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Carcinogenesis by Avian Sarcoma Viruses[1]

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Dr. Habel has presented an excellent summary of current work on the biology of viral carcinogenesis. As is appropriate in view of the work from his own laboratory, he has emphasized the DNA tumor viruses. Using as a base the work from our laboratory with the RNA-containing avian sarcoma viruses, which, as Dr. Habel has indicated, have a simpler and more direct action than do the DNA tumor viruses, I should like to reiterate some of Dr. Habel's points and to extend the discussion to support the following hypothesis of the mechanism of carcinogenesis:

> A genetic change which alters the properties of a cell so that less is required of a specific serum factor(s) necessary for cell multiplication.

Therefore, in conditions in which the amount of this serum factor(s) is limiting for cell multiplication, the altered cells have a selective advantage over the normal cells.

The actual change from a normal cell to a tumor cell can be separated into the following steps: (*a*) interaction of oncogen and normal cell; (*b*) alteration of cell genome; (*c*) activation of altered genome; (*d*) alteration of cell biochemistry; (*e*) alteration of cell behavior; and (*f*) multiplication in a selective environment. For the avian sarcoma viruses these steps are: (*a*) infection of a sensitive cell; (*b*) formation of the DNA provirus; (*c*) cell division; (*d*) (unknown); (*e*) conversion; (*f*) multiplication under conditions where the amount of insulin-like activity of serum is limiting for cell multiplication.

Most of the work on which this hypothesis is based was carried out with the Rous (RSV) or Fujinami (FuV) viruses and chicken embryo fibroblasts. Some additional work with similar results has involved duck embryo fibroblasts.

When RSV is added to competent cultures of chicken or duck embryo fibroblasts and the infected cells are allowed to multiply normally, areas of altered cells or foci appear (6, 9, 21). The foci contain cells infected with RSV, although other cells in a culture may be infected with RSV and are not altered (14). It is interesting to note that these RSV-altered cells differ from uninfected cells by only one of the five criteria listed by Dr. Habel for transformation, i.e., the production of tumors on transplantation to immunologically compatible hosts.

The discovery in 1960 of viral mutants controlling the morphology of the infected chicken cell enabled a direct demonstration of virus control of properties of the infected cell (1, 9, 10). The alteration induced by the virus was, therefore, called conversion, by analogy with lysogenic conversion. Use of these genetically marked viruses for superinfection and mutation experiments led to the concept of the provirus, one or two units per cell of virus information controlling cell morphology and virus production (10–12). It is of interest to note that superinfection with RSV can lead to addition of a second virus to a cell without alteration of the phenotype of that cell. It can also lead to replacement of the original virus by the superinfecting virus. As Dr. Habel mentioned, superinfection with DNA tumor viruses leads only to addition of the second virus and both viruses are expressed.

Work using various inhibitors of nucleic acid synthesis, supported by nucleic acid hybridization studies, had led to the hypothesis that the provirus of RSV is DNA (see Ref. 16). More recent work using stationary and synchronous cultures has confirmed and extended this idea. (It should be noted that no evidence has been found which conflicts with this hypothesis of a DNA provirus and supports an alternative mode of replication for RSV.)

If stationary cultures of chicken embryo fibroblasts are exposed to virus and the cultures kept in the stationary phase by the removal of serum from the medium, it is found that after serum is replaced, even as long as 15 days after infection, the cells divide, produce virus, and become converted (Table 1). (RSV infection does not stimulate cell division.) By contrast, stationary cultures infected with SV40 rapidly lose the ability to become transformed (22). Therefore, RSV can form a provirus in nondividing cells.

Experiments were then carried out to see whether DNA synthesis was needed for formation of the provirus in these stationary cells. It was found that inhibitors of DNA synthesis when applied immediately after the virus prevented formation of the

Table 1

Number of cells plated	8	4	2	1
Number positive				
Total	4/4	4/4	4/4	4/4

Infection of stationary cultures. Stationary cultures were exposed to SR-RSV at a multiplicity of infection of 1 infectious unit/cell. After incubation for 40 minutes, the cultures were washed, and 5 ml of Eagle's medium was added. Each day from 4 to 15 days after infection two cultures were transferred to serum containing medium. Within 3 to 4 days after serum was added all cultures were converted. The cells from the culture left for 15 days without serum were plated as infectious centers 4 days after serum was added.

