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VIRAL ONCOGENESIS AND CHEMICAL CONTROL*

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INTRODUCTION

It may be useful to introduce the system in which we are attempting to study the process of the conversion, or transformation, of living cells into a condition which simulates, or models, the cancerous one. It has been known for about 50 years. Raus was the first to clearly demonstrate that it was possible to produce a tumor in the chickens by the use of a clean virus preparation. It took almost 40 years before it was recognized that this experiment constituted the beginning of a really important era in virology and in oncology. Following this, about a dozen years ago, it became possible to count viruses (virions) by a technique called focus formation. This technique resembles very much the phage assay technique. In this, a gel containing the uniform bacterial inoculum is first prepared. A virus assay is made by taking a dilute aliquot of the virus suspension, infecting the bacterial plate, and wherever a single virus infects a bacterium, that bacterium will eventually lyse, liberate more virus (phage)
particles, resulting in a small clear spot in which would otherwise be a crowded plate uniformly clouded with a bacterial population. This results in the ability to actually count the phage particles. In the case of animal viruses, a related technique is used, particularly with the oncogenic viruses. In this case, the overall plan is very much the same, but instead of a pour plate of bacterial culture which looks cloudy and where the clear spots are counted, the technique is quite the reverse. A smooth, uniform layer of animal cells is infected with an aliquot of the virus which is being assayed, and allowed to grow from 3-7 days. Then, those cells which have been infected by the virus and which are transformed by the virus show, not as clear spots in a uniform background, but as highlights, because the cells which have been infected by the oncogenic virus and transformed grow rapidly and build up in little mounds which can be seen as high, dense cell populations instead of a single monolayer of cells. Figures 1 and 2 will show this phenomenon. The original use of the technique of focus formation was to count the oncogenic virions, just as the bacterial plate was used to count phage particles. In recent years the system has been turned around: Not only is it used to count virions, but the idea that these foci might be considered as models of a cancer system has gained some significance; this is the kind of a system which I will be discussing.

Figure 1 shows cells in tissue culture, through an optical microscope, showing the nucleus of each cell, with the cell membrane around the outer edge. You can see that the cells are in some kind of communication with each other; you can see the strands of contact between them. Actually, this picture shows the beginning of a growth on the plate; when the plate is completely filled, the cells will be in contact all around, on all
sides of their cytoplasmic membrane, and growth will stop when the cells reach that particular stage of confluence in which they are in contact with each other. At that time apparently some "signals" are passed between them which stop the cell growth. This is a model for normal growth by normal cells. When the cells are infected by oncogenic viruses, either RNA or DNA viruses, the signaling system is somehow broken down, and the cells do not cease growing when they reach confluence. They overgrow each other, and the "foci" develop, as shown in Figure 2. The piles of cells (foci), which might be taken to represent models of tumors in the whole animal, appear as light-reflecting piles on the plate; on the right-hand side of the figure it is possible to see individual cells piling on top of each other. The foci can be counted, each one representing a virus infection which has transformed a cell which then grows into a group of cells failing to show contact inhibition. In a sense, this tissue culture represents a model for the whole animal, and the focus represents the cancer.

What type of viruses are we talking about? We use oncogenic viruses, some DNA, and some RNA viruses. A common DNA virus which infects mammalian cells is SV40, the simian virus No. 40, which will infect and transform tissue cultures of monkey cells. A common RNA virus is murine sarcoma virus (MSV), which, in turn, infects and transforms a mouse tissue culture. We will be concerned almost exclusively with the RNA type viruses, although some of the discussion will be relevant to the DNA viruses as well.

Figure 3 shows an electron micrograph of a section of a special strain of Balb cells infected with murine leukemia virus (MLV). The more standard type viruses are shown on the left-hand side of the figure. These are
the Type C viruses, with the nucleoid in the center and capsid around the outside, representing almost complete murine leukemia viruses. In the upper right-hand corner of Figure 3 are visible four invaginations of the vacuolar membrane showing the initiation of the encapsulation of the Type C viruses which ultimately close to give what appears to be a cellular membrane coated virion, mentioned earlier. This particular strain of cells, called Balb UCl-B, is a variant of the ordinary strains and is subject to transformation by MLV alone. We will speak more of the significance of this fact later.

