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STUDIES ON THE INFECTION OF MOUSE CELLS WITH FRIEND LEUKEMIA VIRUS: MACROMOLECULAR SYNTHESIS, CELL GROWTH AND VIRUS PRODUCTION IN FRIEND LEUKEMIA VIRUS INFECTED FIBROBLASTS

by

NORMAN GABELMAN

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the City University of New York.

1972

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

April 24, 1972
date

Charlotte Friend
Chairman of the Examining Committee

April 24, 1972
date

H. Burlington Fuson
Executive Officer

Professor C. Friend

Professor W. Scher

Professor L. Ornstein
Supervisory Committee

The City University of New York

Abstract

STUDIES ON THE INFECTION OF MOUSE CELL WITH FRIEND LEUKEMIA VIRUS: MACROMOLECULAR SYNTHESIS, CELL GROWTH AND VIRUS PRODUCTION IN FRIEND LEUKEMIA VIRUS INFECTED FIBROBLASTS

by

Norman Gabelman

Adviser: Professor Charlotte Friend

Viruses which infect mammalian cells are known to cause changes in host-cell physiology. Most of the studies of Friend Leukemia Virus (FLV) carried out in tissue culture had involved chronically infected host cells. In order to study the effects of viral infection on host cells and the replicative cycle of the virus, the events following infection of secondary mouse embryo fibroblasts (MEF-2) in tissue culture with FLV were investigated. FLV infection was confirmed by electron microscopic examination and the subsequent effects on macromolecular synthesis, cellular growth and virus production were studied.

The rate of DNA synthesis increased in MEF-2 cultures following infection with FLV as compared with cultures which were untreated or exposed to heat-denatured virus (D-FLV). DNA synthesis reached a peak between 24 and 48 hours after infection.

Radioautographic studies of dividing and "resting" (nondividing) cells revealed that the percentage of cells which incorporated thymidine into their nuclei was greater in FLV inoculated cultures than in untreated cultures or in cultures treated with D-FLV. The observed increase in the percentage of DNA synthesizing cells in infected cultures appears to be independent of the ability of the cells to divide.

The rate of cellular RNA and protein synthesis did not appear to be affected by FLV infection.

The generation time (T) in FLV infected MEF-2 was significantly shorter than in control cultures. Cell cycle analysis revealed that the observed shortening of the generation time was a result of a decrease in the time required for the FLV infected cells to traverse the G1 and S phases of the cell cycle. By 72 hours post infection, the cultures had grown to a significantly greater population density than controls.

Studies of the kinetics of virus production indicate that virus is present in the medium by 4 hours after infection and that the maximum rate of virus release occurs between 24 and 96 hours after infection.

Cell-free tissue culture filtrates were found to have a low leukemogenic potential and to induce leukemia in mice only sporadically as had been noted by others.

DEDICATION

To Joan, Andrea and Micah
With Love

ACKNOWLEDGEMENTS

I would like to express my profound gratitude to Dr. Charlotte Friend, whose gracious support, generosity with time, guidance and encouragement made this work possible. Dr. Friend's abilities as teacher and scientist made the time spent in her laboratory a most pleasurable personal as well as intellectual experience.

I would also like to record here my debt of gratitude to Dr. William Scher whose imagination, critical insight and stimulating guidance helped bring this work to a fruitful end.

A debt of gratitude is also owed to Drs. Leonard Ornstein and Shalom Z. Hirschman for their patient interest, helpful discussions and valuable guidance.

In addition, I would like to thank all of the people in the laboratory who offered help and encouragement.

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I. INTRODUCTION

Historical Background

There is considerable information dating back to the turn of the century which establishes a viral etiology for many of the neoplastic diseases of higher vertebrates. The possible role of viruses in the etiology of cancer was considered as early as 1908, when Ellerman and Bang reported the transmission of an erythromyeloblastic leukemia in chickens by the inoculation of a cell-free filtrate (Ellerman and Bang, 1908). Rous in 1911 showed that a fowl sarcoma which originated spontaneously in a Plymouth Rock hen and which had been transmitted several times by cell grafts to birds of the same strain could be transmitted by cell-free filtrate (Rous, 1911). In 1939, Bittner showed that certain mammary carcinomas of mice were transmitted to the progeny through the milk and that the infectious agent would pass through a bacterial filter (Bittner, 1939).

Since mouse leukemia is in many respects similar to human leukemia many investigators attempted to determine whether mouse leukemia was transmitted by a filterable agent. In 1938, Engelbreth-Holm and Frederiksen reported the induction of leukemia in AK mice with cell-free filtrates of lymph nodes of leukemic mice of the same strain (Engelbreth-Holm and Frederiksen, 1938). In 1951, Gross demonstrated in a more conclusive way, the production of a lymphatic leukemia in C3H mice inoculated soon after birth with an extract of

leukemic tissues from an AK mouse (Gross, 1951). Thereafter, a number of murine leukemias having viral etiologies were reported by Friend (1957), Graffi (1957), Buffet and Furth (1959), Maloney (1960), Rauscher (1962), and Leiberman and Kaplan (1966).

The murine leukemia induced by Friend Leukemia Virus (FLV) is of considerable interest in that it resembles DiGuglielmo's disease (DiGuglielmo, 1946), an erythroleukemia (erythremic myelosis) of man. Inoculation of susceptible mice with FLV results in an erythroleukemia characterized by massive splenomegaly, proliferation of abnormal cells in the spleen, liver, bone marrow and peripheral blood, anemia (Friend, 1957) or polycythemia (Mirand and Grace, 1967). The review of the literature presented below is concerned with what is known about the structure, chemical composition and replication cycle of FLV and related oncogenic viruses and their effect upon macromolecular synthesis and growth in mammalian cells in tissue culture.

Viral Morphology

Ultrastructural studies of FLV show that the virions are roughly spherical particles about 90-100nM in diameter having a well defined envelope and a centrally located nucleoid (DeHarven and Friend, 1958). Two types of particles are generally seen in association with infected cells, termed the enveloped type A and C (Bernhard and Guerin, 1958; DeHarven, 1968). The C particle differs from the enveloped A particle in that it has an electron-dense nucleoid, about 70nM in diameter,

while the enveloped type A particle has an electron-luscent nucleoid of similar size.

The arrangement of the nucleic acid in the nucleoid is not completely understood, however recent investigations with Rauscher Leukemia Virus (RLV) suggest that the viral genome is coiled into a hollow ball within the viral core and enclosed within a filamentous capsid rather than bunched into a compact mass (Luftig and Kilham, 1971).

Physical and Chemical Characteristics of the Virus

The virions of the murine leukemia virus group have a buoyant density of 1.16g/ml (O'Connor, Rauscher and Zeigel, 1964; Orozlan, Johns and Rich, 1965; Duesberg and Robinson, 1966). Overall quantitative data on the chemical composition of FLV does not seem to have been reported. However, Francker and Reibeck (1968) reported that when FLV labeled with tritiated precursors of RNA, protein and lipid is analyzed on a sucrose density gradient, the radioactivity and the infectivity are found at the same position in the gradient. The data given below are a construct obtained by piecing together the data from several different laboratories on different, but related oncogenic RNA viruses.

Lipid

The lipid of the oncogenic RNA viruses particle is found mainly in the envelope. It constitutes about 35% of the dry weight of avian myelo-

blastosis virus (AMV) (Bonar and Beard, 1959) and about 27% of the dry weight of mammary tumor virus (MTV) (Lyons and Moore, 1965). About 80% of the lipid found in the viral envelope is phospholipid, which is among the regular components of eukaryotic cell membranes. The specific lipid components are very similar to those found in the host cell (Johnson and Mora, 1967). The large amount of lipid in the envelope accounts for both the low density of the virions and their sensitivity to phospholipases and lipid solvents (de Tkaczevski, et al., 1968).

RNA

Although FLV has ribonucleic acid (RNA) as its genetic material (Francker and Reibeck, 1968), its percentage in the virus is as yet unknown. It has been determined that 2% of AMV is RNA (Bonar and Beard, 1959). Density gradient sedimentation of the RNA derived from purified FLV virions resolves a fast moving component with an estimated $S_{w,20}$ of 68S (about 10^7 daltons of RNA) and a slower moving component with a sedimentation value of less than 10S (Francker and Reibeck, 1968).

Analysis of the base composition of FLV-RNA suggests that the RNA is single stranded (Francker and Reibeck, 1968). In all other oncogenic RNA viruses in which the RNA has been characterized it has been found to be single stranded (Robinson, et al., 1965; Mora, et al., 1966; Robinson, et al., 1967).

Although no analysis of FLV-RNA has been reported, Duesberg and others (Duesberg, 1968; Erickson, 1969; Bader and Steck, 1969;

Biswal and Benyesh-Melnick, 1969) have reported that the "70S" RNA of other oncogenic RNA viruses can be converted to 37S pieces (MW about 2.5×10^6 daltons) by urea, dimethylsulfoxide (DMSO) or heating; treatments known to break hydrogen bonds. This finding is especially interesting since the target size of Rous Sarcoma Virus (RSV) in x-ray inactivation experiments is about the same as the infectivity of Tobacco Mosaic Virus (TMV) under similar conditions (Latarjet and Chamaillard, 1962). Therefore, the oncogenic capacity of RSV may be coded by an RNA subunit the size of the TMV-RNA (about 2.5×10^6 daltons). These findings appear to be in agreement with those reported by Duesberg (1968) for influenza virus RNA which has been shown to consist of five or six pieces by electrophoretic analysis. Watson (1971) has been unable to demonstrate the presence of "70S" viral RNA in the cytoplasm of RLV infected mouse cells, but has been able to isolate a single homogenous 37S peak of viral RNA from cytoplasmic extracts treated with DMSO. It is speculated that 70S RNA is synthesized from 37S pieces at the time that the virus is assembled.

In addition to the "70S" RNA of the viral genome, RNAs thought to be of cellular origin have been reported (Wollman and Kirsten, 1968) to be present in purified mouse leukemia virions. Of particular interest in this regard, is the presence of a methylated 4S RNA, which appears to be similar to host cell tRNA. (Messenger and viral RNAs are not methylated, whereas tRNAs and ribosomal RNAs are methylated.) (Erickson, 1969).

DNA

Although the bulk of the nucleic acid of the oncogenic RNA viruses is known to be RNA, Levinson, et al. (1970) have reported finding DNA located in the interior of the RSV virion. The significance of the presence of the DNA in the RSV is not known. It may be a product of the RNA dependent DNA polymerase or host DNA incorporated during virus maturation, however, DNA inactivation experiments using 5 bromo-2-deoxyuridine indicate that the DNA inclusion, whatever its source, is not required for virus replication (Levinson, et al, 1972).

Proteins

FLV, when disrupted by sodium dodecyl sulfate, yields 3 polypeptides by polyacrylamide electrophoresis (Francker and Reibeck, 1969; Francker and Gruca, 1969). Schafer and coworkers isolated a single homogeneous polypeptide with group specific activity from Tween-ether treated FLV (Schafer, et al., 1969). This polypeptide is antigenically related to protein present in Gross Leukemia Virus (GLV) and RLV (Gregoriades and Old, 1969).

Carbohydrates

In addition to lipid, RNA, DNA, and protein, some carbohydrate moieties may be integral parts of the structure of FLV. Glucosamine has been found in AMV (Bonar and Beard, 1959) and the presence of several carbohydrates in addition to the ribose from the RNA have

been established in the influenza viruses. They amount to 5 to 8% of the dry weight of the virus (Ada and Gottschalk, 1956). They include galactose, mannose, fucose, and glucosamine. It is probable that in FLV as in influenza virus, such substances contribute to the antigenic character of the virus (Laver and Webster, 1966).

Effects of Oncogenic Virus Infection on the Host Cell

Infection of susceptible host cells with oncogenic viruses results in changes in host cell physiology. The cellular alterations which result from infection with oncogenic DNA viruses have been extensively studied (Green, 1970). Cells infected with oncogenic DNA viruses exhibit (a) loss of density-dependent inhibition, which results in striking changes in the growth of the infected cells; (b) changes in cell morphology; (c) changes in karyotype; (d) autonomy and indefinite life in culture; (e) changes in enzyme synthesis; (f) changes in macromolecular synthesis; and (g) acquisition of new virus-specific antigens.

Less is known about the effects of FLV and other oncogenic RNA viruses upon the cells they infect. None appear to be cytopathic. Infection of host cells with certain avian and murine sarcoma viruses (e.g., Rous Sarcoma Virus, Maloney Sarcoma Virus) result in morphologic changes (transformation) similar to those seen with oncogenic DNA viruses, however infection with the murine leukemia and avian leukosis viruses (e.g., FLV, RLV and Avian Leukosis Virus [ALV]) does not result in transformation (see Boiron, et al., 1967; Vigier,

1970).[1] Although no obvious morphological changes in the cell follow infection with FLV, changes in (a) macromolecular synthesis; (b) enzyme synthesis; (c) growth rate; (d) antigenic character of the cell; and (e) the production of infectious virus can be detected.

Macromolecular Synthesis

Among the changes in cellular physiology that may be detected after infection with oncogenic RNA viruses are alterations in DNA, RNA and protein synthesis.

DNA synthesis. Synthesis of DNA in mammalian cells in tissue culture is stimulated by DNA containing oncogenic viruses such as Polyoma Virus (Dulbecco, et al., 1965), Simian Virus 40 (Gershon, et al., 1966), and Adenovirus (Ledinko, 1967).

