STUDIES ON MOUSE EMBRYO CULTURES INFECTED WITH POLYOMA VIRUS

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ABSTRACT

After exposure of mouse embryo cultures to high concentrations of Py, a variable fraction of the cell population is converted to virus producers, but a fraction also survives and proliferates. The surviving fraction can be 20% of the population at input virus:cell ratios of 500 pfu/cell. Resistance to the cytocidal action of the virus in mouse embryo cultures is due neither to interferon nor to genetically resistant cells; it appears to be due to a transient physiological state of the cells.

No transformed cells have been found among the cells surviving a brief exposure to high concentrations of virus. Cultures derived from these cells by growth in antiviral medium resemble uninfected cultures in cell morphology, growth pattern, and sensitivity to reinfection. Transformed cells arise only in cultures which are exposed to Py over a period of two to five weeks. It has been shown that clonal cultures respond in the same way to Py infection as do uncloned mouse embryo cultures; thus, transformation does not result from the infection of rare "transformable variants" preexisting in the cell population.

Changes similar to the transformation which takes place in infected mouse embryo cultures also occur , and rapidly, in uninfected cultures. The occurrence of these changes complicates the analysis of Py induced transformation. It has been shown that "spontaneous" and

virus-induced transformation are two different phenomena, since transplantable cells arising in infected cultures differ antigenically from those arising in uninfected cultures. The relationship between alterations of cell lines observable in vitro and the ability of these lines to produce tumors upon implantation have been studied; definite correlations have been demonstrated between these properties. These facts have been discussed in the light of various theories of Py induced transformation.

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GENERAL INTRODUCTION

The study of the induction of tumors by viruses is a part of the much broader area of research aimed at understanding the initiation and maintenance of the neoplastic state of the cells. In both viral and non-viral tumors, the cells fail to respond to one or more of the growth regulating mechanisms which prevent unlimited and anarchic cell division in the normal organism. Tumors produced by viruses, a unique class among tumors, can be initiated and maintained in vitro, where they can be studied in a preferred way by using modern virological and tissue culture techniques. In the study of the neoplastic transformation of cells in vitro by tumor-producing viruses, two main model systems were developed: one is based on the ribonucleic acid (RNA)*-containing Rous sarcoma virus, the other on the deoxyribonucleic (DNA)-containing polyoma virus. A variety of cells were used in both cases. This thesis is concerned primarily with the effects of the polyoma virus on cultures of mouse embryo cells.

Perhaps the first question of tumor virology is: do virus-induced tumors arise as a result of a genetic interaction between the virus and a normal cell? The concept of virus-cell interactions as genetic interactions arises from the study of bacteriophage virology. Abundant evidence makes it clear that the process of phage multiplication can be regarded as the functioning

* See Glossary (p. 36) for a list of the abbreviations used in this thesis.

and replication of phage genes within an environment largely created and governed by the activities of host cell genes.[1] In the case of temperate bacteriophage, it is well-known that phage genes can interact with the bacterial genome in a recombinational as well as in a functional sense. For example, genetic determinants of temperate phage can become linked to the bacterial chromosome in the form of prophage,[2,3] and bacterial genes can become incorporated into the phage chromosome, thus giving rise to transducing phage.[4,5] In fact, temperate phages have been placed in the class of bacterial genetic elements known as episomes.[6] Episomes are able either to become integrated with the bacterial chromosome and replicate in strict synchrony with it, or alternatively, to multiply autonomously in the cytoplasm. In addition, they may be completely absent from the cell, in which case they can be acquired only from an external source. Temperate phages are episomes which have the genetic information necessary to specify the elaborate mechanism of extracellular genetic transfer known as infection. When a temperate phage exists in the integrated state, a regulatory gene of the phage synthesizes a substance, elaborated throughout the cell, which represses the functioning of the genes concerned with the production of the infectious virus particle.[7] It is this intracellular repressor which is held to be responsible both for the maintenance of the lysogenic state and for the resistance of lysogenic bacteria to superinfection with genetically related phage. Some phage-associated

genes, which have no obvious relation either to the integrated state or to the state of autonomous replication of the virus, function in both states. The changes in cells caused by the function of these genes are known as conversion; they include such phenomena as alteration of the cell wall of phage-infected Salmonella[8] and toxin production in the diptheria bacillus.[9]

In the light of these considerations, we may rephrase our question: are the cells of virus-induced tumors descended from cells within which virual genes have functioned? The alternative hypothesis is that tumor cells descend from cells which were affected indirectly -- for instance, by substances released from other infected cells. If the first alternative is correct, we are led naturally to further questions, such as these: Is the infecting virus genome lost from the neoplastically transformed cell after its genes have functioned, or is there some form of intra-cellular transmission from mother to daughter cell? Can viral genes become integrated with the cell genome in a manner analogous to the integration of temperate phage in lysogenic bacteria? Does the escape of the tumor cells from regulatory mechanisms require the continued presence and functioning of viral genes within the affected cells and their descendants?

The answers to some of these questions are probably different for different tumor viruses. A survey of the results obtained by using the two model systems of tumor virology will make this point clear. We shall begin with a brief review of the observations made with the

Rous sarcoma virus (RSV); polyoma virus (Py), the principal object of this study, will then be considered in more detail.

A. Summary of the Findings with RSV

RSV is a member of a class of viruses--the Avian leukosis group--whose members show serologic cross reactions.[10] RSV contains RNA, protein and lipid.[11, 12] It is a medium sized virus, with a diameter of approximately 70 mμ. Neither RSV, nor any other of the Avian leukosis viruses is known to undergo a 'lytic' cycle of multiplication on cultured fowl cells with rapid and large production of virus and consequent cell death.[13, 14] Virus is synthesized in the cytoplasm of RSV-infected cells and assembled at their surface;[15] it is released from the infected cells continuously rather than in a burst.[13] An infected cell retains the capacity to divide and produce progeny cells, all of which retain the capacity of releasing virus.[13] Recent evidence shows, however, that infectious RSV is released only when the cells are superinfected by another virus of the Avian leukosis complex.[16, 17] This phenomenon has suggested that RSV is defective and requires a helper virus to produce active progeny.[17]

Colonies formed by RSV infected cells can be distinguished in a background of uninfected cells by virtue of their altered morphology and growth characteristics.[18] These altered cells are known as "transformed" cells. Transformation occurs in the absence of

helper virus.[17] The fraction of transformed cells in a population of susceptible cells is directly proportional to the virus input. This observation has led to a convenient in vitro assay for the biological activity of the virus. The study of transformation by this method has had two important conclusions: (1) the majority, if not all, of the cells of cultures infected by high concentrations of virus can become transformed;[19] and (2) different virus mutants induce recognizably different morphological alterations of the infected cells.[20] All of these findings strongly indicate that RSV has a direct and continuing role in the transformation of cultured cells.

B. The Findings with Polyoma Virus

1. Experiments in the animal

Py is a member of the Papova tumor virus group which includes the simian virus 40 (SV_{40}), the rabbit papilloma virus and the human warts virus.[21] These viruses are similar in size--about 45 mμ in diameter--, in composition--DNA and protein--, and in the symmetry of the protein shell or capsid--icosahedral with a similar number of morphological subunits. All of these viruses produce tumors in susceptible hosts.

Other interesting properties of some of these viruses will now be summarized. The relative DNA content of Py is 13% (corresponding to a molecular weight of 5×10^6).[22] Infectious DNA can be extracted from preparations of Py and papilloma.[23,24] The virus capsid of Py

can adsorb to and agglutinate suspensions of the erythrocytes of certain species.[25] This property, known as hemagglutination, affords an extremely useful method of assaying virus concentration. Apparently the other viruses of the Papova group do not share this property. The site of replication of Py, SV_{40} and papilloma in cytocidal infections is the cell nucleus, as shown by fluorescent antibody staining and electron microscopy.[26,27]

Py was originally isolated from leukemic tissues of mice.[28] Apparently, the virus existed as a contaminant in these tissues, because it was subsequently shown to have no leukemogenic activity.[29] However, when injected into susceptible animals (rodents), the virus is able to induce a wide variety of solid tumors. These tumors are frequently localized and non-invasive, although occasional invasive and metastasizing growths are produced. The types of tumors found depend upon the dose of virus and the species and strain of animal injected.[30] Probably no tissue in mice and hamsters, at least, is completely immune, although the parotid gland in some strains of mice,[31] and the kidney in hamsters, are especially susceptible to tumor induction.[32]

Py multiplies extensively and rapidly with accompanying degenerative changes when it is injected into baby mice and baby hamsters.[33,34] The kidneys of infected hamsters illustrate especially clearly the effects of the virus: profound degenerative

changes and a rapid neoplastic response both occur.[35] In fact, discrete microscopic foci of neoplastic growth can be found in the kidneys as early as one week after injection of virus.[36] The number of these foci is approximately proportional to the virus dose, a fact which suggests that one particle is sufficient to induce a neoplastic change. This, in turn, suggests that the virus has a direct and immediate role in tumor induction.

Mice, and to a lesser extent, hamsters, develop, with age, a very strong immunity to the carcinogenic effects of Py.[37] It is known that the rapid immunological response of adult mice to Py reduces virus proliferation in the tissues of older animals, and, in addition, that X-irradiation allows the production of tumors in adult mice.[38] The effect of the irradiation is probably to suppress the immune response. These facts suggest that the immunity of adult mice to Py carcinogenesis may be due to their greater immunological competence.

In spite of the increased reaction, Py does multiply to a certain extent in adult mice: minute quantities of virus, when injected, can proliferate and thereby induce the synthesis of large quantities of antibody. On this fact is based a very sensitive end-point method of assay for the biological activity of the virus--the mouse antibody production test.[39]

To sum up, the experiments in the animal show that the virus can affect cells in two ways--by causing either cell proliferation or cell destruction. The dose response of the production

of discrete foci of neoplastic growth in the kidneys of infected hamsters suggests that the virus has a direct and immediate role in the induction of neoplasia. However, the inability of the virus to induce tumors in adults suggests either that Py may be very inefficient in promoting neoplasia and/or that mice may have efficient means for suppressing such change once it has occurred; immunological mechanisms appear to participate in this suppression.

It seems clear, however, that conclusions about the nature of the cell-virus interaction leading to the neoplastic state can only be suggestive when drawn from experiments with intact animals. The immunological defenses of infected animals, their wide variety of target cells, and the unknown multiplicity of infection of each variety create difficulties for quantitative virology. Therefore, we shall turn to experiments with tissue culture systems which offer better opportunities for these studies.

2. Experiments in tissue culture

The immediate consequence of Py virus in cultures of mouse cells is to cause cell destruction (called cytopathic effect [CPE]).[40] The virus multiplies extensively in these cultures; their fluids have high hemagglutinating titers and high infectious titers, whether determined by production of tumors in the animals or of CPE in tissue

cultures.[40] The CPE is utilized in a plaque assay of the virus,[41,42] a method of bioassay which is as sensitive as, but more accurate than, the mouse antibody production test.[43]

Several workers have described the transformation of cultures of mouse, hamster and rat cells by infection with Py.[44,45,46] Before this phenomenon is discussed in more detail, however, the more general problem of how neoplasia can be defined and detected in vitro will be considered.

a) Definition and detection of neoplasia in vitro. The only direct test for neoplasia in cultured cells is the ability of the cells in question to give rise to a tumor upon implantation into an intact animal of the same histocompatibility genotype. For various reasons, some of which will be discussed more fully in a later section, this test is often inconclusive, and it is always inconvenient. Therefore, other indirect tests will also be used in this work to determine the state of the cells.

It will be recalled that cells respond to RSV infection by a transformation of cell morphology and of growth pattern.* Both are qualities which can be determined visually in living cultures with the microscope. Cell morphology refers to the shape of the cell and to its refractility. The growth pattern of the cells refers to their tendency to grow either as a monolayer (regulated growth[47]) or as a multilayered mat (non-regulated growth) on the surface of a petri dish.

* For a more complete definition of these terms see Glossary (p. 36).

Cells showing regulated growth grow on the substrate rather than on top of one another and, most important, stop dividing once they have exhausted the surface area of the substrate. Cells showing non-regulated growth, on the other hand, continue to divide actively when they reach confluency. It is this uncontrolled cell division which causes the formation of a multilayered mat.

In general, freshly explanted embryo cells show regulated growth. When these cells, as will be seen below, are infected with Py, they develop changes in cell morphology and a non-regulated growth pattern. This process will be called transformation and the resultant cells transformed cells.

It should be pointed out that transformed cells, as defined above, are operationally distinct from neoplastic cells, which are defined by their ability to produce tumors upon implantation. In fact, Py transformed hamster cells are neoplastic in the animal,[44] whereas transformed mouse cells are frequently unable to produce tumors when implanted.[48,49,50] The correlation of the morphology and growth pattern of mouse cells with their ability to produce tumors when implanted is one of the subjects of this thesis.

b) The transformation of cell cultures. A fraction of the cells in mouse embryo cultures do not degenerate after infection with a large dose of virus. A period of about four to eight weeks ensues, in which cell killing is approximately balanced by cell division, so that

there is very little or no net increase in cell number. This period is known as the steady state period. Virus production is abundant through the entire steady state period. Finally, a new type of cell appears and overgrows the culture, the transformed cell, with changed morphology and growth pattern. Coincident with this, the amount of CPE and virus production decreases.[44]

In comparison with mouse embryo cultures, Py-infected cultures of hamster and rat cells show much less cell degeneration and virus proliferation[44,45,46]-- in fact, rat cells have been reported to be incompetent to support the multiplication of the virus.[46] Flourescent antibody staining of hamster cells infected with high concentrations of virus indicates that a small proportion -- perhaps 1%-- does synthesize viral capsid antigen, and the amount of virus multiplication observed supports this conclusion.[51] Unlike infected mouse cultures, infected hamster and rat cultures can be subcultured, but they too are eventually overgrown with transformed cells. The delay between infection and overgrowth of the culture with transformed cells is about three to four weeks for hamster cultures.

The transformed cells which finally overgrow Py infected mouse and hamster cultures have been extensively characterized.[52,53] They tend to grow in interwoven, netlike arrays and form multi-layered mats when they come to confluency. Uninfected cells, on the other hand, grow in parallel bundles and do not continue to divide when the cell sheet covers the available area of the petri dish. Transformed cells

arising from infected hamster cultures are generally free of virus and are completely refractory to reinfection. Transformed mouse embryo cells, on the other hand, continue to release virus at about 1 to 10 per cent of the rate observed during the initial steady state period of degeneration. The analysis of the yields of single Py-infected transformed mouse cells shows that Py is released in bursts -- each infected cell releasing about one thousand plaque forming units (pfu) of virus. Although the proportion of infected cells in transformed mouse cultures (about 1 to 2 per cent) was not reduced by treatment with anti-viral antiserum, transformed, non-virus releasing cultures could be obtained by the expedient of picking a single cell from an infected culture and growing it into a clone. Neither the hamster nor the virus-free mouse transformed cultures could be induced to release virus by treatments known to be effective in inducing phage development in lysogenic bacteria, or by super-infection by a mutant of Py. In addition, it has thus far been impossible to extract infectious nucleic acid from such cultures or to demonstrate the presence of virus capsid antigens.

This situation is quite distinct from that of cells transformed by RSV. In the latter case, it has been shown that every transformed cell is capable of releasing virus when superinfected by a helper virus, and that this ability is transmitted to its progeny.

The finding that Py-transformed cells cannot be induced to produce virus shows that these cells do not behave like a population of

bacteria lysogenic for an inducible prophage. Nevertheless, it has been pointed out by Vogt and Dulbecco[53] that some or all of the genetic material of the virus may exist in the transformed cells in a firmly integrated (i.e., non-inducible) state. Some support for the idea that viral genetic material is present in transformed cells has come from experiments on the transplantability, in mice and hamsters, of Py-induced tumors and of cells transformed in vitro. These experiments indicate that there is a new and apparently virus specific antigen in these cells. Some of the work which has led to this conclusion will be reviewed in detail.

It has already been noted that adult mice and hamsters usually do not develop tumors when infected with Py. It has been found that these tumor-free, infected animals are more resistant to subsequent grafts of Py-induced tumors and of transformed cells than are uninfected animals.[54,55,56] This resistance is specific: Py-infected mice do not become more resistant than uninfected mice to transplants of isologous spontaneous or chemically induced tumors.[54] Nor do mice immunized with other tumor viruses acquire resistance to Py induced tumors. Such mice do, however, show resistance to grafts of tumors originally induced by the immunizing virus.[57,58,59]

It has been shown by Sjogren[60] that neither the resistance nor the new antigen depend on the presence of antiviral antibodies in the graft recipient or on infectious virus in the tumor cells. This was done by demonstrating that mice which had been immunized by homografts of a virus-free, Py-induced tumor were resistant to subsequent

isografts of other virus free Py-induced tumors. (Homografts are grafts between individuals of the same species; isografts, between individuals of the same inbred strain.) This experiment also provides direct evidence that different Py-induced tumors are antigenically cross-related. The Py specific antigen is present in most Py-induced tumors and is maintained even when the tumors are serially transferred in Py-immunized animals.[61] For all these reasons, it has been suggested that the event leading to the production of the new virus-specific antigen is intimately connected with the initiation and maintenance of the neoplastic state. Moreover, this event may be analogous to the phenomenon of conversion in lysogenic bacteria.[62]

What can be said about the role of the virus in the initiation of these apparently specific transformed cells? It has recently been shown that a small proportion of transformed cells appears very shortly after the infection of hamster cell cultures with Py.[63,64] These cells can be detected by the distinctive morphology and growth pattern of the colonies they form. Stoker and MacPherson, plating freshly isolated hamster cells after infection with about 24 plaque forming units of virus per cell, found that about 0.006% of the cells formed transformed colonies.[63] The number of transformed colonies was approximately proportional to the input multiplicity.

Vogt and Dulbecco, using colonial morphology and trans-

plantability as their criteria of transformation, did not find typical transformed colonies when they plated hamster cells immediately after infection.[65,66] Instead, it was possible to isolate "foci of persistent mitotic activity" which, unlike colonies from uninfected hamster embryo cultures, multiplied continuously. But they did not show in full degree the non-regulated growth which is characteristic of established transformed cultures, nor did they produce rapidly growing tumors when implanted into hamsters. As these "early" transformed lines were transferred, a great many abnormal mitoses and chromatid breaks were observed -- events which caused a high frequency of dead cells to be thrown off. Finally there arose typical established or "late" transformed cells which formed dense, piled-up colonies, which had a low frequency of chromatid breaks and of dead cells, and which were fully neoplastic in the animal. Since the infecting virus had been diluted out, reinfection could be excluded as the inducer of the late transformed cells. Vogt and Dulbecco conclude that the late transformed cells derived from the early transformed cells "by a secondary variational process" as a consequence of the original infection with Py.

It is not known whether the transformed cells observed by Stoker and Macpherson were similar to the early or to the late transformed cells of Vogt and Dulbecco.

Stoker and his co-workers have also studied the Py-induced transformation of a permanent tissue culture line of hamster cells.[67,68]

Infection of this line induces transformed cells which display fully neoplastic behavior with respect to transplantability and tissue culture characteristics. This "one-step" transformation need not be at variance with the two-step process proposed by Vogt and Dulbecco, since it is quite possible that the first step had occurred spontaneously during the period needed to produce a permanent line from freshly explanted tissue.[66] In addition, Stoker has recently reported that several cell divisions occur between infection of this line and the appearance in it of transformed cells.[69]

In conclusion, it can be seen that the events observed after infection of either mouse or hamster cells with Py contrasts with the situation in cultures of chicken embryo cells infected with RSV. In the latter case, there is an immediate morphological transformation of a large fraction of the cells, and in a matter of days, virtually the entire culture consists of these altered cells. We have noted that transformed Rous cells can transmit virus, or the ability to produce virus, directly to their progeny without external transmission. In addition, RSV transformed cells can release virus or can be induced to release virus. Finally, we may recall the intracytoplasmic site of synthesis of viral antigen and the slow trickle of progeny virus released from infected cells.

In the case of Py, there appears to be a complete distinction between cells which produce virus and transformed cells. The properties of the transformed cells which arise from infected cultures

are maintained in the absence of infectious virus. Nevertheless, the transformed cells do show evidence of a prior interaction with the virus in the form of a new and specific transplantation antigen. Finally, the relative inefficiency of Py in inducing neoplastic transformation should be pointed out: this is shown, on the one hand, by the very small proportion of cells in hamster cultures infected with very high multiplicities which form transformed colonies, and, on the other hand, by the extended period between infection and the overgrowth of transformed cells in infected mouse populations.

C. Plan of This Thesis

In Part I, experiments measuring the proportion of cells that yield virus and of cells that are killed in freshly infected mouse embryo cultures will be described. It will be shown that, even with very high inputs of virus, it is impossible to convert all of the cells into virus yielders or to kill all of the cells. With the aid of special antiviral media, the survivors of a brief exposure to virus will be examined in the absence of the complications caused by reinfection. In this way, it will be shown that transformed cells appear in the population only after a prolonged exposure of the culture to virus.

In Part II, a study of the response of clonal cultures to virus infection will be described. The main purpose of the study is to determine whether genetic heterogeneity in the culture plays a role in the delayed appearance of transformed cells. The properties of infected and uninfected clonal cultures will be examined with respect to their cell morphology,

their growth pattern, their ability to produce tumors upon implantation, and their antigenicity.

MATERIALS AND METHODS

A. Materials

1. Media

For routine culturing of cells and for plaque assays, Eagle's Medium[70] with a four-fold increase in the concentration of amino acids and containing 10% calf serum was used. Other modifications of Eagle's original formula were also used: they include an increase in the glucose concentration to 0.45% and in the bicarbonate concentration to 0.37%. The partial pressure of CO_2 in our incubators was adjusted to give a pH of 7.4 - 7.6 in the medium at this bicarbonate concentration. For cloning of cells, for routine maintenance of cloned lines and, in later work, for plaque assays, two media were used: One was CMRL-1066[71] with an $NaHCO_3$ concentration of 0.37% and with 12-14% calf serum added. The other (designated PEM) consisted of 42% N 16,[72] 42% modified Eagle's medium, 4.2% NCTC109,[73] and 12% calf serum. The base of PEM was Earle's balanced salt solution[74] (with the increased glucose and bicarbonate noted above) instead of Saline F.[75] For dispersal and subculture of cells, 0.05% trypsin dissolved in tris buffered saline[53] without divalent cations was used. Tris buffered saline (TBS) was used as a diluent for virus and to wash plates before virus infection.