It is believed that the outside membrane of the virus has many of the same qualities as the cell membrane itself. Finally, we believe that the elongated peculiar shapes in the center of Figure 3 are the unfinished or aberrant sarcoma virus particles in this same cell. As far as we can tell, the cell does not produce infectious sarcoma virus, but the aberrant particles are visible, indicating that at least some of the virus genome is present. In this figure, you can see representatives of the two RNA viruses -- the sarcoma virus and leukemia virus -- both of which are RNA-based. The sarcoma virus is the one which produces the transformation of ordinary tissue culture cells; the leukemis virus by itself, in an ordinary tissue culture, does not transform the cells.
A MECHANISM OF CELLULAR TRANSFORMATION

With this introduction, I wish now to turn to the discussion of the possible way in which the RNA oncogenic Type C viruses were conceived of as introducing the necessary information into a cell to transform it from normal to neoplastic growth. The basic idea is that somewhere in the RNA of this sarcoma virus is contained a suitable collection of genes (one gene or several) which code for certain kinds of proteins, which, in turn, transform, or make, the necessary components of the cell membrane so that it no longer can receive the message to cease growing, with the result that it continues to grow and overgrows its neighbors. This last process is pretty far along in the phenotype, or read out, of the results of the onco-gene which is presumed to be present in this RNA virus. How can such a transformation in an animal cell be produced, in which the genetic information is not stored in RNA but, rather, in DNA? The suggestion was made, about six years ago, that RNA viruses, in order to reproduce themselves, had to first make a copy of their RNA into DNA, and then from that DNA, using a DNA-dependent RNA polymerase, could make more RNA, which, in turn, can create more virus.\(^1\) RNA virus replication was blocked by actinomycin D, which we know complexes only with DNA and not with RNA or the enzyme, and, therefore, there must be a DNA molecule in the cycle of RNA virus replication. This was the basic idea for introducing the suggestion. About three years ago, it was shown that the RNA viruses contained in their protein structure an enzyme which was capable of copying an RNA template into a DNA strand.\(^2,3\) This was then called RNA-instructed DNA polymerase (RDP), been given the name reverse transcriptase; for a while, it appeared that only tumor viruses and only tumor cells contained the reverse transcriptase activity; however, it has since turned out that this
type of activity has been shown to reside in several different kinds of protein, from different sources. There appear to be multiple enzymes, with different template specificities, and different substrate specificities.

I will use the term "reverse transcriptase activity" to cover the whole group, and refer to it only in terms of being able to copy an RNA strand into a DNA strand, without trying to specify the details of the template specificity, the primer specificity or substrate specificity.

How can this enzyme be important in the transformation process? One can imagine its importance in the viral replication process, but how can it be important in transformation? Figure 4 shows, schematically, a possible function of reverse transcriptase (RDP) in oncogenesis by RNA viruses.

You can see herein also the cell replication process, with the DNA of the cell being copied in bits and pieces into RNA by a DNA-dependent RNA polymerase (DRP), which gives the messenger RNA, which, in turn, through multiple steps makes the necessary proteins for structure, enzyme and cell construction. This is the normal flow of information in the replication of a cell. If, however, you infect the cell with an oncogenic RNA virus --i.e., insert some RNA information, if there is such a thing as reverse transcriptase activity, then RNA could be copied back into DNA, and it could also copy the viral RNA. If the genes responsible for the lifting of the restraint on growth are contained in that viral RNA, it would be copied into the DNA by the enzyme. Then, there are at least two other enzymes involved. One of them would have to break open the DNA; this is called a clipping enz in Figure 4. Another would seal up the new piece of DNA from the virus into the cellular DNA; this is called a ligase enz in Figure 4. It so happens that enzymes of this type have been known for some time, but in the last
few months I have heard descriptions of very specific enzymes from the SV40 virus, for example, and from other sources. There appears to be an enzyme which clips a piece of DNA at a specific place and allows the right sets of base pairs to be exposed to complete a covalent linkage so a piece of new DNA can match up with it. This would be followed by another enzyme. There are thus at least two more enzymes involved in this insertion process to get the viral RNA information into the cellular DNA and thus make a permanent change in the cell. If the information came from an oncogenic RNA virus it would have to go through the enzyme (RDP) to get there. This is what focused our attention on that particular enzyme. You will see later that it will be necessary to expand that view somewhat and involve the other enzymes as well.

The basis for this additional concern lies in the discovery here in Berkeley a year or two ago of a variant strain of Balb/3T3 mouse cells which had been carried in tissue culture for many years. Normally this Balb strain can undergo transformation, that is, focus formation, by infection of a mouse sarcoma virus (MSV) only. When the sarcoma virus is diluted out of the mixed preparation of MSV and murine leukemia virus (MLV) to the point where no MSV and only MLV virus is present, the ordinary strain of Balb cells is not transformed. The new cell type which was developed at the Naval Biomedical Research Laboratory and has been given the name UCl-B is a strain which differs from the parent in that it is susceptible to transformation and focus formation by MLV alone without the co-infection with MSV.

