

Are Animal Tumor Viruses Always Virus-Like?

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ABSTRACT Three tumors initiated by well characterized viruses, but in which virus is not detectable by ordinary virological techniques, are discussed. The question of the possible state of the virus within these seemingly non-infectious tumors is considered, largely from the standpoint of findings with the rabbit papilloma virus. This agent in its natural host, the cottontail rabbit, is infective, can be seen as virus bodies with the electron microscope, and can be visualized with fluorescent antibody only in the upper keratinizing cells of individual papillomas. At the growing bases of such papillomas, where neoplasia is in active progress, no infective virus is demonstrable and viral bodies cannot be visualized by either the electron microscope or fluorescent antibody. A hypothesis is presented that rabbit papilloma virus exists in cottontail papillomas in two forms—one, the complete mature virus, composed of nucleic acid and protein, and the other, immature virus, composed of naked viral nucleic acid without its protein coating. The function of the mature papilloma virus is to initiate tumor formation,—that of the immature virus, to maintain neoplasia. In the non-infective domestic rabbit papilloma, the viral nucleic acid and protein fail to combine to form mature infective virus and, as in the cottontail papilloma, neoplasia is maintained by the activity of the viral nucleic acid alone.

During recent years there has been an increasing body of opinion that viruses may play a role in the etiology of human cancer. One of the main reasons for this growing belief has been the finding, since Rous' initial discovery of a viral sarcoma of fowl, that there exist within the animal kingdom numerous other tumors caused by viruses. The great diversity of species in which viral carcinogenesis is known to occur has led many to believe that there is no good reason for excluding man as a possible host in which neoplasia due to viruses might take place. It is probably because of this opinion that so many investigators in laboratories all over the world are today directing their efforts toward determining whether or not viruses play a causal role in human cancer.

And what sort of a virus are these investigators looking for in human

malignancies? I think that it is quite natural that the vast majority of them are seeking a typical virus, one possessing those properties through which a virus may be detected by presently available virological tools. This approach is based on the knowledge that at least several of the well studied animal viral tumors have associated with them agents possessing the properties of typical viruses. It might be assumed from this that hypothetical human tumor viruses would also have similar properties that could be exploited in detecting their presence.

The characteristic properties by which viruses causing tumors are detected are well known to workers in the viral tumor field. However, since I intend later to make a point of the presence or absence of these various properties in determining whether or not a neoplasia-inducing agent is virus-like, I must, at the outset, outline them. The properties through which a tumor virus may be detected, and which are considered typically virus-like according to our current concepts, are as follows:—

1. The agent, which is filterable, is infective for the species of animal or bird in which the initial naturally occurring tumor was observed and in such hosts induces tumors like the original growth.
2. The agent usually induces an immune response in the host to which it is administered such that antibodies capable of neutralizing it appear in the blood serum and the host becomes resistant to reinfection by the agent.
3. The agent is visible as a “viral body” in thin sections of the growth examined with the electron microscope.
4. The agent may be made visible within the cells of the tumor by treatment with fluorescent antibody.

If a tumor-inducing agent possesses all four of the properties just mentioned, it is similar, aside from the fact that it causes tumors, to viruses encountered as the established causes of non-neoplastic viral infectious diseases of man and animals and there would, therefore, be every reason to look upon it as completely virus-like and as the initiating cause of the tumor in which it was found.

As mentioned earlier, viruses possessing all four of these properties are known to be the causes of a number of animal tumors, but for the present discussion I shall consider only three of these, the Rous sarcoma of fowl, the polyoma of mice, and the papilloma of cottontail rabbits. My reasons for selecting these are that all have been extensively studied, their causative viruses have been well characterized, and there is general agreement that the agent responsible for inducing each is indeed a typical virus. The matter of determining the viral etiology of cancer would be simple if all tumors were as straightforward in revealing the nature of their etiological agents as those I have just mentioned.

But even with these three apparently typical tumor viruses, we run into

difficulties of detection under certain circumstances and it is these situations that I want to discuss more fully because they seriously raise the question as to whether even the established tumor viruses are always virus-like during the time that they are actually inducing neoplasia. If we can satisfy ourselves concerning the aberrant and unvirus-like behavior of well recognized tumor viruses, we may be in a position to know better what to seek in tumors of as yet unknown etiology.

