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THE RELATIONSHIP OF GROWTH AND DIFFERENTIATION IN DIMETHYL SULFOXIDE-SENSITIVE AND RESISTANT ERYTHROLEUKEMIA CELL LINES

by

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Abstract

THE RELATIONSHIP OF GROWTH AND DIFFERENTIATION IN DIMETHYL SULFOXIDE-SENSITIVE AND RESISTANT ERYTHROLEUKEMIA CELL LINES

by

HARRIETTE HAUBENSTOCK

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Cell lines derived from mice infected with Friend Leukemia virus differentiate in vitro in response to treatment with DMSO. Experiments with these cells have been used to examine the relationship between hemoglobin production and growth arrest.

Hemoglobin production in tissue culture lines varies with the inducer and with the cell line. The percentage of cells producing hemoglobin under different conditions has been determined by scoring the number of benzidine-positive (B+) cells.

DMSO and glycerol (0.95% DMSO together with 3.5% glycerol) were found to act synergistically to induce hemoglobin synthesis in erythroleukemia line 5-86. After treatment with the optimal concentration of DMSO (1.9%), the combination of half the optimal concentration of DMSO and 3.5% glycerol, half the optimal concentration of DMSO alone, and 3.5% glycerol alone, the average percentages of B+ cells were 50.5%, 37.5%, 2.1%, 0.4% respectively, in preliminary experiments.

A DMSO-resistant variant cell line, designated H 1.9, was selected by prolonged treatment of line 5-86 with DMSO. This line resembled line 5-86 in growth pattern and virus production but was not induced to

produce hemoglobin by DMSO.

Assays both in vivo and in vitro were used to detect growth arrest. In one type of experiment, cell density was adjusted every other day to 1×10^5 cells/ml. by transferring the cells to fresh, treated medium. This procedure separates the effect of the inducer from any possible effect due to depletion of nutrients in the medium. Line 5-86, which is DMSO-sensitive, and line H 1.9, which is DMSO resistant, were treated with the set of inducing compounds mentioned above. The maximum percentage of B+ cells, observed eight days after continued transfer of line 5-86 cells in 1.9% DMSO or the combination of DMSO and glycerol, was 92% or 89% respectively. The peak percentage of B+ cells, observed 10 days after identical treatment of line H 1.9, was 27% or 20% respectively, indicating that line H 1.9 is not completely resistant to hemoglobin induction by these agents. Treatment of line 5-86 cells with 1.9% DMSO or the combination of DMSO and glycerol led to apparent cessation of growth. The growth of line H 1.9 was also markedly decreased by these treatments.

In a second assay, line 5-86 cells which had grown in medium containing inducing agents or unsupplemented medium for four days were injected subcutaneously in syngeneic DBA/2 mice. Tumors developed in 53% of the mice injected with 5×10^5 cells which had grown in 1.9% DMSO and in 93% of the mice injected with the same number of cells which had grown in unsupplemented medium. Furthermore, mice which developed tumors after the injection of cells treated with 1.9% DMSO or the combination of DMSO and glycerol survived longer than mice injected with cells grown in unsupplemented medium.

In addition, the effect of treatment with DMSO on the malignancy of the cells was evaluated using four cell lines, 5-86, H 1.9, 707R, and F4-1. These lines differed from each other in their ability to produce hemoglobin or virus. When mice were injected with cells grown in unsupplemented medium, tumors developed in 90% or 80% of the mice injected with cells of the DMSO-responsive lines, 5-86 or F4-1, respectively and 40% of the mice innoculated with cells of the DMSO-resistant lines, H 1.9 and 707R. In all four lines, mice injected with cells which had been treated with DMSO tended to survive longer than mice injected with cells which had not been treated.

Table of Contents

List of Figures

List of Tables

Abbreviations Used

ATPase adenosine trophosphatase

cAMP cyclie adenosine monophosphate

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

FLV Friend leukemia virus

gp 71 glycoprotein 71

HMBA hexamethylene bisacetamide

L.D. 50 lethal dose for 50% of the population

p 30 protein 30

RNA ribonucleic acid

t-RNA transfer ribonucleic acid

v/v volume per volume

Introduction

Treatment of Friend Virus-infected erythroleukemia cell lines in vitro with DMSO leads to changes associated with normal differentiation in erythroid cells. These transformed cells have been used as a model system for studying the induction of differentiation. Hemoglobin synthesis increases and cell growth is arrested after treatment with DMSO. Harrison has suggested that these responses are coupled because they are both lost in DMSO resistant variant lines.[1]

In the studies to be presented, this correlation was investigated in the following way:

1) The level of hemoglobin produced by line 5-86 was varied by changing the concentration of DMSO or by adding glycerol to the medium together with DMSO. The media used included (v/v) 1.9% DMSO, 0.95% DMSO, a combination of 3.5% glycerol and 0.95% DMSO, 3.5% glycerol alone and unsupplemented medium. Differences in treatment were also reflected in differences in growth during the induction period.

2) A DMSO-resistant line, designated H 1.9, was selected by continued treatment of line 5-86 with DMSO.

3) In order to separate the effects of inducing compounds from a possible effect of depletion of nutrients in the medium, some experiments were done in which cell density was adjusted to 1×10^5 by transferring the cells into fresh, treated medium every other day. Under these conditions, the growth rate was uniform in untreated cultures. A DMSO sensitive line, 5-86, and a DMSO resistant line, H 1.9, were treated with the media mentioned above, and the growth rates and changes in hemoglobin production were observed for 12 days.

4) If a majority of the treated cells were terminally differentiated, growth would be limited in vivo as well as in vitro. Therefore, syngeneic mice were injected with treated cells and their survival was compared to that of mice injected with untreated cells. Two sets of experiments were done. In the first, cells of line 5-86 were grown for four days in the media mentioned above before injection into mice. In addition, cells of four different lines were grown in control or DMSO supplemented medium for four days before injection into mice.

These lines differed in capacity to produce hemoglobin in response to treatment with DMSO and in virus production. They were characterized as follows:

(a) Line 5-86 responds to DMSO by producing hemoglobin. It produces virus in control medium and produces a higher level of virus after treatment with 1.9% DMSO.

(b) Line H 1.9 in DMSO resistant on the basis of hemoglobin synthesis but shows the same pattern of increased virus synthesis after treatment with 1.9% DMSO as its parent line. Both line 5-86 and H 1.9 show an initial lag in growth after treatment.

(c) Line 707R produces a low level of virus as detected by the reverse transcriptase assay but produces the same level of virus antigens gp 71 and p 30 as lines 5-86 and H 1.9. When this line is treated with 1% DMSO (2% DMSO is toxic for this line), no change in growth, hemoglobin production or virus production is observed.

(d) Line F4-1 is defective in virus production measured by reverse transcriptase or p30 in the medium. Treatment with 1.5% DMSO leads to hemoglobin production and production of a low level of p 30 but no increase in reverse transcriptase activity.

In general, the results of these studies are consistent with an association between increased hemoglobin synthesis and terminal growth arrest in the induced cells. However, some differences in growth in vitro were observed which were not correlated with an increase in benzidine positive cells. Furthermore, the ability of the cells to grow in vivo varies with the cell line as well as with the treatment and did not always correlate with growth in vitro.

Background

The Cells

Friend leukemia virus (FLV)[2] causes a leukemia in adult mice of susceptible strains 1-2 weeks after injection of virus. The spleen, liver, and bone marrow are infiltrated with primitive cells but the lymph nodes and thymus are not involved. This disease appeared similar to de Guglielmo's disease in man whose features include chronic refractory anemia, enlargement of the spleen and liver, proliferation in the marrow of young erythroid cells that fail to mature, and nucleated red cells in the blood.[3]

The cell lines used in this study were derived from subcutaneous tumors in DBA/2J mice produced by implanting small fragments of spleen or liver from leukemic animals late in infection.[4,5] Other laboratories isolated similar lines from tumors induced by Friend virus infected cells, suggesting that cells of the erythroid series are preferentially malignantly transformed. Evidence for the late committed erythroid cell as the target of Friend leukemia virus has been reviewed.[1] Tissue culture lines which respond to DMSO by producing hemoglobin have been derived from cells of FLV-infected DBA,[4,5] DDD,[6] SIM,[7] Balb[8,9a,b] and hybrid mice.[8]

Morphologically, the tumor cells resemble cells of a reticulum cell sarcoma. Friend noted that in tissue culture some cells had the morphology of stem cells while others appeared to be proerythroblasts or basophilic erythroblasts. The Friend cells in tissue culture accumulated Fe^{59} and extracts from these cells contained benzidine-positive material as determined by absorption spectra.[4]

Effect of DMSO on FLV Infected Tissue Culture Cells

The stimulation of erythroid differentiation in Friend leukemia virus infected cells by DMSO was first described in terms of hemoglobin production and altered morphology as demonstrated by a decrease in cell size and in the nuclear cytoplasmic ratio. The differentiated cells also showed a decrease in malignancy.[10]

DMSO induced changes which seem to parallel differentiation in normal erythrocyte precursers are now known to include increases in iron transport,[11] activity of enzymes of the heme pathway and heme synthesis,[12] and increases in globin RNA sequences in the nucleus[13,14,15,16,17] and messenger RNA in the cytoplasm.[15,16,17,18] There are changes in the activity of carbonic anhydrase,[19,20,21] in purine metabolism,[22] and a decrease in the transport of 2-deoxyglucose.[23] Changes in cell membrane components include an increase in red cell antigens[14,24] and a decrease in H2 antigens in some lines.[24a] Although the nucleus is retained, a majority of the benzidine-positive (B+) cells undergo growth arrest[25,26] and remain in the G1 phase of the cell cycle, even after transfer to fresh medium without DMSO.[27]

The effects of DMSO treatment also include changes in the rates of phospholipid,[28] protein, RNA and DNA synthesis.[29,30] There is a temporary accumulation of cells in G1[31] resulting in a growth lag during the first 24 hours of treatment. There is evidence of DNA breakage,[32,33] a temporary increase in intracellular cAMP,[34] changes in the levels of certain tRNAs,[35] changes in transport[36] which include sodium-mediated transport,[37] and, in some cell lines,.changes in virus synthesis.[6,38,39,40]

Other Inducers

A number of other inducers have been reported whose effects appear similar to those of DMSO in this system. These include hexamethylene bisacetamide[41] and other compounds which have polar properties similar to DMSO[42,43,44] as well as ethionine,[45] hemin,[46,47] hypoxanthine,[48] butyric acid,[49] and other short chain fatty acids,[50] ouabain,[51] Bromodeoxyuridine,[9b,42] and some metabolic inhibitors,[52] including actinomycin D.[52,53]

Glycerol, which was reported to be a moderately effective inducer of erythrocyte membrane antigen in T3Cl2 cells,[54] was not an effective inducer of hemoglobin synthesis although it reduced growth and caused increased virus production.[52] The ineffectiveness of glycerol was confirmed and the effect of glycerol added together with DMSO was mentioned as being additive.[55]

Mechanisms for Inducer Action

The mechanism of action for DMSO or any of the inducers has yet to be explained. The subject has been discussed[1] and recently reviewed.[56] The suggestions include changes in the cell membrane, changes in transport, changes in enzyme activity and changes in chromosome structure. It is also possible that differentiation is induced by the pathway suggested for erythropoietin, a change in a cytoplasmic molecule which moves to the nucleus.[57]

Evidence for a Membrane Effect

The idea that an effect on the membrane promotes differentiation is supported by the fact that three types of agents which affect membranes (certain phorbol diesters which are known to promote tumors,[58,59] local anesthetics[1] and amphotericin,[36] block the induction of differentiation by DMSO and other inducers tested.[59] Furthermore, Rb^+ transport decreases beginning after two hours of treatment with DMSO, dimethyl acetamide or hypoxanthine. This change is accompanied by a decrease in sodium mediated transport and is additive with respect to the effect of ouabain.[37] Ouabain is known to bind to the membrane-associated Na^+, K^+-associated ATPase.[51] The decrease in transport occurs earlier than, and might be related to, the relative decrease in cell size[60] and the reported decreases in 2-deoxyglucose transport[23] and RNA and protein synthesis[29,30] which are other early effects of treatment with some inducing compounds.

Two studies in which unsynchronized cells were removed from medium containing DMSO or HMBA after increasing exposure to the inducer showed no permanent commitment to differentiation within the first 16 or 12 hours of incubation respectively with the concentrations of inducers used for long term studies.[26,34] In contrast, Friend et al. found an increase in B^+ cells compared to controls four days after the cell were exposed to 2% DMSO for one hour and then transferred to fresh medium without the inducer.[10] A model in which an initial event at the cell membrane is followed by a number of subsequent events leading to irreversible commitment to differentiation was suggested by Rifkind.[34]

It is possible that an increase in the level of hypoxanthine, which is a potent inducer, is involved in the action of some inducing agents. This is suggested by a number of studies. Benoff and Skoultchi[61] noted that line 745(from which line 5-86 was derived) has no discernible X chromosome, although it has not been deliberately selected for this characteristic, and that somatic hybrids made with it and which lost the X chromosome from the other parent cell responded to DMSO. Harrison et al.[1] had also found that somatic hybrids of DMSO-responsive cells with non-responsive cells were generally resistant to DMSO and that responsive lines had lost chromosomes. McBurney[62] found that six of seven somatic hybrids between Friend cells and a teratocarcinoma responded to both DMSO and ouabain but the chromosomes were not studied. However, three of the DMSO-responsive lines had the same HGPRT negative teratoma as one parent. Another series of Friend cell x teratoma somatic hybrids only resembled the teratoma.[63] Since the X chromosome carries the HGPRT locus, hypoxanthine might accumulate in these cells. Treatment with agents which cause chromosome breaks[32,33,53] might also raise the level of hypoxanthine.

Evidence of a Nuclear Effect Based on Studies of Globin Message

In recent studies, the time of increase of globin message[18] and the relative amounts of beta minor and beta major chains synthesized[16,64,65] have been compared using different inducers on the same clone of cells. These comparisons showed that heme has the earliest effect on globin message accumulation.[18]

Although the ratios of globin chains may vary somewhat, even between line clones from the same cell line,[16] there are some general patterns. Heme stimulates the production of only beta minor chains.[65] Butyric acid,[16] hypoxanthine,[65] and actinomycin D[53] stimulate the production of approximately equal amounts of both chains. Cells of normal adult DBA mice and a spontaneously differentiating cell line produce ratios of beta major to beta minor close to 4:1[16,65] and 0.3:1[65] respectively. The ratio for DMSO falls higher than that for butyric acid[16,65] and the ratio for HMBA is slightly higher than that for DMSO.[16]

Since the ratios of globins synthesized in a wheat germ system using mRNA from treated cells are the same as the ratios synthesized in the cells, Nudel _et al._[16] concluded that the effect of the inducers is established by the transcription or processing of mRNA. Transcriptional control of globin mRNA induction was suggested by Ross et al. in 1972.[66]

Earlier studies of the synthesis and stability of radioactively labelled globin message[8] and comparison of the levels of globin RNA sequences isolated from the nuclei of control cells and cells which had been treated for three days with DMSO,[14] also provided evidence of an intranuclear effect.

Other Nuclear Changes

Evidence of chromatin breakage[32,33] shortly after the onset of treatment with DMSO was detected by the shift in the size distribution in alkaline sucrose gradients of chromatin from the treated cells which had been labelled with radioactive thymidine. In addition, mixtures of chromatin from prelabelled control cells (^{3}H thymidine) and prelabelled treated cells (^{12}C thymidine) analyzed in neutral sucrose gradients indicated that chromatin density decreased after treatment.[33] One effect of the inducing agent ethionine is to decrease DNA methylation.[45] Since methylation follows DNA synthesis, broken and repaired DNA might also be under-methylated. A new chromatin-associated protein[67] and changes in nuclear histone phosphorylation and nuclear proteins[68] have been observed in cells treated with DMSO.

The Role of Cell Division in the Differentiation of Friend Cells

The relationship between differentiation in Friend cells and events of the cell cycle has been studied with two questions in view; 1) Is a quantal cell division required before differentiation can occur? and 2) How many cell divisions separate growing cells from differentiated, growth-arrested cells?

The quantal division model, proposed by Holtzer,[69] suggests that there is an unequal distribution of some cell component or components such that a daughter cell is capable of responding to a stimulus to differentiate while a stem cell exposed to the same stimulus can not respond. Two groups[1,70] examined this question using semi-solid medium where the number of cells in a colony reflects the number of divisions after a single cell is plated. Both found individual benzidine-positive cells, some of which were binucleate. They concluded that cell division is not a prerequisite for differentiation in this system.

When cells of line T3C12 (from Ikawa's laboratory) were treated with hydroxyurea along with butyric acid, cells produced hemoglobin without dividing, although many of the cells were binucleate. When another Friend cell line was grown with hydroxyurea or cytosine arabinoside in addition to the inducers DMSO, butyric acid, or N-methyl-acetamide, mononuclear cells did not produce hemoglobin, but many doublet colonies and binuclear cells differentiated. When cells were arrested by isoleucine deprivation in the presence of inducer, they did not produce hemoglobin until after isoleucine had been restored. Harrison has suggested that DNA synthesis is required for differentiation.[1,70a]

Tabuse studied Friend's line 745. He found that the number of cells seeded in semi-solid medium with 2% DMSO decreased below the seeding density and many of the benzidine positive cells had not divided. The percentage of benzidine positive cells in semi-solid medium increased with increased seeding density. When the cells were treated in liquid medium, the results were somewhat different. The cells grew, and the percentage of benzidine positive cells decreased when the seeding density was increased.[70]

Two other groups used inhibitors of macromolecular synthesis on cells from Friend's laboratory grown in liquid medium and concluded that at least one cell division is required for differentiation with DMSO as the inducer.[71,72]

The differentiation of Friend cells may not be an appropriate model for a quantal division because the cells are already committed to the erythroid pathway.[1] This commitment is substantiated by the fact that some cells differentiate spontaneously[58] and by the finding that some globin genes of untreated cells are part of the template-active fraction when the chromatin is separated and analyzed by the procedure of Bonner _et al_.[73,74] Similarly, the myeloid leukemia and neuroblastoma cell lines affected by DMSO develop more mature characteristics of myeloid[75] or nerve cells respectively.[76] Furthermore, these cells and Friend cells respond to many of the same inducers.[75,77]

Friend Cell Differentiation as a Stochastic Process

Orkin[14] and others[1] have proposed that differentiation of stem cells in vivo is controlled by a stochastic mechanism rather than a quantal division.