As will be discussed in detail later, drugs have been found which can inhibit this MLV transformation without a correspondingly large inhibition of the replication or multiplication of the leukemia virus
itself. Thus, evidence has been obtained for the participation of additional enzymes in the transformation process, other than those required for replication of the virus, which are sensitive to this class of drugs (rifamycin derivatives). It is these enzymes which have yet to be identified and to which we will refer again later.

RNA-INSTRUCTED DNA POLYMERASE (RDP) AND INHIBITORS

Here I will discuss primarily the nature of the RNA-instructed DNA polymerase and how we can block it from functioning. If we can prevent the RDP from functioning, the information from an RNA oncogenic virus will not be able to get into the DNA of the cell, and, therefore, the cell could not be transformed. The presence of this particular enzyme is not a sufficient condition for transformation but it is a necessary one for transformation by an RNA virus. If this function is stopped, there would then be some way of blocking the transformation of mammalian cells into tumor cells by an exogenous RNA virus.

Drugs

We then turned our attention to chemical methods of inhibiting that particular enzyme action. It did not take long after the description of the RDP to have a number of materials appear which seemed to have some specific inhibitory action on the reverse transcriptase. The material which turned out to be one of the most effective was a modification of an antibiotic which had been developed for TB infections. (The rifamycin B itself was discovered about 1958 in a Mediterranean soil and was found to be particularly effective against tuberculosis.) This was one member of what has turned out to be a class of antibiotics known as the ansa-antibiotics because of the nature of their structure. Four of these
ansa-antibiotics, including rifamycin, are shown in Figure 5. The characteristic structure of these antibiotics is an aromatic ring which is bridged by a large aliphatic ring, which is called the ansa ring because of the nature of its bridging effect. It turned out that the material which first showed up as an inhibitor of the RDP function was a derivative of rifamycin B.

It was also shown that it was possible to make many chemical modifications of the basic rifamycin B structure, with many functional groups. It turned out that most of the derivatives were those of substitution at the No. 3 position of the naphthalene ring, where a whole series of derivatives can be made. Figure 6 shows some of the various derivatives which have been made from the rifamycin B, and most of them are actually derivatives of the 3-position of the rifamycin through the aldehyde. The rifampicin is the material which was finally brought to the market as the drug of choice for tuberculosis, and its mode of action had been delineated as involving the inhibition of bacterial DNA-dependent RNA polymerase. In our studies we have focused on RNA-instructed DNA polymerase from the virus or the cell, and you might wonder what the relationship is between these and the bacterial enzyme. Both of these materials are nucleotide polymerases, and one might expect that the antibiotic inhibitors for nucleotide polymerases might have some relationship to each other.\textsuperscript{12,13} That is at least part of the reason that the rifampicin and its derivatives were tried on the RDP. It turned out that the dimethylbenzyldesmethyl rifampicin (DMB) is very effective on the MSV RDP at almost as low a dose as rifampicin was effective against the DNA-dependent RNA polymerase in \textit{E. coli} and other bacteria. And it is much less effective against other mammalian polymerases.
This type of observation created the opportunity for the chemists and biochemists to synthesize additional active compounds, which, of course, are still basic modifications of the original structure of the rifamycin. With the exception of the dimethylbenzyl compound (DMB) and the rifocyto-Oxime compound, which we obtained originally from the manufacturer (Gruppo Lepetit, Milan, Italy) they were made in the laboratory in Berkeley.\textsuperscript{14} It turns out that I will discuss the DMB, as that is the one we received first and the one which is also non-toxic to the mammalian cell systems which we are using. The rifocyto-Oxime, for example, turned out to be toxic to the mammalian cell. The most important of the various derivatives which have been synthesized in Berkeley is the rifazacyclo-16 which is even more effective as an inhibitor of the reverse transcriptase enzyme activity than the DMB.\textsuperscript{14} I wish to describe more of the chemistry involved in these syntheses and the biological observations as well.