Certain oncogenic RNA viruses are also capable of stimulating DNA synthesis in mammalian cells in tissue culture. Lee, et al. (1968) showed that RSV is capable of stimulating DNA synthesis in chick embryo myotubes, even though it could not stimulate cell division or morphological transformation. Hirschman, et al. (1970) report that while Maloney Leukemia Virus (MLV), which is related to FLV (Friend, 1966) could not stimulate DNA synthesis in contact inhibited 3T3 cells (Todaro and Green, 1963), MLV was capable of stimulating DNA synthesis in actively dividing mouse embryo fibroblasts (MEF). La Cour, et al. (1970) found a similar increase in DNA synthesis in

[1] Osato, et al. (1966) report such transformation with FLV. The result, however, has not been confirmed.

actively dividing chick embryo fibroblasts (CEF) infected with Avian Myeloblastosis Virus (AMV).

RNA synthesis. Different members of the oncogenic RNA virus group appear to have varying effects on cellular RNA synthesis. La Cour, et al. (1970) report an increase in AMV infected CEF. A similar increase in RNA synthesis was reported by Albach, et al. (1970), in GC virus, an oncogenic RNA virus. On the other hand, Hirschman, et al. (1970) report no increase in RNA synthesis in MLV infected MEF. The observed differences in the effects of virus infection on RNA synthesis may result from real differences in the abilities of the various viruses to stimulate RNA synthesis or on the other hand these data may reflect differences in the assay techniques employed.

Protein synthesis. Although little is known about the effects of FLV infection on protein synthesis in vitro, Budillon, et al. (1964) reported increased protein synthesis in the spleens of infected mice.

Enzyme Synthesis

Stimulation of nucleic acid synthesis in cells infected with oncogenic RNA viruses is accompanied by changes in enzyme activities associated with nucleic acid metabolism. FLV infection results in a stimulation of phosphoribisylamidotransferase activity (Reem and Friend, 1967), RNA methylase activity (Silber, et al., 1967), DNA dependent RNA polymerase activity (Lin and Rich, 1968; Munson, et al., 1970), nucleoside deaminase activity (Silber, et al., 1971) and a

depression of RNAase activity (Charkrabarty, et al., 1970) in mouse spleens following FLV infection.

Cell Growth

In the usual tissue culture medium, serum is a limiting factor for the multiplication of fibroblasts in monolayer culture. Holley and Kiernan (1968) have demonstrated that the saturation density of 3T3 cells depends in large part upon the serum concentrations used in the medium. In experimental situations where the serum in the medium is limited, as for example when the medium is not changed during the course of the experiment, the rate at which the cells grow and the final density which they achieve are apparently dependent upon the efficiency with which the serum growth factors are metabolized (Temin, 1971).

Oncogenic RNA virus infection appears to increase the efficiency with which cells in monolayer culture utilize the growth factors present in serum. Colby and Rubin (1969) reported that under conditions where serum was limited RSV infected CEF grew to greater densities than did the uninfected controls. La Cour, et al. (1970) also reported a stimulation of the growth rate in AMV infected CEF cultures.

The increased growth rate in virus infected cells may reflect an ability on the part of such cells to double their number (generation time) more quickly than uninfected cells under the conditions of the experiment. Lajtha, et al. (1954) first demonstrated that several dis-

tinct processes occur during each normal generation time and that each process only occurred once in a single time period. Mitosis and major DNA synthesis do not occur simultaneously and the cellular life cycle (cell cycle) can be divided into a mitotic phase (M-phase) which is typically separated from S-phase during which cellular DNA is replicated by a gap of several hours called G-2. Similarly, the gap, also typically of several hours between the end of M-phase and the beginning of S-phase is called G-1.

Methods for estimating the fraction of the cell population in each phase of the cell cycle and the time spent there have been devised by Quastler and Sherman (1959), Stanners and Till (1960), Puck and Steffan (1963), and Watanabe and Okada (1967).

Some reports have indicated that the ability of a virus to establish an infection in a host cell is dependent upon the stage of the cell cycle (Yoshikura, 1970; Hobom-Schnegg, et al., 1970). However, little is known about the effects of oncogenic RNA virus infection upon the cell cycle or the cellular growth rate. Alterations in the generation time may reflect alterations in the time required for the infected cell to traverse one or more divisions of the cell cycle.

Antigenic Character of the Cell

FLV induces novel antigens in infected host cells which are similar to those found in the virus, and which can be detected by antisera against viral antigens (Old and Boyse, 1965; Hartley, et al., 1965). For ex-

ample, neutralization and agglutination tests have revealed the existence of a viral coat antigen(s) (FMR antigen) shared by MLV, RLV, and FLV (Friend, 1966; Mayyassi, et al., 1966; Steeves and Axelrod, 1967). In addition to the FMR antigen which is found in the envelope of the virus, the mouse leukemia viruses share a common group specific (gs) "soluble" antigen which is thought to be internal to the envelope, and which can be detected by precipitin tests (Geering, et al., 1966).

Production of Infectious Virus

Early investigations of FLV multiplication in vitro were hampered by the absence of a convenient method for titrating tissue culture passaged virus and had been confined largely to immunologic, physical and electron microscopic examinations of viral production. Recently however, assays based on complement fixation (Hartley et al, .1965), viral interference (Yoshikura et al, 1969), syncytium formation in RSV induced rat tumor (XC) cells after cocultivation with murine leukemia virus (MuLV) infected MEF (Klement, et al., 1969), or the enhancement of focus formation by defective MSV (Fischinger and O'Connor, 1968) have begun to facilitate investigations of FLV multiplication.

Studies of the life cycles of FLV and other oncogenic RNA viruses indicate that infection of susceptible cells is followed by (a) adsorption

and penetration of the virions into the cell; (b) passage of the virions into a noninfectious state (latent or eclipse phase during which the viral components are thought to be replicated and assembled), (c) maturation and release of progeny into the extracellular medium, in which the virus titer increase during a few days to a plateau of relatively constant titer.

Adsorption and penetration of virions into the cell. Events which take place at the surface of the cell appear to determine the ability of a virus to adsorb to the host cell membrane and therefore its ability to infect the host cell. These events are not well understood. Interference studies seem to indicate that specific interactions between the viral envelope and cellular receptors must occur before further steps in the replicative process can occur. For example, prior infection of cell cultures with FLV will substantially reduce the ability of a murine sarcoma virus to produce foci of morphologically altered cells (Sarma, et al., 1967; Yoshikura, et al., 1969).

After adsorption has occurred the next event in the infectious process is the penetration of the virion into the cell. The mechanism(s) by which viruses enter the cell after adsorption have been studied by a number of investigators. Earlier studies with influenza virus, which like FLV has an ether sensitive envelope, suggested that the virus was ingested intact (viropexis) (Fazekas de St. Groth, 1948) or entered by a process involving fusion of the cell membrane and the viral envelope (Hoyle, 1962; Morgan and Rose, 1968). More recent investigations of

the method by which RLV enters the cell (Miyamoto and Gilden, 1971) suggest that penetration of the virus results from either (a) simultaneous dissolution of both the viral envelope and cell membrane resulting in the passage of the viral nucleoid directly into the cytoplasm, or (b) dissolution of the cell membrane with direct penetration of the intact enveloped virus into the cytoplasm followed by intracytoplasmic disruption of the envelope resulting in release of the nucleoid into the cytoplasm. The authors further propose that the penetration of the virion into the cell requires the presence of an enzyme localized in the viral envelope, cell membrane, or both.

Latent or eclipse phase. The virus genome having gained access to the cellular cytoplasm encounters the host metabolism which appears to determine the fate of the virus. There may be, for example, a close relationship between the stage of the cell cycle and the efficiency with which the virus can infect it (Hobom-Schnegg, et al., 1970). There is also a requirement for host cell DNA synthesis which need not be typical cellular synthesis during the first 8-12 hours of the viral replication cycle (Bader, 1967; Temin, 1967; Yoshikura, 1968). Cell division is required for viral expression (Murray & Temin, 1970) and continued viral replication requires a functioning DNA at all times after infection. For example, inhibition of transcription by actinomycin D will inhibit oncogenic RNA virus replication (Yoshikura, 1968; Bases and King, 1967). Viruses which are not related to the oncogenic RNA viruses do not have such requirements (Bader, 1967; Bases and King, 1967; Hobom-Schnegg,

et al., 1970).

A model which would satisfy all of the requirements of the experimental data previously discussed was proposed by Temin (1964; 1971). It postulated the transfer of the information resident in the viral RNA to a newly synthesized DNA copy which would upon integration into the host genome serve as a template for the synthesis of viral RNA. Such a model implies the existence of an enzyme capable of synthesizing DNA from an RNA template - an RNA dependent DNA polymerase.

Temin and Mizutani (1970) discovered this enzymatic activity resident in the virions of RSV. Baltimore (1970) independently discovered a similar enzymatic activity in RLV. Because the input is single stranded, an early intermediate should be an RNA:DNA duplex. Speigelman et al, (1970) reported finding such an intermediate in their incubation mixtures after 20 minutes. Examination of the incubation mixture after no further increase in the incorporation of tritiated thymidine into an acid insoluble polymer were detected (2-6 hours) disclosed the presence of short (5-8S) pieces of double stranded DNA (Mizutani et al, 1970). This result suggests the presence of a DNA dependent DNA polymerase activity in the virion in addition to the RNA dependent DNA polymerase, and this activity was reported by Mizutani et al. (1970).

Recently, the purification of an RNA dependent DNA polymerase from AMV was reported (Kacian, et al., 1971). The purified enzyme preparation had the ability to utilize viral RNA, native DNA or synthe-

tic homopolymer duplexes as templates. In addition, it was found that the enzyme will accept genetically unrelated RNA molecules as templates (Speigelman, et al., 1971. Apparently the sequence of steps from input viral RNA to double stranded DNA is mediated by one enzymatic entity.

Mitzutani and coworkers have also demonstrated the presence of an endonuclease (Mitzutani, et al., 1970), an exonuclease and a DNA ligase (Mitzutani, et al., 1971) in RSV. The virions of RSV and perhaps other members of the oncogenic RNA viruses, appear to contain all of the enzymatic machinery necessary for the translation of the information resident in the viral RNA into double stranded DNA and perhaps subsequent incorporation of the DNA into the host genome. However, integration of the DNA replica of the viral information into the host genome has not been definitively demonstrated and there is still a great deal that is unclear about the steps leading from the synthesis of double stranded, virus coded DNA to the final assembly of the intact virion.

Maturation and release of progeny. Immunofluorescence studies indicate that the gs antigen is synthesized in the nuclei of spleen cells of infected mice (Rapp and Friend, 1963) or the perinuclear area of FLV infected MEF (Osato, et al., 1964). The viral antigen is then seen to move into the cytoplasm at subsequent times. In the E.M. studies the membrane and the nucleoid of the particle first appear at early stages of budding and the blebbing envelope is continuous with the cell

membrane (Bader, 1969). Infectious particles are completed by a budding process during which the envelope is acquired from the plasma membrane of the infected cell (DeHarven and Friend, 1960). Other than the budding particles, no structures can be seen which can be identified as virus specific and which can be shown to be part of the replication process (Bader, 1969).

The kinetics of intracellular synthesis has been studied for several oncogenic RNA viruses. Infectious virus can be detected in the tissue culture fluid of MEF cultures directly infected with RLV (Schlom, et al., 1971) and budding from the thymus of FLV infected mice (DeHarven and Friend, 1965) as early as 24 hours after exposure to the virus. Bader (1966) and Hanafusa and Hanafusa (1966) reported a minimum interval of 8 hours between initial infection with RSV and the appearance of infectious progeny.

Studies using cultures persistently infected with Rich Virus (Okano and Rich, 1969) and RLV (Bader, 1970) appear to indicate that synthesis of viral RNA requires about one hour and that synthesis of completed infectious virions requires about two hours.

Attenuation of tissue culture passaged virus. DeHarven and Friend (1966) reported that viruses harvested from chronically infected long term suspension cultures, were highly immunogenic, but only weakly leukemogenic. Similar losses in leukemogenicity have been reported by Moore (1963), Barski and Youn (1966), and Wright and Lesfargues (1966). These investigators reported that viruses passaged

in tissue culture undergo "attenuation" or reduction in leukemogenic potential.

Recent papers by Schlom, et al. (1971) and Yoshikura, et al.(1969), report that RLV and FLV lose leukemogenicity, but not infectivity as judged by their ability to act as "helpers" for defective MSV (Fischinger and O'Connor, 1968). Schlom, et al. (1971) report that attenuation is detectable after only one passage in tissue culture. This suggests that RLV and FLV which are closely related viruses that either (a) are mixtures of leukemogenic and nonleukemogenic virions or (b) that passage in tissue culture converts the leukemogenic population used as an inoculum into a nonleukemogenic population.

In the present studies further information on the effects of FLV infection upon macromolecular synthesis, cell multiplication and upon the kinetics of viral replication is provided. The data to be described indicate that:

1. infection of MEF-2 with FLV results in an increase in the rate of DNA synthesis which is not accompanied by an increase in the rate of RNA and protein synthesis;

2. the observed increase in DNA synthesis is at least in part the result of an increase in the number of cells in the population which synthesize DNA;

3. the increase in the rate of DNA synthesis observed in FLV infected MEF-2 may occur in the absence of cell multiplication;

4. cells infected with FLV have a significantly shorter T than uninfected cells, i.e., faster multiplication rate;

5. infected cells grow to greater population densities in tissue culture than uninfected cells;

6. the time required for infected cells to traverse the G1 and S

phases of the cell cycle is reduced in FLV infected cells;

7. MEF-2 can be productively infected by FLV. The time course of virus production will be described;

8. the virus released is attenuated, i.e., nonleukemogenic in mice.