The Rous, polyoma, and papilloma viruses all yield, in unnatural hosts, tumors in which viruses cannot regularly be detected by the means at present at our disposal. Since these particular tumors may represent the situation prevailing in human cancer more realistically than do the tumors in their natural hosts, they may be more appropriate for study as prototypes of human cancer than are the natural tumors that are rich in typically detectable virus. The point to establish is just how unvirus-like an animal tumor virus may be in inducing neoplasia and I propose to discuss this next.

The fact that a tumor virus might lack the properties permitting its detection has been known almost since the time of discovery of the first viral tumor. In fact, it was 3 years after Rous' discovery of the fowl sarcoma virus that he and Murphy pointed out that in some known viral tumors of chickens, virus could not be demonstrated on occasion by either filtration or desiccation (1). Gye and Andrewes (2), Duran-Reynals and Freire (3), and Bryan, Calnan, and Moloney (4) later defined more closely the conditions under which Rous virus became non-detectable in the tumors of chickens that the agent was known to have initiated. It was concluded that such things as the ages of the chickens inoculated, age of the tumor at harvest, or the amount of virus injected determined the detectability of virus in the resulting tumor. But the more interesting "non-viral" virus tumors are three that have come to light in relatively recent years and they are the ones that are most instructive in emphasizing the unvirus-like nature of their causative agents.

When Rous virus is administered to young turkeys, polyoma virus to baby hamsters, or cottontail papilloma virus to domestic rabbits, tumors result in all instances. These are similar to the tumors caused by these three viruses in their natural hosts and thus superficially have the appearance of what we have come to consider typical virus tumors. However, though viruses are known to have initiated each, virus detectable by the infectivity test as ordinarily applied, is not present in them (5-8). Furthermore, in those cases in which the matter has been studied, "virus bodies" are not seen in thin sections of the tumor by means of the electron microscope (9-11) and treatment of tumor sections with fluorescent antibody does not result in the demonstration of fluorescent virus particles of the type seen in sections of infective tumors (12-14). In all three cases, with only occasional exceptions, antibodies capable of neutralizing the initiating tumor viruses are induced in

the hosts bearing the non-infectious tumors (6-8, 15-17). It is thus apparent that in the tumors under discussion, three of the four properties by which tumor viruses are ordinarily detected in the growths they cause are absent and only the immune response to the virus remains as tangible evidence that the tumor was indeed virus-induced. This property alone, however, would be of little value in identifying a tumor as viral were the host in which the tumor was first encountered one of those from which no infective virus could be extracted, for, with no virus available, an immunological test would be impossible to conduct.

It would appear from the examples I have cited that three of our tumor viruses, and these among those considered to have acceptably virus-like properties, give rise to tumors in unnatural hosts in which virus recognizable as such cannot be detected. In fact, if a freshly harvested Rous turkey sarcoma, a polyoma-induced hamster tumor, or a domestic rabbit papilloma were presented to a skilled virologist for determination of etiology, he would be unable to demonstrate a viral cause in any of the three tumors using the virological tools at present at his command. He would get nothing when he attempted transmission by cell-free preparations of the tumors to other hosts, he would see nothing suggesting the presence of "virus bodies" with the electron microscope, and, if he prepared fluorescent gamma globulin and treated sections of the tumors with this, he would not detect a localization of viral particles as in infectious tumors. All these negative findings would be gotten with tumors that had been induced by typical tumor viruses in hosts that were susceptible to these viruses. They are truly viral tumors and the hosts bearing them demonstrate this to be the case by usually becoming immune to reinfection with the virus or by developing virus-neutralizing antibodies in their sera.

What then has happened to the viruses initiating these particular tumors, and why have they lost the properties identifying them as viruses in the tumors they cause in turkeys, hamsters, or domestic rabbits? Is this phenomenon of a virus losing those properties by which it can be identified as a virus peculiar to tumors induced only in certain unnatural hosts, or does it hold also in the natural hosts of these viruses under certain circumstances? The findings of Bryan *et al.* (4) with the Rous sarcoma virus make a positive answer to the last question seem likely. However, it was the ingenious and brilliant experiments of Noyes and Mellors with the cottontail rabbit papilloma that first suggested the fundamental importance of the unvirus-like virus in the neoplastic process and made answers to the two questions just posed seem even worth speculating upon.

