Viral Carcinogenesis*

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Viral carcinogenesis presents many problems which are the object of very active investigation; to condense in a lecture the present status of all these problems and to do justice to them would be impossible; I shall therefore limit the discussion to a few of them, which concern mainly the mechanism of carcinogenesis by viruses. In this discussion the attention will be focused on experimental results obtained *in vitro*, since these can be evaluated in a simpler and more direct way than results obtained in organisms.

Most of the experimental work to be referred to was carried out by using the DNA-containing polyoma virus and cultures of Syrian hamster or mouse embryo. We shall therefore first summarize the relevant characteristics of this system. Within 2 or 3 weeks following infection with polyoma virus, the hamster cultures undergo a progressive conversion characterized by the replacement of the normal cells with cells of a new type, having special morphological and growth characteristics. In fact the normal cells are broadly fusiform and well spread on the solid substrate on which they are cultivated; in crowded cultures they are more elongated and often assume a parallel arrangement in well ordered bundles. After forming a continuous cellular monolayer these cells do not grow any further, although sometimes two bundles of cells may intersect each other at an angle: thus the cultures are essentially two-dimensional. Descriptively, these cultures can be defined as "regulated." On the contrary, the cells of the converted cultures are much more elongated; they grow at all stages without any regular arrangement: the orientation in respect to each other is nearly random. In crowded cultures the cells form a three-dimensional felt constituted by many layers of criss-crossing cells. In contrast to normal cells, they can be described as "non-regulated."

A similar but slower conversion is caused by polyoma virus in cultures of mouse embryo cells.

The cells present in the hamster embryo cultures converted by the virus are neoplastic. In fact, the subcutaneous inoculation of $2–5 \times 10^6$ converted cells into young adult hamsters gives rise regularly to tumors which become noticeable at the site of inoculation within 1 or 2 weeks, and reach the size of a walnut within a month. In contrast, the inoculation of 10^7 normal hamster embryo cells or of polyoma virus at high titer does not produce any tumor or nodule at the site of inoculation within 1 month (21).

It is also clear that the conversion caused by the polyoma virus in the cultures differs unambiguously from the mysterious spontaneous transformation repeatedly observed in cultures of animal cells, since the latter occurs after a much longer period of cultivation (17).

We shall now pass to the consideration of the mechanism of viral carcinogenesis. The first point is whether the role of the virus in this process is direct or indirect. The evidence on this point stems mainly from experiments in which cultures of cells derived from hamster kidney or hamster embryo were infected with polyoma virus and then transferred to feeder layers of noninfected cells. A proportion of the transferred cells gave rise to converted clones; in parallel experiments, in which the cells were not infected, no such clones were formed (Stoker, personal communication). Although some of the cells of the feeder cultures may have become infected, their proportion cannot be large, and therefore selective conditions due to virus infection should have been considerably weaker for the transferred cells than for cells left in the culture originally infected. Yet the proportion of converted cells appeared to be the same in the two cases.

Other evidence bearing on the same point derives from the time relationship between infection and the appearance of converted cells. The time of appearance of converted cells can be estimated in hamster embryo cultures by examining these cultures at various times after infection. Since the

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converted cells are recognizable for their morphology and growth property, their number can be roughly estimated. These observations suggest that the first converted cells arise within a few days after infection. Thus, the neoplastic conversion is expressed within the same time period required for the virus to reproduce itself within the cells.

These observations tend thus to demonstrate that the neoplastic conversion of hamster cells caused by polyoma virus *in vitro* is the direct consequence of the penetration of the virus into some cells; conclusive proof is, however, still missing. In other systems also one can demonstrate a direct relation between virus infection and cell conversion *in vitro*, on the basis of changes in morphological and growth properties. For instance, chicken embryo cells infected *in vitro* with the Rous sarcoma virus undergo a characteristic conversion which occurs at most one cell division after infection (Ting, personal communication); although there is no evidence, based on transplantation into the animal, that the cells converted by the Rous virus are neoplastic, this is most likely, since they display "non-regulated" growth *in vitro*.