II. MATERIALS AND METHODS

Tissue Culture Media

Dehydrated basal medium (Eagle, 1959) with Earle's balanced salt solution (Earle, 1943), diluted with glass distilled water, sterilized by membrane filtration (BME) and supplemented with 10% fetal calf serum (FCS) (BME10FCS) was employed for the propagation of primary and secondary mouse embryo fibroblasts and XC cells (see section on Tissue Culture).

McCoy's 5A (modified) medium (McCoy, et al., 1959), supplemented with 15% FCS (McC15FCS) was employed for the propagation of 3T3 cells (see section on Tissue Culture).

The FCS, dehydrated BME and McCoy's 5A (modified) medium were obtained from Grand Island Biological Company, Grand Island, N.Y.

All media contained penicillin (250 units/ml), streptomycin (0.2 mg/ml) and mycostatin (50 units/ml).

Tissue Culture

Primary mouse embryo fibroblast cultures (MEF-1) were prepared according to the method described by Hartley, et al. (1965) from the embryos of National Cancer Institute (NCI) or random-bred Swiss mice obtained from Taconic Farms (Germantown, N.Y.). Briefly, 10-13 day old embryos were obtained by Caesarian section and the heads and

limbs removed. The carcasses were then washed with mammalian Ringer's solution (Abbott Laboratories, N. Chicago, Ill.) and minced with scissors. Cellular suspensions were then prepared by incubating the minced tissues with 50 ml of 0.25% trypsin (Diffco 1:250) in Hank's balanced salt solution (calcium and magnesium free) (HBSS-CMF) (Grand Island Biological Company, Grand Island, N.Y.) in a trypsinization flask (Bellco) equipped with a teflon coated magnetic stirring bar (ca. 180 rpm) at 37°C. The first aliquot of trypsin-cell suspension was decanted through sterile gauze into a 50 ml sterile glass conical centrifuge tube containing 1 ml of FCS. An amount of fresh 0.25% trypsin in HBSS-CMF equal to the aliquot decanted was added to the trypsinization flask. The incubation was continued for an additional 30 minutes and the resulting trypsin-cell suspension was collected in the same manner as described for the first aliquot of trypsin-cell suspension. In general, the second aliquot of trypsin-cell suspension was used as a source of cells.

The cell concentration of the trypsin-cell suspension was determined with a hemacytometer and the ability of the cells to exclude trypan blue was used as a measure of their viability (Blaker, et al., 1971). The cells were seeded into BME10FCS at a concentration of 4×10^5 viable cells per ml. Primary cultures were usually planted in 40 ml of BME10FCS in 32 ounce prescription bottles or in 20 ml of BME10FCS in 75cm^2 plastic tissue culture bottles (Falcon Plastics). The bottles were then gassed with 5% CO_2 in air and incubated at 37°C until the

monolayers were confluent (usually 5-6 days).

Secondary mouse embryo fibroblast cultures (MEF-2) were prepared by dispersing confluent primary mouse embryo fibroblasts with 0.25% trypsin in HBSS-CMF and thenseeding the resulting cellular suspension in BME10FCS. MEF-2 monolayer cultures were used routinely in these experiments except where otherwise specified.

The 3T3 cells (Todaro and Green, 1963), an established line developed from random bred Swiss mouse embryos with a confluent density of 10^6 cells per 50 mm petri dish were the kind gift of Dr. C. Basilico. They were grown in monolayer culture in $75cm^2$ plastic tissue culture flasks (Falcon Plastics) containing 20 ml of McC15FCS. The 3T3 cells were subcultured every third or fourth day by dispersing the monolayer with 0.25% trypsin in HBSS-CMF and seeding the resulting cellular suspension in 20 ml of McC15FCS at 5×10^4 cells per ml. When used in the mixed culture cytopathogenicity assay (Klement, et al., 1969), they were seeded into 6cm plastic tissue culture dishes (Falcon Plastics) containing 4 ml of McC15FCS at 5×10^4 cells per ml and incubated.[1]

The XC cells, an established rat tumor cell line originally induced by the Prague strain of RSV (Svoboda, et al., 1963), used in these assays were grown in monolayer culture in $75cm^2$ plastic tissue culture flasks containing 20 ml of McC15FCS. They were subcultured every third or

[1] This and all subsequent incubations were carried out in humidified 5% CO_2 in air at 37°C unless otherwise noted.

fourth day by dispersing the monolayer with 0.25% trypsin in HBSS-CMF and seeding the resulting cellular suspension at 5 x 10^4 cells per ml into 75 cm^2 plastic tissue culture flasks containing 20 ml of McC15FCS.

Radioactive Precursors

(Methyl-^{3}H) Thymidine (specific activity 1.9 Ci/mM) was purchased from Schwarz Bioresearch, Orangeburg, N.Y. (SBR) (^{3}H-TdR).

5-^{3}H-Uridine (specific activity 21.0 Ci/mM) was purchased from SBR (^{3}H-UR).

(4,5-^{3}H (n))-1-leucine (specific activity, 38.5 Ci/mM) was purchased from New England Nuclear Corp., Boston, Mass. (^{3}H-leu).

Virus Stock

The FLV used in these experiments was obtained by inoculating randomly bred Swiss mice (Taconic Farms) intraperitoneally (IP) with 0.2 ml of leukemic spleen filtrate prepared as described previously (Friend, 1957). After 7 days the blood of the inoculated mice was collected from the axilla, immediately diluted 1:1 with mammalian Ringer's solution (Abbott Laboratories) containing 4 units of heparin (Upjohn) per ml and was chilled in ice. The pooled, diluted blood was first centrifuged at 1500 rpm for 10 minutes at 4°C. The plasma was then decanted, filtered through a 0.45 micron membrane filter (Millipore) and then filtered through a 0.22 micron membrane filter, sealed

in glass ampoules and stored at -78°C until used.

Where denatured FLV (D-FLV) was required in these experiments FLV was heated in a water bath for 40 minutes at 56°C.

In Vivo Viral Titration

Samples were titered by incoulating 0.2 ml of each serial 10-fold (whole log) dilution IP into groups of 5 Swiss mice. The inoculated mice were periodically examined for the development of leukemia. Splenomegaly was used as the major indicator of leukemia and was determined by wet spleen weight. At the end of eight weeks any mouse with a spleen weight of 600 mg or more was considered positive (Moore, 1963). Selected spleen, liver and sternal sections were examined histologically throughout the course of this study. The viral titer was calculated according to a modification of the method of Reed and Meunsch (Friend, 1959).

Cell free supernatant tissue culture fluids as well as pooled plasma virus samples were titered as described above.

In Vitro Viral Titration

The presence of FLV in various samples of pooled plasma or cell-free tissue culture fluid was also determined by a modification of the mixed-culture cytopathogenicity test as described by Klement, et al., (1969).

The titration was performed as follows: 6 cm plastic tissue culture dishes containing 4 ml of McC15FCS were seeded with 2 x 10^5 3T3 cells and the dishes were incubated for 24 hours. In order to enhance the efficiency with which the 3T3 cells could be infected, the tissue culture medium in each plate was removed and replaced with 2 ml of McC15FCS containing 0.050 mg of diethylaminoethyl-dextran (DEAE-D) (Pharmacia) of molecular weight ca. 2 x 10^6 and returned to the incubator for one hour. The DEAE-D was decanted and the cell sheets washed with McCoy's medium. Duplicate plates were inoculated with 0.2 ml of the sample to be tested for the presence of virus and returned to the incubator. After 30 minutes, 4 ml of McC15FCS was added to each dish and the dishes returned to the incubator.

On the fourth day, the dishes were subcultured by detaching the cell sheet from each tissue culture dish with 1 ml of 0.25% trypsin in HBSS-CMF and adding 0.5 ml of tissue culture suspension to each fresh tissue dish containing 3.5 ml of McC15FCS. After five or six days further incubation, the tissue culture fluid was removed and several longitudinal strips scraped out of the cell sheet with a sterile wooden stick. The plates were then overlayed with 10^6 freshly trypsinized XC cells and 4 ml of McC15FCS added. The plates were then returned to the incubator and examined for syncytium formation two and four days after the plates were overlayed with XC cells.

When the cultures showed evidence of syncytium formation on examination with the inverted microscope, the tissue culture fluid was

decanted, the plates fixed for 10 minutes with absolute methanol and stained with Harris Hematoxylin (Fisher Scientific). Plates showing syncytium formation were scored as positive (Figure 1) and the titer was calculated by the method of Reed and Meunsch (1938). The titer was reported as the 50% tissue culture infective dose (TCID50) (see legend to Figure 14).

Macromolecular Synthesis

The extent of incorporation of ^{3}H-TdR, ^{3}H-UR or ^{3}H-leu into 5% TCA precipitable radioactive material during a one-hour interval was used as a measure of the rate of DNA, RNA or protein synthesis respectively (Schneider, 1945).

1.5×10^{6} viable MEF-2 cells were seeded into 10 cm plastic tissue culture dishes each containing 15 ml of BME10FCS and incubated for 72 hours. The supernatant medium was removed and 1 ml of FLV, D-FLV, or BME10FCS and 4 ml of BME10FCS added. The plates were allowed to incubate for 90 minutes. The supernatant fluid was removed, replaced with fresh BME10FCS and returned to the incubator. This was considered zero time post infection (PI). At various times PI cultures were sampled in triplicate and the extent of the incorporation of radioactivity following a one-hour pulse determined as follows:

After the desired incubation period, the tissue culture fluid was removed and 5 ml of fresh BME10FCS containing 2 microcuries (μCi) per ml of the appropriate radioactive precursor was added to each plate.

The plates were incubated for one hour. The medium was then decanted and the cell sheet washed with BME (where the rate of DNA synthesis was determined, 5 ml of BME supplemented with 0.05 mg per ml of thymidine was added to each plate and the plates allowed to incubate for 10 minutes). The cell sheets were washed twice with BME, scraped from the culture plates with a rubber policeman and suspended in 5 ml of BME. The cellular suspension was transferred to a 12 ml heavy walled conical centrifuge tube and the procedure continued at 4°C unless otherwise noted. An equal volume of 10% trichloracetic acid (TCA) was added. After 10 minutes the resulting precipitate was collected by centrifugation at 2000 rpm for 10 minutes. The precipitate was washed twice in 2 ml of 5% TCA and once in ethanol-ether (1:1). In order to minimize nucleic acid interference in determinations of the rate of protein synthesis the 5% TCA precipitate was heated to 90°C for 10 minutes in a water bath before washing with 5% TCA. After each wash the precipitate was collected by centrifugation at 2000 rpm for 5 minutes and the supernatant fluid discarded.

1 ml of 0.2N NaOH was then added to the washed precipitate. After 24 hours of incubation at room temperature, 0.5 ml of the resulting solution was added to 20 ml of a scintillating fluid (DND) (Beckman Instruments, 1967) containing:

2,5-Diphenyloxazole (PPO)	5.0	g
Naphthalene	100.0	g
Dioxane to	1000.0	ml

and counted in a Beckman LS250 spectrometer. The counting efficiency was 44%.

Protein Determination

Protein determinations were made according to the method of Lowry, et al. (1951) using bovine serum albumin (Fraction V, Pentex) as the standard.

Determination of the Percent of the Cell Population Engaged in DNA Synthesis

The proportion of the cell population engaged in DNA synthesis (Labeling Index)(LI) was determined in the following manner:

Sterile glass 22mm^2 coverslips were placed into 3-5 cm plastic tissue culture dishes. Each dish was then seeded with approximately 10^5 MEF-2 cells in 2 ml of BME10FCS and the plates incubated for 72 hours. The tissue culture medium was then removed and 0.2 ml of FLV, D-FLV or BME10FCS was added followed by 1.8 ml of BME10FCS. The plates were returned to the incubator for 90 minutes. The supernatant fluid was then removed and replaced with fresh BME10FCS and again placed in the incubator. This was considered zero time PI. At various times PI, the tissue culture medium was decanted from triplicate cultures and 2 ml of fresh BME10FCS containing 2μCi/ml was added to each culture. The plates were incubated for one hour. The ^{3}H-TdR containing medium was then decanted and 2 ml of BME10FCS supplementing with 0.1 mg of unlabeled thymidine was added to each culture.

The cultures were returned to the incubator for 10 minutes. The supernatant fluid was removed and the plates were washed twice with cold BME. The coverslips were fixed for 20 minutes in absolute methanol, air dried and cemented with Harleco Synthetic Resin (Scientific Supply Co.) to clean 1 x 3" microscope slides with the cell monolayer up. After the cement had dried the slides were washed twice with absolute methanol, air dried, dipped in darkness in undiluted NTB-2 nuclear track emulsion (Kodak). The emulsion was air dried and exposed for an appropriate length of time (generally 4-7 days), in a light-tight box. The slides were developed in D19 developer (Kodak) for three minutes and fixed in Kodak fixer for three minutes. The slides were air dried and stained with Harris Hematoxylin (Fisher Scientific Co.). Randomly, at least 500 interphase nuclei were examined on each slide. Every interphase nuclei over which 5 or more grains were seen, was considered radiolabeled. The labeling index was the percentage of labeled nuclei in the sample counted.

When experiments were undertaken which required stationary cultures, such cultures were produced as described in the Stationary Cultures below. The ^{3}H-TdR was added to the cultures in BME.

