Summary. Following intranasal inoculation with rhinovirus NIH 1059, 9 of 9 volunteers developed a respiratory infection and 7 of 9 had illness ranging from mild rhinitis to febrile upper respiratory tract disease. Only 1 of many stool and rectal swab specimens from these men yielded rhinovirus. No detectable intestinal infection occurred in 3 volunteers who received NIH 1059 in enteric-coated capsules, nor in 5 volunteers inoculated directly into the duodenal lumen *via* Rehfuss small intestinal tube. Effects of gastric and duodenal secretions, trypsin, and body temperature (37°C) on growth and survival of NIH 1059 were tested *in vitro,* and their role in preventing intestinal infection and excretion of rhinoviruses evaluated.

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Immunologic Responses in Hamsters to Homologous Tumor Antigens Measured *in vivo* and *in vitro.** (31991)

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Efforts in our laboratories(1-6) to prepare and to assay vaccines against tumor led to the development of a model system in which oncogenic virus is given to hamsters when newborn, and homologous virus-free tumor antigen is given sometime later, prior to first appearance of virus-induced tumor. This is called the SV_{40} virus-newborn hamster system. Irradiated and idodeoxyuridine (IUDR) treated SV_{40} tumor cell antigens proved highly effective (as great as 100%) in preventing the appearance of tumor when given 34 to 76 days after homologous virus. Though very dependable for measurement of vaccine ef-

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ficacy, the test is of excessively long duration and methods were sought whereby immunizing preparations could be assayed in a shorter time period. We present here the findings in a comparative study in which vaccine efficacy was measured by *in vitro* and *in vivo* procedures. In the tests, the capacity for peritoneal exudate cells from immunized hamsters to destroy tumor cells was determined or the immunized animals were challenged directly with viable tumor cells given by transplant. Though the experiments were aimed primarily at the development of a simplified vaccine assay method which would exhibit specificity and sensitivity comparable to that of the SV_{40}-newborn hamster system, the findings are of added interest in presenting information relative to the mechanism of immunity against virus-induced tumor.

Materials and methods. Virus-induced hamster tumor cells. The F5-1 line(2) of SV_{40} and the Cl line(7) of adenovirus type 7 (Pinckney strain) hamster tumor cells were cultivated *in vitro* in medium 199 containing 5 or 10% heat-inactivated calf serum plus antibiotics. Cell cultures from passage 16 to 33 of SV_{40} and 32 to 110 of adenovirus tumor were used. These lines were free of virus and of pleuropneumonia-like organisms, possessed specific T antigen, and were highly neoplastic on transplant to hamsters.

Preparation of tumor cell immunizing antigens. To prepare gamma irradiated cell antigen, the cells from 5 to 7 day-old cultures of SV_{40} or adenovirus tumor were harvested with the aid of trypsin, and were suspended to a concentration of 5×10^7 viable cells per ml in Hanks' balanced salt solution (HBSS) following washing. The tumorigenicity of the cells was destroyed by irradiation in open siliconized petri plates employing a Caesatron model E therapy unit equipped with a 10 cm $\times$ 10 cm cone and delivering 100r of gamma rays per minute in air at 22 cm distance. After treatment with 3000 to 4000r, the cell suspensions were diluted in HBSS and kept at 4°C until inoculated. To prepare formalin-treated cell antigen, a portion of the cell suspension was treated with 0.5% formaldehyde (37% HCHO) for 4 hours at 4°C. The other kinds of antigens were described previously(6).

