seen; their lamellae have become ruptured. At (c) the reinforcing filaments are pushed apart and then break (d). Thus, the inclusion bodies finally burst, and the ground substance (e) pours into the cytoplasm, mixed with fragments of the broken-down filaments (f). As a matter of special interest, it can be seen rather distinctly that the escaping ground substance is composed of fine basophilic granules.

The study of osmium preparations from the same tumor gave the opportunity to analyze the nature of these granules. The Sjövall preparation (Fig. 16) shows that the expanding and finally escaping ground substance chiefly consists of lightly stained granules of the inclusion substance. Osmiophilic granules are seen only in the periphery. On the right side they still mark the original contour of the inclusion body, which in this section has a triangular shape. On the left side the inclusion body has burst, so that here the mass of the granulated inclusion substance has escaped into the cytoplasm. The unstained capsules (f) around groups of osmiophilic granules are remnants of the lamellae. Such an expansion and bursting of inclusion bodies with liberation of granules of the inclusion substance has likewise been observed in Stizostedion cells of 450–500 μ.

On the basis of the data obtained for Acerina on the basophilic staining of the granules of the inclusion substance, it appears probable that mosaic of basophilic granules observed in some inclusion lamellae of the experimentally infected young Stizostedion specimen on the 77th and 99th day of the experiment contain granules of the inclusion substance but no osmiophilic granules at this stage (Fig. 17).

**DISCUSSION**

Mosaics of granules have been described in the intracytoplasmic inclusion bodies of a number of viruses, e.g., in psittacosis (1–4), fowl-pox (6), and in the advanced stages of the inclusions of lymphogranuloma venereum (8). Such granules have been interpreted either as elementary bodies of the viruses or as preceding virus stages which multiply by fission and finally divide into elementary bodies. It is in conformity with these interpretations of the nature of granular structures observed in cytoplasmic inclusions in other virus diseases that the granules of the inclusion substance in the lymphocystis cells are interpreted as developmental stages of the lymphocystis virus. The type of their arrangement, especially their appearance as tetrads or short chains, suggests that the increase in their number is brought about by division of the granules. Apparently, they represent vegetative stages of the virus which participate essentially in the growth of the inclusion bodies and thus increase the virus mass within the host cell. Later, many of the granules of the inclusion substance are transformed into osmiophilic granules.

The bursting of expanded inclusion bodies with the escape of granules of the inclusion substance into the cytoplasm has been demonstrated in Figures 15 and 16 in Acerina cells. Such findings resemble the disintegration of enlarged inclusions with dissemination of elementary bodies which has been described for several macroviruses, especially when cultivated in chick embryos (4, 8). In such cases, virus elements liberated after the breakdown of the host cell are supposed to be the source of the infection of adjacent cells. Certain aspects of the lymphocystis disease may be explained on a
similar basis. In the immediate neighborhood of medium-sized lymphocystis cells newly developed small lymphocystis cells are often found (11, 19). Sometimes a young lymphocystis cell has even been observed within the membrane of a degenerated larger lymphocystis cell (7). In an attempt to interpret such findings, the possibility has to be considered that a new infection by virus units from an outside source may have established itself at the same location as the previous one (11). However, the alternate interpretation, first given by Joseph (7), that infectious agents may have invaded adjacent cells or phagocytes after their development in the larger cells, now gains credence because of the observations in lymphocystis cells of perchs showing escape of granules of the inclusion substance into the cytoplasm. There are two possibilities by which the infected cytoplasm of lymphocystis cells might come into direct contact with other cells: (a) when the cytoplasm protrudes through the damaged cell membrane; or (b) when phagocytes invade a degenerating lymphocystis cell. Both phenomena are frequently observed in lymphocystis tumors.

In contrast to the possible role which granules of the inclusion substance may play in the infection of adjacent cells, the osmiophilic granules apparently represent the stages of the virus which serve to transmit the infection from host to host. Tumors containing either full-grown cells or almost full-grown cells have proved to be very infectious as suspensions of ground cells. In such material, granules of the inclusion substance, if still persisting, would be present in small amounts, whereas the osmiophilic granules would have accumulated as large masses.