Noyes and Mellors (14), using fluorescent antibody, made a study of the cellular distribution and intracellular localization of papilloma virus antigens in papillomas of wild cottontail rabbits. Their findings with regard to the

localization of the viral antigens were completely surprising and unanticipated. They found that in cottontail rabbit papillomas, known to be rich in infective papilloma virus, much material, reacting with the fluorescent antibody and presumably virus, was localized in the keratinized and keratohyaline layers of the papillomas and virtually none was present in the basal proliferating layers where neoplastic cell growth was occurring. Later Noyes (18), employing a microcautery technique to selectively destroy either keratinized or proliferating layers of cells, demonstrated that virus activity, as tested by infectivity, was associated with the keratinized layers of cells and not with the actively proliferating ones. The results of these two studies were completely contrary to what might have been expected. Had one guessed where most of the infective virus should have been, cells of the actively growing base of the tumor, where neoplasia was in actual progress, would have been selected.

Noyes and Mellors, in discussing their observations, expressed the opinion that fluorescent antibody, applied to infective cottontail warts, demonstrated the complete papilloma virus. It was their belief that no complete virus was present in the proliferating cells of cottontail papillomas and that none was encountered until the keratohyaline layer of cells was reached. Since the virus presumably stimulates cell division in the proliferating cells and not in the differentiated cells of the keratinized layers, they postulated that it must be present in the germinal and proliferating cells in an early stage of development, consisting mainly of nucleic acid and deficient in protein. Because of this deficiency or lack of protein, the early stage virus, though probably responsible for stimulating cell proliferation at the growing base of a papilloma, was non-antigenic and, therefore, not demonstrable by fluorescent antibody.

Fluorescent antibody studies of domestic rabbit papillomas revealed the presence of much less viral antigen than had been found in the cottontail warts. However, as in the case of the cottontail warts, what there was lay in the upper keratinized layers and none was found in the proliferating cells of the growing base of the papillomas. It was postulated that in domestic rabbit warts, as in the case of the cottontail papillomas, large amounts of early stage virus, which was not demonstrable by fluorescent antibody, were present in the proliferating cells of the germinal layers. The domestic rabbit papillomas did not yield infective virus because they did not present as favorable a situation as the cottontail papillomas for the development of incomplete virus to complete virus. The virus in the domestic rabbit papillomas was visualized as being for the most part nucleic acid without a protein coat and hence without protection against rapid inactivation outside the infected cell and therefore not capable of transmission.

One deficiency of the fluorescent antibody technique is that it does not

differentiate between viral antigen and complete virus. Thus there is no way of knowing just how much of the large amount of specifically fluorescent material demonstrable in the nuclei of cells of the cottontail papillomas represents complete virus and how much, if any of it, is merely viral antigen. Conversely, there is no way of knowing, from the use of this technique with domestic rabbit papillomas, whether the small amount of fluorescent material in the nuclei of cells of these growths all represents viral antigen, or whether perhaps some of it represents complete virus. The failure of domestic rabbit papillomas to yield infective virus would suggest that the material detectable in them by fluorescent antibody is viral antigen not associated with complete virus. Also Mellors' later observations (19) with two carcinomas derived from the domestic rabbit papilloma would suggest the same thing. Thus of the two carcinomas in which no infective papilloma virus can be demonstrated, one, the VX7, contains material reacting with specific fluorescent antibody in the nuclei of some of its malignant cells and the growth engenders the production of papilloma virus-neutralizing antibodies in rabbits to which it is transplanted. The other rabbit carcinoma, the VX2, in contrast, contains no material reacting with specific fluorescent antibody and does not render animals to which it is transferred immune to papilloma virus. In the usual absence of demonstrable infectivity in the cases of either the domestic rabbit papilloma or the VX7 carcinoma derived from it, a likely speculation concerning the nature of the fluorescent antibody-reacting material demonstrable in both is that it represents viral antigen rather than complete infective virus.