Vaccine assays. Hamsters. Random bred Syrian hamsters from the Lakeview Hamster Colony, Newfield, N. J., were employed. Both tumor cell lines were developed from virus-induced tumors in Lakeview hamsters. *Immunization.* Irradiated tumor cell suspensions which had been freshly prepared and diluted to the desired concentration were given intraperitoneally at weekly intervals in 1 ml amount to hamsters of an age indicated in the text. These animals were a source for sensitized peritoneal exudate cells or were challenged subcutaneously with live tumor cell suspension as described later. *Peritoneal exudate cells.* Each immunized or nonimmunized control hamster was injected intraperitoneally on alternate days on 3 occasions with 3 ml of 1% sterile oyster glycogen solution (Nutritional Biochemicals). Fifteen ml of medium 199 containing 0.5 unit of heparin per ml were given to each hamster 3 to 5 hours after the third injection of glycogen and the exudate was collected by paracentesis with the aid of an 18 gauge needle 5 minutes later. The exudate from several hamsters was pooled, and centrifuged for 20-25 minutes at 900-1000 rpm at 4°C employing a horizontal head. The resuspended cells were then washed in fresh heparinized medium 199 and finally suspended to the desired concentration in medium 199 with or without added calf serum, as desired. Microscopic examination of cell smears stained with Giemsa's stain revealed predominantly cells of the mononuclear series which appeared to be small lymphocytes. *Tumor cells.* Nonirradiated SV_{40} or adenovirus 7 tumor cells were employed in both *in vitro* and *in vivo* assays. The cells were harvested in HBSS following brief exposure to trypsin, washed twice with medium 199 and resuspended in medium 199—5% heat-inactivated calf serum medium to give the desired cell concentration. In vivo *and* in vitro *tests.* Details are presented in the appropriate sections in the text. The animals employed in the *in vivo* tests were examined once weekly for tumors which were confirmed by pathologic examination. Unless otherwise stated, serum complement was not added to the cells in the experiments.

Results. In vitro *assay using exudate cells.* Tests for *in vitro* destruction of SV_{40} or adeno-

TABLE I. Tests for Destruction, *in vitro*, of SV_{40} or Adenovirus 7 Hamster Tumor Cells by Peritoneal Exudate Cells from Hamsters Immunized with Homologous or Heterologous Hamster Tumor Antigen.

Exudate cells* from hamsters immunized with irradiated tumor antigen	Target test cell	Guinea pig complement added†	Result, post-incubation target cell count, nuclei/ml $\times 10^3$
Test:			
SV_{40} tumor antigen	SV_{40} tumor	Yes	103‡
" " "	" "	No	103
Controls:			
Adenovirus 7 tumor antigen	SV_{40} tumor	No	108
Hanks' BSS	" "	No	119
None	" "	Yes	96
"	" "	No	98
SV_{40} tumor antigen	None	No	0
Test:			
Adenovirus 7 tumor antigen	Adenovirus 7	Yes	106
" " " "	" "	No	113
Controls:			
SV_{40} tumor antigen	Adenovirus 7	No	109
Hanks' BSS	" "	No	128
None	" "	Yes	122
"	" "	No	112
Adenovirus 7 tumor antigen	None	No	0

* 2×10^6 mononuclear cells were added to 1×10^5 target cells in tube culture for 24 hr.
† Lyophilized guinea pig complement—0.1 ml of 1:30 dilution.
‡ Average count from 6 tubes.

virus 7 target cells in culture by peritoneal exudate cells from immunized hamsters were carried out according to the procedures described by Rosenau and Moon(8). Donor hamsters 4 to 5 weeks of age were each injected intraperitoneally on 4 occasions at weekly intervals with 2 to 3×10^6 irradiated homologous tumor cells in 1 ml volume, and peritoneal exudate cells were collected 14 days following the last injection of antigen. Appropriate control animals were included. For assay purpose, tube cultures which had been planted 24 hours previously and which contained 10^5 cells per tube, were exposed to 2×10^6 mononuclear exudate cells (1:20 ratio) in serum-free medium 199 for 24 hours at 37°C in the presence or absence of 0.1 ml of 1:30 guinea pig complement. The exudate cells were removed by gentle rinsing and the numbers of surviving tumor cells were enumerated by nuclear count following treatment with citric acid and staining with crystal violet as described by Rosenau and Moon(9). The findings presented in Table I show that there was no destruction of SV_{40} or adenovirus target cells by the exudate cells obtained from hamsters immunized with homologous or heterologous irradiated tumor cells and tested in the presence or absence of added complement.

The specific immune status of the exudate cell donors was proved by subcutaneous challenge with 2×10^4 SV_{40} tumor cells 32 days after the last dose of antigen and observation for development of tumor. None of 5 immunized hamsters developed tumor following challenge whereas all of the 4 surviving control animals developed subcutaneous tumor.