Although Rifkind _et al_. wrote "...MELC are an essentially homogeneous population of transformed erythroid cells."[34] Orkin noted a heterogeneiety of response even at the clonal level. An increasing percentage of the cells produced hemoglobin as the culture continued to grow in the presence of an inducer. Examination of individual cells showed "the extent of induction of a population reflected the number of cells undergoing differentiation rather than the extent to which individual cells in the population could be induced." The rate at which cells become B^+ varies with the clone, the inducer, and its concentration, factors which determine a "probability of differentiation".[14]

Gusella _et al_. transferred cells from medium containing DMSO to plasma clots without DMSO after increasing exposure time to the inducer. They observed a good correlation between the number of committed cells which would be expected on the basis of a stochastic model and the number actually observed. Exposure to DMSO for more than one cycle was necessary for differentiation of a maximum number of cells.[26] A subsequent paper indicated that cells in G1 or G2 at the start of treatment have a higher probability of commitment than cells in S.[78]

If commitment to differentiation is a stochastic process, the relationship between growth and differentiation must be phrased in terms of the number of cell divisions between commitment and growth arrest. Reports from different investigators indicate between three[27] and four[26] divisions occur in cells treated with DMSO and five divisions occur in spontaneously differentiating lines.[58]

Variant Cell Lines and Development of a New Variant Line

DMSO-resistant variant cell lines have been used to study the induction of differentiation. DMSO resistant variant lines have been selected by testing clones from mass cultures[6] or by taking advantage of the selection effected by prolonged culture in DMSO.[1] Cells of these lines may differentiate in response to other inducers or when heme[1] or NDV (Newcastle Disease Virus) interferon is added in addition to DMSO.[6] Some variant cell lines produce a low percentage of B+ cells after treatment with DMSO.[25]

The TG-13 line was derived from cells cultured in a low concentration of 6-thioguanine. Cells of this line do not respond to DMSO but respond to a higher concentration of 6-thioguanine and to hypoxanthine as inducers.[48] The changes in transport observed in responsive cell lines are also observed when this line is treated with DMSO,[37] although another DMSO-resistant line and the DMSO responsive revertant line derived from it show the changes which would be expected if a decrease in transport is associated with differentiation.[37] Three variant DMSO-resistant lines fail to show the increase in transferrin binding capacity observed in line 745.[11] Although treatment with DMSO induces a program of differentiation, the elements of the program may be under independent control. Thus resistant variants[1,24] have been found in which DMSO induced spectrin alone, or a new chromosomal protein alone, or spectrin, glycophorin and the chromosomal protein but no hemoglobin, or no discernible change, or all the erythroid markers with a small amount of hemoglobin but no terminal differentiation. These data, plus the fact that lines selected for high constitutive levels of hemoglobin production revert quickly unless they are prevented from producing hemoglobin by treatment with phorbol esters, led Harrison to write "...it seems difficult to uncouple hemoglobin production from terminal differentiation."[1]

Some of the experiments to be discussed deal with characteristics of line H 1.9, a line developed in this laboratory by prolonged culture in DMSO.

Commitment to Differentiation

The decrease in susceptibility to thymidine suicide,[27] the decrease in colony forming ability in solid media[25,26,79,80] and the decrease in tumorigenicity[10] indicate that DMSO-treated differentiating cells have limited growth capacity. Non-differentiating cells in DMSO-treated cultures are killed by high doses of ^{3}H thymidine but committed cells, in which hemoglobin can not yet be detected, are spared because most of

Differentiation after treatment with DMSO has been correlated with decreased colony size after treated cells were plated into semi-solid medium without the inducer. The largest completely B+ colonies found by Gusella *et al.* 84 hours after transfer of DMSO-treated cells to solid medium without DMSO consisted of between 17 and 32 cells. In contrast, benzidine negative (B-) colonies and B- cells in mixed colonies had continued to grow with a doubling time of approximately 12 hours.[26] A decrease in colony forming ability after four days of of DMSO treatment had also been noted by Goldstein *et al.*[79] and Rovera and Bonaiuto.[25] If one compares the data from these three laboratories in an effort to determine what percentage of the cells are committed to terminal differentiation, one finds that at the time 50% of the cells are B+,90% have a limited capacity to proliferate. However, an experiment has been described in which line 745 cells were grown in DMSO for four days and then cloned in agar and only 0.4% of the cells were able to form undifferentiated colonies.[80]

After four days of treatment with DMSO,66% of line 707 cells produced hemoglobin. The L.D. 50 of syngeneic mice injected subcutaneously with cells which had grown in DMSO for four days was approximately one log higher than the L.D. 50 of mice injected with cells grown in unsupplemented medium for four days.[10] The results of the majority of experiments in which growth capacity after treatment with DMSO was measured by colony formation in semi-solid medium without DMSO are consistent with the earlier findings *in vivo.*

Materials and Methods

Cells: Mouse erythroleukemia cell line 5-86 used in these experiments was derived by cloning line 745A in semi-solid agar. Line 745A[29] is a tissue culture line from a DBA/2J mouse infected with Friend leukemia virus (FLV). DMSO-resistant line H 1.9 was selected from line 5-86 by prolonged culture in DMSO as described below. Line 707R is a DMSO resistant line obtained from Reem. This line was derived from line 707[1] which was isolated from an FLV-infected mouse by Friend.[10] Line F 4-1 was a gift from Eisen[24] and was derived from the line isolated from a DBA/2J mouse infected with polycythemic FLV by Ostertag *et al.*[36]

Medium: Except where indicated, the medium used was dehydrated Eagle's Basal Medium with Earle's salts (BME/E) (Grand Island Biological Co., Grand Island, N.Y.) reconstituted with distilled water and buffered with sodium bicarbonate. Line 707R was grown in RPMI 1640 medium (Flow Lab., Rockville, Md.) because DMSO is less toxic to these cells in RPMI 1640 than in BME/E. Line F4-1 was grown in RPMI 1640 medium because it appeared to be less adherent to the culture vessel in this medium. The medium was supplemented with 15% (v/v) fetal bovine serum (Reheis Chem. Co., Kanakee, Ill.), 250 units/ml. of penicillin and 0.2 mg./ml. of streptomycin. In addition, the RPMI 1640 medium was supplemented with 2mM glutamine.

Reagents: Note; all percentages of reagents are given as v/v except where indicated. Certified reagent grade DMSO lot #733788 (Fisher Sci. Co., Fair Lawn, N.J.) was stored in a brown bottle at room temperature and added unsterilized to the medium to a final concentration of 1.9% for lines 5-86 and H 1.9 or as noted. Line 707 was treated with 1% DMSO and line F4-1 was treated with 1.5% DMSO. Glycerol (U.S.P.) was sterilized by autoclaving. Hexamethylene bisacetamide (HMBA) was a generous gift from R. Reuben. A solution in BME/E was filtered through a 20 micron Nalgene filter (Sybon Corp., Rochester, N.Y.) and diluted to 5 mM. N-butyric acid (Aldrich Chem. Co., Inc., Milwaukee, Wisc.) was diluted with BME/E to 100 x the desired concentration, filtered through a 20 micron Nalgene filter, and stored at 4 degrees centigrade. The stock was added to the medium before the cells were added to yield a final concentration of 2 mM. (H^3) methyl thymidine (specific activity 54 Ci/mM was purchased from Schwarz-Mann (Orangeburg, N.Y.) lot #ZR 1347. All other compounds were reagent grade.

Mice: DBA/2J mice were purchased from Jackson Laboratories (Bar Harbor, Me.) or bred in this laboratory. Swiss mice were purchased from Taconic Farms (Germantown, N.Y.). Except for experiment I testing DMSO and glycerol treated cells, where three month old mice were used, mice were used within one month after weaning. Mice were housed no more than 10/cage in a temperature-controlled room with reversed light cycle and had access to water and chow ad lib.

Cell culture: Cells were grown in a humidified atmosphere containing 5% CO_2 in air at 37 degrees C. Stocks were maintained by passage twice weekly in 3 ml. of medium in plastic plates (35 x 10 mm. style, Falcon, Div. of Becton Dickinson and Co., Oxnard, Calif.) by seeding at approximately 10^5 cells/ml. For experiments, cells which had been passed three or four days previously were seeded at 10^5 cells/ml. in 3 ml. of medium as above except where noted. The number of cells was determined by counting an appropriate dilution in a hemocytometer with 0.1% (w/v) trypan blue added to estimate the ratio of living and dead cells.[81] When cells were transferred, three ml. of the appropriate medium were added to the plate, a volume equal to the volume of treated cells and medium containing 3×10^5 cells was removed, and the treated cells and medium were added. Experiments were initiated by adding the reagents at an appropriate higher concentration and diluting to the desired concentration by the addition of the cells in untreated medium.

Heme determination: Cells containing large amounts of heme were enumerated using a modification of the method of Orkin et al.[33] Two volumes of a cell suspension were mixed with one volume of benzidine-peroxide solution which was kept on ice. The benzidine-peroxide solution consisted of 1.0 ml. of stock benzidine dihydrochloride solution (0.2% w/v benzidine dihydrochloride (Fisher Sci. Co., Springfield, N.J.) in 0.5 M acetic acid which was stored at 40 degrees C.) plus 0.02 ml. freshly added 30% H_2O_2. The mixture of cells and benzidine-peroxide solution was examined immediately in a hemocytometer chember. Cells stained light and dark blue were counted as positive. Between 100 and 200 cells were examined for each treatment.

DNA synthesis: DNA synthesis was estimated by the determination of H^3 Thymidine incorporation into acid precipitable radioactive material. The pool sizes of thymidine were not determined. (Pool sizes have been found to decrease or remain the same in response to treatment with DMSO in cell lines derived from line 745A.)[82] Cells were grown with various test reagents and in control medium. After designated time intervals, the cells were counted. A volume containing 5×10^5 cells was pipetted into a snap-cap tube (Falcon plastic 12 x 75 mm.), centrifuged (200 x g at 4 degrees C. for 10 minutes in an International centrifuge), and resuspended in 0.5 ml. of fresh medium which contained the same reagents as the original treatment. The tubes were gassed with 5% CO_2 in air and incubated at an angle of approximately 45 degrees in a shaking water bath (Gyrotory Shaker, New Brunswick Scientific, New Brunswick, N.J.) at approximately 220 r.p.m. at 37 degrees C. for 20 minutes. After the addition of 0.5 microcurie/tube of (H^3) thymidine, the tubes were gassed again and incubated for an additional 40 minutes. At the end of the incubation period, the tubes were placed in ice and 0.05 ml. samples were spotted on 3 mm discs (Whatman). The discs were rinsed once in 10% TCA (4 degrees C.), 3x in 5% TCA (4 degrees C.) twice in 95% ethanol (room temperature) and once in ether (room temperature). The discs were dried, transferred to vials, and counted in toluene-based scintillation fluid. This method is a modification of that used by Friend et al.[29] and Tsuie (personal communication.)

Selection of line H 1.9: Cells of line 5-86, pass 145, were seeded in 1.9% DMSO at 10^5 cells/ml and allowed to grow for two days. They were then seeded again at 10^5 cells/ml. in 1.9% DMSO. Four days later they were seeded a third time at 10^5 cells/ml. in 3 ml. of 1.9% DMSO. After an additional ten days, the entire contents of the plate was centrifuged (200 x g for 10 minutes) and the cells were resuspended in three ml. of medium without DMSO. After 10 passes the mass culture was tested for response to DMSO by growing a sample of cells in 1.9% DMSO and staining with benzidine after four days of treatment. There was little response. A month after the cells had been transferred to control medium they were cloned in 96 well, round bottom Linbro plates (Flow Laboratories, Inc.). Cells were cloned by diluting them in medium to a concentration of one cell/0.9 ml. and pipetting 0.12 ml. of cell suspension into each well. Wells were not checked for individual cells after the pipetting step but the probability of having pipetted more than one cell in a well was less than 0.05 since there were fewer clones than would be expected from a dilution adjusted to give one cell/five wells. Several clones were tested for DMSO-stimulated heme synthesis and reverse transcriptase activity in the medium. Clone 1, later designated H 1.9, was selected for further study because the change in reverse transcriptase activity in response to DMSO treatment was similar to that of the parent line, 5-86.

Subcloning of line H 1.9: Cells of line H 1.9 were cloned as above with the difference that both control medium and medium containing 1.9% DMSO were used, and one plate was cloned at a concentration of 2 cells/5 wells in addition. Cells which grew in 1.9% DMSO were transferred to another well by removing 0.1 ml. and adding 0.08 ml. DMSO-supplemented medium. The cells which remained in the well were tested for heme production by adding benzidine-peroxide solution to the well and counting under the binocular microscope.

Test of malignancy of tissue culture cells and of virus from conditioned medium: Cells were seeded in plates and grown for four days in the medium noted as described above. Viability and percentage of benzidine positive cells were determined. The cells were centrifuged (200 x g, 10 minutes at 4 degrees C.) and the supernatant fluid was saved and kept at 4 degrees C. The cells were resuspended in cold BME, counted again, and the concentration was adjusted to 5x the desired concentration/ml. with additional BME. The cells were kept on ice for the short interval before they were injected subcutaneously in a volume of 0.2 ml./mouse. The supernatant fluid samples which had been saved were filtered (Swinnex 0.22 micrometers, Millipore Corp., Bedford, Mass.) kept on ice and 0.2 ml. was injected intraperitoneally (i.p.)/ mouse. Mice were observed weekly and, after death, they were autopsied. Experiments were terminated after six months except as noted.

Test of immunogenicity: A 20% (w/v) spleen suspension in buffered Ringer's with glucose (BGR) was prepared from a mouse which had been infected with polycythemic Friend Leukemia virus. The filtered (Nalgene) suspension was diluted with BGR and injected i.p. as noted using five mice/dilution. Groups of mice which had survived previous treatment with cells or conditioned medium were subdivided into two groups. Mice of one group were innoculated i.p. with 0.2 ml. of the spleen filtrate diluted 10^{-2} in BGR and the second group was left untreated as a control. Mice were observed and autopsied as above.

Cell cycle analysis: Cells for cell cycle analysis were grown in 80 ml. of medium in glass prescription bottles (32 oz.) which were gassed with 5% CO_2 in air. At the times noted, cells were counted and appropriate volumes were treated for the propidium iodide and acridine orange methods. Separate bottles were used for each time point. Both methods of cell cycle analysis were used on the same set of cell cultures.

Preparation of cells for cell cycle analysis by flow microfluorometry with propidium iodide: All centrifugations were 200 x g for five minutes. A volume of cell suspension containing 2 x 10^6 cells was centrifuged, resuspended in 50% methanol in 1x Earle's Basic Salts without Ca^{++} and Mg^{++} (EBS), and fixed for 30 minutes on ice. The cells were then centrifuged and resuspended first in 25% methanol in EBS and then in 12.5% methanol in EBS. After another centrifugation, they were resuspended in 1 ml. 0.2 sodium phosphate buffer at pH 7.

Worthington 9A RNA-ase A was added to 1 mg./ml. and the cells were incubated for 30 minutes at 37 degrees C. The cells were then centrifuged and resuspended in 2 ml. cold EBS and refrigerated. Immediately before testing, the cells were centrifuged and resuspended at 7 x 10^5 cells/ml. in EBS and 0.025 ml. of propidium iodide (2 mg./ml. H_2O) was added. The rest of the procedure was carried out as described.[27]

Preparation of cells for cell cycle analysis by flow microfluorometry with acridine orange or propidium iodide: A volume of cell suspension containing 10^7 cells was washed once in phosphate buffered saline (PBS) containing 2 mM $MgCl_2$. The cells were resuspended in the ratio of one part cell suspension to 9 parts 1:1 ethanol:acetone and kept at 4 degrees C. The rest of the procedure was carried out as described.[83]

Reverse transcriptase assay: The cells were sedimented by centrifuging at 200 x g. and the supernatant fluid was centrifuged at 10,000 r.p.m. in a Sorvall centrifuge using rotor ss 34 at 4 degrees C. The resulting supernatant fluid was then spun at 27,000 r.p.m. in a Spinco 30 rotor for 90 minutes at 4 degrees C. (After the cells were removed from the incubator all materials were kept on ice.) The virus pellet was resuspended in NTE buffer (0.2 ml. 0.1 M NaCl, 0.001 M Tris, pH 7.6). Exogenous enzyme activity associated with virus was assayed by the method of Mayer *et al.*[84] with modification. The reaction mixture in a final volume of 50 µl contained: 50 mM Tris, pH 8.0, 30 mM NaCl, 2 mM dithiothreitol, 0.1 mM dATP, 0.8 mM $MnCl_2$, 0.02% (v/v) Nonidet P40 (Shell Oil Co., N.Y., N.Y.), 10 µg/ml. poly rA. dT_{10} (Collaborative Res. Inc., Waltham, Mass.), 1 µCi (^3H)-thymidine-5-triphosphate (TTP)

(New England Nuclear, 51 Ci/mmole), 0.01 mM unlabelled TTP and the virus suspension. The mixture was incubated at 37°C. for 30 min., stopped with 10 μl of 0.2M EDTA and chilled in ice. A volume of 50 μl was spotted on a 25 mm disc of DE-81 filter paper (Whatman, Inc., Clifton, N.J.) which was washed 6 times with 5% $NaHPO_4$, twice with water, twice with 95% ethanol and twice with ethyl ether. Filters were dried and counted in a toluene-base scintillation fluid.

Competitive radioimmunoassay: Purified viral gp71 and p30 from FLV infected Eveline STU, a generous gift from Dr. D. Bolognesi, have been described.[85] Virus protein tyrosine residues were labelled with ^{125}I by the lactoperoxidase[86] or the chloramine-T iodination[87] procedures. Specific antisera tc FLV-gp71 or p30, generous gift from J. Gruber, were prepared in goats as described.[88] The procedure for competitive radioimmunoassay follows. Briefly, 200 μl fluid from daily cultures of different FL lines were clarified and centrifuged at 10,000 rpm in Sorvall SS34 rotor. The supernatant fluid was then incubated with 50 μl of anti-FLV p 30 or anti-FLV gp71 sera (1:1000 to 1:250) at 37°C. for 30 min., then overnight at 4°C. To the mixture, 50 μl of ^{125}I-labelled gp71 or p30 was added and incubation was carried out at 37°C. for 1 hr. and then 4°C. for 4 hrs. Donkey gamma globulin, generous gift of Dr. J. Gruber, was added to precipitate the antigen-antibody complex. The mixture was further incubated at 37°C. for 1 hr. and then 4°C. for 1 hr. The precipitate was collected, washed with TNE buffer and ^{125}I-labelled antigen present in the precipitate was measured in a gamma counter.