Following the work of Noyes and Mellors, Stone, Moore, and I (20), using the technique of thin section electron microscopy, studied the matter of distribution of virus visible by this method in cottontail papillomas. Our findings supported the conclusions reached by Noyes and Mellors concerning the localization of virus within infective cottontail papillomas. Thus we found that viral bodies were present only within the differentiated cells well up in the keratohyaline and keratinizing areas of the papillomas. No viral bodies were to be found in cells of the basal germinal layers. However, cells in these areas appeared abnormal in that their nuclei were swollen and the nucleoli had a granular appearance. A little higher in the papillomas were cells containing nucleoli in which there were viral bodies surrounded by zones of low density. In such cells, viral bodies were limited to the nucleoli and none was to be seen in the nuclei. Still higher in the papillomas, in more differentiated cells, viral bodies were present throughout the nuclei as well as within the nucleoli. We postulated from our findings that the first morphological evidence of the presence of the virus is in the cells of the lower stratum spinosum and consists in the appearance of finely granular material in the nucleolar area. The first definite morphological evidence of the presence of virus was

to be seen in presumably more mature cells higher in the papilloma in the form of spherical viral bodies seemingly developing within a reticulum which formed out of the granular matrix of the nucleolus. As did Noyes and Mellors in demonstrating viral antigen, we found morphologically typical viral bodies only in the upper differentiated and keratinizing cells of the cottontail papillomas.

It would appear, therefore, from studies utilizing two different technical approaches, that no virus identifiable as such, either by fluorescent antibody or electron microscopy, is present in proliferating cells of the growing bases of cottontail rabbit papillomas. Furthermore, Noyes has demonstrated by his microcautery technique that there is no demonstrably infective virus in this area either. On the other hand, in the differentiated and keratinized portion of the papilloma, above the actively proliferating zone of cells, virus is known to be present by the infectivity test, by examination under the electron microscope, and either virus or viral antigen or both, by the fluorescent antibody test. Thus we have a situation in which the upper non-proliferating portion of a tumor contains an agent that is typically viral by three criteria that can be applied. In the lower proliferating neoplastic portion of the tumor, on the other hand, virus detectable by infectivity, electron microscopy, or fluorescent antibody, is not demonstrable. Virus typical in all respects is thus certainly present within cottontail papillomas, but it is not present in that portion of the tumors that is growing. It seems apparent, therefore, that if virus is contributing to the neoplasia in the case of the rabbit papilloma, and I do not think anyone would seriously doubt that it is, it is functioning in a completely unvirus-like form.

It should perhaps be pointed out that for the first 25 years the rabbit papilloma was under study, it was generally assumed and accepted by investigators that virus was probably distributed throughout infective cottontail papillomas in a uniform manner. It was only after the application of two relatively new viral techniques to a study of this tumor that the unusual and unexpected distribution of its virus came to light and that the existence of more than one form of virus within an infectious papilloma was even suspected.

It is true, of course, that since the beginning of work with the rabbit papilloma, it has been known that papillomas initiated by cottontail virus in domestic rabbits contained little or no virus that was demonstrably infective for other rabbits. They all did, however, contain what I chose to refer to as "masked" virus (17). Masked papilloma virus was antigenic in that when it was injected subcutaneously or intraperitoneally into either domestic or cottontail rabbits, it not only rendered these animals immune to infection with cottontail virus, but also stimulated within them the formation of specific virus-neutralizing antibodies. Despite this, masked virus from domestic

rabbit papillomas, in contrast to infective virus from cottontail warts, failed either to induce papillomas or to engender immunity when applied to the scarified skin of domestic or cottontail rabbits. The exact nature of the non-infective but antigenic masked papilloma virus has remained unknown for a long time. It seems likely that in the light of an interpretation of the experiments of Noyes and Mellors, and our own with cottontail papillomas, the nature of masked virus can now be postulated and I shall do this in a later section of this paper.

To recapitulate the evidence concerning the state of the virus within infective cottontail papillomas, the work of Noyes and Mellors, as well as our own, suggests that it exists there in two forms: One of these is fully infective virus visible with the electron microscope as round bodies in the nuclei of differentiated keratinizing cells in the upper portions of the papillomas; the other is non-infective virus, not visible with the electron microscope except perhaps as granular material within nucleoli of cells of the basal germinal layers. Noyes and Mellors visualize from their findings with fluorescent antibody that the non-infective virus in the proliferating cells of the papilloma is probably incomplete virus comprised of naked viral nucleic acid deficient or totally lacking in a protein component. It is suggested, from our electron microscope studies, that the process of virus maturation proceeds from the cells of the growing base of the papilloma to those in the higher differentiating and keratinizing layers. The incomplete virus of Noyes and Mellors is the same as the virus which we have termed immature. It is visualized that such incomplete or immature virus develops with the growth and differentiation of the maturing cells of the papilloma to become the virus of our studies, visible as round viral bodies in the differentiated keratinizing cells in the upper portions of the papilloma. Our mature virus, in turn, corresponds to the complete virus of Noyes and Mellors, comprised of viral nucleic acid coated with specific viral protein capable of reacting with fluorescent antibody.

