INDUCTION OF VIRUS FROM TRANSFORMED CELLS : THE SV40 MODEL

by

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ABSTRACT

In the present work we describe experiments on the rescue of SV40 by cell association techniques (fusion or co-cultivation). Evidence is presented on the application of electron microscopy for the rapid detection of SV40 particles rescued by cell association. The utility of the electron microscope as a rapid means for the detection of virus eventually recovered from human or animal tumors, as well as from other diseases where viral etiology seems the most probable, is also discussed. Different factors concerning the cell fusion and the biology of fused cells are examined. Main features of successive generations of tumors produced in hamsters by inoculation of SV40-transformed cells are described. Studies on tumor cell cultures are also reported. A theory is proposed to explain, in part, the local growth of the tumor as well as the occurrence of metastases, particularly those at the lymph node level.

In whole, we present a general view of the main biological problems concerning the virogenesis and some other biological features of SV40-transformed cells and the tumors produced in hamsters by these cells.

Je dédie ce travail

à mes Parents,

à mes Professeurs,

et

à mes Amis.

FOREWORD

Before undertaking the studies described below, we were in search of a research topic correlated with SV40 which would not demand any special knowledge in biochemistry and biophysics. This research project would also be of a kind which could be worked out without any special technical assistance and whose results would contribute, possibly, to the growing knowledge of comparative virology or tumor biology. By then evidence had been presented for the first time, on the inductibility of the SV40-genome from transformed cells which did not yield virus by any other techniques known then. We, then, thought that the SV40-hamster system could be used as a model for preparing ourselves for later study on virus rescue from other systems, particularly from man. This should include virogenic studies on the Burkitt's lymphoma, leukemia and possibly neoplasms of various types. Incidentally, a considerable number of workers had started reporting the establishment of cell lines from some of those malignancies. We then wrote to eight of those authors to enquire if samples of those cell cultures could be obtained for further studies with them. Surprisingly, six of those workers never answered our request, one did send cells which were not the same as described in his paper and furthermore these cells, grown only in suspension, were perishable at low speed centrifugation (i.e. 120g/10min). Another author did send us samples in two irrational ways: (1) bottles one third filled and shipped like any other object, and (2) frozen monolayers. This was done by a "scientist" from the National Cancer Institute, N.I.H.,

Bethesda (U.S.A.). Thus, we started to investigate other aspects using our own system. Since there are still many more interesting things to be learned with the SV40-hamster system we felt the work was worth continuing.

Most of our results described in the present work have been communicated in some scientific meetings, published, submitted or in preparation for publication as indicated below:

1 - Menezes, J., Pavilanis, V., and Dubreuil, R. Etudes sur l'induction virale, par la fusion cellulaire, de deux lignées cellulaires d'hamster transformées par le SV40. Ann. de l'ACFAS, 35, 164 (1968).

2 - Menezes, J. Studies on the recovery of SV40 virions from SV40-transformed cells. Program and Abstracts, 19th Ann. Meeting, Can. Soc. Microbiol., p.48, Univeristy of Ottawa (1969).

3 - Menezes, J. and Perry, E. Can SV40 be a helper for Picodnaviruses? Program and Abstracts, 20th Ann. Meeting. Canad. Soc. Microbiol., p. 70, Dalhousie University, Halifax (1970).

4 - Menezes, J. Electron microscopic detection of transforming viruses induced by cell association. I. The SV40 model. Int. J. Cancer, 7, 331-338 (1971).

5 - Menezes, J. and Perry, E. Main features of successive generations of Cl_2TSV_5 and RHaT produced tumors and their cell cultures, and the inductibility of SV40. Int. J. Cancer (submitted for publication).

6 - Menezes, J. and Perry, E. Evidence of SV40 helper action for a parvovirus. (In preparation).

In this thesis we present first a literature survey on the main characteristics of SV40, the cell fusion process and its main applications for virus rescue. We then describe in chapter II studies performed on the rescue of SV40 by using permissive as well as non-permissive cells. In this work, electron microscopy is used as the main tool for the detection of rescued SV40. In the chapter III different features of tumors induced by SV40-transformed cells are described; different properties of tumor cell cultures are also discussed. An introduction and a discussion are given at the beginning and the end, respectively, of these two chapters.

Although it might seem that a few techniques referred to in the chapter II are repeatedly described in chapter III, we wish to make it clear that this is not exactly the case. In chapter II those techniques used were described by other workers, while in chapter III we investigated a certain number of points which we found had not yet been described by other authors. Moreover, we have also decided to present these two chapters as two successive steps of our research work. A short general discussion combined with conclusions follows these chapters.

Finally, an appendix is also given in which we summarize observations concerning the evidence of SV40 helper action for a parvovirus which was surprisingly found during the present work. A list of cited references will, as usual, close this thesis.

ACKNOWLEDGEMENTS

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We are particularly indebted to the Medical Research Council of Canada for a fellowship awarded to us to undertake these studies.

"Prenez intérêt, je vous en conjure, à ces demeures sacrées que l'on désigne du nom expressif de laboratoires. Demandez qu'on les multiplie, qu'on les orne; ce sont les temples de l'avenir, de la recherche et du bien-être; c'est là que l'humanité grandit, se fortifie et devient meilleure".

Louis PASTEUR

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A - THE SIMIAN VIRUS 40 : AN ONCOGENIC DEOXYRIBOVIRUS.

THE DISCOVERY OF SV40 : A HISTORIC NOTE.

In 1960, Sweet and Hilleman reported the "detection of a "non-detectable" simian virus (vacuolating agent) present in rhesus and cynomolgus monkey kidney cell culture material". This vacuolating agent was, thereafter, called "the vacuolating virus, SV40" by the same authors (Sweet and Hilleman, 1960b).

It is, however, interesting to note that independently of Sweet and Hilleman (1960a,b), Eddy et al.,(1961) set out to determine whether viruses found in rhesus monkey kidney cells (Hull et al.,1958) could produce tumors when inoculated in baby hamsters; they did not test isolated viruses, but merely extracts from frozen and thawed cultures of rhesus monkey kidney cells. Thus, Eddy et al.,(1961) described tumor production in hamsters inoculated with such extracts. Subsequently, in 1962, Girardi et al., Eddy et al., and Rabson et al., described for the first time the oncogenicity of SV40. In fact, after the inoculation of SV40 into newborn Syrian hamster (Mesocricetus auratus) (Eddy et al., 1962; Girardi et al., 1962) or rat (Rattus natalensis) (Rabson et al., 1962), these authors observed development of malignant tumors in those animals within a few months; moreover, these tumors also proved to be transplantable to an homologous host.

Thus, SV40 was found to be the first virus not to express its oncogenic potential in the natural host. This very particular and interesting feature was later also demonstrated with some members of the adenovirus group of human (Huebner et al., 1962; Girardi et al., 1964;

Pereira et al., 1965; Sohier et al., 1965; Trentin et al., 1962;), simian (Hull et al., 1965), bovine (Darbyshire et al., 1968) and avian (Sarma et al., 1965) origin.

After these general considerations, we will now examine the main properties of SV40 and some interesting features of SV40-cell interactions, which might be of relevance. Other molecular and biological aspects concerning SV40, which have been recently reviewed in detail by Green (1970), Crawford (1968), Fenner (1968), Dubreuil (1970), Dulbecco (1969), Eckhart (1969) and Yoshikawa-Fukada and Ebert (1971) will not be considered in the present review.

THE SV40 VIRION: STRUCTURE AND BIOCHEMISTRY

SV40 is a small icosahedral deoxyribovirus which is classified as a member of the papovavirus group, (Melnick, 1962; Lwoff and Tournier, 1966). The virion has a molecular weight of about 17 million daltons (Koch et al., 1967), and contains 12% DNA and 88% protein (Green, 1966, 1970). The SV40 capside appears to reveal 72 morphological units or capsomeres (Anderer et al., 1967) and the DNA has a guanine + cytosine (G+C) content of 41% (Green, 1965); this base value is very close to that of mammalian cell DNA's (42-44% G+C). Seven to nine percent of the SV40 DNA hybridyzes with monkey cell DNA, and a similar complementarity is also found between the SV40 molecule and mouse cell DNA (Aloni et al., 1969). The molecular weight of the SV40 DNA is about 2.5×10^6 daltons, (Anderer et al., 1967), and it has, in the virion, the tertiary structure of a covalently closed twisted cyclic double-stranded molecule. Since at least one strand of the duplex must be opened for the DNA to

be replicated semi-conservatively, it is thought that the DNA exists in the completely linear form for at least some of its time in the cell (Vinograd and Lebowitz, 1966). The structural protein of SV40 consists of three polyeptide chains designated A, B, and C (Anderer et al., 1967, 1968), with a combined molecular weight of about 16,350 in a ratio of 45,5 : 45,5 : 9. Ninety-one per cent of the protein in the whole virus consists of the A and B chains, and these two chains make up the icosahedral shell of the virus. The arginine rich C chain represents the remaining 9% of the virus particle protein. This chain is apparently associated intimately with the DNA inside the intact virus, since a stable DNA-C chain complex may be isolated. Antibodies produced against purified, whole virus antigen do not react with this polypeptide chain, thus indicating that it is inside the shell. Since empty virus shells which contain no DNA do contain C protein, it has been suggested that the C chain ensures the proper binding of the DNA to the A and B shell chains (Anderer et al., 1968). It can be calculated, moreover, from the molecular weight of the protein coat and the number of sub-units found therein, that between one-third and one fourth of the genetic information of the virus is required to specificity for coat protein. The remaining genetic information, is sufficient to code for between four and seven new proteins, depending on their size. The virus is known to code for a T-antigen found in the nucleus early in infection (Black et al., 1963); Pope and Rowe, 1964, Rapp et al., 1964; Gilden et al., 1965), a transplantation antigen found on the cell membrane (Defendi, 1963; Habel and Eddy, 1963; Koch and Sabin, 1963; Goldner et al., 1964), and probably for its own thymidine kinase (Carp, 1967).

Polyoma virus, another oncogenic papovavirus, is very similar in these respects.

PRODUCTIVE (LYTIC) CYCLE : SV40 can infect either productively or abortively, depending on the type of host cell (Fig. 1). Cell transformation occurs only in abortive infection and its mechanism is unknown. On infection of highly susceptible African green monkey kidney (AGMK) cells, the virus is absorbed to the cell surface, phagocytosed and uncoated (Green, 1966, 1970). The SV40 then goes into an eclipse phase. The earliest events indicative of SV40 infection are the appearance of T-antigen in the nucleus at about 16 - 20 hours after infection (Mayor et al., 1962; Gilden et al., 1965; Knowles et al., 1969), and an increase in the level of several of the enzymes necessary for DNA synthesis. Cellular DNA synthesis increases, and increased viral DNA synthesis begins at this time (Green, 1966). Viral coat protein is then synthesized and by approximately 40 hours after infection (assuming a large multiplicity of infection), the AGMK cells show characteristic vacuolar lesions (Knowles et al., 1969) and lyse, yielding infectious progeny. However, in similar conditions, extracellular virus has been detected 24 hours after infection (Mayor et al., 1962). Depending on the virus strain, one SV40-infected cell can yield up to 10^6-10^7 virus particles (Koch and Eggers, 1967; Davis et al., 1968). The virus can also replicate in human cells, but when normal human fibroblasts are infected with a high input multiplicity of SV40 less than 10% of these cells produce virus (Knowles et al., 1969). These two cell systems, monkey and human, are referred to as permissive to SV40 replication. All cells or cell lines which do not support virus growth are called nonpermissive.

FIG. 1

(see explanation on page 7)

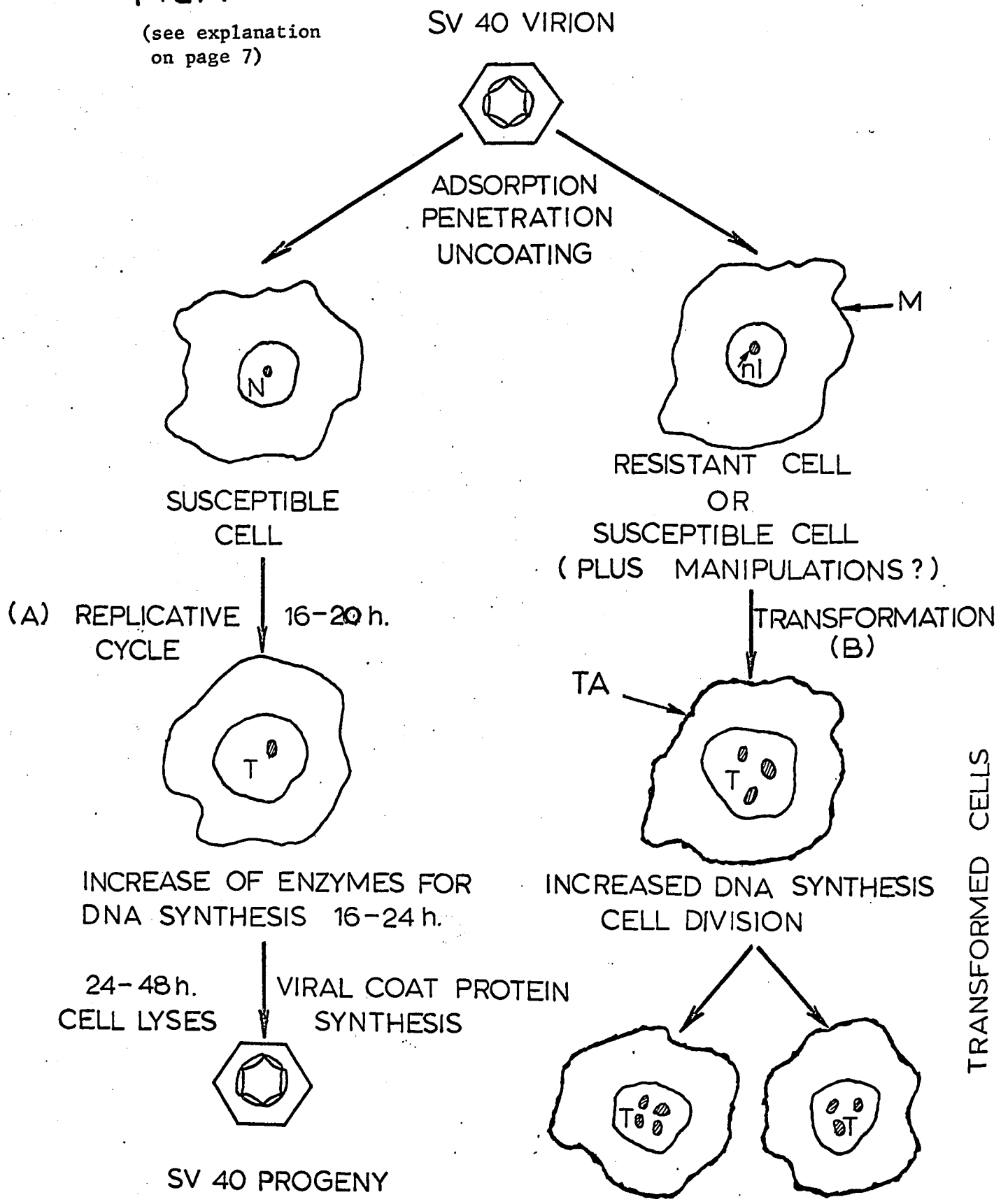


Fig. 1 (cont.) Schematic representation of SV40 lytic (productive) cycle (A) and the cell transformation (B). M: cell membrane; N: nucleus; nl: nucleolus; T: T-antigen in the nucleus; TA: transplantation antigen.

VIRAL FUNCTIONS IN SV40-CELL INTERACTIONS:

By far the most thoroughly studied systems as far as host cell-virus interactions and viral functions are concerned are those dealing with the SV40 and polyoma viruses (Braun, 1970; Yoshikawa-Fukada and Ebert, 1971). Of the seven viral functions that have thus far been specified, two are particularly suspected as being etiologically involved in the transformation process. The first of these is a virus-specific antigen, the transplantation antigen, that is present on the surface of transformed cells. According to Dulbecco (1969) this may be important since it may reflect surface changes which alter the response of cells to exogenous regulatory influences. A second and perhaps highly significant function identified with viral activity is activation of the synthesis of cellular DNA and the enzymes required for that synthesis, as well as synthesis of chromosomal proteins (Hancock and Weil, 1969). It has been suggested (Dulbecco, 1969) that the viral gene which directly or indirectly induces cellular DNA synthesis may be the main agent involved in the transformation process in these systems.

A third viral function that has been identified but does not now seem to be relevant to transformation is that concerned with the synthesis of the T-antigen, which differs in immunological specificity from both the transplantation antigen and the protein of the viral coat. For two reasons it was suggested (Braun, 1970) that the T-antigen may not be necessary, and is certainly not in itself sufficient, to maintain the neoplastic state. First, it appears to be absent in 3T3 cells transformed by the mutant Ts-a strain of the virus at 31°C (Dulbecco, 1969) when the

transformed cells are incubated at 39.5°C. Second, during normalization of cells transformed by either SV40 or polyoma viruses the T-antigen continues to be detectable in the cells.

The other four viral functions that have thus far been detected appear to be irrelevant to transformation (Braun, 1970). Thus the problem might be narrowly restricted to one or two viral functions, which can not as yet be pointed to with certainty, as far as significance in the etiology of transformation is concerned.

CELL TRANSFORMATION

If SV40 nonpermissive hamster or mouse cells (Shein et al., 1963; Todaro and Green, 1966) are infected, or if permissive cells are properly manipulated upon infection, completion of the replicative cycle does not take place and transformation can occur (Koprowski et al., 1962; Shein and Enders, 1962; Fernandes and Moorhead, 1965; Carp and Gilden, 1966). However, nonpermissiveness of the cells is important for transformation because it prevents the expression of late functions which are lethal for the cells (Dulbecco, 1969). Normally, it appears that permissive cells can be transformed only by viral mutants in which the mutation abolishes the expression of these late functions (Dulbecco, 1969). The events leading to transformation vary according to the type of cell used, but the essential outcome is the selection of a population of transformed cells in which cellular DNA synthesis is elevated. The majority of these cells are no longer susceptible to contact inhibition of growth. In addition, SV40-specific T- and transplantation antigens can be demonstrated in those cells. The presence of these antigens and/the elevated DNA synthesis is

attributed to early functions of the viral genome (because the late functions of this genome, that is, viral coat protein and whole virus production are not expressed in the transformation process) (Dulbecco, 1969).

SV40-transformed cells can be divided into four classes on the basis of the type of cellular environment which may be permissive or nonpermissive and the type of viral genome they contain (viral genomes can be defective or non-defective)(Eckhart, 1969): (1) Nonpermissive cells containing non-defective viral genomes do not yield virus when superinfected, but can be induced to yield virus by fusion with sensitive (permissive) cells. (2) Nonpermissive cells containing presumably defective viral genomes do not yield virus when superinfected, and can not be induced to yield virus by fusion with sensitive cells. (3) Permissive cells containing presumably defective viral genomes do yield virus when superinfected, but cannot be induced to yield virus by fusion with sensitive cells. (4) Permissive cells containing non-defective viral genomes do yield virus when superinfected, and can be induced to yield virus by fusion with sensitive cells. Infectious virus is not detectable in these cells unless they are fused with sensitive cells. (Eckhart, 1969).

TYPES OF TRANSFORMATION AND THE STATE OF VIRAL GENOME IN THE TRANSFORMED CELL

As seen above, the relation of SV40 (or that of polyoma virus) to the target cell may be of several kinds (Dulbecco, 1969; Eckhart, 1969; Braun, 1970; Yoshikawa-Fukada and Ebert, 1971). In the productive type of infection the virus multiplies essentially unchecked and eventually lyses and kills the cells. In the

second or abortive type there is no productive infection but instead a transformation leading to the tumorous state. Transformations occur with a low frequency of from one to five percent when such cells are plated in the usual manner. When, on the other hand, the cells are cultured in a viscous solution of Methacel (Stoker, 1968), up to half of them form colonies and behave as though they were transformed. When such colony-forming cells are removed from the Methacel and cultured further, the majority revert to normal cell types. These results were interpreted to mean that transformation may either be temporary, in which case the cells become normal again (abortive transformation), or be permanent if the viral DNA becomes integrated into the genome of the host cell and replicates with this genome (stable transformation). It was suggested further, that although the physiology of the transformed cell does not depend on integration, transformation can be perpetuated only if the viral DNA is integrated (Stoker, 1968). That integration occurs, is now a well established fact (Gelb et al., 1971, Westphal and Dulbecco, 1968; Sambrook et al., 1968; Tai and O'Brien, 1969). However, the number of SV40 sequences per transformed cell reported by each of these authors' group is different from others. The viral DNA molecules are linked to cellular DNA by alkali-stable covalent linkages.

There appears, then, in these two rather special cases of transformation, to be evidence for an alteration in the integrity of the genetic information in the host cell (Braun, 1970). Yet, recent studies (Pollack et al., 1968; Rabinowitz and Sachs, 1968; Hitotsumachi et al., 1971; Macpherson, 1970) have indicated that the transformed state is not

irreversibly fixed. Rabinowitz and Sachs (1968) have found, for example, that clones of cells in which the transformed state had become a hereditary cellular property produced a high frequency (18 percent) of revertants. Thus, even in the case of the SV40 and polyoma viruses, where the viral genome is integrated into the genome of the host, the expression of neoplastic behavior, once established, is not irreversibly fixed in a cell but is subject to change, this despite the fact that the T-antigen continues to be found in the revertant cells.