Results

Treatment of Line 5-86 with DMSO, Glycerol and Combinations of DMSO and Glycerol

Effect of Glycerol on Growth and Percentage of B+ Cells

Initial studies of the effects of increasing concentrations of glycerol on line 5-86 showed a small increase in the percentage of benzidine positive (B+) cells and a decrease in cell growth (Table 1). However, cells treated with 1% glycerol grew faster than cells plated in control medium for the first day (Figure 1) and grew to 3×10^6 cells/ml. after seven days compared with 2.3×10^6 cells/ml., the level reached by cells grown in control medium. Cells treated with 6% glycerol, the most effective concentration for this cell line, averaged 12% B+ cells after six days of treatment although cells treated with 2% DMSO in the same experiment averaged 59% B+ cells. Cells treated with 6% glycerol showed a lag in growth on day one and a decrease in growth rate after day 2.

Effect of Glycerol Plus DMSO on Growth and Percentage of B+ Cells

Since the optimum concentration of glycerol stimulated 12% of the cells to become B+ ,it appeared that this compound had some ability to induce differentiation. The fact that it decreased cell growth led us to test the effect of low concentrations in combination with DMSO.(Table 1). After four days of treatment, the percentage of B+ cells in cultures treated with 2% or 1% DMSO alone were 65.6% and 13.6% respectively. The concentrations of glycerol in order of decreasing effectiveness in combination with 1% DMSO were 3%,4%,2%, and 1%, which induced hemoglobin synthesis in 58%,53%,43.9%, and 34.6% of the cells respectively. The

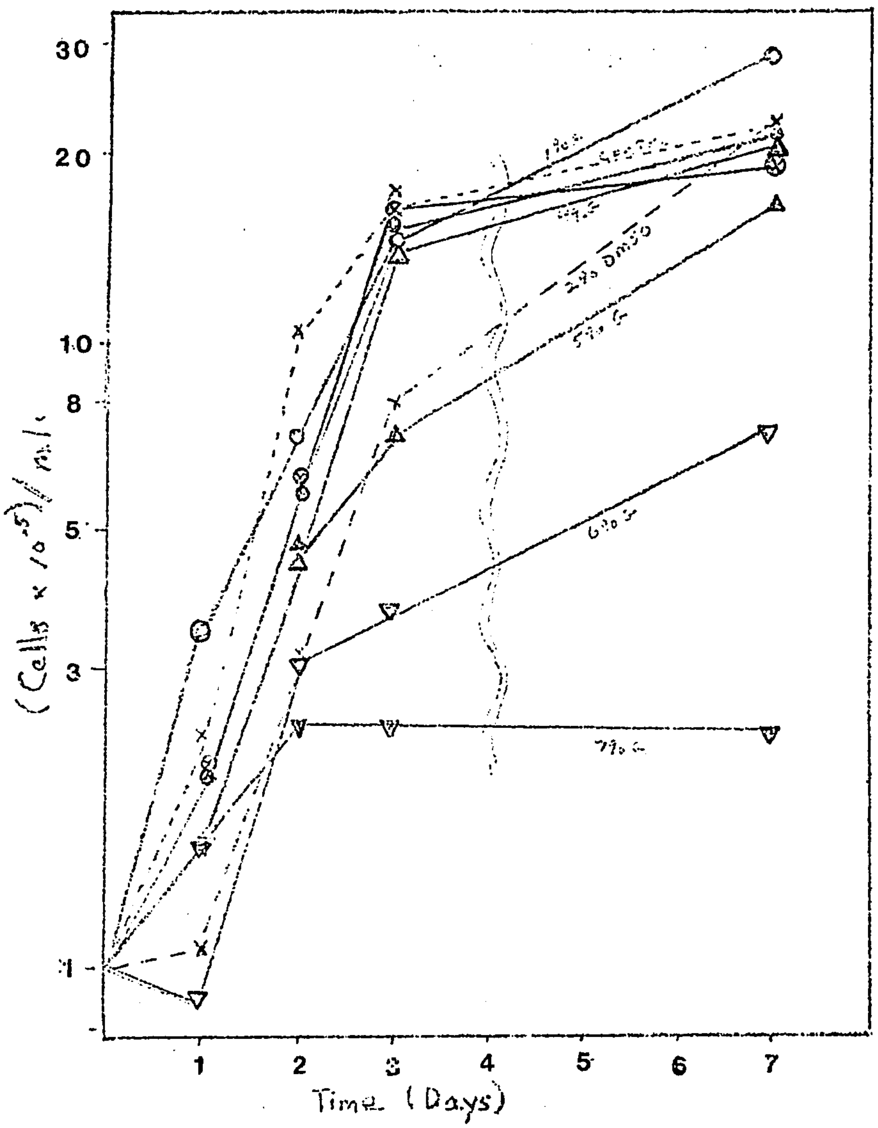


Figure 1. Growth in Increasing Concentrations of Glycerol

control _ _ _ _ _ _ X
1% Glycerol _ _ _ _ o
2% Glycerol _ _ _ _ o
3% Glycerol _ _ _ _ ⊗
4% Glycerol _ _ _ _ Δ
5% Glycerol _ _ _ _ Δ
6% Glycerol _ _ _ _ ∇
7% Glycerol _ _ _ _ ∇
2% DMSO _ _ _ _ _ _ X

Cells were seeded at 1×10^5 cells/ml. and counted using a hemocytometer.

Table 1

Cell Counts and Percentages of B+ Cells After Four Days of Treatment of line 5-86 cells with DMSO, Glycerol and Combinations of DMSO and Glycerol.

Treatment	Cell count cells x 10^{-5}/ml.	%B+ cells
control	18	0.5
2% DMSO	13	65.6
1% DMSO	20	13.6
1% Glycerol	20	0
2% Glycerol	18.5	2.2
3% Glycerol	14	2.7
4% Glycerol	11	4.3
5% Glycerol	6.8	7.5
6% Glycerol	4.4	12.0
7% Glycerol	2.5	7.0
1% DMSO + 1% Glycerol	20	34.6
1% DMSO + 2% Glycerol	16.5	43.9
1% DMSO + 3% Glycerol	10	58.3
1% DMSO + 4% Glycerol	2.3	53.0
2% DMSO + 1% Glycerol	3.7	62.8
2% DMSO + 2% Glycerol	2.6	77.0
2% DMSO + 3% Glycerol	0.9	39.5
2% DMSO + 4% Glycerol	0.4	0

concentrations of glycerol in order of decreasing effectiveness in combination with 2% DMSO were 2%,1%, 3%, and 4% which induced hemoglobin synthesis in 77%,62.8%,39.5%, and 0% of the cells respectively.

Cells treated with glycerol and 1% DMSO grew more slowly than cells treated with the same concentration of DMSO or glycerol alone. Thus, cells grown in 1% DMSO, 4% glycerol, and 1% DMSO plus 4% glycerol grew to 20.5, 11, and 2.3 x 10^5 cells/ml. respectively. Although cells treated with 1% glycerol plus 1% DMSO grew to 20 x 10^5 cells/ml., early growth was delayed (Figure 2). The decreased rate was accompanied by an increased percentage of B+ cells (Table 1).

The combination of glycerol with 2% DMSO reduced growth to less than two doublings compared with the more than three doublings observed after treatment with 2% DMSO alone. Cells treated with the combination of 1% glycerol and 2% DMSO grew to less than 35% of the control value compared with the 77.5% of control value reached by cells treated with 2% DMSO. However, the two treatments induced hemoglobin synthesis in 62.8% and 65.6% of the cells respectively (Table 1).

Since seeding density had been shown to affect hemoglobin induction by DMSO[72], the effects of seeding density were compared using cells treated with 2% DMSO or 1% glycerol plus 2% DMSO(Figure 3). Cells treated with 1% glycerol in addition to 2% DMSO consistently grew more slowly than cells grown in 2% DMSO alone except when the cells were seeded at 8 x 10^5 cells/ml. and the growth curves were parallel for the first two days. In cultures treated with 2% DMSO alone at seeding densities of 4-8 x 10^5 cells/ml., there was an average decrease in B+ cells of slightly more than 10% compared to cells treated with DMSO at lower densities or cells treated with 1% glycerol plus 2% DMSO (Table 2).

The effect of seeding density was also studied by comparing cells

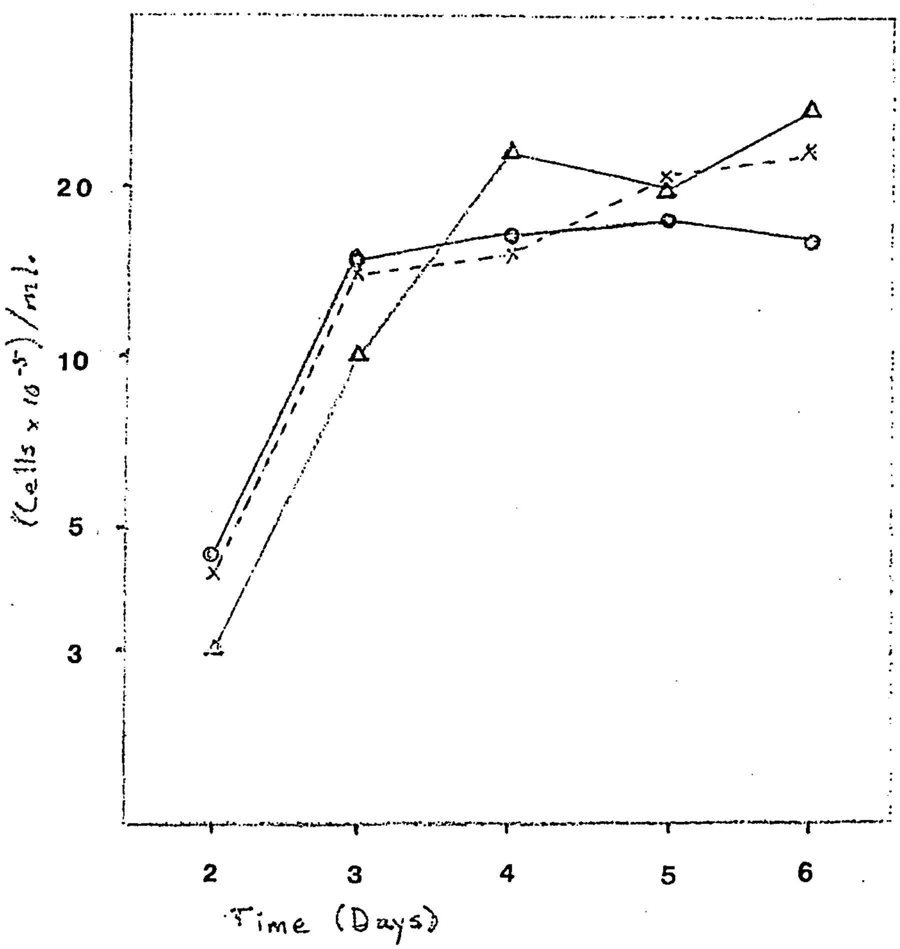


Figure 2. Growth in Glycerol plus DMSO

control ×
2% Glycerol ○
1% Glycerol plus
1% DMSO △

Cells were seeded at 1 x 10^5 cells/ml. and counted using a hemocytometer.

Table 2

Effects of Treatment with 2% DMSO or 2% DMSO Plus 1% Glycerol

on Percentage of B+ Cells Evaluated on Day 4

		Seeding Density (cells x 10^{-5})									
Treatment	Experiment	0.3	0.67	1.0	1.3	2.0	2.7	4.0	6.0	8.0	Average
2% DMSO	1			42.0		42.6		29.5	30.1	26.3	34.1
	2	41.6	42.2	50.1	56.5						49.3
2% DMSO	1			38.6		34.7		47.0	44.6	36.6	40.4
plus 1% glycerol	2	48.6	53.7	53.8	49.5						52.1

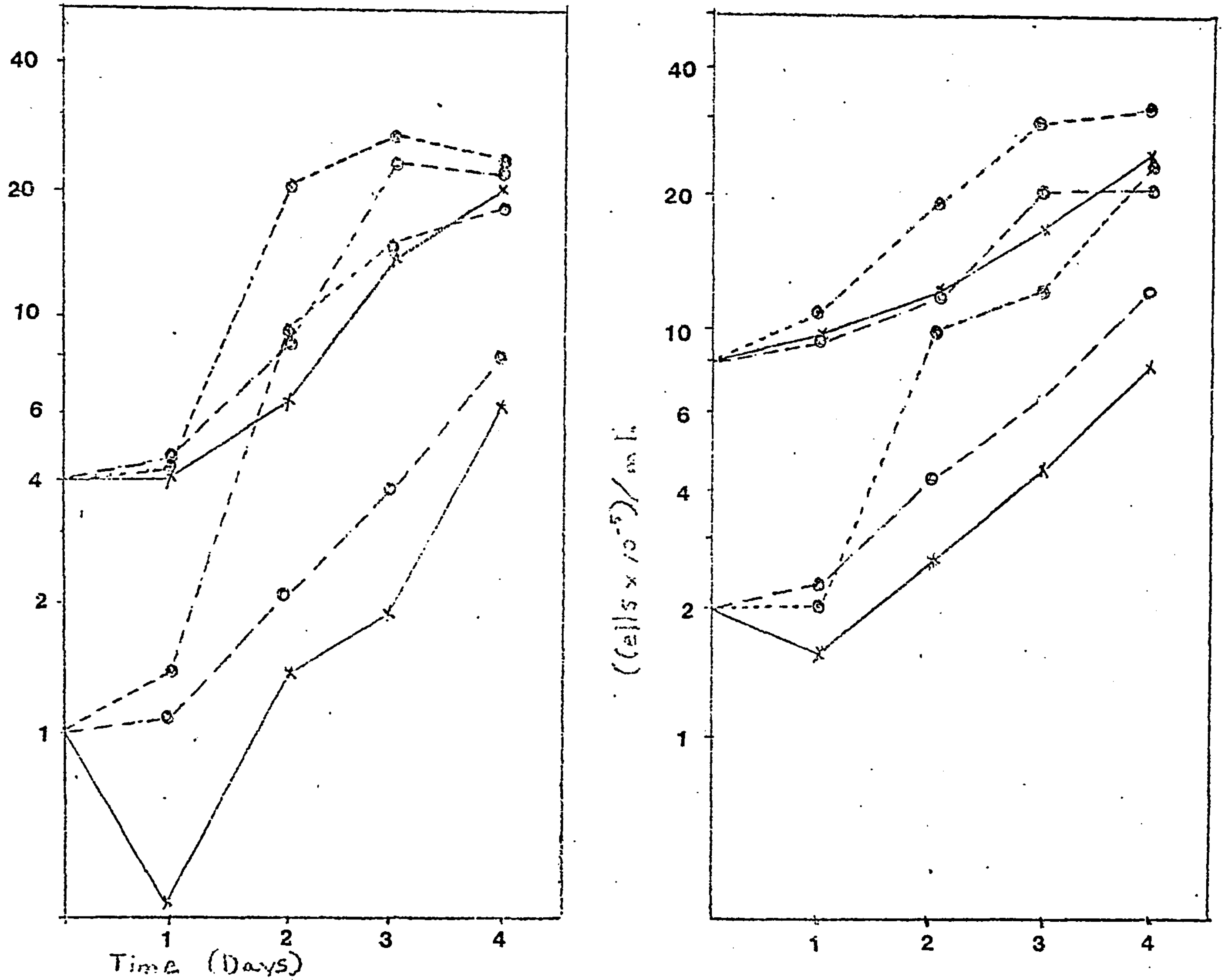


Figure 3

Effect of Seeding Density On Cell Growth

Control ⊕
2% DMSO o
1% Glycerol plus 2% DMSO ×

treated with 3% glycerol plus 2% DMSO to cells treated with 2% DMSO alone. Table 3 (column 3 on day 4)shows that an average of 45.5% and 7.5% of the cells seeded at 1 x 10^5cells/ml. were B+ after four days of treatment with 2% DMSO and 2% DMSO plus 3% glycerol respectively. Comparable results were obtained in a similar experiment,(Table 4). Cultures treated with DMSO and seeded 1-2 x 10^5cells /ml. had an average of 40% B+ cells while cultures seeded above that level had an average of 25% B+ cells. Cultures seeded in 3% glycerol plus 2% DMSO amd seeded at 1-2 x 10^5 cells/ml.had an average of 22.5% B+ cells and an average of 19.6% B+ cells if seeded above that level. Removing the conditioned medium by spinning the cells before seeding appeared to increase growth as well as differentiation. The average cell density on day 4 was increased by 5.5 x 10^5 cells/ml. for cells grown in control medium or 2% DMSO and 3 x 10^5 cells/ml. for cells grown in 3% glycerol plus 2% DMSO. The average percentage of B+ cells was increased by 10% and 6% respectively (Table 4).

Percentages of G1 Cells and ^{3}H Thymidine Incorporation

As the data in Table 1 and Table 4 indicate, after 4 days of treatment with 3% glycerol plus 2% DMSO approximately 20% of the cells differentiated.Figure 4 shows the growth of control and treated cells. Whether the slight increase in cell number at most represents one doubling of all the cells or two doublings of those cells which differentiated is not clear. Since there was a definite effect of treatment on growth, the distribution of cells in regard to the cell cycle and DNA synthesis were studied. When the length of the cell cycle increases,the major change is an increase in G1. In unsynchronozed cells,the percentage of cells in a phase of the cycle is proportional to the time required to traverse that phase, and high G1 values reflect slow growth.[89]

The experiment was started using cells which had been grown for

Table 3

Effect of seeding density on cell growth and differentiation of line 5-86 cells treated with 2% DMSO or 3% glycerol plus 2% DMSO.

