factors involved in the development of splenomegaly are different with these two murine leukemias.

Summary. The production of porphobilinogen and porphyrins by spleen extracts of Friend virus (FV) infected mice depends on the initial enzymic activity in the organ and varies with the amount of virus injected and the strain of mice employed. The increase in the enzymic activity appears to be as sensitive a measure of FV infection as was the splenomegalic response. Only minor changes in the enzymic activity were observed during the development of splenomegaly induced by leukemia L1210.

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Viral Inhibition in Adenovirus 5 Treated Hamster Cells (33559)

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It has been shown by Pereira *et al.* (1) that human adenovirus type 5 causes a fatal infection of newborn hamsters with liver cell necrosis and characteristic nuclear changes of adenovirus infection. However, these authors could not make a categorical statement regarding virus multiplication in this host. This is the only human adenovirus thus far reported to be lethal for the newborn hamster. This effect is in contrast to that caused by other human adenoviruses [types 3 (2), 7 (3), 12 (4), 18 (5), and 31 (6)], namely, malignant cellular transformation. A study was undertaken to investigate the interactions between adenovirus 5 and hamster embryo fibroblasts (HEF) in culture and to determine whether this virus would behave *in vitro* as it did *in vivo*.

Materials and Methods. Cells. Pregnant golden Syrian hamsters (*Mesocricetus auratus*) were obtained from the Animal Production Section, Laboratory Aids Branch, NIH. Embryos were harvested on days 14–16 of gestation and cell cultures were prepared by the Tissue Culture Section, Laboratory of Virology and Rickettsiology, DBS, by seeding cells into 2-oz bottles. Chick embryo fibroblast (CEF) monolayers were prepared from 10–12-day-old embryos and seeded in a similar manner. Cell sheets were used for testing only when they had become confluent monolayers. The human diploid cell strain, WI-38, was supplied as confluent monolayers of cells by the Tissue Culture Section. Human embryonic kidney (HEK) cell cultures were obtained as complete monolayers from Flow Laboratories, (Rockville, Md.). A human epithelial cell line (KB strain) and human amnion cell culturees (HA strain) were obtained from Dr. K. O. Smith, NIH.

Media. Eagle's basal medium with 2% fetal calf serum containing 400 units of penicil-

lin/ml, 0.4 mg of streptomycin/ml, and glutamine, 4.8 m*M* was used for maintenance of chick and hamster cells, and to prepare all dilutions.

Viruses. Adenovirus 5, prototype strain, adeno-associated virus (AAV)-free, was kindly supplied by Dr. K. O. Smith, NIH, and was propagated initially on KB cells. This material was used as one of the pools in the following experiments and was also used as seed virus for subsequent pools grown on HA cells. The titer of the pools used in the following experiments ranged from $10^{7.8}$ tissue culture infectious doses 50% ($TCID_{50}$) to $10^{9.5}$ $TCID_{50}$/ml. The Indiana strain of vesicular stomatitis virus (VSV) was propagated in CEF. When it was found that this strain consisted of a population of mixed plaque size, selection of the large plaque size variant was made and pools prepared (titer, $10^{7.9}$ plaque forming units (pfu)/ml). Herpes simplex virus (HSV), that had been propagated on H-Ep II cells (titer, $10^{7.3}$ pfu/ml), was obtained from Dr. B. G. Young, NIH. The pool of Semliki Forest virus (SFV) was grown in CEF (titer, $10^{7.2}$ pfu/ml).

Virus titrations. Plaquing of VSV and SFV was accomplished by inoculating 0.1 ml of the viral preparation onto confluent cell monolayers and allowing incubation at 37° for 1 hr. Following this, Eagle's plaque base, with 1.7% of 1:500 neutral red, and Noble's agar 1.8%, were mixed in equal parts and the cell sheets were overlaid with 7 ml/bottle. After hardening of the agar at room temperature, the bottles were inverted and incubated at 37°. Plaques were counted at 48 and 72 hr. Plaquing of herpes simplex on WI-38 cells was initiated by allowing the virus to adsorb for 2 hr at room temperature. The bottles were then fed with Eagle's minimal essential medium (MEM) with penicillin, streptomycin, glutamine, and 4% fetal calf serum to which 0.5% pooled human gamma globulin had been added. Forty-eight hr later the medium was decanted and the bottles were stained with crystal violet (1:5 dilution of 10% crystal violet in 1% aqueous ammonium oxalate) for 15 sec, washed, and plaques were then counted under a dissecting microscope.

TABLE I. Titer of Adenovirus 5 in Hamster Cells at Several Intervals after Virus Inoculation.

	Log_{10} ID_{50} per 0.1 ml of adenovirus 5 in	
	Hamster cell cultures	Culture bottle containing medium but no cells
Input	7.8[a]	7.8
Time 0	5.8	5.9[b]
Day 3	6.0	ND[c]
Day 5	6.0	5.8

[a] Titers determined in HEK cells.