In vivo *assays using exudate cells. Intraperitoneal inoculation of tumor cells mixed with exudate cells.* Peritoneal exudate cells from SV_{40} tumor immune or control hamster donors described in the section on *in vitro* assay results above were mixed in varying proportions with live SV_{40} tumor cells and inoculated intraperitoneally into 1 month or 1 week-old hamsters after 37°C incubation for 30 minutes. The animals were observed for Development of transplant tumors and the findings are summarized in Figs. 1, 2 and 3. Figure 1 shows that 45,000 immune exudate cells suppressed the development of tumor in 60% of the one month-old hamsters when the SV_{40} tumor cell challenge dose was 10,000

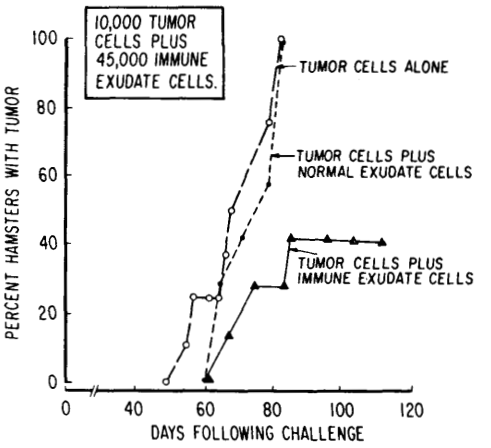

Fig. 1. Intraperitoneal Challenge Tests in one month old hamsters for destruction of F5-1 SV_{40} hamster tumor cells (10,000 cell challenge) by peritoneal exudate cells from hamsters immunized with homologous tumor antigen.

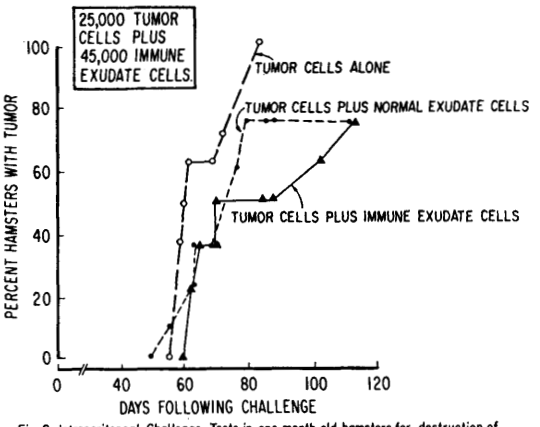

Fig. 2. Intraperitoneal Challenge Tests in one month old hamsters for destruction of F5-1 SV_{40} hamster tumor cells (25,000 cell challenge) by peritoneal exudate cells from hamsters immunized with homologus tumor antigen.

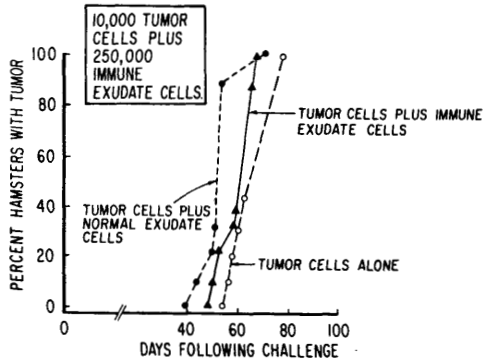

Fig. 3. Intraperitoneal Challenge Tests in one week old hamsters for destruction of F5-1 SV_{40} Hamster tumor cells (10,000 cell challenge) by peritoneal exudate cells from hamsters immunized with homologous tumor antigen.

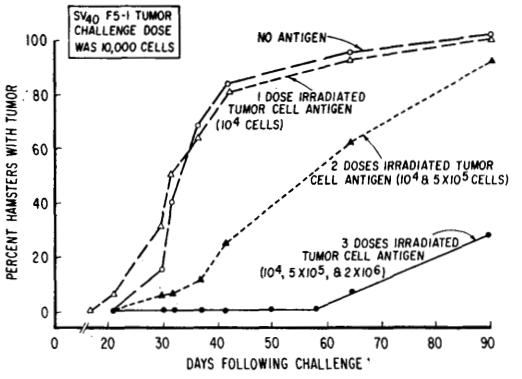

Fig. 4. Occurrence of F5-1 SV_{40} Hamster tumor following Subcutaneous challenge in hamsters which received 1, 2 or 3 intraperitoneal doses of homologous irradiated tumor cell antigen.

cells. Exudate cells from nonimunized donors failed to afford any apparent protection. As seen in Fig. 2, increase of the SV_{40} challenge cell dose to 25,000 cells without increase in immune exudate cells resulted in slight delay in time of tumor appearance but no significant reduction in percentage of animals eventually developing tumor compared with controls. The importance of age of test animals was shown in Fig. 3. In striking contrast to the findings with 1 month-old hamsters in Fig. 1, no protection was afforded by the immune exudate cells when assayed in 1-week-old hamsters even though 250,000 exudate cells were added to the 10,000 SV_{40} tumor cell challenge.