In order to study the effect of these drugs on the activity of the enzyme, it was necessary, of course, to have some purified enzyme available. The typical method by which the enzyme was first demonstrated and on which most of the work in the literature today has been based, was dependent upon a preliminary purification of the virions themselves. Then, the suspension of the virus particles is broken up, disassembled, with suitable detergents, thus liberating the enzyme. The incorporation of the various nucleotides into nucleic acid can be measured upon various templates, such as poly rA:oligo dT. The assay is actually not very definitive, except that it does demonstrate that an RNA strand is copied into a DNA strand. It was difficult to obtain clean enzyme from the virion itself, partly because the virion contains in it its own RNA as well. The cells
which had been transformed by the MSV (MLV) was our principal source, but here also the presence of virion is not eliminated.

Figure 7 shows the extraction procedure. The cell layers, which are grown in the cell medium, are scraped off the bottles in which they are grown; the cell-free supernatant contains the virus. Most of our work has been done with the cell extracts. After the cells are washed, we get a cell pellet which can be broken up and precipitated with ammonium sulfate. The ammonium sulfate pellet was resuspended in a detergent (Triton X-100, for example) and the material was then centrifuged. The supernatant is called the cell extract. In effect, the result is a Triton X-100 supernatant either from the cell or from the virion. However, the cell extract has a much higher total enzyme activity than the virion extract. 15

Further purifications to obtain more highly purified material have turned out to be not as simple as we had hoped. The enzyme itself is very lipophilic, which is one of the reasons for the use of detergent in the first place. It turned out, however, that the activity of the enzyme itself seems to be dependent—in fact, it is very dependent—on the presence of the detergent. 16 This is not due to solubilization in detergent micelles. The detergent, of course, does help to solubilize the enzyme and take it out of the particulates so it won't centrifuge down, but it also is essential for the full activity of the enzyme itself. We learned of the effect of the detergent on enzyme activity by trying to separate out the enzyme activity from the detergent extract. When we performed that operation, we lost the enzyme activity. Then, we considered that perhaps that was merely the enzyme precipitating, but that turned out not to be the case. It was the enzyme being inactivated by the absence of a
suitable lipophilic material. We were able to show this by adding detergent back again to the extract and recovering the enzyme activity.

**Detergents**

This phenomenon, showing the recovery of enzyme activity with detergent addition, is shown in Figure 8. The detergents we used for this experiment were nonionic and all of them contain in them a polyethylene glycol chain. So, we then introduced polyethylene glycol itself as a material in the solution, and the polyethylene glycol does not activate the enzyme. However, the detergents which contain the polyethylene glycol do activate the enzyme. The recovery of enzyme activity is approximately one-third to one-half of the original activity present. The same effect is observed no matter which nonionic detergent is used, and the activation curve of the enzyme activity is dependent upon the molarity of the detergent and not on the critical micelle concentration.\(^{16}\) The formulas of the nonionic detergents which we have used in these experiments are shown in Figure 9. The first two detergents (Triton X-100 and Triton X-1017) are alkyl aromatics on polyethylene glycol; the Triton DN-65 is a mixed alkyl on a mixture of ethylene and propylene glycols. Brij-35 is the simplest detergent which we used, but it has a long ethylene glycol tail. All of the nonionic detergents do have an effect on the enzyme activity and all of them activate the enzyme to the same extent on a molar basis.

This led us to the conviction that there was a lipophilic site in the enzyme which perhaps represented, and might be identical with, a site at which the enzyme is bound to the membrane. This would also be the reason that detergents are required to remove the enzyme from the cell membrane.\(^{16}\)
The fact that variable detergent was always present in the enzyme assays (in the lit.) helps to account for some of the variability of activity and of inhibitor qualities which were reported for various drugs. The ability of the drug to inhibit the enzyme activity is also dependent upon the presence of detergent, but it is at a completely different level (critical micelle concentration) of detergent that the drug inhibition is affected. The relative activity of some of the rifamycin derivatives on the enzyme action, at two different detergent levels, is shown in Figure 10. You can see that the rifocytloxime and the rifazacyclo-16 are the most active. Even the large dimeric molecules are active. Notice also the amount of drug required to reduce the enzyme activity to one-half in the presence of the detergent (Triton X-100). The amount of drug required for inhibition is higher at the higher detergent level. This gave us some concern, because in the literature you will find that there are many reports of drug activities without careful specification of how much detergent was used. You can see how sensitive this inhibition is to the presence of detergent.