SIMILARITY BETWEEN TRANSFORMATION AND LYSOGENIZATION

The state of cellular transformation was accordingly compared with the lysogenization of bacteria by phages, and numerous unsuccessful attempts were made to activate infectious virus from transformed cells. Extracts of transformed cells were always found to be non-infectious when they were plated on AGMK cells (Sabin and Koch, 1963). However, infectious SV40 virus could occasionally be recovered when undamaged non-permissive transformed cells were cocultivated with AGMK cells (Gerber, 1963, 1964; Gerber and Kirschstein, 1962; Sabin and Koch, 1963). Originally it was felt that the recovery of small amounts of virus was due to the presence of complete genomes in a small proportion of the transformed cell population. Suspicion as to the validity of this hypothesis arose though when cloned populations of cells gave the same result. The introduction of the technique of cell fusion by inactivated Sendai virus confirmed the supposition that the low yield of virus after cocultivation was due to a limited amount of fused cells in the culture (Gerber, 1966; Cassingena et al., 1969; Dubbs et al., 1967; Takemoto et al., 1968; Koprowski et al., 1967). Thus, the induction of virus replication in fused

cells by using the inactivated Sendai virus to impose a form of artificial sexuality on these animal cells can be favourably compared to the phenomenon of "zygotic induction" observed in lysogenic bacteria.

Application of the cell fusion technique between various transformed cell lines and SV40-permissive cells has also allowed the examination of some other interesting aspects of the cell-virus relationship in transformed cells (Dulbecco, 1969; Eckhart, 1969; Svoboda and Hlozanek, 1970).

B - THE "IN VITRO" PRODUCTION OF HETEROKARYOCYTES AND THE INDUCTION OF VIRUSES FROM TRANSFORMED CELLS.

DEFINITIONS Before entering into further consideration, it might be necessary to define here the following terms. The term "heterokaryon" (Harris and Watkins, 1965) implies a cell with more than one type of nucleus as a result of fusion of previously separate cells. "Polykaryocyte" was introduced by Roizman (1962) to describe any multinucleate cell, regardless of its mode of formation. Polykaryocytes, in which the nuclei have fused together to form a single nucleus, are called "synkaryons": "homosynkaryons" where nuclei of the same type have fused together, and "heterosynkaryons" where nuclei of different types have fused together (Harris and Watkins, 1965). The term "heterokaryocyte" is used as synonym of heterokaryon. The term "giant cell" is being widely, although incorrectly, used by many workers to name multinucleate cells, particularly those observed in various pathological conditions.

SHORT HISTORIC NOTE ! It is interesting to note here that the first observation of multinucleate cells in vertebrates was described in 1838 by Müller from his studies of tumors. At that time, the introduction of the cell theory by the botanist Schleiden (1838) and the zoologist Schwann (1847) provided the basis for the interpretation of the structure of plant and animal tissues in terms of cells. The very first descriptions of polykaryocytes observed in virus infections appear to be those of Luginbühl (1873) and Weigert (1874) in smallpox, Unna (1896) and Tyzzer (1905) in varicella and Kromayer (1889) and Hecht (1910) in measles. Since then, all kind of information on multinucleate cells or polykaryocytes has been accumulating through the years; Harris et al., (1966) and Poste (1970) have recently reviewed this information in detail. Therefore, we will examine here only the problem concerning cell fusion by Sendai virus and the application of heterokaryocytes for the induction of transforming viruses, mainly the SV40.

FUSION INDUCING VIRUSES AND THE FUSION FACTOR As a result of a considerable amount of work done with viruses which are known to cause cell fusion, these viruses have now been categorized into three distinct groups: herpes viruses, pox viruses and myxoviruses or paramyxoviruses (Steplewski and Koprowski, 1970).

Although many viruses are capable of fusing cells, more extensive investigations were carried out with two viruses exhibiting the fusion factor, Newcastle disease virus (NDV) and a paramyxovirus, Sendai virus (or HVJ, as it is called by Japanese workers). The Sendai virus offers

several advantages as a cell fusing agent (Okada, 1969): 1) it can be propagated easily in embryonated eggs, yielding a high virus titer; 2) it aggregates cells in suspension under suitable laboratory conditions; 3) and, the most important, receptors for Sendai virus are widely found on membranes of animal cells of different origins (Okada, 1969). Unlike the Sendai virus, NDV appears to fuse only cells of continuous cell lines but not primary human, chick and mouse cells (Kohn, 1965).

Thus, the production of heterokaryocytes in tissue culture became a tool available to laboratory investigations only after a fusion factor, produced by Sendai virus, permitted fusion of cells "in vitro" under established conditions (Okada, 1962; Harris and Watkins, 1965). The nature of this fusion factor is still unknown; it is, however, closely associated with the virus particle and with the lipoprotein envelope of the virion, and is not destroyed by some agents inactivating infectivity (Okada, 1969). It is destroyed by lipases (Kohn, 1965) and by various lipid solvents and detergents (Koprowski, 1971). Since some substances which destroy the fusion factor have no effect on viral hemagglutinin, it can be concluded that the fusion factor is not related to the viral hemagglutinin. Therefore, it can be said that the determination of fusing ability of the virus preparation, based on its hemagglutination titer, is not a right procedure; it is also clear that we do not have, at present, a better test for determining concentration of the fusion factor.

THE CELL FUSION PROCESS

Since the cell fusion technique will be described in "Material and Methods" of our experimental work, we would like to present now a schematic description of the cell fusion process (see Fig.2 and page 16) as modified from Hosaka and Koshi (1968).

FIG. 2 (see explanation on page 16)

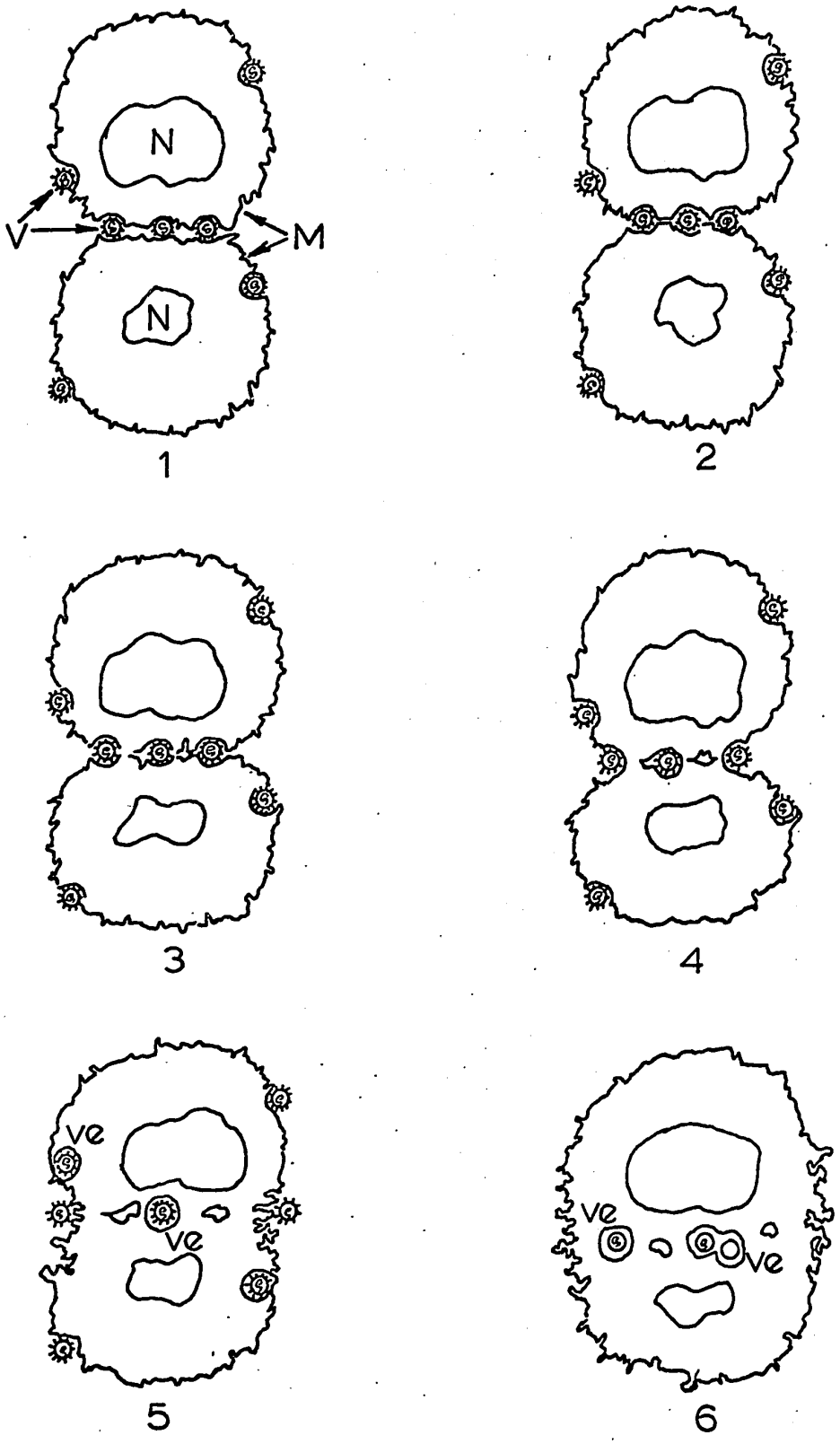


Fig. 2 (cont.)

Schematic representation (modified from Hosaka and Koshi, 1968) of the sequences of fusion, between two types of cells in the presence of Sendai virus (V). N = nucleus. Step "1" takes place at 4°C and steps 2-6 at 37°C. 1 - Agglutination of cells by Sendai virus, at the points where virus particles are absorbed on the respective cell membranes (M). In an ice bath (4°C), this step occurs almost immediately after mixing the (two) cell populations at a suitable concentration. 2 - Close contact of adjacent cell membranes near absorbed virus. 3 - Degradation of cell membranes by virus and communication of the cytoplasms at the points of breakage (at 2 minutes). In some cells, the degradation of cell membranes begins to occur as early as 1 minute at 37°C. 4 - Connection of the edges of broken cell membranes and the formation of cytoplasmic bridges (1-2 minutes or more). 5 - Enlargement of the cytoplasmic bridges and engulfing of virus within vesicules (Ve) (at about 5 minutes). Some virus particles elute and regions of the cell surface exhibit a rich formation of microvilli. 6 - Rounding of the fused cells which merge into one; formation of larger vesicules containing Sendai virus and the alteration of these particles can be observed (after 5 minutes) (Hosaka and Koshi, 1968).

RECOGNITION OF HETEROKARYOCYTE

Once the heterokaryocyte is formed, the parental cells can often be quite easily identified because of the morphological difference of their nuclei; staining of the preparation in a routine fashion (e.g. Giemsa) and observing it under the ordinary optic microscope is the general procedure followed for such purpose. The nuclei of (tumor or) virus-transformed cells are often pleomorphic and possess generally 3 or more nucleoli of different size; if one parental cell population used for fusion is of normal type, it becomes easy to recognize the type of cells which formed a (poly- or) heterokaryocyte. If the parental cells nuclei are morphologically indistinguishable, one of these parental cell cultures (assuming there are only two) is grown, for at least one cell generation time, in a medium containing thymidine -^{3}H

for the labeling of its nuclei; after fusion (12-24 h) the cells are fixed and subjected to autoradiography. The details of these techniques have been described by Harris and co-workers (1965,1966); Koprowski et al., (1967) and Steplewski and Koprowski (1970).

SOME APPLICATIONS OF HETEROKARYOCYTOSIS

At this point one must recognize that, first of all, virus-induced heterokaryocytes have permitted a totally new approach to learn many aspects of cell function, cytogenetics and biochemistry which have hitherto not been experimentally possible in animal cells. Moreover, cell fusion is a good example of fusion between two biological membrane systems. According to Weiss (1967), the cell periphery reflects much of the cellular organization. Recent progress in the biology of cell fusion, its mechanisms and applications have been widely reviewed by Poste (1970) and Steplewski and Koprowski (1970).

One of the most interesting observations with cell fusion in the medical field is that concerning the reduction of the oncogenic potential of tumor cells by fusing them with normal cells (Harris et al., 1969; Watkins and Chen, 1969). This might also indicate a new approach to cancer immunotherapy. Highly significant is also the study recently reported by Pontecorvo (1971) in which cell fusion by Sendai virus has been employed, together with other physical or chemical treatments, to induce directional chromosome elimination in somatic cell hybrids. Thus, it is now possible to predetermine which of the two sets of parental chromosomes will be preferentially lost in cell hybrids.

CELL FUSION AND THE RESCUE OF VIRUSES

The cell fusion technique by means of inactivated Sendai virus has been the tool responsible for proving that the complete viral genome is present in a reactivable form in many virus-free SV40-transformed cell lines. Gerber (1966) was the first author to report the use of inactivated Sendai virus to rescue SV40 from hamster tumor cells by fusing them with monkey kidney cells. Koprowski et al., (1967) proved that infectious virus could be rescued only after fusion of SV40-transformed human and monkey cells with susceptible AGMK cells. Takemoto et al., (1968), and Watkins and Dulbecco (1967) confirmed in their studies the role of cell fusion in rescuing infectious virus from SV40-transformed cells of mouse origin; these cells did not yield virus by simple co-cultivation with susceptible cells.

Knowles et al., (1968) reported the rescue of infectious SV40 after fusion between different SV40-transformed cells; these cells did not yield virus after fusion with susceptible AGMK cells in the presence of Sendai virus. Thus, those authors concluded that SV40 is, in this case, rescued either by recombination or by complementation.

Takemoto et al., (1968) found, after a careful study, that SV40 virions rescued from transformed cells have the same genetic markers as the original transforming strains. Todaro and Takemoto (1969) reported that virus rescued from transformed 3T3 cells was more efficient at transformation than the parental type; this property of "high efficiency "transformation" is stable through at least two passages in AGMK cells.

The increased transforming efficiency could be shown for each plaque type, and in human as well as in mouse cells.

It has been reported that, in general, malignant cells fuse better with themselves or with normal cells than do two normal cell lines with each other (Steplewski and Koprowski, 1970; Okada and Tadokoro, 1963; Kohn, 1965). This observation explains the rescue of SV40 by simply co-cultivating transformed cells with permissive cells (Gerber and Kirschstein, 1962; Gerber, 1963, 1964; Dubbs et al., 1967; Tournier et al., 1967). Cassingena and Tournier, (1968a) observed 6% virogenic heterokaryons by just co-cultivating SV40-transformed cells (Cl_2TSV_5) with those of BSC-1. It is known that low temperatures alone favour cell fusion (Scaletta and Ephrussi, 1965; Yoshida and Ephrussi, 1967). Cassingena and Tournier (1968a) and Cassingena et al., (1969b) have observed up to 12.7% of fused cells when BSC-1 cells were co-cultivated at low temperature (29°C) with those of the Cl_2TSV_5 line. However, the highest number of heterokaryons was observed in the presence of inactivated Sendai virus (Cassingena et al., 1969b).

It is very interesting to note here that Brackett et al., (1971) fused SV40-DNA-infected rabbit spermatozoa with AGMK cells; these authors found that SV40-DNA which had escaped from the spermatozoa was activated in AGMK cells, and caused production of infectious virus.

Recently, Barbanti-Brodano et al., (1970) reported the rescue of a paramyxovirus and also of papova-like virus particles from cases of subacute sclerosing panencephalitis; cells from patient brain tissue

were fused in the presence of Sendai virus with either AGMK or human diploid brain cells. The rescued paramyxovirus produced plaques on AGMK cells, but the papova-like virus was detected only by electron microscopy (Koprowski, 1971).

It is also of great interest to note here that Baranska et al., (1971) infected mouse ova with the Moloney sarcoma virus (MSV) through fusion with MSV-infected fibroblasts. Seventy-two hours later these mouse ova were fused with indicator Rous sarcoma XC rat cells; thus, lesions characteristic of MSV infection were observed. Mouse ova were also infected with either SV40 or SV40 DNA, and infectious virus was rescued by fusion of those ova with monkey cells (Baranska et al., 1971).

Although polyoma virus is very similar to SV40, infectious virus can not be rescued from polyoma-transformed (virus free) cells by fusion technique (Burns and Black, 1969; Watkins and Dulbecco, 1967). Attempts to rescue virus from adenovirus-transformed cells have also been unsuccessful (Burns and Black, 1969). These negative results with polyoma- and adenovirus-transformed cells could be explained by the fact that the viral genome is present in those cells either in defective form or, if complete, it is not reactivable - at least under the experimental conditions attempted to date. However, an increase of virus titer up to 100 times was observed by fusion, from polyoma-transformed cell population which spontaneously used to release virus in small quantity (Fogel and Sachs, 1969).

Cell fusion by Sendai virus has also been used to increase considerably the amount of oncogenic RNA viruses recovered by co-cultivating transformed cells with susceptible cells (Svoboda et al., 1968; Svoboda and Hlozanek, 1970). It appears that, in most cases, those RNA viruses have the capacity to generate endosymbiotic virus-cell complexes (Davis et al., 1968; Macpherson, 1970).

Finally, our knowledge of the role played by cell fusion, as applied to virus induction, can be summarized by saying that the fusion technique is a unique tool which contributed to the rescue of: 1-SV40 virions from transformed cells; 2- an increased amount of virus from cultures of transformed cell populations, which appear to exhibit a virus-cell interaction of the steady-state type; 3- a paramyxovirus, and also papova-like virus particles from brain cells of patients with subacute sclerosing panencephalitis; 4- viral genome from spermatozoa experimentally exposed to SV40-DNA; 5- infectious virus from mouse ova infected (a) with Moloney sarcoma virus, and (b) with SV40 or SV40 DNA.

II. DETECTION OF (TRANSFORMING) VIRUSES INDUCED BY CELL ASSOCIATION : THE SV40 MODEL.

As seen in the previous chapter, the use of Sendai virus to induce cell fusion (Okada, 1962; Harris and Watkins, 1965) has proven so far to be the best technique for the recovery of infectious virus from several lines of transformed cells (Gerber, 1966; Koprowski et al., 1967; Watkins and Dulbecco, 1967; Takemoto et al., 1968; Cassingena et al., 1969). In some cases when virus could be rescued simply by co-cultivation of transformed cells with permissive cells, cell fusion between these two cell types proved to recover virus up to 100 times the titer obtained by cell co-cultivation (Svoboda et al., 1968; Fogel and Sachs, 1969). In all these cases classic techniques were employed for the detection of rescued viruses.

It has been shown that DNA or RNA isolated from animal viruses can be infectious not only for permissive cells but also for naturally insusceptible cells (Diderholm and Wesslén, 1963; Gerber, 1963; Holland et al., 1959; Mountain and Alexander, 1959). In the latter case (of naturally insusceptible cells) only one cycle of virus replication, originated by infectious nucleic acid, takes place. Thus, in principle, it seems also possible to use naturally insusceptible cells (when the permissive cell type or line for the virus to be rescued may be yet unknown) for fusion with cells of a human or animal tumor for virus induction. Furthermore, in any situation the recovery of non-infective virus particles cannot be excluded. In both cases, whether the rescued virus particles are infectious or not, electron microscopy could make available information on features such as virus shape, size, envelope, symmetry or capsomeres, which are among the basic criteria for the taxonomic characterization of any virion. Moreover, with recent progress in the diagnostic field (Doane et al., 1969; Joncas et al., 1969; Spradbrow and Francis, 1969; Tyrrel and Almeida, 1967) it becomes

evident that electron microscopy can be successfully employed for rapid detection and characterization of viruses from different sources. Therefore, using the SV40 experimental model, a study was undertaken on the application of electron microscopy for the rapid presumptive detection of viral particles rescued by cell association (fusion or co-cultivation).

MATERIAL AND METHODS

Cell cultures

Transformed cells: (1) SV40-transformed cell line (Cl_2TSV_5), derived from a tumor in a Syrian hamster, was supplied by Dr. P. Tournier (Laboratoire de Virologie, Institut de Recherches Scientifiques sur le Cancer, Villejuif, France) and maintained in this laboratory, grown as described by Tournier et al., (1967). (2) RHaT-126, referred to as RHaT, a cell line which was derived from a SV40-transformed hamster kidney cell culture by Dr. R. Dubreuil of the Institut de Microbiologie et d'Hygiène de l'Université de Montréal, Canada. This cell line was grown in Eagle's minimum essential medium (MEM)(Eagle, 1959) with 10% fetal calf serum (FCS). Both lines proved not to produce SV40 virions spontaneously.

Non-transformed cells: I. SV40-permissive cultures: (1) BSC-1 (Meyer et al., 1962), referred to as BSC, were grown in MEM containing 10% FCS and 10% tryptose phosphate broth (TPB). (2) African green monkey kidney (AGMK), secondary culture was grown in MEM with 5% FCS and 5% TPB. To avoid contaminant simian viruses only secondary cultures from selected AGMK monolayers were used. II. SV40-nonpermissive cultures: (1) MDBK (CCL22) a bovine kidney cell line (Madin and Darby, 1958) was maintained in MEM

with 10% FCS. (2) PK-15 (CCL33), a pig kidney cell line grown in MEM with 5% FCS; these last two lines were obtained from the American Type Culture Collection. (3) Bovine (calf) kidney (BK) cells, primary culture. (4) Hamster kidney (HK) cells, primary culture. (5) Pig kidney (PK) cells, primary culture and (6) baby rabbit kidney (RK) cells, primary culture; all these last four primary cultures were grown in MEM with 10% FCS and 10% TPB.

For cell co-cultivation or fused cultures the growth medium employed was MEM, supplemented as required by the more fastidious of the two cell types. Penicillin (100 units/ml) and streptomycin (100 micrograms/ml) were added to the medium in all cultures. Kanamycin (100 micrograms/ml) was also added during four consecutive passages for all continuous cell lines, prior to their use for experiment. FCS used in the medium was heat-inactivated (56°C/30 min).