[b] Calculated value following dilution in 7 ml of media.

[c] Not done.

Assays for adenovirus were performed on HEK cells using 4 tubes/virus dilution. Final readings were taken on day 14 after virus inoculation and graded 0–4 + for cytopathic effect (CPE). Tubes showing 2 + CPE or greater were considered to be positive, and the titer was calculated using the Kärber-Spearman method (7).

Results. Lack of demonstrated replication of infectious adenovirus 5 in hamster embryo fibroblasts. In order to demonstrate whether adenovirus 5 multiplied in hamster cells, HEF monolayers were inoculated with 0.1 ml of the adenovirus pool at a multiplicity of infection (MOI) of 15. Following adsorption for 1 hr at 37°, the cell sheets were washed four times and refed with 7 ml of maintenance medium. At time 0 and selected times thereafter samples were quickly frozen at —70°, thawed, and aliquots were sonicated for 30 sec in a 10 kc Raytheon sonic oscillator set at the maximum plate setting and output current. The resulting material was then titrated on HEK cells. As shown in Table I, there is no significant net increase in the amount of infectious adenovirus during the 5-day observation period. To rule out the possibility that replication had occurred but that a balance had been reached between production of small amounts of infectious virus and inactivation of the virus, $10^{7.8}$ $TCID_{50}$ of adenovirus 5 were placed in media without

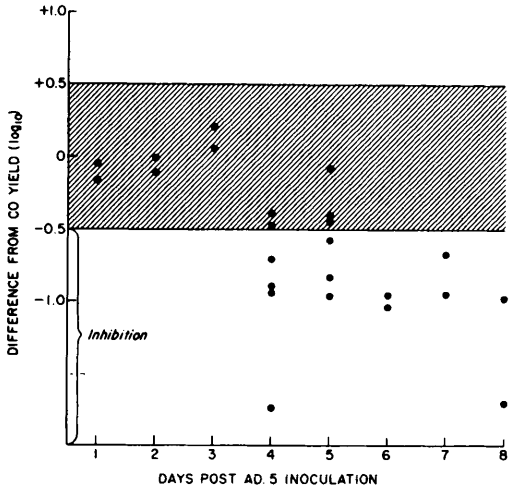

FIG. 1. Intracellular inhibition: comparison of VSV titers from control cells and cells previously infected with adenovirus 5.

cells, held at 37° for 5 days, and then titrated (Table I). The virus is not readily inactivated under these conditions. Therefore, if replication had occurred, this would have been reflected as a significant net increase in yield.

Failure to detect an extracellular viral inhibitor. It seemed possible that, despite the lack of replication of adenovirus 5 in HEF, the virus did have some effect on the cells such as the production of interferon. However, supernatant fluids from cell cultures inoculated with adenovirus 5 1–5 days previously failed to exhibit any interferon-like activity when tested.

Detection of viral inhibition in adenovirus 5 treated cells. In an attempt to determine if an intracellular inhibitory effect was present in cells previously inoculated with a 10^{-1} dilution of an adenovirus 5 pool (MOI, 1.5), these cells were challenged directly with approximately 50–200 pfu of either VSV or SFV. Fluids were harvested 18-hr postinfection with the challenge virus, and their titers were determined. Inhibition was significant ($p \leq 0.05$) if the yield from the adenovirus-treated cells was less than the yield from control cells by 0.5 $\log_{10}$ or more (8). Figures 1 and 2 demonstrate a series of tests and plot the difference from yields in control cells when VSV and SFV were used as the challenge viruses. Inhibition can be detected on the fourth day postadenovirus 5 treatment but is not present prior to this. Inhibition, when it does appear, is generally in the range of 0.5–1 $\log_{10}$. A corresponding decrease in CPE due to the challenge virus and significant inhibition of plaque formation (50% of plaques in control cultures or less) has also been noted. (Data not presented.) The development of inhibition in one cell lot through days 4 and 6 for VSV and SFV, respectively, is shown in Fig. 3. In the other cell lot shown in Fig. 3 inhibition of VSV was noted on days 6–10.