2. Virus

Py was originally obtained from Dr. Rowe of the National

Institutes of Health. This strain of virus will be referred to as large plaque (lp) virus or, more frequently, simply as Py. In several experiments two other strains of virus were used: the small plaque (sp) mutant, isolated in this laboratory[52] and the P16 strain[68] which was sent to us by Professor M. Stoker of the Institute of Virology, Glasgow. The latter strain is a small plaque forming variant of the Toronto strain of Py.

Virus prepared by three different methods was used: (1) Tissue culture virus was the supernant medium of infected mouse embryo cultures. These stocks generally contained 2 to 10 x 10^7 plaque forming units (pfu) per cc; (2) Occasionally, tissue culture virus was concentrated and partially purified by two cycles of adsorption to and elution from guinea pig erythrocytes; (3) In some experiments, concentrated and purified virus prepared according to the method of Winocour[76] was used.

Virus stocks were generally stored in a deep freeze at -20°C.

3. Receptor destroying enzyme (RDE)

RDE, an enzyme which destroys the receptor sites for Py[77] (and other hemagglutinating viruses) on the surface of erythrocytes, was obtained as a lyophilised powder from Behringwerke AG. The powder, supplied in serum bottles, was dissolved in 2.5 cc TBS / 0.1% $CaCl_2$ and stored, for up to one month, in the refrigerator. When used, RDE was added directly to the culture medium. RDE concen-

trations will be expressed as the reciprocal of dilutions from the original solution. The preparations of RDE obtained from Behringwerke were effective in inhibiting the agglutination of guinea pig erythrocytes by Py, at a concentration of 0.002 units under conditions similar to those described by Burnet and Stone.[78]

4. Antivirus serum (AS)

Two rabbits, No. 1 and No. 2, were given two series of one intravenous followed by two subcutaneous injections of virus, over the course of two months. Five-tenths to 1.0 cc of a virus stock titering 5×10^8 pfu/cc, prepared by adsorption to and elution from guinea pig erythrocytes, was administered at each injection. Blood was collected by heart puncture 10 days after the last injection. Blood was collected once more from these rabbits after another series of three intravenous injections spaced at intervals of three to five days. In the later series of injections, 0.5 cc of purified "empty shells"[76,79] at a concentration of 5×10^5 hemagglutinating (HA) units was used as antigen.

The sera thus obtained were absorbed with 2 to 8×10^7 mouse embryo cells per cc of serum. Sera obtained from the second bleeding were also absorbed with 5×10^7 cells/cc of a Py induced tumor.* After absorption, the sera were heated at 56°C for one half hour, centrifuged

* This tumor, designated SESF, was sent to us by Dr. H.O. Sjogren.

to remove cell debris, and sterilized by filtration through a millipore filter. The sterile sera were distributed into tubes and stored at -20°C. Hemagglutination inhibition (HI) tests performed on these sera by the method of Rowe, et al.[80] gave HI titers in the range of 1:40,000 to 1:100,000.

When the serum was used to treat cells, the calf serum which is incorporated into our tissue culture media was heated at 56°C for one-half hour in order to destroy complement. AS concentrations will be expressed as the reciprocal of dilutions.

B. Methods

1. Primary mouse embryo cultures

Mouse embryo cells were obtained from 12 to 14 day old embryos of noninbred Swiss or Py free A/Sn strain mice* according to the method of Dulbecco and Freeman.[41] Generally, the disaggregated cells of one embryo would be explanted on one or two 100 mm polystyrene petri dishes (obtained from Falcon Plastics) in Eagle's medium. Cells from these primary cultures were subcultured 3 to 6 days later, generally on 65 mm petri dishes.

2. Virus titration

Plaque assays were performed according to the method of Dulbecco and Freeman[41] except that a one and one-half hour

* These mice were kindly sent to us by Dr. H.O. Sjogren from the colony of Dr. G. Klein at the Institute for Tumor Biology, Stockholm. They were kept and bred in a restricted room on a different floor from the laboratory where virus experiments were performed. Hemagglutination inhibition tests (see below) were regularly performed on randomly selected mice from the colony, but no mouse with a positive serum (according to the criteria of Sjogren and Ringertz[81] was ever found.

adsorption period was used. If the infected cells were to be used subsequently for cloning or for an infective center assay, the cultures were washed before infection with PEM, and the virus was diluted in PEM. Otherwise, TBS was usually used for these steps.

Hemagglutinations were performed according to the method of Rowe, et. al.[80] The reciprocal of the highest dilution showing positive agglutination was taken as the concentration of that virus stock in HA units.

3. Determination of total virus yield

In order to determine the total virus content of an infected culture, it was necessary to assay the virus present in the supernatant medium and the virus associated with the cells. The latter virus fraction -- which consists of virus adsorbed to the cell surface and virus present inside the cell -- is known as cell associated virus (CAV). It was measured by removing the cells from the petri dish with a policeman, disrupting them by three cycles of rapid freeze-thawing, and assaying the lysed cells for plaque formers or for hemagglutinin. The supernatant medium, of course, could be assayed directly.

In order to determine virus production in an infected culture after a single cycle of virus growth, its total virus content was measured at 40 to 44 hours after infection. This procedure is justified by the data of Winocour and Sachs[43] which show that the latent period for Py in mouse cells is 22 to 24 hours.

4. Cell cloning

a) Feeder layers. Feeder layers of secondary or tertiary mouse embryo cells were prepared as follows: A dose of 5000 r was administered to a cell suspension in 5 cc of medium in a petri dish.* The irradiated cells were sedimented, resuspended in fresh medium, counted, and 5×10^5 cells were plated per 65 mm petri dish. Special precautions were taken to eliminate clumps from the suspension as described below.

b) Cloning. One to two days after plating, the medium was removed from the feeder layers and 2 cc of PEM or CMRL-1066 were added. The suspension of cells to be cloned was added in a small volume of medium and the plates were incubated for 8 to 12 hours to allow cell attachment.

The following precautions were taken to avoid plating clumps: the suspensions were allowed to stand in a centrifuge tube for 5 minutes, and the top layer was removed and placed in a paraffin coated tube. The cells were counted using a technique which allowed the proportion of clumps to be determined. A minimum of 200 cells was counted. The number of clumps larger than 4 cells was always less than 0.5%. A variable number of cells, generally less than 10% were in clumps of 2-4 cells. On occasion, the cells to be cloned were added to the feeder

* The physical factors were 0.38 mm Al filtration, 50 KVP. 30 MA, 5.7 cm target to sample distance, Machlett OEG 60 tube with a beryllium window, and a dose rate of 2500 r/min.

plates with a micropipette. Generally, only one cell was added to each plate in these cases. The single cell was picked from a cell suspension under the dissecting microscope.

After the cells had attached to the feeder plate, a molten solution of 0.6% agar in PEM or CMRL was added directly to the 2 cc of medium already on the plate. Four days later, 3 cc of liquid cloning medium was added and at the 8th to 10th day after plating, the agar was poured off and 5 cc of fresh medium was added.

c) Picking clones. One day after the agar was poured off, the colonies were counted and their position marked. Several control plates which received no cells were always counted to check the efficiency of the irradiation. Cloning efficiencies of 7 to 20% were routinely obtained with mouse embryo secondary cells.

On plates with four or fewer well-separated colonies, as many as three might be picked and transferred to a new plate with a feeder layer prepared as noted above. The clones were picked using the following procedure: The medium was removed from the plate and a glass cylinder, 10 mm in diameter, was affixed around the colony with sterile silicone grease.[82] Trypsin was added, and, after most of the cells had detached, they were transferred to the fresh plate with a pasteur pipette. The feeding routine for these secondary plates was the same as for the primary plates. Ten days to two weeks later, most of the clones could be transferred without a feeder layer. Generally, subcultivation could be attempted from 4 to 6 weeks after the original

cloning. Overall, about 60% of the clones that were picked gave rise to continually growing cultures; most of those that did not showed no growth at the first transfer.

5. Routine culture methods

In view of the known danger of cross contamination of cell lines carried in the same laboratory (see reference 82, for instance), it may be well to give a brief account of the methods used for cultivation of these cells. Every two to three days, plates of each line were scanned under the inverted microscope, and it was decided whether the plate should be transferred or fluid changed. Generally, plates were transferred when the cell sheet neared confluency. Only a fraction of the cell population was used to reseed new petri dishes. Since the growth rate of many cell lines falls off rather sharply at suboptimal cell densities,[84] several plates were initiated at different cell densities at each transfer. The plate containing the minimum cell number judged adequate to maintain the maximal growth rate was selected for the following transfer.

After the plates were scanned, media and trypsin solution were distributed in tubes, one for each culture, in a section of the laboratory where no virus work was performed. All lines were fluid changed and transferred in the virus section, but no virus infected material was introduced into the working area before uninfected cells were transferred. No instance of virus contamination was ever

detected. It is impossible to rule out contamination of one cell line with another by chromosome cytology since all the cultures derived from mouse tissue. However, whenever any suspicion of contamination arose -- that a pipette was not changed, for instance -- the lines concerned were immediately discarded.

6. Overall growth curves

These curves[52] describe the net increase in cell mass of cultures which are being transferred at regular intervals as a function of time. When a confluent culture is transferred, generally only a fraction of the total cell population is used to seed the new plate (see 5 above). The net increase in cell mass in the intervals between transfers is set equal to the reciprocal of this fraction. If the cells are always maintained under optimal growth conditions, and if there is no long lag period after transfer, the slope of the overall growth curve will closely reflect the average generation time of the cells.

7. Implantation tests

Occasionally, cultures derived from A/Sn embryos were tested for their tumor-inducing potential by implantation into irradiated and unirradiated A strain mice.* A confluent but not overcrowded plate of the culture to be tested was trypsinized, centrifuged and resuspended

* Most of the mice used for the implantation test were irradiated with a whole-body dose of 425 r. The physical factors were: 1 mm Al filtration, 250 KV, 15 MA, and a dose rate of 125 r/min.

in 0.1 - 0.2 cc of TBS. Cell counts were not routinely made, but by experience it is known that from 2 to 6 million cells can be recovered from such cultures. The volume of the centrifuged cell suspension was always checked, and if any doubt existed about the cell number, a count was made. Any excess over 6 million cells was discarded and fewer than 1.5 million cells were not injected. The cells were injected subcutaneously along the flank and the mice were regularly observed for at least 3 months after injection. A mouse was judged positive if a tumor arose at the site of implantation within 10 weeks of injection and grew to the size of a walnut (or killed the mouse before it did). Several of the tumors resulting from the injection of Py-infected transformed cells were sent to a laboratory for examination. Dr. Dennis Shillam of the Pasadena Clinical Laboratory found that 5 out of 5 of these tumors could be diagnosed as chondrosarcomas of subcutaneous tissues.

8. Detection of the Py specific antigen

The mice to be used were selected from cages containing no more than about five litters, of age one to two months. Half of these mice were injected subcutaneously with Py. Generally three injections of 0.1 cc of purified virus containing 5×10^8 pfu/cc in TBS were administered at weekly intervals. At the time of the third injection, the control mice were given a single injection of 0.1 cc of TBS. The cells to be tested for the antigen were dispersed by trypsin, centri-

fuged and resuspended in TBS. One tenth cc of buffer containing a known number of cells was injected subcutaneously into the virus-infected and virus free groups of mice. In most cases, three different doses of cells were employed.

The mice were observed at four to seven day intervals for the two months following the cell injection. Developing tumors were measured with calipers and the mean of two measurements was reported as the average tumor diameter.

9. Cell freezing

The cells to be frozen were suspended in a tube containing 3 to 4 cc of medium plus 6 to 8% sterile glycerol. The tubes were placed in an alcohol bath at 5°C and the temperature was lowered by a programmed temperature controller obtained from Conalco, N.Y. at a rate of 1°C min. to -30°C. Thereafter, the temperature was allowed to fall at an uncontrolled rate to the sublimation point of CO_2. The cells were stored in a freezer at this temperature.

The cells were thawed rapidly in a water bath at 37°C, and the contents of the thawed tubes were immediately poured onto petri dishes. Equal volumes of fresh medium were slowly added and the plates were then placed in the incubator. They were generally fluid changed after 1 or 2 days. Cell recovery by this technique is somewhat variable -- it ranges from 10% to 90%. If, when the cells were first fluid changed, a substantial number had not attached, the old supernatant medium was

centrifuged and the sedimented cells were resuspended in fresh medium and added back to the plate.

10. Antiviral medium

In several of the experiments to be described later, Py-infected cells were treated with medium containing antivirus serum and RDE (AS and RDE treatment). The function of the antiserum was to inactivate virus which was free in the medium (free virus) and virus which was superficially associated with the cells (superficially adsorbed virus). RDE, by destroying the receptors for virus adsorption, converts virus which is associated with cells to free virus[85] and also prevents the infection of uninfected cells.* We shall summarize several experiments performed to assess the efficiency of AS and RDE treatment in performing its functions.

a) Inactivation of free virus. The multiplicity curves[86] for the two antivirus sera we have used have been presented in Fig. 1. In these experiments, various dilutions of serum were added to aliquots of a tissue culture stock of virus at a concentration of 5×10^7 pfu/ml. This mixture was incubated at 37° for two hours, then diluted and plated for plaques. This incubation time should be long enough to allow the inactivation reaction to go to completion.

These results indicate that at the serum concentrations used, and at the virus concentrations we shall encounter, we can expect to

* The use of RDE to prevent reinfection was originally suggested to the author by Mr. Michael Fried.

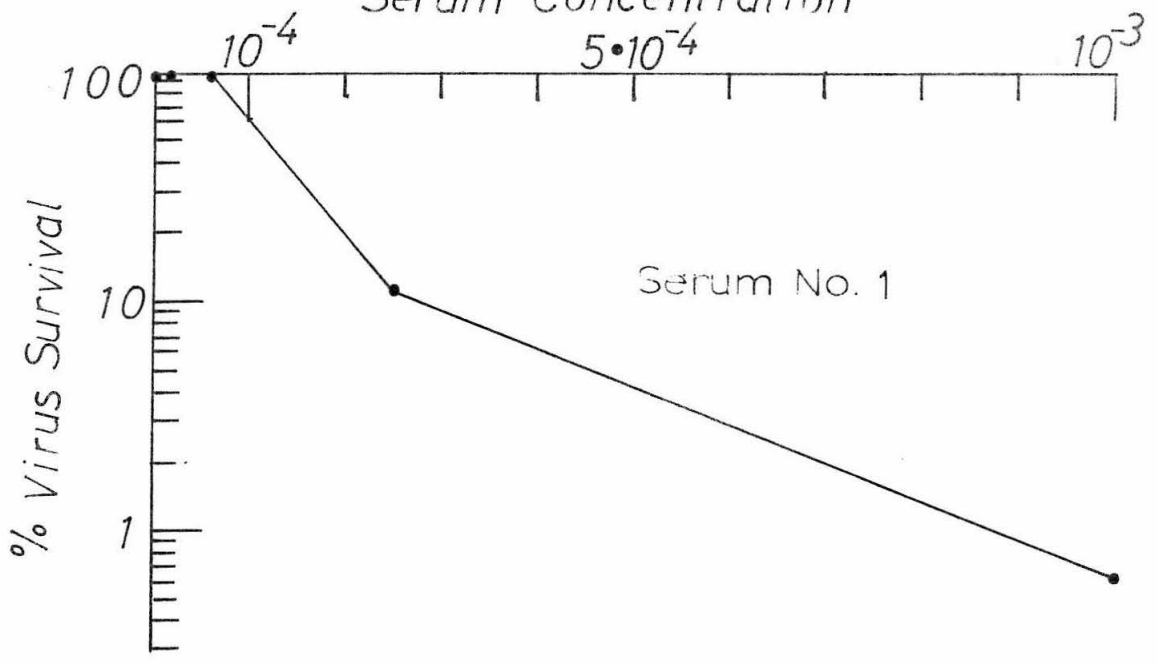
Figure 1. Multiplicity of Neutralization Curves for Antipolyoma Sera.

Various dilutions of sera were added to aliquots of a Py stock containing 5×10^7 pfu/cc. The mixtures were incubated at 37° for two hours, then diluted and plated for plaques.

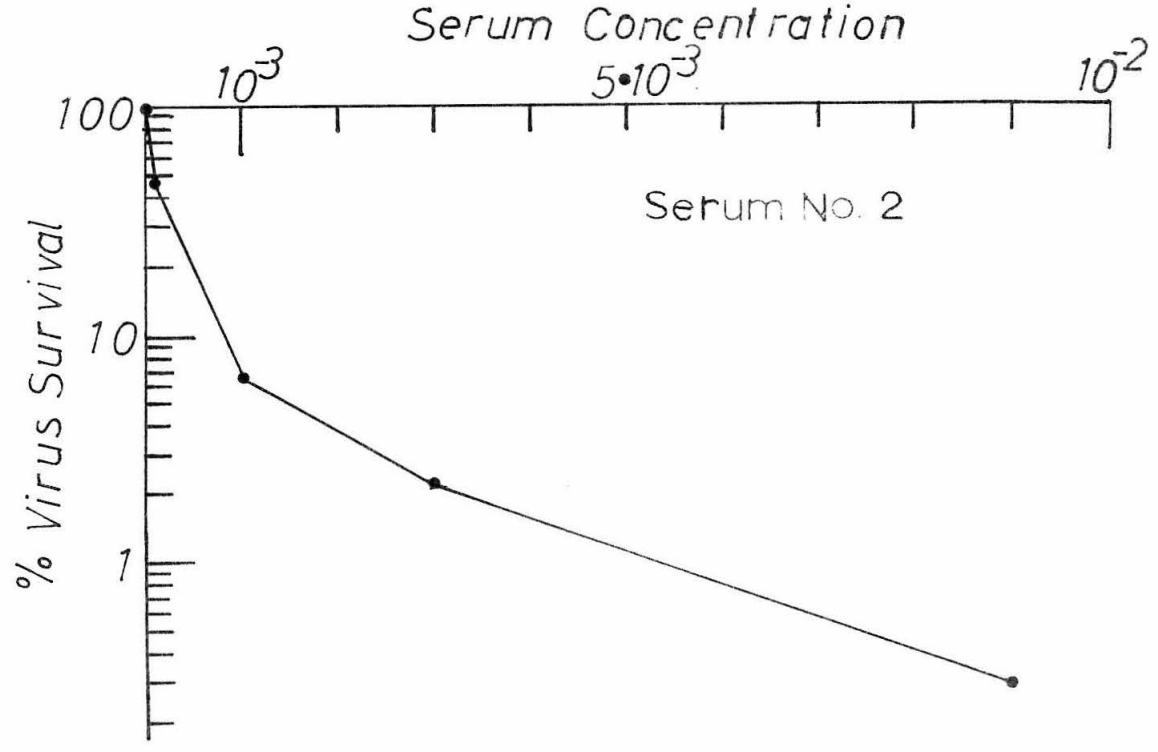
a) Antiserum from rabbit No. 1.

b) Antiserum from rabbit No. 2. (Note that the scale of the abscissa is decreased by a factor of ten.)

Fig. 1


Serum Concentration
10^{-4}
$5 \cdot 10^{-4}$
10^{-3}
100
10
1
% Virus Survival
Serum No. 1

(a)


Serum Concentration
10^{-3}
$5 \cdot 10^{-3}$
10^{-2}
100
10
1
% Virus Survival
Serum No. 2

(b)

neutralize at least 99% of the free virus.

b) Inactivation of superficially adsorbed virus. Confluent monolayers were infected with high concentrations of virus. After adsorption, the monolayers were extensively washed to remove loosely bound virus. Nutrient medium containing high concentrations of AS and RDE was added to one series of plates, and medium without AS and RDE was added to a control series. The cell sheets were washed and dispersed by trypsin before any progeny virus had appeared, disrupted by freeze-thawing, and assayed for their virus content. It was found that 80-90% of the superficially adsorbed virus could be eliminated by AS and RDE treatment.

c) Protection of cells from infection. Confluent plates of mouse embryo cells were treated with RDE for five hours, then washed, infected and observed for plaques. The results (Table 1) show that 80 to 95% of the infectible cells became resistant to infection at RDE concentrations of 0.004 to 0.02 units. In another experiment, at an RDE concentration of 0.006 units, the number of plaques after one hour of pretreatment was 15%, and after seven hours of pretreatment it was 10% that of an untreated control.

This reduction in cell infectibility was probably not due to some general deleterious effect of RDE for the following reasons: (1) The plaque size on RDE treated plates was the same as the plaque size on

Table 1. RDE Treatment of Mouse Embryo Cells

Various dilutions of RDE in Eagle's medium containing 10% calf serum were added to confluent monolayers of mouse embryo cells. Five hours later, the medium was removed, the plates washed once with TBS, and the monolayers used for plaque assay in the usual manner. Three plates were used for each RDE dilution. No reduction in plaque size was noted when the plates were read.

Table 1

Dilution of RDE	1:10	1:50	1:250	1:6250	1:∞
Average plaque number as a fraction of the untreated control	0.04	0.05	0.21	0.53	1.00

untreated plates and (2) cloning experiments performed in the presence and absence of RDE show that RDE does not lower the cloning efficiency of mouse cells.

C. Glossary of Descriptive Terms

1. Descriptive terms

a) Cell morphology and cell orientation. Mouse embryo cells which have been freshly explanted from the animal (Fig. 2c and d) have a rather broad, flat, irregular shape. They appear non-refractile* when viewed with non-phase contrast optics. The cells tend to grow side by side when the cultures near confluency: this will be denoted as an oriented or parallel configuration.