The infective papilloma virus, visible with the electron microscope, detectable with fluorescent antibody, and antigenic as evidenced by its induction of viral immunity in animals that it infects, therefore, represents mature complete virus. It would be generally considered by virologists as a typical virus. However, located as it is high in the papilloma in differentiated keratinizing cells that are no longer capable of dividing, it is obviously playing no further role in stimulating the neoplastic change in the tumor in which it is found. As an individual virus particle, it has reached the end of its developmental cycle in the cottontail papilloma in which it has developed and it has no further purpose than to serve to initiate a papilloma in a new host. If the new host it reaches is a cottontail rabbit, it will repeat its developmental cycle. Its fate, if it reaches a domestic rabbit, will be speculated upon later.

The non-extrinsically infective, immature virus is present in the actively proliferating cells of the growing base of the papilloma. It is probably naked viral nucleic acid which evidence that has been outlined indicates will acquire a coating of viral protein higher in the cottontail papilloma and become then both visible with the electron microscope and detectable by fluorescent antibody. However, in its immature state in the cells of the neoplastic portion of the papilloma, this agent is very unvirus-like; it is not infective for other hosts, it is not visible as a recognizable viral body under the electron microscope, and it is not detectable with fluorescent antibody. Despite its lack of virus-like properties, however, the location of this immature virus within cells of the actively proliferating portion of the tumor makes it seemingly the important agency through which neoplasia in the growing cottontail papilloma is maintained. It would appear to be the cause of cell proliferation in the basal layers of the papillomas and, although not infectious from animal to animal, may still maintain infection of new cells as they divide and may serve as the continuing cause of neoplasia. Thus the properties by which we recognize rabbit papilloma virus, and that we use to characterize it as a typical tumor virus, are in reality all lost when it gets down to the grim business of maintaining the malignant growth which may eventually destroy its host.

The situation as regards the state of the virus in the papillomas in domestic rabbits and the mechanism by which neoplasia is maintained in these non-infective tumors would seem to be similar to that which prevails in the basal actively growing portions of cottontail papillomas. The similarity in the manner of growth and the progression to malignancy of domestic rabbit papillomas, when compared with cottontail tumors, indicates that the extrinsically non-infective virus responsible for their maintenance is immature virus, just as it is in the neoplastic areas of cottontail tumors. It would appear likely, therefore, that the entity responsible for stimulating cell proliferation with consequent papilloma formation in both cottontail and domestic rabbits is the naked nucleic acid component of the agent we recognize in its protein-coated complete form as the papilloma virus. The domestic rabbit, however, seems incapable of developing immature virus to the mature infective form achieved in most cottontail papillomas. That the capacity to transform immature to mature papilloma virus is not absolutely host-dependent is indicated by the occasional failure of cottontail papillomas to yield infective virus and the very infrequent instances in which domestic rabbit papillomas may yield infective virus. Because domestic rabbit papillomas are known to be antigenic and capable of inducing immunity against infective papilloma virus (17), it becomes necessary to assume that specific viral protein is present in the warts of domestic rabbits. In fact, Mellors, with the fluorescent antibody technique, has shown the presence of viral antigen in small amounts in both domestic rabbit papillomas and in the VX7 carcinoma of domestic

rabbits (19). It is apparent from this that both viral nucleic acid and the viral protein moiety are produced in domestic rabbit warts. What seems a possible explanation to account for the failure of formation of complete infective virus is that though both the nucleic acid and protein components of the virus may be produced in domestic rabbits, they usually fail to combine to form mature virus as they almost always do in the cottontail, and remain as separate components with the nucleic acid moiety serving to maintain active neoplasia and the protein component serving to render the animal immune to further onslaughts by infective complete virus. If this visualization of the situation in domestic rabbit papillomas is correct, then the masked virus they contain, detectable experimentally mainly by immunological means, may be merely the non-infective protein component of the virus whose synthesis has continued without incorporation into complete mature virus. Some work by Sachs and Fogel (13) suggests that, in hamster polyoma tumors also, viral antigen may accumulate without being incorporated into complete infective virus.