Why should the drug inhibition be dependent upon the detergent concentration? The obvious answer turned out to be that the detergent forms micelles and the micelles are, in effect, a lipid phase. The drug has lipid solubility and goes into the micelles in competition with the enzyme. In effect, there is a competition between the micelles and the enzyme for the lipophilic drug. This is actually what the situation is, and the inhibition titration of the RDP by DMB and rifazacyclo-16 is shown in Figure 11. There were two different levels of detergent concentration and two different drugs. You can see that the rifazacyclo-16
(the circles) at very low detergent levels is extremely active. This accounts to some extent for the arguments that the chemists and bio-
chemists have been having as to which of the drugs are effective and which ones are not. The question now arose as to whether we could show this was micelle extraction of the drug from the enzyme. The results of this experiment are shown in Figure 12. Here we have only one drug present (rifazacyclo-16) and three different detergents, and we are showing the percent of inhibition of the control activity as the function of the amount of detergent used. We have a constant amount of drug and are changing the amount of detergent. As we increase the amount of detergent, the drug becomes totally inactive, and almost 100% of the enzyme activity is exhibited; at lower detergent levels, the drug is working. The same effect shows for all of the various detergents tried--Brij-35, Triton X-100, and Triton DN-65. You can see also that the drug ceases its activity in proportion to the amount of micelle formation. I believe that there is little doubt that these two phenomena are related to each other. The drug is removed from the enzyme by extraction into the micelles. The evaluation of a drug-enzyme interaction, therefore, depends a great deal upon nature of the drug and the nature of the detergent which is in the medium and the concentration of the detergent in the medium.

The next step was to show that the drug actually is in the micelles. The results of an experiment in which the drug is passed through a gel permeation column in the presence of detergent is shown in Figure 13. When there are no micelles, the drug comes out as free drug; as the amount of detergent is increased to where most of the detergent is in the micelles, most of the drug comes out in the detergent micelles.
We thought for some time that other components of the assay mixture might affect the drug activity, and, in fact, they do. The bovine serum albumin (BSA) has a very high lipid content, and it can act in the same way that the micelles do, and the effect of solubilization of DMB with BSA is shown in Figure 14. The tritiated drug (DMB), without any BSA, comes out as a simple molecule very late. As BSA is added, the drug comes out with the BSA peak and the higher BSA ratios give more of the drug in the peak. Here, again, is another variable which must be considered in these enzymatic assays.

We have shown that there are several drugs which are potent enzyme inhibitors. Is this a clue as to the ability of the drug to prevent the focus formation, i.e., to prevent the transformation of a cell into a cancer cell by the virus? This would require a measurement of the ability of the virus to transform cells in the presence of different amounts of drug, without affecting the viability of the cells themselves. In other words, you mustn't hurt the ability of the cell to grow and divide, i.e., relative insensitivity of DDP and DRP. Table 1 shows some of the more recent results, comparing RDP inhibition by seven different rifamycin derivatives and three different detergent concentrations. Table 2 shows the focus inhibition of MSV in Balb/3T3 cells by DMB. In each case, the level of 50% inhibition is of the order of 2-3 μg/ml, which gives pretty good control, and when the concentration of drug is increased to 8 μg/ml, there are no foci present at all. We have studied the ability of cells to grow and multiply in the presence of these drugs, and the drugs are not cytotoxic at low concentration. Having done this experiment with DMB, we decided to compare the effects of DMB and rifazacyclo-16 on the inhibition of focus formation of MLV on the special cell line, UCl-B, and
Table 1. Concentration (µg/ml) Yielding 50% Inhibition or the RDP

<table>
<thead>
<tr>
<th>Rifamycin Derivative</th>
<th>Triton X-100 Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.005%</td>
</tr>
<tr>
<td>DMB</td>
<td>22</td>
</tr>
<tr>
<td>Rifaldehyde octyloxime</td>
<td>6</td>
</tr>
<tr>
<td>Rifamazine</td>
<td>21</td>
</tr>
<tr>
<td>Rif-urea</td>
<td>27</td>
</tr>
<tr>
<td>Dirifampin</td>
<td>18</td>
</tr>
<tr>
<td>Rifazabicyclo-9a</td>
<td>12</td>
</tr>
<tr>
<td>Rifazacyclo-16</td>
<td>4</td>
</tr>
<tr>
<td>Dansyldesmethylrifampicin</td>
<td>11</td>
</tr>
<tr>
<td>Aminodesmethylrifampicin</td>
<td>55</td>
</tr>
<tr>
<td>DMB-oxidizedb</td>
<td>--</td>
</tr>
<tr>
<td>Desmethylrifampicin</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Spin-labeled rifampicin 1</td>
<td>--</td>
</tr>
<tr>
<td>Spin-labeled rifampicin 2</td>
<td>--</td>
</tr>
</tbody>
</table>

aTetrahydrofuran was used instead of DMSO.
bQuinone form of DMB.