Cells from cultures infected with Py (Fig. 2a and b) frequently have a rather elongated and generally triangular shape. They appear refractile under non-phase optics. The cells tend to lie across one another in a netlike array when the cultures near confluency: this will be called a random or netlike configuration. Cells from such cultures will be said to have transformed morphology.

b) Growth pattern. If a culture remains flat and two dimensional after it has been confluent for at least a week, it will be said to have a regulated growth pattern. If, on the other hand, it

* Refractility refers to the property of some cells of acting as lenses. These cells appear light, and then dark, as one focuses through them with a microscope, and their edges are sharply outlined.

Figure 2. Cell Morphology and Cell Orientation in Py Transformed Cultures and in Freshly Isolated Mouse Embryo Cultures.

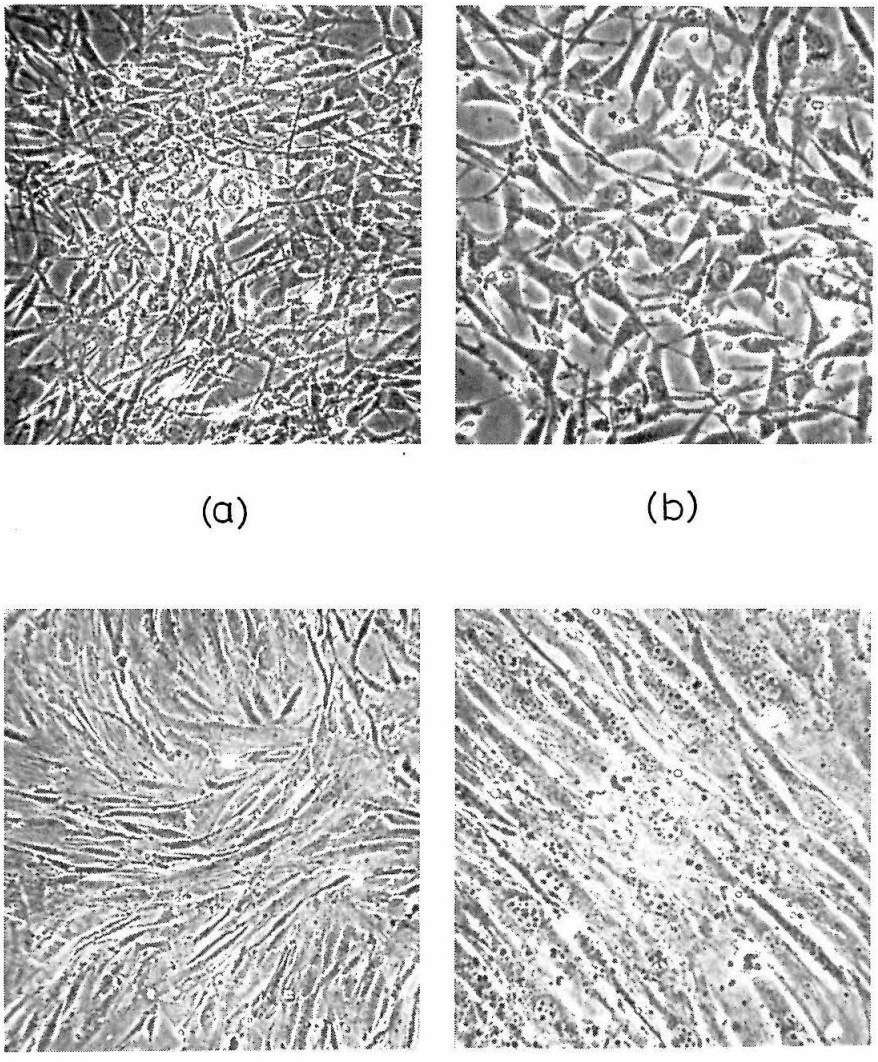
(a) Transformed Culture. Phase contrast, x 90.

(b) Transformed Culture. Phase contrast, x 150.

(c) Freshly isolated, uninfected culture. Phase contrast, x 90.

(d) Freshly isolated, uninfected culture. Phase contrast, x 150.

The typical criss-cross cell orientation and sharply defined cell shape of the transformed cells contrasts with the parallel cell orientation and rather ill-defined cell shape seen in the freshly isolated cultures.



(a) (b)

(c) (d)

Figure 2

displays areas where cells are proliferating on top of the monolayer, it will be said to have a non-regulated or multilayered (ML) growth pattern. (See Fig. 3 for examples). Cells with transformed morphology invariably have a non-regulated growth pattern.

c. Colonial morphology. Cells from freshly explanted cultures form colonies in which the cells grow strictly in two dimensions. Such colonies will be described as flat or regulated. Cells from transformed cultures frequently form colonies with thick, multilayered centers. Unlike flat colonies, these colonies are visible to the naked eye, without staining, by eight days after plating. They will be referred to as dense colonies. Two illustrations of a flat colony next to a dense colony are given in Figure 4.

2. Abbreviations

RNA, ribonucleic acid; DNA, deoxyribonucleic acid; Py, polyoma virus; RSV, Rous sarcoma virus; AS, antiserum; RDE, receptor destroying enzyme; CAV, cell associated virus; MOI, multiplicity of infection; IC, infective center; ML, multilayered; pfu, plaque forming unit.

Figure 3. Microphotographs of Areas of ML Growth Appearing in Confluent Cultures.

(a) Unstained, x 20.

(b) Unstained, x 60.

(c) Phase-contrast, x 130.

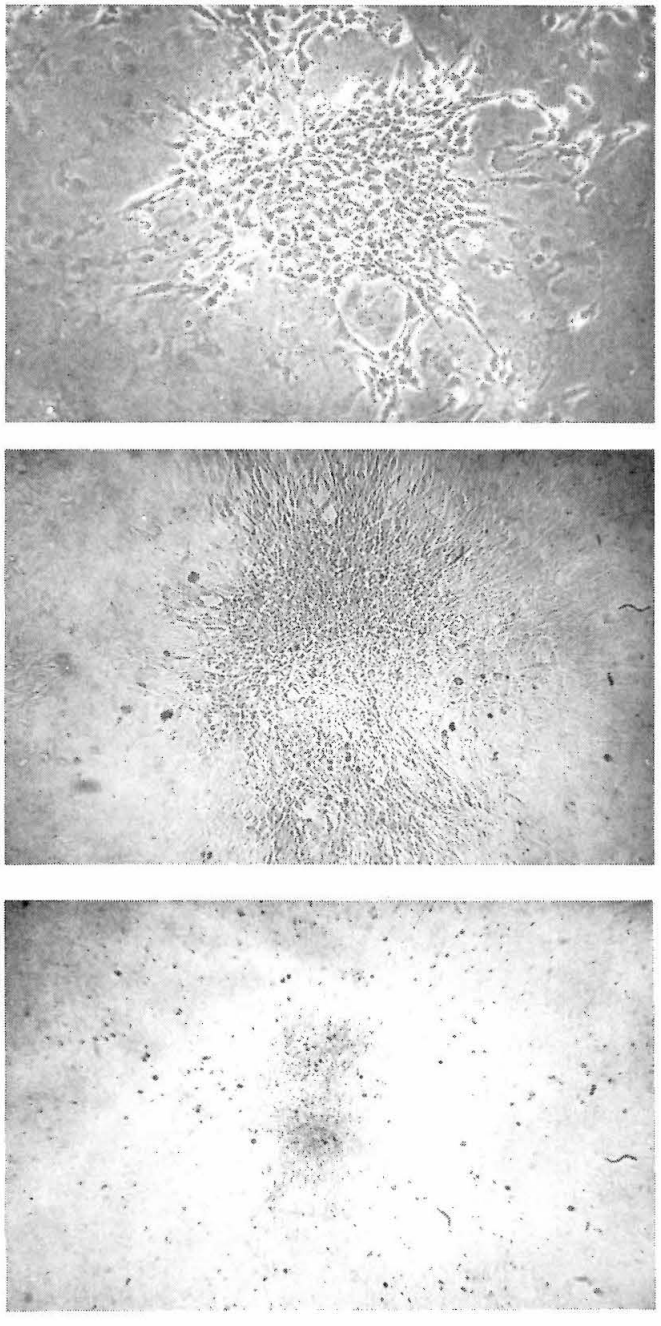


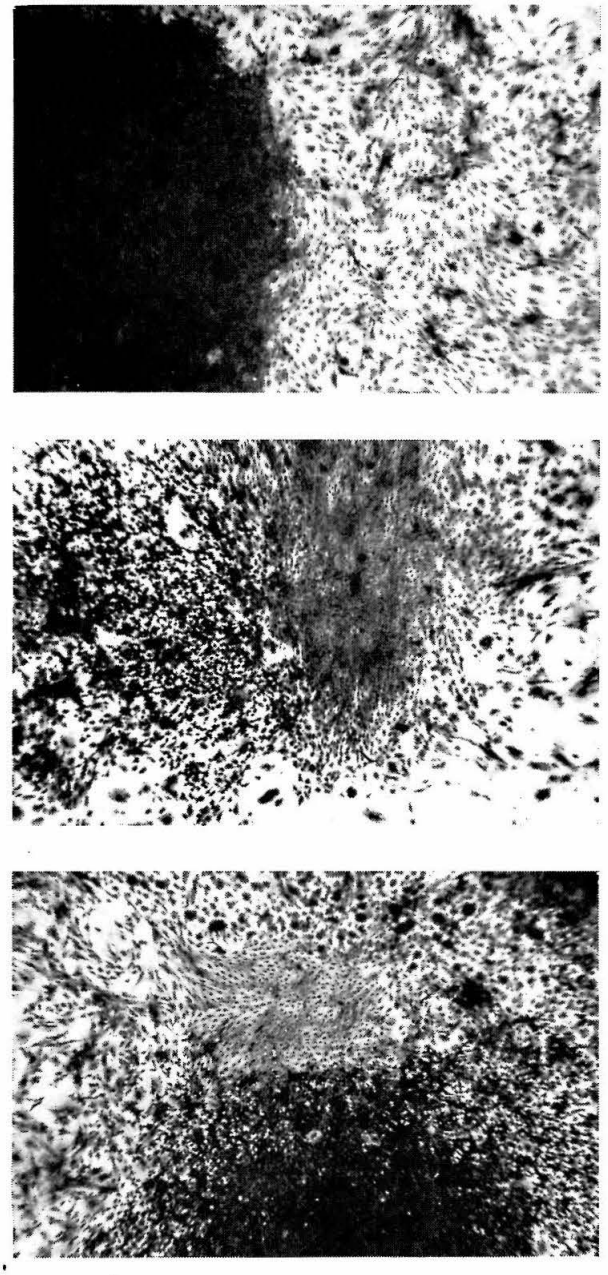
Figure 3

Figure 4. Microphotographs of Dense Colonies and Flat Colonies. Stained with Methylene Blue. x 20.

(a) The dense clone is at the left of the photograph, and the flat clone to the right.

(b) The dense clone is at the top of the photograph, and the flat clone toward the bottom.

(c) The dense clone is at the top of the photograph, and the flat clone at the bottom.



(a) (b) (c)

Figure 4

PART I THE IMMEDIATE EFFECTS OF POLYOMA VIRUS INFECTION ON MOUSE EMBRYO CULTURES

A. Virus Multiplication and Cell Killing

It was noted in the Introduction that mouse embryo cultures show a heterogeneous response to infection with Py: some cells degenerate and others survive and continue to divide. Quantitative information concerning this phenomenon is, however, lacking. Experiments were therefore undertaken to determine the mode of virus release, the proportion of cells yielding virus and the proportion of cells killed after exposure of cultures to known concentrations of virus.

1. Mode of virus release

This was studied in single cell yield experiments carried out by a method similar to that described by Lwoff, et. al.[87] Single cells obtained by trypsinization from infected cultures were transferred under the dissecting microscope to small volumes of nutrient medium. The medium contained, as feeders, Py-transformed hamster cells incapable of supporting virus growth. After several days' incubation the medium was assayed for virus.

The results of three single cell yield experiments are summarized in Table 2. In experiment (1), a culture was infected with Py at a virus:cell ratio of 100 pfu/cell; single cells were isolated before

Table 2. Analysis of Virus Yields from Single Cells.

Single cells were isolated from infected cultures and tested for virus production as described below.

Experiment (1). A confluent monolayer of mouse embryo secondary cells was infected at an input virus:cell ratio of 100 pfu/cell. Fifteen hours after infection, single cells were transferred to plastic tubes containing 0.5 cc of medium which had been preseeded with $5 - 10 \times 10^3$ Py transformed hamster cells. Seventy two hours after infection, the tubes were freeze-thawed three times, and the contents assayed for virus.

Experiments (2) and (3). Two monolayers of mouse embryo cells were each infected at an input virus:cell ratio of 1 pfu/cell. At three and five days after infection, respectively, single cells were isolated in small drops of medium[87] which had been preseeded with 50 - 100 irradiated Py-transformed hamster cells. Three days after isolation, the medium of the drops was collected and fresh medium was added. Six days after isolation, the medium was collected again, pooled with the previous collection and the virus content of the drops determined.

Note (1): The figure denotes the number of cells producing the indicated amount of virus.

Note (2): A sample of the infected cell suspension containing at least 5×10^5 cells was plated on a petri dish. The virus production of these cells was determined after 46 (experiment 1) and 24 (experiments 2 and 3) hours of incubation.

Note (3): Single infected cells were placed in 0.5 cc of medium at the beginning of the experiment and immediately disrupted by freeze-thawing. A 0.1 cc sample of this fluid was assayed for CAV.

Note (4): This figure denotes the number of samples containing the indicated quantity of virus.

Note (5): Pooled results of experiments (2) and (3).

Experiment	Distribution of pfu produced per cell: 0-5	5-100	101-200	201-300	301-400	401-500	501-600	601-700	701-800	801-900	901-1000	(1) Number of cells	(2) % of cells yielding virus	(3) Total virus production (pfu)	Average yield per producing cell (pfu) (4)	Average virus production (5) (pfu/cell) [(3) ÷ (1)]	Average virus yield in (6) mass culture (pfu/cell) ②	(7) Distribution of CAV ③ (pfu/sample) 0 1 2 3 4	(8) Total CAV (pfu/cell)
(1) 52①	0	0	1	0	2	0	0	1	0	0	0	60	13.3	5586	698	93	172		2
(2) 43	0	0	0	0	1	0	0	1	0	0	0	45	4.4	940	470	21	40	25 4 1 1 0	2
(3) 52	0	0	0	0	1	0	0	0	0	0	1	54	3.7	1320	660	24	30	23 5 0 0 0	1
$(2)^5$ + (3) 95	0	0	0	0	2	0	0	1	0	0	1	99	4	2260	565	23	35		1.5

Table 2

any progeny virus had appeared. About 13% of the cells released virus (column 2) with an average yield of about 700 pfu per producing cell (column 4).

In experiments (2) and (3), cultures were infected at virus:cell ratios of 1 pfu/cell. Cells were taken from them at three and five days after infection, respectively, i.e., after several cycles of virus growth had already occurred. In these experiments, about 5% of the cells released virus (column 2) with an average yield per producing cell of 565 pfu (column 4).

These experiments were controlled in several ways: (1) The cell associated virus (CAV) carried over from the infecting virus was determined by assaying a known number of the trypsinized cells disrupted by freeze-thawing. As seen from a comparison of columns five and eight (Table 2), CAV was about 1/20th of the virus produced by the cells and therefore did not obscure the study of virus production. This is also shown in experiments (2) and (3) by the fact that the distribution of CAV (column 7) is quite unlike the distribution of pfu produced per cell. (2) To check whether virus production was depressed in isolated cells, the virus yield of infected cells kept in "mass culture" -- i.e., at a cell density of greater than 5×10^5 cells per plate -- was determined. A comparison of columns 6 and 5 (Table 2) shows that the virus yield of isolated cells was 54 to 80% that of cells in mass cultures. Therefore, isolation of the cell did

not markedly depress the virus yield.

The principal conclusions to be drawn from these single-cell experiments is that the major proportion of virus production in freshly infected and in steady state cultures of mouse embryo cells occurs in large yields produced by a few cells. Thus the mode of virus release in these cultures is similar to that observed by Dulbecco and Vogt[52,53] in cultures of persistently infected Py-transformed mouse cells.

2. The relationship between the proportion of cells producing virus and the multiplicity of infection

The results of the previous section show that only a small proportion of the cells of steady state or of freshly infected cultures were virus yielders even when the input virus:cell ratios were high. The reasons for the high proportion of non-yielding cells were further investigated by using the infective center (IC) technique.

The plan of the experiments was similar to that employed by Winocour and Sachs.[43] Confluent monolayers of mouse embryo secondary cells were infected with various concentrations of purified virus under conditions identical to those used in the plaque assay. The average input multiplicity of infection (MOI or simply multiplicity) was therefore given by the ratio of the virus input expressed in pfu's to the number of cells on a confluent plate. At times ranging from five to twenty hours after infection, the cells were dispersed with trypsin,

counted, diluted and plated in known numbers on plaque assay cultures (Winocour and Sachs (43), Method A).

In experiments of this type with Py the large amount of CAV represents a grave danger since by desorption it can give rise to spurious "infective centers". In order to decrease this danger, the infected monolayers were washed several times and treated with AS and EDE. CAV was furthermore determined in each experiment by measuring the amount of virus released from aliquots of the infected cells by freeze-thawing. In all experiments with multiplicities of less than 100 pfu/cell, the residual CAV was less than 25% of the number of infective centers. At multiplicities of more than 100 pfu/cell, however, the amount of CAV was so large as to preclude meaningful infective center experiments.

The expectation in these experiments is the following. If the cell population is homogeneous with regard to the probability that a virus particle will initiate infection, the relation between the MOI and the proportion of virus yielding cells is given by the zero term of the Poisson distribution,

$$\text{Proportion of IC} = 1 - e^{-\text{MOI}}.$$

At MOI $\leq$ 0.1 pfu/cell, this equation reduces to:

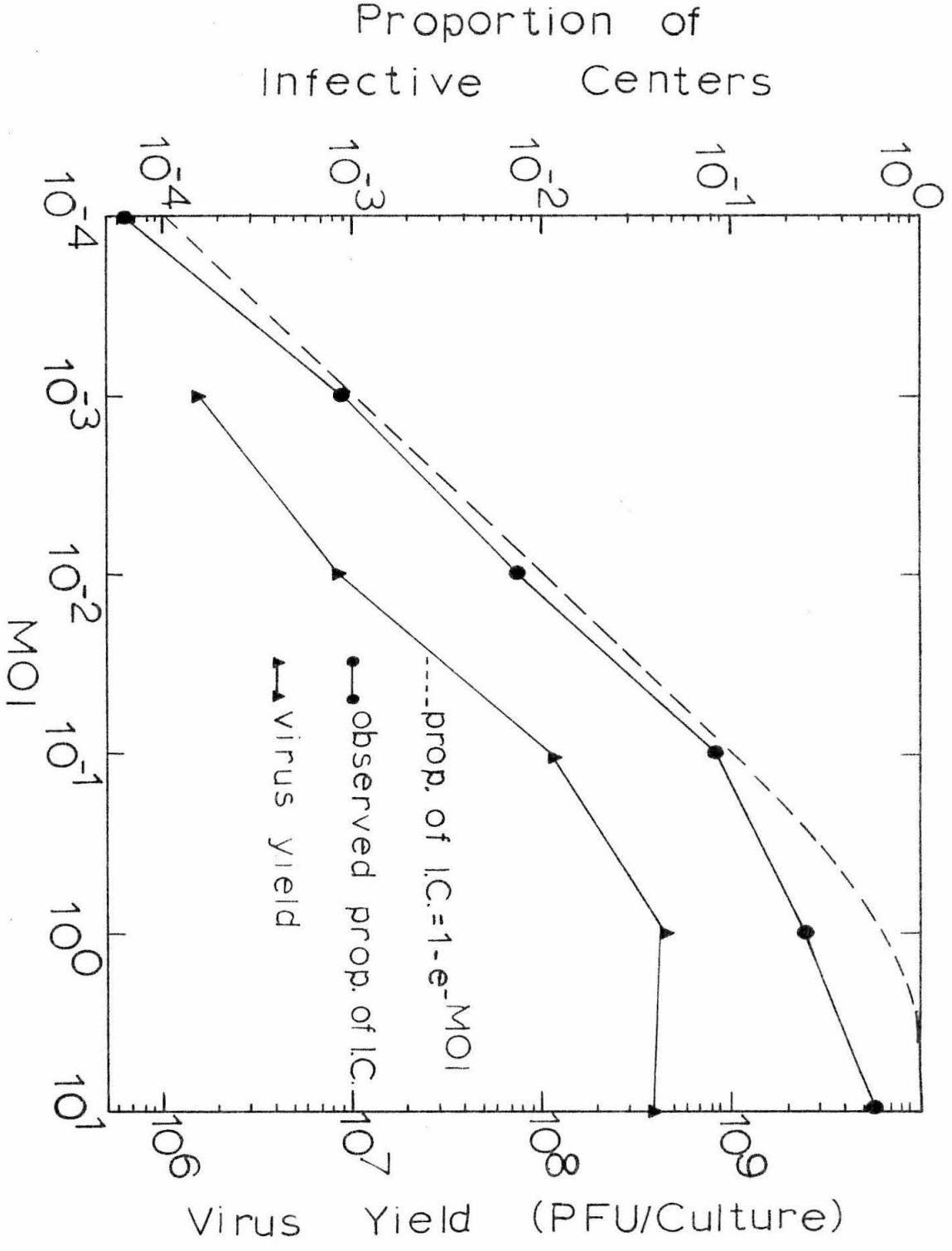
$$\text{Proportion of IC} \approx \text{MOI}.$$

The results obtained are not in complete agreement with this expectation. The type of deviation from expectation is shown by Fig. 5

Figure 5. The Relationship Between the Multiplicity of Infection and the Proportion of Infective Centers.

Mouse embryo secondary cultures were infected with serial dilutions of a purified Py stock as described in the text and in Table 3, experiment PIC-11. AS and FDE were added at concentrations of 0.02 and 0.01 units, respectively, at four hours after infection. The cells were plated for infective centers at six hours after infection. An aliquot of each infected cell suspension containing 2.0×10^6 cells was plated on a petri dish, and the total virus yield from these cells was determined after forty-one hours of further incubation.