Prior to their use, all the cell cultures described above (SV40-transformed and non-transformed) were tested by electron microscopy and infectivity assay for the presence of any detectable virus.

Viruses

Sendai virus: a strain was supplied by Dr. H.G. Pereira of the National Institute for Medical Research, Mill Hill, England. This virus strain was propagated in the allantoic sac of 10-day-old chick embryos, harvested, inactivated by exposure for 5 min. to ultra-violet light and stored at -70°C as described by Harris and Watkins (1965). This inactivated Sendai virus is referred to as UV-SeV.

SV40 strains: the two strains used in control experiments were obtained by cell co-cultivation of Cl_2TSV_5 and RHaT, respectively, with BSC cells.

Cell fusion technique

The procedure followed was essentially the same as described by Harris and Watkins (1965) and Koprowski et al., (1967). Five million cells in o.5 ml of each of the two cultures to be fused were mixed in the presence of 4,ooo hemagglutinating units of UV-SeV, suspended in 1 ml of MEM. The mixture was kept at 4°C for 10 min, shaken gently at regular intervals, then incubated in a 37°C water bath for 20 min. MEM with 10% FCS was then added to the mixture and the cells were recovered after a low-speed centrifugation. These cells were then resuspended in growth medium and seeded in tissue culture tubes or 1-oz bottles. Two to three plastic chambers (Sattar and Westwood, 1967) were also seeded with each mixture for later observation, by staining the slides with Giemsa, of the formation of heterokaryons and/or any eventual cytopathic effect. As controls, mixed cells without UV-SeV (co-cultivation) and cells of each type with and without this virus were also prepared. The percentage of heterokaryons induced by UV-SeV varied from 9 to 37 in different experiments, while in control cultures it was about 1-2(%).

Electron microscopy

The method used is essentially the same as described by Pinteric and Fenje (1966) and Doane et al., (1969). The cells were removed from tissue

culture flasks or tubes by scraping and centrifuged at 120 x g/10 min. After the supernatant had been decanted the cell pellet was transferred with a Pasteur pipette to a stainless steel spoon. It was then successively frozen and thawed four times by alternately placing the spoon on dry ice and on the palm of the hand or gently heating over a Bunsen flame. A drop of sterile distilled water was then added and the cells were frozen and thawed two more times. A small drop of the resulting fluid was applied to a formvar-carbon-coated grid with a Pasteur pipette and after about 30 seconds a drop of 2.5% neutral phosphotungstic acid (PTA) was added and left for a few seconds. Excess fluid was then removed by touching it with filter paper and the grid was allowed to dry in air. Two grids were prepared from each sample and examined with a Philips 200, or Siemens Elmiskop 1A electron microscope. The rest of the fluid from the spoon — which was increased by adding 1-2 drops of decanted supernatant or sterile distilled water, then frozen and thawed once — was used as antigen for preparing droplets for fluorescent antibody staining and immunodiffusion (see below).

Studies on the relative efficiency of the infectivity test and electron microscopy

a) To determine the sensitivity of electron microscopy in terms of the number of infected cells required in a monolayer, the following experimental approach was used: monolayers of AGMK and BSC cells were exposed to SV40 at a multiplicity of infection of 100 PFU/cell and after an adsorption period of 1 h the inoculum was removed and fresh medium was added. Twenty hours after inoculation the medium was removed and the

cultures were rinsed twice with MEM. Culture medium containing 0.5% rabbit anti-SV40 serum (neutralizing titer/ml:4,000) was then added and the monolayers incubated at 37°C until a 50% cytopathic effect was observed. The cultures were then rinsed three times, trypsinized, and the cells dispensed at a concentration of about 50 cells per ml of MEM, in a 50 mm Petri dish. While being observed with an inverted microscope, various numbers of these cells were individually collected with a capillary tipped Pasteur pipette and transferred to a pellet containing about 100,000 non-infected cells. Two identical series of cell pellets were thus prepared. Pellets from one series were used for preparing grids for electron microscopy; the other series was frozen and thawed six times in a small amount of MEM and the resulting fluid passed onto monolayers of AGMK or BSC cells. Either in the presence or in the absence of CPE in these cells (in negative cases the cultures were observed until the 21st day) grids were prepared from the cellular material for electron microscopy.

b) To determine whether SV40 particles could be detected earlier by infectivity testing or by electron microscopy, experiments were performed using BSC and RHaT cell lines. BSC cells grown in 1-oz bottles were exposed to SV40 (virus strain obtained from RHaT) at a multiplicity of infection of 10 PFU/cell and after an adsorption of one hour the inoculum was removed and the cells were rinsed three times with MEM. Fresh medium with o.5% rabbit anti-SV40 serum was then added, this medium being replaced with an identical one after 12h. Sixteen hours after infection and every 2 h thereafter a pair of monolayers was rinsed

and pellets prepared, one being used for infectivity assay on BSC cells and the other prepared for electron microscopy.

BSC cells were co-cultivated and also fused with those of RHaT line, samples being tested as above for infectivity and electron microscopy.

Fluorescent antibody (FA) staining technique

The indirect method was used. Droplets of crude cell lysate were prepared on slides following a procedure developed in this laboratory for FA staining (Menezes and Perry, in preparation). This procedure involves basically the same early steps of preparation of droplets on slides and drying them in air as for the acridine orange staining of virus suspension (Menezes, 1971). The slides with droplets were then fixed in acetone for 3 min, rinced in phosphate-buffered or tris-buffered saline and treated according to the classic technique of FA staining with the respective controls (Vogt, 1969). Further details are given in the next chapter. Rabbit and horse anti-SV40 sera (Grand Island Biological Co.) and goat anti-rabbit anti-globulin conjugated with fluorescein-isothiocyanate (Microbiological Associates, Inc.) were used. The fluorescence microscope and equipment were the same as described by Chaudhary and Westwood, (1970).

Immunodiffusion technique

The procedure followed is that described by Johnson et al., (1964) with cellulose acetate. The antigen to be identified (virus or crude cell lysate) was applied in the centre of the template and the known antisera in the surrounding wells.

Inoculation of hamster with SV40-transformed cells

Five-week-old Syrian hamsters were injected subcutaneously with 10^6 cells of either Cl_2TSV_5 or RHaT suspended in MEM. Tissue culture bottles were seeded at the same time with samples of these cells to check cell viability. When the tumor diameter reached 1 cm or more — in the earliest case it was 3 weeks after cell injection — the animal was killed and the tumor excised. Part of the tumor was placed in MEM containing 10% FCS and 10% dimethylsulphoxide and frozen in liquid nitrogen and the remaining portion was trypsinized; an aliquot of the cells obtained after trypsinization was fused with SV40-permissive cells (BSC or AGMK) and the other part seeded in tissue culture bottles or tubes for further observations. Control animals were injected with MEM only and did not show any signs of tumor development.

RESULTS

Relative efficiency of electron microscopy compared to the infectivity test

a) As shown in Table I, under the experimental conditions described, an average of about two SV40-infected cells containing virus particles is sufficient in a cell pellet for positive detection of this virus by electron microscopy while the infectivity test seems to be slightly more sensitive.

TABLE 1

MINIMUM NUMBER OF SV40-CONTAINING CELLS REQUIRED FOR POSITIVE VIRUS DETECTION BY:

	Infectivity test	Electron microscopy
AGMK	2[1]	2
	1	2
	1	2
	2[2]	2
	1	3
	1	1
BSC	1[1]	2
	2	3
	1	2
	2[2]	3
	1	1
	1	2

1 Trials using the SV40 strain rescued from RHaT cells.

2 Trials using SV40 from Cl_2TSV_5 cells.

b) The results of the experiments performed to determine the earliest time at which SV40 could be detected by electron microscopy and by infectivity testing are shown in Table II. From these data it appears that virus particles can be detected a little earlier by electron microscopy than by the latter technique. Thus, this observation might suggest that SV40 particles require a certain period of time to become infectious from the moment their virion-like morphology can be detected by electron microscopy. Since in this work samples were taken only every two hours, it appears that further experiments with sampling at shorter intervals could give more detailed information in this matter.

Rescue of SV40 from transformed cells

Cells of Cl_2TSV_5 and RHaT lines were co-cultivated or fused with non-transformed cells from different sources. The results of these experiments are summarized in Table III. The positive results indicated imply the detection of virus by both infectivity assay and electron microscopy, whereas negatives resulted from virus being detected by neither method. No case of conflict between the two methods was encountered in this series.

Preliminary experiments performed in this laboratory by infecting kidney cells of bovine, hamster, porcine and rabbit origin with SV40 virions did not show any evidence of virus multiplication. However, it is important to note that virus could be rescued by fusing these

TABLE II

MINIMUM TIME (HOURS) REQUIRED FOR VIRUS DETECTION AFTER INFECTION, CO-CULTIVATION AND CELL FUSION

	Infectivity test[1]	Electron microscopy
Infected cells	26	24
(BSC)	24	24
	24	22
Cell co-cultivation	72	70
(BSC-RHaT)	72	70
	70	70
Cell fusion	34	32
(BSC-RHaT)	36	34
	34	34

[1] The times listed here correspond to earliest period after infection, co-cultivation, or cell fusion at which the samples taken proved to be virus-positive when inoculated into BSC cultures.

TABLE III

RESCUE OF SV40 VIRIONS BY CO-CULTIVATION OR FUSION BETWEEN TRANSFORMED AND NON-TRANSFORMED CELLS

	Cl_2TSV_5		RHaT	
	Co-cultivation	Fusion	Co-cultivation	Fusion
AGMK or BSC	+	+	+	+
B K	-	+	-	+
H K	-	+	-	+
P K	-	+	-	+
R K	-	+	-	+
MDBK	-	-	-	-
PK-15	-	-	-	-

cells with those of SV40-transformed lines (Table III). This observation supports the idea that naturally insusceptible cells could be successfully used for the induction, by cell fusion, of a virus whose genome is present in a transformed cell or even an apparently normal cell.

SV40 virions were also rescued by fusing BSC or AGMK cells with those obtained by trypsinization of tumors produced by injecting two Syrian hamsters, one with RHaT and the other with Cl_2TSV_5 cells. It was also found that cells prepared by trypsinization of tumor fragments which had been preserved in liquid nitrogen could be successfully used for SV40 induction by fusing them with BSC cells.

In one case when cells of a tumor induced by Cl_2TSV_5 cell inoculation were co-cultivated with those of the BSC line, SV40 particles were detected only by electron microscopy, while the infectivity test gave negative results. Virus could not be detected by fusing Cl_2TSV_5 with RHaT cells.

General comments on the electron microscopic examination for virus detection

During this work it was found that in positive cases virus presence could generally be detected within a few minutes of observation in the electron microscope. This observation might be explained by the fact that virus particles were generally present in clump-type arrangements (Fig.3). Since virus from supernatant fluids of infected tissue cultures or concentrated suspensions is seen in a more dispersed pattern under the electron microscope, the procedure as described in this work appears to be

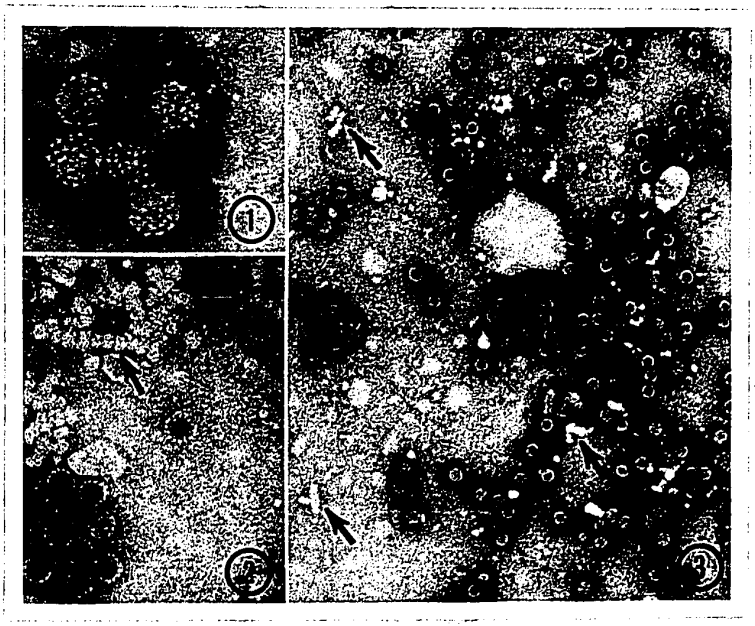


FIG. 3

(1) Typical SV40 particles rescued from RHaT cells. x 190,000.

(2-3) SV40 particles and oval structures showing some chain arrangements (arrows). (2): x 110,000; (3) x 45,000.

a method of choice for virus detection or diagnosis when the nature of the infected material is tissue culture, at least for viruses containing double-stranded nucleic acid, which tend to remain cell-associated after their maturation.

Furthermore, it is important to note here that structures, generally oval-shaped and measuring about 20-24nm, were regularly observed in connection with virus presence and that they were often seen in chain-like arrangements (Fig.3). Similar structures were also observed in grids prepared from cells infected (in the presence or absence of FCS) with other viruses such as poliovirus, coxsackie virus, reovirus, parvovirus H-1, and vesicular stomatitis virus (Menezes, unpublished observations); they were only observed in grids prepared with virus-infected cells. Since it has been reported that in specimens which are negatively stained with phosphotungstic acid some cellular components may not be visible at all and that ribosomes, unless previously fixed, are degraded and invisible (Howatson, 1969), it seems reasonable to think that these structures or bodies might be some cellular product connected with virus multiplication. Further studies on these structures are in progress.

Results of FA staining and immunodiffusion

The identity of the papova-like particles detected by electron microscopy was confirmed as being that of SV40 by FA staining of droplet bands of crude cell lysate. Droplets first treated with horse anti-SV40 serum and then with rabbit anti-SV40 serum and goat anti-rabbit anti-globulin conjugated with fluorescein-isothiocyanate did not show any specific

fluorescence, while those treated with the last two reagents only were positive.

The results of immunodiffusion assays using cellulose acetate strips were not consistent. Although the precipitation lines were present only between anti-SV40 sera and SV40-containing crude cell lysate, they could not always be reproduced. However, it seems that crude cell lysate, obtained as described above, could eventually be used as antigen for immunodiffusion in order to have another serological approach for the identification of the virus detected by electron microscopy, provided that the problem of reproducibility could be solved in this technique. Since the immunodiffusion microtechnique has not yet been employed for virus identification from crude cell lysate obtained as described above, it might be interesting to undertake comparative studies with this material on agar and on cellulose acetate strips.

DISCUSSION

It has been shown that electron microscopy can be employed for the rapid diagnosis of viral infections (Doane et al., 1969; Joncas et al., 1969), and in this work evidence is presented on its use for the rapid detection of a transforming virus, SV40. These results also suggest that virus detection or characterization could be done much faster and more economically by electron microscopy than by infectivity testing.

Further, and perhaps even more significant, is the possibility that naturally insusceptible cells could be successfully employed in cases when the permissive cell type for multiplication of the transforming virus is unknown. The results described in this work using bovine, hamster, porcine and rabbit primary cells are an example of such cases, in which electron microscopy is without a doubt the best technique for the detection or characterization of the rescued virus.

It is known that the complete integrity of the viral genome is not required for transformation by polyoma virus (Benjamin, 1965; Basilico and DiMayorca, 1965; Latarjet et al., 1967), and that defective SV40 particles are as tumorigenic as plaque formers in the hamster (Uchida and Watanabe, 1968). Therefore, the recovery of a defective non-infectious virus from this type of transformed or tumor cells cannot be excluded. Furthermore, as shown with SV40-transformed cells (Knowles et al., 1968), the recovery of an oncogenic virus by complementation as a result of fusion between two non-yielding cells is also a matter which cannot be neglected at the present time. Electron microscopy could be very useful for detection or characterization of virus or viruses rescued in similar cases.

Recent progress in the preservation of tumor and normal tissues by freezing (Kline and Trapani, 1964; Mouriquand et al, 1966, 1969) makes it possible to use such tissues at any future time for culture. Experiments performed during this work after preserving fragments of SV40-induced tumors not only support these results but also show that cells of the preserved tumor fragments could be used for virus induction by cell association techniques.

Recently ZuRhein (1969) suggested that electron microscopic examination of cell pellets from leukemic patients with progressive multifocal leukoencephalopathy (PML) could reveal a viral etiology of this disease. Papova-like virus particles have been found associated with PML and their role as its possible etiological agent has been discussed (ZuRhein, 1969; Koprowski et al., 1970). Barbanti-Brodano et al., (1970) reported the rescue, by cell fusion, of a paramyxovirus and also of papova-like virus particles from similar cases; while the paramyxovirus produced plaques on AGMK cells, the papova-like particles were detected only by electron microscopy. This observation stresses again the importance of electron microscopy for virus detection. Thus, it might be worth while to undertake further studies using direct electron microscopic examination of cultures resulting from fusion between brain cells of patients with PML and normal cells of human, simian or other origin. It might also be worth while to undertake similar studies in cases such as Burkitt tumor, Hodgkin's disease, leukemia, neoplasms of various types and even in infectious mononucleosis and systemic lupus erythematosus, in which viral etiology might be a possibility.

Because of latent, defective or associate-type virus which could be present in any cell it might be also of value to employ the electron microscope to investigate the possibility of virus presence in cell cultures regularly used for studies in virology or virus oncogenesis. Recently an SV40-transformed cell line (RHa 3-T), derived from hamster kidney (Dubreuil et al., 1964) was found to be in the carrier state

for a parvovirus after its presence had been detected by electron microscopy (Menezes and Perry, 1970; see Appendix).

It might also be worth mentioning here that, following the method described above for preparing grids, cellular material from the same pellet or sample could possibly be used to determine the antigenic nature of the detected virus particles, thus facilitating their identification.

In conclusion, the experimental approach such as described in this work using the SV40 model holds some promise for the rapid detection of other transforming viruses, and may also produce further evidence in several conditions where viral etiology is suspected.

RESUMÉ

DETÈCTION DE VIRUS (ONCOGÈNES) INDUITS PAR ASSOCIATION CELLULAIRE : LE MODÈLE SV40

Le présent chapitre décrit une étude portant sur l'utilisation du microscope électronique pour la détection rapide des particules SV40 induites par association (co-cultivation ou fusion) entre les cellules transformées et les cellules sensibles ou naturellement insensibles au SV40. On y discute également l'utilité de la microscopie électronique en tant que moyen de détection rapide des virus provenant de tumeurs humaines ou animales ainsi que d'autres maladies où l'origine virale semble très probable.

III. MAIN FEATURES OF SUCCESSIVE GENERATIONS OF Cl_2TSV_5 AND RHaT PRODUCED TUMORS AND THEIR CELL CULTURES, AND THE RESCUE OF SV40.

Although the cell fusion technique has been widely used to rescue viruses from transformed cells, and mainly SV40 (Svoboda and Hlozanek, 1970), most authors have been very cautious in describing details for cultivation of fused cells as well as any eventual modification of the standard fusion procedure which was described in the preceding chapter. During the studies reported in that chapter on SV40 detection after cell fusion, and during some preliminary experiments performed under different circumstances with cultures of transformed and non-transformed cells a few unexpected observations stimulated further research in this field.

It is, moreover, of interest to note that experiments on the rescue of SV40-genome from tumor cells, after various number of passages of transformed cells in hamster, have not yet been described. Therefore, a study was also undertaken on the rescue of SV40 from transformed cells after passages "in vivo" and on some features of these tumor cells.

MATERIAL AND METHODS

Cell cultures and media

SV40-transformed cells: (1) Cl_2TSV_5 same as grown and described in the preceding chapter. (2) CH_4, a cell line derived from a tumor in a Syrian hamster (Dubreuil et al., 1964) was originally obtained from Dr. R. Dubreuil. (3) RHaT same as reported earlier. Cells derived from both these cultures were grown separately in two different media: (a) MEM with 10% FCS and 10% TPB, and (b) medium 199 (M.199) (Morgan et al., 1950) supplemented with 10% FCS. (4) RLT_2, referred to as RLT, a cell line derived from SV40-

transformed rabbit kidney cell culture was supplied by Dr. R. Dubreuil and maintained grown in M.199 with 10% FCS. None of these cultures produced SV40 virions spontaneously when tested.

Non-transformed cells: (1) AGMK, secondary cultures, one of which was grown in M.199 with 5% FCS and the other in MEM with 5% FCS and 5% TPB. (2) BSC cultures; cells derived from this line were grown separately in two different media: (a) MEM with 10% FCS and 10% TPB, and (b) M.199 containing 10% FCS. (3) PK-15 (CCL 33) grown as described previously. (4) Mouse embryo cells, primary and secondary cultures, grown in MEM supplemented with 10% FCS. (5) Hamster kidney cells, primary culture, grown in M.199 with 10% FCS.

For cell co-cultivation or fused cultures the growth medium employed was a mixture (ratio: 50 : 50) of the media supplemented as used for separate cultivation of the two cell types; only the serum concentration in this growth mixture was as required by the more fastidious of the two cell types. Antibiotic solutions were the same as described in the preceding experiments. Cell cultures for microscopic examination (whether stained or not) were prepared in tissue culture Petri dishes containing coverslips which were made from microscopic slides (ESCO, Erie Scientific) by cutting them in pieces of a suitable size; preliminary observations in this laboratory proved that this type of coverslip shows the best monolayer when Petri dishes are incubated at 37°C in a gas mixture of 5% carbon dioxide in air, or when those coverslips are introduced into a tissue culture flask. However, due to their thickness, unless properly manipulated, the cells on these coverslips cannot be examined under many high power objectives when the coverslips are mounted in the usual inverted position.