Adenovirus dose and appearance of inhibition. Confluent monolayers of HEF in 2-oz bottles were inoculated with 0.1 ml of tenfold dilutions of an adenovirus 5 pool (Mycoplasma-free, see below) containing $10^{7.3}$ $TCID_{50}$. The average total cell count in the 2-oz bottles at the time of adenovirus inoculation was 1.3×10^6 (range, 1.1×10^6 to 1.8×10^6). These treated cells were then challenged with VSV as described above on day 6 postadenovirus inoculation and the inhibition in yield of the challenge virus was determined. Multiplicities of infection of adenovirus ranged from 15 to 0.015. The results of these experiments (Table II) indicate that a MOI of adenovirus 5 of between 0.15 and 1.5 is necessary to achieve significant depression of VSV replication in the

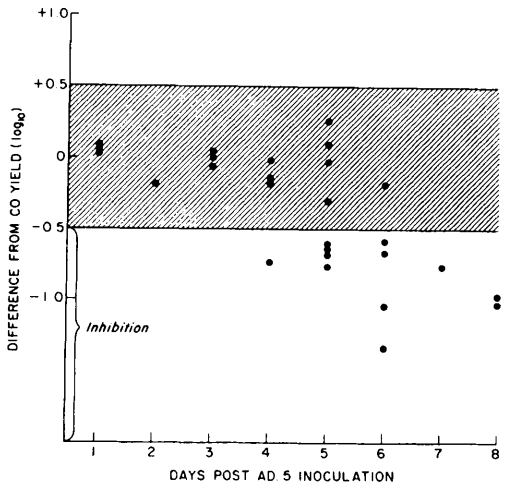

FIG. 2. Intracellular inhibition: comparison of Semliki Forest virus titers from control cells and cells previously infected with adenovirus 5.

TABLE II. Multiplicity of Infection of Adenovirus 5 and Appearance of Intracellular Inhibition.

Dilution of Ad. 5 pool	Multiplicity of infection	Inhibition[a] (log_{10} difference from control)
10^{0}	15	—1.8
10^{-1}	1.5	—0.7
10^{-2}	0.15	—0.3
10^{-3}	0.015	—0.2

[a] Average of three tests performed on three separate cell lots.

cell. Furthermore, there is a direct correlation between multiplicity of infection and the degree of inhibition noted. In these experiments it was noted that in a high percentage of bottles inoculated with undiluted virus pool (MOI, 15) detachment of some cells from the glass had occurred. This was presumed to be due to the cytotoxic action of the soluble protein factor found in adenovirus 5 preparations (9). However, some monolayers remained intact and these were the ones chosen for the test. Cell counts did not differ significantly between these and control bottles. A 10^{-1} dilution of the pool was noted to eliminate this difficulty.

Range of intracellular inhibition. It seemed possible that the intracellular inhibition noted might be due to a low level of interferon which was undetectable in our assay system, but which was present in sufficient amounts to cause an intracellular biological effect. In order to determine if this were so, a strain of herpes simplex virus, first found to be relatively insensitive to exogenously applied hamster interferon, then was used for direct challenge of the adenovirus-treated

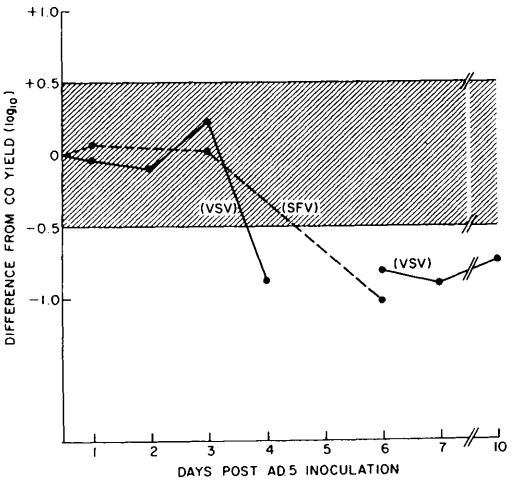

FIG. 3. Inhibition of challenge virus as a function of time in adenovirus 5 treated cells.

cells. Table III indicates that a dilution of hamster interferon which caused greater than one log_{10} inhibition of VSV had no significant effect on the herpes virus. However, definite inhibition of replication of herpes simplex was seen in the adenovirus-treated cells. It therefore seems unlikely that the intracellular inhibition seen in these cells is interferon mediated.

Attempts to extract an inhibitor from the cells. Freeze–thawing and sonication of monolayers exhibiting significant inhibition of challenge virus replication failed to release any substance into the supernatant which was capable of exhibiting antiviral activity when tested on HEF. Extraction of cell monolayers with Genetron 113, using the methods of Gessler (10) and Pereira (11), also failed to yield an inhibitor.

Adsorption experiments. Experiments were

TABLE III. Comparative Sensitivity of Herpes Simplex Virus to Exogenous Interferon, and Intracellular Inhibitor in Adenovirus 5-Inoculated Cells.