Fig. 5


Proportion of
Infective Centers
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
MOI
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
prop. of I.C. = $1-e^{-MOI}$
observed prop. of I.C.
virus yield
10^{6}
10^{7}
10^{8}
10^{9}
Virus Yield (PFU/Culture)

which reproduces the results of one extensive experiment (Experiment PIC-11). At a MOI of 0.1 pfu/cell or less, the results are in agreement with expectation; they deviate at higher multiplicities because the proportion of infective centers is lower than expected. Similar conclusions can be deduced from the relationship of total virus yield to multiplicity. This deviation suggests an inhomogeneity of the population with respect to the probability of giving rise to infective centers when exposed to virus.

The relationship between the MOI and the proportion of infective centers was therefore investigated in detail at multiplicities above 1 pfu/cell. Table 3 and Fig. 6 give the results of several experiments of this type. These results confirm that in a fraction of the cell population (the resistant fraction), the probability of giving rise to an infective center after exposure to virus is lower than the average over the whole population. The size of the resistant fraction varied from experiment to experiment but was relatively constant for a given batch of secondary cultures. The resistance is not absolute since resistant cells could be converted to infective cells by increasing the virus input, as shown in Fig. 6. It can also be seen (Experiment CIC-19, Table 3) that in one experiment an sp line of Py was not appreciably more effective than the standard lp line in converting resistant cells to virus yielders in contrast to what had been previously suggested.[88] Further experimentation, however, will be needed to confirm this point.

Table 3. The Relation Between MOI and % I.C. at MOI ≯ 1.

Mouse embryo cells were infected as described in the text. AS and FDE at concentrations of 0.02 and 0.01 units, respectively, were added to the culture medium at the indicated times. At the end of the treatment period, the cells were plated for infective centers.

Note ①: The % IC figure is uncorrected for the amount of CAV. The CAV figure is given as a percentage of the proportion of infective centers.

Note ②: A purified stock of sp virus was used at the indicated multiplicity in experiment CIC-19.

Table 3

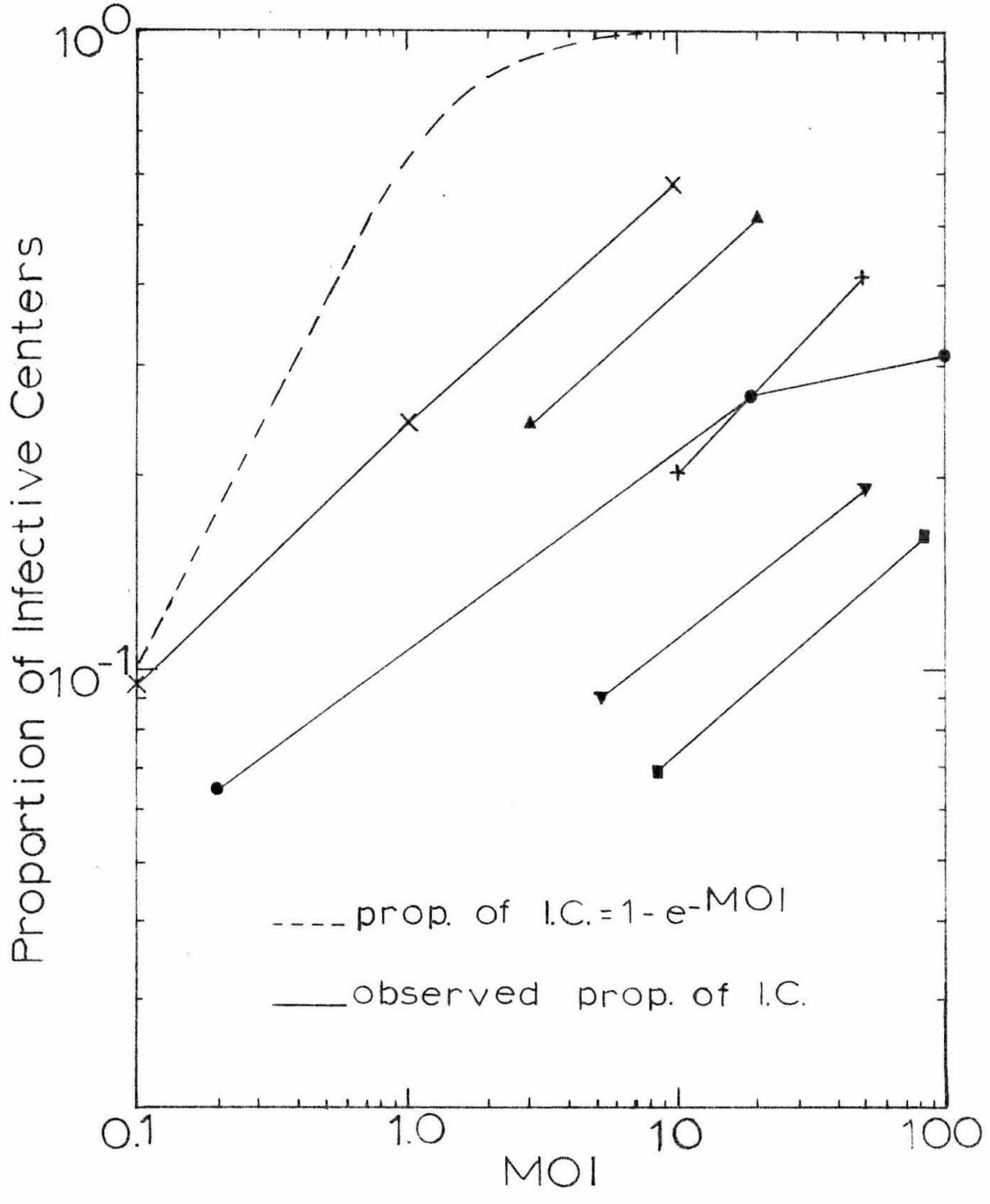
Experiment	MOI	% I. C.	CAV	% I. C. (corrected for CAV)	Use of AS and RDE Duration of treatment (hrs. after infection)	
					From	To
CIC-8	2.5	4 ①	2 ①	4	3	6
CIC-10	80	20	20	16	2-1/2	10
	8	8	8	7		
CIC-11	50	25	25	19	2-1/2	10
	5	10	10	9		
PIC-3	10	15	8	14	1-1/2	20
CIC-19	50	43	2	42	1-1/2	6
	10(sp) ②	20	2	20		
PIC-10	10	26	3	25	1-1/2	5
	10	18	3	17	3-1/2	5
	10	35	15	30	--	-
CIC-21	20	52	2	51	10	13
	4	25	1	25		
PIC-11	10	65	13	57	4	6
	1	27	7	25		
CIC-24	100	18	8	17	2	16
	100	35	12	31	13	16
	100	100	103	--	--	--
	20	28	3	27	13	16

Figure 6. Multiplicity of Infection - Proportion of Infective Centers Relationship at Multiplicities Greater than One pfu/cell.

Mouse embryo cells were infected and plated for infective centers as described in Table 3.

Symbol		Experiment
■——■	----------	CIC-10
▼——▼	----------	CIC-11
+——+	----------	CIC-19
x——x	----------	PIC-11
▲——▲	----------	CIC-21
●——●	----------	CIC-24

Fig. 6


10^0
10^{-1}
Proportion of Infective Centers
0.1
1.0
10
100
MOI
---- prop. of I.C. = 1 - e^{-MOI}
____ observed prop. of I.C.

Several types of controls were carried out. The first control was made to test whether the measures taken to remove superficially adsorbed virus prevented the infection of cells that would otherwise have been infected. Repeated washing appears to be safe because when applied to plaque assay cultures it did not decrease the number of plaques. AS and EDE treatment of plates infected at low multiplicities (Table 4) or at high multiplicities (Table 3, experiments PIC-10 and CIC-24) caused a reduction in the proportion of infective centers, particularly when the treatment was applied immediately after the end of the adsorption period. The maximum loss observed was about 30% of the number of infective centers in the first case and about 45% in the second. These losses alone cannot account for the failure of the results to agree with expectation at high multiplicities because they would not be expected to be multiplicity-dependent.

Other controls were made to investigate whether the size of the resistant fraction depends on the initial cell density or on the time at which the infected cells were dispersed and replated. The data presented in Table 5 show that neither of these factors has any large effect.

In conclusion, the infective center experiments show that the proportion of virus yielding cells under our experimental conditions is lower than expected. This reduction cannot be attributed to experimental complications. It is likely that it is caused by the presence of a class

Table 4. Effect of Antiviral Serum and RDE on Infected Cells.

Confluent secondary cultures of mouse embryo cells were infected with virus at the indicated dilutions. After a two hour adsorption period, the plates were washed two times with medium, and two cc of PEM + 10% inactivated calf serum were added. AS and RDE were added at the indicated times after infection to the appropriate plates, at concentrations of 0.02 and 0.01 units, respectively. The plates were washed two more times at fifteen hours and then, either treated as ordinary plaque assay plates or trypsinized and plated as infective centers at a dilution of 1:10. The numbers of plaques formed are given as the average of the numbers obtained on three plates.

Table 4

Virus dilution	AS and RDE treatment Begin	AS and RDE treatment End (hrs.)	Procedure at 15 hrs. after infection	Average No. of plaques per plate	% of Untreated control
2×10^6	2	15	Plate 10% of cell sheet as I.C.'s	21	70%
2×10^6	13	15	Plate 10% of cell sheet as I.C.'s	26	87%
2×10^6	--	--	Plate 10% of cell sheet as I.C.'s	27	90%
2×10^7	2	15	Remove AS and RDE, wash, and add agar	24	80%
2×10^7	13	15	Remove AS and RDE, wash, and add agar	28	93%
2×10^7	--	--	Wash and add agar	30	100%

Table 5. The Effect of Cell Density and Time of Plating on the Proportion of Cells Registering as Infective Centers.

The cells were infected with 0.2 cc of purified Py at a concentration of 10^8 pfu/ml (MOI = 10 for plates with 2×10^6 cells). The virus was adsorbed for 1-1/2 hours and the plates were washed and treated with AS and RDE at concentrations of 0.02 and 0.01 units, respectively. At the indicated times after infection, the cells were trypsinized and plated as infective centers. The level of superficially adsorbed virus was below 8% of the % I.C. in all cases.

Table 5

Initial Cell Density (cells/plate)	Time of Plating (hrs. after infec.)	% I. C.
2×10^6	8	12
2×10^6	15	10
2×10^6	20	15
1×10^6	20	9
5×10^5	20	13

of cells with a lower than average probability of producing a virus yield when exposed to virus. The cells that do produce virus, however, yield relatively large quantities: on the order of 500 pfu/cell.*

3. The relationship between the proportion of cells killed and the multiplicity of infection

In many of the infective center experiments of the previous section, an attempt to estimate the proportion of cells killed was also made. The presence of CAV is less important for this determination than for the determination of the proportion of infective centers, and therefore higher MOI can be used. Cell killing is a more accurate measure of the proportion of cells infected than are infective centers

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* The resistant fraction could be a cause of inefficiency in the plaque assay. Whether or not this is so depends on the nature of the resistant fraction.

Model (1): The resistant fraction consists of cells whose ability to adsorb virus is unimpaired. In this case, potential plaque formers are being lost by adsorption onto resistant cells. With respect to this factor alone, the efficiency of the assay is essentially given by the proportion of infective centers at the highest MOI for which the Poisson law accurately describes the number of infective centers in the population. This proportion was about 10 - 20% in experiment PIC-11, and this is probably a maximum estimate (Fig. 3).

Model (2): The resistant fraction consists of cells whose ability to adsorb virus is impaired. In this case, the resistant fraction does not cause inefficiency in the plaque assay.

The true MOI on the most easily infected fraction of the population is higher than the estimated input MOI in both cases. Under model (1), this is because the inefficiency of the plaque assay causes an underestimation of the input virus titer. Under model (2), this is because the cells adsorbing virus are fewer than the cells counted.

when this proportion is large. The approach suffers, however, from one important disadvantage: since the cloning efficiency of uninfected mouse embryo cells is of the order of 10%, we are observing the behavior of only a minority of the cells.

The technique of infection used in these experiments is the same as that used for the infective center experiments. In fact, in several experiments, the proportion of cells killed and the proportion of infective centers were determined on the same infected population. To determine the proportion of cells killed, the cloning technique described under Methods was utilized, with one exception: the incorporation of AS and RDE into the cloning medium at concentrations of 0.02 and 0.01 units, respectively, in order to prevent secondary virus infection on the cloning plate. The cloning efficiency of uninfected cells was determined in all experiments. This efficiency varied from 6 to 24% with different batches of secondary cultures, but was relatively constant for cells selected from different plates of the same batch. The cloning efficiency was not significantly affected by the AS and RDE treatment.

The results of four cell killing experiments, performed with three different strains of Py, are presented in Fig. 7. As in the infective center experiments, the relation between MOI and proportion of surviving colony formers deviates appreciably from Poisson expectation:

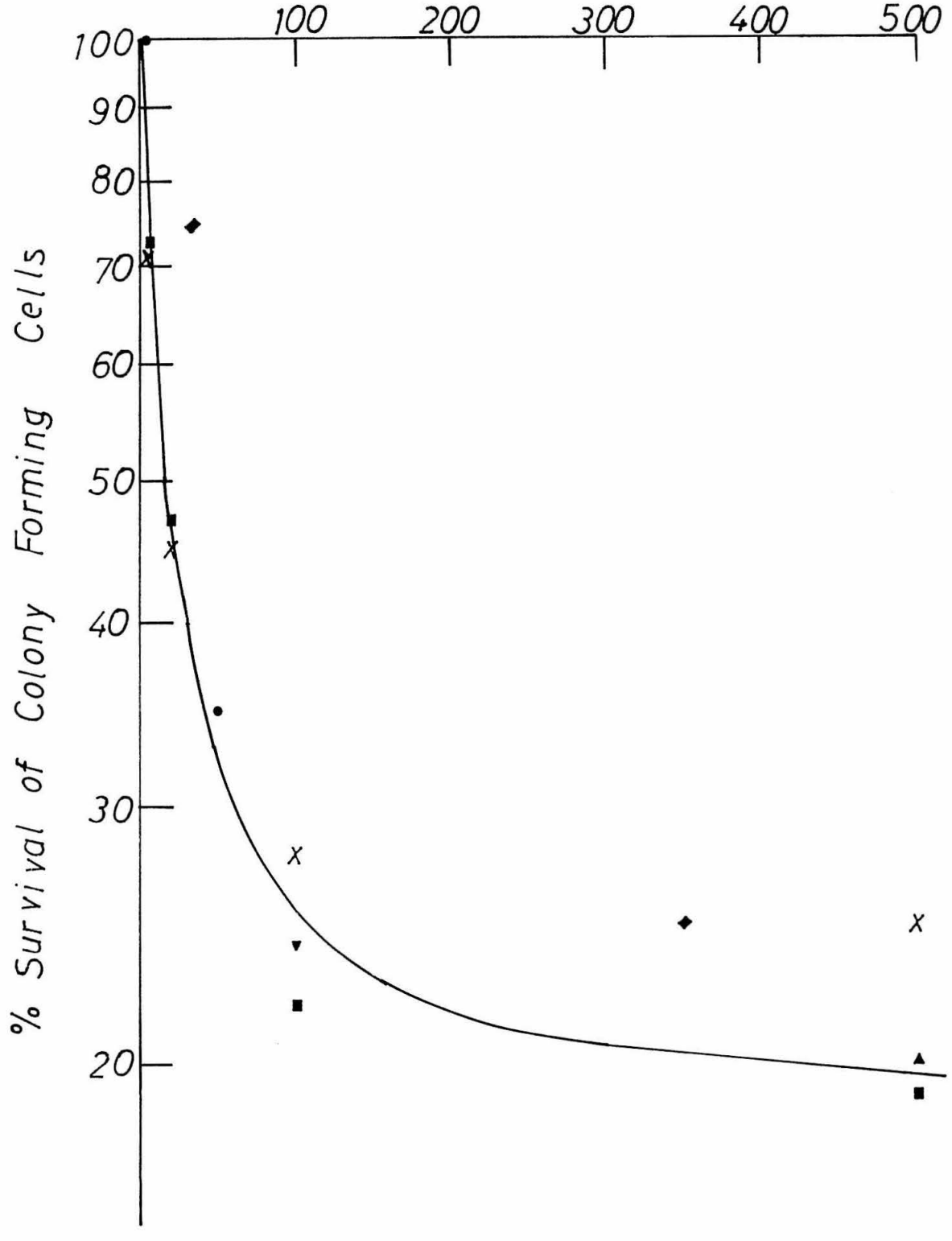
$$\text{Fraction of surviving cells} = e^{-MOI}.$$

Figure 7. Multiplicity of Infection - Cell Survival Relationship

Mouse embryo cells were infected at the indicated multiplicities and then cloned on feeder layers in the presence of AS and RDE. A "mock-infected" control plate was plated at the same time to estimate the cloning efficiency of uninfected cells (this ranged between 6% to 25%, but was relatively constant for a given batch of cells). In several experiments, the sp and P16 strains of Py were used.

Symbol	Experiment Number	Virus Used
●——●	CIC-11	LP
▲——▲	CIC 18	LP
▼——▼	CIC-18	sp
◆——◆	CIC-18	P16
x——x	CIC-21	LP
■——■	CIC-24	LP

Fig. 7


MOI
100
200
300
400
500
100
90
80
70
60
50
40
30
20
% Survival of Colony Forming Cells

On the scale of Fig. 7, the curve of the equation would be a straight line passing through the origin and almost parallel to the ordinate. At multiplicities above 100 pfu/cell, the curve appears to plateau at about 20 to 30% survival. However, in an experiment in which cells were infected with a purified stock of the p 16 strain of Py at a MOI of 5000 pfu/cell, the survival decreased to 3%. This suggests that the resistant cells can be killed if the multiplicity is high enough.

Two other conclusions can be derived from these data: (1) The three different virus strains used -- lp, sp and p16 - have a similar cell killing ability per pfu; and (2) In some experiments the proportion of cells killed and the proportion of infective centers were in disagreement (Table 6). The latter result suggests that in some cases cells are killed without becoming virus producers. This point cannot be conclusively established from this type of experiment, however, because the proportion of cells killed is determined in a selected fraction of the population; whereas the proportion of infective centers is determined in the whole population.

A possible objection to the results of the cell killing experiments concerns the possibility of cell loss by secondary infection on the cloning plate. The most likely source of such loss is through the conversion of superficially adsorbed virus into infecting virus. Because the level of superficially adsorbed virus per cell after extensive washing and AS and RDE treatment is about 0.1% of the input

Table 6. Relation Between the Proportion of Infective Centers and Cell Killing.

Mouse embryo cells were infected and then plated for infective centers and cloned -- both as described in the text. The proportion of infective centers is uncorrected for superficially adsorbed virus. "A & P early" or "A & R late" refers to the time at which AS and RDE treatment was begun. These times are given in Table 2. An uninfected sample of the same batch of cells was always cloned at the same time in order to determine normal cloning efficiency.

Table 6

Experiment	MOI	% IC	% Cells Killed	AS and RDE Treatment (See Table 2)
CIC-11	50	19	65	A & F early
	5	9	10	
CIC-21	500	--	75	A & R late
	100	--	72	
	20	52	55	
	4	25	29	
CIC-24	100	17	79	A & R early
	100	31	81	A & F late
	100	--	81	No A & R (except on the cloning plates)
	20	27	53	A & R late
	4	--	28	A & R late
	0.2	7	--	A & R late

multiplicity, the proportion of cells killed at MOI greater than or equal to 500 pfu/cell should be regarded as a maximum value.

B. The Properties of Cells Surviving Infection with Py

The results of the previous section have shown that a substantial fraction of the clone forming cells in mouse embryo cultures is capable of proliferation after a single cycle of virus growth. Among the cells which escape killing by virus in spite of continued exposure to it, transformed cells finally arise.[44] These transformed cells, the properties of which have already been extensively described (see Introduction and Glossary) have a well defined morphology, growth pattern and resistance to the cytocidal effects of Py infection, quite distinct from those of uninfected cells. In this section, an attempt will be made to define the time of appearance of morphologically transformed cells in the infected cultures. A secondary, but related, goal will be to define the time of appearance of virus-resistant cells in the infected cultures.

Several technical achievements facilitated this work. One of these is the method for purifying and concentrating Py which allows the use of very high multiplicities of infection and the removal of inhibitors from virus preparations:[76] the other is the development of the methods, described in Section A, which enable us to cut down reinfection and thus to define more precisely the condition of infection of the cells. These developments allowed us to probe more deeply and

more critically into the induction of the initial transformed cells.

Before proceeding, the results will be summarized: (1) Transformed cells were detected in steady state cultures at thirty to forty days after infection. However, exploratory studies have suggested that under certain conditions, these cells can arise as early as two weeks after infection. (2) Numerous experiments designed to reveal the presence of transformed cells arising after a single cycle of infection were uniformly negative. (3) The surviving cells of cultures in which only one or a few cycles of virus growth had taken place did not develop the transformed character or segregate transformed cells even if they were cultivated for an extended period in antiviral media. (4) It proved possible by such cultivation to free these surviving cells completely of infective virus and to test their sensitivity to reinfection. Their sensitivity to reinfection with Py was similar to that of uninfected cells.

We shall now proceed to the documentation of these observations.

1. The properties of the early descendants of cells surviving infection with Py

The general procedure here has been to clone cells from infected cultures and to score for the presence or absence of transformed colonies: those colonies showing non-regulated growth, and a transformed cell type (see Glossary).

In an early series of experiments designed to determine the

time of appearance of transformed cells in steady state cultures, cells were cloned from infected cultures at various times after infection. The results, represented in Table 7, show that no transformed cells were detected in samples taken as late as seventeen days after infection, irrespective of the experimental conditions employed. Transformed cells were, however, detected in a culture which was cloned at forty days after infection.

