MEETING REPORT

THE BIOLOGY OF ONCOGENIC VIRUSES*
A report on the 2nd Lepetit Colloquium held at the Pasteur Institute, Paris, during 6–8 November 1970

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1. Introduction

Since the discovery that certain viruses can induce malignant tumours in experimental animals and can transform cells in culture giving them oncogenic properties, the study of such viruses has become one of the most interesting and fruitful approaches to the control of human cancer. The viral etiology of animal leukaemias and possibly a human lymphoma gives support for this view. The colloquium provided a report on current progress and emphasised that molecular biology is at last being put to use in medicine. For none of the exciting results described, particularly with regard to the diagnosis of human leukaemias by RNA-dependent DNA polymerase assays and the activity of rifampicin derivatives to inhibit this viral enzyme, would have been possible without knowledge of cellular and viral macromolecular mechanisms of replication and information transfer. The advances that have been made recently in this complex field, which are reported below, are the product of many years of virological and biochemical work. When such results are presented elaborate nomenclature is used and conceptual assumptions are made that scientists in other fields find difficulty in understanding. Perhaps a discussion of such words and concepts would be useful before a report on the meeting.

Oncogenic viruses, that is viruses which can transform (convert) normal cells *in vivo* or *in vitro* into "cancer" cells which multiply and proliferate in an uncontrolled manner, are classified as to whether they contain DNA or RNA. The genetic information carried in the nucleic acid of the virus particle (virion) is termed the virus genome. Although other DNA-containing viruses have been shown to cause tumours in experimental animals, current research into the mechanism of oncogenesis by DNA viruses is almost entirely restricted to simian virus strain 40 (SV40) isolated originally from monkey kidney cells, and polyoma, originally isolated from mouse tumour tissue. There are several reasons why these two are so popular. They can be grown easily and will transform cells without difficulty in the laboratory. Also they are both small and their DNA probably does not code for more than ten small proteins. Yet in this small piece of nucleic acid lies the information to cause a malignant tumour. When a virus of this type enters a cell, it can either grow lytically, that is, multiply within and eventually lyse and destroy the cell to liberate daughter particles, or it can transform the cell. In the latter response, the viral DNA is integrated into the DNA of the host cell chromosomes. The viral DNA becomes part of the chromosomal DNA and is replicated during the normal process of cell mitosis. Thus the cell has a covalently fixed molecular parasite which is reproducing itself by making the cell multiply repeatedly. In the laboratory, normal cells grow on the wall of the culture dish to form an even monolayer and then stop dividing. In this state they are said to be confluent. The same cells when transformed grow in multilayered heaps (foci) on the dish wall.

The probable molecular biology of these processes

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may be summarized as follows. After entering the cell, the virus particle loses its protein capsid (outer coat) and the viral DNA passes into the nucleus. There host DNA-dependent RNA polymerase binds to the viral DNA, probably at a specific promoter (the site at which RNA synthesis is initiated) and transcribes the DNA, forming complementary viral RNA. The latter then passes back into the cytoplasm to be translated into viral specific protein on the cell ribosomes. One of these proteins is known and is called the tumour antigen (T-antigen). It can be detected immunologically. Depending on the nature of the cell or other factors, the lytic or transformation interaction may occur. In the lytic response, viral DNA is replicated presumably by viral enzyme(s) and packaged with virus capsid protein to form new virions. If transformation is elicited the viral DNA is integrated by a mechanism as yet unclear. All viral specific activities which happen in the cell before viral DNA replication begins are termed early functions. Those occuring during replication are called late. Besides the induction of T-antigen, other early functions are: stimulation of host DNA synthesis; a change in the host cell surface properties; and stimulation of the synthesis of certain host cell enzymes concerned with DNA replication.