Treatment	Seeding Density x 10^{-5}	Cell Count day 4			% B+ Cells day 4			Cell Count day 5			% B+ Cells day 5		
		exp.1	exp.2	av.	exp.1	exp.2	av.	exp.1	exp.3	av.	exp.1	exp.3	av.
Control	1	27	23	25	0.5	0	0.3	26	19	22.5	0	0.9	0.5
2% DMSO	1	10.1	10.1	10.1	35.6	55.3	45.5	10.1	19.5	14.5	59.5	86.9	73.2
2% DMSO	1	1.1	0.8	1.0	6.2	9.8	7.5	1.6	1.5	1.6	32.2	15.6	23.9
plus	2	1.7	2.5	2.1	26.5	18.6	22.6	2.4	2.8	2.6	38.8	70.9	54.9
3% Glycerol	4	4.2	3.5	3.9	39.5	24.5	32	4.3	4.3	4.3	50.2	62.9	56.6

Table 4

Effect of Plating Density and Procedure on Differentiation of Cells Treated with 2% DMSO or 2% DMSO Plus 3% Glycerol Evaluated on Day 4

Seeding density at plating x 10^{-5}	Treatment before plating	Control medium cell count x 10^{-5}	DMSO medium cell count x 10^{-5}	DMSO medium % B+	D + G* medium cell count x 10^{-5}	D + G* medium % B+		Pooled DMSO medium % B+	Pooled D + G* medium % B+
1	spun	15	19	40.5	1.5	10.2			
	not spun	7.4	16	34.6	1.3	20.4			
							spun	44.9	22.8
							not spun	35.5	22.0
2	spun	17	15	49.2	5.7	35.4	average	40.1	22.5
	not spun	25	8.4	36.4	3.1	23.7			
4	spun	26	18	24.6	14	14.7			
	not spun	25	17	24.4	8.4	25.9			
6	spun	38	36	27.0	14	31.6	spun	29.5	24.6
	not spun	25	30	14.7	11	0	not spun	20.8	14.7
							average	25.2	19.6
8	spun	39	46	36.9	16	27.4			
	not spun	25	34	23.3	12	18.1			
Average (4.2)	spun	27	26.8	35.6	10.2	23.9			
	not spun	21.5	21.1	26.7	7.2	17.6			

*D + G indicates 2% DMSO plus 3% glycerol

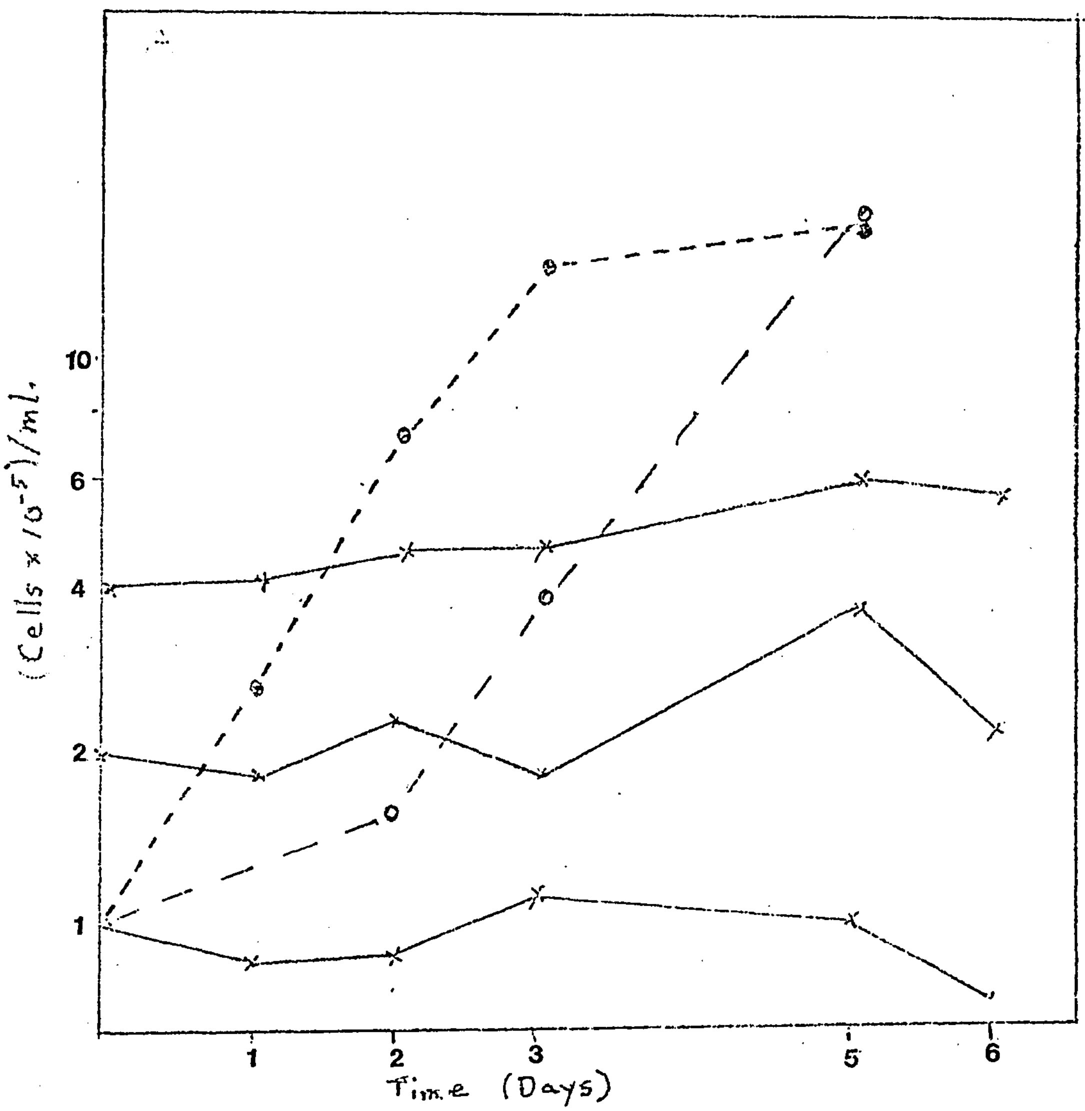
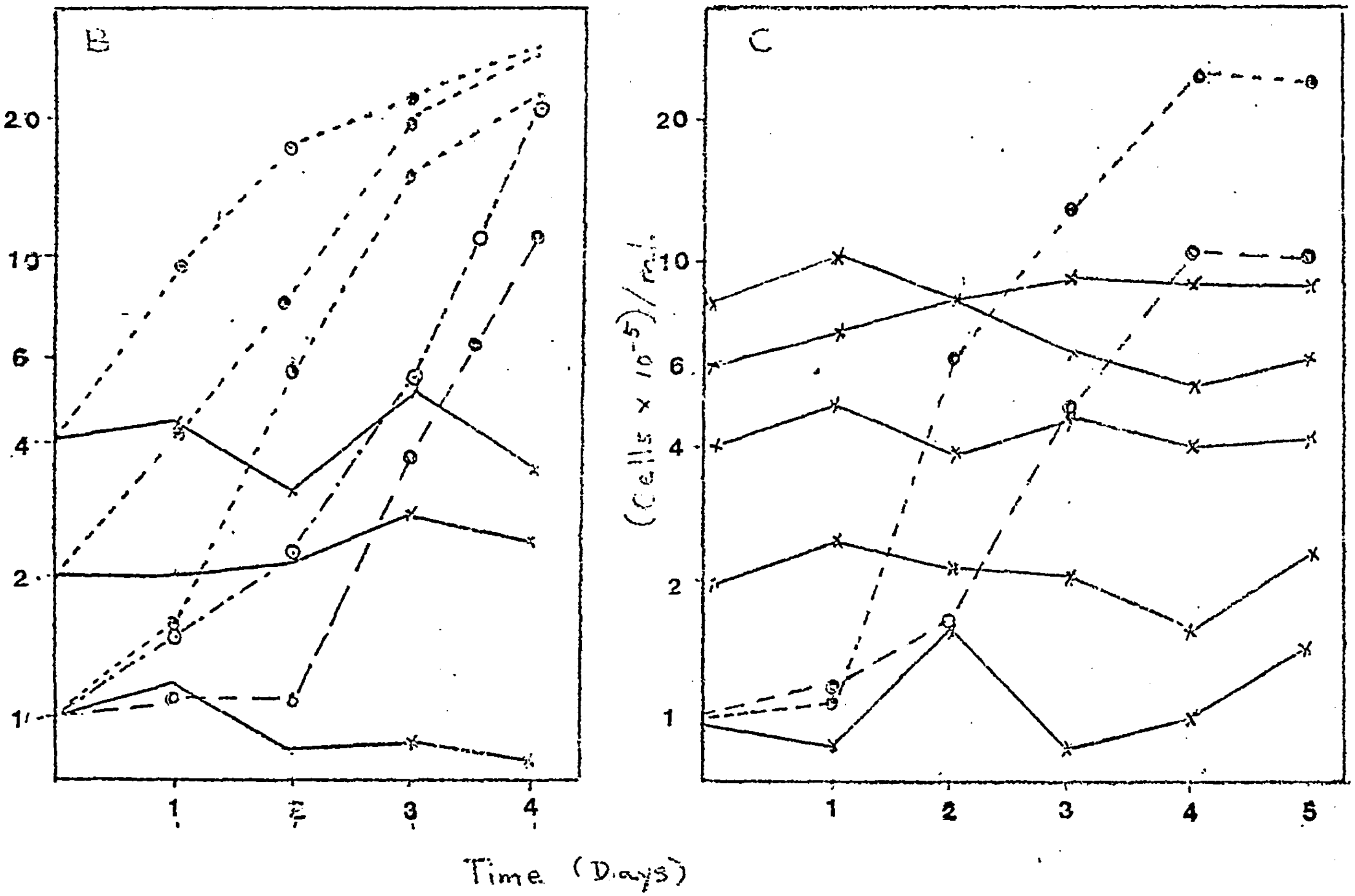


Figure 4A. Growth of Cells in 3% Glycerol plus 2% DMSO Experiment 1

control ⊖
2% DMSO ○
3% Glycerol
plus 2% DMSO ×

Cells were seeded as indicated and counted using a haemocytometer.



Figures 4B and C.

Experiments 2
and 3

control
2% DMSO
3% Glycerol
3% Glycerol
plus 2%
DMSO

four days in control medium. At that point they had been in the plateau phase of growth for one day and were parasynchronized in G1 (Z. Darzenkiewicz, unpublished data). After 18 hours, (Table 5) the percentage of cells in G1 for cells grown in control, 3% glycerol, 2% DMSO, and 3% glycerol plus 2% DMSO medium was 32.7, 46.6, 78.8, and 66.8 respectively. This distribution is consistent with the growth lag observed after treatment with DMSO.[31] After two days, slightly less than 50% of the control and DMSO-treated cells were in G1 compared to more than 85% of the cells observed in G1 after growth in 3% glycerol plus 2% DMSO. After 90 hours of treatment, the level of G1 cells in cultures which were treated with 2% DMSO or 3% glycerol plus 2% DMSO was higher than in culture grown in unsupplemented medium. After five days, cultures grown in 3% glycerol or unsupplemented medium had a distribution of G1 cells close to that observed at the beginning of the experiment and culture treated with 2% DMSO had an even higher proportion of G1 cells.

Measurements of ^{3}H thymidine incorporation (Figure 5A,B) also indicated that cells treated with both 3% glycerol and 2% DMSO synthesized little DNA. The average value of counts above background for the first four days of treatment with both compounds was 4% or 10% of the peak value for DMSO on day two in two different experiments. ^{3}H thymidine incorporation in cells treated with 1.9% DMSO was low on day 1 and peaked on day 2 as reported.[29] The rate of DNA synthesis of cells treated with 3 % glycerol was slower than that of cells grown in unsupplemented medium on day 1 but higher than the rate for cells treated with 2% DMSO, and remained constant through the second day. On day three, the rate of DNA synthesis decreased for cells in unsupplemented medium or 2% DMSO or 3% glycerol. Since the level of ^{3}H thymidine

Table 5

Cell Cycle Distribution of Treated Cells

Hours post seeding	Medium treatment	Percent G1 Cells: Propidium iodide	Acridine orange	Acridine orange without RNA-ase
0	–	74.2	72.4	
6	control	74.7	69.8	
	2% DMSO	71.6	67.3	
	3% glycerol	68.2	71.4	
	3% glycerol + 2% DMSO	72.8	68.3	
13	control	56.6		
	2% DMSO	64.7		
	3% glycerol + 2% DMSO	62.5		
18	control	42		32.7
	2% DMSO	71		78.8
	3% glycerol	41.6		46.6
	3% glycerol + 2% DMSO			66.8
48	control	43		49.8
	2% DMSO	47		47.1
	3% glycerol + 2% DMSO			88.2
90	control	53		57.3
	2% DMSO	80		85
	3% glycerol + 2% DMSO	64		87.5
118	control	70.5		69
	2% DMSO	87.5		87.2
	3% glycerol	73.7		74.1

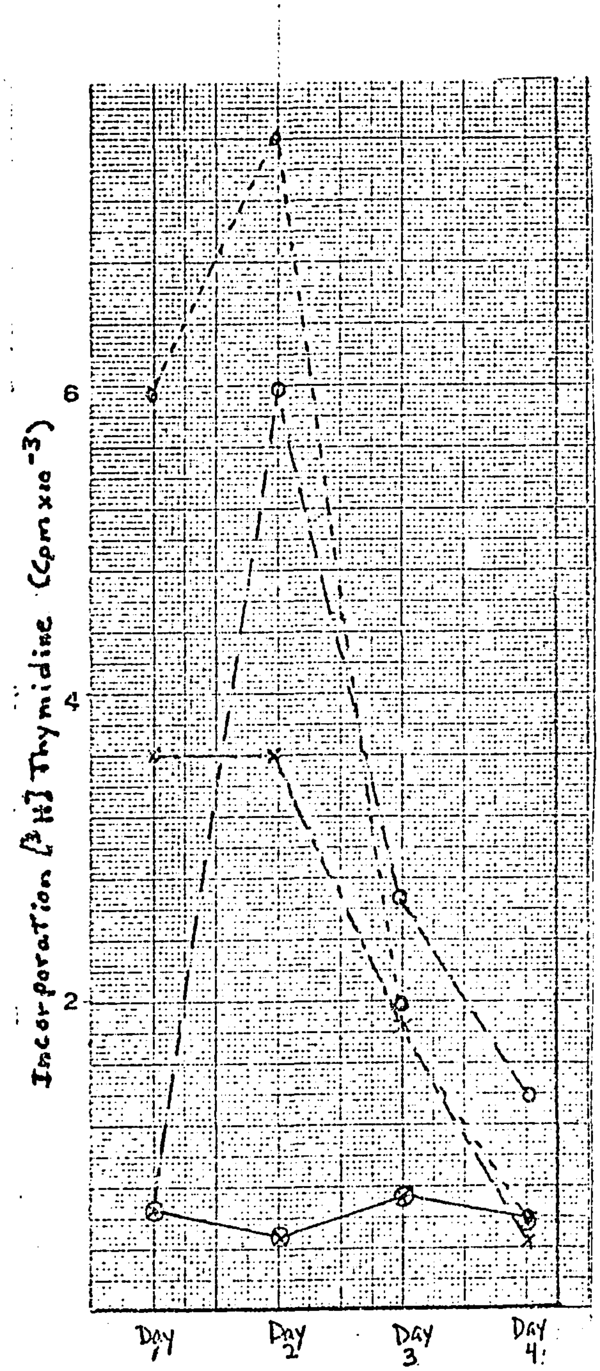
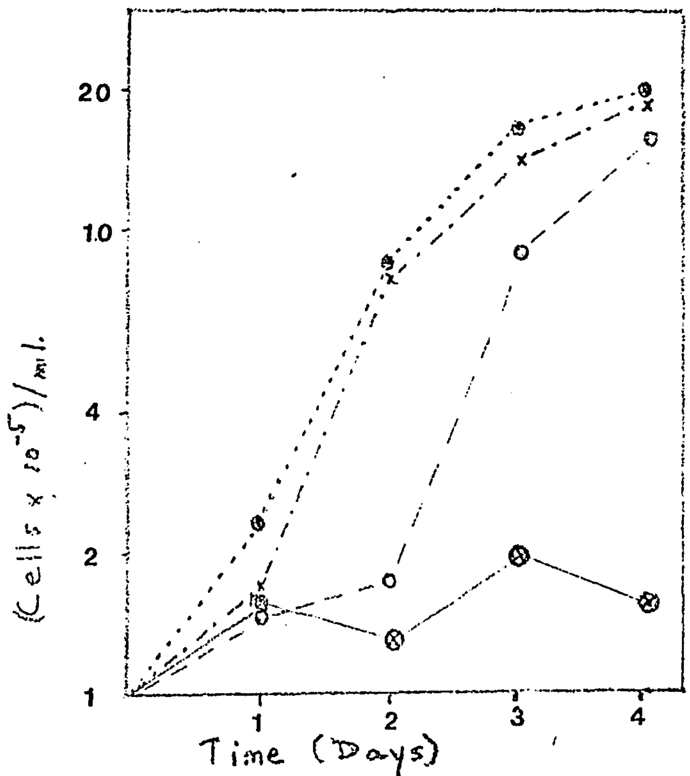


Figure 5A. ^{3}H Thymidine Incorporation of Treated Cells

control
2% DMSO
3% Glycerol
2% DMSO plus 3% Glycerol

5A. Cells were seeded in media as described. Cultures were resuspended at 5×10^{5} cells in 0.5 ml. of fresh, treated medium before testing. After a 20 minute incubation, 0.5 microcurie of ^{3}H Thymidine was added per sample, tubes were gassed and incubated for an additional 40 minutes. Samples were spotted on 3 MM Whatman discs, rinsed as described, and counted in toluene-based scintillation fluid.



5B. Growth of Treated Cells Evaluated for ^{3}H Thymidine Incorporation

Cells were seeded as described and counted in a haemocytometer.

control ⊕
2% DMSO o
3% Glycerol x
2% DMSO plus 3% Glycerol ⊗

incorporation was uniformly low throughout the experiment and the level of G1 cells was high, it is unlikely that the limited increase in cell number during treatment with 3% glycerol and 2% DMSO represented a balance resulting from a combination of growth and cell death rather than limited growth. It should be noted that no dead cells as assayed by the trypan blue exclusion test were observed.