The considerable delay in the appearance of transformed cells may be attributed to either of two characteristics of these experiments: either the low virus input which may render transformation improbable, or the continued presence of virus in the cultures, which could kill the transformed cells if they were virus-sensitive. It was possible to test both of these hypotheses by infecting cultures with high titer virus, treating them with antiviral medium, and then plating the cells for colonies. In these experiments, the effects on transformation of omitting feeder layers were also investigated. The results of a series of such experiments are given in Table 8. The results show that among the cells surviving infection, none were found which were capable of forming transformed colonies, irrespective of the conditions used.

Two points should be emphasized concerning these experiments: (1) A line derived from the survivors of infection of a culture at high MOI (line CIC-11-37) failed to show transformation when infected for a second time (Table 8, experiments CIC-14 and CIC-15); and (2) In several of these experiments the sp and P16 strains of Py were used.

Table 7. The Appearance of Transformed Cells in Py-Infected Mouse Embryo Cultures.

The cells were infected at an input multiplicity of approximately 5 pfu/cell. At the indicated times, they were washed, in order to reduce the background virus level, and then cloned. The conditions of plating, other than those specified in the table, are described under Methods. RDE was added to the specified cloning plates at concentrations of 0.005 to 0.01 units.

Note (1): The infected culture used in experiment CIC-6 was a cloned mouse culture (clone 143) which had been growing in vitro for 19 weeks at the time of infection. (The isolation of this clone is described in Part II of the Results section of this thesis.) At the time of infection, clone 143 was growing as a regulated culture composed of rather broad, flat cells, with a doubling time of 2.5 - 3 days.

Note (2): The "controls" used in experiments CIC-5, CIC-19, and CI-9 were freshly isolated, uninfected mouse embryo secondary cells. Therefore, their only function is to serve as a check on the cloning conditions.

Note (3): The cloning efficiencies of the infected cultures are only approximate values. It was difficult to count colonies deriving from such cultures, because many colonies contained a large proportion of degenerating cells and they were generally smaller than colonies deriving from uninfected cultures.

Table 7

Experiment	Time of plating (days after inf.)	Conditions of cloning: medium	Conditions of cloning: feeder	Eff. of cloning (3) %	No. cells plated	No. of transformed clones
CIC-1	<1	Eagle's	None	1.8×10^{-2}	3.7×10^{5}	0
	Controls	Eagle's	None	3.1×10^{-2}		
	<1	CMRL	None	0.44	3.7×10^{5}	0
	Controls	CMRL	None	0.8		
CIC-6 (1)	<1	PEM+RDE	None	5×10^{-3}	2.6×10^{5}	0
C1-18	<1	PEM	Mouse	8.3	7.1×10^{2}	0
	Controls	PEM	Mouse	8.6		
	<1	PEM	Chick	2.0	7.1×10^{2}	0
	Controls	PEM	Chick	4.1		
CIC-5	6	PEM+RDE	Mouse	0.56	1.4×10^{4}	0
	Controls	PEM+RDE	Mouse	15.6		
	6	PEM+RDE	None	5×10^{-3}	2.5×10^{5}	0
CIC-2	12	Eagle's	None	2×10^{-3}	2.5×10^{5}	0
	12	CMRL	None	5×10^{-3}	2.5×10^{5}	0
CIC-19	17	PEM	Mouse	1.2	3×10^{3}	0
	Controls (2)	PEM	Mouse	2.5		
	17	PEM	Hamster	0.1		
	Controls (2)	PEM	Hamster	0.3		
C1-9	40	Eagle's	Mouse	0.3	1.1×10^{4}	6
	Controls (2)	Eagle's	Mouse	0.5		

Table 8. Transformation After Infection of Mouse Embryo Cultures with High Titer Virus.

Confluent monolayers of mouse embryo cells were infected with virus at the indicated multiplicities. After an adsorption period of 1-1/2 to 2 hours, the inoculum was removed, the plates washed three times with warm medium and 2 cc of PEM containing 10% inactivated calf serum added. Anti-viral serum and RDE were then added to final concentrations of 1:50 and 1:100, respectively, except in the case of experiment CIC-21 when serum and enzyme were added at 10 hours after infection. The cells were washed again, trypsinized, counted, diluted and plated between the eighth and twentieth hour after infection. The irradiated feeder layers were prepared as indicated in Methods and were preincubated for at least two hours in medium containing antiserum in the concentrations used above. The infected cells were plated directly into this medium. When no feeder layer was used, the cell suspension was added to a bare petri dish containing this medium. Colonies were counted under the microscope at 8 to 10 days after plating and scanned again for the presence of transformed colonies at 14 days. The number of cells plated per dish never exceeded 1.5×10^5.

Note (1): The presence of multilayered, criss-cross growth in a loose network, or the presence of dense, piled, foci were used as criteria for transformed colonies. When there were more than 200-300 colonies/plate, individual colonies could not be distinguished, but the entire plate was inspected (both microscopically and macroscopically) for the presence of brownish, raised areas.

Note ②: Line CIC-11-37 (see Table 11, experiment (b)) was derived from the survivors of 1.2×10^4 infected mouse embryo secondary cells which were plated under antiserum and F DE on a bare dish. When it was used in experiments CIC-14 and CIC-15, it was free of any carried virus. Confluent plates of this line generally contained about 10 times fewer cells than confluent mouse embryo plates.

Table 8

Experiment	Input MOI (pfu/cell)	Virus Type	Eff. of Cloning (%)	No. of Transformed Clones (1)	No. of Cells Plated	Special Conditions
a. Plating of freshly infected mouse embryo cells on feeder layers in the presence of RDE and antiserum.						
CIC-11	500	lp	1.9	0	4.5×10^3	
	0	-	5.5			
	0	-	6.0			No RDE or AS
CIC-15	500	lp	0.48	0	1.1×10^4	Line CIC-11-37 (2) was used in its 9th transfer
	0	-	2.2			
CIC-18	500	lp	1.8	0	2.3×10^4	
	200	sp	2.1	0	2.3×10^4	
	500	P16	2.2	0	2.3×10^4	
	0	-	9.1			
	0	-	8.9			No RDE or AS
CIC-21	500	lp	3.7	0	2.9×10^4	
	5000	P16	0.5	0	4.6×10^5	
	0	-	14.9			

Experiment	Input MOI (pfu/cell)	Virus Type	Eff. of Cloning (%)	No. of Trans-formed Clones	No. of Cells Plated	Special Conditions
b. Plating of infected mouse embryo cells on bare dishes in the presence of RDE and antiserum.						
CIC-11	500	lp	c0.2	0	$3x10^5$	
	0	-	c1.5			
CIC-14	500	lp	$1.3x10^{-3}$	0	$1.8x10^4$	Line CIC-11-37 ② was used in its 8th transfer.
	0	-	c0.4			
CIC-18	200	sp	c0.15	0	$3.1x10^5$	
	500	P16	c0.15	0	$3.1x10^5$	
	0	-	c0.9			
CIC-21	5000	P16	$c7.9x10^{-3}$	0	$5.5x10^5$	
	0	-	0.8			

These strains appear to have a higher probability to cause transformation per pfu than does lp virus when they are tested on hamster embryo cells.[89] Nevertheless, transformation was not observed.

In an effort to increase the efficiency of transformation, a series of experiments was carried out by utilizing a technique that has proved useful in the detection of transformed cells in the hamster system (transformation in situ).[89] The cells were infected, washed and overlaid with nutrient medium containing agar. Thereafter, the plates were treated as cloning plates. The object of this procedure was to avoid the use of trypsin, which may have deleterious effects on the cells. In this experimental series, the possibility that AS and RDE treatment might be obscuring or preventing the formation of transformed colonies was investigated: to test this point, experiments were performed in which the AS and RDE treatment was omitted. Other experiments of this series were designed to investigate a range of virus and cell concentrations. In fact, no transformed colonies appeared under any of the described conditions (Table 9).

By using transformation in situ, as described above, we tested for the possibility of transforming a clonal line of mouse embryo cells, clone 143 (see Table 12, Part III), which had been cultivated in vitro for approximately nine months at the time of the experiment: again there was no evidence of transformation.

From the experiments presented above, it is concluded that no transformed cells, as previously defined, can be detected after exposure

Table 9. Attempts to Induce Transformation in situ.

Plates of mouse embryo cells were prepared at the indicated cell densities. Twelve hours after plating, the cells were infected with 0.1 cc of the P16 strain of Py at the indicated concentrations. After adsorption, the plates were washed three times, and 2 cc of cloning medium containing 10% inactivated calf serum was added. AS and FDE (where indicated) were added to the plates at concentrations of 0.02 and 0.01 units, respectively, 10 to 12 hours later. After a 1 to 2 hour AS and FDE treatment, 4 cc of medium containing 0.5% agar (plus AS and FDE where indicated) was added to each plate. The plates were scanned for colonies at 9-10 days and again at 14 days after plating.

Note ①: No transformed colonies appeared in any of these experiments.

Note ②: NT = Not Tried.

Note ③: In experiment CIC-22, when confluent (cell conc. = 2×10^6/plate) and near confluent (cell conc. = 10^6/plate) cultures were infected and then treated with FDE and AS, the resulting low grade, but generalized, degeneration made it difficult to observe the morphology and growth pattern of the surviving cells. The same plates in the untreated series showed much more severe degeneration, but the killed cells tended to detach from the dish and were largely removed when the plates were fluid changed. When fewer than 10^6 cells/plate were used, fewer total cells degenerated and observation of the surviving cells was not obscured.

Note ④: A permanent clonal line of mouse embryo cells, line 143, was used in experiment CIC-23. This line consisted of relatively broad, flat cells which displayed strictly regulated growth. Approximately 10^6 cells could be recovered from a confluent 65 mm petri dish of line 143. The generation time of these cells was about 1 division every 2 to 3 days which is about 5 times slower than freshly isolated mouse embryo cells.

Experiment	Virus Titer (pfu/cc)	Cell conc. (no. cells/ plate)	Number of Surviving Colonies ① AS and RDE added	AS and RDE not added
CIC-20	$5x10^{10}$	$4x10^5$	NT ②	2
	$2.5x10^{10}$	$4x10^5$	54	0
	0	$4x10^5$	NT	Confluent
	$5x10^{10}$	$2x10^5$	NT	0
	$2.5x10^{10}$	$2x10^5$	8	0
CIC-22 ③	$2x10^{10}$	$2x10^6$	Confluent	35
	$5x10^9$	$2x10^6$	Confluent	25
	$1.3x10^9$	$2x10^6$	Confluent	NT
	$2x10^{10}$	$1x10^6$	200-300	5
	$5x10^9$	$1x10^6$	Confluent	12
	$1.3x10^9$	$1x10^6$	Confluent	NT
	$2x10^{10}$	$5x10^5$	200-300	0
	$5x10^9$	$5x10^5$	Confluent	6
	$1.3x10^9$	$5x10^5$	Confluent	NT
	$1.3x10^9$	$2.5x10^5$	200-300	4
	0	$2.5x10^5$	Confluent	Confluent
CIC-23 ④	$2x10^{10}$	$8x10^5$	0	NT
	$1x10^{10}$	$8x10^5$	7	NT
	$5x10^9$	$8x10^5$	29	NT
	10^{10}	$4x10^5$	0	NT
	$5x10^9$	$4x10^5$	0	NT
	$5x10^9$	$2x10^5$	0	NT
	0	$2x10^5$	Confluent	NT

Table 9

of mouse embryo cultures to a single cycle of virus growth.

2. The properties of the distant descendants of the surviving cells in Py-infected cultures

The results of the previous section raise the question of whether the infection produces unexpressed transformed cells from which recognizable transformed cells derive after a certain number of cell generations.

An attempt to answer this question was made by cultivating infected cultures or selected groups of cells from infected cultures in antiviral medium. This procedure has two advantages: (1) by suppressing virus multiplication, it provides favorable conditions for observing the morphology and growth pattern of the infected cultures and (2) it ensures that any unexpressed transformed cells present at the start of the treatment will not be destroyed due to reinfection.

In our first experiment, six "islands" of surviving cells in a seven day old infected culture were isolated and grown in the presence of 0.005 units of RDE. These islands are colony-like areas which contain both healthy and degenerating cells and which are frequently observed in steady state cultures. Each of the islands selected for study initially consisted of one to two thousand cells. The isolation procedure was similar to that used for picking colonies (see Methods) except that feeder layers were not used. These six cultures are described in Table 10. The results show that no transformed cells

Table 10. Growth of Infected Cultures in the Presence of PDE.

"Islands" composed of healthy and degenerating cells were picked as described in the text from a culture infected seven days previously with Py. The cells were cultivated in the presence of 0.005 units of PDE and regularly observed.

Note(1): The supernatants of the indicated cultures were sampled at 26 days after infection.

Table 10

Culture number	Virus release① (pfu/cell/day)	CPE	Predominant cell type at the final observation	Growth pattern of cells at the final observation	Observation period (days)
1	0.05	-	Broad, flat fibroblasts	Regulated	33
2	0.05	-	Broad, flat fibroblasts Perhaps a small proportion of more elongated, refractile cells at 81 days after infection.	Regulated	81
3	0.05	-	Broad, flat fibroblasts	Regulated	33
4	0.05	-	Broad, flat fibroblasts	Regulated	33
5	2.5	+	Mostly degenerating cells	No diagnosis possible	33
6	18	+	Surviving cells seemed to be rather broad and flat.	A few regulated colonies	42

arose in cultures derived from these islands of surviving cells. For reasons not understood at present, virus multiplication was less efficiently inhibited in cultures 5 and 6 than in the other four cultures. Little net cell growth was observed in these two cultures.

The failure to detect unexpressed transformed cells in the previous experiment may be attributed to the cultivation of selected groups of cells from the infected culture. Therefore, a non-selective experiment was carried out. RDE (0.005 units) was incorporated into the culture medium of an entire plate of infected cells on the sixth day after infection. The culture was maintained and regularly observed. It was found that there was little or no cell killing during a fifty-two day period and virus production was considerably reduced. At the last observation, no areas of non-regulated growth or cells with transformed morphology could be observed in the culture. Therefore, it is concluded that no unexpressed transformed cells were present in the culture when the treatment with antiviral medium began. This statement should be qualified by the recognition that if unexpressed transformed cells were rare and selected against, they might not have been detected.

Other experiments designed to detect transformed cells were carried out by employing two technical modifications: (1) AS, in addition to FDE, was incorporated into the antiviral medium in order to obtain a more complete inhibition of virus multiplication and (2) at

each transfer of an infected culture, several extra cultures were initiated with 2 to 8 x 10^4 cells. It was found[65] that these sparse cultures were useful for detecting Py-transformed hamster cells. The main lines of the cultures, however, were maintained by transferring heavily seeded cultures.

The results of four experiments in which these modifications were utilized are described in Table II. The results of these experiments are summarized as follows:

(1) In a culture maintained in the presence of non-antiviral medium, the first transformed colonies were found in sparse cultures initiated at thirty-two days after infection (experiment a).

(2) After a brief exposure to virus, followed by growth in antiviral medium, neither selected survivors (experiment b) nor an entire culture of infected cells (experiment c) showed transformation. Therefore, unexpressed transformed cells were not present in these cultures.

(3) In a culture exposed to antiviral medium at thirteen days after infection, transformed cells were immediately detected (experiment d).

The results of the first three experiments confirm the results previously obtained concerning the delayed appearance of transformed cells. Experiment (d), however, suggests that transformed cells can, under some circumstances, appear within two weeks of infection. Two

Table 11. The Time of Appearance of Transformed Cells in Py-Infected Mouse Embryo Cultures.

Cells from four different infected cultures were used to seed sparse plates at the indicated times after infection. Antiviral or regular medium was added to these sparse plates as indicated. The plates were inspected microscopically and with the naked eye for colonies of transformed cells up to two weeks after they were initiated. The four infected cultures from which the cells were taken are listed below:

Experiment (a): Transformation of infected cells grown in the presence of regular medium. - A mouse embryo culture was infected with Py at a multiplicity of 500 pfu/cell and thereafter maintained in regular medium.

Experiment (b): Transformation of cells selected from an infected culture and grown in the presence of antiviral medium. - A culture was infected with Py at a multiplicity of 500 pfu/cell, and then immediately treated with AS and RDE and plated for transformed colonies in the absence of a feeder layer as described in Table 8, Experiment CIC-11. A cell line (CIC-11-37) was initiated from a plate containing twelve surviving colonies (the efficiency of plating was 0.1%). This line was thereafter carried in antiviral medium.

Experiment (c): Transformation of cells first exposed to antiviral medium at four days after infection. - A culture was infected with the P16 strain of Py at a multiplicity of 200 pfu/cell. Four days later, the culture was transferred and was thereafter maintained in antiviral medium.

Experiment (d): Transformation of cells first exposed to antiviral medium at thirteen days after infection. - A duplicate of the culture used in experiment (c) was transferred at thirteen days after infection and was thereafter maintained in antiviral medium.

Note (1): Mouse embryo cells tend to form rather diffuse colonies in liquid medium. Therefore, only a rough estimate of the number of colonies can be made.

Note (2): These transformed colonies were counted macroscopically as raised papillae in the monolayer.

le 11

		Number of colonies[1] on sparse plates	Number of transformed colonies on sparse plates
		0	-
		0	-
		0	-
		5	1
		> 200	47[2]
	∓	> 200	38[2]
47	-	> 200	200[2]
	+	> 2 00	200[2]
Experiment (b)			
20	+	> 200	0
27	+	> 2 00	0
34	+	> 2 00	0
	-	> 200	0
39	+	> 200	0
45	+	> 200	0
50	+	> 200	0
	-	> 200	0
56	-	> 200	0
61	-	> 200	0

Time after infection (days)	+ or - AS and RDE on sparse cultures	Number of colonies on sparse plates	Number of transformed colonies on sparse plates
Experiment (c)			
4	+	> 200	0
8	+	> 200	0
13	+	> 200	0
18	+	> 200	0
22	+	> 200	0
	-	> 200	0
27	+	> 200	0
	-	> 200	0
Experiment (d)			
13	+	100	4
	-	~15	0
20	+	200	40 ②
	-	~20	0

conditions of this experiment which could have been important in determining the results should be emphasized: (1) The use of antiviral medium to protect early arising transformed cells; and (2) the use of the P16 strain of Py which, as already mentioned, may be more efficient than the large plaque type in causing transformation of hamster cells. Further experiments are needed to decide whether any of these conditions were crucial for the result obtained.

3. The sensitivity to reinfection of cells surviving infection

In order to assess properly the results just presented, it is desirable to know the sensitivity to Py infection of the earliest appearing transformed cells. In the next paragraphs, evidence will be presented which shows that the morphologically unchanged descendants of cells surviving a brief exposure to Py are not resistant to the cytocidal effects of reinfection. This fact suggests that a brief exposure of a culture to infection does not detectably induce or select for genetically virus-resistant cells. Further experimentation, however, is necessary to determine the virus sensitivity of the earliest detectable transformed cells.

To document these statements, two experiments will be presented:

(1) The initiation of culture CIC-11-37 from the selected survivors of an infected culture has been described (Table 11, experiment (b)). After cultivation in antiviral medium for one month, it no

longer produced infective virus. A cell killing experiment, carried out at 56 days after the original infection (Table 7, experiment CIC-15), showed that the cured line CIC-11-37 was as sensitive to cell killing by Py as were comparably infected mouse embryo cultures.

(2) A cell line was derived from the unselected surviving cells of a culture which was exposed to infection for four days (Table 11, experiment (c)). After cultivation in antiviral medium for three weeks, it no longer produced infective virus. When this cured culture was reinfected at four weeks after the original infection, its total single cycle virus yield was similar to that of comparably infected mouse embryo cultures. Therefore, there could be no gross difference between the proportion of virus resistant cells in the infected cell line and that in uninfected mouse embryo cultures.

These findings indicate that the resistance to the cytocidal effects of Py displayed by a fraction of the cells in infected cultures (section A) is not a heriditary property of the cells. In fact, colonies and mass cultures deriving from cells which survive a brief exposure to Py at high MOI are not detectably different from freshly infected mouse embryo cells in cell morphology, growth pattern and sensitivity to Py infection. The findings also show that in the steady state culture, the virus-cell relationship is essentially that of a carrier culture.

PART II. TRANSFORMATION IN CLONAL MOUSE EMBRYO CULTURES OCCURRING AFTER PROLONGED EXPOSURE TO POLYOMA VIRUS

The experiments presented in this section were designed to determine some of the factors responsible for the delayed appearance of transformed cells after virus infection and to further characterize the transformed cells themselves. In the course of these studies, it was found that uninfected mouse embryo cultures frequently underwent a rapid transformation to a non-regulated growth pattern. Therefore, experiments designed to investigate the difference between the changes occurring in infected and those occurring in uninfected cultures were also carried out.

The experiments were initially designed to test the following hypothesis concerning the delay in the appearance of transformed cells following Py infection: that the capacity to be transformed by Py is a heritable trait of mutational or embryological origin possessed by only a few cells preexisting in the uninfected population. By "embryological origin" we mean a cell trait which arises as a result of differentiation during embryological development. These cells can be defined as "transformable variants." The hypothesis can be tested by observing the response to infection of cultures recently derived from a single cell. If the hypothesis were correct, such clonal cultures would be expected either to show very rapid transformation or no transformation depending on whether the original cells were or were not transformable variants.

A. Preliminary Study

In a preliminary experiment, cells from a secondary Swiss mouse embryo culture were cloned. A single colony was picked and grown into a new culture: culture 1. Five weeks after cloning, when the clone had attained a size of 2×10^6 cells, the culture was divided in half: one half (culture Py 1) was infected with a tissue culture stock of Py at an input multiplicity of five pfu/cell; the other half (culture 1) served as a control. The overall growth curves of these two lines are given in Fig. 8. The rate of virus production, the time at which multilayer growth was first observed, and the time at which culture Py-1 was judged to consist mainly of transformed cells are also shown on this graph.

The curves show that for approximately the first four weeks, culture Py 1 remained in the steady state with abundant virus production. By the eighth week, its doubling time was twenty-four hours. This behavior is typical of Py infected, uncloned mouse embryo cultures.[52]

Culture 1 grew slowly with a generation time of three to four days for the first five weeks, then, over a period of about two weeks, increased its generation time to forty hours. By the fifteenth week, culture 1 was growing as rapidly as culture Py 1.