Tumor production in hamsters by inoculation of SV40-transformed cells.

Five- and fifteen-week-old inbred Syrian hamsters were injected subcutaneously with 10^6 cells of Cl_2TSV_5, RHaT, or RLT suspended in MEM. Hamsters bearing tumors with 1 cm or more in diameter were sacrificed and the tumors excised. Parts of the tumors (primary and metastases) were trypsinized and some portions fixed in Bouin (Formalin 125 ml; Sat. aq. sol. picric acid 375 ml; acetic acid, glacial 2.5 ml) for histological examination after sectioning and staining with hematoxylin-eosin. Fragments of some primary tumors as well as of some metastases were preserved frozen (as described in the preceding chapter) to use at any future time for culture. Eight to ten-week-old rabbits were also injected with up to 10^7RLT, Cl_2TSV_5, or RHaT cells in MEM.

Controls for cell viability and animal inoculation were established as indicated in previous experiments.

Preparation of Sendai virus

Sendai was propagated as described in the former chapter. The pooled allantoic fluid with a titer of about 8,000 hemagglutinating units (HAU)/ml. was clarified by centrifugation at 2,000 g. for 10 min. and part of the supernatant was then centrifuged at 30,000 g. for 30 min. and the sediment resuspended in MEM at one-twentieth of the original volume. These preparations were stored in 0.5 ml. and 1 ml. lots at -70°C. Prior to use for cell fusion, the virus preparation was titrated for HAU/ml with guinea-pig erythrocytes and suitably diluted in phosphate-buffered saline (PBS)

or MEM. Virus inactivation was carried out by placing the final suspension in a 50 mm tissue culture Petri dish within 10-12 cm of a germicidal lamp (Westinghouse Sterilamp G36T64, or Westinghouse F20T 12/BL). The loss of infectivity was ascertained by inoculation of embryonated eggs or AGMK cell cultures.

Cell fusion technique

Unless otherwise indicated below, most steps of the fusion procedure were the same as described earlier. In a preliminary experiment to determine the best concentration of inactivated Sendai virus (UV-SeV) for cell fusion, suspensions of UV-SeV at different concentrations (HAU/ml, ranging from 1×10^3, 2×10^3 ... to 1×10^4) were added to mixtures of two cell populations (5×10^6 cells /0.5 ml of each type). Thus, the most suitable concentration (4,000 HAU/ml, see results) of UV-SeV was thereafter employed for further cell fusion experiments. The presence of heterokaryons and/or any eventual CPE in fused cultures was ascertained by microscopic examination of coverslips stained with Giemsa or Paragon multiple stain (Paragon G. & C. Co.Inc.). The percentage of fusions was calculated, after 24 h, on an average of about 1,300 cells (3-5 fields) from each culture. Control cell mixtures, treated as above but not exposed to Sendai virus, were also examined to rule out spontaneously occurring fused cells. In fused cultures, the growth medium was replaced with an identical one after 24 h. Thereafter the medium was changed every 3 days and the serum concentration in the growth mixture was reduced to 50%.

Fluorescent antibody staining

FA staining of SV40-capside antigens: The antisera are the same as described in the preceding chapter. The coverslip with monolayer was rinsed in PBS, drained and air dried; it was then fixed in acetone for

5 min. at room temperature. Once dried, the coverslip was dipped into PBS and the excess fluid drained before staining. If the monolayers were to be stored (at 4°C) for a few days before staining, the cells were then fixed at -20°C for 10 min. The staining was carried out by the indirect method; the coverslip was placed on slide in a humid plastic chamber (37°C) and the cell sheet flooded for 30 min. with a drop of nonconjugated rabbit or horse anti-SV40 serum. After having been washed in three changes of PBS and drained, the coverslip was returned to the humid chamber and the cells were covered with a drop of goat anti-rabbit anti-globulin conjugated with fluorescein-isothiocyanate. Thirty minutes later, the coverslip was washed as above and mounted in buffered glycerol (90% glycerol in PBS).

FA staining of T-antigen in SV40-transformed cells: The procedure for fixation of cells, washing and mounting was as described above. For direct FA staining, the cell sheet was covered with a drop of FA preparation (hamster anti-T globulin conjugated with fluorescein-isothiocyanate) and placed in a humid chamber at 37°C for 60 min. For indirect FA staining, the cells were first treated for 30 min. with the serum of hamsters bearing SV40-induced tumors, and later with rabbit anti-hamster anti-globulin conjugated with fluorescein-isothiocyanate for 30 min.

Controls included normal cells, as well as normal serum from the same species as the immuneserum. The staining reaction was also blocked by nonconjugated antiserum (horse anti-SV40 serum for SV40 capside antigens and rabbit or hamster immuneserum for T-antigen).

Electron microscopy

The procedure for preparing grids for virus detection from cell cultures was the same as described before. The preparations were negatively stained with 2.5% neutral PTA and the grids were examined with a Siemens Elmiskop IA or Philips 300 electron microscope.

Detection of Interferon production in cell cultures treated with UV-SeV.

Supernatant medium harvested from cell cultures 48 h after exposure to 4,000 HAU of UV-SeV was acidified to pH 2.0 by dropwise addition of concentrated HCl. It was then kept at 4°C for 48 h; after this holding time, the pH was brought to 7.0 with saturated NaOH.

The viruses used for testing the presence of interferon in such treated fluids were SV40 (rescued from RHaT) and vesicular stomatitis virus (VSV, a stock strain from this laboratory grown in BSC or AGMK cells). Since these viruses give a characteristic CPE in the test cells, the assay procedure followed was that of inhibition of viral CPE (Sellers and Fitzpatrick, 1962), because the method is simple, reliable and can give reproducible results (Finter, 1966). The culture medium to be tested was serially diluted 2-fold and 0.2 ml amounts were added to groups of 4 cultures in tubes; after 24 h of incubation at 37°C all these cultures together with untreated controls were challenged with 50 TCD50 of the virus, SV40 or VSV (one TCD50 represents the amount of virus which causes typical CPE in 50% of cultures). The cultures were examined daily under the microscope and the final reading of the test was carried out when 75-100% of the monolayer in the control tubes had a characteristic CPE.

OBSERVATIONS

Cl_2TSV_5 and RHaT produced tumors and their cell cultures

Since SV40 virions could be rescued from tumors by cell association technique (see preceding chapter) experiments were performed to determine whether the SV40-genome could be induced from tumor cells after various (alternate) "in vitro"-"in vivo" passages. After the first "in vivo" passage and for the subsequent inoculation into hamsters, the cell culture was derived from the tumor which developed at the site of injection. In most cases one or more animals (from a group), which were injected with cells of the same origin, showed metastases. In the latter case the cell culture to be inoculated into a new hamster for tumor production was derived from the tumor grown at the site of inoculation and not from metastases. Thus, a uniform method for production of tumor generations in hamsters was followed in the present work. At each animal passage level, tumors were cultivated "in vitro" and cells from the 3^{rd}-6^{th} passage were used for subsequent animal inoculation. During this study, observations on the nature of tumors and some features of these tumor cells were also recorded. Table IV summarizes some of these observations.

Tumor production: One hundred per cent of 5-week-old and over 90% 15-week-old hamsters inoculated with Cl_2TSV_5 and RHaT cells developed tumors within a period of 3-20 weeks (Fig.4-5). Most of the 5-week-old animals showed large (1 cm and more) tumors, with or without metastases, in a time 10-30% shorter than did the older hamsters. In practically all cases metastases were observed only 8-9 weeks after cell inoculation.

TABLE IV

MAIN FEATURES OF DIFFERENT GENERATIONS OF Cl_2TSV_5 AND RHaT PRODUCED TUMORS AND THEIR CELL CULTURES

Cells	Passage level "in vivo" (a)	Metastases (b)	Presence of polykaryocytes in culture (c)	T antigen (d)	Spontaneous production of SV40 or SV40 capsid antigens	Rescue of SV40 by cell fusion (e)	% of fusion with BSC cells
Cl_2TSV_5	1	+ (2/10)	+ (7%)	+	-	+	23
	2	+ (2/8)	+	nd	-	+	nd
	3	+ (2/5)	+	nd	-	+	39
	4	+ (4/6)	+ (16%)	nd	-	+	67
	5	+ (11/13)	+ (27%)	+	-	+	84
RHaT	1	- (0/5)	+ (4%)	+	-	+	9
	2	- (0/3)	+ (6%)	nd	-	+	11
	3	+ (1/5)	+	+	nd	nd	nd
	4	+ (2/4)	+	+	-	+	nd
	5	+ (3/4)	+ (18%)	+	-	+	47

(a) Five-week-old hamsters were used for tumor passage, except for passage 3 of RHaT where 15-week-old animals were used.
(b) The numbers in brackets indicate the number of animals with metastases/total number of animals with tumors in a given group.
(c) The % given are only from experiments in which counting was done from primary cell cultures of the respective tumors.
(d) The presence of T antigen was ascertained by FA staining, except in one case of Cl_2TSV_5 cells where complement fixation technique was used.
(e) SV40 was detected by infectivity assay (CPE) and/or electron microscopy.
nd: Not done.

Fig.4

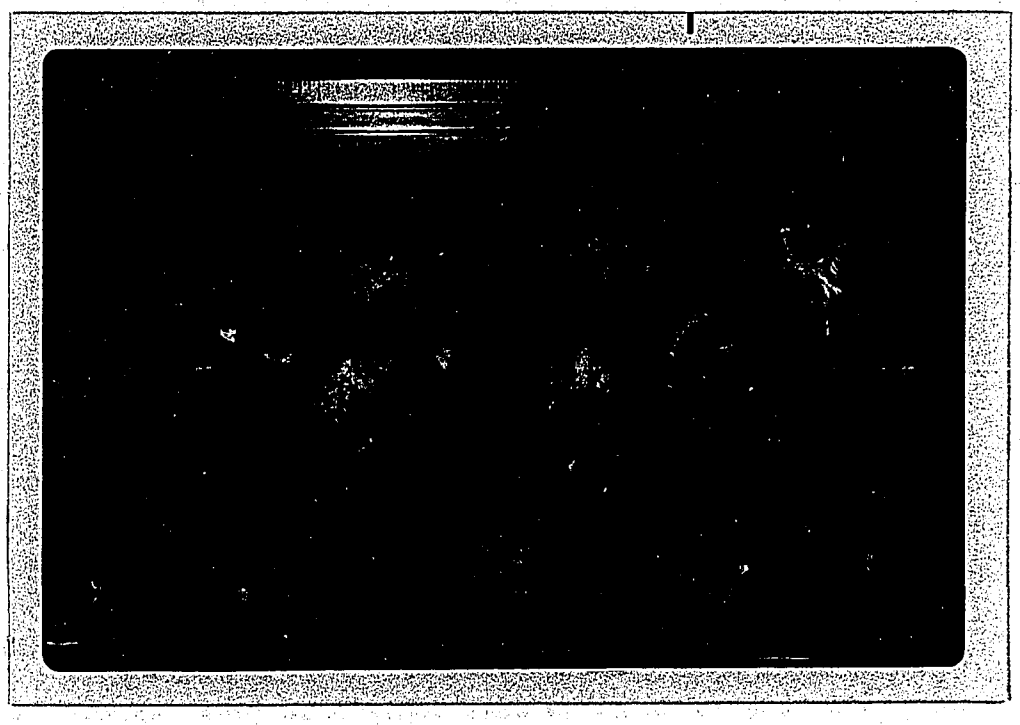


Fig.5

Fig. 4 Hamster with Cl_2TSV_5-produced subcutaneous tumors, and the control animal, 12 weeks after inoculation.

Fig. 5 Tumor-bearing hamsters showing large tumor masses, and the control animal (left), 12 weeks after inoculation. The two animals on the left side are the same as shown in Fig.4; note the difference between these two animals here as compared to Fig.4.

Hamsters dying earlier with tumors did not show any apparent sign of metastases, but only a solid proliferating tumor mass at the site of injection. No differences were observed in development of metastases between the two age groups.

Hamsters which received RLT cells, and rabbits injected with RLT, Cl_2TSV_5 and RHaT cells did not show any sign of tumor development. However, their sera contained anti-T antibodies, as proved by the results of FA staining reaction performed by using these sera as blocking agents.

The cases of metastases reported here are those detected with the naked eye at the time of necropsy of hamsters. Viscera or other organs were not examined microscopically. However, it is important to note here that even in case of animals bearing several invasive SV40-tumor masses, metastases were seldom detected macroscopically at the visceral level; when observed, they were localized in the peritoneal cavity and exceptionally in the pulmonary area. In many animals with metastases, liver, spleen, kidneys and lungs often appeared inflammed. Most metastases observed were localized subcutaneously, the lymph nodes being the main organs invaded. Subcutaneous tumors were seen surrounded by highly congested areas and some necrotic zones. In many cases of animals without metastases, the large tumor mass which developed at the site of inoculation had almost incorporated the closest lymph node; this was particularly evident with Cl_2TSV_5 cells which, throughout the passages "in vivo", proved to produce increased number of metastases at the lymph node level (Fig.6-7).

Fig.6

Fig.7

Fig. 6 Hamster referred to in Fig. 4 and 5, showing several metastases. Arrow indicates the site of inoculation. Cultures were derived from tumor masses a, b and c, and SV40 was rescued from all three cultures.

Fig. 7 Same animal as in Fig. 6. No metastases were macroscopically seen at the visceral level. Most viscera appear highly congested.

After successive "in vivo" passages, Cl_2TSV_5 and RHaT tumor cells exhibited an increased potential to produce metastases in all inoculated hamsters and they appeared in about 30% shorter time at the last "in vivo" passage level than for the first time they appeared. As shown in table IV two other parameters appear to parallel this observation: (1) the increased capacity of cells for fusion, and (2) the increased number of polykaryocytes in tumor cell cultures.

Tumor morphology: Tumors produced by both cell lines (Cl_2TSV_5 and RHaT) at different "in vivo" passage levels had the histological spectrum of sarcomas (Fig. 8-10) similar to those described by other authors with SV40-hamster system (Diamandopoulos, 1968; Diamandopoulos et al., 1969; Easton et al., 1970). Lymphosarcomatous areas were present in sections from metastases. The tumor cells had round to oval nuclei which were densely hyperchromatic to vesicular, and varied considerably in size. Giant cells and mitotic figures were present in all sections examined. Areas with necrosis, and zones containing inflammatory cells and degenerating nuclei were regularly observed. Small zones of relatively acellular connective tissue and necrosis were also found. In sections of a few primary tumors a capsule-like connective tissue formation was observed.

"In vitro" cultural characteristics. Cells of both lines (Cl_2TSV_5 and RHaT) showed in culture, and throughout the passages("in vitro"-"in vivo"), a predominantly triangular to polygonal epithelioid morphology, similar to that described by other authors with SV40-transformed hamster cells (Black and Rowe, 1962; Easton et al., 1970). A few epithelial-like cells were

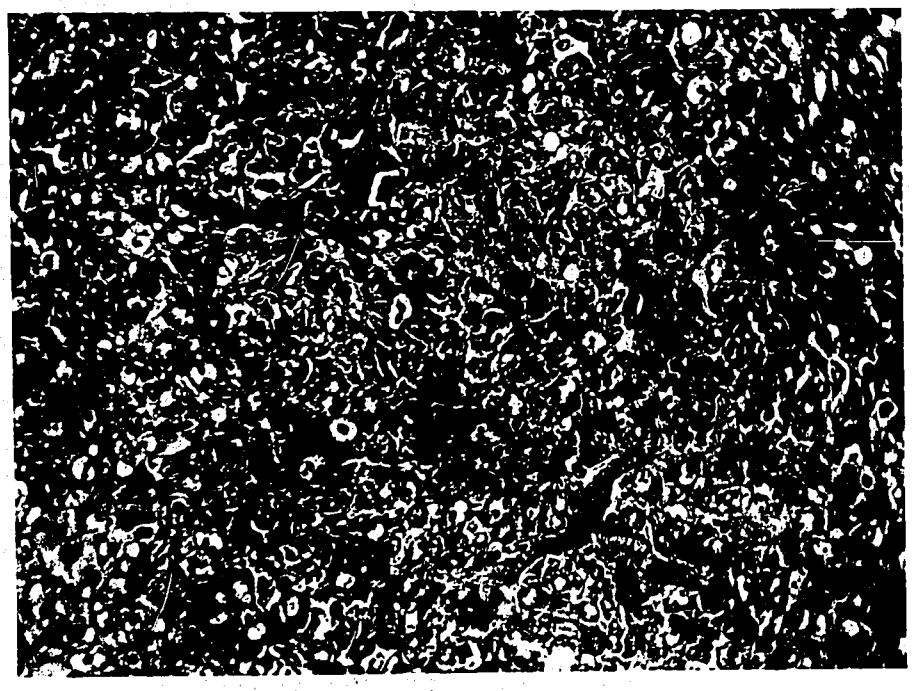


Fig. 8

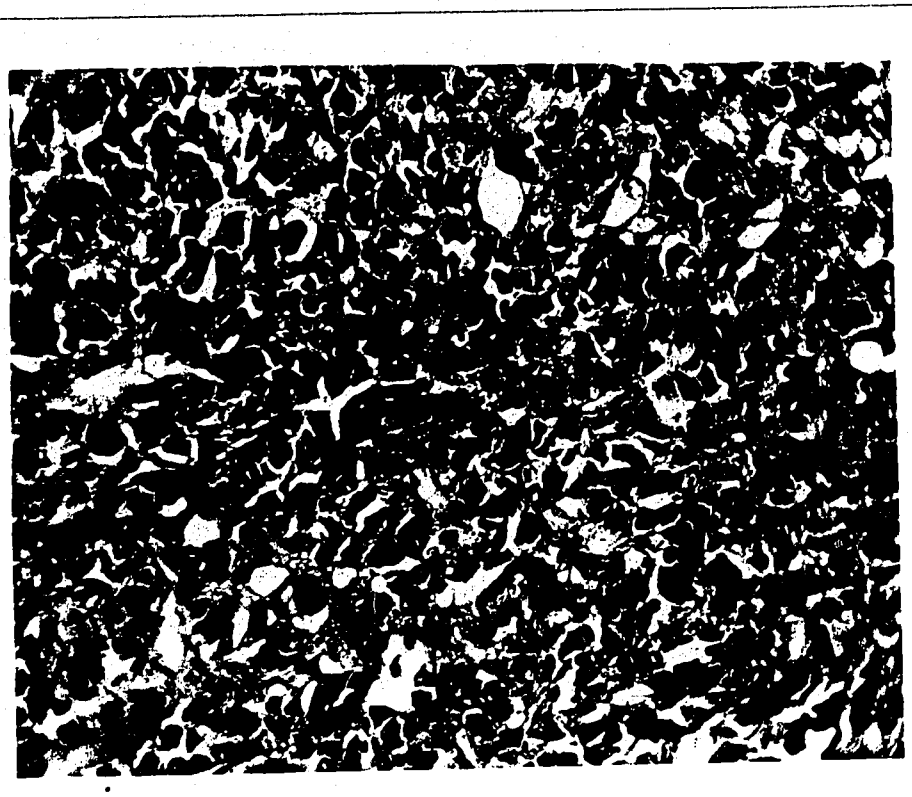


Fig. 9

Fig. 8 Cl_2TSV_5-produced sarcoma with vesicular nuclei and prominent nucleoli, and mitotic figures. Hematoxylin-eosin stain. x 800.

Fig. 9 Very anaplastic sarcoma with pleomorphic and hyperchromatic nuclei. Hematoxylin-eosin stain. x 800.

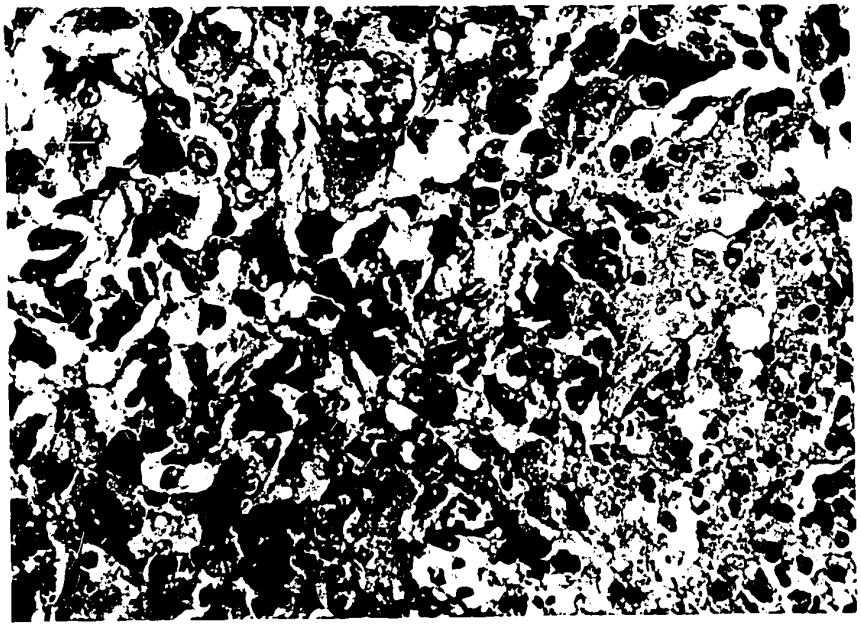


Fig. 10 Sarcoma with vacuolization, giant cells and necrosis. Hematoxylin-eosin stain. x 1,000.

sometimes observed in RHaT cultures but their number did not increase in successive generations and the cultures consistently showed the same basic features. Three clones of RHaT and two of Cl_2TSV_5 cells cultivated "in vitro" for 10-30 passages showed the same epithelioid morphology. Cultures derived from 2 generations of tumors produced by cells from two of these clones (one of Cl_2TSV_5, the other of RHaT cells) also had the morphological features of the parental cells. In many cases of tumor primary culture cells from Cl_2TSV_5 produced tumors, a fibroblastoid morphology was predominantly observed. In the next "in vitro" passages the cultures gained the epithelioid morphology.