We can, therefore, conclude that most probably viruses play a direct role in causing the neoplastic conversion of cells in cultures. This result raises new questions: is this effect due to a specific contribution of the virus to the cells or to an influence of the virus on some cellular function? We shall now explore these two possible mechanisms.

Concerning the possibility of a specific contribution of the virus, it will be recalled that a virus brings in contact with the cell, and in part introduces into it, two or three main chemical components: nucleic acid, proteins, and, for some viruses, lipides. Since we know too little about the role of lipides, we shall concentrate our attention on the other two components. Both nucleic acids and proteins are made up of molecules of high informational content; their main contribution to the cells should, therefore, be the information they contain.

Let us consider the contribution of the nucleic acid of the virus. As a carrier of information, the viral nucleic acid is equivalent to a fragment of genetic material. Thus, if neoplastic conversion is caused by the introduction into the cell of the special information present in the viral nucleic acid, it is a process similar to either transduction or conversion of bacteria by bacteriophage. This similarity has been pointed out and discussed in the past (5, 11), and it is not necessary to discuss it again. It should, however, be remarked that, if this is the mechanism of carcinogenesis, the viral nucleic acid should be permanently present within the transformed cells.

Recent experimental developments with polyoma virus have made it possible to test this prediction in several ways. To analyze the available evidence, we shall assume that the viral nucleic acid may be present in the converted cells in one of three states which were first defined for bacteria-bacteriophage complexes. The first state is that of replicating nucleic acid, which is present in all cells in which the virus multiplies: we shall call this a *vegetative* state. The second, *proviral*, state is comparable to the prophage of the lysogenic bacteria. The third state is one resulting from the incorporation of the viral nucleic acids into some permanent cell constituent, from which it cannot be readily separated in its original form: we shall call this an *incorporated* state.

The possibility that the converted cells contain a viral nucleic acid in the vegetative state has been extensively tested by using the polyoma virus system. A series of experiments was devoted to the question whether the converted cells spontaneously release polyoma virus (6).

A continued production of infectious virus was found to be a property of all polyoma-converted mouse embryo cultures which were not cloned after infection, and of some similarly uncloned hamster embryo cultures; cultures of this type, not cloned, will be referred to as *primary* converted cultures. However, clonal cultures derived from the primary virus-releasing cultures under conditions excluding carried-over virus did not continue to release virus, although they retained the morphological and growth properties of the parental cultures. Thus, the ability to release virus is not transmitted hereditarily from cell to cell and cannot be, therefore, an intrinsic property of the converted cells. Other observations showed that the continued virus release of primary converted cultures is due to a "virus carrier state," in which certain cells become infected and produce virus, which in turn infects other cells; the cells participating in this persistent virus production show characteristics similar to those shown by infected sensitive mouse embryo cells both in amount of virus production and in the localization of virus synthesis, as detected by fluorescent antiviral antibody. Fundamentally, the cells converted by polyoma virus are not releasing virus, as is shown by the production of many clonal lines of nonvirus-releasing converted mouse embryo cells, as well as by the many converted hamster embryo cultures which are not releasing virus from the start.

An identical situation may exist in carcinomas deriving from virus-induced rabbit papillomas, as

deduced from observations carried out with fluorescent antiviral antibodies (12). In fact, one of these carcinomas was found to contain a small proportion of fluorescent nuclei, as we found in the virus-carrier polyoma-converted cultures; furthermore, the rabbit carcinoma also continued to produce small amounts of infectious virus (15). A critical evaluation of the state of the virus in this system would, however, depend on the study of clonal populations of cancer cells produced under conditions preventing viral reinfection of the cells.