Cell Growth

3.5 cm plastic tissue culture dishes containing 2 ml of BME15FCS were seeded with 10^{5} viable MEF-2 cells. After 24-48 hours incubation, the medium was decanted and 0.2 ml of FLV, D-FLV or BME10FCS

was added, followed by 1.8 ml of fresh BME10FCS. After 90 minutes of incubation the inocula were decanted and replaced with an equal amount of fresh BME10FCS. This was considered zero-time PI. At various times thereafter, triplicate samples of virus inoculated and control cultures were removed from the incubator and the supernatant fluid from each plate transferred to an individual tube. Each plate was rinsed with 1 ml of 0.25% trypsin in HBSS-CMF. The trypsin solution was removed and added to the appropriate test-tube containing the supernatant fluid. 1 ml of fresh trypsin solution was again added to each plate. The plates were incubated for 5 minutes and the remaining cells detached from the plate with a rubber policeman. Finally, the cellular suspension was transferred to the appropriate test tube with a Pasteur pipette and the cellular suspension counted with a hemacytometer. The ability to exclude trypan-blue was used as a measure of their viability.

The time required for the cell population to double (doubling time) during the logarithmic phase of growth was considered equivalent to the generation time (T) since a very small percentage of the population was found to be nonviable during these determinations (Cleaver, 1967). The number of cells per plate at 72 hours PI is referred to as the "maximum cell density" (MCD).

Cell Cycle Analysis

The duration of the individual phases of the cell cycle were measured according to the method of Stanners and Till (1960).

MEF-1 cells were seeded in 6 cm plastic tissue culture dishes at 10^5 cells per ml in 4 ml of BME10FCS and incubated for 24 hours. The tissue culture fluid was decanted and 0.5 ml of FLV, D-FLV or BME10FCS added to each plate. 4 ml of BME10FCS was added to each plate and the plates returned to the incubator for 90 minutes. The supernatant fluid was removed and replaced with 4 ml of BME10FCS to which 1 μCi of ^{3}H-TdR had been added. This was considered zero time. At hourly intervals thereafter, tissue culture dishes were removed from the incubator and the fluid and cells from each plate harvested as previously described in the section Cell Growth. Slides were prepared from the cellular suspension with the aid of a cytocentrifuge (Shandon). Approximately 5 x 10^4 cells in 0.3 ml of BME10FCS were used to prepare each slide. The slides were then fixed for 20 minutes in absolute alcohol methanol, washed twice with absolute methanol, air dried and dipped in darkness in NTB-2 nuclear track emulsion (Kodak) and stored in a light-tight container at 4°C for 10 days. The slides were then developed and fixed as described in Cell Growth section (page 29-30), and stained with Harris Hematoxylin.

The duration of mitosis was calculated from the generation time and the average mitotic index (see Appendix A). The percentage of cells in mitosis (Mitotic Index) (MI) was determined by counting the number of cells showing a single group of clearly discernible chromosomes in the absence of a nuclear membrane per 100 cells counted. One thousand cells were counted in each determination.

The duration of G-2 (T-G2) was determined from counts of the percentage of mitoses which were labeled with ^{3}H-TdR as determined by autoradiography. Fifty mitoses were usually examined and any cell over which five or more grains were seen was considered to be labeled. Labeled mitoses curves were made by plotting the percent of labeled mitoses on the ordinate and time on the abcissa. The T-G2 was estimated by plotting the first derivative of the labeled mitosis curves. The time at which the peak of the derivative curve occurred was reported as the modal T-G2. The duration of S phase was determined as follows:

The LI was determined at hourly intervals and the values obtained were plotted versus time. The Y intercept of the line thus described was determined by linear regression analysis (Hill, 1966). The value obtained for the Y intercept was taken as the L(t) corr. (Stanners, J. personal communication). (The L(t) corr. is the percentage of labeled cells that would have been observed had no cell division taken place and the labeling had been instantaneous.) The length of S phase was then calculated by inserting the experimental values obtained into the equation:

$$(1) \quad S = (T/\ln 2)\,[L(t)\ \text{corr.} + e^{\ln 2 G2/T}] - G2$$

The duration of G1 was obtained by difference.

Stationary Cultures

When stationary cultures were required they were produced as follows: 48-72 hours after subculture, the supernatant tissue culture

fluid was removed from the MEF-2 cultures and replaced with BME. The BME was replaced every second day for 5 or 6 days, at which time the cultures were judged to be stationary by cell counts.

Electron Microscopy

The electron microscopic examination of FLV infected MEF-2 was done by Dr. E. DeHarven of the Sloan-Kettering Institute for Cancer Research. The cultures were fixed in glutaraldehyde and post-fixed in osmium tetroxide before ethanol dehydration and embedding in Epon. Ultra-thin sections cut with a diamond knife were stained with uranyl acetate, post-stained with lead hydroxide and viewed in a Siemens Elmskop I electron microscope.

III. RESULTS

Macromolecular Synthesis

DNA Synthesis

The extent of incorporation of a one-hour pulse of ^{3}H-TdR into TCA precipitable radioactive material was taken as a comparative measure of the rate of DNA synthesis in MEF-2 cultures. The rate of DNA synthesis was measured at 1, 4, 24, 48, 72, and 96 hours post infection (PI) and the results reported in cpm per mg protein.

The data from a typical experiment are shown in text figure 2. The rate of incorporation of ^{3}H-TdR into DNA appears to reach a maximum within 24 hours in both experimental and control cultures. At 24 and 48 hours the rate of DNA synthesis is significantly greater in FLV-treated cultures than in either of the controls ($p \leq 0.05$)[1] (Hill, 1966).

Text figure 3 compares the rate of DNA synthesis in FLV and D-FLV treated cultures as a percentage of the rate of DNA synthesis observed in untreated control cultures. The data represents the average of at least three experiments (including the data in text figure 2) in which there were triplicate samples. The rate of DNA synthesis in FLV treated cultures is 161% of uninfected control at 24 hours ($p < 0.002$) and 202% of uninfected controls at 48 hours ($p < 0.03$). This stimulation of DNA synthesis, which is not seen in cultures treated with D-FLV appears to be due to virus action.

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[1] This, and all succeeding "p" values were determined by student's "t" test (Hill, 1966).

RNA Synthesis

The extent of incorporation of a one-hour pulse of ^{3}H-UR into TCA precipitable radioactive material was taken as a comparative measure of the rate of RNA synthesis in MEF-2 cultures. The rate of RNA synthesis was measured at the same time intervals used for DNA and the data reported as cpm per mg protein.

The data from a typical experiment are shown in text figure 4. The rate of incorporation of ^{3}H-UR appears to decline throughout the experimental period and no significant difference in the rates of incorporation of precursor between the values obtained from experimental and control cultures is seen during the experimental period ($p > 0.05$).

Text figure 5 compares the rate of RNA synthesis in FLV and D-FLV treated cultures as a percentage of the rate of RNA synthesis in uninfected control cultures. The data represent the average from triplicate samples of at least three experiments (including the data from text figure 4). The rate of RNA synthesis in experimental and control cultures is not significantly different.

Protein Synthesis

The extent of incorporation of ^{3}H-leu into TCA precipitable radioactive material during a one hour pulse was taken as a measure of the rate of protein synthesis in MEF-2 cultures. The rate of protein synthesis was measured at 1, 4, 24, 48, 72, and 96 hours PI and results reported in cpm per mg protein. Data for a typical experiment are shown in text figure 6.

Text figure 7 compares the rate of protein synthesis in FLV and D-FLV treated cultures and the results are reported as a percentage of the rate of protein synthesis in the uninfected control cultures. The data represent the average of three experiments that were sampled in triplicate (including the data in text figure 6).

The data in text figures 6 and 7 show that no appreciable difference in the rate of protein synthesis occurs as a result of infection with FLV ($p > 0.05$).

Labeling Index

The percentage of cells in the population which displayed nuclear labeling following a one hour exposure to ^{3}H-TdR (LI) was determined for FLV treated MEF-2 cultures, D-FLV treated cultures, and uninfected control cultures by autoradiography. The LI was determined at 1, 4, 24, 48, 72, and 96 hours PI.

The data for a typical experiment are shown in text figure 8. Although the LI appears to reach a maximum at about 24 hours PI in both experimental and control cultures, it appears to be significantly greater in FLV treated than in D-FLV treated or untreated cells ($p < 0.005$).

Text figure 9 compares the LI in FLV and D-FLV treated cultures. The data obtained from three experiments, each with triplicate samples, including that which appears in text figure 8, are expressed as a percentage of the LI observed in untreated control cultures. The LI in FLV treated cultures was 171% of untreated controls at 24 hours PI

and 159% of untreated controls at 48 hours PI ($p < 0.05$). Since a significant increase in the number of cells synthesizing DNA was not seen in cell cultures exposed to D-FLV when compared with untreated controls, active virus appears to be necessary for stimulation.

In order to determine whether similar effects on the LI were encountered in cell cultures which were not actively dividing, "resting" cells were prepared according to the method described on page 20 (Tissue Culture Media) and the effect of FLV and D-FLV on the kinetics of the LI in these "resting" cultures was determined. The data for a typical experiment are shown in text figure 10. The LI appears to reach a maximum at about 24 hours PI in both infected cultures and controls. The LI, however, is significantly greater in FLV infected cell cultures than in controls ($p < 0.05$).

In text figure 11 the LI of FLV and D-FLV treated "resting" cultures are compared. The data from three experiments, each sampled in triplicate, including the data in text figure 10, are expressed as a percentage of the LI observed in untreated control cultures. The LI in FLV exposed cultures was 277% of control ($p < 0.001$) at 24 hours PI and 216% of control at 48 hours PI ($0.05 < p < 0.10$). Active virus again appears to be necessary for this response also since an increase in the LI was not seen in the control cultures.

Cell Growth

Generation Time

Cell growth was followed by counting the number of viable cells in

triplicate cultures exposed to FLV, D-FLV, or BME10FCS at various intervals PI. The viable cell counts were taken to be equivalent to the total cell number since few dead cells were observed in these cultures during the experimental period; in repeated autoradiographic experiments the LI was found to be 95-99% after 24 hours. In these experiments the times required for the cell populations to double was determined during the logarithmic phase of growth (usually after 4 to 6 hours PI). The time required for the cell population to double during logarithmic growth was considered the generation time (T). The data for a typical experiment are shown in text figure 12. The average T obtained from three separate experiments, each sampled in triplicate, was 15.8 ± 0.44 hours for FLV treated cultures; 26.3 ± 2.3 hours for D-FLV treated cultures, and 22.9 ± 1.56 hours for untreated cultures (Table 1). T for FLV treated cultures was 67% of the untreated cultures ($p < 0.02$), whereas T for D-FLV inoculated cultures was 115% of untreated control cultures ($p > 0.2$). The experiments indicate that FLV inoculated cultures have a significantly shorter T than either D-FLV inoculated or untreated cultures.

Cell Density

Since all of the cultures sampled in these experiments were in stationary phase by 72 hours PI, the cell counts obtained 72 hours after treatment were considered to be a relative measure of the cell densities in experimental and control cultures. Cell densities in FLV

treated cultures were significantly higher than in D-FLV inoculated cultures when compared as a percentage of the uninfected controls ($p < 0.05$) (Table 2).

Multiplication of "Resting" Cells

Since both T and cell density were altered by treatment with FLV, its effect on proliferation of "resting' cells in serumless medium was studied. "Resting" cultures were prepared according to the method described in the first section of "Methods and Materials." The cultures were treated with FLV, D-FLV, and BME, and the number of cells determined at various times subsequent to treatment. (The number of dead cells never exceeded 7.5% of the total cell count and was usually zero during the period of the experiment.) No significant increase in cell number occurred during the 72 hour experimental period (the cell population did not double during the experiment) and no apparent differences between the growth rate in experimental and control cultures were observed during the time in which the cultures were followed as has been found by others (Murray and Temin, 1971).

Cell Cycle Analysis

Since FLV inoculation was found to reduce T, experiments were performed to determine which segments of the cell cycle were affected. The duration of the phases of the cell cycle were determined by the method of Stanners and Till as described on page 30 (Cell Cycle Analysis). The data for a typical experiment are reported in Table 3.

The duration of G2 (T-G2) and mitosis (T-M) in FLV treated cells was not found to be significantly different from controls.

The duration of G1 (T-G1) was found to be 5.28 hours in FLV treated MEF-2, 9.78 hours in D-FLV treated cells, and 8.65 hours in untreated cells. Therefore T-G1 in FLV treated cells was 60.5% of untreated control and in D-FLV treated cells it was similar to (113% of) untreated controls.

The duration of S-phase (T-S) is 5.55 hours in FLV treated cells, 11.67 hours in D-FLV treated cells, and 9.67 hours in untreated cells. T-S in FLV treated cells is 58% of that in untreated cells, while in D-FLV inoculated cells, it is 120% of the value found in untreated controls.

Virus Production

FLV Infection of MEF-2 Cultures

In order to determine whether inoculation of MEF-2 cultures with FLV resulted in productive infection, subconfluent cultures were inoculated with FLV, refed on day 3 and subcultured on day 7 of each week.

Samples of cells obtained on the second, seventh, and twenty-first days PI were examined under the electron microscope by Dr. E. DeHarven of the Slaon-Kettering Institute for Cancer Research for the presence of virus. No virus was seen on day 2 or day 7. The FLV treated 21 day samples showed virus budding from cell membranes (text figure 13). No virus was seen budding from the controls at 21 days PI.

The Kinetics of Virus Production

The time course of FLV production by MEF-2 cells was determined by a modification of the method of Klement, et al. The ability of XC cells to undergo syncytium formation when placed in contact with MEF-2 cells inoculated with tissue culture fluids from FLV treated cultures, D-FLV treated cultures, and untreated cultures was determined. Log dilutions of the tissue culture fluids were used in order to determine their titer. The data in a typical set of titrations of the tissue culture fluids is shown in text figure 14.