Another very interesting observation was made during some preliminary experiments with Cl_2TSV_5 cells, which are normally cultivated in a relatively rich medium (MEM supplemented with 4 x amino acids, 8 x vitamines, 10% TPB and 10% FCS). Attempts to reduce amino acid, vitamin and TPB concentrations in this medium resulted in the selection of a cell population with a typical fibroblastic morphology and a slow growth rate. These features were again observed when an attempt was made to grow Cl_2TSV_5 cells in M.199 supplemented with 10-15% FCS. Thus, it appears, at least in the case of Cl_2TSV_5 cells, that the composition of the growth medium can play an important role in determining the cell morphology through some selection mechanism in the cell population.

The most important finding during cytological studies was observed in the cultures of different generations of tumors: an increase in percentage of polykaryocytes was found in tumor cell cultures after "in vivo" passages (Table IV). In fact, some primary cultures of Cl_2TSV_5 tumors at the 5th "in vivo" passage level showed up to 40-60% multinucleate cells containing multiple prominent nuclei. In many of these cells the nuclei appeared to undergo fusion (Fig. 11-12). In subsequent "in vitro" passages the number of polykaryocytes, containing more than 5-6 nuclei, gradually decreased in the cultures. However, after about 6-8 of these passages the number of multinucleate cells remained constant in a given cell population. This observation appears to indicate that these large polykaryocytes cannot survive,or do not attach to the tissue culture flask, or cannot multiply after their trypsination for subculture. These multinucleate cells were also extremely sensitive to freezing procedure for tissue culture preservation. Thus, they were drastically reduced in sub-

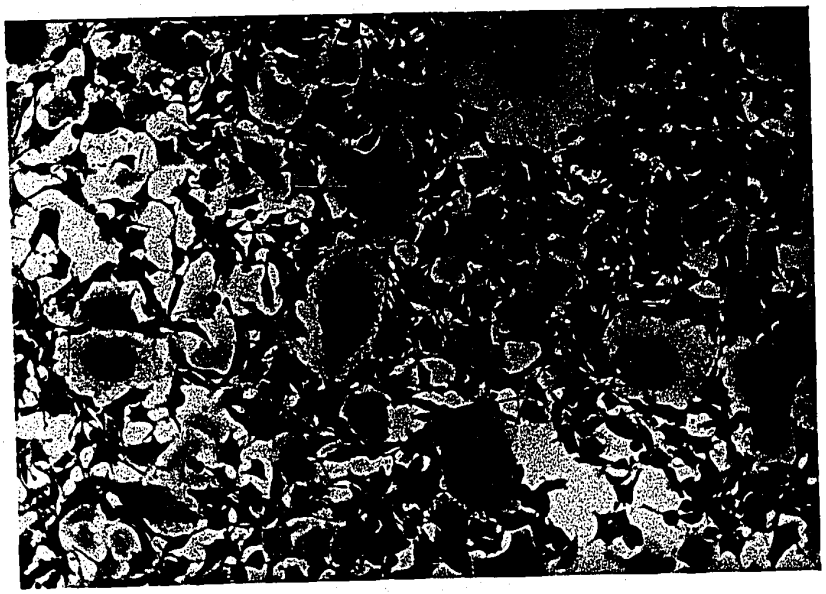


Fig. 11

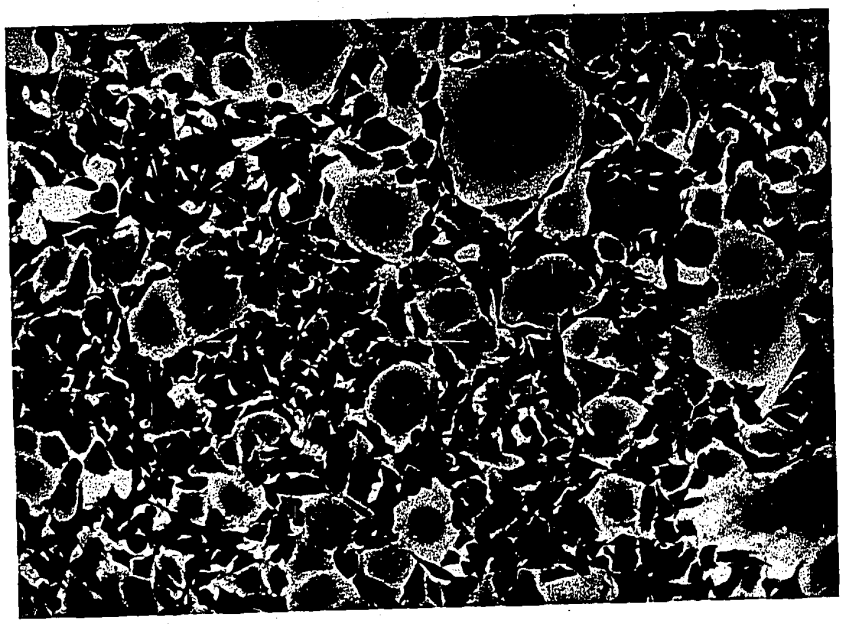


Fig. 12

Fig. 11-12 Cells from two $C1_2TSV_5$-produced tumors at the 5th "in vivo" passage level. Note the number of polykaryocytes in these 3-day-old cell populations. In many of these cells the nuclei appear to undergo fusion. Note the pleomorphism of all nuclei and the fibroblastoid to epithelioid morphology of the cells. Paragon multiple stain. x 400.

cultures of tumor primary cells which were kept frozen (suspended in MEM with 10% FCS and 10% glycerol). It is also important to note that they were not seen in cultures of fused cells, a fact which leads one to suspect that they may not be viable after the fusion procedure.

Attempts to detect or isolate any virus or a fusion factor from cell cultures containing the above described polykaryocytes or tumor extracts were unsuccessful. Also the results of FA staining showed that they do not synthesize SV40 capsid antigens. Therefore, it is believed that these polykaryocytes are not a product of fusion by a virus. Since the cytoplasm of these multinucleate cells is relatively large in size, it may be concluded that these polykaryocytes are the result of fusion of previously separate cells. The mechanism of such fusion is unknown.

Detection of a growth stimulating factor in SV40-transformed cell cultures.

In a preliminary experiment where Cl_2TSV_5 tumor cells were co-cultivated with those of PK-15 line an overgrowth of these pig cells was observed after 3 days of culture. Although by this time a complete sheet had been formed by the co-cultivated cells and the medium was very much acidified by those cells, the overgrowth continued until 7-8 days. The loss of density-inhibition in those cultures was evident at that time. Cl_2TSV_5 cells started degenerating after 8-10 days but the PK-15 cells did not show any such sign. When the medium was changed after 4-6 days of co-cultivation, Cl_2TSV_5 cells started rounding after 8-10 days and detached gradually from the flask wall; in contrast, most PK-15 cells

remained viable for subculture until about 18th day. When cultivated separately (controls), Cl_2TSV_5 cells degenerated in a 20-25% shorter time than did the PK-15 cells.

Experiments performed by adding to PK-15 culture medium, aliquots of cell-free supernatant from 6-day-old Cl_2TSV_5 cultures gave similar results. Stimulating activity was observed even when this supernatant was present in PK-15 cultures at a concentration of 1/20. The highest overgrowth of PK-15 cells was observed when they were co-cultivated with those of Cl_2TSV_5 or with cells of tumors produced in hamsters by cultures of Cl_2TSV_5.

On another occasion, BSC culture tubes were inoculated with 0.2 ml amounts of (virus-free and cell-free) supernatant from Cl_2TSV_5 tumor cultures and placed on a roller drum. The tubes were then examined daily under the microscope (without changing the medium) for any eventual CPE. Unexpectedly, the cell sheet in those tubes which received 0.2 ml of supernatant underwent degeneration only one week later than the monolayer in controls,(a): tubes which did not receive supernatant, and (b): tubes which received 0.2 ml of growth medium for Cl_2TSV_5 cells). Further research is needed to determine the nature of this factor and its production or presence in tumor cells.

Studies on cell fusion

Notes on the use of Sendai virus preparations for cell fusion. The concentration of Sendai virus used by different authors for cell fusion has

varied from 400 HAU to 80,000 HAU (Okada, 1962; Harris and Watkins, 1965; Gerber, 1966; Koprowski et al., 1967; Watkins and Dulbecco, 1967; Takemoto et al., 1968; Cassingena et al., 1969b; Steplewski and Koprowski, 1970). In a preliminary experiment during this study with a monkey-hamster system for SV40 induction, different concentrations of UV-SeV were employed to determine the most suitable concentration of this virus for cell fusion. Cells were fused according to the following combinations: (1) Cl_2TSV_5 + AGMK grown in M.199; (2) Cl_2TSV_5 + AGMK grown in MEM; (3) Cl_2TSV_5 + BSC grown in M.199; (4) Cl_2TSV_5 + BSC grown in MEM; (5) RHaT + BSC grown in M.199. It was then found that with over 4,000 HAU of UV-SeV there was no significant increase of fusion. Moreover, at 24 h, cultures treated with 7,000-10,000 HAU showed many more non-viable cells than the cultures exposed, under the same conditions, to lower concentration of UV-SeV. At those virus concentrations (7,000-10,000 HAU) AGMK and BSC control cells appeared to show cytotoxic effect when observed after 24 h of culture. No significant differences in percentage of fusion was observed between BSC and AGMK cultures maintained in different growth media (M.199 or MEM).

Although ultraviolet light (UV) is widely used to inactivate virus preparations, claims have been made that inactivation of Sendai virus by UV does not remove 100% of infectivity from the pool of virus used for fusion experiments; to overcome this problem Steplewski and Koprowski (1970) used beta-propiolactone to inactivate the pool of virus for fusion experiments. During this study, following the procedure described above, it was found that virus inactivation by UV is an easy method which gives fully reliable results and does not require any che-

mical, or time-consuming and laborious operations. It was also observed that an overexposure of virus preparation to UV for a period exceeding 20 min.(1,000 ergs/cm$_2$/sec) does not produce any detectable change in the fusing capacity of Sendai virus. When the virus was inactivated by UV and kept at 4°C for 8-10 h its fusing ability was found to be practically unchanged. Moreover, this study suggests that most germicidal lamps available in tissue culture laboratories could successfully be employed for UV-inactivation of virus preparations to be used for cell fusion, provided that they can supply a desirable UV-activity.

Fused cell cultures. When cells of two lines grown in different media are fused together, the main problem is to determine the ideal composition of the medium in which to cultivate this Sendai-treated mixed cell population. Furthermore, if one adapts a cell line to another medium (different from that in which it has been regularly maintained) there is a possibility of selecting a population or "variant" with decreased fusion capacity. A similar observation has been reported with SV40-transformed SV_3T_3 cells (Watkins, 1970).

During the present study, when AGMK or BSC cells were fused with those of the Cl_2TSV_5 line and the cell mixture was then cultivated in Cl_2TSV_5 growth medium, an unequal growth of non-fused Cl_2TSV_5 cells was observed within 24 h. This resulted in a very small population of monkey cells in the mixed culture. If the growth medium employed was the one regularly used for AGMK or BSC cultures then the growth of non-fused Cl_2TSV_5 cells was considerably reduced. However, when the medium used was a mixture (ratio 50 : 50) of these two media, the fused cells did not appear to suffer although the growth of the Cl_2TSV_5 cells was relatively reduced and the BSC or AGMK cells grew well.

It is also important to note that tumor cells having an accelerated growth caused the medium to become acidic more quickly than did the normal cells. Under such metabolic conditions (i.e. reduced pH) AGMK and BSC cells degenerated much faster than they did when cultivated separately. Since the main goal of these experiments was the induction of virus, and since SV40 is known to give a relatively slow CPE, it was important to obtain a balanced growth of the two types of cells in culture or to slightly favor the SV40-permissive type without, however, affecting the viability of the heterokaryons. Therefore, after the above observation, the medium adopted to grow the fused cells was a mixture of both growth media in which the serum concentration was that required by the more fastidious of the two cell types. Under these conditions the polykaryocytes showed the longest survival time. Other preliminary experiments also showed that, under these conditions, the yield of SV40 by fused or co-cultivated cultures was the highest. Thus, the problem of medium selection appeared to be at least partially overcome by using the ratio mixture described.

The observations which will now be described concern some features of fused cells produced in Cl_2TSV_5 - BSC cultures. In all these cultures, the medium was first changed after 24 h of culture and thereafter every 3 days. Polykaryocytes with more than 3-4 nuclei did not appear to multiply. However, a few of the heterokaryocytes with 8-20 nuclei remained alive, under suitable conditions, as long as 2 weeks. All these heterokaryocytes with a longer survival time contained many more nuclei of BSC origin than of Cl_2TSV_5. Moreover, homokaryons of Cl_2TSV_5 cells

degenerated faster than those of BSC cells and after about 6-7 days only BSC homokaryons could be seen in the culture. Most heterokaryocytes with 40-60 nuclei began to degenerate after 5 days. In a few cases, within the polykaryocyte, some nuclei undergoing fusion were also seen.

Heterokaryocytes and SV40-production. In some cases of SV40-producing heterokaryocytes which had only 2-4 nuclei a typical CPE was observed under the microscope 20-24 h after fusion. This observation was consistent with results of FA staining of SV40 capsid antigens in those cells. Most heterokaryocytes with 2-4 nuclei were SV40-productive, but their number in the cultures was generally very low (Table V). In contrast, a large number of heterokaryocytes had over 10 nuclei and most of these nuclei were SV40 non-productive.(Fig.13-24) Heterokaryocytes with extremely large numbers of nuclei in a diffuse mass of cytoplasm were often seen. In this particular type of polykaryocytes only a small number of nuclei showed sign of SV40-production after 3 days in culture and there was very little vacuolization in the cytoplasm. FA staining (on 3rd day) of heterokaryocytes with over 40 nuclei showed that a maximum of 6% of these nuclei were SV40-positive. It was also observed that among the large heterokaryocytes, those showing SV40-CPE remained attached to coverslip longer than those which were non-productive.

To determine the yield of SV40 in a culture of fused cells an experiment was performed by using cells of BSC line and those of a culture derived from a Cl_2TSV_5-produced tumor at the 1st "in vivo" passage level. Tissue culture (2-oz) flasks were seeded with approximatively 1 x 10^6 cells suspended in 6 ml of growth medium and incubated at 37°C. Twenty

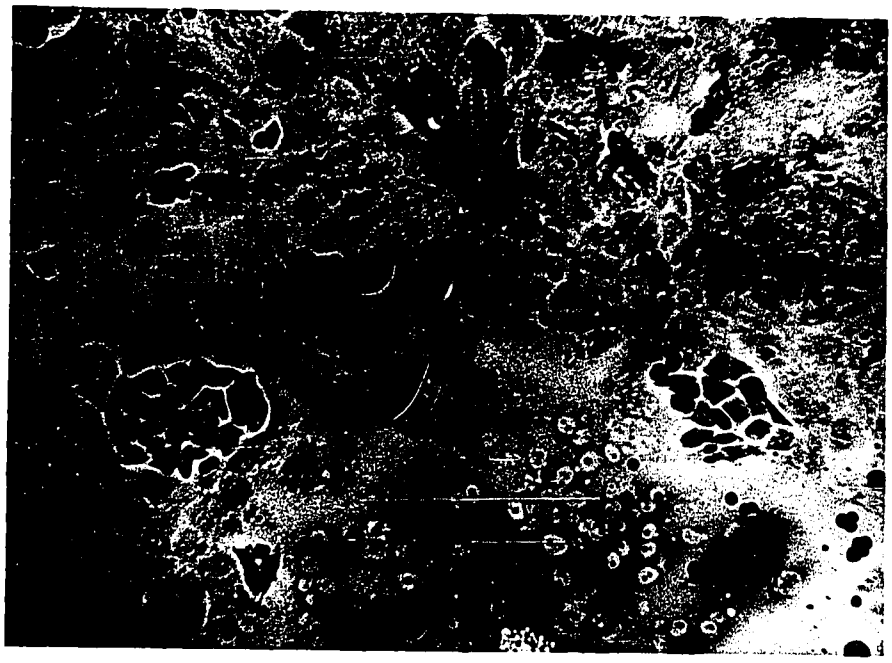


Fig.13

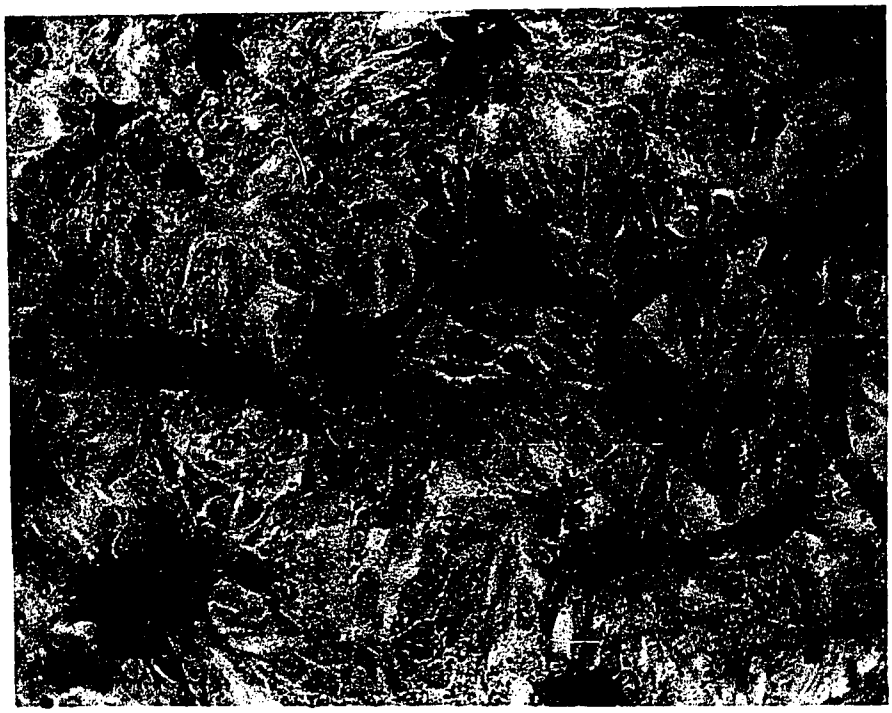


Fig.14

Fig. 13 Three-day-old UV-SeV-treated Cl_2TSV_5-BSC culture. Note the vacuolization in the lower part of the cytoplasm of large multinucleate mass. Typical clone-like non-fused Cl_2TSV_5 cells are present in this field. BSC nuclei with 1-2 prominent nucleoli can be easily recognized in the cytoplasm of the large fused cells. x 400.

Fig. 14 Three-day-old Cl_2TSV_5-BSC co-cultivated cell culture. Nuclei of both cell populations can be recognized in this cell sheet. a: BSC cells; b: Cl_2TSV_5 cells. Paragon multiple stain. x 400.

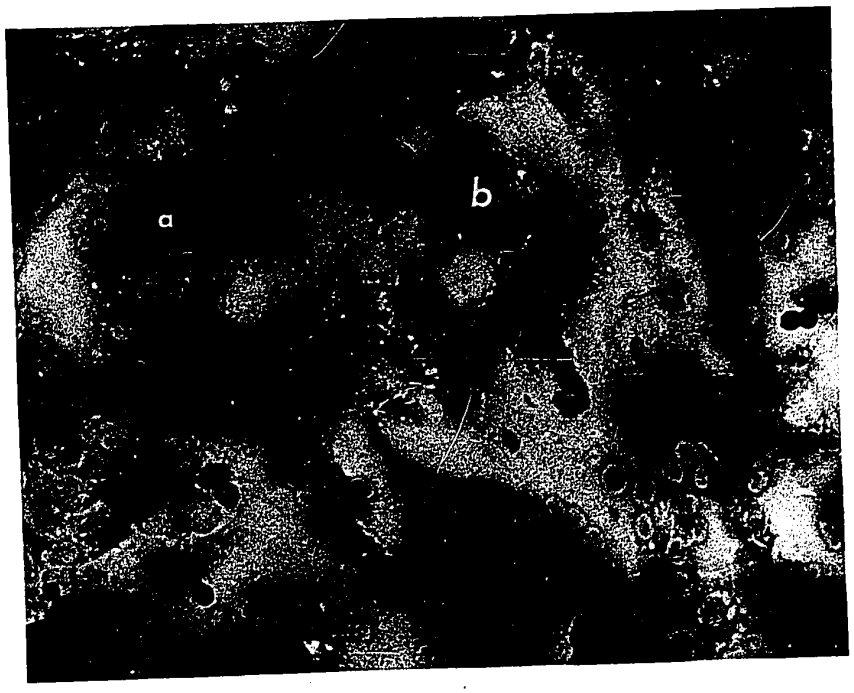


Fig.15

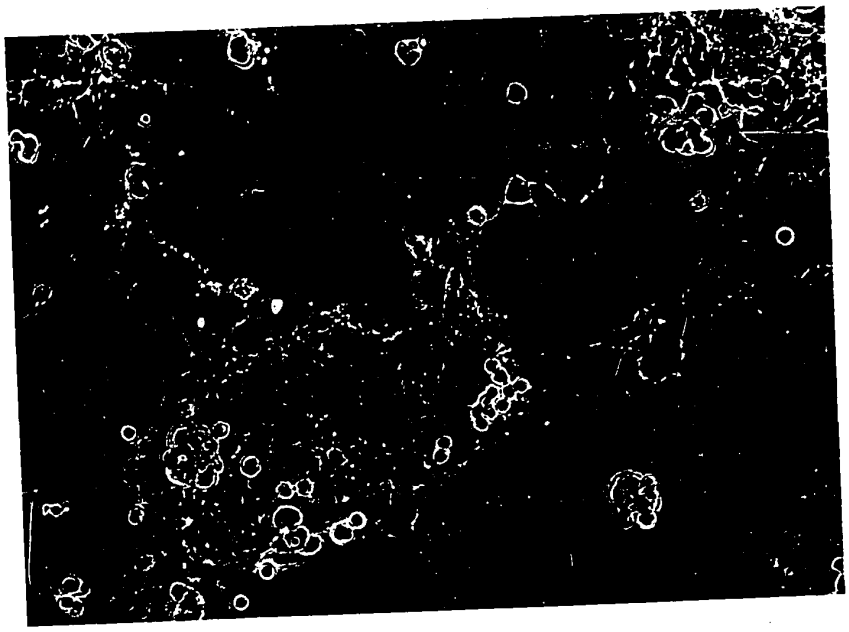


Fig.16

Fig.15 Four-day-old Cl_2TSV_5-BSC fused cells. Note the SV40-positive BSC (a) and Cl_2TSV_5 (b) nuclei and small areas of vacuolization in the cytoplasm. Paragon multiple stain. x 900.