Numerous RNA tumour viruses are under study and some, notably the Schmidt-Ruppin strain of Rous sarcoma virus, will transform cells in culture. The molecular biology of these viruses has become more clear recently. The RNA replication of RNA bacteriophages and many RNA viruses, such as influenza, is mediated by an RNA-dependent RNA polymerase and proceeds by transcription of the viral RNA to form a second strand of RNA bound to the first. This is called the double strand viral RNA intermediate. The second strand, termed minus strand, in then replicated to form the daughter strands which are identical with the virion RNA. However, the work of Temin and others has shown that RNA tumour virus nucleic acid is replicated by an RNA-dependent DNA polymerase which produces an RNA–DNA hybrid (containing single stranded viral specific DNA), then a single stranded viral DNA, and finally, double stranded viral DNA. This viral DNA possibly may then act as template for production of daughter viral RNA or may be integrated into host cell chromosomal DNA during transformation of the cell. Production of daughter viral RNA from the integrated DNA also almost certainly occurs.

The meeting was divided into sessions at which either DNA or RNA tumour viruses were discussed. We shall start with the DNA virus reports.

2. Biochemical studies on the gene products of DNA viruses responsible for transformation

As the initial product of gene expression is RNA, several papers dealt with viral RNA synthesis, *in vitro*, in the lytic response, and in transformed cells. Following this, viral protein synthesis was discussed.

2.1. *In vitro transcription*

Transcription of SV40 DNA by *Escherichia coli* RNA polymerase, as described by H. Westphal (Cold Spring Harbor), can proceed along the entire genome without release, although rho, the bacterial factor which causes specific termination of RNA synthesis at the end of a gene, slightly reduces the size of the product RNA. In agreement with this finding of polycistronic transcription, E. Winocour (Weizmann Institute) reported electron microscope studies which showed that SV40 DNA binds only one molecule of monkey or rat RNA polymerase per molecule DNA indicating that only one promoter site for initiating RNA synthesis is present. So it would appear that the whole of the viral DNA is transcribed in one pass of the enzyme, producing a piece of RNA that is the same length as the DNA.

2.2. *Viral transcription during the lytic response and in the integrated state*

The viral RNA species detectable in cells lytically infected with SV40 have been characterised with respect to size and time of synthesis after infection by M. Herzberg (Weizmann Institute) and E. Winocour and by S. Tonegawa et al. (Salk Institute). The size of the RNA produced correlates well with the size of some of the viral proteins formed at the same time after infection. So it seems likely that these RNA species are in fact the viral RNA messengers that are translated to protein on the cell ribosomes. Cells transformed by SV40 synthesise viral RNA which is found initially in the high molecular weight nucleoplasmic RNA fraction. J.E. Darnell and T.R. Wall (Columbia) showed this to consist mainly of cell RNA with a 10–25% content of viral RNA. Tonegawa reported that the viral RNA is degraded to half genome size

pieces before transfer to the cytoplasm.

All these results bode well for the possible isolation and purification of the viral mRNAs. Of particular importance is the isolation of the messengers necessary for viral integration and its maintenance.

2.3. *Virus specified proteins*

Isolation of the virus-specified proteins whose action mediates transformation is still a pipedream to most oncogeneticists. One therefore cannot but admire the courage of L.V. Crawford (Imperial Cancer Research Fund), B. Hirt, R.F. Gesteland and G.M. Rubin (Cold Spring Harbor) in having the temerity to attempt to synthesise such proteins *in vitro*. Early results look encouraging in that a combined RNA and protein synthesising system containing RNA polymerase, ribosomes and factors from *E. coli* will make large polypetides, some up to 35,000 daltons, coded for entirely by SV40 or polyoma DNA. The polypeptides are being characterised by tryptic digestion and electrophoresis. The electrophoretic patterns they yield will then be compared with those given by virion and *in vivo* produced viral proteins.