The 50% tissue culture infective dose (TCID50) at each PI period was determined by methods described by Reed and Meunsch (1938). Briefly, the cumulative percentage of infected cultures was plotted versus the negative log of the virus sample dilution. The TCID50 was then considered to be the dilution of virus which infected 50% of the cell cultures (text figure 14).

The TCID50s such as obtained from text figure 14 were plotted versus time in text figure 15. The number in parenthesis indicates the number of experiments performed in which 4 to 6 replicate samples per point were assayed.

No curve is shown for either denatured virus or uninfected control cultures since syncytium formation was not seen at any dilution of the tissue culture fluids from either of the controls at any time PI.

Virus can be detected in the medium harvested from FLV treated cultures as early as one hour PI. Increases in viral titer above the

initial values began at some time between one and four hours PI and reached a maximum between 96 and 168 hours PI. The titer at 96 hours PI was $10^{3.9}$ TCID50s per 0.2 ml of tissue culture supernatant.

An experiment was performed in which fresh leukemic mouse plasma was used as the source of virus. The virus plasma was titered in mice by the method of Reed and Meunsch and in tissue culture by the method described by Klement, et al. The virus yielded a titer in mice of $10^{3.5}$ 50% lethal doses (LD50s) per 0.2 ml and $10^{5.3}$ TCIDs/0.2 ml *in vitro*. Denatured virus and untreated controls gave no evidence of the ability to promote syncytium formation.

The ability of cell free tissue culture fluids obtained at various times PI from FLV treated and control MEF-2 cultures to cause leukemia when inoculated intraperitoneally into young adult female Swiss mice was investigated. Our data confirms that of Moore (1963) in that tissue culture virus was found to have low leukemogenic potential and to induce leukemia sporadically.

A. Uninoculated Control Culture.

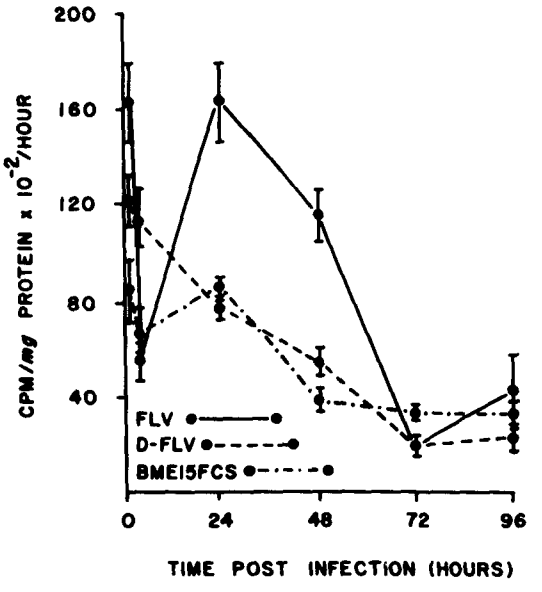
B. Culture inoculated with FLV on day 1.

Text Figure 1. MEF-2 cells 12 days after subculture and two days after addition of XC cells. Harris Hematoxylin x 100.

Text Figure 1. MEF-2 cells 12 days after subculture and 2 days after addition of XC cells, Harris Hematoxylin x 100.

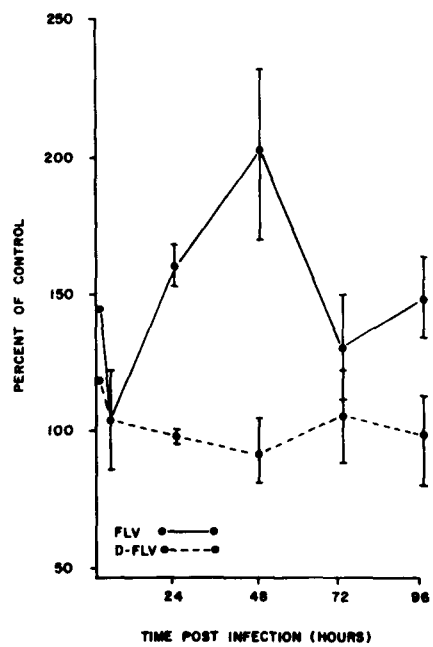
A. Uninoculated control culture.

B. Culture inoculated with FLV on day 1.



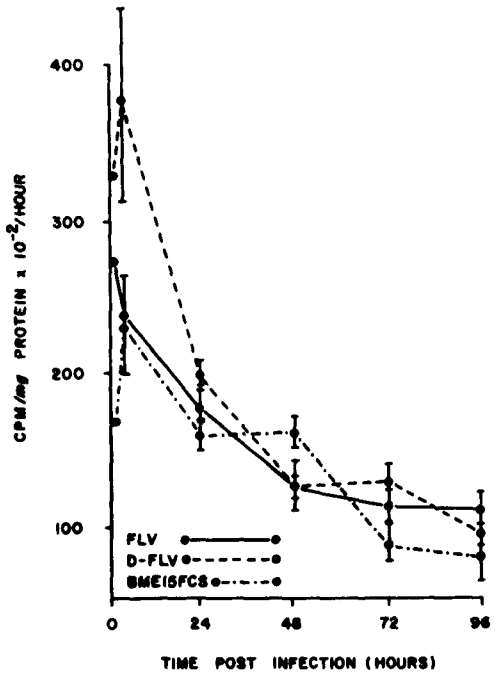
TEXT FIG. 2 RATE OF INCORPORATION OF ^{3}H-TdR INTO MEF-2

Text Figure 2. Rate of incorporation of ^{3}H-TdR into MEF-2. 1.5×10^{6} MEF-2 cells were seeded into 10 cm plastic tissue culture dishes each containing 15 ml of Basal Medium Eagle supplemented with 15% Fetal Calf Serum (BME15FCS) and incubated for 72 hours. The medium was then decanted and 0.0 ml Friend Leukemia Virus (FLV) titrate was added to some dishes. Other dishes received either virus denatured by heating at 56°C for 40 minutes (D-FLV) or BME15FCS. The dishes were incubated for 90 minutes after which the supernatant was removed and replaced with fresh BME15FCS (zero time post-infection). At 1, 4, 24, 48, 72, and 96 hours PI the cultures were sampled in triplicate and the extent of incorporation of ^{3}H-TdR (1.9 Ci/mM, 2μCi/ml) into 5% TCA insoluble product during a one-hour pulse was determined as described in the Materials and Methods section (page 26).
——— FLV; ------- D-FLV treated; -.-.-.- BME15FCS treated.
Vertical arrows in all figure legends indicate one standard error.



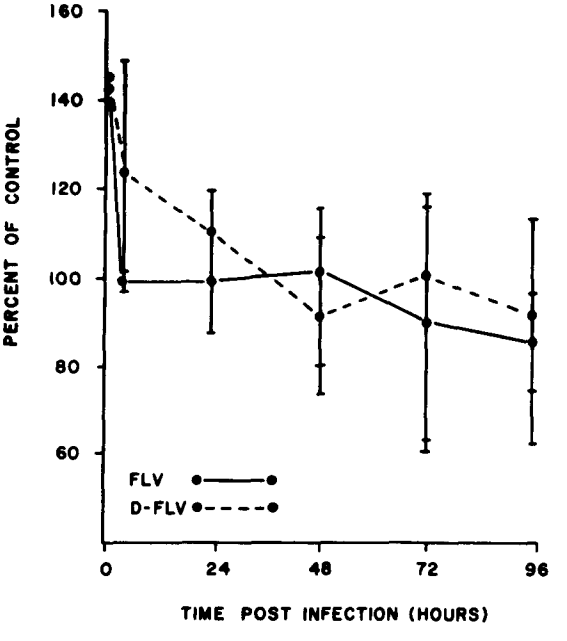
TEXT FIG. 3 RATE OF INCORPORATION OF ^{3}H-TdR INTO MEF-2

Text Figure 3. Rate of incorporation of ^{3}H-TdR into MEF-2. The extent of incorporation of ^{3}H-TdR into acid-insoluble product during a one-hour pulse was measured as described in the legend to text figure 2 and Methods and Materials section (page 26). The rate of DNA synthesis in FLV treated and D-FLV treated MEF-2 cell cultures are compared as a percentage of the untreated controls. ———FLV; - - - - -D-FLV.



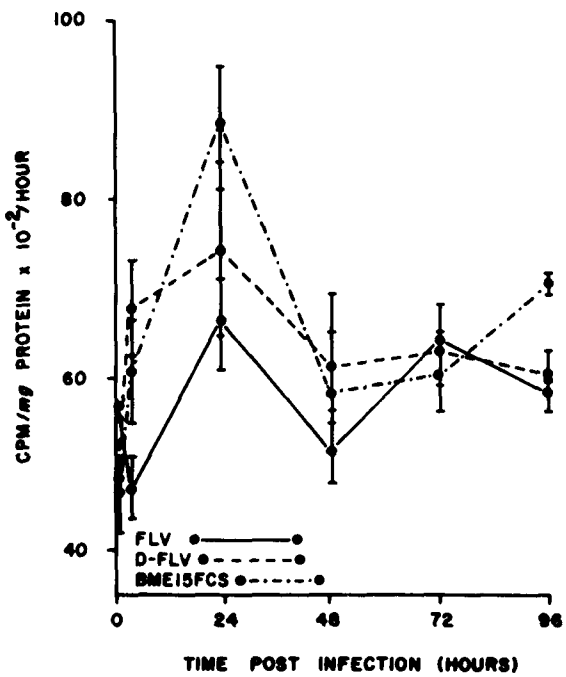
TEXT FIG. 4 RATE OF INCORPORATION OF ^{3}H-UR INTO MEF-2

Text Figure 4. Rate of incorporation of ^{3}H-UR into MEF-2. At 1, 4, 24, 48, 72, and 96 hours PI the cultures were sampled in triplicate. The extent of incorporation of ^{3}H-UR (21.0 Ci/mM; 2μCi/ml) into acid insoluble product during a one-hour pulse was determined as described in text figure 2 and Methods and Materials section (page 26). ——— FLV; ------- D-FLV; -·-·-·- BME15FCS.



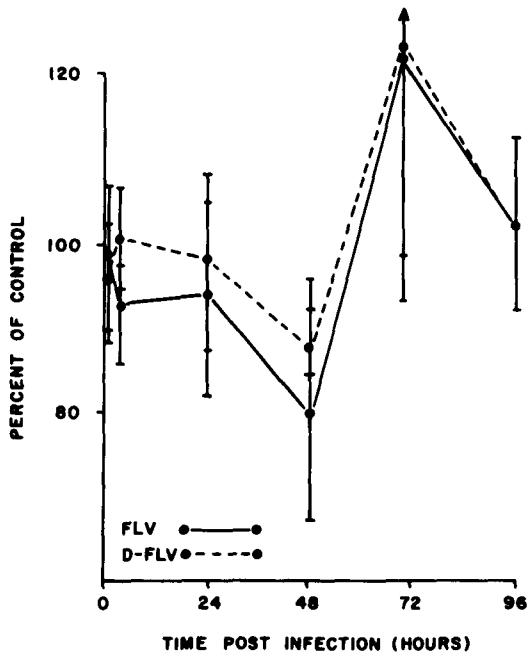
TEXT FIG. 5 RATE OF INCORPORATION OF ^{3}H-UR INTO MEF-2

Text Figure 5. Rate of incorporation of ^{3}H-UR into MEF-2. The extent of incorporation of ^{3}H-UR into an acid insoluble product during a one-hour pulse was measured as described in text figure 3 and in the Methods and Materials section (page 26). The rate of RNA synthesis in FLV and D-FLV treated cell cultures are compared as a percentage of the untreated controls. ——— FLV; - - - - - - - D-FLV.



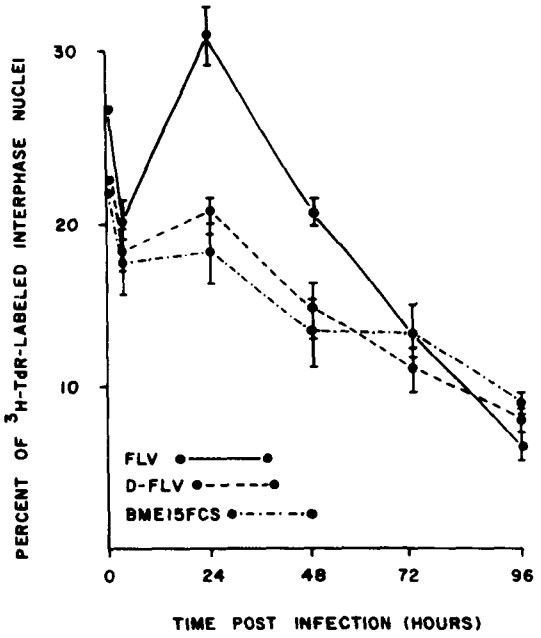
TEXT FIG. 6 RATE OF INCORPORATION OF ^{3}H-LEU INTO MEF-2

Text Figure 6. Rate of incorporation of ^{3}H-LEU into MEF-2. At the times described in text figure 2 the cultures were sampled in triplicate and the extent of incorporation of ^{3}H-1-LEU (38.5 Ci/mM; 2μCi/ml) into hot 5% TCA (90°C for 10 minutes) insoluble, product during a one-hour pulse was determined as described in the Materials and Methods section (page 26). ——— FLV; ------- D-FLV; -.-.-.-.-.- BME15CFS.