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provirus. These same inhibitors had no effect if applied before the virus was added or after the provirus was formed (Chart 1).

Infected stationary cultures do not make virus or undergo conversion until serum is added. When serum was added to these cultures for 1 to 3 days, in order to see how many cell divisions were required for activation of the provirus, it was found that two divisions were needed to get maximum conversion of morphology and maximum rate of virus production (Chart 2).

Similar conclusions about the need for new DNA synthesis to form the provirus and for cell division to activate the provirus were first drawn from a study of infection of partially synchronized cultures (16).

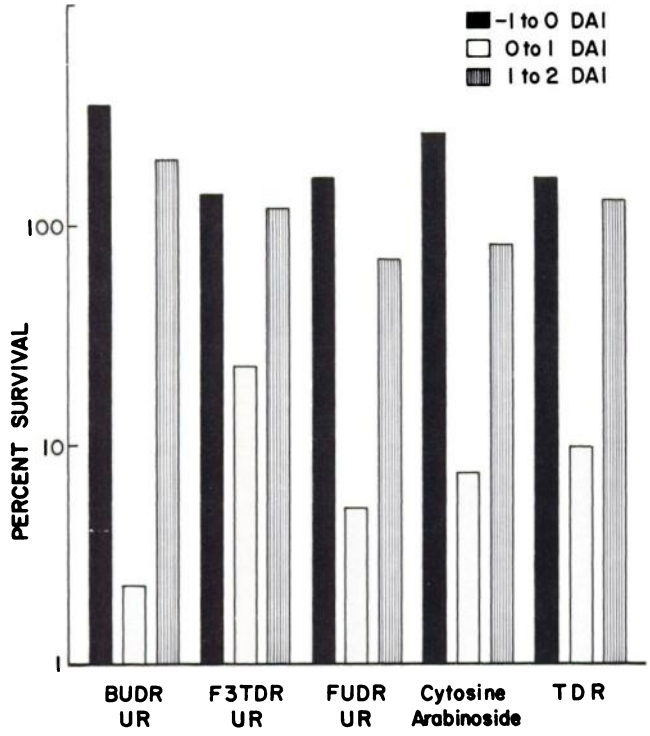

Chart 1. Effect of inhibitors of DNA synthesis on infection of stationary cultures. Secondary cultures containing 10^6 chicken embryo fibroblasts were prepared in 3 ml of Eagle's medium with no serum. Two days after preparation all cultures were exposed to 100 focus-forming units of Schmidt-Ruppin virus for 40 minutes, and unabsorbed virus was removed by washing. The indicated compounds were added to one third of the cultures from 24 hours before infection to infection, to one third from infection to 1 day after infection (DAI), and to the rest from 1 to 2 days after infection. Two days after infection all the cultures were overlaid with Eagle's medium containing 20% tryptose phosphate broth, 10% calf serum, 0.5% agar, 2×10^{-5} M deoxycytidine, and 2×10^{-5} M thymidine. After 10 days of further incubation, foci were counted and their number expressed as a percent of the controls. All values are the average of those for duplicate cultures. Bromodeoxyuridine (BUDR) was used at a concentration of 200 μg/ml; trifluorothymidine (F3TDR), 5 μg/ml; cytosine arabinoside, 5×10^{-5} M; thymidine (TDR), 5×10^{-3} M; and uridine, 5×10^{-5} M; fluorodeoxyuridine (FUDR), 5 μg/ml.

After formation and activation of the provirus, the infected cells may or may not be converted (7, 8, 14, 18). The conditions which determine whether or not the cells are converted are as yet not known.

Chart 3 presents a summary of some of the properties of uninfected and converted chicken and duck embryo fibroblasts and of chicken iris cells. The comparisons of uninfected and converted cells were made from cultures with similar cell densities and rates of multiplication.