3. Cell surface changes induced during transformation by DNA viruses

Cell surface changes are induced early after infection and are essential if the cell is to exhibit oncogenic growth. The demonstration by Max Burger and coworkers (Princeton) that wheat germ agglutinin reacts with cell membrane receptor sites exposed during infection and transformation by polyoma or SV40 has provided an important tool to examine the role of the cell membrane in oncogenesis. Trypsin treatment exposes the receptors in normal cell membranes. Burger presented results which indicate that receptor exposure is linked with cell DNA replication and that the agglutinin, which kills the cells, acts by sticking them together thereby preventing essential movement. He suggests that the normal receptor-covering material stops growth of confluent cells in a similar way. When this material is lost during transformation the multilayer heaped growth characteristic of oncogenic cells can occur, as the outer membranes do not then interact.

Walter Eckhart (Salk Institute) reported that in cells transformed by a temperature sensitive (ts) mutant of polyoma, ts-3, the state of the plasma membrane was controlled by the mutant gene. At permissive temperatures which allow expression of the gene the receptors were exposed, whilst at non-permissive temperatures which inactivate the gene they were covered; switching from one condition to the other could be demonstrated by altering the growth temperature. However, J.F. Sambrook (Cold Spring Harbor) described the isolation of mutant transformed cells which had acquired resistance to concanavalin A, a substance with similar properties to wheat germ agglutinin, showing that these cells have regained genetic control of this feature of the cell surface without affecting the transformed state.

A difference in the topography of the agglutination sites on the cell surface was found by Leo Sachs (Weizmann Institute) and he claimed that all carcinogens, chemical or viral, produce the same changes. Biochemical changes in the cell plasma membrane on transformation have been detected. Rose Sheinin (Toronto) showed that transformation causes an increase in the amount of basic protein present and a loss of 2 of the 4 gangliosides detectable. Also I.A. Macpherson (Imperial Cancer Research Fund) found that two glycolipids, aminoglycolipid and ceramide trihexoside, are similarly lost.

4. Genetic analysis of the virion and of the intergrated viral genome of tumour viruses

Genetic analysis is a powerful tool with which to define the genes responsible for transformation and study their mode of action. Unfortunately the relative inefficiency of the growth of cells in culture and the growth of virus in cells compared to bacteria–bacteriophage systems have made its application difficult. However, progress is being made. Temperature sensitive and host range mutants have been discovered.

4.1. *Temperature sensitive (ts) mutants*

Eckhart described the range and properties of available ts mutants of polyoma which do not replicate their DNA at the non-permissive temperature and showed that certain cellular characteristics are controlled by these mutant viral genes both during infection and during the maintenance of the trans-

formed state; for example, the cell surface changes described earlier. Tumor viruses containing RNA have yielded ts mutants affecting their alibity to transform cells. Reports of their isolation came for the Schmidt-Ruppin strain of Rous sarcoma virus by P.Vigier (Radium Institute) and by G.S. Martin (Berkeley) and for another avian sarcoma virus by Peter Vogt (Seattle). Evidence concerning the nature of the ts-a gene of polyoma was presented by Jesse Summers (Salk Institute). The gene may control the maintenance of the integrated state of the viral genome since, if grown at the non-permissive temperature, cells previously transformed by this mutant will yield virus if fused to mouse cells at the permissive temperature. This phenomenon is not seen with cells transformed by wild type virus or when the polyoma ts-a transformed cells are cloned at the permissive temperature. Attempts by A. Ishikawa and G. di Mayorca (Nebraska) to set up an efficient recombination system to map the ts mutants of polyoma proved unsuccessful though recombination was demonstrated.

4.2. *Host range mutants*

Mutants of polyoma which show lytic growth in transformed mouse cells but which do not grow in normal cells have been isolated by Thomas Benjamin (New York Public Health Research Institute). Happily, they all show loss of transforming ability as well as lytic growth in normal mouse cells. This indicates that the mutations lie in a gene whose activity is needed for lytic growth and for integration and which is also expressed during the maintenance of the integrated viral DNA. Benjamin reported that loss of this gene activity did not prevent adsorption, penetration, uncoating, T-antigen induction, or stimulation of host cell DNA synthesis when non-permissive, that is, normal cells were infected with the mutants. However, no cell surface changes are induced and the virus capsid protein is not made.