By nine weeks after infection, the two lines differed both in cell morphology and growth pattern. Culture Py 1 cells (Fig. 9a)

Figure 8. Overall Growth Curves of Cultures 1 and Py 1.

The overall growth curves of cultures 1 and Py 1 are given. The virus production, time of appearance of non-regulated growth, and the time of appearance of morphologically transformed cells are given for culture Py 1.

Fig. 8

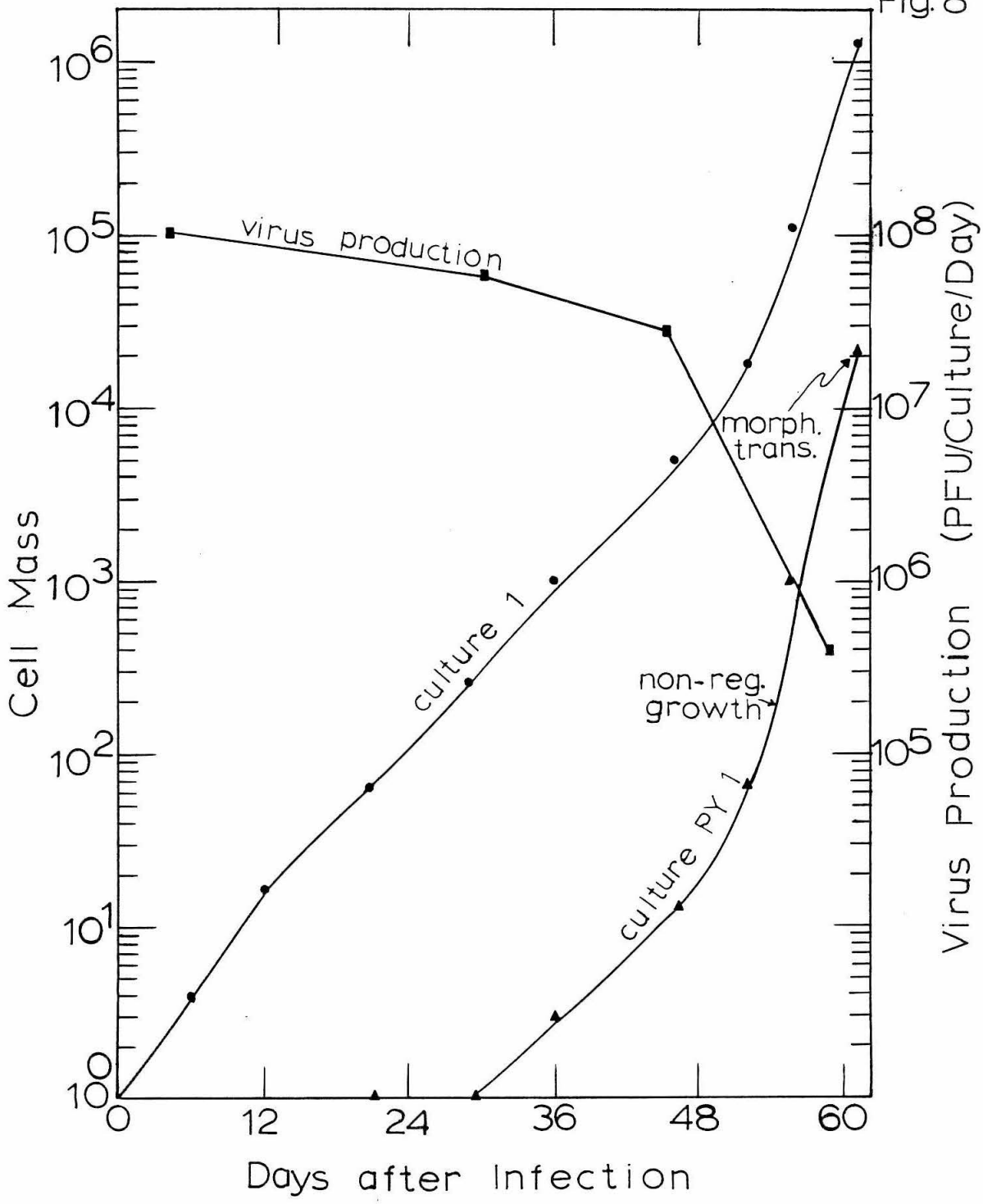
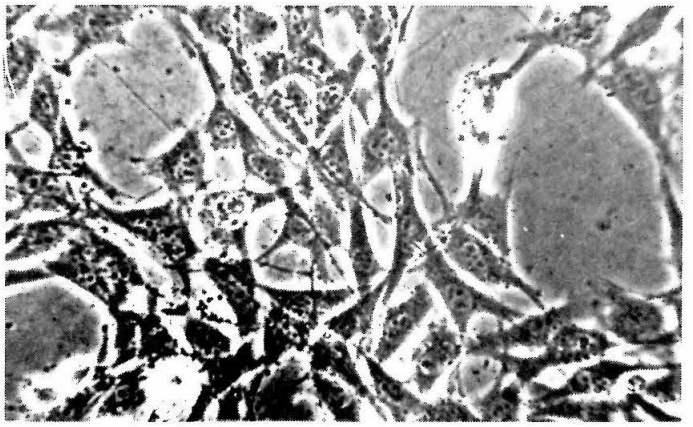

Cell Mass
Virus Production (PFU/Culture/Day)
Days after Infection
virus production
morph. trans.
culture 1
non-reg. growth
culture PY 1
10^0
10^1
10^2
10^3
10^4
10^5
10^6
10^5
10^6
10^7
10^8
0
12
24
36
48
60

Figure 9. The cell Morphology and Cell Orientation of Clonal Cultures 1 and Py 1.

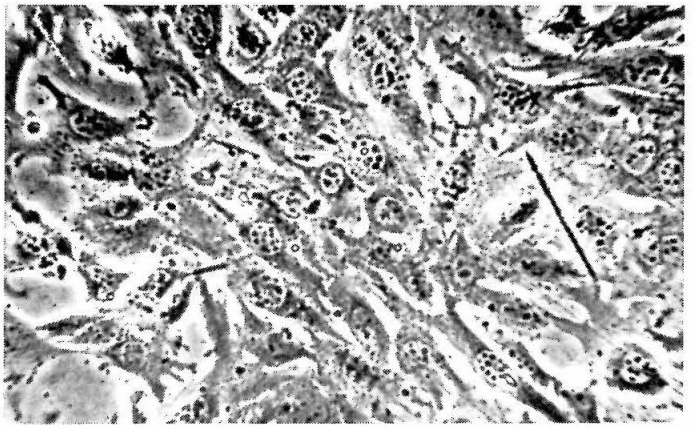
Phase contrast, x 200.

(a) Culture Py 1

(b) Culture 1.



(a)



(b)

Figure 9

looked refractile, were elongated with a triangular shape, and tended to grow in netlike, randomly oriented arrays. If culture Py 1 was not transferred when it became confluent, its cells piled up and formed a mat several layers thick. Thus, the cell morphology and growth pattern of culture Py 1 were identical to those of Py-infected, uncloned mouse embryo cultures.

Culture 1 cells, on the other hand, looked flat, were similar in shape to freshly isolated cells, and tended to grow side-by-side in parallel bundles rather than in a netlike array. For the first eighteen to twenty weeks, culture 1 showed regulated growth. At this time, however, it was noted that a confluent plate of culture 1 showed discrete areas of multilayered growth; after a few more transfers of this line, confluent plates showed generalized non-regulated growth. In spite of the acquisition of a non-regulated growth pattern, the cells of culture 1 retained their oriented cell arrangement. The morphology of the cells was that of rather short, broad fibroblasts (Fig. 9b), quite unlike that of Py-transformed cells.

The observation that a clonal culture shows the same general behavior after Py infection as do uncloned mouse embryo cultures renders doubtful the hypothesis that the delay in transformation following Py infection is due to the low frequency of transformable variants in the population. However, if it is assumed that transformable variants arise frequently in the population by mutation, it could then be argued

that culture 1 had, by chance, as low a proportion of transformable variants as have uncloned mouse embryo populations. Therefore, further experiments were needed to rule out this hypothesis.

The rapid occurrence of spontaneous changes in culture 1 raised another type of question concerning Py-induced transformation: i.e., whether Py transformation is due to an induced cell change following infection of a cell with the virus or due to selection of a spontaneously changed cell.

B. Further Studies on Transformation in Clonal Cultures

In view of these arguments, it seemed desirable, on the one hand, to extend the studies of the neoplastic transformation of clonal cultures to a much larger number of cultures and, on the other hand, to broaden the approach by using cells derived from an inbred mouse strain. The latter precaution allows the study of an additional criterion of transformation, both Py-induced and spontaneous: tumor production in the animal.

The studies were primarily designed to test the previously presented hypothesis of "transformable variants" with the additional assumption that transformable variants can arise by mutation during the growth of a clone. This hypothesis can be tested by observing the time interval between infection and transformation in clonal cultures: this interval should depend on the frequency of transformable variants in the clonal culture. If the variants arose by mutation and were not

strongly selected against, then their frequencies ought to fluctuate widely from clone to clone.[90] The critical finding in this test is the presence, among the various clones, of a clone which transforms much more rapidly than the average owing to an early occurrence of the mutation. In this experiment, a number of uninfected clonal lines were also observed in order to detect and study spontaneously occurring changes.

The experiment was initiated with a single embryo of the A/Sn strain, explanted in vitro. Cell suspensions obtained from this initial culture were plated for colonies at one and two weeks after explantation from the animal. Fifty colonies were picked and grown into clonal cultures of six to ten million cells. Plates of each clone containing two million cells were then infected with Py at a multiplicity of 10 pfu/cell. Control plates were treated with medium under identical conditions. Thereafter, the infected and uninfected portions of each clone were kept separate but were maintained under the same regime. The infected subline of each clone is designated by the prefix "Py" before the clone number.

The infected and the uninfected cultures were scanned microscopically every two or three days in order to check cell morphology, growth pattern, growth rate and virus-induced cell degeneration. The supernatant medium was regularly assayed for virus. Some of the uninfected and infected cultures were implanted into adult animals of the same inbred strain. The results, presented in Table 12, will be examined below.

Table 12. Some Properties of Py-Infected and Uninfected Clonal Mouse Lines

The clonal lines were maintained with cloning medium.

Note ①: Clones were numbered serially, but only certain clones were maintained in culture. Numbers prefixed by "Py" designate Py infected sublines.

Note ②: The recovery time of an infected culture is defined as the time between infection and the first subculture (in weeks).

Note ③: Non-Regulated (Non-Reg.) and regulated (reg.) growth patterns are described in the Glossary. Briefly, a plate is kept for 7 to 10 days after the cells have grown to confluency with regular fluid changing. If, at this time, brown, raised areas are seen upon macroscopic and microscopic inspection, the culture is said to have a Non-reg. growth pattern. If the cells have remained in a monolayer, the culture is said to have a regulated growth pattern.

Note ④: A morphologically transformed culture (see Glossary) is one in which the majority of the cells possess the triangular shape, high refractility and random cell orientation characteristic of transformed cells. All morphologically transformed cultures show Non-reg. growth.

Note ⑤: Unless noted, mice were given 425 r of X-irradiation and injected subcutaneously with 0.1 cc of a suspension containing 1.5 to 6 x 10^6 cells. (See Methods.) The results are expressed as number positive over number of mice injected. Mice were judged to be positive if they bore a palpable tumor by 10 wks. after injection. Mice developing tumors after this period are specifically noted. The mice were observed for at least 10 wks. after injection.

Note ⑥: A walnut-sized tumor is one with an average linear dimension of approximately 3 cm.

Note ⑦: Cell line frozen and thawed before infection (see Methods).

Note ⑧: Py 76" was maintained in Eagle's medium.

Note ⑨: Tumors arose more than 10 weeks after implantation in mice inoculated with cell lines Py 104 and Py 181.

Note ⑩: An autopsy of the mouse inoculated with Py 140 showed a large, firm whitish tumor in the liver.

Note ⑪: Cl 27 (clone 27) was a virus-free clonal subclone of line Py 143.

Note ⑫: Py 143 (at 20 wks. after infection), Cl 27, and Py 143, were injected into unirradiated mice.

Note ⑬: Py 160 and Py 204 were injected after freezing and thawing of the infected cell line.

Note ⑭: The time scale is reckoned as "weeks after explantation" for the uninfected lines and as "weeks after infection" for the infected lines.

Table 12

Clone No. (1)	Wks. in vitro before Py inf.	Recovery time for inf. cultures (2)	Type of growth: (wk. observed) (3) (14)	Morph. transformation of culture: (wk. observed) (4) (14)	Results of implantation (5)	Wk. of injection	Latent period (wks.)	Time to walnut size (wks.) (6)
70 (7)			Non-reg.:(24)	0: (25)	1/1	25	3	8
PY70	17	4	Non-reg.:(4)	+: (7)	1/1	10	6	10
71			Non-reg.:(25)	0: (25)	1/1	25	8	11
PY71	15	4	Non-reg.:(4)	0: (7)	-			
72			Reg.:(18)					
PY72	10	5	Non-reg.:(5)	+: (8)	-			
PY72'	16	6	Non-reg.:(6)	+: (8)	1/1	8	6	9
73			Reg.:(15)		-			
PY73	13	4	Non-reg.:(5)	+: (10)	1/1	10	6	9
76			Reg.:(25)		0/1	25		
PY76	7	No recovery						
PY76'	11	6	Non-reg.:(7)	+: (13)	0/1	14		
PY76'' (8)	13	3	Non-reg,:(9)	+: (10)	-			
77			Reg.:(15)		-			
PY77	9	9	Non-reg.:(9)	+: (13)	0/1	14		
85			Reg.:(26)		0/1	26		
PY85	16	8	Non-reg.:(10)	0: (10)	0/1	10		
88			Not Carried					
PY88	8	13	Non-reg.:(13)	+: (15)	1/1			
94			Reg.:(14)		-			
PY94	12	5	Non-reg.:(5)	+: (10)	0/1	8	3	Regressed

Clone No.	Wks. in vitro before Py inf.	Recovery time for inf. cultures	Type of growth: (wk. observed	Morph. transformation of culture: (wk. observed)	Results of implantation	Wk. of injection	Latent period (wks.)	Time to walnut size (wks.)
98			Reg.:(15)		–			
PY98	7	7	Non-reg.:(7)	+: (10)	–			
104			Reg.:(14)		–			
PY104	6	11	Non-reg.:(11)	+: (14)	0/1 ⑨	16	(11)	(14)
105			Reg.:(15)		–			
PY105	12	4	Non-reg.:(4)	+: (6)	1/1	8	4	6
114			Not Carried					
PY114	11	7	Non-reg.:(7)	0: (14)	0/1	14		
117			Reg. :(13)		–			
PY117	11	6	Non-reg.:(6)	+: (6)	1/1	10	3	7
118			Reg.:(14)		–			
PY118	10	5	Non-reg.:(5)	+: (8)	1/1	16	4	7
121			Not Carried		–			
PY121	14	Immed.	Reg.:(14)		0/2	9 & 11		
125			Reg.:(15)		–			
PY125	8	No recovery						
PY125'	11	3	Non-reg.:(5)	+: (8)	1/1	10	5	8
130			Reg.:(14)		–			
PY130	11	13	Non-reg.:(13)	+: (13)	0/1	16		
131			Non-reg.:(22)	0: (26)	0/1	26		
PY131	15	3	Non-reg.:(8)	+: (12)	1/1	12	6	9

Clone No.	Wks. in vitro before Py inf.	Recovery time for inf. cultures	Type of growth: (wk. observed	Morph. transformation of culture: (wk. observed)	Results of implantation	Wk. of injection	Latent period (wks.)	Time to walnut size (wks.)
132			Reg.:(14)		–			
PY132	9	4	Non-reg.:(5)	+: (8)	1/1	19	4	8
135			Reg.:(14)		–			
PY135	6	6	Non-reg.:(8)	+: (11)	1/1	15	3	7
137			Reg.:(15)		–			
PY137	12	5	Non-reg.:(5)	+: (5)	–			
140			Non-reg.:(15)	+: (17)	0/1	25		
PY140	11	7	Non-reg.:(7)	+:(11)	1/1	14	Cells inj. died at i.p. 8 wks. (10)	
143			Reg.:(22)		0/2	9 & 15		
PY143	7	6	Non-reg.:(6)	+: (9)	1/1	13	1	4
					2/2 (12)	20	1	4
C127 (11)	-	-	- -	- -	3/3 (12)	-	1	4
PY143' (7)	11	3	Non-reg.:(5)	0: (5)	0/1 (12)	5		
144			Reg.:(15)		–			
PY144	8	7	Non-reg.:(8)	+: (12)	–			
150			Non-reg.:(23)	0: (25)	1/1	25	4	8
PY150	16	3	Non-reg.:(4)	+: (5)	1/1	5	2	5
151			Reg.:(14)		–			
PY151	10	6	Non-reg.:(6)	+: (10)	0/1	16		
155			Not Carried					
PY155	9	9	Non-reg.:(9)	+: (13)	0/1	18		

Clone No.	Wks. in vitro before Py inf.	Recovery time for inf. cultures	Type of growth: (wk. observed)	Morph. transformation of culture: (wk. observed)	Results of implantation	Wk. of injection	Latent period (wks.)	Time to walnut size (wks.)
156			Reg.:(14)					
PY156	7	6	Non-reg.:(9)	+: (14)	1/1	20	1	4
159			Reg.:(14)					
PY159	10	9	Non-reg.:(11)	+: (13)	0/1	14		
160			Reg.:(14)					
PY160	10	6	Non-reg.:(10)	+: (14)	0/1 [13]	14		
161			Reg.:(27)		1/1	27	3	7
PY161	10	9	Non-reg.:(9)	+: (14)	1/1	15	6	8
165			Reg.:(15)					
PY165	10	7	Non-reg.:(7)	+: (12)	0/1	13		
171			Reg.:(16)					
PY171	11	4	Non-reg.:(4)	+: (7)	1/1	10	3	4
178			Reg.:(15)					
PY178	11	4	Non-reg.:(4)	+: (7)				
181			Non-reg.:(21)	0: (25)				
PY181	7	5	Non-reg.:(10)	0: (17)	0/1 [9]	13	(12)	(16)
182			Non-reg.:(19)	0: (24)				
PY182	9	3	Non-reg.:(5)	+: (10)	1/1	13	1	6
194			Reg.:(14)					
PY194	7	5	Non-reg.:(7)	+: (10)	0/1	15	2	Regressed
195			Reg.:(15)					
PY195	10	7	Non-reg.:(8)	+: (12)				

Clone No.	Wks. in vitro before Py inf.	Recovery time for inf. cultures	Type of growth: (wk. observed)	Morph. transformation of culture: (wk. observed)	Results of implantation	Wk. of injection	Latent period (wks.)	Time to walnut size (wks.)
204			Reg.:(15)		–			
PY204	7	6	Non-reg.:(7)	+: (9)	0/1 (13)	15		
210			Non-reg.:(19)		–			
PY210	10	3	Non-reg.:(8)	+: (9)	–			
213			Reg.:(15)		–			
PY213	9	8	Non-reg.:(9)	+: (11)	1/1	13	2	4
216			Reg.:(14)		–			
PY216	9	9	Non-reg.:(9)	+: (10)	1/1	13	2	6
218			Reg.:(15)		–			
PY218	11	6	Non-reg.:(9)	+: (15)	1/1	16	1	4
221			Non-reg.:(21)	0: (26)	1/1	26	3	7
PY221	9	8	Non-reg.:(9)	+: (15)	1/1	18	1	4
224			Non-reg.:(22)	+: (25)	1/1	25	9	12
PY224	9	5	Non-reg.:(8)	0: (11)	–			
232			Not Carried					
PY232	10	5	Non-reg.:(6)	+: (8)	1/1	12	2	6
234			Not Carried					
PY234	10	5	Non-reg.:(5)	+: (11)	–			
235			Reg.:(14)		–			
PY235	7	8	Non-reg.:(8)	+: (10)	–			
236			Reg.:(15)		–			
PY236	11	3	Non-reg.:(9)	+: (12)	1/1	14	3	7
87			Reg.:(25)		0/1	26		
222			Non-reg.:(16)	0: (27)	1/1	26	4	8

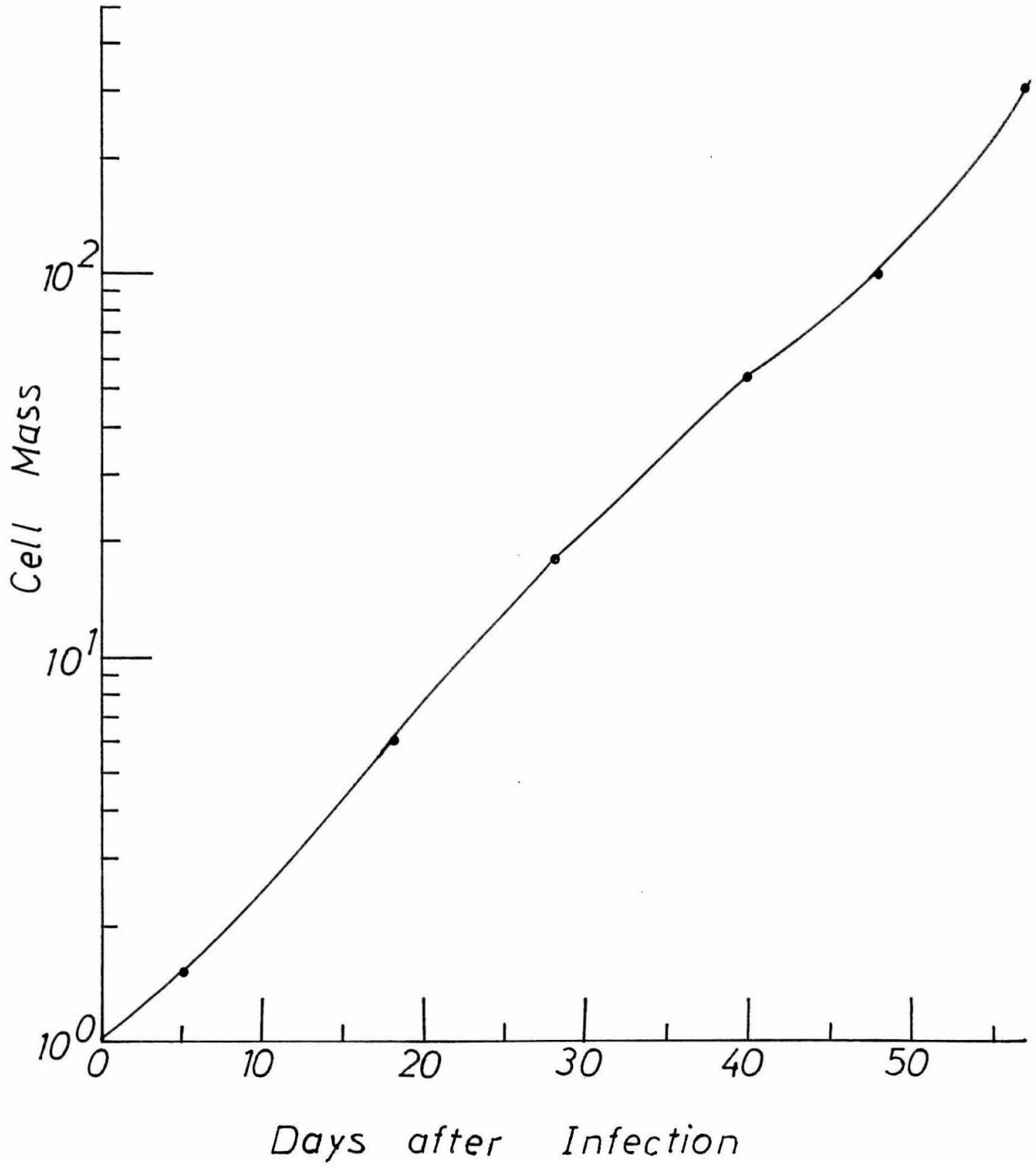
1. Changes in infected clonal lines

The clonal cultures were infected with virus between six and sixteen weeks after the original explantation from the animal. Forty-nine out of 50 tested cultures underwent a steady state period of extensive cell killing and abundant virus proliferation, at least three weeks in duration, during which they could not be subcultured. In all cultures susceptible to the cytocidal effects of Py, cells capable of producing areas of non-regulated growth arose. This occurred in most but not in all cases within three weeks after the first subculture, but never before four weeks after infection. In all but two cases, the non-regulated cultures were overgrown by morphologically-transformed cells within one to four weeks after the areas of non-regulated growth were first observed.