Assays were done as described in Methods. DMSO was 1.0% or 1.0%/100 g derivative, whichever is greater. Protein was 0.45 µg-2.5 µg with an activity of 200-62 pmol/hr/g.
Table 2. Focus Inhibition of MSV in Balb/3T3 Cells by DMB

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>DMB* (μg/ml)</th>
<th>Average # Foci</th>
<th>% of Control Foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>597</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>301</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>70</td>
<td>47</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>159</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Drug was present in the medium throughout the experiments, the duration of which was 7 days.
how it was affected by amphotericin B. The results are shown in Table 3. We have expected the rifazacyclo-16 to be an even better inhibitor of focus formation than DMB because, as seen in Table 1, it will inhibit the RDP activity at about one-fifth the concentration which was required for DMB to produce the inhibition. We were surprised to note in our first experiment with rifazacyclo-16 that equal amounts of rifazacyclo-16 (in this case 6 μg/ml) produced much poorer inhibition (14% inhibition) of focus formation than DMB at the same level (46% inhibition). It was at this point that we suspected that the failure of rifazacyclo-16 to live up to its promise as indicated by its enzyme inhibiting capability when used on whole animals might be attributed to its inability to penetrate the cell membrane. It had been previously shown that amphotericin B, a fungicide, when used on yeast on conjunction with other antibiotics enhanced the effect of antibiotics; this was believed to be by virtue of its ability to allow the antibiotic to penetrate the cell membranes in this case. It was for this reason that amphotericin B was tried in the focus inhibition test as well.

Other Enzymes

We have just now begun to expand our efforts, now that we have determined a method for getting the drug into the cells. We now want to extract the enzyme to which the drug has been bound. There is a reason to believe that one of the other enzymes, other than the RDP, is even more sensitive to the drugs than is the reverse transcriptase. There are at least two other enzymes involved--one that breaks the DNA strand and the other that inserts information into the DNA. One or the other of the second stage enzymes in the insertion of new information from an RNA or DNA source is
Table 3. Effects of Amphotericin B and Rifampicin Derivatives on Moloney Leukemia Virus Transformation of UCl-B Cells

<table>
<thead>
<tr>
<th>Rifampicin Derivative</th>
<th>Average # Foci Formed</th>
<th>With Amphotericin B (1 μg/ml)</th>
<th>Without Amphotericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylbenzyl-desmethyl rifampicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>298 (100)</td>
<td>287 (100)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>180 (60)*</td>
<td>234 (80)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>42 (14)</td>
<td>157 (54)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Rifazacyclo-16</td>
<td>298</td>
<td>287</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>298 (94)</td>
<td>291 (91)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30 (10)</td>
<td>251 (86)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

*Figure in parenthesis: percent of control.

Focus inhibition assay was done as described in Table 2.
apparently even more sensitive to the drug than is the reverse transcriptase.

Reference to Figure 4 clearly shows the place in which these additional enzymes play their role. We have called them, in Figure 4, "clipping" enzymes, of which the restriction enzymes are a specific example. Of course, the restriction enzymes are only a specific type of a general class of nucleases called endonucleases which will clip one strand of a double strand. If, however, there is a specificity at the site of clipping such that both specific sites are within a limited number of bases of each other, then both strands of the double strand will be clipped in such a way as to provide sticky ends with a specific base sequence to be exposed, of anywhere from four to eight nucleotides. These specific sticky ends would then be found in both the parent DNA as well as in the DNA copy of the viral RNA if they are both clipped by the same clipping enzyme with this same specificity. This would provide a specific mechanism for inserting the viral information in the form of the DNA copy with the proper sticky ends to find a site in the cell DNA. Following this annealing would come the ligase enzyme which would then link the newly found matching base sequences by a homopolar linkage, completing the insertion process.

CHEMICAL AND VIRAL CARCINOGENESIS

Our primary concern has not been viral oncogenesis but chemical carcinogenesis. At about the same time that I learned of the reverse transcriptase I also learned that a tumor which had been generated by dimethylbenzanthracene and carried as an ascites for some time had the
RDP activity in it. It was this information which really began our work on viral enzymes, which was preparatory for chemical carcinogenic research.