5. Studies on DNA virus replication, integration and rescue

Evidence concerning the mechanism of polyoma DNA replication was presented by Pierre Bourgaux (Sherbrook University, Quebec). Polyoma DNA, which is circular, is replicated at a single growing point which travels along the DNA unidirectionally. This generates an intermediate resembling two circles of DNA partially fused together such that a portion of the DNA is common to both circles. Elegant electron micrographs were shown of the replicative intermediate before and after treatment with an endonuclease from *Neurospora crassa*. This enzyme is highly specific for single stranded nucleic acid. The endonuclease cleaved the intermediate first at one branch point and then at the other.

Studies on the use of the cell fusion technique to test the dependence on host chromosomes of the lytic or transforming response of polyoma in mouse–hamster cell hybrids are underway in Claudio Basilico's laboratory (New York University). As found previously, permissiveness to lytic growth is dominant, that is, these hybrids almost always support lytic growth and are not transformed. In hybrids of transformed hamster and normal mouse cells, superinfection of the hybrid cells with mutant ts-a polyoma virus appears to rescue the transforming virus, though recombination effects, that is, exchange of genes between the integrated and infecting virus DNA, are difficult to rule out. Rescue, which means excision of integrated viral DNA from the host chromosome, of SV40 from transformed hamster cells may involve a helper virus. F.C. Jensen (Wistar Institute) reported that a new foamy virus (hamster syncytia forming virus) always appears with the rescued SV40. Intracellular factors affecting the replication of SV40 were discussed by S. Kit (Baylor) and Roland Cassingena (Villejuif) and the confused state of knowledge concerning the regulation of interferon production and action was described by C. Chany (Villejuif).

6. Rifampicin, an anti-transformation drug?

On the anti-DNA tumour virus drug scene, J.H. Subak-Sharpe (Glasgow) reported a greatly depressed polyoma transformation frequency with rifampicin-resistant BHK cells in the presence of the drug. This might indicate that the aminopiperazine family of drugs has some activity against oncogenic transformation that could be enhanced by a proficient organic chemist. An interesting and possibly highly significant finding was presented by L. Thiry (Pasteur, Brussels). The AP4 derivative of rifampicin is able to reduce the Epstein-Barr virus antigen by over 80% in a Burkitt

cell line derived from human Burkitt's lymphoma tissue. The DNA-containing Epstein-Barr virus, a type of herpes virus, may be the causal agent of this human cancer.

7. The mechanism of oncogenesis caused by RNA tumour viruses

Probably the major scientific event of the year was the discovery of RNA-dependent DNA polymerase activity in the virions of many RNA tumour viruses. The validity of H.M. Temin's ideas on the role of virus-specific DNA in the replication and integration into host chromosomes of the viral nucleic acid is now generally accepted. At this meeting "Le Déluge" continued.