The length of the steady state period was variable: the cultures which recovered most rapidly from the cytocidal effects of infection could be subcultured at three weeks, but some required a much longer time. The main causes of this variability are not known, but one clone (121) responded in such a way as to suggest that genetically virus resistant variants in the cell population can play a role in the response of the culture to infection. The cultures of this line responded to Py infection with neither marked cell degeneration, nor transformation: they continued to grow regularly after infection as shown by the over-all growth curve (Fig. 10). Virus was continually produced also in

Figure 10. Overall Growth Curve of Culture Fy 121.

Fig. 10



this culture, although the amount of cell degeneration was small. Thus, the resistance of the cells to the virus was not absolute, and a stable virus carrier state was established. The single cycle virus yield of the cells of this clone after infection at an input multiplicity of 100 pfu/cell was less than 1% that of other cloned or uncloned cultures.

2. Changes in uninfected clonal lines

Changes in growth rate, in cell orientation and morphology, and in growth pattern were observed in the uninfected lines. The growth rate of all clones carried in vitro for longer than 16 weeks (Table 13) increased by a factor of two to five during this period. The cell orientation in several older clonal cultures became more disorderly. Disorder usually increased gradually and did not become extreme. However, two cultures acquired the morphological characteristics of Py-infected cultures: random cell orientation and triangular, refractile cell morphology.

Among the seventeen clonal lines that were carried in vitro for longer than sixteen weeks (Table 13), eleven acquired a non-regulated growth pattern. The time at which this change was first observed was as early as fifteen weeks. Six of the clones showed regulated growth when they were discontinued.

We shall examine the significance of these results for the two hypotheses already advanced, that of the transformable variants, and

Table 13. Characteristics of Long Term Clonal Cultures.

In this table we have abstracted data from Table 12 which pertain to the characteristics of those clonal cultures that were observed for more than sixteen weeks after explantation from the animal.

Table 13

Clone No.	No. of wks. in vitro when infected	No. of wks. in vitro when ML growth was observed		Final cell morphology		Results of implantation	
		Inf.	Control	Inf.	Control	Inf.	Control
70	17	21	24	Tr	Untr	1/1	1/1
71	15	19	25	Untr	Untr	NT	1/1
76	11	18	>25	Tr	Untr	0/1	0/1
85	16	26	>26	Untr	Untr	0/1	0/1
87	--	--	>25	--	Untr	--	0/1
131	15	23	22	Tr	Untr	1/1	0/1
140	11	18	15	Tr	Tr	1/1	0/1
143	7	13	>22	Tr	Untr	3/3	0/2
150	16	20	23	Tr	Untr	1/1	1/1
161	10	19	>27	Tr	Untr	1/1	1/1
171	11	15	>16	Tr	Untr	1/1	NT
181	7	17	21	Untr	Untr	0/1	NT
182	9	14	19	Tr	Untr	1/1	NT
210	10	18	19	Tr	Untr	NT	NT
221	9	18	21	Tr	Untr	1/1	1/1
222	--	--	16	--	Untr	--	1/1
224	9	16	22	Untr	Tr	NT	1/1

that of the selection of spontaneous transformed variants. We shall consider in the first place the relevance of the failure of finding rapidly transformable clones to the hypothesis of transformable variants.

This result, taken at face value, would show that transformable variants do not exist. There are, however, several conditions under which the transformable variants might not have been detected by the experiment if they existed. These conditions are: (1) the low probability of occurrence of a mutation leading to transformable variants; (2) killing of the early transformed cells by reinfection with Py; and (3) contra-selection of transformable variants. Further experiments are required to test the role of these conditions.

The relevance of the results so far obtained to the selection hypothesis can be discerned as follows. At first sight, the association of morphological transformation with virus infection (Table 13) seems to speak against selection; this is not so, however, because the transformed morphology could be a secondary characteristic of Py-resistant cells which tend to become selected for during the steady-state period. In fact, a similar phenomenon has been discovered in a different system: HeLa cell mutants which are resistant to infection with polio virus frequently possess an unusual morphology as well.[91] The observation that a non-regulated growth pattern usually appears earlier and more reproducibly in infected than in uninfected sublines of the same clone (Table 13) could also be explained in the same way.

However, more decisive evidence on the selection hypothesis was obtained from the study of the transplantability of infected and uninfected cell lines which will be considered in the next section.

3. The transplantability of uninfected and infected cell lines

A difference was found between the transplantability of uninfected and of Py infected cultures. Among the thirteen uninfected lines tested (Table 13), there was a definite, but not absolute correlation between the acquisition of a non-regulated growth pattern and the ability to give rise to a tumor upon implantation. In fact, seven of the thirteen lines were transplantable. One (line 161) of six lines showing regulated growth produced tumors, and six of eight lines showing non-regulated growth produced tumors. In evaluating the finding that a culture with a regulated growth pattern produced a tumor, it should be considered that a tumor can result from the growth of a very small proportion of tumorogenic cells in the population. For this reason, tumorogenic cells might have been present in line 161 although they were not detectable. Inasmuch as this line is no longer available, we are unable to test this possibility.

A strikingly different behavior was found in the infected clonal lines in which there was virtually no correlation between non-regulated growth and transplantability. Only a little over half of the forty tested cell lines were able to induce tumors, even though almost all showed non-regulated growth and were composed of morphologically-

transformed cells (Table 12). Thus, transplantability is not necessarily associated with Py-induced transformation.

The results indicate that some further change must occur, in addition to the acquisition of a non-regulated growth pattern and of morphologically transformed cells, before the infected lines become transplantable. This finding is substantiated by the results of experiments with the uncloned, parental culture (Py A/2). This culture was overgrown with morphologically transformed cells within eight weeks after it was infected. Nevertheless, implantation tests performed at various times after transformation had occurred did not give a positive result until nineteen weeks after infection.

In order to assess the significance of this finding, it must be recognized that a positive reimplantation test is much more significant than a negative result. A positive result is evidence that the cell line in question has acquired a new property: the ability to grow autonomously in vivo. A negative result, on the contrary, does not show that this property is absent: in fact a negative result could follow from a number of causes, of which some may be irrelevant to the response of the cells to the regulatory influences of the host. Prime among these causes is the possibility of an immune reaction by the host directed against a foreign antigen of the graft. This possibility must be considered very seriously since, as will be shown later, transplantable, Py-transformed cells have acquired a new, specific

antigen. That the immune reaction can decrease the transplantability of infected lines is shown by the observation that two lines (Py 161 and Py 182) out of five infected lines tested produced tumors in irradiated mice but failed to produce them in unirradiated animals. This result suggests that these lines differed antigenically from the hosts. Whether the failure of transplantation experiments with other infected lines in irradiated animals was due to inefficient suppression of the immune response by irradiation is unknown.

Since the infected lines were all virus carrier cultures, the immunological defenses of the hosts could have been enhanced in some cases by the virus injected with the cells. This possibility was tested experimentally by curing two non-transplantable lines (Py 130 and Py 159) of virus. This was done by a combination of passage at low cell concentrations and treatment with antiviral medium. The cured lines were found still unable to produce tumors upon implantation; thus the role of the carried virus cannot be too important.

On the basis of the previous considerations and findings, one may suspect that the infected lines to become transplantable must acquire some additional changes, in addition to the loss of response to regulatory influences of the host. For instance, the infected line may have to acquire a decreased sensitivity to the immunological defenses of the host. It is conceivable that the morphological characters for transformation as previously defined are adequate to detect the loss of response to regulatory influences but not the other

additional changes. It is therefore reasonable to search for other in vitro characters able to reveal these additional changes by their correlation with transplantability.

This was done with a number of lines which were selected as representative of various classes. These lines were selected on the basis of the following criteria (Table 14): Lines 70 and 150 were chosen because they seemed typical of uninfected cultures that had acquired transplantability. Five lines were selected among the transplantable, Py transformed lines as representative of various classes. Py 161 gave rise to tumors only after a long latent period; the other four lines produced rapidly growing tumors with a short latent period. Of these, clone 27 was a virus free line grown from a single cell of line Py 143. Finally, two lines -- Py 130 and Py 159 -- represented Py transformed, but non-transplantable lines.

C. Studies on the in vitro Characteristics of Selected Transformed Cell Lines

The lines were characterized by their cloning efficiency, colonial morphology and the cell density attained by crowded plates (saturation density). The results of these studies are presented in Table 14.

It can be seen that there are marked differences between transplantable and non-transplantable lines in several of the characteristics studied. The cloning efficiencies of the transplantable lines are five

Table 14. The Properties of the Cell Lines Selected for Further Study.

The data for the first three columns of this table are taken from Table 12. The cells were cloned as described in Methods. The test for saturation density was performed as follows: Five x 10^5 cells of the line to be tested were plated on a 65 mm petri dish. The cells were fluid changed regularly, and then, after they had grown to confluency, they were dispersed by trypsin and counted in the hemocytometer. The cells of lines Py 130 and Py 159 were counted six days after they had grown to confluency, and the cells of the other lines tested were counted at four days after they had grown to confluency. The reason for this is that the cell sheets formed by these latter lines had a tendency to detach from the plate after prolonged incubation.

Note (1): Definitions of the terms used to describe colonial morphology are given in the Glossary except for the term "piled" which is described in the text (see also Fig. 11).

Note (2): N. T. = not tried.

Note (3): Clone 27 is a virus free line derived from a single cell of line Py 143.

Note (4): Lines Py 161 and Py 182 were transplantable with difficulty or not at all in unirradiated hosts.

Table 14

Cell Line	Cell Morphology	Growth Pattern	Transplantability	Cloning Efficiency (%)	Colonial Morphology (1)	Saturation Density (cells/plate)
Uninfected Lines:						
70	Untransformed	Non-regulated	Transplantable	NT (2)	NT	NT
150	Untransformed	Non-regulated	Transplantable	NT	NT	NT
Infected Lines:						
PY 130	Transformed	Non-regulated	Non-transplantable	10	Small, piled colonies of randomly oriented cells.	$3-4 \times 10^6$
PY 159	Transformed	Non-regulated	Non-transplantable	10	Small, piled colonies of randomly oriented cells.	$3-4 \times 10^6$
PY 143	Transformed	Non-regulated	Transplantable	90	About 90% dense colonies. All colonies had randomly oriented cell arrangement.	$1-2 \times 10^7$
Clone 27 (3)	Transformed	Non-regulated	Transplantable	100	100% dense colonies.	$1-2 \times 10^7$
PY 160	Transformed	Non-regulated	Transplantable	100	About 90% dense colonies. All colonies had randomly oriented cell arrangement.	$1-2 \times 10^7$
PY 161	Transformed	Non-regulated	Transplantable (4)	30	Small, piled colonies with rather dense centers. About 5% were typical dense colonies.	NT
PY 182	Transformed	Non-regulated	Transplantable (4)	80	About 90% dense colonies. All colonies had randomly oriented cell arrangement.	$1-2 \times 10^7$

to ten times those of the non-transplantable lines. In addition, the lines of the two groups form colonies of different types. The cells of the transplantable lines tend to form densely heaped up colonies in which the cells seem to grow equally well in contact with the substrate or on top of neighboring cells. Most of these colonies are visible to the naked eye at seven days after plating (Fig. 11a and b). On the contrary, the cells of the non-transplantable lines make colonies which are much less heaped up (Fig. 11c and d). The cells tend to grow in a rather loose, netlike configuration and do not form a many-layered felt. These colonies are invisible to the naked eye, unless stained, at seven days after plating, but acquire a much thicker, piled-up appearance by two weeks ("piled" colonies).

Line Py 161 is somewhat atypical since it gave rise to heterogeneous colony types. About 5% of these colonies were identical to the dense clones of the other transplantable lines. It is likely that only these cells gave rise to tumorous growth when transplanted, since the latent period for tumor production with the cells of this line was long.

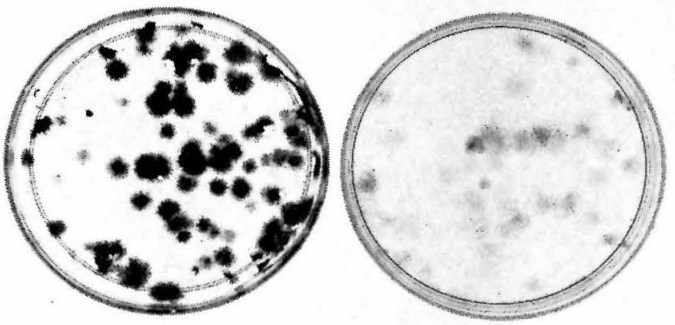
The saturation density was determined by counting the cells in mass cultures which were incubated for several days past the time they became confluent. The transplantable cultures reached saturation densities of 2×10^7 cells per plate; in non-transplantable lines, it did not exceed 4×10^6 cells per plate.

Figure 11.

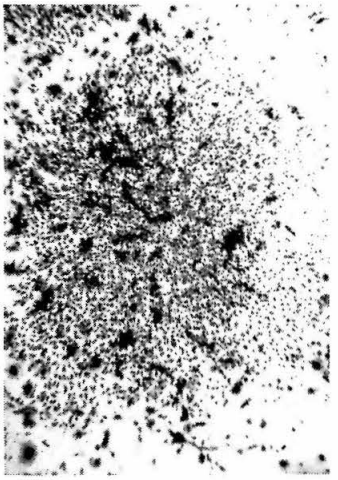
(a) Photograph of a plate containing "dense" colonies (left) and of a plate containing "piled" colonies (right). Stained with methylene blue.

(b) Microphotograph of "dense" colonies appearing in (a). x 20

(c) Microphotograph of "piled" colonies appearing in (a). x 20



(a)



(b) (c)

Figure 11

These studies show that marked differences in behavior in vitro do exist between transformed lines of different transplantability. The observations agree with similar observations made with Py-transformed hamster cells.[65,66] From the combination of these observations the following generalization can be derived: that the ability of a Py-infected cell line to grow autonomously in vivo results from the presence in it of cells which are able to form dense colonies in vitro. As in the case of the Py-transformed hamster cells also in the mouse system these cells could arise as a result of some secondary change in cultures of cells primarily transformed by the virus which are not themselves able to produce tumors. The role of the secondary change may be mainly that of rendering the cell less susceptible to the immunological defenses of the host.

D. Presence of the Py-specific Antigen in the Transformed Lines

The results so far obtained indicate that there is some fundamental difference between infected and uninfected transformed lines that affects their transplantability. The presence of a Py-induced antigen in the infected transformed cells[56] strongly suggests that the main difference between the two types of lines is the presence or absence of the antigen. Experiments were therefore carried out to test the generality of the differences.

Six lines, two uninfected and four infected, were tested for the Py specific antigen by comparing their transplantability into virus-free and into virus immunized animals (see Methods). The re-

sults of these experiments, which are reported in Table 15, can be summarized as follows: (a) Although the number of mice used in these tests was small, the infected cell lines showed definite evidence of decreased transplantability and retarded growth in virus-infected animals; the magnitude of this effect is similar to that observed by other workers.[54,55,56] (b) The uninfected lines did not show evidence of decreased transplantability in immunized mice; therefore, lines that acquired transplantability without virus infection do not possess the virus specific antigen; this result also shows that the effect of virus infection on the transplantability of the infected lines cannot be due to some nonspecific effect, such as on the immunological apparatus of the host. (c) Immunity to the infected lines was induced in the animals by injection of purified virus, so that any suspicion that resistance is induced by non-viral materials present in the lysate can be ruled out. (d) The antigen has been retained in the lines through a clonal isolation (clone 27); this finding suggests that most of the cells in transformed cultures possess the antigen. More work will be needed to establish this point, however. (e) The antigen could be demonstrated even in a line which had low transplantability in unirradiated mice (line Py 182) by irradiating some of the host mice before injection of cells and by injecting unusually large numbers of cells (Table 15).

These results tend to support the generalization that the infected transformed lines possess the Py-specific antigen, whereas the

Table 15. Tumor Incidence and Tumor Growth in Virus-Immunized and Unimmunized Mice.

Py-immunized mice were given three weekly subcutaneous injections of 5×10^7 pfu of virus. Virus free mice were cage mates or litter mates of the virus-treated mice. Inoculations of cell suspensions were given subcutaneously at four to five weeks after the last virus injection. The mice were inspected every four to seven days for at least sixty days and tumors, if present, were measured with calipers. The "average tumor diameter" is the mean of two different measurements.

a) Tumor incidence at two months after inoculation: The final tumor incidence is given as the number of tumor-bearing mice over the number injected for each group of recipients.

b) Growth of tumors: The average tumor diameter is given as a function of time after injection for the lowest tested cell dose that produced 100% tumors in non-virus treated animals.

Note (1): The mice receiving Py 182 were irradiated with a whole body dose of 425 r on the same day they were grafted with tumor.

Note (2): The "final" tumor incidence for the mice injected with Py 182 cells is the tumor incidence at fifty days after injection.

a) Tumor Incidence at Two Months after Inoculation

Cell line	Number of cells injected	Mice	Tumor incidence at two months
70	$2x10^6$	Py-infected	5/5
		Virus Free	5/5
	$2x10^5$	Py-infected	2/3
		Virus Free	3/3
	$2x10^4$	Py-infected	1/2
		Virus Free	0/2
150	10^6	Py-infected	4/4
		Virus Free	4/4
	10^5	Py-infected	4/4
		Virus Free	4/4
	10^4	Py-infected	1/2
		Virus Free	0/2
Py 143	10^6	Py-infected	0/4
		Virus Free	3/4
	10^5	Py-infected	1/4
		Virus Free	4/4
	10^4	Py-infected	0/4
		Virus Free	2/3
Clone 27	10^6	Py-infected	2/3
		Virus Free	4/4
	10^5	Py-infected	1/3
		Virus Free	4/4
	10^4	Py-infected	1/7
		Virus Free	7/7
Py 150	10^6	Py-infected	2/5
		Virus Free	5/5
	10^5	Py-infected	2/5
		Virus Free	5/5
Py 182	$4x10^6$	Py-infected	0/3 ②
		Virus Free	2/3
		Py-infected (Irrad. ①)	0/2
		Virus Free (Irrad.)	2/2

Table 15

(b) Growth of Tumors

Tumor Incidence: (average diameter in cm.).