Inoculation into hamster cheek pouch of tumor cells mixed with exudate cells. Exudate cells from hamsters immunized with SV_{40} tumor cells or from nonimmunized controls were prepared as described for the *in vitro* assays above and mixed with live SV_{40} tumor cells in the proportions shown in Table II. Following incubation at 37°C for 30 minutes, 0.1 ml amounts of the mixtures were inoculated into each cheek pouch of groups of ten 30 to 35 day-old hamsters according to the procedure of Foley and Handler(10). The cheek pouches were examined weekly for detection and measurement of tumor. The findings presented in Table II show quite clearly that there was suppression in both number and mean size of tumor in animals which received SV_{40} tumor cells mixed with immune exudate cells contrasted with those which were given tumor cells with exudate cells from nonimmunized animals or no ex-

TABLE II. Cheek Pouch Route of Challenge. Tests in *one-month-old* hamsters for destruction of F5-1 SV_{40} hamster tumor cells by peritoneal exudate cells from hamsters immunized with homologous tumor antigen.

	No. of cells per pouch			Development of tumor according to day following inoculation							
				11		25		32		50	
	SV_{40} tumor cells	Peritoneal exudate cells		No. pos. /total*	Mean tumor size (mm)	No. pos. /total	Mean tumor size (mm)	No. pos. /total	Mean tumor size (mm)	No. pos. /total	Mean tumor size (mm)
		Immune	Normal								
Tests:	56,000	420,000	—	5/10	2.1	5/10	4.2	6/8	8.5	7/8	25
	120,000	420,000	—	9/10	3.3	9/10	11.0	10/10	30	10/10	>30
Controls:	56,000	—	420,000	10/10	2.1	10/10	11.2	10/10	>30	10/10	>30
	120,000	—	420,000	9/10	3.0	10/10	14.4	10/10	>30	10/10	>30
	56,000	—	—	9/10	1.8	10/10	14.9	10/10	>30	10/10	>30
	120,000	—	—	10/10	3.8	10/10	12.8	10/10	30	10/10	>30

* No. pos. = Number positive, *i.e.*, number of animals with tumor.

udate cells at all. This was more evident in the tests in which the challenge tumor cell dose was only 56,000 cells rather than 120,000 cells.

Direct subcutaneous tumor cell challenge of immunized hamsters. Groups of 32 hamsters which were 4 to 5 weeks of age were immunized intraperitoneally at weekly intervals with one, two, or three doses of irradiated tumor cell antigen containing increasing numbers of irradiated cells. Two to 4 weeks following the last dose of immunizing antigen, each immunized or nonimmunized control animal was challenged subcutaneously in the right scapular region with 10,000 or 20,000 live SV_{40} tumor cells in 1 ml of HBSS. The findings presented in Figs. 4 and 5 clearly demonstrate the protective effect of the immunization procedure, whether 10,000 or 20,000 cells were used as challenge. Little or no effect was found following the first dose of vaccine and the degree of protection after the second dose was only modest. The degree of suppression following the third dose, however, was of the order of 75 to 95%. Protective efficacy might have been more closely related to the numbers of irradiated cells given in a particular dose than to the numbers of doses administered. Only a few tumors occurred subsequently in the animals which received 3 doses of vaccine and it was apparent that the high level of protective efficacy of immunization was retained for at least 90 days following challenge.

This same subcutaneous tumor cell challenge procedure was used to assay the immunizing capability of tumor antigens which had been tested previously(4,6) in the SV_{40}-newborn hamster system. Tumor cells were treated with formalin (A), disrupted in a French pressure cell (B), gamma irradiated (C) or treated with IUDR (D). The tumor cell antigens were given intraperitoneally as 3 weekly doses (2.5×10^5, 2.5×10^6, 2.5×10^6 cells) into groups of 16 or 32 four to five week-old hamsters. Challenge was 7 days following the last dose of tumor cell antigen. The live SV_{40} virus vaccine (E) was given intraperitoneally as 2 weekly doses of $10^{7.5}$ $TCID_{50}$ (0.1 ml) and the animals were challenged 14 days after the last vaccine dose. Ap-