7.1. *Role of RNA-dependent DNA polymerase in viral integration and replication*

David Baltimore (M.I.T.), M. Green (St. Louis) and S. Spiegelman (Columbia) each described the latest properties of their respective polymerase enzyme(s) which synthesise double strand viral DNA from single strand viral RNA. The consensus appears to be that a single enzyme or multienzyme complex catalyses the reaction, with RNA–DNA hybrid and single strand DNA as intermediates. This was demonstrated by Baltimore with the mouse leukaemia virus system utilising the *N. crassa* endonuclease which specifically breaks down single stranded nucleic acid. Here the polymerase(s) act on viral RNA to produce a complex containing all the above intermediates and products. When treated with the endonuclease, the complex yields an RNA–DNA hybrid and double strand viral DNA. Green showed similar results using hydroxyapatite to separate single and double stranded DNA and RNA–DNA hybrid. In all cases the final products are small pieces of DNA which sediment at less than 10 S. It was left to Peter Duesberg and coworkers (Berkeley), however, to clear up the troublesome problems of the extent of virus specific DNA in the DNA product and of the extent of RNA transcription – is the whole viral genome transcribed into DNA or just a small portion? Firstly, the RNA–DNA hybrid of the Schmidt-Ruppin Rous sarcoma virus, after treatment with RNase, was shown to have a density in gradients close to that calculated for a complete double strand RNA–DNA hybrid. Since RNase breaks down single stranded RNA, the majority of the DNA present must be in the hybrid form (single stranded DNA could easily be distinguished at a lower density). Secondly, in saturation studies, 60–70% of the RNA strand could be annealed with DNA indicating extensive transcription of the viral genome. The ribbon to tie up this neat package could be a ligase activity which would link up the bits of viral DNA to make a complete viral DNA genome. This could then be used for infection, replication, and integration studies. Unfortunately the small size range could be due to contamination of the *in vitro* system with a cellular endonuclease artifactually degrading the DNA product to the small pieces observed.

G.J. Todaro (Bethesda) questioned the belief that the RNA-dependent DNA polymerase system is unique to tumour viruses since he has also found the activity in visna and in primate syncytia forming virus (foamy) type 3. A voice in the wilderness was that of L.J.L.G. van Griensven (St. Louis Hospital, Paris) who dared to suggested that an RNA tumour virus might replicate not via DNA but through a double stranded viral RNA intermediate. Though out of fashion this summer, his group have isolated such material from cells infected with mouse sarcoma virus and have shown that interferon inhibits its production and the formation of complete virus particles. This would not be expected if a DNA intermediate were involved. The paper, however, drew heavy criticism since little is known of the mode of action of interferon and the results could perhaps be explained by cellular changes induced by the antiviral agent. Also, Temin pointed out that interferon has little effect on cell transformation by these viruses. N. Biswal and M. Benyesh-Melnick (Baylor), with feet firmly in both camps, reported that double strand viral RNA can be demonstrated in cells transformed and infected with the murine sarcoma–murine leukaemia virus complex, the virions of which contain DNA, in addition to the RNA genome, which is complementary to the viral RNA. They suggest that whilst transformation is DNA dependent, replication is not.

7.2. *Clinical approach*

Perhaps the most spectacular achievement reported at the colloquium was the clinical application of these findings. S. Spiegelman described 9 cases of untreated human leukaemia (the total examined so far) in which this probably unique viral polymerase could be detected in the membrane fraction of blood buffy coat cells

(leukocytes). Tests on 30 controls, 10 normal and 20 from patients with a non-malignant leukocyte proliferation, were all negative. A clear cut result, positive or negative, was obtained in all tests. The assay can be performed on as little as 5 ml of blood and uses poly dT:rA or poly dC:dG as template for the enzyme. The use of the technique as a diagnostic tool is therefore well on the way to being established. Possibly a more meaningful test would be to assay the blastoid cells in the bone marrow and this approach is being examined currently.

One of the hopes promoted by Temin's prediction and discovery is that drugs may be found to antagonise this apparently unique viral enzyme activity. In this regard, M. Green reported that derivatives of rifampicin will inhibit the polymerase at concentrations lower than are required for inactivation of normal bacterial and cellular polymerases. Since rifampicin is already in clinical use, this could be a most important finding. Gallo has already partially purified the viral polymerase from the lymphoblasts of leukaemic blood. This shows a 60–70% inhibition with 50 μg/ml of *N*-dimethylrifampicin. These drugs seem to have promise in the treatment of leukaemia, provided that the enzyme is important in the maintenance of the leukaemic state.