As this work progressed, we were pleased to learn that a relationship between chemical carcinogenesis and viral carcinogenesis has long been known. Perhaps some of the earliest work in this field was first unequivocally demonstrated by F. Duran-Reynals as early as 1957 in which the combined effects of chemical carcinogens and virus infection were described. Another unequivocal demonstration that the administration of both chemical carcinogen and oncogenic virus enhances the probability of appearance of neoplastic transformations appeared in 1965. Much of this work has recently been reviewed by Maria Duran-Reynals. Most recently, this synergism has been described on tissue cultures from a variety of cell lines. Here, again, not only was a synergism demonstrable but in some cases in which the virus was not added exogenously the administration of the chemical was able to produce the release of the virion into the medium, suggesting that the viral information was present in the cell in some form, perhaps as a plastid or even in the nuclear DNA itself.

A rather comprehensive review of the relationships of various endogenous viral informational fragments, particularly in chick embryo cells, has been prepared by Weiss. Here, again, it is evident that a variety of chemical carcinogens, including condensed ring systems, can induce the release of various forms of virions from such cells, depending upon the particular informational content of the cells. Apparently these bits of virion information are widely distributed in
the fowl population, both domestic and wild, and are normally not visible in the phenotype until some special coincidence of circumstance makes their appearance possible.

Perhaps one of the most illuminating bits of tissue culture work indicating the relationship between a chemical carcinogen of the type in which we are interested, namely, the condensed ring hydrocarbons, and viral oncogenesis is some very recent work describing this synergism as a function of the order of addition of the two materials. Using rat embryo cells maintained for as many as 40 passages it has been shown that the chemical carcinogen 3-methylcholanthrene (MCA) at suitably chosen doses is not effective in producing transformation of this tissue culture unless the culture has previously been exposed and infected by a rat-adapted Rauscher leukemia virus (RLV). Furthermore, since treatment by the MCA prior to RLV infection does not produce the synergism, even after a significant number of passages with the virus, the indication is that no long-lived effect of the MCA, either in the form of an activated intermediate or in the form of a preliminary action on the cellular components is crucial. On the other hand, since treatment with MCA following infection with this RLV will show transformation and usually not before several passages after the treatment with MCA, the suggestion here is clearly that the carcinogen acts directly on the package of oncogenic information contained in the RLV. It is for reasons such as this, but long before this particular information appeared, that our chemical carcinogenic activities were directed toward the interaction of the chemical carcinogen with a package of cancer producing information as represented by the oncogenic virus.
In the work reported here, I have another bit of evidence to add, demonstrating the relationship between viral and chemical carcinogenesis. That data is shown in Figure 15. We have a strain of rats which upon two injections of dimethylbenzanthracene will within six weeks all develop mammary tumors. This is a good animal system for examining chemical carcinogenic effects. I was anxious to see if the drug would have any effect in the live animals, and the results of this experiment show what happens when the drug is given to the animals after the injection of the carcinogenic material. The carcinogen is injected during the first week, and the drug is given at several different concentrations and rates for the following 10-12 weeks. The number of control animals (the animals which received no drug) are shown in the circles. The animals in the upper part of Figure 15 are Sprague-Dawley rats with the carcinogen and DMB compound injected intraperitoneally; the animals in the lower part of Figure 15 are Long-Evans rats injected with trimethylbenzanthracene and DMB-rifampicin injected intraperitoneally.

You will note that in both cases the onset of tumors is later with the injection of the drug and the average lifetime of the animals is from about eight weeks to eleven to thirteen weeks. The onset of the tumors is slower and the development is slowed up. The drug does not cure the tumors, or stop them, but it slows them down in their onset and progress. This may be due to the fact that the chemical carcinogenesis does not involve the insertion of a new piece of RNA or DNA into the cells, but the chemical allows it to become expressed in some way.

The reason that the drug acts at all in the chemical carcinogenesis is that in order to keep the cancer growing at its full speed, the RNA-instructed DNA polymerase must play a role in the replication and division
of the tumor cells. This gives us some justification for saying that the
drug does indeed slow down the onset of the chemically induced tumor and
slow down its ultimate development.
REFERENCES

FIGURE CAPTIONS

Figure 1  Nontransformed Balb/3T3 cells, 5500 x, showing contact between cells (optical microscope)

Figure 2  MSV foci on Balb/3T3 cells (two magnifications: 10 x and 40 x) (optical microscope)

Figure 3  Section through UCl-B cells showing both normal (MLV) and aberrant (MSV) Type C virus in cells (magnification 240,000 x) (electron microscope)