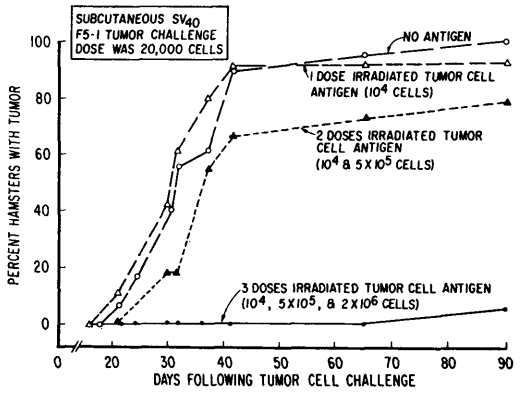

Fig. 5. Occurrence of F5-1 SV_{40} Hamster tumor following Subcutaneous challenge in hamsters which received 1, 2 or 3 intraperitoneal doses of homologous irradiated tumor cell antigen.

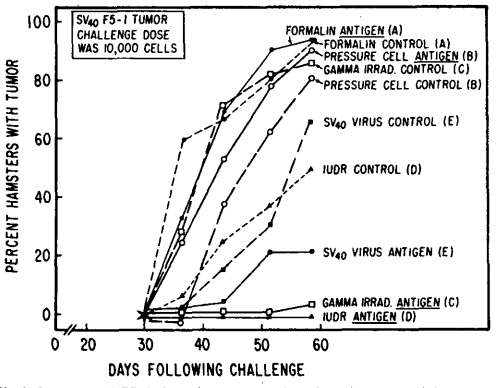

Fig. 6. Occurrence of F5-1 SV_{40} hamster tumor following subcutaneous challenge in hamsters which had received various homologous antigens.

propriate nonimmunized control animals were always included.

The findings presented in Fig. 6 showed close agreement with those obtained when the same kinds of antigen were assayed in the SV_{40} virus-newborn hamster test system(4,6). Thus, formalin-treated and pressure cell disrupted antigens gave no protection whereas, by contrast, gamma irradiated and IUDR-treated antigens afforded near 100% protection. The SV_{40} virus antigen effect was likely due to conversion of a number of cells to tumor cells *in vivo* with resultant immunization against tumor cell challenge. The effect was only modest compared with that obtained using the gamma irradiated or IUDR treated antigens.

The range of tumor occurrence following challenge was 50 to 93% in animals vaccinated with control fluids. This revealed the degree of variability in results which can occur in the subcutaneous challenge test.

Immunologic specificity of the subcutaneous challenge assay. The immunologic specificity of the protection shown in Figs. 4 and 5 was examined in tests in which 5 week-old hamsters were given 2 doses intraperitoneally of irradiated SV_{40} or adenovirus tumor cell antigen and were challenged subcutaneously with 100,000 live homologous or heterologous tumor cells. The findings summarized in Table III show that there was marked suppression of tumor development resulting from prior immunization with homologous tumor antigen but not with heterologous antigen.

Discussion. It is clear from the present data that cell mediated immunity against homologous SV_{40} tumor cells was demonstrable in the *in vivo* hamster system but not *in vitro*. Thus, the immune quality of the peritoneal exudate cells from vaccinated hamsters against tumor cells was demonstrable in peritoneal or cheek pouch tests in adult hamsters but not on exposure of the tumor cells to the same exudate cells in culture. The ratio of exudate cells to tumor cells was clearly of consequence in determining outcome of the *in vivo* tests since the protective effect was lost when an overwhelming challenge dose of tumor cells was used. The age of hamster used to demonstrate the protective effect was also important since *in vivo* neutralization could not be demonstrated when 1 week-old hamsters were employed for testing. Factors contributing to the effect of age of host might be that the baby hamsters were more susceptible to tumor cell growth or that there was a contribution on the part of the mature host to the weak or temporary protective effect given by the immune exudate cells. The immunity which was shown was clearly cell-mediated rather than due to a humoral factor since the peritoneal exudate cells were thoroughly washed. This did not preclude the possible contribution, however, of cell associated antibody.

In contrast to the *in vivo* results, no effect could be demonstrated when the immune exudate cells were tested with homologous tumor cells *in vitro*. This suggests a possible contribution, by the intact host, of an

TABLE III. Immunologic Specificity Demonstrated in Hamsters Immunized with Two Doses of SV_{40} or Adenovirus Tumor Antigen and Challenged Subcutaneously with Homologous or Heterologous Tumor Cells.