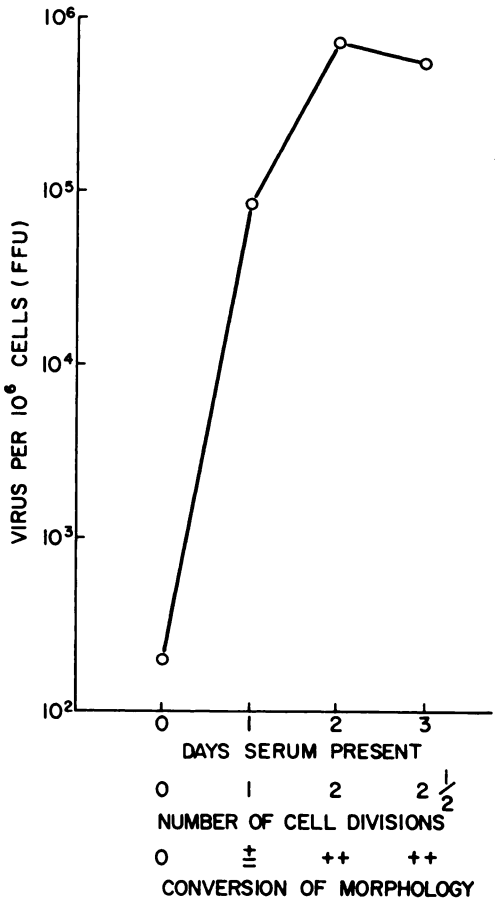

Chart 2. Effect of cell division on infection of stationary cultures. Secondary cultures containing 10^6 chicken embryo fibroblasts were prepared in 3 ml of Eagle's medium without serum. After overnight incubation, they were exposed to a multiplicity of infection of 2 infectious units/cell of *morph*^{r} Fujinami virus, washed, and 5 ml of fresh Eagle's medium without serum added. Four days after infection 5 ml of fresh Eagle's medium containing 20% tryptose phosphate broth and 10% calf serum was added to the cultures for 0, 1, 2, or 3 days. At the end of this time the cultures were washed and 5 ml of fresh Eagle's medium without serum was added. Ten days after infection the cultures were examined for morphologic conversion, the supernatant medium harvested and assayed to determine virus production, and the number of cells and amounts of protein and DNA per culture determined. The cultures with no serum were assumed to have had no divisions.

SPECIES	CHICKEN				DUCK	
Cell Type	Fibroblast		Iris		Fibroblast	
Infection	0	+	0	+	0	+
Shape	△	◯/	□	◯/	△	/
Pigment	0	0	+	0	0	0
AMPS Synthesis	+	++	0	+	+	++
Collagen Synthesis	+	+	0	0	ND	
Requirement for SILA for multiplication and glycolysis	+	±	ND		+	±

Chart 3. Effect of conversion on properties of cells. Cultures of chicken fibroblasts or iris cells and of duck fibroblasts were exposed to virus, and the properties of the infected cultures were compared with those of parallel uninfected cultures. 0, the absence of property; +, a normal amount; ++, a larger amount; ±, a lesser amount; ND, not determined; AMPS, acid mucopolysaccharide; SILA, serum insulin-like activity. Details are given in the papers cited in the text.

It is obvious that many changes occur in converted cells. It is probable that many other changes could be found. It is also obvious that there is specificity in the changes. The shape of the infected chicken fibroblasts or iris cell depends on the virus genotype, but the shape of the infected duck cell seems to be independent of the differences in viral genotype. Some cell functions, e.g., hyaluronic acid synthesis, are increased; others, e.g., pigment production in iris cells, are decreased; and other functions, e.g., collagen synthesis, are unaltered.

Two different mechanisms could be postulated for the control of the specificity of these changes. In one, the provirus directly affects each of the altered characters; in another, the provirus directly affects one or a few primary characters, and the other effects are pleiotropic in nature. (The change in cell shape, round or fusiform, is independent of the other alterations.) Since several of the changes observed seem to have a common locus at the cell membrane, the latter hypothesis is probably to be favored. Some of the changes have not yet been related to virus or tumor growth, but may be related to invasion and metastasis. Work with more viral mutants may resolve this problem by showing whether or not separate viral genes control each alteration.

The change from an uninfected cell to a converted cell is not instantaneous even in a rapidly multiplying culture. After formation and activation of the provirus, about a day elapses before maximum virus production is reached (16), and another day or two elapses before conversion is complete (19). Since all of these alterations occur at about the same time, none of them can be the primary virus action.