The upshot of a consideration of the differences and similarities between papilloma virus in its natural host and in an unnatural host has indicated that though this agent in an unnatural host is not virus-like by the criteria selected as typical of a tumor virus, the agent actually responsible for the truly neoplastic process, even in the natural host, is not virus-like either by the same criteria. The cottontail papilloma has offered a microanatomically unique situation in which to demonstrate the presence of two forms of virus within a single infective tumor and the domestic rabbit papilloma has fortuitously supplied supporting evidence that it is the unvirus-like agent that is responsible for the neoplastic process in the tumors of both the natural and unnatural hosts. The tumors induced in chickens and turkeys by Rous sarcoma virus and in mice and hamsters by polyoma virus do not allow for a clear-cut microanatomical differentiation, such as exists in the case of the rabbit papilloma, between fully developed tumor areas and areas in which active neoplasia is still progressing. For this reason it is impossible to determine whether or not, as in the rabbit papilloma, two forms of virus exist in the infective Rous tumors of chickens or polyoma tumors in mice. However, the finding that the neoplasia-inducing agent in an unnatural host of the rabbit papilloma virus, though very unvirus-like, is indeed the same as the one responsible for the true neoplastic process in the natural host would, at least to me, suggest strongly that a situation similar to this may also hold in the Rous and polyoma tumors. It is true that from both the turkey tumors and the hamster tumors, by appropriate procedures usually involving the use of tissue culture or grafting, infective virus can sometimes be derived (21, 22). Such findings only serve to emphasize that a very unvirus-like agent, capable of being

converted to characteristic virus under certain conditions, has been acting as the carcinogenic agent in these turkey and hamster tumors.

I think it is obvious by now that the answer to the question posed in my title, "Are Animal Tumor Viruses Always Virus-Like?" is definitely in the negative. Evidence from three well studied animal tumors suggests that, in seeking the actual cause of viral neoplasia, we should think in terms of an agent quite unlike our current visualization of a typical virus. It has been pointed out that in the cases of these tumors in unnatural hosts, initiated by typical tumor viruses, no agent with characteristic virus properties can be demonstrated. Furthermore it has been indicated that in one tumor in its natural host, the papilloma in the cottontail rabbit, though typical virus can be shown to be present in the tumor, it is not located within the tumor in the area of active neoplasia and is hence not the continuing cause of the tumor.

Some of what I have presented is speculative in that certain of the observations that I have interpreted in one way may be capable of interpretations in other ways that will, in the end, prove to be more correct. However, since the ultimate interests of all of us who work in the animal tumor field relate to a better understanding of human carcinogenesis, it has seemed worthwhile to indicate that viruses, even in animal tumors, may not always have the stereotyped properties that we have come to consider characteristic of this group of infectious agents. It would appear, therefore, that in attempting to learn whether viruses play an etiological role in human cancer, we should perhaps broaden our approach a bit to include a search for unvirus-like agents as well as those having the properties of typical viruses.

Ito, in some very recent work with the rabbit papilloma virus, has perhaps indicated one direction that a study of unvirus-like tumor-inducing agents might take. Using a method of phenol extraction, he demonstrated, in cottontail warts, the presence of viral DNA possessing the infective properties of papilloma virus (23). Of more importance, however, was his subsequent demonstration, by a similar chemical method, of infective viral DNA in domestic rabbit warts that, without such chemical treatment, were devoid of viral infectivity (24). Ito, in recovering infective viral DNA from non-infectious domestic rabbit warts by the methods he employed, may have shown the way for the similar demonstration of viral tumor-inducing activity in other tumors not containing viruses detectable by currently used methods. It seems likely that Ito's success in obtaining infective viral DNA from domestic rabbit warts entailed the removal or destruction, by the chemical procedures he followed, of some substance, perhaps DNase, that was inhibitory or deleterious to the naked DNA of papilloma virus outside the protection of its intracellular environment.

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