Irradiated tumor cell immunizing antigen		Tumor cell challenge		Result—days after challenge			
				Day 42		Day 48	
Kind	No. irradiated cells/dose	Kind	No. viable cells	No. with tumor /total	% with tumor	No. with tumor /total	% with tumor
SV_{40} (F5-1)	5×10^5 and 3×10^6	SV_{40} (F5-1)	1×10^5	2/13	15%	5/13	38%
"	"	Adenovirus 7 (Pinckney)	"	10/14	71%	11/14	78%
Adenovirus 7 (Pinckney)	5×10^5 and 3×10^6	SV_{40} (F5-1)	"	11/15	73%	13/15	87%
"	"	Adenovirus 7 (Pinckney)	"	1/13	7%	1/13	7%
Control (none)	—	SV_{40} (F5-1)	"	21/24	87%	23/24	96%
"	—	Adenovirus 7 (Pinckney)	"	14/22	64%	18/22	81%

immunity cofactor or other immune response which facilitated tumor cell destruction. Strong *in vitro* or *in vivo* destruction of target cells by immune mononuclear cells has been routinely demonstrated by others(8,9,11,12) employing allogeneic or xenogeneic transplant immunity cell systems but there are few reports of positive effect in syngeneic chemical or polyoma virus induced tumor-lymphocyte systems(13-15).

Direct subcutaneous challenge as employed previously by others(16-22) with tumor cells of immunized hamsters was by far the most efficient method for demonstrating potency of immunizing antigens. The protective efficacy was large approaching 100%, the tests were rapidly and simply carried out, and definitive results were available within 2 months following initiation of vaccination. Further, the findings were highly specific to homologous virus-induced tumor, and were in agreement with those obtained in the SV_{40} virus-newborn hamster test system. Variation in the test results, as evidenced by occurrence of tumor in 50 to 93% of control fluid vaccine animals given the same challenge dose suggested that the assay might not detect weakly potent antigens and indicated that further refinement in the challenge procedure should be sought.

Summary. Cell mediated immunity against homologous SV_{40} tumor cells was demonstrable in the homologous *in vivo* hamster system but not in cell culture. The effectiveness of exudate cells from immunized hamsters was clearly influenced by the ratio of target cells to immune cells and by the age of test hamsters used with protective effect demonstrable in adult but not in 1 week-old hamsters. High levels of immunity against homologous tumor were also demonstrable by direct subcutaneous challenge. The latter system was desirable for assay of vaccine potency because of simplicity and rapidity of obtaining results.

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Viability of Cell Cultures Following Extended Preservation in Liquid Nitrogen. (31992)

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Swim and coworkers(1) found that a number of cultured cells could be recovered for periods up to 3 years storage at —70°C but there was gradual loss of viability throughout the storage period. The success of storage was affected by the glycerol concentration, the method of freezing and strain of cells employed. Peterson and Stulberg(2) found that storage temperatures somewhat colder than —70°C resulted in an appreciable decay in cell viability in a matter of months, if not weeks. They recommended that the lowest temperature practicable should be used for long term storage of cells, and many investigators now store frozen cells at —196°C in liquid nitrogen and obtain good viability on recovery.

Although no changes have been noted in biochemical markers, tumorigenicity or antigenic composition due to freezing and storage in liquid nitrogen, there have been recent reports of deterioration of viability (3), change in LDH enzyme activity(2) and increase in generation time(4) after preservation in liquid nitrogen. These reports prompted a detailed viability study of 22 batches of frozen cells stored from 570 to 1530 days in liquid nitrogen and the results are reported below.

Materials and methods. The experimental cell lines studied are listed in Table I together with the time they were stored in liquid nitrogen and the composition of the culture media. The majority of frozen cells studied were candidates for the Cell Repository at the American Type Culture Collection. The frozen ampules of cells were prepared according to the procedures established by the Cell Culture Collection Committee(5). The cells were cultivated as monolayers in antibiotic-free media. They were removed from the flasks with 0.25% trypsin, centrifuged and resuspended in the original growth media to which 5% glycerol was added. Approximately 2 to 5 $\times$ 10^6 cells in 0.7 ml were deposited in 1.2 ml thick-walled glass ampules and flame sealed. Ampules were slow-frozen in a Linde BF-1 apparatus at the rate of 1-2°C/min to —50°C and then transferred to a Linde 300 Tank

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