Fig.16 Three-day-old RHaT-AGMK fused culture. Note vacuolization (arrow). Phase-contrast. x 600.

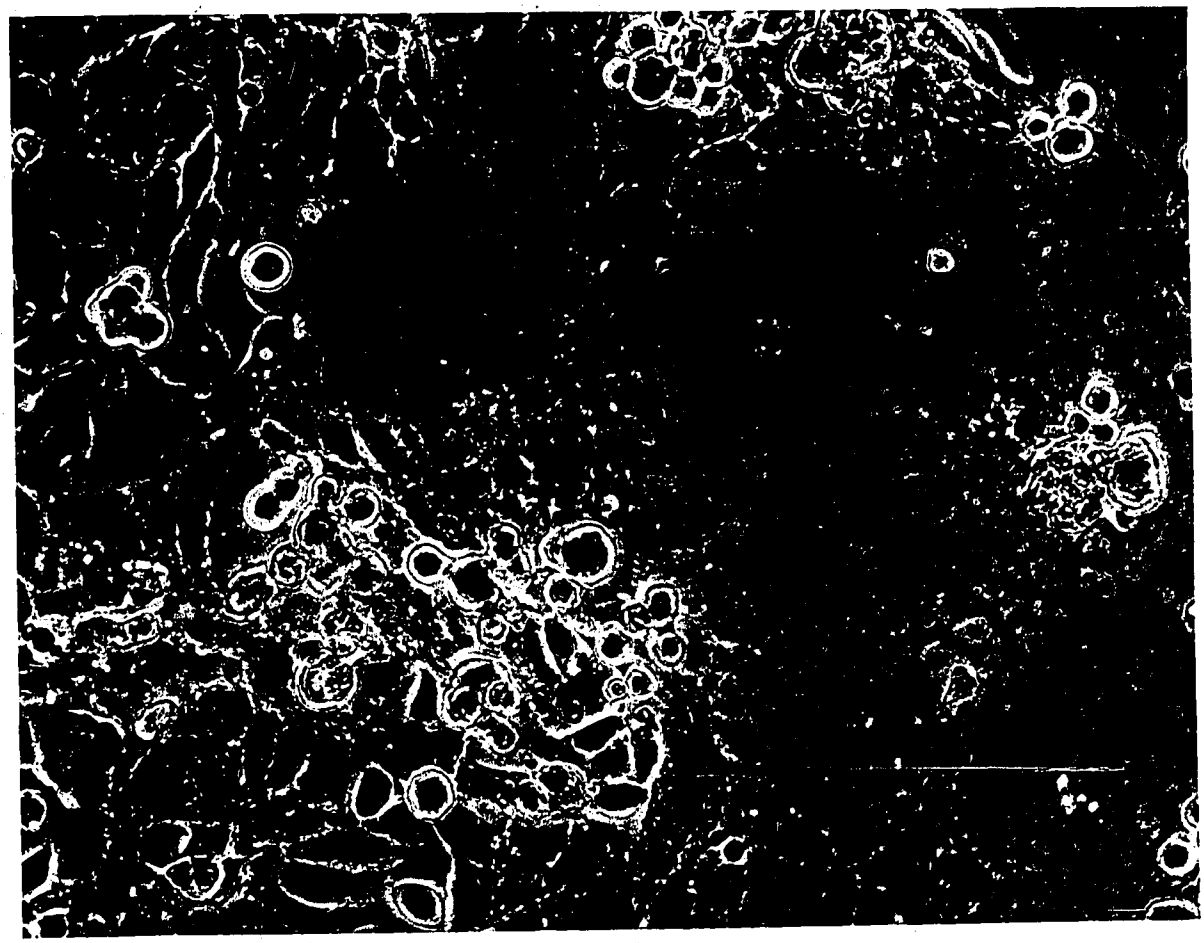


Fig. 17 Large multinuclear masses with vacuolization (arrow) in a fused culture of Cl_2TSV_5-BSC cells. Note both Cl_2TSV_5 and BSC nuclei, in the heterokaryocytes. Phase-contrast. x 1,500.

Fig.18 Six-day-old AGMK culture, 60 h after infection with Sendai virus. Note the CPE with polykaryocytes and the bridge-like figures typical of a paramyxovirus infection. One or two nucleoli are present in the nuclei. Hematoxylin-eosin stain. x 300.

hours later the medium was decanted and the cultures were rinsed three times with MEM. New medium with a reduced concentration (50%) of FCS was added and the cultures were returned to the incubator. Until the end of the experiment, the cultures were maintained in this same medium. Under these conditions all cultures degenerated 9-10 days after fusion.

Before virus titration, the cell sheets were detached from the flasks' walls by scrapping and, together with the culture medium, they were alternately frozen and thawed five times. After the cell debris sedimented by a low-speed centrifugation, the supernatant was then used for virus titration. SV40 titer was calculated by plaque assay procedure (Takemoto et al., 1966) using BSC cells. The results of these titration

TABLE V

SOME CHARACTERISTICS OF HETEROKARYOCYTES PRODUCED BY FUSION OF BSC CELLS WITH $C1_2TSV_5$ CELLS OF 5th "IN VIVO" PASSAGE CULTURE WITH HIGH FUSION CAPACITY (85%).

HETEROKARYOCYTES WITH	THEIR % AMONG HETEROKARYOCYTES	APPROXIMATIVE % OF BSC NUCLEI IN THE HETEROKARYOCYTES	AVERAGE LIFE TIME (DAYS) OF HETEROKARYOCYTES	EARLIEST SV40-CPE IN DAYS
2-4 nuclei	7	50	12	1
5-8 nuclei	12	57	6	2
9-25 nuclei	43	55	4	2
26-40 nuclei	26	55	3	3
over 40 nuclei	12	60	2	3

Fig.19

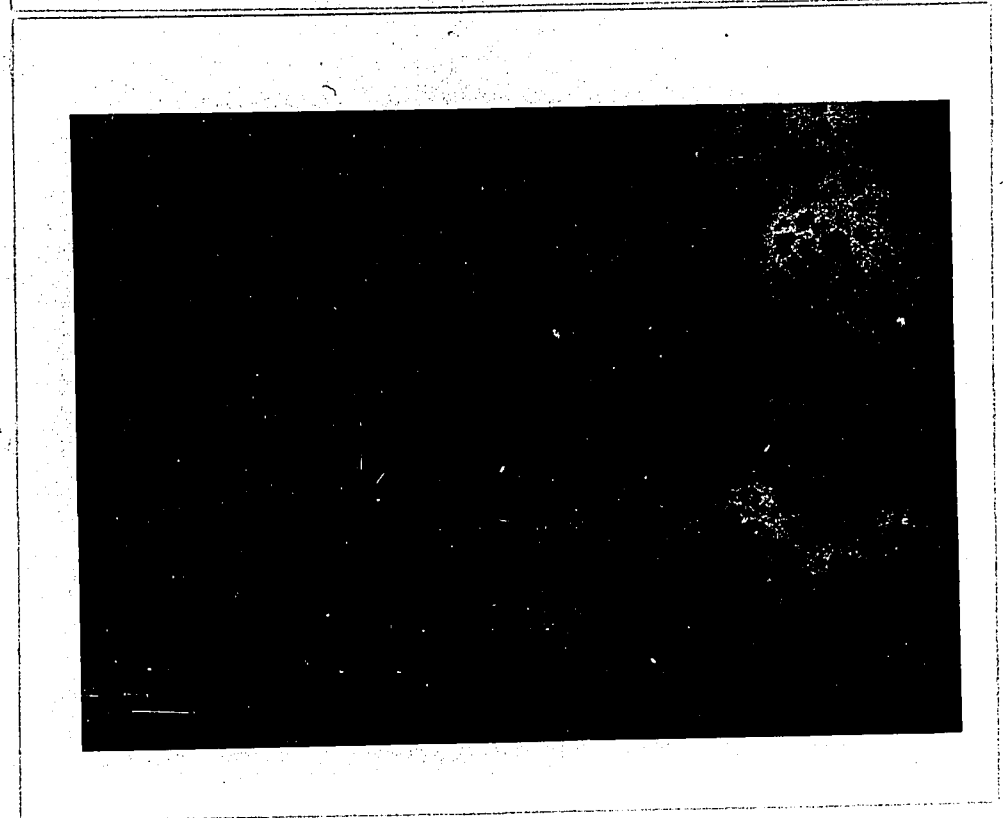


Fig.20

Fig.19 FA staining of SV40 capsid antigens, 48 h after fusion between Cl_2TSV_5 and BSC cells. Note the SV40-positive nuclei. x 2,000.

Fig.20 BSC cell monolayer (control for SV40 antigens) stained with FA. x 600.

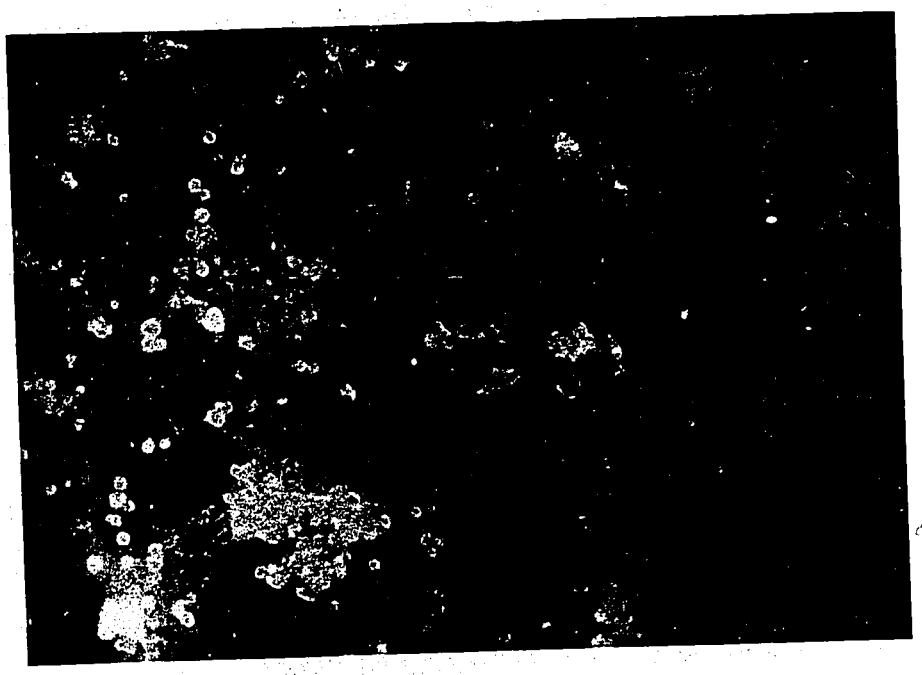


Fig.21

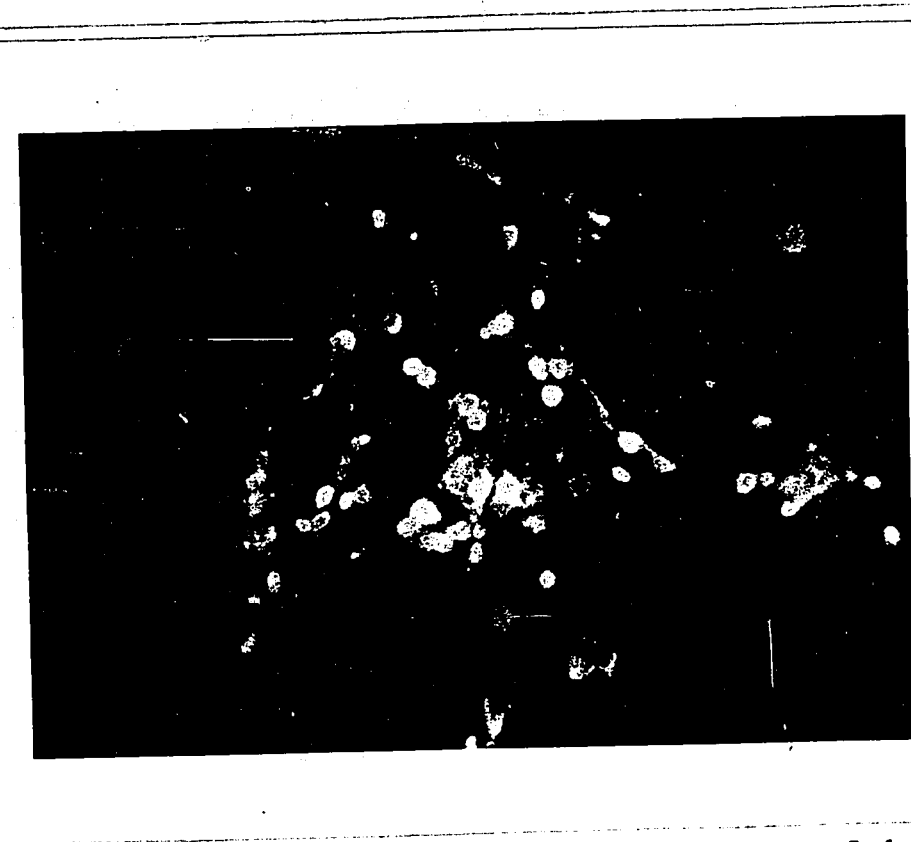


Fig.22

Fig. 21 Cl_2TSV_5-BSC cells stained with FA for SV40 antigens 5 days after fusion. Nuclei at different stages of positivity are present. x 450.

Fig. 22 BSC cells (control) stained with FA for SV40 antigens 5 days after infection with SV40. x 600.

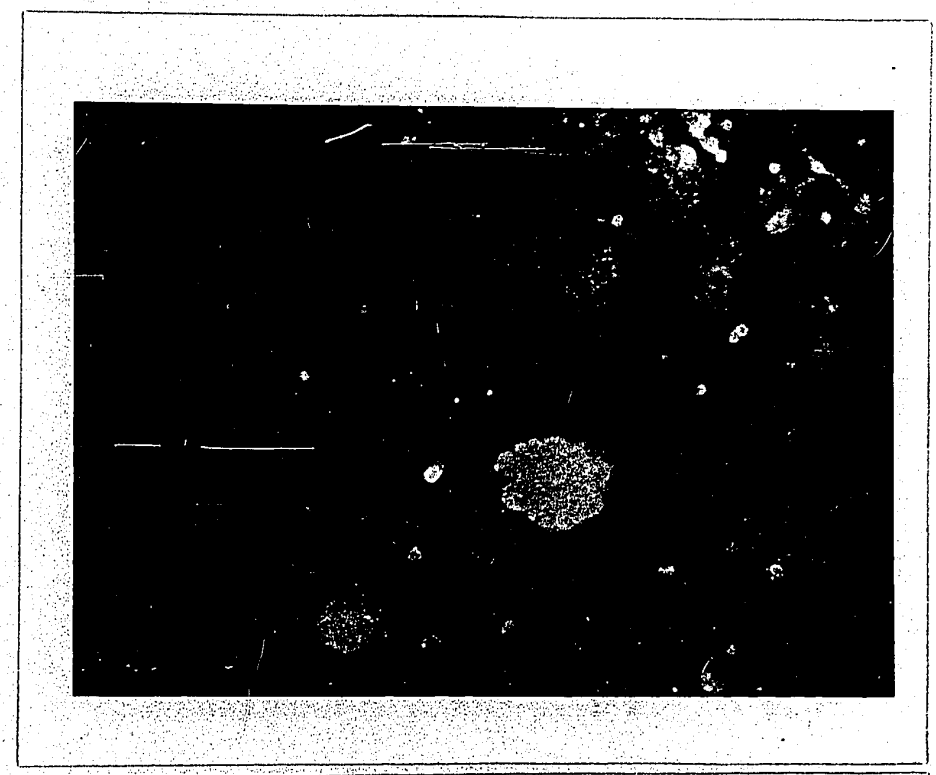


Fig.23

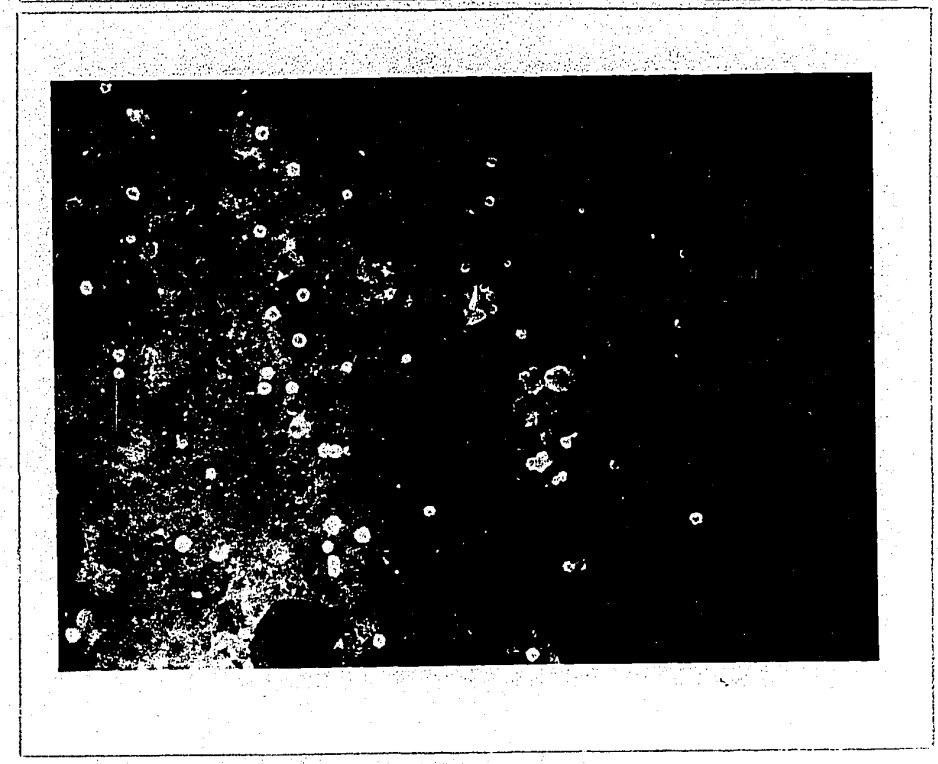


Fig.24

Fig. 23 FA staining of SV40 antigens in BSC-RHaT culture 65 h after fusion. Note the two polykaryocytes showing very positive staining reaction in the nuclei. x 600.

Fig. 24 FA staining of RHaT-BSC cells 6 days after co-cultivation (control). Note the SV40-positive nuclei dispersed in the field. x 600.

experiments are given in Table VI. As shown in this table, under the experimental conditions described, the maximum yield of SV40 occurred not later than one week after fusion experiments. Considerable amounts of SV40 were detected in fused cultures after 3 days; in contrast, the yield of virus by co-cultivated cells was extremely low at this time. Although the degeneration of fused cell cultures started earlier and was more marked than that of the co-cultivated cells, the yield of SV40 after a 10-day-period was comparatively low in these controls.

The medium decanted from the cultures 20 h after fusion, did not contain any infectious virus when tested on BSC cells.

Observations on the detection of interferon in UV-SeV-treated cultures.

Interferon might make the rescue of SV40 from fused cultures more difficult possibly by two mechanisms: first, by its production and antiviral action in fused cells, and, second, by its action on the next generation(s) of non-fused SV40-permissive cells present in culture of Sendai-treated cell mixture. Cassingena et al., (1969b) described an anti-SV40 action of interferon in fused cultures and Takemoto et al., (1968) reported that SV40 appeared to be as sensitive to interferon as VSV.

In the present study, experiments were performed to detect the production of interferon in UV-SeV-treated cultures. Culture fluid of the following cell mixtures was tested: (1) Cl_2TSV_5-AGMK; (2) Cl_2TSV_5-BSC; (3) RHaT-AGMK; (4) RHaT-BSC. Two identical cultures of each cell mixture were prepared and incubated at 37°C for 48 h without changing the medium which contained 0.5% of heat-inactivated rabbit anti-SV40 serum. Co-cul-

TABLE V1. RESULTS OF EXPERIMENTS ON THE YIELD OF SV40 AFTER FUSING CELLS OF BSC LINE WITH THOSE OF A CULTURE DERIVED FROM A Cl_2TSV_5-PRODUCED TUMOR AT 1st "IN VIVO" PASSAGE LEVEL (1).