Effect of Treatment with Glycerol Before Transfer to Other Media

Since treatment with glycerol and 2% DMSO at the same time decreased cell growth, the effect of preliminary growth in glycerol was studied. In one experiment (Table 6), the cells were grown in unsupplemented medium, 3.5% glycerol or 4.75% glycerol for three or four days and then transferred to unsupplemented medium, 2% DMSO, 3.5% glycerol or a combination of 3.5% glycerol and 1% DMSO. Cells treated with 4.75% glycerol for three days and then seeded at 1×10^5 cells/ml. in unsupplemented medium or 2% DMSO grew to 7.3 or 4.5×10^5 cells respectively four days later. In contrast, cells grown in unsupplemented medium before transfer grew to 16 or 15×10^5 cells/ml. in unsupplemented medium or medium with 2% DMSO. The percentage of B+ cells observed after treatment with 4.5% glycerol followed by treatment with 2% DMSO was almost 14% lower than the percentage observed when cells were grown in control medium before transfer to 2% DMSO. Three days of exposure of the cells to 3.5% glycerol decreased subsequent growth in 2% DMSO and 1% DMSO plus 3.5% glycerol by 13% and 94% respectively as compared to untreated cultures after four days of growth. The effect on growth rate did not affect the continued differentiation of the cells which increased from day 4 to day 5. (Table 6).

In a second experiment (Tables 7,8), cells were transferred to unsupplemented medium or 2% DMSO after growth in unsupplemented medium

Table 6

Effect of Three or Four Days of Treatment with

Control Medium or 3.5% Glycerol or 4.75 % Glycerol on Response to Subsequent Treatments

Days of treatment	Treatment one	Treatment two	% B+ Cells		Cell Count x 10^{-5}	
			day 4	day 5	day 4	day 5
3	control	control	0	0	16	22
		DMSO*	46.2	59.4	15	11
		glycerol**	0	N.D.	13	N.D.
		D + G***	21.2	30.6	8.7	8.2
	glycerol (3.5%)	control	0	0	12	11
		DMSO	27.9	45.9	13	16
		glycerol	0	N.D.	13	N.D.
		D + G	8.6	32	0.58	0.42
	glycerol (4.75%)	control	0	0	7.3	9.3
		DMSO	32.6	43.9	4.5	4.0
4	control	control	0.3	0	17	17
		DMSO	38.3	65	14	13
		glycerol	1.2	N.D.	12	N.D.
		D + G	17.6	29.6	8.5	9.0
	glycerol (3.5%)	control	0	0	13	18
		DMSO	41.9	61.9	7.4	13
		glycerol	0.5	N.D.	11	N.D.
		D + G	6.3	27.7	0.53	0.78
	glycerol (4.75%)	control	0	0	8.2	8.8
		DMSO	22.7	52.8	3.5	4.1

*DMSO = 2% DMSO
**glycerol = 3.5% glycerol
***D + G = 1.0% DMSO + 3.5% glycerol

Table 7

Effect of Growth in Control Medium or 4% Glycerol for Increasing Amounts of Time on Subsequent Differentiation in 1.9% DMSO

		Percent B+ Cells				
Hours	Medium	Day 1	Day 2	Day 3	Day 4	Day 5
20	control	N.D.	12.9	48.5	66.9	87.2
	glycerol	N.D.	37.4	39.6	65.7	84.2
44	control	0.5	21.2	25.3	71.2	79.8
	glycerol	1.4	34.5	23.1	58.6	80.9
68	control	0.8	15.4	47.8	71.9	84.2
	glycerol	4.1	26.9	20.3	64.2	75.0
92	control	0	7.5	36.9	60.9	89.0
	glycerol	1.7	15.2	42.5	54.0	80.2

Table 8

Effect of Growth in Control Medium or 4% Glycerol for Increasing Amounts of Time on Subsequent Growth in Control Medium or 1.9% DMSO

Hours treatment one	Treatment one	Treatment two	Cells x 10^{-5}/ml. day 1	day 2	day 3	day 4	day 5
20	control	control	N.D.*	11	18	23	15
		DMSO		4.5	7.4	12	16
	glycerol	control		8.4	7.7	12	18
		DMSO		1.5	5.0	13	14
44	control	control	3.1	15	19	18	22
		DMSO	2.4	4.1	12	15	17
	glycerol	control	1.7	3.5	2.3	9	23
		DMSO	2.0	1.4	0.87	6.7	8.6
68	control	control	2.2	8.6	19	11	19
		DMSO	0.98	2.1	4.3	5.7	14
	glycerol	control	1.5	1.9	8.9	6.7	9.2
		DMSO	0.97	0.87	2.2	5.3	9.3
92	control	control	1.2	4.2	13	13	22
		DMSO	1.1	1.1	3.3	9.6	8.2
	glycerol	control	1.3	3.1	8.2	8.4	7.0
		DMSO	0.57	0.55	1.3	4.2	4.7

*Cells were seeded at 1 x 10^5/ml. This group was not counted on day 1.

or 4% glycerol for 20, 44, 68, or 92 hours. Cells grown first in glycerol showed earlier increases in B+ cells, with the highest relative values, 37.4% and 34.5% for cells transferred to DMSO from glycerol one or two days earlier. By the third day, cells which had grown in unsupplemented medium had 8% more B+ cells than cells which had grown in glycerol. Cells grown in glycerol before transfer consistently grew less than cells which had grown in control medium up to day 5 when cells which had been incubated with glycerol for 20 and 44 hours caught up to the control cells.

The values for cell growth and differentiation after transfer from unsupplemented medium from the experiment above and the values from two additional experiments in which 1.9% DMSO rather than 2% DMSO was used were averaged. Cell counts for cultures grown in unsupplemented medium, 3.5% glycerol, 1.9-2.0% DMSO, or 3.5% glycerol plus 1.9-2.0% DMSO were 16.6, 14, 13, or 8.5 x 10^5 cells/ml. respectively and the percentages of B+ cells were 0.4, 2.1, 50.5, or 37.5 respectively.

Response of Lines H 1.9 and 5-86 to DMSO, HMBA, and Butyric Acid

The DMSO-resistant line H 1.9 was developed in this laboratory by prolonged culture of line 5-86 in DMSO. It showed a slightly longer growth lag than line 5-86 when treated with HMBA or DMSO and a similar growth pattern to line 5-86 during treatment with butyric acid (Figure 6A, B,C,D). In contrast to the growth pattern in response to these inducers, line H 1.9 developed no increase in B+ cells in response to treatment with 2% DMSO and 2% B+ cells on day three after treatment with HMBA although no B+ cells were apparent on day 4. Line 5-86 developed 34% and 51% B+ cells after four days of treatment with DMSO or HMBA respectively in the same experiment. Treatment with butyric acid induced 10% B+ cells in line H 1.9 and 47% B+ cells in line 5-86

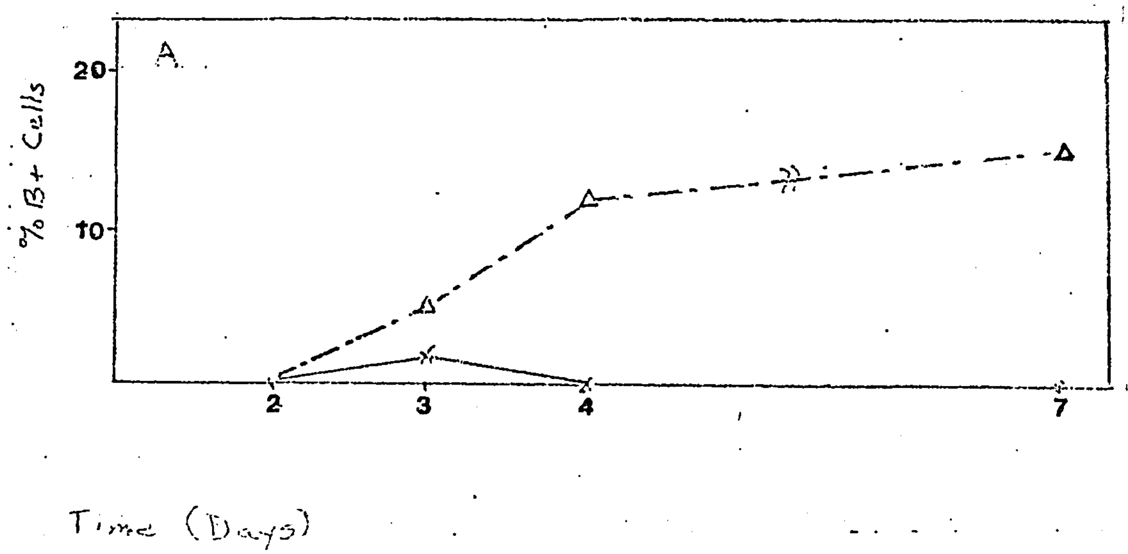


Figure 6A. Differentiation of line H 1.9

1.9% DMSO O 5 mM HMBA × 2 mM Butyric acid △

Cells were seeded at 1×10^5 cells/ml. Samples of 0.1 ml. were mixed with 0.05 ml. of benzidine-peroxide reagent described in materials and methods and counted in a haemocytometer.

Line H 1.9 had less than 2% B+ cells after growth in 1.9% DMSO or unsupplemented medium.

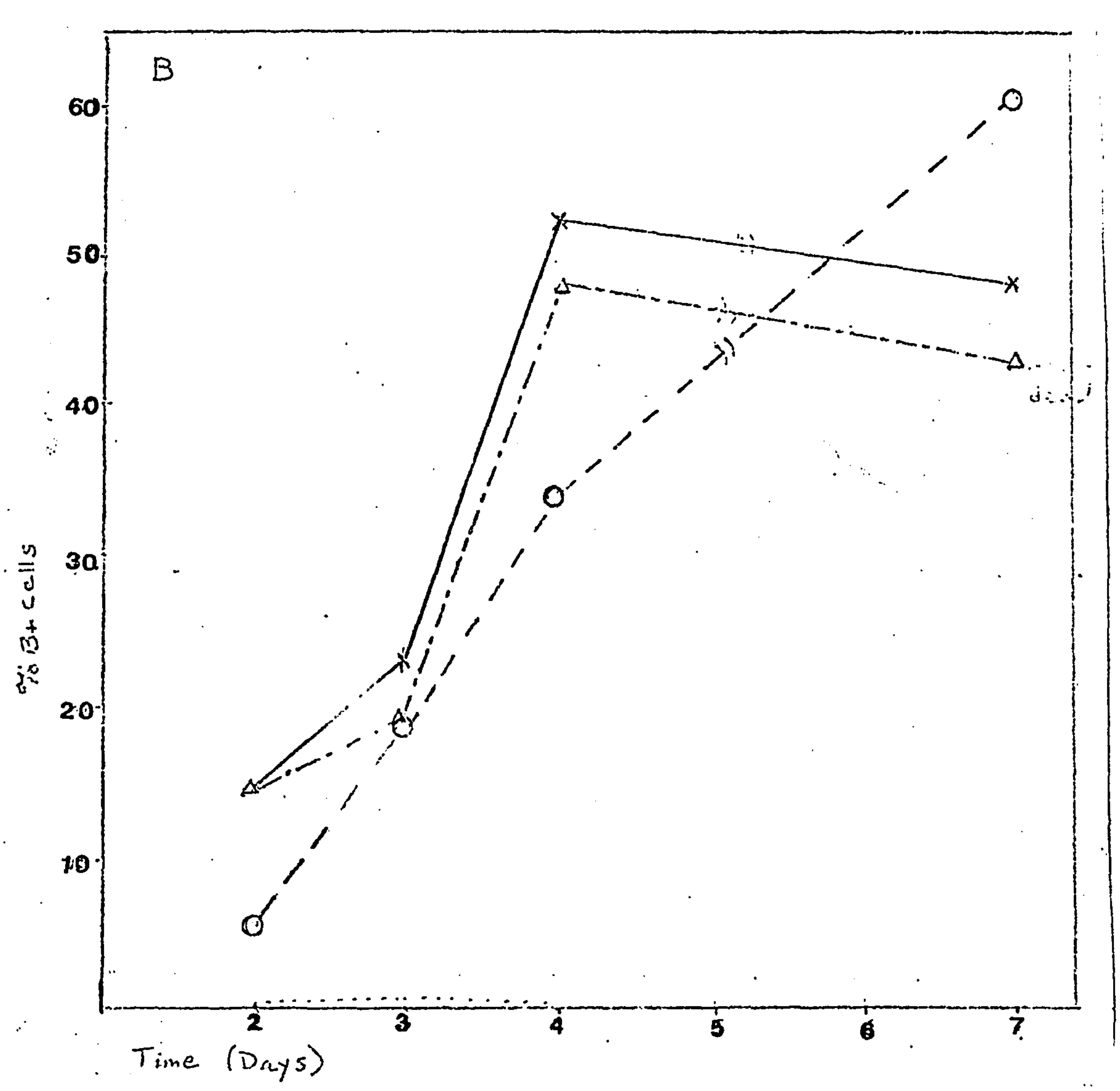


Figure 6B. Differentiation of line 5-86

1.9% DMSO ○ 5 mM HMBA × 2 mM Butyric acid △

Line 5-86 had less than 2% B+ cells when grown in unsupplemented medium. Cells were seeded at 1 x 10^5 cells/ml. and evaluated as in 6A.

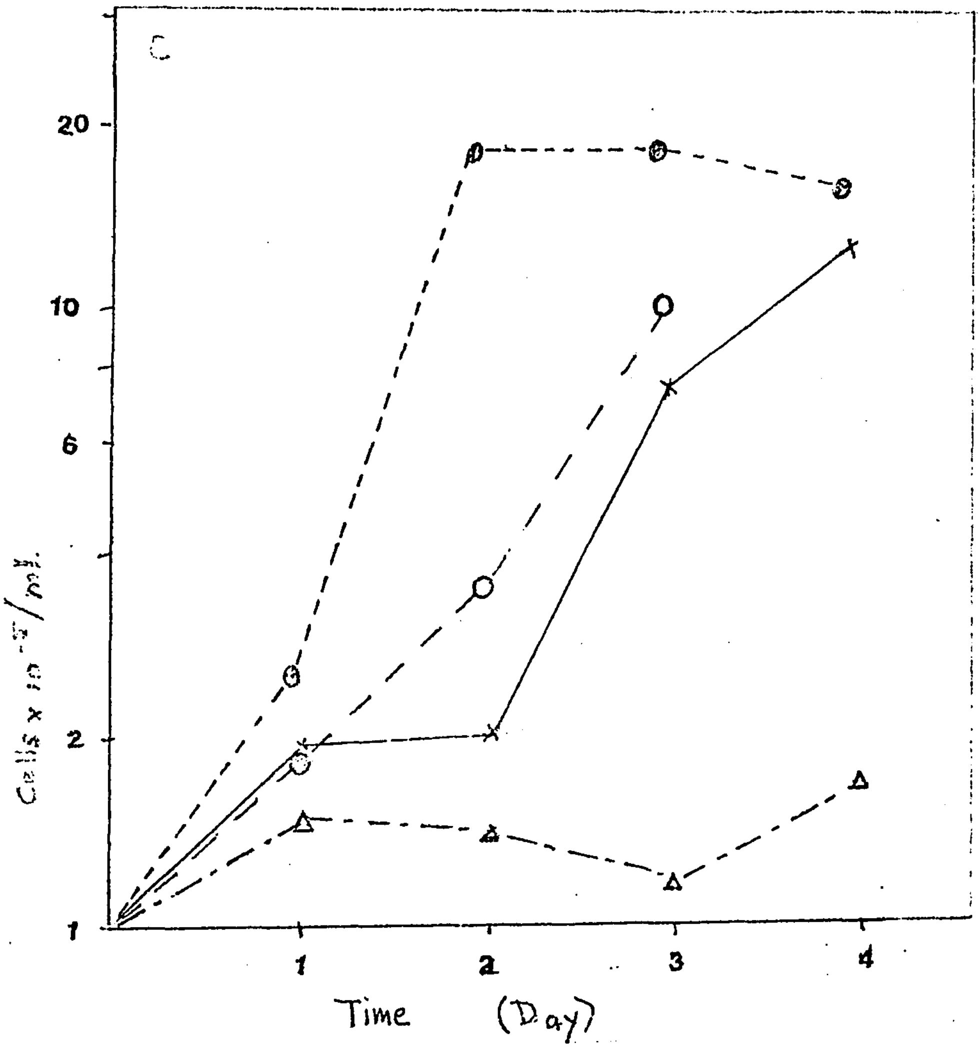


Figure 6C. Growth of line H 1.9 in DMSO, HMBA, and Butyric acid

control ⊘ 1.9% DMSO ○ 5 mM HMBA × 2 mM Butyric acid △

Cells were seeded at 1×10^5 cells/ml. and counted in a haemocytometer.

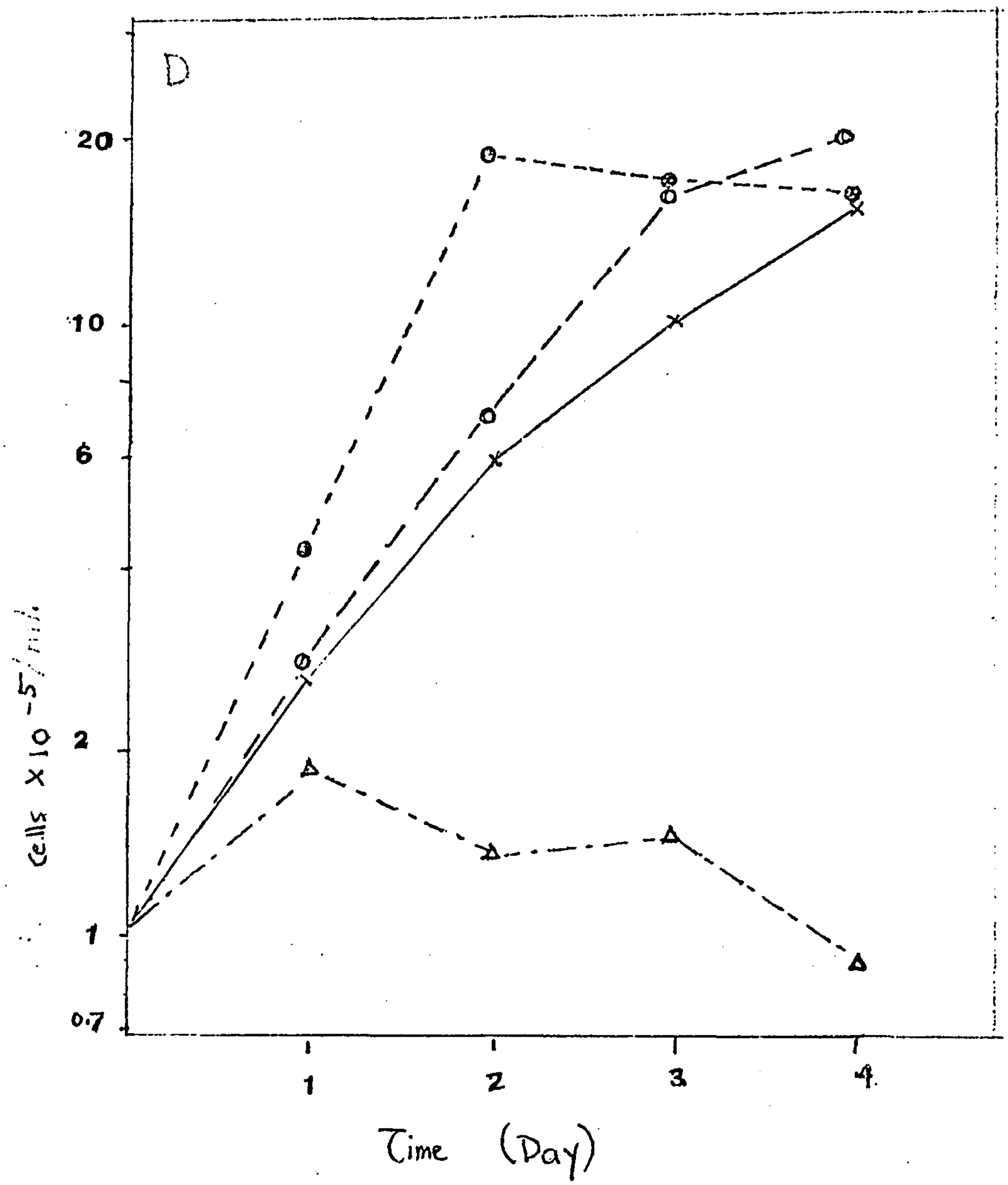


Figure 6D. Growth of line 5-86 in DMSO, HMBA, and Butyric acid

control ○ 1.9% DMSO ○ 5 mM HMBA × 2 mM Butyric acid △

Cells were seeded at 1 x 10^5 cells/ml. and counted in a haemocytometer.