Cell line	Number of cells injected	Mice	Days after injection. 6-8	10-13	16-19	22-25	28-31	35-38	42-44	48-51	53-57	61-66
70	$2x10^5$	Py	0/3:(-)	0/3:(-)	0/3:(-)	0/3:(-)	0/3:(-)	1/3:(0.8)	1/3:(0.7)	2/3:(1.1)	2/3:(1.6)	2/3:(2.0)
		Virus free	0/3:(-)	0/3:(-)	0/3:(-)	0/3:(-)	0/3:(-)	2/3:(1.1)	3/3:(1.0)	3/3:(1.2)	3/3:(2.0)	3/3:(2.4)
150	10^5	Py	0/4:(-)	0/4:(-)	0/4:(-)	1/4:(1.1)	3/4:(0.6)	3/4:(1.0)	4/4:(1.4)	4/4:(1.8)	4/4:(2.0)	4/4:(2.3)
		Virus free	0/4:(-)	0/4:(-)	0/4:(-)	1/4:(1.1)	3/4:(0.9)	4/4:(1.1)	4/4:(1.2)	4/4:(1.5)	4/4:(1.6)	4/4:(2.0)
Pyl43	10^4	Py	0/4:(-)	0/4:(-)	0/4:(-)	1/4:(0.6)	1/4:(0.8)	1/4:(1.9)	1/4:(1.7)	1/4:(2.0)	1/4:(2.7)	0/3:(-)
		Virus free	0/4:(-)	0/4:(-)	1/4:(0.7)	2/4:(0.8)	2/4:(1.1)	3/4:(1.4)	3/4:(1.9)	3/4:(2.4)	3/3:(2.0)	1/1:(1.5)
Clone 27	10^4	Py	0/7:(-)	0/7:(-)	1/7:(1.1)	1/7:(1.8)	1/7:(2.7)	1/7:(3.2)	0/6:(-)	0/6:(-)	0/6:(-)	0/6:(-)
		Virus free	0/7:(-)	1/7:(0.8)	7/7:(1.4)	7/7:(1.8)	7/7:(2.5)	5/5:(3.1)	2/2:(3.3)	--	--	--
Pyl50	10^5	Py	0/5:(-)	0/5(-)	0/5:(-)	1/5:(0.7)	2/5:(0.6)	2/5:(1.0)	2/5:(1.3)	2/5:(1.7)	2/5:(2.5)	1/4:(2.4)
		Virus free	0/5:(-)	0/5(-)	5/5:(0.9)	5/5:(1.1)	5/5:(1.4)	5/5:(2.0)	5/5:(2.9)	2/2:(3.5)	--	--

uninfected transformed lines do not. They therefore afford a very strong argument against the hypothesis that Py-induced transformation is due to the selection of spontaneously altered cells. This conclusion appears especially strong for cases in which an uninfected and an infected line derived from the same cell (such as 150 and Py 150) showed a difference in antigenicity.

We do not wish to claim, however, that the selection of spontaneously occurring changes plays no role in the neoplastic transformation of Py-infected cultures. This subject will be considered in greater detail in the discussion.

DISCUSSION

A. Recapitulation of Results

The results obtained in this thesis and their significance can be outlined as follows. The quantitative studies on the infection of mouse embryo cultures with Py have shown that the proportion of virus producers and the proportion of cells killed in cultures infected at high input multiplicity are smaller than expected. The descendants of cells surviving infection at high multiplicities are similar to uninfected cells in cell morphology, growth pattern, and sensitivity to reinfection. These findings suggest that the mouse embryo cell population contains a fraction of cells with a lower than average probability of being infected by Py. The resistance of these cells to infection is probably not of genetic origin, but is determined by a transient, physiological state of the cells.

If virus growth is allowed to continue unhindered for many generations in the population of survivors, however, a new type of cell--the transformed cell-- appears and overgrows the culture. These transformed cells are not necessarily malignant in the animal, since in many cases they do not give rise to a continuously growing tumor upon implantation into an isologous host. It is only after continued *in vitro* cultivation that many transformed cell lines acquire the property of transplantability. Transplantability is associated with special growth properties detectable *in vitro*.

Another noticeable result obtained is the converse of the one just mentioned: mouse cells, in the absence of deliberate virus infection, frequently acquire a non-regulated growth pattern. In general, there was a correlation, in uninfected cultures, between non-regulated growth and the ability to give rise to a tumor upon implantation. The correlation between the in vitro and in vivo properties of both uninfected and infected cells lines is complex and interesting for an understanding of the process of tumor formation by viruses.

The delayed appearance of transformed cells in infected cultures and the occurrence of changes in uninfected cultures raise the question of whether the transforming action of the virus is due to induction of cell change or to selection of spontaneously changed cells. Transplantable cells arising in infected cultures were found to have a new antigen which was absent in those arising in uninfected cultures. This fact and other features of the transformation process suggest that the transforming action of the virus is due to the induction of cell change and not to selection.

Other possible explanations for the delayed appearance of transformed cells in infected cultures have also been considered: the results obtained suggest that the delay in transformation is not primarily due to the delayed expression of the transformed character or to the low frequency of transformable variants preexisting in the cell population.

B. Discussion of Results

The results on the proportion of virus yielding cells in freshly infected cultures qualitatively confirm those reported by Winocour and Sachs.[43] These workers used antiviral flourescent antibody to determine the number of cells in which virus was synthesized. However, the multiplicities of infection used in their experiments are not known since they did not use uniform conditions for adsorption of the virus to the cells. Therefore, the present results cannot be compared exactly with their results. The highest proportion of infective centers observed in the present study was 60%; Winocour and Sachs found 80%. These observations show that a fraction of the cell population has a lower than average probability of being converted into virus producers upon exposure to virus. This conclusion is supported by the results of single cell yield experiments in which the proportion of cells releasing virus was also lower than expected.

A similar conclusion derives also from the relationship between proportion of cells killed and multiplicity of infection: it has been shown in this work that 20% to 30% of the clone forming cells in mouse embryo cultures were able to survive infection at input multiplicities of 500 pfu/cell. This finding suggests that a substantial fraction of the cell population has a lower than average probability of being killed upon exposure to high concentrations of virus.

In comparing the proportion of cells killed with that of cells forming infective centers we assume that every cell which is killed

by virus is a virus producer. This is not known with certainty, however: in particular, it is not excluded that certain cells may be killed without producing virus. Therefore, the proportion of cells killed may exceed that of infective centers at the same multiplicity of infection.

The results of the infective center experiments could be interpreted in a different way; namely, that all cells in cultures exposed to high concentrations of virus become virus producers, but that a fraction of them does not register as infective centers because they produce virus too late to initiate plaques. This interpretation is suggested by the following findings of Rubin with RSV:[19] only a small proportion (c. 10%) of the cells of cultures infected with RSV at high virus:cell ratios is able to produce foci when they are plated immediately after infection on focus assay cultures. If the plating of the infected cultures is delayed, however, the proportion of focus forming cells increases substantially. The explanation of this phenomenon lies in the fact that as the initial cell density of focus assay cultures increases, the number of foci formed in the cultures after virus infection decreases, possibly as a result of changed physiological conditions in the denser cultures. This decrease in the sensitivity of RSV assay cultures suggests that if a focus is not initiated soon after infection, it will not be detected at all. Rubin concludes, therefore, that (1) freshly infected cells show a variable delay before they become able to initiate a focus and (2) incubating an infected culture before

plating its cells for focus formers increases the probability that an infected cell will initiate a focus on the assay plate soon after plating.

An interpretation of this kind is not likely to hold for the Py-mouse embryo system for the following reasons: (1) Incubation of the cells until seventy-two hours after infection did not increase the proportion of cells releasing virus in a single cell yield experiment; in general, there was no correlation between the proportion of cells registering as infective centers and the time of plating from five to twenty hours after infection; (2) The plateau observed in the proportion of cells killed vs. MOI curve cannot be explained simply by a delay in the cytocidal effect of the virus and (3) The size of Py plaques increases for many days after they are first observed; this suggests that old plaque assay cultures remain sensitive to virus.

These arguments indicate that failure to demonstrate virus synthesis in a fraction of the population is due to complete inhibition and not to retardation.

What factors determine the existence and size of the resistant fraction? We can consider the presence of resistant mutants, of interfering substances and of special transient physiological states of the cells.

The evidence on the apparently normal reinfectibility of the survivors of infection indicates that the resistant fraction is not principally composed of resistant mutants.

A role of interfering substances (interferon) produced by the infected cells has been suggested.[92] It has been shown that Py-infected cells produce interferon and that interferon produced in response to infection with other viruses can protect cells against infection with Py. It seems, however, unlikely that interferon is the cause of the resistance to infection observed in these experiments, although it may play some role in the dynamics of virus growth and cell infectibility in steady state cultures. In fact, the resistant fraction was demonstrated in cultures infected with a purified virus preparation, which cannot contain interferon, under conditions in which interferon produced after infection could not play any role.

If the resistance is not a heritable property of the cells and is not due to the action of interferon, it is probably determined by some transient physiological state of the cells. The nature of the block to virus synthesis in the resistant cells is unknown; it could lie either in a decreased probability of adsorption or in some subsequent step. If poor adsorption is not the cause of resistance, potentially infective virus particles must be lost in the infected cultures by adsorption to the resistant cells. This loss could be partially responsible for the 70:1 physical particle to pfu ratio observed in the Py system.[93]

Physiological resistance to the cytocidal effects of Py as observed in mouse embryo cells is neither a necessarily general phenomenon nor the exclusive type of resistance encountered. In fact,

on one hand, Winocour[76] has reported that in infected baby mouse kidney cultures, the proportion of cells degenerating and the production of progeny virus is considerably larger than in mouse embryo cultures. On the other hand, Sanford, et. al.[94] and Dawe and Law[95] have obtained cultures from adult mice which show a permanently greater resistance to the cytocidal effects of the virus, apparently as a hereditary character of the cells.

Cells hereditarily resistant to the virus are also present or can arise in mouse embryo cultures, as shown by the results presented for clone 121. The behavior of this clone toward Py infection is remarkable also in another way: in fact, this was the only one out of fifty tested clones which did not show any symptoms of transformation after infection. Owing to the regular occurrence of transformation in the infected clones which showed normal susceptibility to the cytocidal action of the virus, it is tempting to suggest that there is a correlation between resistance to the cytocidal effect of the virus and lack of transformation. This correlation, in turn, would suggest that the initiation of transformation and the initiation of virus production proceed to a certain extent through a common pathway which is blocked in the resistant cells.

There is yet another similarity between the cytocidal and transforming interactions of Py: this is found by comparing the reported results concerning the proportion of infective centers with the

results of Stoker and Abel[68] concerning the proportion of transformed cells in infected hamster cultures. There are two similarities between the two sets of results: (1) the proportion of transformed cells saturates at high virus inputs at values well below 100% of the cells in the culture and (2) the proportion of transformed cells at a given virus input is relatively constant within a single experiment, but varies over a range greater than tenfold from experiment to experiment. This behavior was also noted in the infective center experiments except that the proportion of infective centers was, in general, ten times larger than the proportion of transformed cells.

It is interesting that the cytocidal and transforming interactions of Py show these similarities. Some of the similarities, such as the experimental variability, may be due to technical details rather than to an inherent property of the system; all together, however, they seem to point to some more profound relationship between the two phenomena.

The next question to be considered is whether the transformed cells found in infected cultures descend from cells which have undergone a direct interaction with the virus. To discuss this question, we shall first consider some alternative hypotheses for transformation.

Two models of indirect transformation by Py will be considered. In the first model, the role of the virus would be purely passive, i.e., it would simply select for transformed cells already preexisting in the tissue from which the cultures were derived. This model has been considered and ruled out by results reported in this thesis, which show that transformation occurred in infected clonal lines which were untransformed before infection.

The second model could be described as selection plus mutation. It has been shown that changes to non-regulated growth occur regularly in uninfected cultures. In infected cultures, such changes might occur at a higher rate as an indirect result of virus proliferation. The typical transformed cell morphology, which is so characteristic of Py-transformed cultures, but which appears relatively infrequently in uninfected cultures, could be explained purely on the basis of indirect selection: the specific morphology could, in fact, be a secondary attribute of cells selected for virus resistance.

At this time, it is impossible to rule out conclusively the selection plus mutation hypothesis. This is because it is difficult to differentiate a rarely occurring change induced by the virus from a spontaneous mutation. The hypothesis is, however, unlikely for the following reasons. In the first place, the rapid occurrence of transformation in hamster cell cultures after Py infection and its linear dose response suggest that the first event in transformation is the induction of cell change by a single virus particle. It is possible, however, that transformation occurs by a different mechanism in mouse than in hamster tissue.

The second line of evidence comes from recognition that Py-induced tumors and transformed cells possess a foreign characteristic antigen. The Py-specific antigen differs from the foreign antigens

sometimes found in non-viral tumors: in fact, the antigens of different tumors induced by Py in animals of the same species show a strong cross reaction; whereas the antigens of tumors induced in cells of the same inbred line by a given carcinogen show no detectable degree of cross reaction.[62] This uniformity in the antigenic structure of different Py induced tumors is difficult, although not impossible, to reconcile with their supposedly mutational origin.

There are, then, two lines of evidence which strongly suggest that transformation is not the selection of a spontaneous cell mutant but is a consequence of the introduction of viral genes into a potentially transformable cell. In the case of transformation in the mouse system, we must enter a caveat. It is conceivable that Py-transformed mouse cells originated as spontaneous mutants but acquired the Py-specific antigen after a subsequent infection with the virus. Strictly speaking, therefore, the presence of the virus specific antigen in transformed mouse cells is evidence for the occurrence of a non-cytocidal virus cell interaction but not necessarily for the origination of transformation in such an interaction. This caveat does not apply to transformation in the hamster system because (1) transformation here is known to occur as the result of a single virus-cell interaction and (2) there is little virus proliferation and hence only a small probability of secondary infection in infected hamster cultures.

In view of the conclusion that transformation in the mouse system is a direct result of a virus-cell interaction, it is noteworthy that

transformation could not be detected after a brief exposure of the cells to virus. In the next paragraphs we shall consider some explanations that have been advanced to explain the delay in the appearance of transformed cells.

A. Delayed Integration

Vogt and Dulbecco[44] proposed that most of the surviving cells in infected mouse embryo cultures had been infected with virus. The association of the virus genome with these cells was metastable: it could either enter into a phase of rapid replication which resulted in cell lysis, or it could enter into a stable, integrated relationship with the cell, analogous to lysogeny, which resulted in transformation. In order to explain the continuous high level of virus production and the delayed appearance of transformation in steady state cultures, it was postulated that the metastable virus-cell relationship could persist for some time and that the frequency of integration was low.

A strict analogy between transformation and lysogenization has already been rendered doubtful by subsequent work of Vogt and Dulbecco[52,53] on the nature of virus release in the transformed cells. In fact, it was shown that the presistent virus release of Py-transformed mouse cultures is due to a virus carrier state and not to induction of a provirus. Moreover, virus production could not be induced in, and virus-related materials could not be isolated from, virus-free transformed cells.

The results presented here have shown that virus growth and cell killing in steady state cultures could be reduced or eliminated if the spread of extracellular virus was hindered by antiviral medium. This finding indicates that the principal type of virus:cell association in steady state cultures in which no transformed cells have appeared is also that of a carrier culture.

The concept of delayed integration, however, may still be applicable if it is assumed that virus in the metastable state is not able to enter into a phase of rapid replication. Thus modified, the theory can be tested by its prediction that transformation is a delayed result of infection. The results reported in this thesis showed that no unexpressed transformed cells can be detected in cultures which were briefly exposed to virus and then grown in antiviral medium. Therefore the delay in transformation of infected mouse embryo cultures cannot be due to delayed integration unless the unexpressed transformed cells are very rare and selected against.

Nevertheless, the finding of Stoker[69] that transformed hamster cells do not appear immediately after infection indicates that delayed integration may play some role in Py-induced transformation. It may be possible to demonstrate the occurrence of delayed integration in infected mouse cultures by an analysis of the following form: infected cultures could be treated with antiviral medium at various times after infection. At various times after this treatment had begun, the cultures

could be plated to detect transformed colonies. This approach would enable us to determine at least an upper limit to the time interval between infection and transformation. It would have the further advantages that we would be able to determine both the length of virus exposure needed to induce transformation and the properties of the earliest appearing transformed cells.

B. Transformable Variants

It has been shown here that the response of a clonal culture to Py infection was similar to that of uncloned cultures: an extended steady state period was followed by the appearance of transformed cells. From this finding it follows that transformable variants pre-existing in the animal are not necessary for virus induced transformation.

A modified fluctuation test[90] was performed to test the hypothesis that transformation results from the infection of transformable variants of mutational origin. In this test, the frequency of transformable variants in clonal cultures was approximately inferred from the time of appearance of Py-transformed cells. In fact, no clonal culture was found that showed any symptom of transformation earlier than four weeks after infection. The conclusion that the hypothesis is incorrect must, however, be qualified as follows: (1) if the mutation rate of the transformable variants is extremely low, then the probability of finding a rapidly transformable clone would be correspondingly low;

(2) if resistance to the cytocidal effects of infection is a secondary and not a primary attribute of transformed cells, then the time between infection and transformation would reflect, in part, the time needed to select for virus resistant transformed cells and not the frequency of transformable variants; and (3) if the transformable variants were selected against, their frequencies in clonal cultures would not be expected to show much fluctuation.

Since this work was started, a number of other workers have also investigated the possibility that susceptibility to transformation might be a heritable trait.[67,94,96] Their conclusions were similar to our own. Stoker and Macpherson,[67] using a quantitative assay for transformation, have found that cloned sublines of a permanent hamster kidney culture show the same proportion of transformed cells after Py infection as does the uncloned parental population. Sachs et. al.,[96] working with cloned lines of freshly isolated mouse and hamster cells have obtained similar data.

It is of interest that the latter workers detected transformation by plating the infected cultures for transformed colonies on feeder layers of virus resistant rat embryo cells at seventeen days after infection. We were unable to detect transformed cells by plating on mouse feeders at seventeen days after infection. This discrepancy might possibly reflect a loss of virus sensitive transformed cells in the present experiments as a result of using virus sensitive feeder cells.

Since it has not been possible to show that the delayed appearance of transformation in infected mouse embryo cultures results either from the delayed integration of the viral genome or from a low frequency of transformable variants, it is worthwhile considering other hypotheses which could explain this phenomenon. First, transformation could be due to infection by a rare transforming mutant in the virus population: prolonged exposure of the culture to infection would increase the opportunities for a mutant:cell interaction. There is one finding which could provide some support for this idea; namely, that the P16 strain of Py is more efficient per pfu than the lp strain in causing transformation in the hamster system.[89] Second, transformation could be due to a rare type of virus-cell interaction which is conditioned by the physiological state of the cell: prolonged exposure of the culture to infection would increase the opportunities for such a rare virus:cell interaction. Since we might expect the physiological state of the cells to vary with environmental conditions, Stoker and Abel's finding that the concentration of Mg ions influences the transformation rate of hamster cells provides support for this theory. Further experimentation will be required to decide which one of these models we have mentioned best explains the process of Py induced transformation of mouse embryo cells.

The broader issue of the relationship between the in vitro properties and the transplantability of cultured cells will now be

examined. Py-infected cultures will be discussed separately from uninfected cultures because of the antigenic differences which have been found between them.

Infected cultures. Almost all established lines of Py-transformed hamster cells are able to produce tumors upon reimplantation.[44] However, many workers have reported difficulty in producing tumors with Py infected mouse lines.[48,49,50] The lack of transplantability of some of our clonally derived transformed lines agrees with the findings of these workers. Some of the factors which may influence the transplantability of infected mouse cultures have been examined.

It has been shown here that the failure of virus carrying lines of transformed cells to produce tumors on transplantation is not solely due to the injection of virus with the cells. However, it is likely that the presence of the Py specific antigen in the cells influences their transplantability. The work of Habel[56] and the work presented here have shown that this antigen is present in transplantable transformed cultures; presumably it is also present in the non-transplantable lines, although further experiments will be needed to prove this point.

Our findings indicate that the transplantability of infected lines is correlated with certain of their in vitro properties: non-transplantable and transplantable lines differ in colonial morphology and efficiency of cloning. The enhanced transplantability of the cell lines which form dense colonies could arise from a number of causes: for instance, the transplantable cells could have an increased capacity to withstand the immunological defenses of the host, or a decreased anti-

genicity, or a more marked indifference to growth controlling mechanisms of the host.

The morphologically transformed but non-transplantable mouse cells are reminiscent of the early transformed hamster cells of Vogt and Dulbecco.[65,66] These workers found that the initial result of Py induced transformation was a non-transplantable but morphologically changed cell; only upon continued in vitro cultivation did fully transplantable, or "late" transformed cells arise. The difference in the colonial morphology of the early and late transformed hamster cells resembles the difference between our non-transplantable and transplantable mouse lines. To establish this analogy on a firmer basis, it will be necessary to show in the mouse system that transplantable cells possessing the Py specific antigen derive from non-transplantable cells in the absence of reinfection.

Uninfected Cultures. It has been found that a correlation exists between the growth pattern of uninfected cultures and their ability to produce tumors: cultures with non-regulated growth patterns frequently produced tumors; regulated cultures, with one exception, did not. We are unable to decide whether the one tumor-producing, regulated culture represents an exception to the rule or merely reflects the insensitivity of the methods used for detecting non-regulated growth in vitro.

The association in uninfected lines of the in vitro character of non-regulated growth with the capacity for autonomous growth in vivo suggests that the non-transplantable, Py-transformed lines also have a decreased sensitivity to the growth controlling influences of the intact

animal. The failure of these transformed lines to produce tumors could be explained, as has been pointed out, by their greater antigenicity.

Many other workers have found that cultured mouse cells can undergo spontaneous changes in the absence of any known virus or carcinogen.[97,98,99] Sanford and co-workers[97,98] have found that the acquisition of transplantability in mouse cultures generally requires at least an eight-month and frequently a longer period of growth in vitro. In the experiments reported here, transformation to non-regulated growth and to transplantability frequently occurred within six months of the explantation of the cultures. In a recent communication, Todaro and Green[84] have reported results similar to our own. They have described the establishment of permanent cell lines from short term cultures of mouse embryo cells. Establishment -- defined by the ability of the culture in question to maintain a constant or rising growth rate upon continued transfer -- frequently occurred within three months of explantation from the animal. The establishment of a cell line was often, although not always, followed by the acquisition of a non-regulated growth pattern. Todaro and Green noted that establishment was often delayed or completely prevented by transferring the cells at suboptimal cell densities: in such cases, the cultures frequently died out. This observation suggests that it might be possible to develop a more sensitive method of studying Py-induced transformation in

mouse cultures: one could infect cells which had been transferred several times at low cell densities and would therefore be less likely to show spontaneous changes.

In closing, we wish to point out that the major details observed in Py-induced transformation seem to hold true for another virus of the Papova group, SV_{40}, in hamster cell cultures. Here too, only a small proportion of the cell population is transformed after infection with the virus [100] and, in addition, SV_{40} induced hamster tumors possess a new antigen -- one which is probably not related to the Py specific antigen.[59] SV_{40} is also known to cause both virus proliferation and transformation when it infects human cell cultures.[101] It is tempting to suggest that the phenomena we have observed in investigating Py infection of mouse embryo cells -- duality of virus action, neoplastic transformation as a rare event, and induction of a new antigen in the transformed cells -- are typical of this group of viruses.

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