EXPT. NO	% OF FUSION (HETEROKARYONS)	3-DAY YIELD OF SV40 IN CULTURE (PFU/ML)	5-DAY YIELD OF SV40/ML	7-DAY YIELD OF SV40/ML	10-DAY YIELD OF SV40/ML
1	23	7.2×10^3	9.6×10^3	7.4×10^4	7.0×10^4
2	25	7.8×10^3	2.4×10^4	2.4×10^5	2.6×10^5
3	21	6.8×10^3	9.4×10^3	6.2×10^4	6.2×10^4
APPROXIMATIVE MEAN	23	7.2×10^3	1.4×10^4	1.2×10^5	1.3×10^5
CO-CULTIVATED CULTURES (CONTROLS)	3	1.4×10^1	1.7×10^2	9.8×10^2	1.1×10^3

(1) Cl_2TSV_5 tumor cell cultures used for these fusion experiments were from the 6th "in vitro" passage.

tivated control cultures as well as cells of each type (treated and non-treated by UV-SeV) were also prepared. Medium harvested from these cultures after 24 h and 48 h was treated as if it was interferon (see "Material and Methods") and applied to cultures of AGMK, BSC and hamster kidney (HK) primary cells. Twenty hours later, these cultures were rinsed twice with MEM and challenged with VSV or SV40. Thus, it was found that all fluids from cultures containing AGMK or BSC cells treated with UV-SeV showed anti-viral activity and both viruses appeared to be equally inhibited. UV-SeV-treated mixed cultures containing AGMK cells produced an interfering substance which produced 100% inhibition of 50 TCD_{50} of the challenging virus when the culture fluid was diluted 1/16. Fluid from AGMK control cultures treated with UV-SeV showed the same degree of interfering capacity at 1/64 dilution. Samples harvested at 24 h and 48 h did not show a significant difference in interferon titer when tested on AGMK cells. Culture fluid also from UV-SeV-treated BSC cultures did exhibit interferon activity. This activity was about 25% less than that of AGMK cultures under the same conditions. Culture fluid of UV-SeV-treated BSC cell cultures harvested after 48 h appeared to show the same interfering capacity as samples taken at 24 h, when tested on AGMK cells.

For interferon assay, AGMK cells proved to be more sensitive (about 25%) than the BSC cells to the interferon preparation from the same source (either AGMK or BSC). Both cell cultures (AGMK and BSC) appeared to show the same sensitivity to SV40 and VSV.

Both Cl_2TSV_5 and RHaT cultures used for these studies were derived from the tumors at the 1st and 4th "in vivo" passage levels, respectively. Culture fluid from UV-SeV-treated Cl_2TSV_5 and RHaT cultures was assayed on HK cells which were then challenged with USV. From this experiment, it appeared that they did not produce interferon or, if produced it was not present in the culture fluid at a detectable concentration. Another possible explanation might be that the HK cells used in the experiment did not constitute the best system for the detection of small amounts of interferon. However, interferon was detected in hamster kidney cell cultures treated with UV-SeV. This interferon produced a 100% inhibition of 50 TCD_{50} of VSV on HK cells when diluted 1/16. It did not, however, protect AGMK cells against the same virus (VSV). Treatment of HK cells with an undiluted interferon preparation from AGMK or BSC cultures did not protect those hamster cells against VSV infection. In contrast, AGMK and BSC cells treated with this same preparation were protected against VSV or SV40 infection. The medium of the UV-SeV-untreated cultures (controls) did not show any interferon activity.

Thus, the species specificity, stability at pH 2.0, lack of toxicity and the lack of virus specificity of the anti-viral substance present in the tested media indicated that this substance has the characteristics of interferon (Lockart, 1966). For this reason, all cultures treated with UV-SeV for virus rescue were thoroughly rinsed 24 h after fusion.

Rescue of SV40 from tumor cell cultures

SV40 virions were rescued from all cultures of Cl_2TSV_5 and RHaT primary tumors at all "in vivo" passage levels (Table IV). Cell fusion was 100% successful in inducing SV40 in these tumor cells (Fig.25-28). Virus could, generally, be rescued also by co-cultivating tumor cells with BSC cells. In the latter case, in 29 different cases of tumor cultures studied, only 3 did not produce infectious SV40 by co-cultivation (2 from Cl_2TSV_5 and 1 from RHaT produced tumors at 3^{rd}, 5^{th} and 3^{rd} "in vivo" passage levels, respectively). It is also worth noting here that in one of those cases, a culture was derived from a Cl_2TSV_5 produced tumor (at 5^{th} "in vivo" passage level) in a hamster without metastasis. This culture contained about 5% small polykaryocytes in the cell population of 1^{st} "in vitro" passage and fused poorly with BSC cells (Fig.29) when treated with UV-SeV (7% of heterokaryons). However, SV40 was rescued from this culture by cell fusion.

It is important to mention here that a cell culture derived from a Cl_2TSV_5 produced tumor, at the 1^{st} "in vivo" passage level, was maintained in this laboratory for many generations. It had been kept frozen (at -80°C in MEM containing 10% FCS and 10% glycerol) for periods averaging 2 months, between the 9^{th} and 10^{th} passages, 19^{th} and 20^{th} passages, and between the 36^{th} and 37^{th} passages. This culture was tested from time to time for SV40 rescue and virus was induced every time by cell fusion. In contrast, similar results were not obtained by cell co-cultivation. Thus, virus was not detected when these Cl_2TSV_5 cells at the 9^{th}, 13^{th} and 19^{th} subculture levels were co-cultivated with those of the BSC line. However,

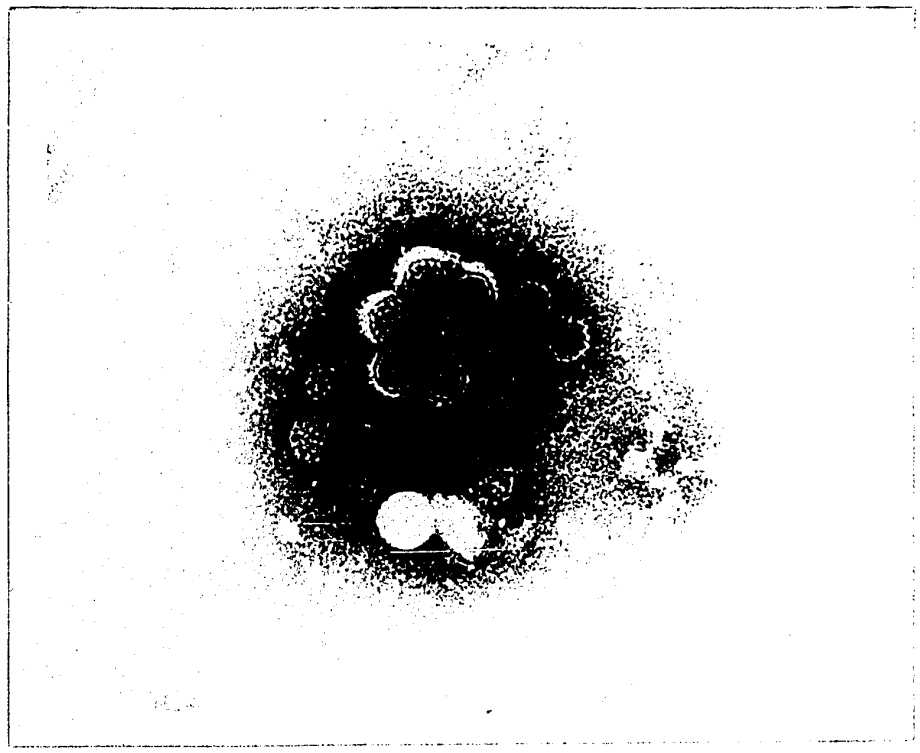


Fig.25

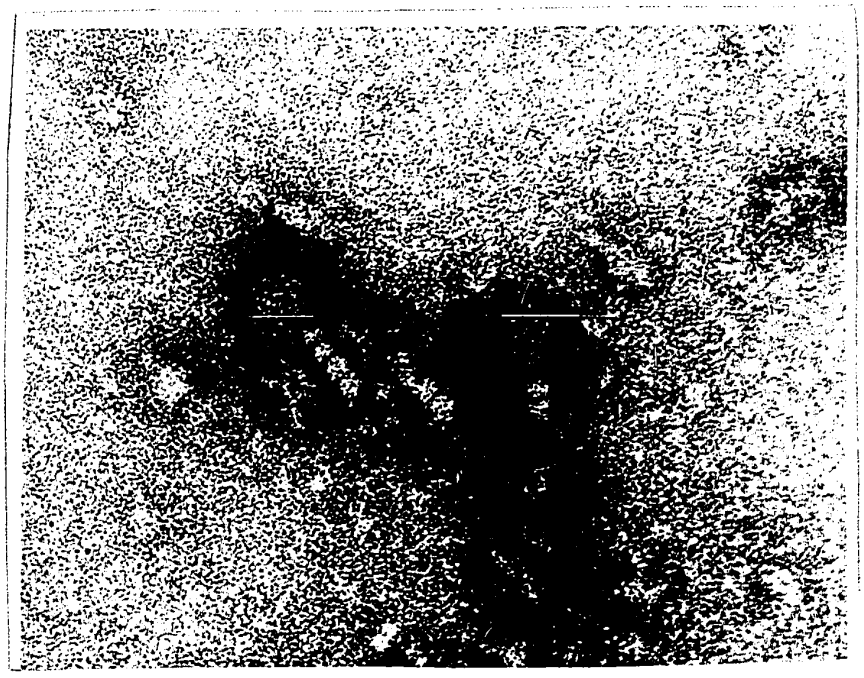


Fig.26

Fig. 25 Typical SV40 virions rescued from a Cl_2TSV_5-produced tumor at the 5th "in vivo" passage level. x 155.000.

Fig. 26 SV40 particles recovered from a Cl_2TSV_5-produced tumor cell culture. Oval structures similar to those described in Fig.3 are also present. x 230.000.

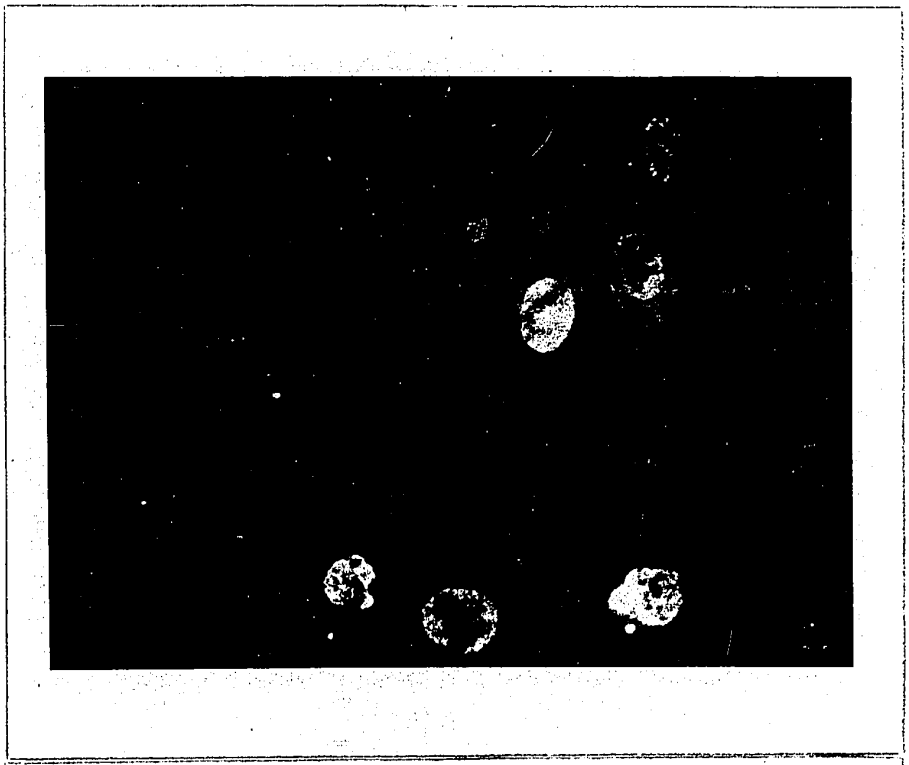


Fig.27

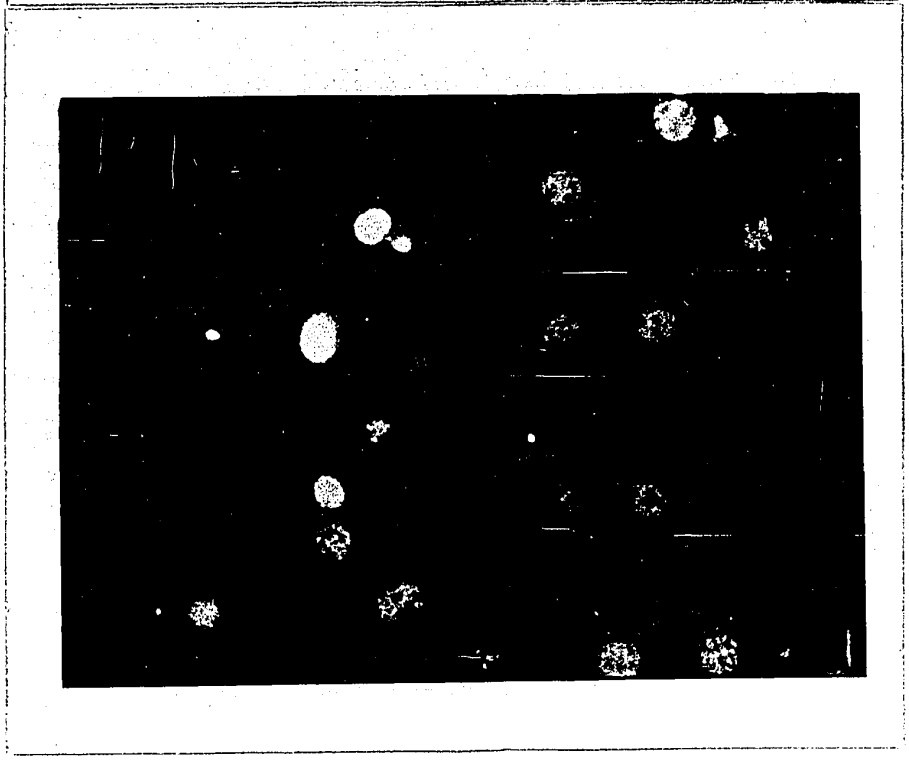


Fig.28

Fig. 27-28 FA staining of T-antigen in RHaT cells at two different "in vivo" passage levels. Note the pleomorphism of nucleoli in the nuclei. Fig. 27: x 2.200; Fig. 28: x 1.800.

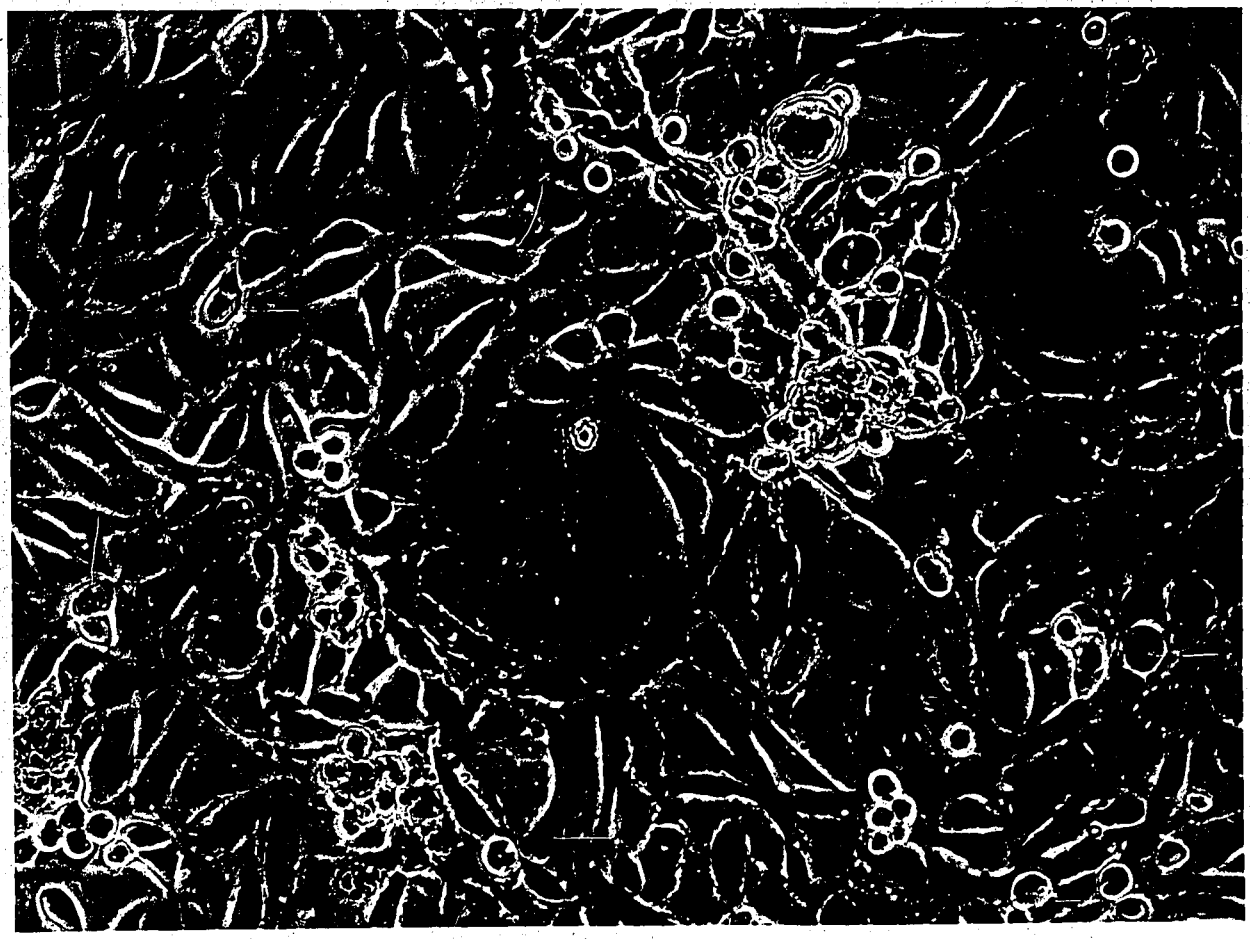


Fig. 29 Culture of BSC and Cl_2TSV_5-produced tumor cells (at 5th "in vivo" passage level), 4 days after fusion. Only one heterokaryocyte appears to be present in the field. A few binuclear homokaryocytes can also be recognized. Phase-contrast. x 1,500.

SV40 was rescued from those cells by simple co-cultivation at the 3rd, 33rd and 36th subculture levels. This observation suggests that the SV40-transformed cells might undergo eventual physiological changes, during some period(s) of their life, under the influence of environmental or other factors.

At no time was spontaneous production of virus detected in Cl_2TSV_5 or RHaT produced tumor cultures.

SV40 non-productive clones were also derived from different tumor cultures (of Cl_2TSV_5 or RHaT origin). Nineteen clones, cultivated for 3-8 passages, were tested for SV40 rescue by cell co-cultivation. It was found that only 4 of them did not produce virus by co-cultivation. Cell fusion was able to rescue SV40 in only one of these 4 clones. All the cell cultures which do produce SV40 by simple cell co-cultivation always yield virus by cell fusion. Therefore, cell fusion became unnecessary for those cell clones yielding SV40 merely by co-cultivation.

Cells of the CH_4 line did not yield virus when fused with BSC cells and were therefore not used for animal inoculation. SV40 was rescued from RLT cells by both methods, fusion and co-cultivation with BSC cells. SV40 could not be detected by fusing cell mixtures of the Cl_2TSV_5, RHaT, CH_4 and RLT lines, at different combinations.

The virus rescued from all the above cases was identified as SV40 by the following criteria: (1) type of CPE in BSC or AGMK cells; (2) morphology and size by electron microscopy; (3) FA staining for capside antigens; (4) neutralization by specific antiserum.

DISCUSSION

An increase in oncogenicity has been attributed to SV40-transformed cells after their passage "in vivo", and it was suggested that the familiar processes of mutation and selection are the basic mechanisms responsible for tumor progression (Enders and Diamandopoulos, 1969). The data presented in this work on the production of tumors in hamsters by SV40-transformed cells appear to bring further information on the matter. In fact, their increased potential to grow, invade locally, and to produce metastases indicates that an increase in the oncogenic potential of those cells takes place by their successive passages in the animal. In our view, the most important observation of all is that two other parameters - not yet described - parallel the above observation: (1) the increased number of polykaryocytes in tumor primary cell cultures, and (2) the increased capacity of cells for fusion. No mechanisms other than the cellular mutation and/or selection of cells of high oncogenicity appear to be responsible for the increased oncogenic potential of those tumor cells. It seems that the enhancement of oncogenic potential in less oncogenic cells depends on cellular changes that occur "in vivo" (Enders and Diamandopoulos, 1969).

The tendency of metastases to be localized at the lymph node level, particularly evident in cases of Cl_2TSV_5 tumors, is also a fact which deserves careful consideration. The observations described on the features of tumor cells suggest strongly that the malignant cells might have a natural tendency to fuse with other cells. Since lymph nodes, particularly the local ones, seem basically involved in the defence mechanism, the

selection of a highly malignant cell population may lead to an increased interaction between these tumor cells and the cellular elements responsible for anti-tumoral immunity. The present knowledge on the physiology of fused cells (Harris et al., 1966; Poste, 1970) shows the possibility of expressing (one or more) previously non-existent function(s) in a cell (by derepression or repression mechanisms?) after its fusion with another type of cell which is in an active state concerning the respective function(s). Thus, if fusion occurs spontaneously between a tumor cell and the lymphocyte (or plasma cell), the possibility of a malignant transformation of this last cell cannot be excluded. Since the nuclei of a fused cell may also fuse between themselves and since the malignant cells show an accelerated growth, the consequences of similar phenomena could be disastrous for animal (or human) health. The very same mechanism might also be responsible to a certain extent for the local growth of a tumor mass, if the malignant cells fuse with normal cells present "in situ". Experiments performed "in vitro" with hybrids of malignant and normal mouse cells proved that incubation at 37°C eliminates rapidly the hybrids from the cell mixture; in contrast, incubation at 28-29°C shows a rapid and continuous increase of the percentage of hybrid cells in the mixed culture (Scaletta and Ephrussi, 1965). For this reason, it might be worth while to undertake experimental studies on the role of (low) temperature in the growth of the tumor mass in the animal.