(Figure 6). Comparison of the two lines and their response to DMSO in RPMI and BME medium showed a slight increase in spontaneous differentiation in RPMI medium (Table 9) for both cell lines but line H 1.9 did not respond to DMSO after five days of treatment in either medium.

Effect of Continued Transfer to Fresh Treated Medium

Growth in the presence of sub-optimal amounts of HMBA had been found to lead to a linear increase in the percentage of differentiating cells.[41] Furthermore, the model suggested by Gusella *et al.*[26] involved the cumulative commitment of a percentage of the uncommitted cells with succeeding cell cycles. Since the percentage of differentiating cells decreased with increased seeding density, a series of experiments was done in which the cell number was adjusted every other day to 1×10^5 cells/ml. so that growth was not limited by lack of some factor in fresh medium.

In an initial study with line 5-86, the highest percentage (91%) of differentiated cells after treatment with 1.9% DMSO was reached at day 10, although there was little difference between days 6-10 (Figure 7A,B). When the cells were treated with 0.95% DMSO, the percentage of B+ cells increased more slowly and peaked at a value almost 10% less than that for cells treated with 1.9% DMSO. In spite of the fact that the cells were transferred to fresh DMSO-supplemented medium, the cells treated with 1.9% DMSO did not double in number between day 8 and 10 and the experiment was discontinued. The data suggest that cultures treated with 1.9% DMSO become terminally differentiated earlier than cultures treated with 0.95% DMSO.

In this experiment, some cells were kept for four days without changing the medium. If the medium was not changed, the percentage of

Table 9

Growth and Differentiation of Lines 5-86 and H1.9 Compared

After Five Days in BME and RPMI Medium

Cell line	Treatment	BME medium % B+	BME medium Count	RPMI medium % B+	RPMI medium Count
5/86	control	0.8	1.8×10^6	6.4	1.6×10^6
	DMSO	72.7	1.35×10^6	79.1	1.0×10^6
H1.9	control	0	2.0×10^6	2.4	9.2×10^5
	DMSO	0	1.6×10^6	2.7	5.4×10^5

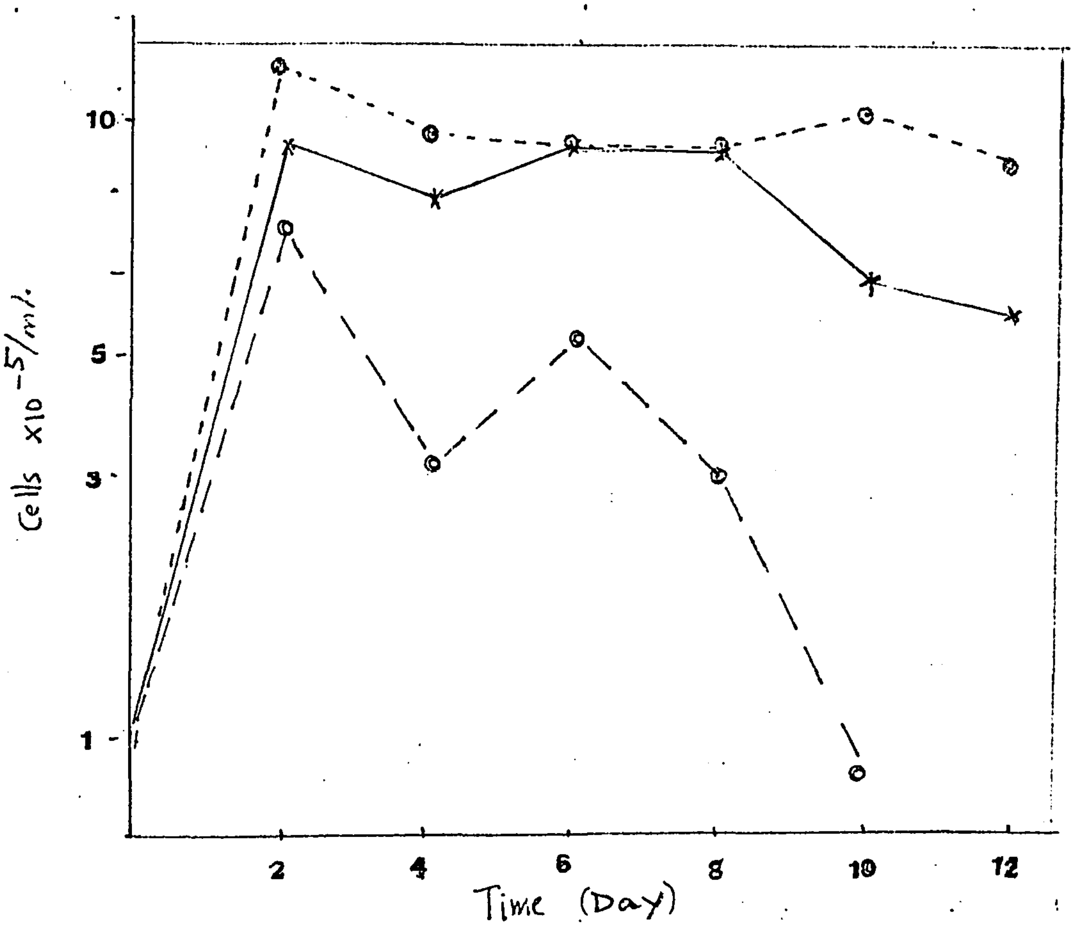


Figure 7A. Growth of line 5-86 passed every second day in 1.9% DMSO, 0.95% DMSO or unsupplemented medium at 1 x 10^5 cells/ml.

control ⊙ 1.9% DMSO O 0.95% DMSO X

Cells were seeded at 1 x 10^5 cells/ml. and counted in a haemocytometer.

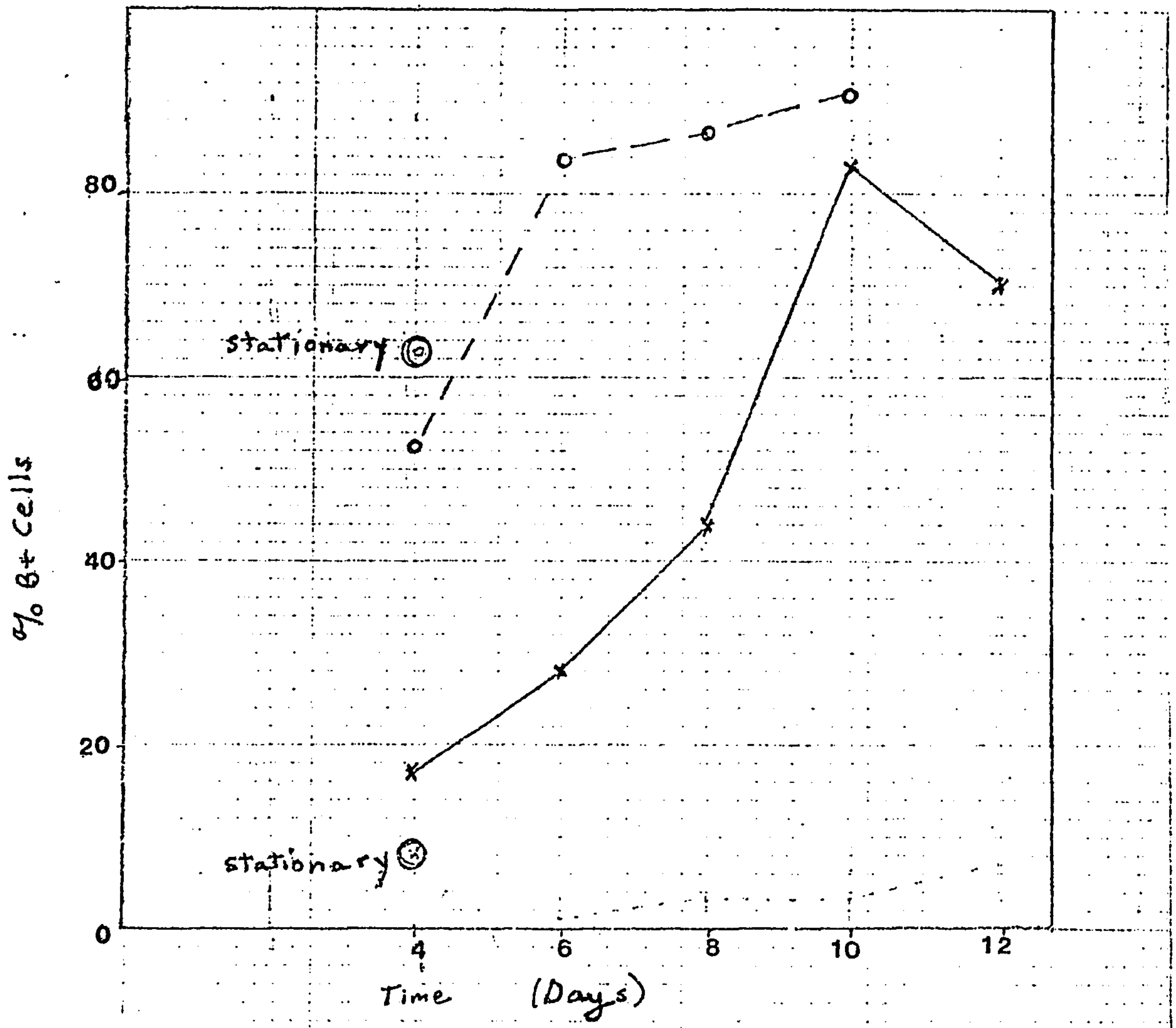


Figure 7B. Differentiation of line 5-86 passed every second day in

1.9% DMSO ○ 0.95% DMSO ×

. Cells were seeded at 1 x 10^5 cells/ml. and evaluated as in 6A.

B+ cells after treatment with 1.9% DMSO was approximately 10% higher than if the medium was changed. In contrast, transferring the cells on day two, when the cells were treated with 0.95% DMSO, doubled the percentage of differentiating cells from 8% to 16%.

In a separate experiment, 3% of cells treated with 0.95% DMSO for four days produced hemoglobin and 25% of the cells produced hemoglobin when the cells were resuspended in fresh medium on day two. Differentiation was the same if the fresh medium had been incubated for two days side by side with the cells or was made up just before use (Table 10).

In a second experiment (Figure 8, B and D), the cells treated with 1.9% DMSO failed to double between days 6 and 8. On day 6, 85% of the cells were B+. The highest value, 90% was observed on day 8. There was a gradual decline in percentage of differentiated cells until day 12 followed by a sharper decrease at that point. Increasing numbers of dead cells were observed after day 8, and the total number of cells decreased between days 10 and 12.

Cells treated with 3.5G D/2 also reached their maximum percentage of differentiated cells on day 8 although the shape of the curve suggests that differentiation appeared later and ended earlier in these cells. There was a sharp decrease in growth between days 6 and 8 followed by a slight recovery between days 8 and 10 and by a loss of cells between days 10 and 12. The decline in the percentage of differentiated cells followed the loss of cells.

Cells treated with 0.95% DMSO showed the same general pattern of a decrease in growth between days 6 and 8 followed by a slight recovery and a second decrease between days 8 and 10 but the decreases were not as sharp and at 10 days the count was 2.5×10^5 compared with less than 1×10^5 for the other treatments already discussed. The peak

Table 10

Effect of Growth Conditions on Response to DMSO

Treatment*	% B+ Cells day 4	day 7	day 8	Cell Count x 10^{-5} day 4	day 7	day 8
control stationary	N.D.	1.4	N.D.	N.D.	17	18
control transferred day 2	0.9	0.7	N.D.	8.7	N.D.	N.D.
control transferred day 3	N.D.	N.D.	N.D.	N.D.	20	N.D.
0.95% DMSO stationary	2.9	5.1	N.D.	14	19	N.D.
0.95% DMSO transferred day 2 to refrigerated medium (A)	26	42	59	6.1	16	19
0.95% DMSO transferred day 2 to incubated medium (B)	24.1	45	N.D.	7.4	13	N.D.
A transferred day 4 to refrigerated medium	–	40.7	63	N.D.	18	13
B transferred day 4 to refrigerated medium	–	46.5	N.D.	N.D.	13	N.D.
1.9% DMSO stationary	37.4	51.7	60	10	12	14
1.9% DMSO transferred on day 3 to refrigerated medium	–	81.5	94	–	5.1	5.8
0.95% DMSO seeded 2 x 10^4	21.8	30.3	–	13	15	N.D.
1.9% DMSO seeded 2 x 10^4	43.9	88.2	99	2.1	7.7	6.8

value, 78% B+ cells, was reached on day 6. The increase in cell growth between days 12 and 16 was accompanied by a decrease in B+ cells and a decrease in cell growth between days 16 and 18 was accompanied by a further decrease in differentiated cells. Cells treated with 3.5% glycerol alone showed slight fluctuations in growth, for the most part parallel to those of cells treated with 0.95% DMSO, and no more than background numbers of B+ cells.

Cells of line H 1.9 were treated identically to the cells of line 5-86 (Figure 8 A and C). These cells treated with 1.9 DMSO or 0.95% DMSO plus 3.5% glycerol showed a growth decrease between days 4 and 6 followed by a recovery between days 6 and 8 followed by a second decrease between days 8 and 12. The maximum percentages of B+ cells were reached on day 10 when 22% of the cells treated with 1.9% DMSO were B+ and 18% of the cells treated with 0.95% DMSO and 3.5% glycerol were B+. Cells treated with 0.95% DMSO alone had two peaks of B+ cells at 8 and 14 days where approximately 6% of the cells were B+. Cells treated with this concentration of DMSO or with 3.5% glycerol showed very slight changes in growth pattern which seemed to follow the pattern for 1.9% DMSO.

In a third experiment (Figure 9A,B,C,D), cells of line 5-86 and line H 1.9 were transferred at a level of 5×10^4 cells/ml. on day two and allowed to grow for three days before the next transfer. On day five there were consistently more cells in all the treated cultures of line H 1.9. Peak values for benzidine positive cells for line 5-86 were 94% for cells treated with 1.9% DMSO on day 7, 88% for cells treated with 0.95% DMSO and 3.5% glycerol on day 9, and 79% for cells treated with 0.95% DMSO alone also on day 9. Peak values for benzidine positive cells for line H 1.9 were 32% B+ on day 9 for cells treated

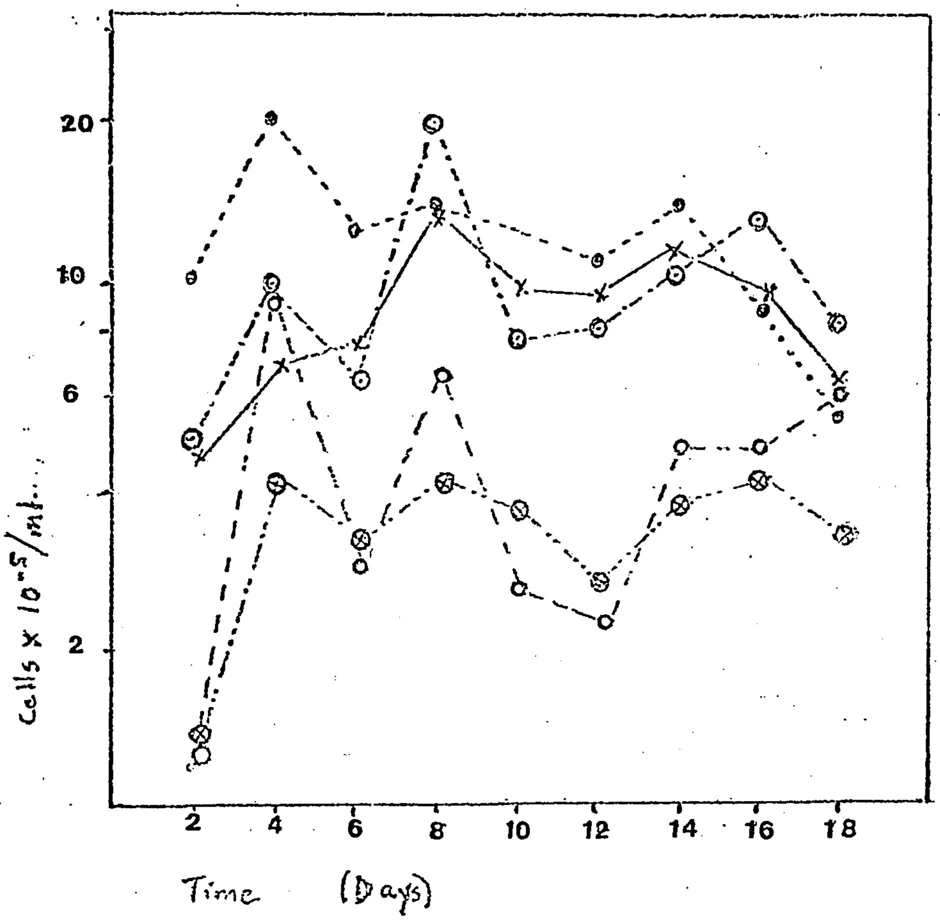


Figure 8A. Growth of line H 1.9 when cells were transferred every other day.

control • 1.9% DMSO ○ 0.95% DMSO plus 3.5% glycerol ⊗

0.95% DMSO ⊙ 3.5 glycerol ×

Cells were seeded as indicated and counted using a haemocytometer.