With the polyoma system it was possible to go one step further and to test whether the converted cells contain vegetative viral nucleic acid incapable of maturation in the converted cells, but able to do so in normal cells (Dulbecco and Vogt, unpublished). This could be done by taking advantage of the property of the polyoma virus DNA to be infectious for normal mouse embryo cultures (4, 23). Many attempts to extract with phenol infectious polyoma virus DNA from the converted cultures were carried out, all with negative results; under identical conditions infectious DNA was extracted from recently infected normal mouse embryo cultures as well as from virus-carrier primary converted cultures. Furthermore, it was also tested whether the converted cells contain a vegetative nucleic acid of a noncytocidal mutant of polyoma virus, able to cause transformation. This was done by testing the transforming capacity for regular mouse and hamster embryo cells both of saline extracts from freeze-thawed converted mouse and hamster cultures, and of DNA preparation from these cultures. No converting ability was found.

It appears, therefore, that vegetative viral nucleic acid is not a regular constituent of cells converted by the polyoma virus, nor is it required for maintaining the neoplastic state. A similar conclusion may also apply to other virus-induced neoplasias, especially those in which the conversion is caused by a DNA-containing virus. For cells converted by an RNA-containing virus, such as the Rous sarcoma virus or the myeloblastosis virus, on the contrary, the opposite situation seems to apply (20); however, with the Rous virus the existence of nonvirus-producing tumors (3) and of clones of converted cells not releasing virus (13) has been reported, so that extensive and exacting cloning experiments with cells converted by these viruses would be desirable.

The possibility that the converted cells contain the polyoma virus nucleic acid as provirus has been tested in extensive attempts at inducing them to release virus by using many different treatments, including all those which are known to induce virus release in lysogenic bacterial cells, either of the "inducible" or of the "non-inducible" type (Vogt and Dulbecco, unpublished). These experiments were carried out by using nonvirus-releasing converted cultures derived either from mouse embryo cells (clonal cultures) or from hamster embryo cells. The release of mature polyoma virus could never be induced. Likewise, negative results were obtained in attempts to induce either the production of viral antigen, recognizable by using fluorescent antibodies and by other immunological tests, or the production of infectious viral DNA extractable from the cells by phenol.

Observations on the degree of susceptibility of cells converted by polyoma virus to superinfection with this virus also have some bearing on the problem whether the converted cells contain the viral nucleic acid as provirus; in fact, lysogenic bacterial cells are immune to superinfection with a virus equal to that carried as prophage. It was found that mouse embryo cells converted *in vitro* by polyoma virus show a greatly enhanced resistance to superinfection with this virus (21); however, cells of mouse tumors induced by the virus in the animal are quite sensitive (8). It seems likely from this and other observations that high resistance to polyoma virus is not a necessary property of all the cells converted by polyoma virus; the high resistance of mouse embryo cells converted *in vitro* can be attributed to the selection caused by the continued presence in the cultures of polyoma virus in fairly high titer. Thus, the converted mouse cells do not show the immunity characteristic of lysogenic bacteria. The hamster cells, whether converted *in vitro* or *in vivo*, are usually not superinfectible by polyoma virus; the cause of their resistance is unknown.

It appears, therefore, that the available experimental evidence does not support the hypothesis that the cells converted by polyoma virus contain the viral nucleic acid in a provirus state, if this term defines a state entirely similar to that of prophage. Likewise, no supporting evidence can be obtained from other systems involving DNA-containing viruses. As far as RNA-containing viruses are concerned, this hypothesis cannot be discussed for lack of experimental evidence, apart from the consideration that it is not known whether RNA could at all assume a provirus-like state.

The presence in the converted cultures of a viral DNA either in a vegetative or proviral state is thus undemonstrated, and probably unlikely.