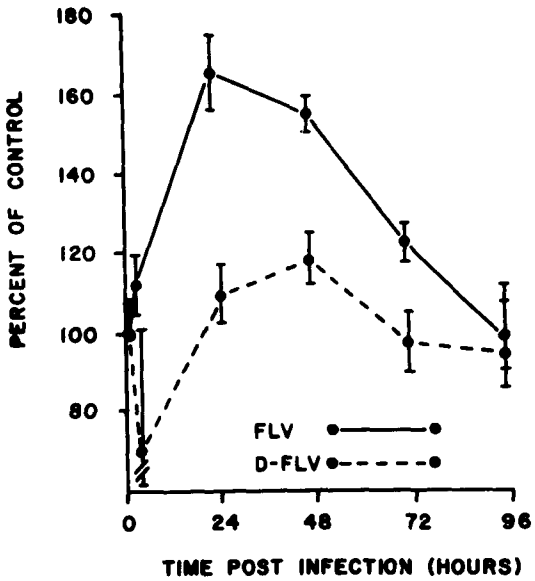
TEXT FIG. 7 RATE OF INCORPORATION OF ^{3}H-LEU INTO MEF-2

Text Figure 7. Rate of incorporation of ^{3}H-LEU into MEF-2. The extent of incorporation of ^{3}H-LEU into hot TCA insoluble product during a one-hour pulse was measured as described in the legend to text figure 6. The rate of protein synthesis in FLV and D-FLV treated cell cultures are compared as a percentage of the untreated controls. ——— FLV; - - - - D-FLV.



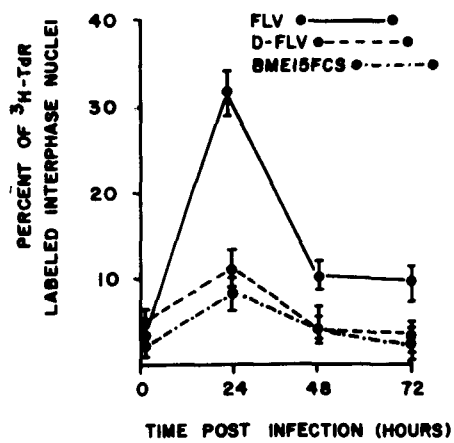
TEXT FIG. 8 EFFECT OF FLV AND D-FLV ON THE NUCLEAR LABELING INDEX IN MEF-2

Text Figure 8. Effect of FLV and D-FLV on the Nuclear Labeling Index in MEF-2. The proportion of the cell population engaged in DNA synthesis was determined in the following manner: 10^5 MEF-2 cells were seeded into 3.5 cm plastic tissue culture dishes containing sterile 22 cm^2 coverslips and 2 ml of BME15FCS and incubated for 72 hours. The medium was decanted and 0.2 ml of FLV suspension was added to some dishes. Other dishes received 0.2 ml of D-FLV or BME15FCS. The dishes were incubated for 90 minutes after which the supernatant was decanted and replaced with fresh BME15FCS. At the times indicated in the figure, the cultures were sampled in triplicate. The culture medium was removed and 4μCi of ^{3}H-TdR in 2 ml of BME15FCS was added to the dishes which were then incubated for one hour. The cells on the coverslips were then fixed and percentage of the cells with labeled nuclei were determined by radioautography as described in the Materials and Methods section (page 28). ——— FLV; ------- D-FLV; -·-·-·-·- BME15FCS.



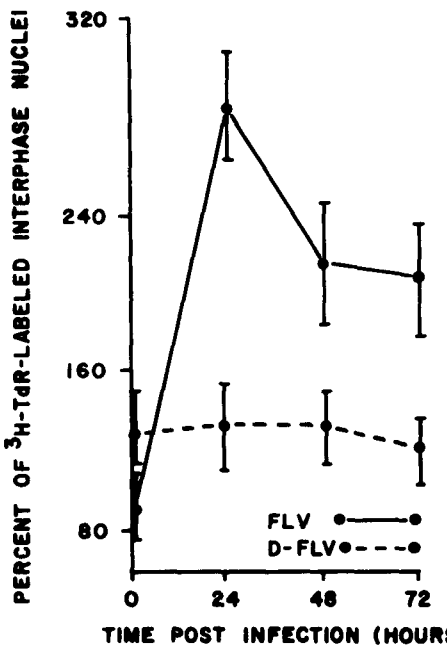
TEXT FIG. 9 EFFECT OF FLV AND D-FLV ON THE NUCLEAR LABELING INDEX IN MEF-2

Text Figure 9. Effect of FLV and D-FLV on the Nuclear Labeling Index in MEF-2. The proportion of the cell population engaged in DNA synthesis was determined in the manner indicated in text figure 8 and in the Methods and Materials section (page 28). The LI in FLV and D-FLV treated cell cultures are compared as percentage of the untreated controls. ——— FLV; ------- D-FLV.



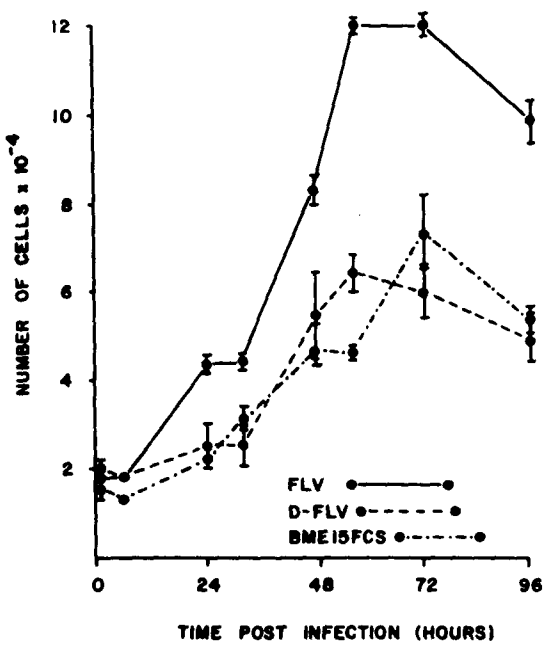
TEXT FIG. 10 EFFECT OF FLV AND D-FLV ON THE NUCLEAR LABELING INDEX IN "RESTING" MEF-2

Text Figure 10. Effect of FLV and D-FLV on the Nuclear Labeling Index in "Resting" MEF-2. The proportion of the cell population engaged in DNA synthesis in "resting" culture was determined in the following manner: 10^5 cells were seeded into 3.5 cm plastic tissue culture dishes containing sterile 22mm^2 coverslips and 2 ml of BME15FCS and incubated for 48 to 72 hours. The culture medium was then decanted and replaced with serum-free BME. The serum-free medium was replaced every two or three days for five to six days, at which time the culture was judged to be "resting." The medium was then decanted and 0.2 ml of FLV was added to some dishes. Other dishes received 0.2 ml of D-FLV or serum-free BME. The dishes were then incubated for 90 minutes after which the supernatant was decanted and replaced with fresh serum-free BME. At the times indicated in the figure the cultures were sampled in triplicate. The culture medium was removed and 4μCi of ^{3}H-thymidine in 2 ml of serum-free BME was added to the plates, which were then incubated for one hour. The cells on the coverslips were then fixed and the percentage of cells with labeled nuclei were determined by autoradiography as described in the Methods and Materials section (page 29). ———FLV; ------- D-FLV; -.-.-.-.-.- BME.



TEXT FIG. II EFFECT OF FLV AND D-FLV ON THE NUCLEAR LABELING INDEX IN "RESTING" MEF-2

Text Figure 11. Effect of FLV and D-FLV on the Nuclear Labeling Index in "Resting" MEF-2. The proportion of the cell population engaged in DNA synthesis was determined in "resting" cultures as described in text figure 10. The LI in FLV and D-FLV treated cell cultures are compared as a percentage of the LI in untreated control cultures. ——— FLV; ------- D-FLV.



TEXT FIG. 12 EFFECT OF FLV AND D-FLV ON THE GROWTH RATE OF MEF-2

Text Figure 12. Effect of FLV and D-FLV on the Growth Rate of MEF-2. 3.5 cm plastic tissue culture dishes containing 2 ml of BME10FCS were seeded with 10^5 viable MEF-2 cells. After 24 to 48 hours the medium was decanted and 0.2 ml of FLV, D-FLV or BME10FCS were added and then 1.8 ml of BME10FCS to bring each dish to its original volume. After 90 minutes, the inoculum was decanted and replaced with an equal volume of fresh BME10FCS. This was considered zero time PI. At the times indicated in the figure the cells in triplicate cultures were counted. ---------- FLV; - - - - - - - D-FLV; -·-·-·-·-·-·-BME15FCS.

Table 1

The Generation Time of MEF-2 Cells*

Exp.	FLV	D-FLV	Unt.[a]
1	15.5[b]	27.8	23.2
2	16.4	23.0	20.2
3	15.4	28.0	25.4
X_3	15.8	26.3	22.9
S.E.	0.26	1.63	1.56

* The time (hours) necessary for the cell population to double during the logarithmic phase of growth. Each experiment reported is the mean of triplicate determinations.

[a] Treated with BME15FCS.

[b] Hours

Table 2

The Cell Density of MEF-2 Cultures*

Exp.[a]	FLV	D-FLV	UNT[b]	FLV/UNT	D-FLV/UNT
1	63.00[c]	53.64	48.00	1.24	1.04
2	98.48	72.80	74.00	1.32	0.99
3	48.00	24.00	29.32	1.64	0.95
X_3				1.40	0.95
S.E.				0.12	0.06

* The cell number at 72 hours PI was taken as a measure of the relative density of the cultures.

[a] Each experiment reported is the mean of triplicate determinations.

[b] Treated with BME15FCS.

[c] Cells per 6 cm culture dish x 10^{-4}.

Table 3

Cell Cycle Analysis*

	EXP	FLV	D-FLV	UNT[a]
T-M	1	0.72[b]	0.83	0.65
	2	0.71	1.10	0.56
	3	0.49	0.63	0.53
	$\overline{X}_3$	0.64	0.85	0.58
	S.E.	0.08	0.14	0.04
T-G1	1	6.00[c]	11.31	8.99
	2	4.33	10.97	8.90
	3	5.50	7.05	8.06
	$\overline{X}_3$	5.28	9.78	8.06
	S.E.	0.40	1.12	0.26
T-S	1	5.08[d]	10.16	9.26
	2	5.76	11.23	9.44
	3	5.81	13.62	10.31
	$\overline{X}_3$	5.55	11.67	9.67
	S.E.	0.19	0.28	0.25
T-G2	1	4.00[e]	4.00	4.00
	2	4.00	3.00	4.00
	3	4.00	5.00	4.00
	$\overline{X}_3$	4.00	4.00	4.00
	S.E.	0.00	0.13	0.00

* The determination of the time (hours) required for MEF-2 cells to traverse the various stages of the cell cycle was made according to the method of Stanners and Till as described in the Materials and Methods section (page 30).

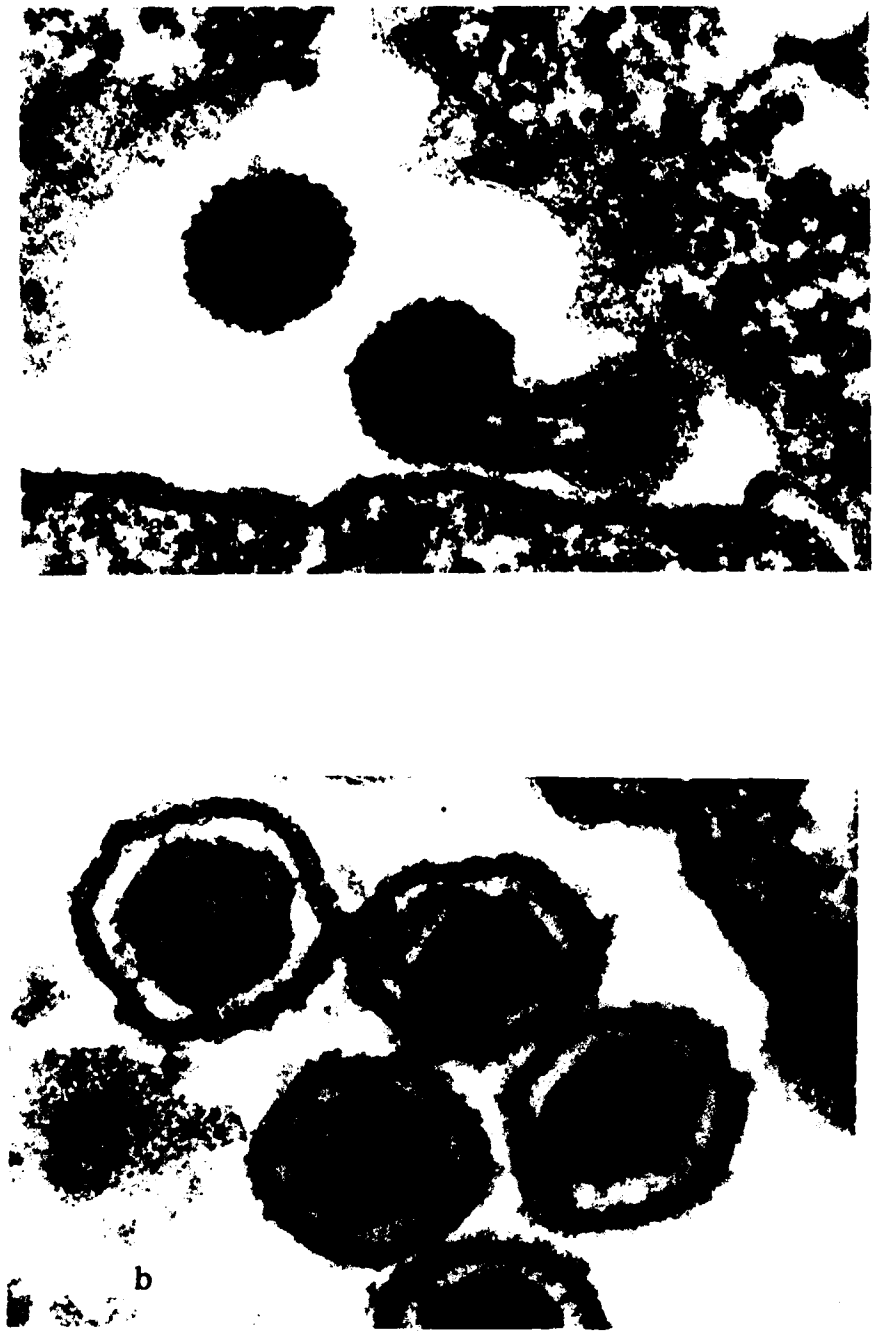
[a] BME15FCS

[b] Calculated from determinations of the mitotic index as described in the Methods and Materials section (page 31).