It is doubtful whether the osmiophilic granules are capable of increasing their number by division after they have developed by transformation of granules of the inclusion substance. An arrangement of them in dyads, tetrads, or short chains, which has been frequently observed (14), could represent the persisting arrangement of the granules from which they have developed by transformation and which in their turn originated as division products from stem granules.

Hitherto it has not been proved that the infection can be transmitted by an inoculation of the contents of large lymphocystis cells into tissues. An effective method developed by the author for experimental infection consists of forcing a suspension of ground large tumor cells into the lumen of the pharynx by a syringe, either through the mouth or through the opercular clefts (12, 13). This method imitates the presumed natural conditions of the infection. Evidently virus particles become liberated when large lymphocystis cells disintegrate in the water after tumor pieces have been shed or after the death of the fish. Probably such particles may float for some time and perhaps pass through other organisms, until finally some of them are sucked into the pharynx of a fish of the host species via respired water, or possibly are swallowed with food. The storage of masses of osmiophilic granules in large numbers in full-grown lymphocystis cells suggests that these granules are the form in which the infection is transmitted from host to host.

The study of the finer structures of the inclusion bodies in the lymphocystis cells has thus led to the conclusion that in the developmental cycle of the lymphocystis virus two types of granules of elementary size can be distinguished: (a) the granules of the inclusion substance which represent vegetative stages of the virus and (b) the osmiophilic granules interpreted as virus particles which transmit the infection. For other viruses it has not been shown that elementary bodies, formed as products of the multiplication of the virus within the inclusion bodies, must undergo a change to become transmission stages of the virus. For two reasons one might expect that the developmental cycle of the lymphocystis virus would show some peculiarities. First, in the lymphocystis cells, which hypertrophy to gigantic dimensions, the vegetative phases of the virus, as components of the inclusion bodies, reach a higher level in growth and differentiation than in other viruses. Secondly, with regard to the transmission of the infection from host to host, the lymphocystis virus represents the rare case of a water-borne virus adapted to parasitism in aquatic animals.

The relation of the granules of the inclusion substance to the formation of the framework of the inclusion bodies, which apparently must be considered as an additional vegetative phase of the virus, will be discussed in another paper.

SUMMARY

The formation of the osmiophilic granules, previously interpreted as elementary bodies of the lymphocystis virus (14), in the outer layer of the inclusion bodies of lymphocystis tumor cells is not an abrupt process. In stages preceding the appearance of the osmiophilic granules, a granular composition of the basophilic inclusion substance can be demonstrated in thin layers of typical inclusion bodies by the Sjövall osmium method as well as in swollen inclusions with ordinary methods of fixation and staining. In the osmium preparations the "granules of the inclusion substance" show a slighter staining reaction with osmic acid.
FIG. 1.—Section through a lymphocystis cell of about 40 μ in diameter from an experimentally infected Pleuronectes specimen on the 30th day of the experiment. Fixation with acetified alcohol (absolute alcohol, 9.5 parts; glacial acetic acid, 0.5 parts). Staining with hematoxylin. X1125. (a), (b), (c), (d), (e) five stages of the growth of the intracytoplasmic inclusion bodies; (m) cell membrane; (n) nucleus.

Figures 2-5 show inclusion bodies from lymphocystis cells of 100-200 μ in diameter of the same fish as in Fig. 1, fixed however on the 68th day of the experiment. X1125.

Fig. 2.—Fixation with acetified alcohol. Staining with hematoxylin. The arrows mark spots where the cortical layer of the inclusions has ruptured.

Fig. 3.—Fixation with fluid of Meves (mixture of 2 per cent aqueous osmic acid, 4 parts; 1 per cent aqueous chromic acid, 15 parts; glacial acetic acid, 3 drops). Staining with safranin and Cajal’s mixture of indigo carmine and picric acid.