The tumors described had characteristics which are predominant in tumors produced by SV40-transformed cells (Igel and Black, 1967; Diamandopoulos, 1968; Diamandopoulos et al., 1969; Easton et al., 1970). The

lymphosarcomatous spectrum of most metastases could be attributed mainly to the direct involvement of lymph nodes. Areas of undifferentiated carcinoma, adenocarcinoma or epidermoid carcinoma, as found in tumors produced by SV40-transformed hamster cells of different types (Rabson and Kirschstein, 1962; Diamandopoulos and Dalton-Tucker, 1969) were absent in the tumor sections examined. However, it also appears that these features are not always present (Black and Rowe, 1963). The pleomorphic features of these tumors can be easily explained if one considers that different histological types of tumors, regardless of original cell type, can be produced by SV40-transformed cells (Diamandopoulos and Dalton-Tucker, 1969), and that a single clone may give rise to a whole histological spectrum of sarcomas, irrespective of whether its oncogenic potential is high or relatively low (Diamandopoulos, 1968).

Our results on the increased percentage of polykaryocytes in tumor primary cell cultures after successive "in vitro" passages, suggest that the observation stating that no correlation can be made between "in vitro" cytological morphology and the degree of oncogenic potential (Diamandopoulos, 1968) may be a little too broad or imprecise. In our view, this attempt to correlate between "in vitro" cytological morphology and oncogenicity, although very interesting, may only be valid if one does not take into consideration the polykaryocytes in the tumor primary cell culture.

The data on the spontaneously occurring polykaryocytes in tumor primary cultures, show that these large multinucleate cells cannot multiply under the laboratory conditions described. It appears that these polykaryo-

cytes are the result of fusion of previously separate cells because: (1) their cytoplasm is relatively large in size, (2) attempts to isolate or detect any virus in cultures containing those polykaryocytes have been unsuccessful, and (3) no fusion factor can be detected in the culture fluids or cell extracts. The mechanism of such fusion is unknown. They seem to represent an unorganized and undifferentiated multinucleate form of unicellular life in which the different regulatory mechanisms responsible for cellular activities may be deffective or incompatible with each other. If those cells occur spontaneously in increasing percentages in highly malignant tumors it might then be speculated that the highest degree of malignancy is not only incompatible with the animal life, but may also stop any indefinite perpetuation of this highly malignant tumoral state because of the non-reproducibility of these increasingly predominant polykaryocytes after successive "in vivo" passages.

Recently, an overgrowth stimulating factor has been described, first from the Rous sarcoma cell cultures and later in density-inhibited chick embryo cells (Rubin, 1970 a,b). It was then suggested that density-inhibited cells contain material in a sequestered location which, when released, stimulate rapid growth in similarly inhibited cultures (Rubin, 1970 b). It appears that a similar overgrowth stimulating factor is also produced by Cl_2TSV_5 tumor cells. Our observations also indicate that Cl_2TSV_5 culture fluids show another activity which results in prolonging the survival time of cell monolayers such as those of BSC line. Further investigation is required to determine the precise nature of the substance(s) responsible for such cell stimulating activities.

Results of experiments performed to determine the most suitable concentration of UV-SeV for cell fusion in hamster-monkey system proved that the use of high concentration (over 7.000 HAU) of UV-SeV results in a marked cytotoxic effect of virus on cells. Similar cytotoxicity of virus preparation on cells has also been reported by other authors (Harris and Watkins, 1965). Since the best results are obtained by using 3,000-4,000 HAU of UV-SeV, it appears evident that concentrations over 4,000 HAU of Sendai virus should not be used for cell fusion. This observation is in agreement with Okada's results (in Svoboda et al., 1968, p. 319). Our data also suggests that the fusion factor is relatively resistant to UV, and it appears that the UV treatment is still the method of choice for inactivation of Sendai virus because of its simplicity and reliability. Since heterokaryocytes, like other polykaryocytes, are relatively sensitive to centrifugation after the cell fusion procedure, our experiments suggest that a relatively low speed (i.e. not over 170 g/10 min) must be used to recover the cells from the UV-SeV-treated mixture.

The present study suggests that little or no interferon is produced in UV-SeV-treated monkey cell cultures after 24 h. Therefore, it seems very important to rinse these cultures 24 h after cell fusion to discard the interferon present in the culture fluid. Thus, the recovery of virus from UV-SeV treated cultures might not be significantly affected especially when a moderate or low concentration of cells is used in the inoculum for these cultures. It appears that in most cases, cellular resistance to virus declines within a relatively short period after the removal

of the interferon inducer (Buckler et al., 1968). It is interesting to note that SV40 appears to be as sensitive as VSV to monkey interferon system, which confirms the observation of other workers (Takemoto et al., 1968).

The absence of detectable interferon in hamster tumor cell cultures suggests that the SV40 genome present in those cells might interfere with the mechanism responsible for interferon synthesis. It has been suggested that decreased interferon production is an early consequence of infection by oncogenic viruses, and a property which persists in many lines of transformed cells (Brailovsky et al., 1969). It would be worth while to see whether a correlation does or does not exist between decreased interferon production and the degree of oncogenicity of a transformed cell population.

When cells of two lines grown in different media are fused together, it becomes difficult to determine the ideal composition of the medium in which to cultivate this mixed cell population. Our studies suggest that this problem of medium selection may be at least partially overcome by using a ratio mixture (50 : 50), particularly in the cell systems used in this work. Another solution to this problem, as determined during some preliminary experiments, consists of decreasing the concentration of transformed cells, i.e. increasing the ratio (permissive cells/transformed cells) in favour of non-transformed cells used for virus rescue. In any case, it seems important to determine the most suitable growth conditions for each system, after taking into consideration the purpose of the experiment.

Studies on SV40-production by heterokaryocytes strongly suggest that the large polykaryocytes (with over 20 nuclei) are very poor producers of SV40. This observation may be easily explained by the fact that they have a short life time (about 3 days). It has been shown by other workers (Gerber, 1966; Watkins and Dulbecco, 1967; Takemoto et al., 1968) that comparatively larger amounts of virus can be recovered by cell fusion than by co-cultivation. Our results on the yield of SV40, by using transformed cells different from those used by the above authors, support their observations. Also, the titer of SV40 after a 10-day-period is comparatively lower in the co-cultivated cultures than it is in the fused cultures. Similar results have also been reported by others (Takemoto et al., 1968). Moreover, as suggested by cytological and FA studies, the SV40 genome might start replicating in the productive heterokaryocyte soon after cell fusion.

From the data from the fusion experiments with different tumor cell cultures, it can be concluded that the two main factors responsible for cell fusion are: (1) the fusion factor present in Sendai virus preparation and (2) the capacity of a cell to fuse with another. As reported by other workers (Okada and Tadokoro, 1963; Kohn, 1965; Steplewski and Koprowski, 1970; Koprowska, in Svoboda et al., 1968, p.320) and from our own observations, it becomes evident that tumor cells naturally show a more increased capacity to fuse with normal cells as well as among themselves than do normal cells under the same conditions. It has also been reported "that cells undergoing mitotic

divisions indent cell membranes of, and possibly fuse with, multinucleated syncytia" (Koprowska, in Svoboda et al., 1968, p.320). Since tumor cells exhibit a relatively higher mitotic activity than the normal cells, it thus appears that the capacity of the cells to fuse might be correlated with their mitotic capacity. Further research could be carried out on this important matter. However, experiments performed during the present work using UV-SeV from the same preparation and BSC cells of the same culture to fuse with cells of different tumors gave different results,(Fig.17 and 29). Since the BSC cells and UV-SeV were from the same source and the experiments were performed under the same conditions, it might be suggested that the percentage of fusion predominantly depends on the type of cells, and that between two cell types in a mixture only one may determine the number of fusions.

It is very important to note that the SV40 genome remains inductible in tumor cells even after several "in vivo" passages. It would, however, be interesting to determine whether its transforming efficiency is the same as it was at the initial cell transformation level.

Evidence for a protein repressor in SV40-transformed cells has been described (Cassingena and Tournier, 1968 b; Cassingena et al, 1969 a, c). The fact that during our experiments, virus could not be rescued by fusing different SV40 transformed cell cultures between themselves appears to support the presence of a repressor-like substance(s) which may block late viral functions.

Finally, from the observations presented in this work on the rescue of SV40, it may be concluded that:

(a) All SV40-transformed cells which produce virus simply by co-cultivation always produce virus by fusion with SV40-permissive cells.

(b) In other transformed cells, the SV40 genome can be reactivated only by fusion with permissive cells.

(c) The viral genome cannot be reactivated by cell fusion from some SV40-transformed cultures.

(d) Malignant clones, non-inductible by cell fusion, can be derived from SV40-transformed cells or tumor cell cultures which do yield SV40 by either one or both of the above methods.

(e) Inductibility of SV40 by cell co-cultivation is a feature which may not be detectable in a cell culture, for unknown reasons, during period(s) of its life time ("in vitro").

RESUME

LES PROPRIETES PRINCIPALES DES GENERATIONS SUCCESSIVES DE TUMEURS PRODUITES PAR LES CELLULES Cl_2TSV_5 ET RHaT ET LEURS CULTURES CELLULAIRES, ET L'INDUCTION DU SV40.

Ce chapitre décrit une étude portant sur les générations successives des tumeurs produites par les cellules transformées par le SV40 (Cl_2TSV_5 et RHaT) et l'induction du virus dans les cellules tumorales par la fusion cellulaire. On y décrit les différentes propriétés biologiques des cellules tumorales ainsi que celles des cultures de cellules fusionnées. Dans cette étude on examine également quelques facteurs qui influencent la fusion cellulaire et l'induction virale. On y discute aussi de la corrélation existant entre le nombre de cellules multinucléaires dans les cultures primaires des tumeurs et la capacité de ces cellules pour fusionner, d'une part, et l'incidence des métastases d'autre part.

"Si la joie est dans le succès
la vertu est dans l'effort".

Louis PASTEUR

IV. GENERAL DISCUSSION AND CONCLUSIONS

The cell fusion procedure, a most recent biological tool, has provided a totally new approach for the examination of many aspects of cell functions, cytogenetics and biochemistry which previously was not experimentally possible in animal cells. The induction of virus replication in fused cells, by using the inactivated Sendai virus to impose a form of artificial sexuality on these animal cells, is among the most recent and most fascinating observation in tumor biology.

The cell fusion technique has been the main tool used during the present work to demonstrate the rescue of SV40 genome from transformed cells of hamster and rabbit origin. We have shown that not only the permissive cells can be used for virus induction but also the naturally insusceptible cells can be used for the rescue of SV40. Whether this observation can be generalized or not is a matter which may only become clear with further experiments in other inductible virus-transformed cell systems. The possibility that normal cells of some lines or species might have repressor-like substances which block the late viral functions necessary for production of infectious virus (SV40 or other viruses) is also a question which should be answered in the near future. However, the fact that virus progeny can be obtained by infecting so-called non-permissive cells with the nucleic acid of many DNA or RNA viruses suggests that such repressor mechanisms, if present, are not very frequent. This observation challenges the validity of the concept of permissiveness so frequently expressed by many virologists.

Since virus or viruses may also be responsible for at least some human tumors, it is too premature to exclude the possibility of rescuing

these oncogenic viruses by cell association techniques. During such experiments, some DNA or RNA viruses with well known markers could also be added to the cell mixture because this might well lead to the rescue of another virus by complementation or helper-type action, as has recently been shown with other viruses.

In this work, evidence has also been presented on the use of electron microscopy for the rapid detection of SV40 rescued by cell association techniques. Due to the reasons given above, it follows that electron microscopy could be a very useful tool for virus detection in cultures resulting from fusion of different normal cells with cells of patients with diseases, in which viral etiology is suspected. An experimental approach is proposed for further studies.

In the preceding chapter we have presented evidence concerning the correlation between the increased percentage of polykaryocytes in the tumor primary cell cultures and the increased capacity of cells for fusion on the one side, and the production of metastases on the other. This correlation was until now unknown in tumor biology.

Our data also suggest that the polykaryocytes from tumor cell cultures cannot multiply and that they are particularly affected by most experimental procedures. They appear to perish after (low-speed) centrifugation, freezing procedure for routine cell preservation and even trypsination. Therefore, they represent an extremely fragile type of cell which makes difficult most biological studies with them.

Some preliminary experiments performed during our studies suggested that an overgrowth stimulating-like factor is produced in SV40-transformed cell cultures. Further research is required to determine the precise nature of the substance(s) responsible of such cell stimulating activities. A possibility that similar factors might generally be produced by tumor cells cannot be neglected at the present time.

An attempt was also made to obtain further data on the fused cell cultures as well as on some factors which may affect virus rescue. The observations thus described showed that the growth medium is an important factor influencing the outcome of fused cell cultures and that interferon is produced in cultures containing normal cells which were treated with Sendai virus. Since it appears that interferon is produced under those culture conditions only for a short period of time, it is suggested that rinsing of the cell sheet and changing the medium 24 h after fusion might give better results with virus rescue procedures.

Different aspects of the inductibility of SV40 genome from the transformed cells are also defined through our own observations along with the present knowledge of this subject.

Moreover, a theory is also proposed to explain, in part, the local growth of the tumor as well as the occurrence of metastases, particularly those at the lymph node level.

In conclusion, in the two preceding studies we have attempted to give a general view of the main biological problems concerning the virogenesis and some other biological features of the SV40-transformed cells and the tumors produced in hamsters by these cells. An experimental approach which may confirm the suspected viral etiology of several pathological conditions, is also proposed.

V. APPENDIX

EVIDENCE OF SV40 HELPER ACTION FOR A PARVOVIRUS

EVIDENCE OF SV40 HELPER ACTION FOR A PARVOVIRUS

Small parvovirus-like particles were first detected by electron microscopy in association with cells of SV40-transformed culture of hamster origin (RHa3-T) (Menezes and Perry, 1970). This culture produced SV40 spontaneously. Identical virus particles were also found in a stock of SV40 virions. Further investigations proved that this small virus multiplies in BSC cells in the presence of SV40 virions only. When the SV40 preparation containing the small virus was treated with rabbit anti-SV40 serum and used to infect BSC, virus production was not obtained. Attempts to produce virus in a SV4o-transformed cell culture (CH_4, which is SV40-non-productive) as well as in BHK-21 cells were unsuccessful. However, the virus multiplied continuously in RHa3-T cultures. In these cells the peak of the virus titer was reached in 8-10 days with a marked CPE in the last two days. This CPE consisted of rounding of the cells, which then detached from the flask's wall. This small virus multiplies in 8-10-day-old hamster embryo primary cells. Hamster embryo cultures after 4 passages (in vitro) were completely resistant to virus infection. However, cell cultures of the same origin (hamster embryos) which were transformed by SV40 during the first passage were found to be a good source for multiplication of this small virus. These cells were obviously in a carrier state for SV40 during those passages (6^{th}, 9^{th}, 12^{th}, 15^{th}) when they were infected by that small virus. The lack of virus production in the above cases was ascertained by FA staining technique (indirect method, using hamster anti-viral serum and rabbit anti-hamster

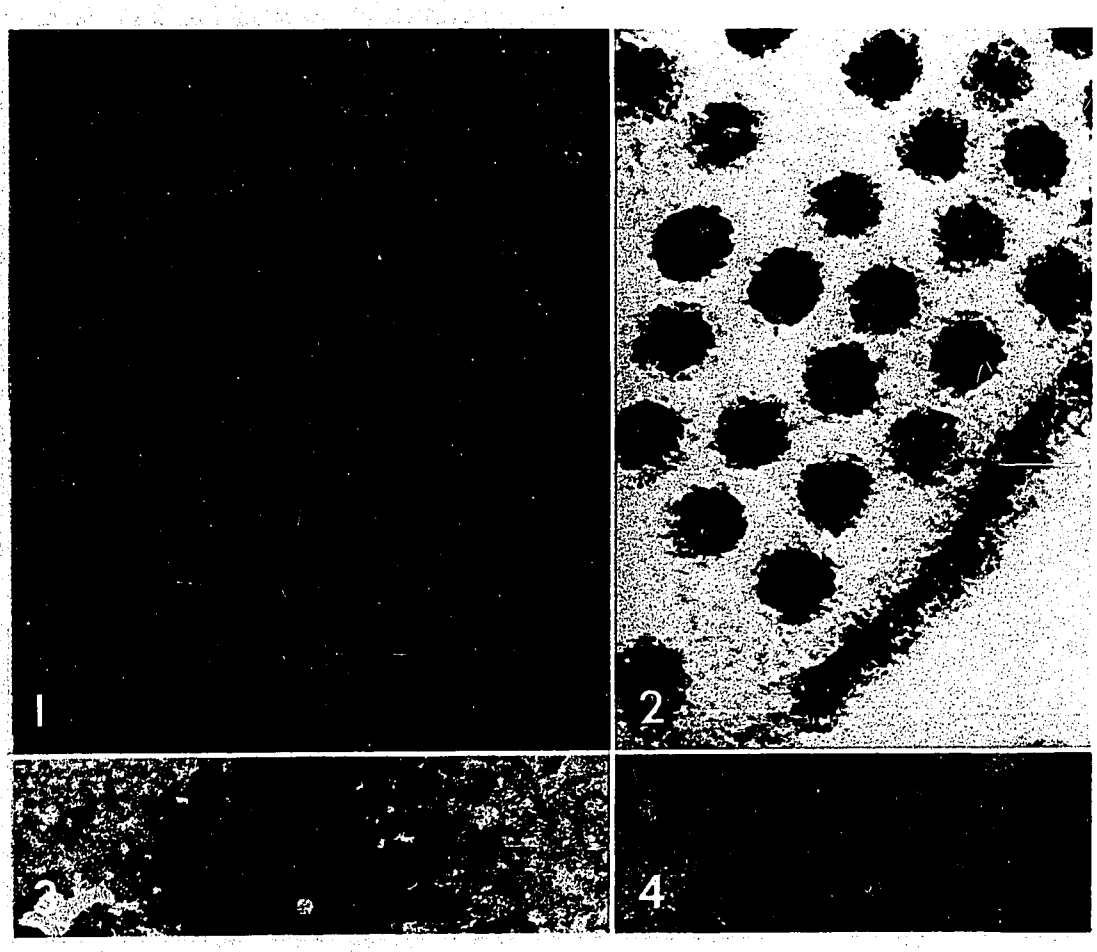
globulin conjugated with fluorescein-isothiocyanate), electron microscopy and hemagglutination technique.

This small virus has a hemagglutinating pattern identical to that of H-1 virus (Toolan, 1968). It agglutinated red blood cells of guinea pig, hamster, man, rat, horse and goose. It did not agglutinate erythrocytes of rabbit, sheep and chicken. The virus gave highest hemagglutination titers when the mixture was incubated at 22°C rather than at 4°C or 37°C.

The small virions have a diameter of approximately 22 nm and appear to show 32 capsomeres (Plate I). The purified preparation (Black et al., 1964) was used for acridine orange staining (Menezes, 1971) and serological studies. It was thus observed that this small virus is a single-stranded DNA virus, whose hemagglutinating activity can be inhibited by anti-H-1 serum (samples obtained from Dr. H.W. Toolan, Putnam Memorial Hospital Institute for Medical Research, Bennington, Vermont, and from Microbiological Associates, Inc.). Its replication was inhibited by the presence of 5-iodo-2'-deoxyuridine (Sigma Chemical Co.) in the culture medium. This supports our observations made after acridine orange staining of this small virus. Antigenic relation was not found between SV40 and these small virions.

Newborn hamsters inoculated subcutaneously or intraperitoneally within 3 days of birth, with 1-4 HAU of virus generally died within 8 days. At necropsy all of them showed an acute enteritis generally localized to colon. Histological examination of sections of the intestine

PLATE I



(1): Virus particles surrounded by a membrane-like structure, obtained by infecting RHa3-T cells. x 200.000. (2): Photographic reversal of some particles shown in the preceding micrograph. x 400.000. (3): SV40 virions and two small virus particles obtained by infecting BSC cells. x 100.000. (4): Parvovirus particles obtained by infecting a hamster embryo cell culture. x 165.000.

did not show any inclusions. The few animals, inoculated within 3 days of birth, which survived showed mongoloid-like deformities, the absence of teeth development being the predominant feature (Fig.30). A few of the animals aged from 3-7 days which were inoculated with this virus showed deformities which were predominantly evident at teeth level. These animals if nourished with powdered food developed a normal stature. When these animals were inbred it was found that mongoloid features were absent in all hamsters of next generation. Therefore, it appears that the mongoloid-like deformities produced by the small virus are not a hereditary feature.

From the data given above it might well be concluded that SV40 acts as a helper for the above virus whose morphological and biological features are identical to those attributed to H-1 virus (Toolan, 1968; Karasaki, 1966). It may then be said that this small virus is a parvovirus and that SV40 acts as a helper for this parvovirus, at least in BSC and some hamster cell cultures.

Fig. 30. Ten-week-old teethless hamster which had been injected with the parvovirus 40 h after birth.

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