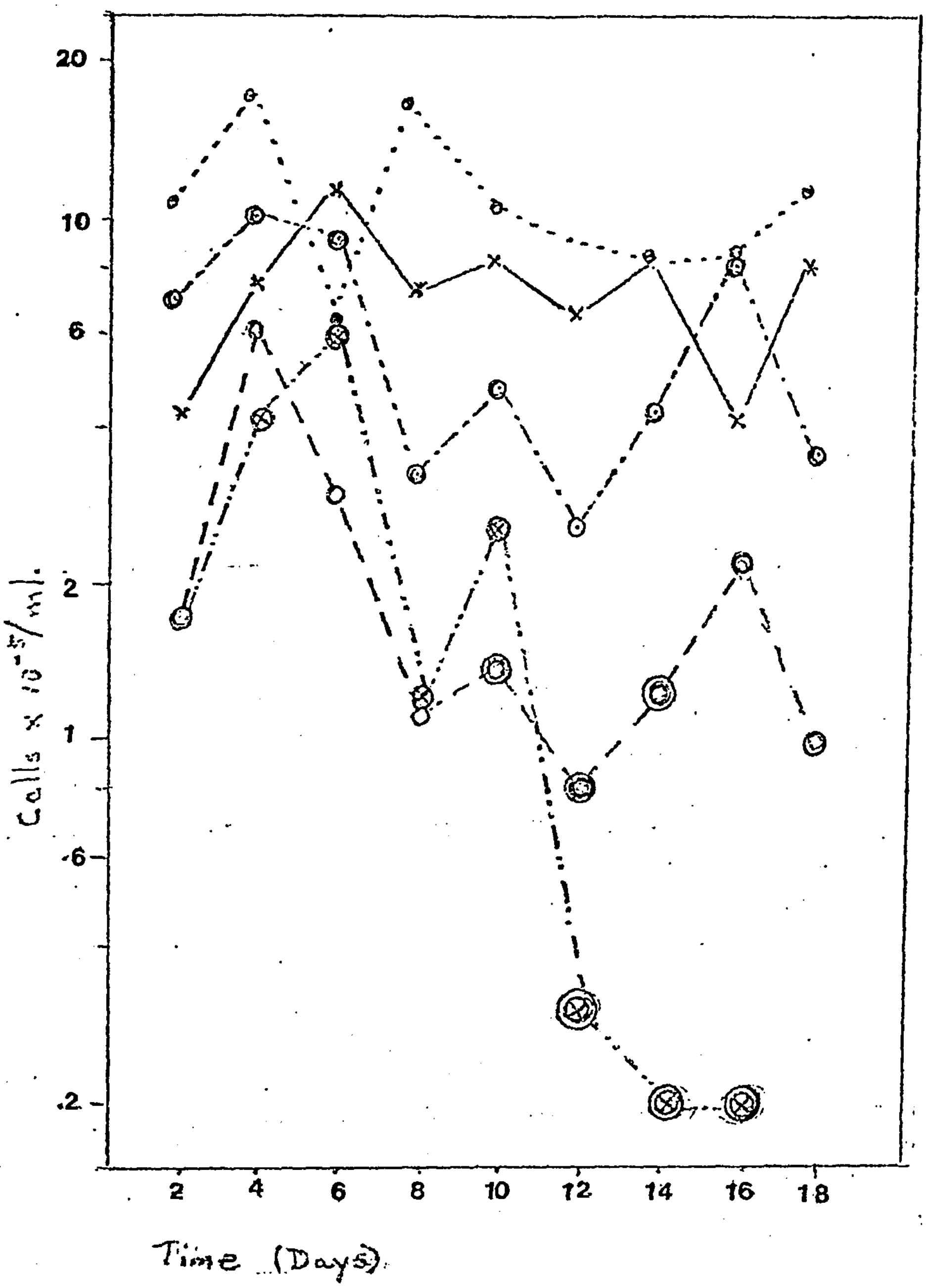


Figure 8B. Growth of line 5-86 when cells were transferred every other day.

control ○ 1.9% DMSO ○ 0.95% DMSO plus 3.5% glycerol ⊗

0.95% DMSO ⊙ 3.5% glycerol ×

Circled symbol indicates cells were not transferred.

Cells were seeded as indicated and counted using a haemocytometer.

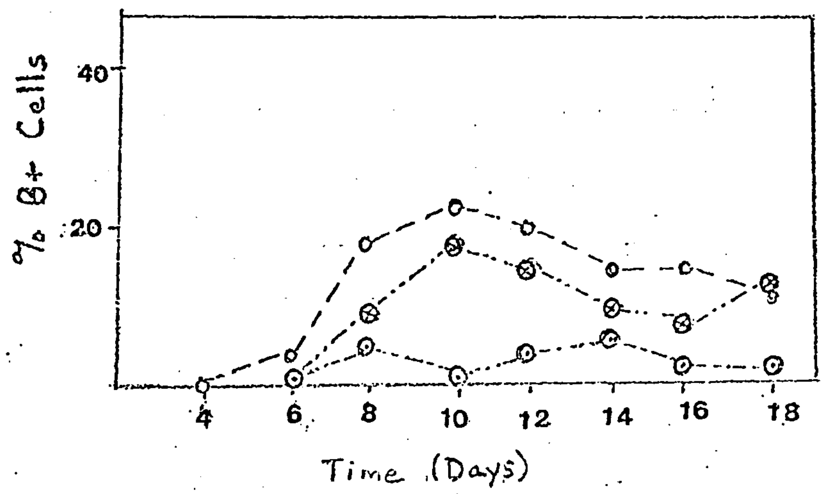


Figure 8 C. Differentiation of line H 1.9 when cells were transferred every other day

⊖ 1.9% DMSO ○ 0.95% DMSO plus 3.5% glycerol ⊗ 0.95% DMSO ⊙

Cells were seeded at 1 x 10^5 cells/ml. Samples of 0.1 ml. were mixed with 0.05 ml. of benzidine-peroxide reagent described in materials and methods and counted in a haemocytometer.

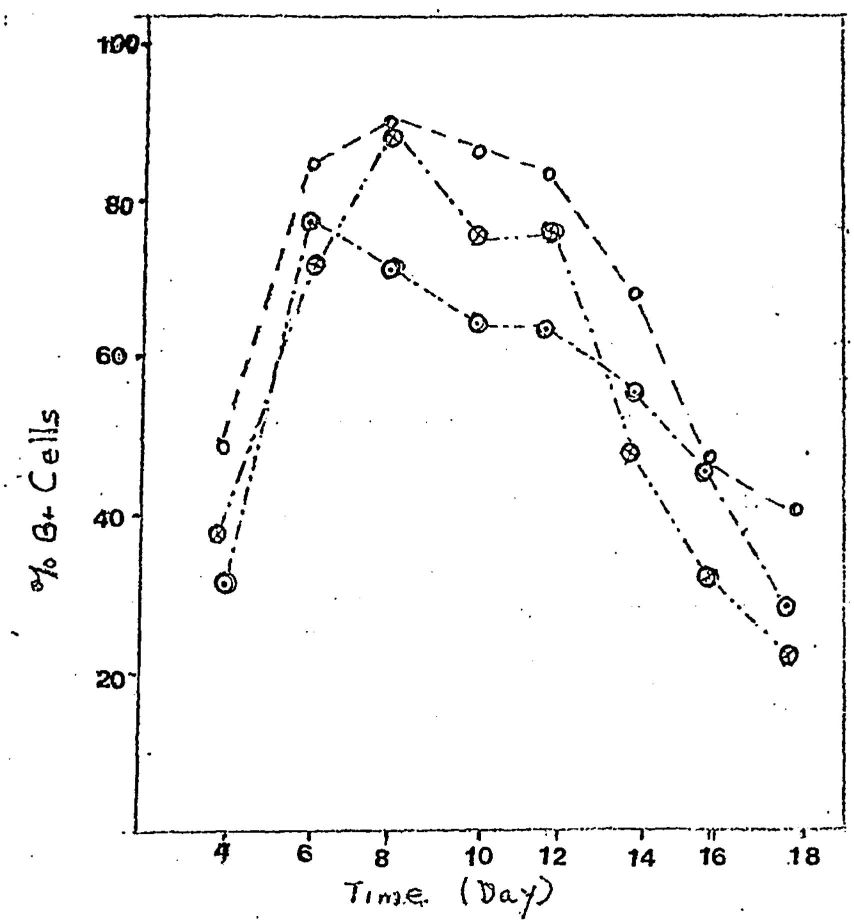


Figure 8 D. Differentiation of line 5-86 when cells were transferred every other day

1.9% DMSO ○ 0.95% DMSO plus 3.5% glycerol ⊗ 0.95% DMSO ⊙

Cells were seeded at 1 x 10^5 cells/ml. Samples of 0.1 ml. were mixed with 0.05 ml. of benzidine-peroxide reagent described in materials and methods and counted in a haemocytometer.

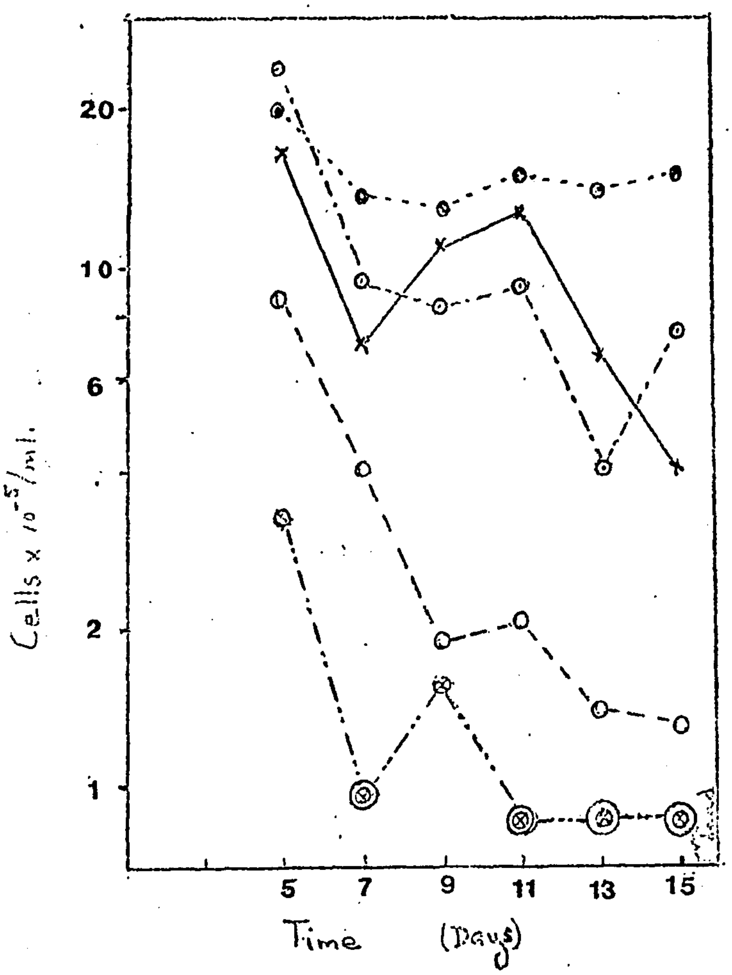


Figure 9A. Growth of line H 1.9 when cells were transferred every other day beginning on day 5 (transferred at 5 x 10^4/ml. on day 2). control ∘ 1.9% DMSO ○ 0.95% DMSO plus 3.5% glycerol ⊗ 0.95% DMSO ⊙ 3.5% glycerol x

Circled symbol indicates cells were not transferred.

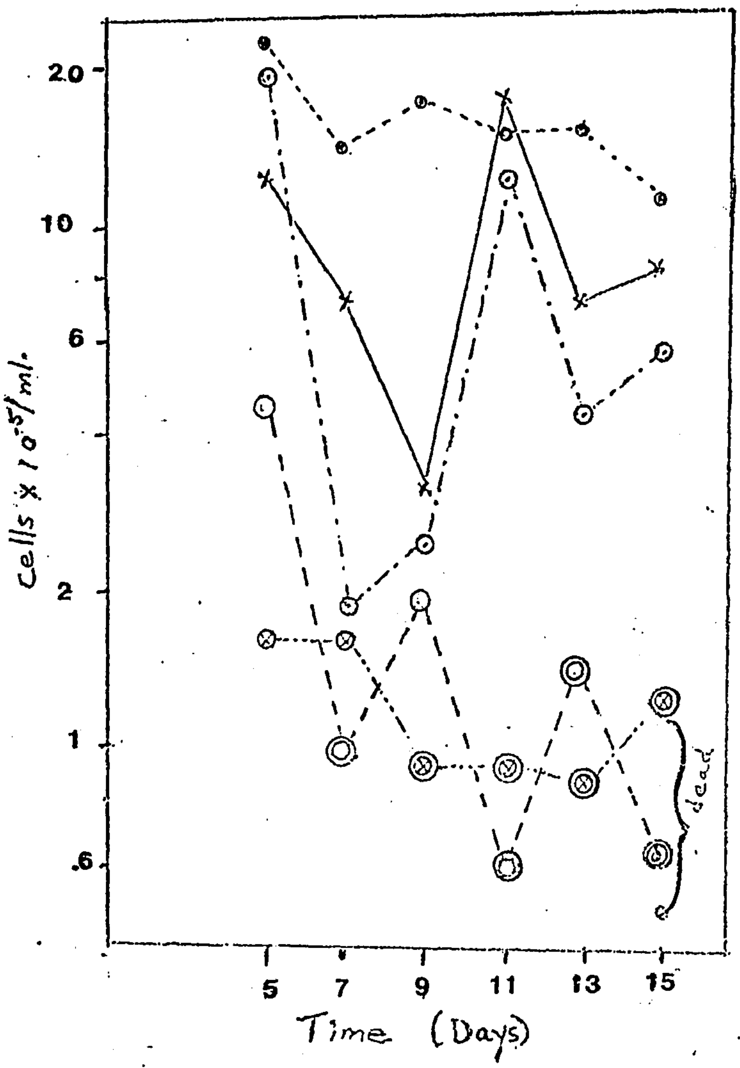


Figure 9B. Growth of line 5-86 when cells were transferred every other day beginning on day 5 (transferred at 5 x 10^4/ml. on day 2). control ⊙ 1.9% DMSO ○ 0.95% DMSO plus 3.5% glycerol ⊗ 0.95% DMSO ⊙ 3.5% glycerol × Circled symbol indicates cells were not transferred.

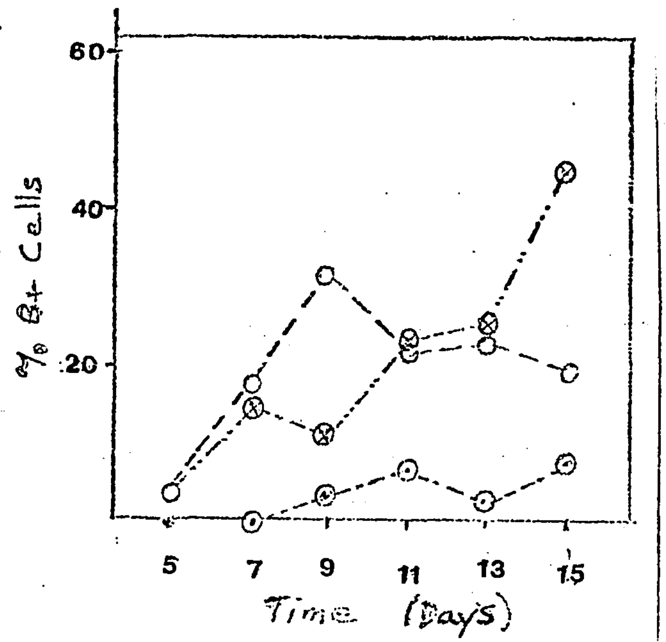


Figure 9C. Differentiation of line H 1.9 when cells were transferred every other day beginning on day 5 (transferred at 5×10^4/ml. on day 2).

1.9% DMSO ○ 0.95% DMSO plus 3.5% glycerol ⊗

0.95% DMSO ⊙

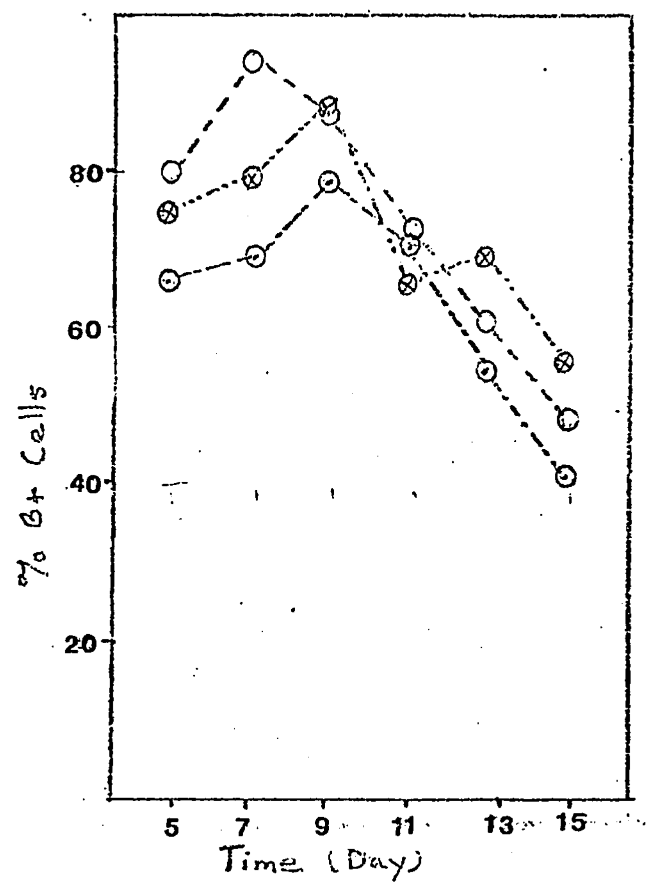


Figure 9D. Differentiation of line 5-86 when cells were transferred every other day beginning on day 5 (transferred at 5×10^4/ml. on day 2).