[c] The difference between T and T-M, T-G2 and T-S.

[d] Calculated from the interphase labeling index according to the method of Stanners and Till as described in the Materials and Methods section (page 30).

[e] Modal T-G2.

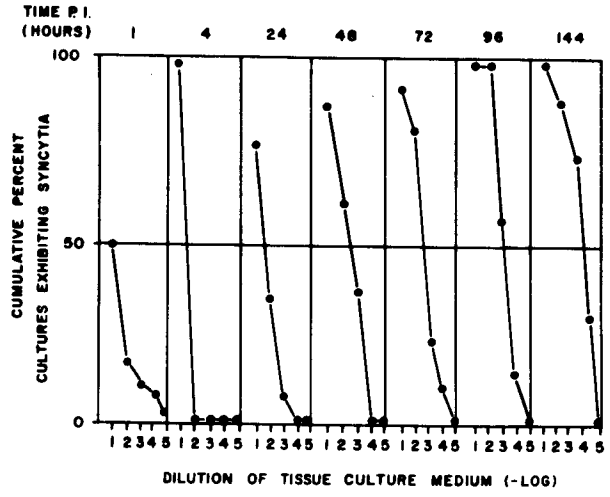


Text Figure 13. Electron micrographs of MEF-2 twenty-one days PI with FLV.

Text Figure 13. Electron micrographs of MEF-2 twenty-one days PI with FLV

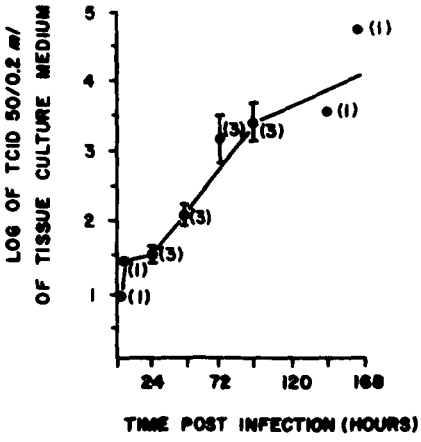
a. An MEF-2 cell 21 days after inoculation with FLV. Note the budding of the viral particle from the cell membrane into the intercellular space x 180,000.

b. Completed virus particles in the intercellular space of FLV inoculated MEF-2 cells 21 days PI x 250,000.



TEXT FIG. 14 DETERMINATION OF TCID50 OF FLV-TREATED CULTURE MEDIUM AT VARIOUS TIMES POST INFECTION

Text Figure 14. A typical determination of the $TCID_{50}$ of the tissue culture medium from FLV infected MEF-2. 6 cm plastic tissue culture dishes containing 4 ml of McC15FCS were seeded with 2 x 10^5 3T3 cells. After 24 hours the tissue culture medium was removed and replaced with 2 ml of McC15FCS containing 0.05 mg of DEAE-D. After one hour of incubation, the DEAE-D was decanted and the cell sheets were washed and exposed to 0.2 ml of the sample to be tested for the presence of virus for 30 minutes. 4 ml of McC15FCS was then added to each dish and the dishes were returned to the incubator. On the fourth day the dishes were subcultured by detaching the cell sheet from the tissue culture dishes with 1 ml of 0.25% trypsin in HBSS-CMF. 0.5 ml of the tissue culture suspension and 3.5 ml of McC15FCS was added to each fresh tissue culture dish. After five or six days the tissue culture fluid was removed and longitudinal strips scraped out of the cell sheet with a sterile wooden stick. The plates were then overlayed with 10^6 XC cells and 4 ml of McC15FCS. The plates were examined for the presence of syncytium formation on the second and fourth day. When the cultures showed evidence of syncytium formation on examination with an inverted microscope, the tissue culture fluid was decanted, the plates fixed with methanol and stained with Harris Hematoxylin. The titer was calculated by the method of Reed and Meunsch and reported as the TCID50.



TEXT FIG. 15 KINETICS OF VIRUS PRODUCTION

Text Figure 15. The kinetics of virus production. The infectivity (TCID50) of samples of tissue culture medium harvested at the times after infection indicated was determined according to the method described in text figure 14 and was plotted versus time PI. The supernatant fluids of cultures treated with D-FLV or BME10FCS showed no ability to induce syncytium formation. The number in parenthesis ( ) indicates the number of experiments performed, in which four to six replicate samples per point were assayed.

IV. DISCUSSION

The experiments reported here indicate that the infection of MEF-2 cultures with FLV result in (1) a stimulation of the rate of DNA synthesis which appears to be independent of a requirement for cell division and which is not accompanied by a similar increase in RNA or protein synthesis; (2) an increase in the maximum population density (cells per plate) which an MEF-2 culture can attain; (3) a shortening of the generation time of MEF-2, which appears to result from a shortening of the time required for infected cells to traverse the G-1 and S phases of the cell cycle; and (4) the production of infectious virus of low leukemogenic potential.

DNA Synthesis

The stimulation of cultured cells by DNA containing oncogenic viruses has been extensively studied (Black, 1968; Winocur, 1969). Induction of DNA synthesis has also been reported to occur under culture conditions which permit little or no DNA synthesis to take place by Polyoma virus (Dulbecco, et al., 1965; Ossovski and Sachs, 1968), SV_{40} (Gershon, et al., 1966) and Adenovirus (Ledinko, 1967). Although RSV infection result in ^{3}H-TdR incorporation into multinucleate myotubes (Lee, et al., 1968), the ability to induce DNA synthesis in cell cultures not already synthesizing DNA does not appear to be a general property of the oncogenic RNA viruses (Vigier, 1970; Green, 1970). However,

infection of actively growing host cells with oncogenic RNA viruses does result in an increased rate of DNA synthesis. This phenomenon was reported for RSV infected CEF (Kara, et al., 1968), GC virus infected MEF-2 (Albach, et al., 1970), MSV-MLV infected MEF-2 (Hirschman, et al., 1970), and AMV infected CEF (LaCour, et al., 1970).

In the present study, the results of experiments designed to measure the rate of DNA synthesis as ^{3}H-TdR incorporation into acid insoluble product revealed that FLV (an oncogenic RNA virus) is also capable of stimulating an increase in DNA synthesis in actively growing MEF-2 cultures (text figures 2 and 13). The incorporation of ^{3}H-TdR into acid insoluble product allows one to measure only total DNA synthesis in the cultures and does not distinguish between increases in DNA synthesis which result from an increase in the percentage of cells per culture which synthesize DNA and those increases in DNA synthesis which result from an increase in the amount of DNA synthesized per cell. The possibility also exists that FLV infection may stimulate DNA synthesis by altering the cell membrane (Pardee, 1971) and thus facilitating nucleoside transport into the cell membrane. Such an effect would probably be reflected by an increase in the size of the cellular nucleotide pool (Plagemann, 1971a, 1971b). The effects, if any, of FLV infection upon the size of the cellular nucleotide pool have yet to be determined.

The data obtained from autoradiographic experiments disclosed that the percentage of cells in FLV infected cultures synthesizing DNA was greater than that observed in either of the controls at times when the TCA precipitable counts in FLV treated cultures was also greater than in controls (te t figures 8 and 9). Although these data do not exclude the possibility that some of the observed increase in the rate of total DNA synthesis may result from an increase in the amount of DNA synthesized per cell, they do suggest that the observed increase in total DNA synthesis, is in large part, the result of an increase in the number of cells per culture synthesizing DNA.

Although the data thus far presented appear to be consistent with the idea that FLV infection results in an increase in the rate of cellular DNA synthesis, probably as a result of an increase in the number of cells which are induced to synthesize DNA, it may be argued that the observed increase in DNA reflects the synthesis of a large number of DNA intermediates copied from viral RNA by the RNA dependent and DNA dependent DNA polymerases (Temin, 1971; Speigelman, et al., 1970; Baltimore, 1970). If this were the case, then more DNA hybridizable to viral RNA should be present in FLV infected cells than in controls.

Although these hybridization experiments have not been done in our system as yet, Harel, et al. (1972), reported that their hybridization studies in AMV infected CEF have failed to reveal any difference of this sort. They conclude that it is enhancement of <u>cellular</u> DNA synthesis which they have observed in their investigations.

In order to determine whether the observed increase in DNA synthesis required actively dividing cells, similar autoradiographic experiments were performed with cells which had been cultured in serum-free medium for 5 or 6 days ("resting" cultures). Although the cell number in such "resting" cultures did not double during the 72 hours that they were examined, DNA synthesis did not cease. Infection of such cultures with FLV resulted in increases in the rate of DNA synthesis over that found in controls which was similar to, if not greater than observed in experiments with serum supplemented cultures (text figures 10 and 11). However, in contrast with the oncogenic DNA viruses, which appear to be capable of inducing DNA synthesis in cells which are not synthesizing detectable amounts of DNA, oncogenic RNA viruses required some minimal level of ongoing DNA synthesis in the host cells before stimulation of the rate of DNA synthesis can be measured. Yoshikura (1968) reported that incorporation of ^{3}H-TdR into FLV infected cells treated with 10^{-4} M cytosine arabinoside, a potent inhibitor of DNA synthesis, was reduced to 5% of control. Similar, but preliminary experiments in this laboratory indicate that treatment of FLV infected MEF-2 and controls with cytosine arabinoside resulted in an almost complete inhibition of DNA synthesis in all cultures and no subsequent FLV stimulated DNA synthesis.

Hirschman, et al. (1970) reported that infection by MLV-MSV of growth inhibited (stationary) 3T3 cells maintained in medium removed from stationary cell cultures (spent medium) did not result in enhance-

ment of cellular DNA synthesis. This finding is also consistent with the idea that a certain minimal level of DNA synthesis in the host cell is required before enhancement of DNA synthesis as a result of infection with an oncogenic RNA virus can be measured, since less than 0.1% of 3T3 cells grown under these conditions demonstrated DNA synthesis, as determined by autoradiography (Renger, personal communication).

Biochemical and autoradiographic experiments with both actively dividing and resting FLV treated and control cultures revealed that the maximum rate of DNA synthesis occurred about 24 hours after infection and that the maximum difference between the rates of DNA synthesis in infected and control cultures occurred between 24 and 48 hours after infection. These results are in substantial agreement with the findings of Hirschman, et al. (1970) in MLV-MSV infected MEF-2, but do not agree with the data reported by LaCour, et al. (1970) in AMV infected CEF-2 which indicate that the maximum increase in the rate of DNA synthesis occurred between 0 and 4 hours and no difference was discernible by 24 hours. The reasons for this difference are not clear but may reflect differences in the virus cell systems employed. These experiments also indicate that the observed increase in the rate of DNA synthesis requires the presence of active virus or of some heat labile viral component since a similar increase in the rate of DNA synthesis is not induced by heat denatured virus.

RNA Synthesis

No significant difference between the rate of ^{3}H-UR incorporation into FLV infected MEF-2 cultures and controls was observed (text figures 4 and 5). This data is in agreement with that reported by Hirschman, et al. (1970) for MSV-MLV infected MEF-2. These results differ from those reported by Albach, et al. (1970) and LaCour, et al. (1970) who reported stimulation of RNA synthesis in GC and AMV infected cultures. The reasons for this difference in ability to stimulate RNA synthesis are obscure. Such differences may originate in the effects of different viruses upon cells in tissue culture or may result from differences in the sensitivity of the assay employed since RNA synthesis in both GC virus infected MEF and AMV infected CEF was monitored by radioautographic methods while RNA synthesis in MLV-MSV and FLV infected cells was measured by gross biochemical counting methods.

Protein Synthesis

The rate of protein synthesis in FLV infected and control cultures was investigated and no significant difference was seen (text figures 6 and 7). Although little is known of the effects of FLV infection on protein synthesis in cultured cells, Budillon, et al. (1964) reported an increase in the rate of protein synthesis in the spleens of FLV infected mice. The observed differences in the ability of FLV to affect protein synthesis may be due to the fact that the measurements were made with

two different kinds of cell populations.

The data in this study indicate that no differences in the rate of bulk RNA and protein syntheses appear to result from infection of MEF-2 with FLV. However, since DNA synthesis is thought to be preceded by several rounds of protein and RNA synthesis (Rovera, et al., 1971), the possibility exists that infection with FLV stimulates an increase in the synthesis of only certain species of protein and RNA (e.g., acidic nuclear proteins) and that this difference, although real, is not detectable by the methods employed in this dissertation.

Cell Growth

Studies of the population density to which MEF-2 grew in monolayer culture revealed that those infected with FLV grew to an average of 1.4 times the population density of uninfected controls (Table 2). The population density of D-FLV inoculated controls was not significantly different from that of uninfected controls.