Challenge virus	Exogenous interferon: Dilution of interferon	Exogenous interferon: Inhibition of yield (log_{10})	Intracellular inhibition; inhibition of yield (log_{10})
Herpes simplex	1:16	0.2	0.8
		0.1	0.6
VSV	1:16	1.7	0.9
		1.1	0.9

carried out to determine if there were any differences between adsorption of the challenge virus to the adenovirus 5-treated cells and untreated cells. Cell counts did not differ significantly between these two groups. In a representative experiment, cells were cooled at 4° for 0.5 hr to permit adsorption but delay penetration, and then inoculated with $10^{6.1}$ pfu of VSV. Following 1 hr of incubation at 36°, the cell sheets were washed repeatedly, frozen, thawed, sonicated, and then titers of VSV were determined. No significant difference between adsorption in control cells ($10^{3.2}$ pfu of VSV) and adenovirus 5-treated cells ($10^{3.4}$ pfu of VSV) was noted.

Effect of Mycoplasma. The adenovirus 5 pools used in the majority of these experiments were found to contain Mycoplasma which was identified by Dr. M. Barile of NIH, as *M. orale,* Type I. An adenovirus 5 pool free of Mycoplasma was subsequently prepared. Inhibition of VSV, SFV, and HSV still was noted using this new virus pool. Also, treatment of HEF cell cultures with $10^{4.3}$ colony forming units of this Mycoplasma failed to produce inhibition of yields of challenge virus. Since the possibility existed that the Mycoplasma had interfered with the replication of the adenovirus 5 in the hamster cells (12), replication experiments as described above were performed again using the Mycoplasma-free adenovirus pool. Again, no evidence for replication of infectious virus was obtained.

Discussion. Following inoculation of adenovirus 5 onto HEF, no overt evidence of infection was observed. Cytopathic effect was not seen and replication of infectious particles in HEF was either nonexistent for at least 5 days or occurred at such a low rate that it could not be detected. Nevertheless, if these cells were challenged on the fourth day or later after adenovirus 5 inoculation, a significant amount of inhibition of replication of the challenge virus was noted. This inhibition has been found to affect both VSV and SFV (RNA viruses), and HSV (a DNA virus). Based on the inhibition of HSV which is relatively resistant to interferon, it is concluded that the inhibition does not appear to be interferon mediated. Blockage of adsorption of the challenge virus did not appear to be the cause of the decreased yields.

Inhibition of replication of adenovirus type 5, poliovirus type 1, and vaccinia virus in HeLa cells previously infected with adenovirus 5, was reported by Pereira (13). The inhibition was not due to interferon but was associated with a type specific structural protein, namely the fiber antigen (14). Levine and Ginsberg (15), working with KB cells and purified adenovirus 5 fiber antigen, showed that the mechanism by which fiber antigen caused inhibition was by decreasing macromolecular host cell synthesis.

It is possible, of course, that although replication of infectious adenovirus 5 particles did not occur, nevertheless, adenovirus 5 fiber antigen was being produced. This, in turn, could be responsible for decreased cellular macromolecular synthesis with a subsequent decrease in replication of challenge virus.

Preliminary studies, using tritiated uridine and valine for measurement of specific activity of RNA and protein synthesis, are underway. Thus far, a consistent effect on protein specific activity has not been observed, but consistent suppression of RNA synthesis (range of 37–70%) was noted. However, there is a poor correlation between the degree of suppression of cellular RNA synthesis and degree of inhibition of replication of challenge virus.

Several other explanations may be considered to account for the decreased replication of a challenge virus in the adenovirus 5-treated cells. A subtle diffuse toxic effect other than that produced by fiber antigen may be present. Evidence against this is the ability of treated cells to remain as a confluent monolayer for periods of time greater than 1 week postadenovirus treatment, as well as the ability of these cells to support some replication of the challenge virus. Another possible explanation of the inhibitory effect is non-interferon mediated direct viral interference. This interpretation is consistent with the finding that an input MOI of at least one was required to cause inhibition of

heterologous viruses (see Table II).

The results presented here can perhaps be applied to an observation made by Peries *et al.* (16) that simultaneous subcutaneous inoculation of hamsters with adenovirus 5 and 12 resulted in inhibition of adenovirus 12 oncogenicity. This is not due to inhibition of replication of infectious adenovirus 12, since it has been reported that this virus does not multiply in hamster cells (17). It is possible that the events initiated by adenovirus 5 in the cell, which result in inhibition of challenge virus replication, are of such a nature as to also inhibit cellular transformation by an oncogenic virus. This possibility is presently under investigation.

Summary. Inoculation of hamster embryo fibroblast cultures with human adenovirus type 5 resulted in inhibition of replication of heterologous challenge virus in these cells. No evidence for replication of infectious adenovirus 5 particles could be found in this system. Beginning on day 4 postadenovirus 5 inoculation, decreased yields of challenge viruses were noted as compared to control yields. This effect was seen with two RNA viruses (vesicular stomatitis virus and Semliki Forest virus) as well as with one DNA virus (herpes simplex virus). The inhibition in the adenovirus 5-treated cells did not appear to be interferon mediated. Adsorption of challenge virus to treated cells did not differ significantly from adsorption to control cells.

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