This conclusion must be compared with some recent observations concerning cells transformed

by polyoma virus, which have been interpreted in the opposite way. A phenomenon resembling induction of virus release was observed when cells of apparently virus-free polyoma-induced primary mouse tumors were transferred to *in vitro* culture (24). This phenomenon can, however, be explained differently. In fact, the tumors employed were highly susceptible to polyoma virus; since they grew in mice infected with large doses of virus, but in the presence of antiviral antibodies, it is likely that they were in a "virus carrier state," although the virus was masked by antibody. Thus, virus release following cultivation of the cells *en masse*, without cloning, can be attributed to the shift in the steady state concentration of virus which occurred when the cells were placed in the antibody-free environment and, moreover, under conditions unfavorable to cell multiplication. A similar result can, in fact, be obtained in cultures of HeLa cells maintained in a virus carrier state by antiviral antibody, when a similar change of environment is made (1).

Another observation of this type is the staining, with fluorescent antiviral antibody, of cells derived from tumors induced by polyoma virus in hamster and other species, in which infectious virus was not demonstrable (16). It is, however, not certain that the stained material was viral protein, for two reasons. In the first place, the stained material was found in the cytoplasm and not in the nucleus, contrary to what is observed during the normal development of this virus in normal mouse or hamster embryo cells as well as in virus-carrier transformed cultures of both species. In the second place, the reported staining was mostly present in degenerating or rounded-up cells and therefore can be attributed to nonspecific penetration and binding of gamma globulin; it is, in fact, known that damaged cells became stained with any fluorescent gamma globulin (9).

In continuing our discussion of the possible role of the viral nucleic acid in viral carcinogenesis, we should discuss the possibility that the nucleic acid may be present in the converted cells in an incorporated state. However, for lack of experimental evidence this point cannot be discussed at present.

We shall now briefly consider the viral protein as the other macromolecular component of the virus which may participate in the process of neoplastic conversion by contributing information. Viral protein can enter in relation with the cells either as a constituent of the infecting virus particle or as a product synthesized under the control of the viral genome in the cells. The question whether the viral protein has a primary role in carcinogenesis is not usually asked. This is not so much the consequence of irrelevance of the viral protein, but rather of the state of our knowledge of biological phenomena: in fact, we can very well formulate hypotheses concerning the utilization of the information present in the nucleic acid, but cannot formulate equally good hypotheses concerning the utilization of the information contained in a protein. Yet, we know that the information carried by antigenic proteins is utilized by the cells in the process of antibody formation.

The role of the viral protein may be particularly important in carcinogenesis caused by RNA-containing viruses; in fact, in the cells transformed by these viruses a vegetative virus genome may be continuously present and may produce viral protein all the time. In cells recently infected by the Rous virus a protein having the antigenic specificity of the viral protein was found associated with the cell membrane initially and within the cytoplasm later (22); if this protein continues to be present in the cell at all times, it may be responsible for some aspects of cellular conversion.

This concludes our discussion of the possible specific contribution of a virus to the cell in the process of neoplastic conversion; we can now turn to a consideration of the possibility of a nonspecific effect of the virus, i.e., through some influence on the functions of the host cell. This point is extremely complex: in fact, the metabolism of a virus-infected cell is abnormal and furthermore very little known. During the multiplication of the virus many kinds of reciprocal influences between the virus and the host cell can develop. For instance, a generalized repression of the synthesis of normal cellular components takes place in bacteria infected by certain bacteriophages. However, if in carcinogenesis an influence of this type plays a dominant role, it should have a more restricted and more specific effect.

The possible nature of these influences is suggested by some recent discoveries in bacterial biology, which are likely to have broad significance. It has in fact been shown that the function of a number of genes is normally prevented by repressor substances; the function becomes expressed only when an inducing substance, able to counteract the repressor, becomes available to the cells. It is probable that these findings are applicable to animal cells which are normally regulated in their growth ability, as shown by their failure to grow freely in spite of the availability of the necessary substrates. This growth regulation may be caused by the operation of special repressor mechanisms. The neoplastic transformation can then be the consequence of the inhibition or inactivation of these mechanisms. Perhaps such an inhibiting

or inactivating action is reflected in the deletion of proteins and enzymes which have been frequently observed in cancer cells (14).