Similar findings have been reported for RSV infected CEF (Colby and Rubin, 1969), AMV infected CEF (LaCour, et al., 1970), MSV infected rat cells (Temin, 1967), and SV40 infected 3T3 cells (Holley and Kiernan, 1968).

Virus infection also influenced the cell cycle (Tables 1 and 3). The T of FLV infected fibroblasts was found to be 67% of the uninfected controls, whereas the T of D-FLV inoculated cultures was found to be similar to uninfected controls. Cell cycle analysis disclosed that the shorten-

ing of T was the result of a shortening of T-G1 and T-S in infected cells. The T-M and T-G2 of FLV uninfected cells were not affected.

There appears to be a difference between the effects of FLV and RSV upon the growth rate of infected cells. The studies reported here indicate that the increased cell density observed in FLV infected cultures is the result of a shortening of T, whereas Leong, et al. (1972) and others (Temin, 1965; Colby and Rubin, 1969; Hanafusa, 1969) report that there is no change in T in RSV infected cells as compared with controls and that the increase in population density observed in RSV infected cells is the result of an increase in the efficiency with which multiplication stimulating factors are utilized (Jainchill and Todaro, 1970; Temin, 1971). Infected but untransformed cells, producing virus do not have this diminished requirement for multiplication stimulating factor (Biquard, 1970).

Little is known about the mechanisms by which the temporal sequence of events in the cell cycle are regulated (Rovera, et al., 1971). Nothing is known about the way in which FLV shortens the time required for MEF-2 to traverse the cell cycle.

Virus Production

When murine leukemia viruses are propagated in tissue culture, the leukemogenicity of the virus produced decreases to near zero (Moore, et al., 1958; Boiron, et al., 1967; Friend and Rossi, 1968; Yoshikura, et al., 1969; Schlom, et al., 1971). There has been, therefore, some question as to whether FLV can infect MEF-2 in a consistent fashion,

and if so, whether the infection is productive. Dr. E. DeHarven of Sloan-Kettering Institutes examined FLV infected MEF-2 under the electron microscope for the presence of budding virus. The electron micrographs clearly indicate that FLV does productively infect MEF-2 (DeHarven, personal communication) (text figure 13).

Since there is a loss in leukemogenicity, tissue culture passaged virus cannot be titered reliably by in vivo methods. Therefore, the course of virus production was followed by the mixed culture cytopathogenicity assay. Evidence of virus in the supernatant tissue culture fluid was found as early as four hours PI (text figures 14 and 15). The maximum rate of virus production occurred between 24 and 96 hours and infective virus was still found in the medium after 168 hours. In agreement with these findings are those of Hirschman, et al. (1969) who reported the detection of MSV-MLV in supernatant fluids earlier than 10 hours after infection, those of Schlom, et al. (1971) who reported high titers of RLV in supernatant fluids within 24 hours PI. Also in agreement with these findings are the studies of Okano and Rich (1969) with cultures persistently infected with Rich virus and those of Bader (1970) using cultures persistently infected with RLV. These studies indicate that synthesis of completed infectious virions requires about two hours.

In vivo titrations of tissue culture supernatant fluids of FLV infected MEF-2 confirm the findings of Moore (1963), Boiron (1967), deTkaczevski, et al. (1968), Yoshikura (1969), and Schlom (1971). FLV does become attenuated as a result of tissue culture passage. Even

after as little as one passage in tissue culture, FLV does not consistently or reliably cause leukemia in susceptible mice.

The rapid loss of leukemogenicity seen in tissue culture passaged FLV is of considerable interest. This phenomenon may be the result of (1) a change in the phenotype of the virus as a result of passage in tissue culture (Schlom, et al., 1971); (2) a genetic change in the virus or host cell resulting in alteration of the host range of the virus (Aaronson, ,1971), or (3) selection for a pre-existing nonleukemogenic variant which forms part of an FLV complex as hypothesized by Steeves and Mirand (1969). The possibility that there is a decrease in the number of virions released, i.e., less than the amount necessary for a leukemogenic dose should also be considered, although the EM evidence makes this most unlikely. Experiments are planned to determine which of these factors plays a role in the attenuation phenomenon.

In conclusion, it has been found that the exposure of MEF-2 to FLV results in (1) an increased rate of DNA synthesis without similar increases in the rates of RNA and protein synthesis; (2) alterations in the growth rate of MEF-2 which were reflected in greater cell density in 72 hour cultures; (3) shortened generation time which resulted from a shortened G1 and S phase; and (4) production of an attenuated virus as early as four hours PI.

The mechanisms of the stimulatory effects observed and their importance in the induction of murine leukemia are not yet understood.

V. SUMMARY

Viruses which infect mammalian cells are known to cause changes in host-cell physiology. Most of the studies of Friend Leukemia Virus (FLV) carried out in tissue culture had involved chronically infected host cells. In order to study the effects of viral infection on host cells and the replicative cycle of the virus, the events following infection of secondary mouse embryo fibroblasts (MEF-2) in tissue culture with FLV were investigated. FLV infection was confirmed by electron microscopic examination and the subsequent effects on macromolecular synthesis, cellular growth and virus production were studied.

The rate of DNA synthesis increased in MEF-2 cultures following infection with FLV as compared with cultures which were untreated or exposed to heat-denatured virus (D-FLV). DNA synthesis reached a peak between 24 and 48 hours after infection.

Radioautographic studies of dividing and "resting" (nondividing) cells revealed that the percentage of cells which incorporated thymidine into their nuclei was greater in FLV inoculated cultures than in untreated cultures or in cultures treated with D-FLV. The observed increase in the percentage of DNA synthesizing cells in infected cultures appears to be independent of the ability of the cells to divide.

The rate of cellular RNA and protein synthesis did not appear to be affected by FLV infection.

The generation time (T) in FLV infected MEF-2 was significantly shorter than in control cultures. Cell cycle analysis revealed that

the observed shortening of the generation time was a result of a decrease in the time required for the FLV infected cells to traverse the G1 and S phases of the cell cycle. By 72 hours post infection, the cultures had grown to a significantly greater population density than controls.

Studies of the kinetics of virus production indicate that virus is present in the medium by four hours after infection and that the maximum rate of virus release occurs between 24 and 96 hours after infection.

Cell-free tissue culture filtrates were found to have a low leukemogenic potential and to induce leukemia in mice only sporadically as had been noted by others.

APPENDIX I

CELL CYCLE ANALYSIS (Stanners & Till, 1960)

Distribution of Cell Numbers at Different Portions of the Mitotic Cycle

According to Stanners and Till, cell cultures are said to be asynchronous if the cell numbers in each culture increase exponentially with time and a constant fraction of cells is found to be undergoing division. For such a culture the frequency distribution of cells around the mitotic cycle (text figure 16) or the number of cells in each portion of the cycle is an exponential function (Powell, 1956) with twice as many cells leaving mitosis as entering it. The mitotic index should remain constant for at least one generation. Our cell cultures were asynchronous as judged by the criterion of constant mitotic index.

In asynchronous, exponentially multiplying cultures, the cell concentration N(t) as a function of time t is given by

$$N(t) = N_0 e \exp at \qquad (1)$$

where N_0 is the initial concentration and $a = \frac{0.693}{T}$, T being the doubling time.

To locate the position of any given cell within the mitotic cycle a variable b, where $0 < b < T$ is assigned, representing the time separating the cell from division. At b = G2 + S, the cell begins DNA synthesis which lasts a time S; at b = G2 the cell ceases DNA synthesis and enters the premitotic nonsynthetic period of duration G2; at $b = b_m$, the cell enters mitosis and at b = 0 the cell divides. Thus, the G2 period is defined as the period from the completion of synthesis to division.

Let n(t, b) be the number of cells per unit time at time t flowing through a point on the cycle which precedes division by a time b. At b = 0 the number of cells dividing in a time interval t to t+dt is dN(t), the number of new cells appearing:

$$n(t, 0)dt = dN(t) \qquad (2)$$

from Equation I

$$n(t, 0) = aN_0 \text{ e exp at} = aN(t) \qquad (3)$$

Assuming a uniform rate of flow of cells around the cycle, a cell which is at position b at a time t will have moved to b = 0 at time t + b, so that

$$n(t + b, 0) = n(t, b) \qquad (4)$$

which yields using equations (1) and (3)

$$n(t, b) = aN(t) \text{ e exp ab} \qquad (5)$$

In text figure 17, n(t, b) is plotted for constant t. It is apparent that

$$N(t) = \int_{b=0}^{T} n(t, b)\, db$$

which is the area between b = 0 and b = T in text figure 17.

Defining the mitotic index, M(t) x 100, as the percent of cells in the interval b = 0 to b = b_m, we have

$$M(t) = \frac{\int_{b=0}^{b_m} n(tb)\, db}{\int_0^T n(t, b)\, db} \qquad (6)$$

$$= \text{e exp ab}_m - 1$$

$$ab_m,\ b_m >\!> T$$

The mitotic index is constant with time.

The percentage of cells taking up label in asynchronous cultures

Referring to text figure 17 and using equation (4) the fraction of labeled cells L(t) as a function of time t is given by:

$$L(t) = \frac{\int_{G_2}^{G_2 + S + t} n(t, 0)\, dt}{N(t)}, \qquad \text{for } t < G_2$$

$$= \frac{\int_{G_2}^{G_2 + S + t} n(t, 0)\, dt + \int_{T + G_2}^{T + t} n(t, 0)\, dt}{N(t)} \quad \text{for } t > G_2 \qquad (7)$$

where label is added at time t = 0.

The second integral is equation (7) for $t > G_2$ arises from the fact that after time G_2 has lapsed, cells that have picked up label begin to divide, thus increasing the percentage of labeled cells. It is assumed in equation (7) that all cells have the same G_2 period. The theoretical treatment is improved by correcting for the division of labeled and unlabeled cells taking into account the variation in G_2 times. The percentage of labeled cells which would have been observed had no cell division taken place can be obtained from the observed labeling indices by employing the following expression:

$$L(t)_{corr.} = 1/No\ [L(t)exper.\ N(t) - \int_0^t P(t - b_m)\ aN(t)\ dt] \quad (8)$$

Where $aN(t)\ dt$ is the increase in cell number over the time interval dt and $P(t - b_m)$ is the fraction of labeled cells entering division. Allowance is made for the fact that metaphase precedes division by time b_m. The first term in the bracket represents the total number of labeled cells that are seen, and the second term in the bracket represents the number of these that are due to division of already labeled cells.

After a time G_1 has elapsed a further correction would be required to allow for cells which have divided after the addition of label and which have not been in the S period in the presence of label. There is little point in extending the treatment for times larger than G_1, however, since the spread in S and G_1 times would have to be accurately known or the uncertainty introduced in making such a correction would be large.

Thus for $t < G_1$ equation (8) becomes

$$L(t)_{corr.} = \frac{\int_{G_2}^{G_2 + S + t} n(t, 0)\ dt}{No} \quad (9)$$

Using equation (3) this gives:

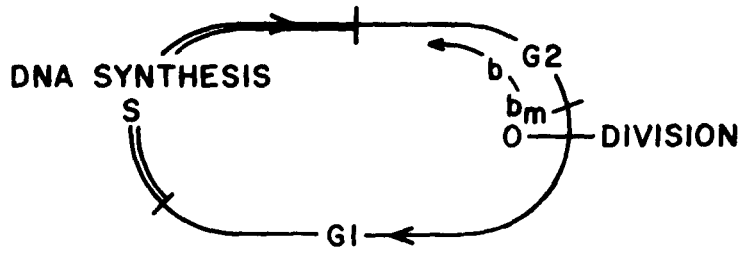
$$L(t)_{corr.} = e\ exp\ aG_2\ [e\ exp\ aG_2\ [e\ exp\ a(s + t) - 1] \quad (10)$$

From which one obtains:

$$S = 1/a\ ln\ [L(t)_{corr.} + e\ exp\ aG_2] - (G_2 + t) \quad (11)$$

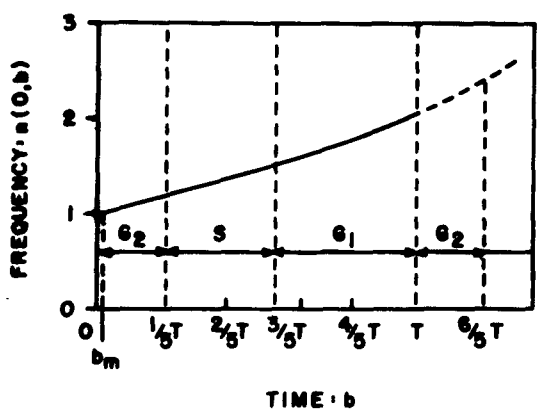
For each corrected experimental L(t) value a value of S can be computed.

At the suggestion of Dr. Stanners the observed labeling indices were plotted versus time. The line described was then extrapolated to zero time and the value of the Y intercept thus obtained was used as the L(t) corr. in this report.



TEXT FIG. 16 THE MITOTIC CYCLE OF ACTIVELY MULTIPLYING CELLS

Text Figure 16. The mitotic cycle of actively multiplying cells, showing the DNA synthetic period and its time relation to division (cells move clockwise around the cycle).



TEXT FIG. 17 THE FREQUENCY DISTRIBUTION OF AN ASYNCHRONOUS CULTURE

Text Figure 17. The frequency distribution of an asynchronous culture giving the relative number of cells in each part of the mitotic cycle and showing the approximate position of the G_2, S and G_1 periods.

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