7.3. *Cellular RNA-dependent DNA polymerase?*

The probability of finding specific antiviral drugs would sink if it were found that the "viral" polymerase is in fact cellular. A cellular polymerase could well be carried in the virion. Evidence that this might be the case is starting the accumulate. Spiegelman finds that the polymerase can be detected in a line of rat ascites tumour cells which has not shown the presence of any virus over several years. Also G. Tocchini-Valenti (Naples) reported that ribosomal DNA syntheis in developing Xenopus oöcytes is inhibited by a rifampicin derivative (AF/ABDMP) which also inhibits RNA-dependent DNA polymerase but which has no effect on the known cellular RNA or DNA polymerases. This result suggests that extrachromosomal gene amplification, such as the production of ribosomal DNA in oocytes, proceeds via an RNA intermediate; whether or not gene amplification occurs in other cells remains to be investigated.

8. Biology of RNA tumour viruses

Since Temin, the workers in this field have found themselves in the spotlight of trendy science. It was apparent that until quite recently this research had suffered through lack of attention by molecular biologists and biochemists. The deficiency is now more than remedied. Temin, far from resting on his laurels, continued to speculate.

8.1. *Further Teminism*

H.M. Temin (Wisconsin) suggested the family name of Rous viruses for those viruses able to replicate and transform cells via a DNA intermediate and went on to discuss the origin of these particles. Sites, which he called protoviruses, in the chromosomal DNA of somatic cells, but not germ cells, might yield RNA capable of passing from cell to cell and acting as a template for re-synthesis of DNA. This in turn could be re-integrated into chromosomal DNA probably at or alongside the protovirus site. The protovirus hypothesis suggests that such a cycle, with its potential for mutation and recombination, could have provided a mechanism for the evolution of RNA tumour viruses.

8.2. *Rous sarcoma virus (RSV)*

This, perhaps the most famous of all tumour viruses, is still yielding highly significant results with regard to virus cancer. Cells from chick embryos are either positive or negative for a genetic factor (called *chf* or COFAL) which enables RSV to grow and produce infectious particles (normally it requires the presence of the helper virus, avian leukosis virus). The work of H. Hanafusa et al. (New York Public Health Research Institute) has shown that the genetic factor is integrated into the host cell chromosome and is inherited in a Mendelian manner. It consists of a defective avian leukosis genome. Uniquely, the RSV can rescue the factor and transmit it from cell to cell. Hanafusa also reported that RNA- and DNA-dependent DNA polymerases are necessary for transformation of cells by RSV but not for the maintenance of the transformed state. Defective RSV called α (0) cannot transform cells unless these activites are supplied by a helper virus. But once integrated into cellular DNA, the viral presence is stable. M.A.Baluda (U.C.L.A.) demonstrated such integration by hybridization tests. Duesberg reported that RSV virion RNA can be separated into two subunits, called *a* and *b*, one of which appears to be as-

sociated with transforming ability. Both avian leukaemia virus which does not transform, and Peter Vogt's non-transforming mutant of RSV lack the *a* subunit of RNA.

8.3. *Tumor antigens*

Hopefully doctors may one day be using agents to attack specifically the integrated viral genome in cells of certain types of cancer tissue. Hence the detection and identification of the virus present is obviously of great importance. W. Schäfer (Tübingen) reported the discovery of an antigen which is interspecies specific for several mammalian leukaemia and sarcoma viruses. Of considerable significance is the finding that the antigen can be detected in bovine and human leukaemic cells in culture and in cultured human breast cancer cells. This is further evidence for the viral etiology of human leukaemia and other human cancers.

In conclusion

The Italian pharmaceutical company Lepetit must be well pleased with the success of their second colloquium, and if the current promise of rifampicin derivatives as anti-tumour virus agents matures, they should be able to afford a third next year. Apart from an overcrowded programme, which sometimes did not allow much time for discussion, and a fiery monolingual French projectionist, the meeting was exceedingly well organised (mainly due to the efforts of Professor L.G. Silvestri of Lepetit) and the hospitality lavish.