Fig. 4.—Sjövall’s method: fixation with mixture of formalin (1 part) and distilled water (3 parts), staining with 2 per cent aqueous osmic acid for 3 days at 37° C. Inclusion body of the clear type.

Fig. 5.—Sjövall’s method. Inclusion body of the brown type which at the areas (a), (b), (c), (d) and partly in the bar (e) shows granules of the inclusion substance.

Figures 6-10 from a specimen of Pleuronectes caught in September with lymphocystis tumors in which the cells have reached 200-470 μ in diameter and often show swollen inclusion bodies. Fixation with acetified alcohol. Staining with hematoxylin.

Fig. 6.—Section through a lymphocystis cell containing inclusion bodies at an early stage of swelling. (a) inclusion body of medium size; (b) group of small inclusion bodies; (n) nucleus; (u) nucleolus. X240.

Fig. 7.—Four of the small inclusion bodies of group (b) of Fig. 6, magnified X1750. (b') and (b') inclusions in which a number of the granules of the inclusion substance are arranged as tetrads.

Fig. 8.—The inclusion body (a) of Figure 6, magnified X1750. The arrow points to a tetrad of granules of the inclusion substance.

Fig. 9.—Section through a lymphocystis cell with inclusions showing an advanced stage of swelling. (f) inclusions swollen into large fluffy bodies; (n) nucleus. X240.

Fig. 10.—Small portion of a swollen inclusion body magnified X1125.

Figures 11-14 from an experimentally infected young specimen of Stizostedion on the 105th day of the experiment. Sjövall’s method.

Fig. 11.—Section through a lymphocystis cell (long diameter, 200 μ) in which the inclusion network has extended through one-half of the cell only. (a) piece of an inclusion bar; (n) nucleus; (u) nucleolus. X240.

Fig. 12.—Piece (a) of the inclusion network of Figure 11, magnified X1750. (f) framework capsules; (i) granules of the inclusion substance; (o) osmiophilic granules.

Fig. 13.—Section through a lymphocystis cell (long diameter 250 μ) in which the inclusion network has extended through a large portion of the cell body around the nucleus. (b) piece of the inclusion network. X240.

Fig. 14.—Piece (b) of the inclusion network of Fig. 13, magnified X1750. (i) granules of the inclusion substance; (o) osmiophilic granules.

Figures 15 and 16 show bursting inclusion bodies from an Acerina specimen in which the lymphocystis cells have reached diameters of 260-320 μ.

Fig. 15.—Fixation with Meves’ fluid. Staining with crystal violet (Benda’s method for the staining of mitochondria). X675. (a), (b), (c), (d) successive stages of expansion and bursting of the inclusion bodies; (e) mass of granules of the inclusion substance escaping into the cytoplasm; (f) fragment of a framework filament; (p) cytoplasm.

Fig. 16.—Sjövall’s method. X1125. (f) framework capsules; (i) granules of the inclusion substance; (o) osmiophilic granules; (p) cytoplasm.

Fig. 17.—Lamella of an inclusion body from a Stizostedion lymphocystis cell on the 99th day of the infection experiment. Fixation with Flemming’s fluid (mixture of 2 per cent aqueous osmic acid, 4 parts; 1 per cent aqueous chromic acid, 15 parts; glacial acetic acid, 1 part). Staining as in Figure 3. X1125.

The lamella shows a network of framework filaments covered by granules interpreted as granules of the inclusion substance.
and a lower index of refraction than the osmiophilic granules. Evidence is presented suggesting that the granules of the inclusion substance multiply by fission and eventually form the osmiophilic granules. Thus, in the developmental cycle of the lymphocystis virus two types of particles of elementary size can be distinguished showing different features in osmium preparations: (a) the granules of the inclusion substance, interpreted as vegetative stages which serve for the growth of the virus within the host cells, and (b) the osmiophilic granules which are considered to be the virus particles which transmit the infection from host to host. Accumulated in large numbers in advanced lymphocystis cells, they are liberated when the tumor cells disintegrate in the water after extrusion or after the death of the fish.

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_Cancer Res_ 1951;11:608-613.

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