Further insight into the specificity of conversion was gained by a study of the increased hyaluronic acid synthetase activity in converted cells (2). When properties of an enzyme fraction from converted cells were compared with properties of an enzyme fraction from parallel cultures of uninfected cells, the kinetic properties, including Michaelis constant and rate of heat inactivation at a number of different pH's, were found to be similar. This evidence supports the hypothesis that the increase is an increase in the activity of a cell enzyme. Since the activity appears to be located on the cell membrane, the alteration in enzyme activity may be secondary to a change in the membrane.

Uninfected and converted chicken fibroblasts multiply at the same exponential rate when the medium is changed frequently (13). In these circumstances, neither type of cell shows any signs of "contact or density inhibition of multiplication." (Cells in a perfusion system also do not show this type of inhibition (3).) When the uninfected and the converted cells are multiplying at the same rate, their rates of glycolysis are the same (20).

However, a very different picture appears when the amount of serum in the medium is reduced, so that its amount is limiting for cell multiplication (13, 15, 17). Under these conditions, the amount of cell multiplication is proportional to the amount of serum in the medium, and the converted cells multiply to a cell density about 50% higher than the uninfected ones. These experiments demonstrate that both uninfected and converted cells require serum for multiplication, but that converted cells require less serum for each cell division.

[This experiment cannot be carried out with a normal medium. Because a toxin is produced by the converted cells, a polyanion, such as agar or dextran sulfate, must be added to the medium (15).]

When the amount of serum is limiting for cell multiplication, the rate of multiplication decreases as the medium is depleted.

The rate of glycolysis varies in the same way as the rate of multiplication in these cultures (20). Thus in cultures where the amount of multiplication is limited by the amount of serum, there is more multiplication and more glycolysis in the cultures of converted cells. The increase in glycolysis in these cultures is at least reminiscent of the classic observations of Warburg.

Another circumstance in which more glycolysis occurs in converted cells is in cultures which are stationary because of depletion or absence of serum. In such cultures, the rate of glycolysis in uninfected cells decreases greatly, while the rate in converted cells does not.

Although serum is a complicated mixture of compounds, pure insulin or insulin-like activity can replace the factor(s) in serum whose amount is limiting for multiplication of uninfected and converted chicken and duck fibroblasts (17, 19). Therefore, it appears that the increased multiplication found in cultures of converted cells as compared with uninfected cells results from a decreased requirement by the converted cells for an insulin-like activity found in serum.

If cells are allowed to multiply until they reach the stationary phase as a result of depletion of serum, addition of insulin will allow multiplication to resume. If insulin is added to the medium of cultures in which the amount of serum is limiting for cell multiplication, a higher cell density is found than in the absence of insulin. These effects of insulin have been found with both chicken and duck cells. Medium depleted by multiplication of cells of one species does not promote multiplication of cells of the other species. Mixtures of duck and chicken cells do not multiply more than these cells separately. Therefore, duck and chicken cells appear to use the same serum factor(s).

Although insulin is not the only factor in serum which is needed by cells in culture, medium containing insulin and no serum supports limited cell multiplication (5). The amount of multiplication is limited by the amount of insulin present, but at each insulin concentration the converted cells multiply more than the uninfected ones (17, 19). Since substances other than insulin which have insulin-like activity are also active in promoting cell multiplication, we cannot yet conclude what is the actual active component of serum.

At this point several obvious questions arise: What is the mechanism of action of serum and insulin on cell multiplication? What change has occurred in the converted cells so that less serum or insulin is needed for cell multiplication? How general is the hypothesis of the mechanism of carcinogenesis presented here that carcinogenesis results from a genetic change which alters a cell so that less is required of a specific serum factor(s) necessary for cell multiplication?

At the present time, I shall not present any more data relating to these questions, but will close with a quotation from Dr. Rachmiel Levine (4) on the mechanism of action of insulin. I believe this thought is very relevant to my discussion: "One cannot attribute some of the effects of insulin to the enhancement of glucose transport, but it is just as clear that one still has to consider seriously the proposal that the interaction of insulin with the cell membrane initiates a set of signals which in turn lead to the other demonstrated effects of insulin, as well as to glucose transport stimulation."

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