Owing to the hereditary character of the neoplastic transformation, the inhibition of the regulatory mechanisms must be caused by changes of the genetic material of the cell. A virus could cause this effect by contributing to the cell its own genetic information, and thus causing in the cell a genetic imbalance. However, this mechanism would require the persistence of the viral genome in the cells, a possibility which could not be proved so far in a general way, as already discussed.

It is interesting, therefore, to explore the possibility of another hypothesis for a heritable virus-induced perturbation of cell regulation.

Certain results obtained in bacteria suggest an interesting although speculative possibility; it has been shown (10) that there exist in bacteria units called episomes, endowed with genetic continuity but distinct from the regular cellular genetic apparatus and having in some cases recognized specific functions, such as sex determination. Bacterial episomes have properties similar to those of proviruses; by undergoing a change of state they can assume properties comparable to those of vegetative viruses; in this state they can sometimes be lost from the cell, perhaps because of an insufficient rate of multiplication. Many different episomes can regularly coexist in the same cell; in some cases, however, exclusion phenomena have been observed, similar to those shown by viruses infecting the same cell.

It could be suggested that in animal cells the genetic material of an episome is involved in regulation and carcinogenesis. This hypothesis appears of potential interest for an interpretation of carcinogenesis in general, since many different agents could alter the function of the episome and cause the neoplastic conversion. For instance, the episome could be altered by mutation caused by mutagenic agents, could be destroyed or inactivated by some chemicals, could be lost by competition with a virus or by dilution or segregation in hormonally stimulated cells. According to this hypothesis the carcinogenic role of the virus would end with the loss of the episome, and therefore later on the neoplastic character of the cell would not depend any longer on the presence of the viral genome; ultimately the virus could be completely lost, as may be the case for polyoma virus.

This hypothesis as applied to viral carcinogenesis would have to be reconciled with results obtained with the Rous virus, which show that the morphology of cells converted by this virus is a function of the virus genome, different mutants of the virus causing the formation of converted cells of different morphology (7, 19). The hypothesis and the observations could be reconciled, for instance, by assuming that the neoplastic character and the morphological cell type are determined by two distinct mechanisms: the former by the inactivation or loss of the episome, the latter by the genome of the virus which, as already discussed, appears to be present in cells converted by the Rous virus. Cell morphology could be related to the type of viral protein synthesized in the cell surface; a new cell shape would correspond to a mutated virus protein, in the same way as a sickle shape of the red blood cells corresponds to a mutated form of the hemoglobin they contain.

Our discussion brings us now to some concluding points. It is clear that, although with viruses we can reproducibly cause a neoplastic conversion of cells, we do not yet know its mechanism. However, the experimental work carried out so far has led to a simplification of the hypotheses, since several of them can be discarded or, at least, considered very unlikely. The remaining hypotheses offer two extreme, contrasting possibilities: one, the addition of a part of the virus genome to the cell genome as an integral part, the other the permanent inactivation or elimination from the cell of a regulatory element endowed with genetic continuity. These hypotheses also are in certain points amenable to experimental verification. For instance, one point to be tested is the prediction that, under either hypothesis, the changes produced in the cells are essentially irreversible, except, in some cases, for the occurrence of mutations. Another point could be tested by experiments of cell hybridization (2, 18), if hybridization of converted, nonregulated cells with normal, regulated cells can be obtained; in fact, the hybrids would be nonregulated under the first hypothesis and regulated under the second hypothesis.

Further progress will now depend to a large extent on increased knowledge in the areas of biology which are connected with the expression and regulation of gene function and with the regulation of cell growth and multiplication. Thus, the problem of carcinogenesis is related to that of cellular differentiation. We can hopefully anticipate that viruses and animal cells cultivated *in vitro* will be very useful tools in furthering this progress.

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