Figure 4  Scheme for possible function of RDP in oncogenesis by RNA viruses

Figure 5  Four ansa antibiotics, including rifamycin

Figure 6  Some synthetic modifications of rifamycin

Figure 7  Extraction scheme for RDP

Figure 8  Activation of RDP by nonionic detergents

Figure 9  Structural formula of nonionic detergents used in RDP activation. Those shown with ethylene oxide components indicate the composition of the starting monomer; those shown with polyglycol formulas indicate materials which have been purified after polymerization

Figure 10  Relative activities of rifamycin derivatives in RDP inhibition inhibition

Figure 11  Titration of RDP inhibition by DMB and RC-16 at three Triton X-100 concentrations

Figure 12  RDP inhibition by RC-16 and detergent micelle formation by Brij-35, Triton X-100 and Triton DN-65 detergents

Figure 13  Solubilization of DMB in Triton X-100 micelles by gel filtration (Sephadex G50)

Figure 14  Solubilization of DMB by BSA, gel filtration (Sephadex G50)

Figure 15  Prophylactic effect of DMB against DMBA and TMBA in rats
Nontransformed Balb 3T3/A31 cells (5500 x)

Fig. 1
MSV Foci on BALB/3T3 Culture

10x Dark field illumination 40x

Fig. 2
Complete MLV C Type
Aberrant MSV cylindrical and incomplete

Balb UCI-B + MLV $\sim 240$ K

Fig. 3
Fig. 4

- DNA<sub>c</sub> (Normal Cell) → DRP → RNA → Proteins
- DNA<sub>v</sub> (Transformed Cell) → RNA<sub>v</sub> (Oncogenic Virus)
- Clipping Enz.
- Ligase Enz.
- DDP (Replication)
- DNA<sub>c</sub> → DNA<sub>c</sub> - DNA<sub>v</sub>
RIFAMYCIN AND CONGENERS

RIFAMYCIN B

STREPTOVARICIN A (X=OH, Y=OCOCH₃)
C (X=H, Y=OH)
D as C + 2H on C₂₇ + C₃₀

TOLIPOMYCIN Y

GELDANAMYCIN

1. R=H
2. R=COCH₃

Fig. 5
Rifampicin

DMB

Rifazabicyclo-9

Rifocytoxime

Rifazacyclo-16

Rifurea

Rifamazine

Dirifampin

Fig. 6

XBL729-4774 A
RIDP EXTRACTION

**cell layers in growth medium**

- **cells**
  - wash scrape
  - centrifuge
  - cell pellet
  - sonicate precipitate centrifuge
  - \((\text{NH}_4)_2\text{SO}_4\) precipitate
  - centrifuge
  - \((\text{NH}_4)_2\text{SO}_4\) pellet
  - resuspend solubilize (Triton X-100) centrifuge
  - \((100,000 \times g; 60 \text{ min})\)
  - cell extract

- **medium**
  - centrifuge
  - cell free supernatant
  - centrifuge \((40,000 \times g; 90 \text{ min})\)
  - virus pellet
  - resuspend solubilize (Triton X-100) centrifuge
  - \((100,000 \times g; 60 \text{ min})\)
  - virus extract

Fig. 7
Fig. 8

TRITON X-100 CONCENTRATION (mM and v/v%) IN ASSAY

% of MAXIMAL ACTIVITY

0 0.04 0.08 0.12 0.16 mM
0 0.0025 0.005 0.0075 0.010 %
Triton X-100  
(Rohm and Haas)

Triton X-1017

Triton DN-65  
(Rohm and Haas)

Brij 35  
(Sigma)

Polyethylene Glycol-400

Fig. 9
<table>
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<tr>
<th>Compound</th>
<th>Partition ratio</th>
<th>$C_{1/2}^*(0.005)$</th>
<th>$C_{1/2}^*(0.025)$</th>
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<tr>
<td>Rifampicin</td>
<td>14</td>
<td>&gt;100</td>
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<tr>
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<td>1700</td>
<td>22</td>
<td>73</td>
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<td>---</td>
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<td>48</td>
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<tr>
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<tr>
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<td>35</td>
</tr>
<tr>
<td>Dirifampin</td>
<td>49</td>
<td>18</td>
<td>35</td>
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</table>

*C_{1/2}^* is the concentration of rifamycin derivative in μg/ml required to produce a 50% inhibition of RIDP activity. The numbers in parentheses are the concentrations of Triton X-100 in percent (v/v).

Fig. 10
Fig. 11
Fig. 12
Fig. 13
Fig. 14
Fig. 15
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