1.9% DMSO ○ 0.95% DMSO plus 3.5% glycerol ⊗

0.95% DMSO ⊙

with 1.9% DMSO, 44% for cells treated with 0.95% DMSO and 3.5% glycerol on day 15 (which had remained in the same medium since day 7 because they didn't grow), and two peaks, on days 11 and 15 of approximately 6% B+ cells for cells treated with 0.95% DMSO alone.

Line 5-86 cells treated with 1.9% DMSO did not grow between days five and seven, grew a little during the following two days in the same medium, lost cells after a transfer on day nine, recovered slightly after remaining in the same medium and then lost cells again. Cells treated with 3.5% glycerol plus 0.95% DMSO doubled only once between days two and five and days five and seven and remained stationary in the same medium from day 7 to day 15. On day 15 roughly half the cells were dead by the criterion of trypan blue positivity. Cells treated with 0.95% DMSO alone did not double between days five and seven, recovered to almost control rate by day 11 and then grew more slowly between day 11 and 15. Cells treated with 3.5% glycerol alone showed the same pattern as the cells treated with the lower concentration of DMSO but with much less decrease in growth rate between days five and seven.

Line H 1.9 cells counted on day 5 after treatment with 1.9% DMSO had twice as many cells as line 5-86 cells treated the same way. Their growth rate decreased more slowly reaching a low point of close to two doublings in 48 hours between days 7 and 9 and then decreased again to less than 1.5 doublings during the intervals ending on days 13 and 15. Cells treated with 0.95% DMSO plus 3.5% glycerol also had approximately twice as many cells as identically treated line 5-86 cells on day 5. However, they did not grow between days 5 and 7, grew less than two doublings when kept in the same medium, and then remained stationary in the same medium between days 9 and 15. Line H 1.9 cells treated with

0.95% DMSO or 3.5% glycerol alone grew very similarly with a decrease in growth rate between days five and seven followed by a recovery to near control rates for glycerol treated cells and a plateau for 0.95% DMSO- treated cells followed by a sharp decrease in growth rate between days 11 and 13 for both groups, followed by a slight recovery for the DMSO treated cells and a continued decline for the glycerol treated cells.

Effect of Cloning Line H 1.9 in 1.9% DMSO

Since line H 1.9 was induced to produce B+ cells after prolonged growth in the presence of DMSO or DMSO plus glycerol, the heterogeniety of the response was examined in a cloning experiment.

In contrast to line 5-86, line H 1.9 can be cloned in control medium or in 1.9% DMSO with similar efficiency. Cells cloned at a density of two cells per 5 wells produced 20 clones/96 wells in DMSO and 24 clones/96 wells in control medium. Line 5-86 cells cloned the same way produced 22 clones/84 wells in control medium and 0 cells in 96 wells in DMSO even when cloned at 20 cells/well. On day 8 after cloning line H 1.9 in DMSO, aliquats from each well which had a colony were transferred to fresh medium and the remaining cells were tested for hemoglobin. Four clones out of 16 showed some B+ cells. The percentage of differentiated cells in these clones ranged from 0.4% to 36%. Three days later, 13 clones out of 14 showed some B+ cells and two of the clones had failed to grow after transfer.(Table 11). The percentage of B+ cells ranged from 5% to 67% and the percentage of B+ cells which would result if the clones were pooled was 16%, a reflection of the percentage of B+ cells which would have been found in treated uncloned cultures of line H 1.9. After 30 days of treatment with DMSO,only four clones survived.

Table 11

Differentiation and Growth of Clones of H 1.9 Grown in DMSO

	Day 11		Day 20		Day 26	
Clone**	% B+	N*	% B+	N*	% B+	N*
1	-					
2	52.6	19	-			
3	5.0	>1,000	0	207	0	52
4	9.75	>1,000	0	148	1.4	37
5	39	300-400	-			
6	4.75	400	0.9	223	0	25
7	32.1	28	10	128	1.8	24
8	21.3	400	-			
9	-					
10	44.7	300	-			
11	0	158	2.3	214	0	27
12	10.3	400	-			
13	10	40	-			
14	17	500	8.4	202	0	36
15	67	6	-			
16	20	10	-			

**Clones were numbered on day 8

*N = number of cells observed after 0.1 ml. of medium was transferred to another linbro well and liquified benzidine reagent was added to what was left in the well. Wells were seeded with .18 ml.

Studies on the Relationship Between Tumorigenicity and Differentiation

A. The effect of treating line 5-86 cells with DMSO, glycerol and combinations of the two before innoculation into syngeneic mice.

The results of the first two experiments evaluating tumorigenicity of line 5-86 cells which had been treated with DMSO, glycerol or combinations of the two have been pooled (Table 12). The DBA/2J male mice injected with 5×10^5 control cells died an average of 35.7 ± 2.5 days later and the survival of all the treated groups except for the mice injected with cells treated with 3% glycerol and 1.9% DMSO was significantly different from that value. Mice injected with cells which had been incubated with 3.5% glycerol or 0.95% DMSO survived 48.9 ± 5.9 and 49 ± 2.5 days respectively. The closeness of these values is not too surprising since cells incubated with 0.95% DMSO for four days without a change into fresh treated medium have a very low level of differentiated cells. The animals which survived the longest were those innoculated with cells which had been incubated in 1.9% DMSO or 3.5% glycerol plus 0.95% DMSO which lived 54 ± 4.4 and 67.3 ± 4.9 days respectively. Tumor incidence was the same (9/10 or 10/10) for all groups except mice innoculated with cells treated with 1.9% DMSO which caused tumors in five out of 10 mice.

In a third experiment (Table 13), female mice were used and the results were similar but not statistically significant. The mice injected with cells grown in unsupplemented medium died 44.2 ± 5.3 days after innoculation and, although the survival of mice innoculated with cells incubated with 0.95% or 1.9% DMSO was close to the times observed in the first two experiments, (45.2 ± 3.8 days and 54 ± 10.2 days respectively in this experiment) these values were not significantly

Table 12

Bioassay of Cells Grown for Four Days in Control Medium or DMSO or Glycerol or a Combination of DMSO and Glycerol.

In both experiments DBA/2J male mice were innoculated subcutaneously with 5×10^5 cells/mouse

Medium treatment	Experiment 1		Experiment 2		Experiments 1 + 2 pooled	
	# mice w. tumor/total	mean days survival	# mice w. tumor/total	mean days survival	# mice w. tumor/total	mean days survival
Control	5/5	34.4 ± 4.4	4/5	37.3 ± 3.0	9/10	35.7 ± 2.5
1.9% DMSO	3/5	55.0 ± 5.3	2/5	52.5 ± 13.4	5/10**	54.0 ± 4.4*
3.5% Glycerol	4/5	46.3 ± 2.8	5/5	51.0 ± 11.4	9/10	48.9 ± 5.9*
0.95% DMSO	5/5	50.0 ± 3.4	5/5	48.0 ± 4.4	10/10	49.0 ± 2.5*
3.5% Glycerol + 0.95% DMSO	5/5	61.8 ± 4.8	4/5	74.3 ± 9.6	9/10	67.3 ± 4.9
3% Glycerol + 1.9% DMSO	5/5	47.6 ± 8.1	5/5	46.0 ± 4.4	10/10	46.8 ± 4.1

**$p \leq .01$ *$p \leq .05$

Table 13

Bioassay of Cells Grown for Four Days in Control Medium or DMSO or Glycerol or a Combination of DMSO and Glycerol 5×10^5 Cells/Mouse Innoculated Subcutaneously in DBA/2J Female Mice

medium treatment	# mice w. tumor/total	mean days survival
control	5/5	44.2 ± 5.3
1.9% DMSO	3/5	54 ± 10.2
3.5% Glycerol	5/5*	55.2 - 9.7
	3/5	64.7 ± 14.3
0.95% DMSO	5.5	45.2 3.8
3.5% + Glycerol 0.95% DMSO	4/5*	53.0 ± 5.8
3.0% Glycerol+	3/5	56.3 ± 6.5
1.9% DMSO	1/5	66.0

*Indicates one or more mice died with red lungs. Second calculation for group omits these mice.

different from the control value in this experiment. Mice treated with cells which had been incubated in 3.5% glycerol survived 64.7 ± 14.3 days, a value subject to question because of its large standard error. Another inconsistent finding was that only one mouse died of the five innoculated with cells incubated in 1.9% DMSO plus 3% glycerol and it lived for 66 days. Two mice died of pneumonia in the group innoculated with cells incubated in 3.5% glycerol and one mouse died of pneumonia in the group innoculated with cells incubated in 3.5% glycerol and 0.95% DMSO. These mice were omitted from the calculations. Treatment of the cells with 1.9% DMSO before innoculation resulted in decreased tumor incidence as in the first two experiments. Three of the five mice injected with cells grown in 1.9% DMSO developed tumors compared to 100% tumor incidence when the cells had grown in unsupplemented medium.

B. Comparison of tumorigenicity of cells of four cell lines differing in virus production and response to DMSO after growth in medium with or without the addition of DMSO.

Differentiation in Response to DMSO and Virus Production of Lines 5-86, H 1.9, 707R and F4-1

When the cells of lines 5-86, H 1.9, 707R and F4-1 were set up in preparation for the first experiment in which their tumorigenicity was compared, additional cultures were set up to allow daily evaluation of cell number, percent of B+ cells, and reverse transcriptase and viral antigens in the medium. These observations established the properties of these cell lines at the time and under the conditions in which they were grown before innoculation. The results follow:

Cell growth: (Fig. 10A)

Over the 6-day period of the experiment, the growth patterns

LEGENDS TO FIGURES 10A-E

Figure 10A. Comparison of growth of line 5-86, H 1.9, 707 R, and F4-1. Cells were seeded at 1 x 10^5 cells/ml. in DMSO or unsupplemented medium as described in materials and methods. control DMSO

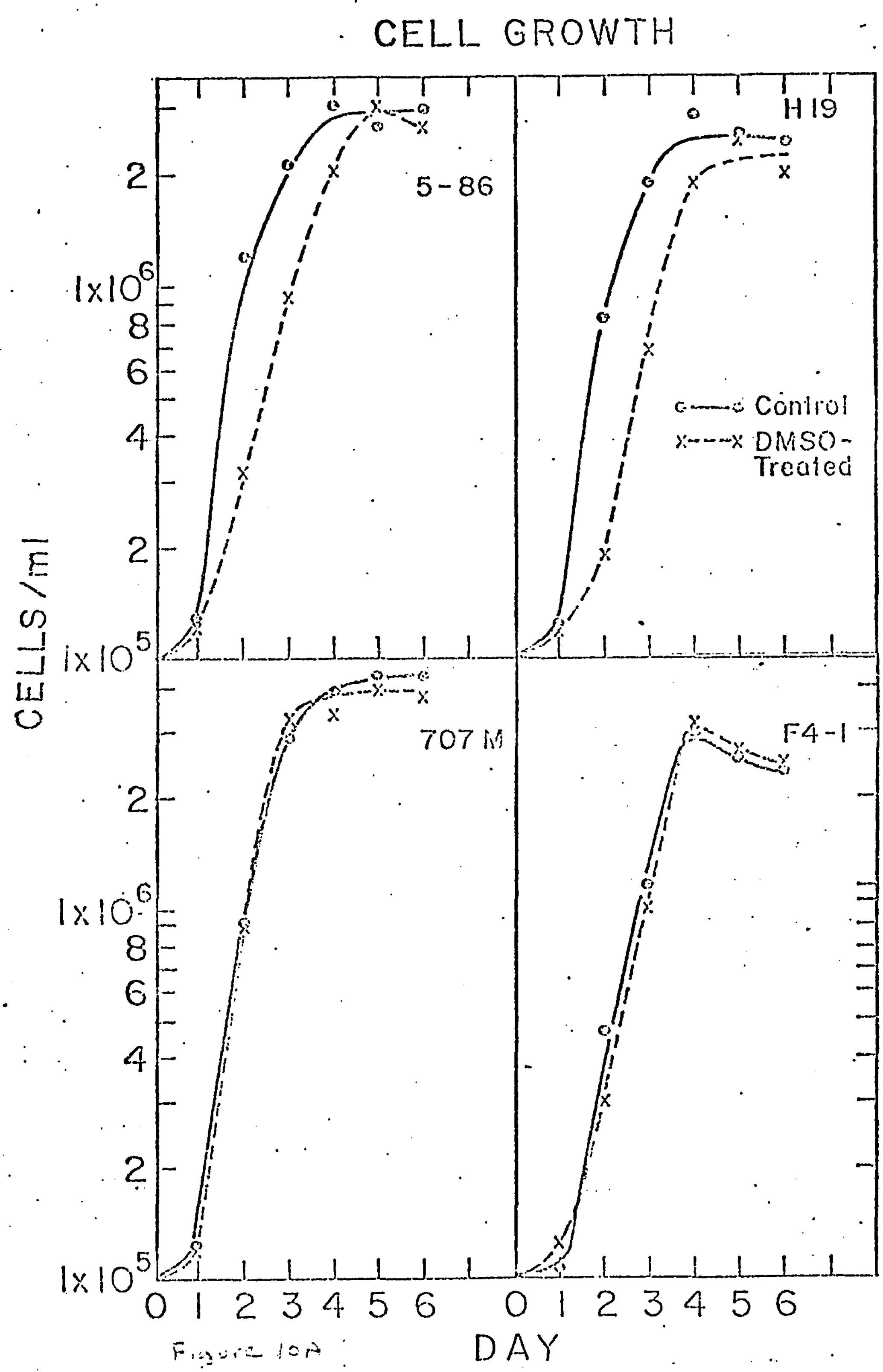
Figure 10B. Percentage of B+ cells of line 5-86, H 1.9, 707 R, and F 4-1.

Treatment of cells as in 10A. control DMSO

Figure 10C. Accumulation of viral reverse transcriptase activity in conditioned medium of line 5-86, H 1.9, 707 R, and F4-1. Culture fluid was harvested daily for reverse transcriptase assay. Each point represents the average of 3 reaction mixtures after zero-time reaction had been subtracted. About 2,000 cpm equals one p mole of (^{3}H)-TMP incorporation. control DMSO

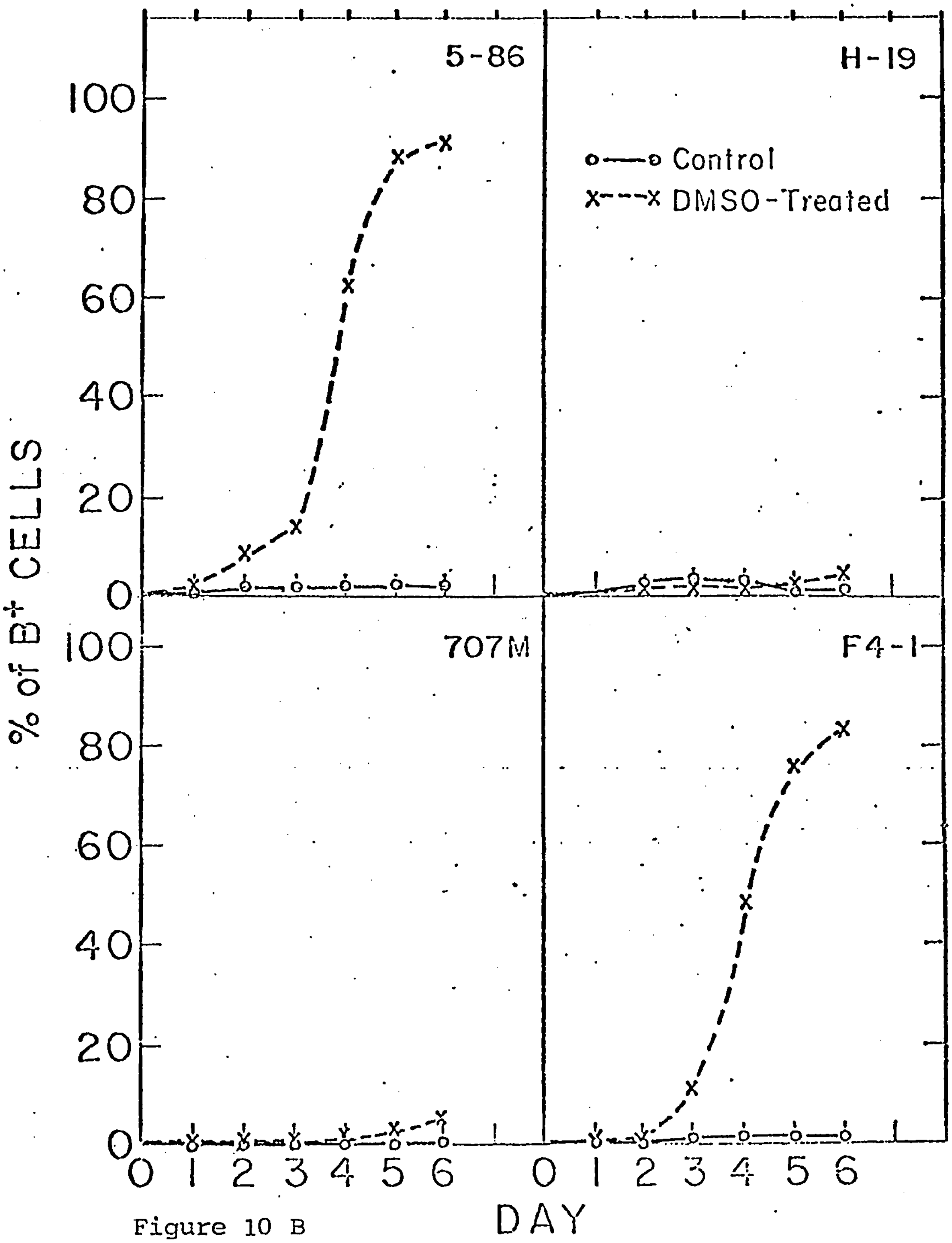
Figure 10D. Competitive radioimmunoassay of gp 71 in conditioned medium of line 5-86, H 1.9, 707 R, and F4-1. Samples of 0.2 ml. fluid were taken daily, clarified, and used to compete with the binding of goat antiserum. control DMSO

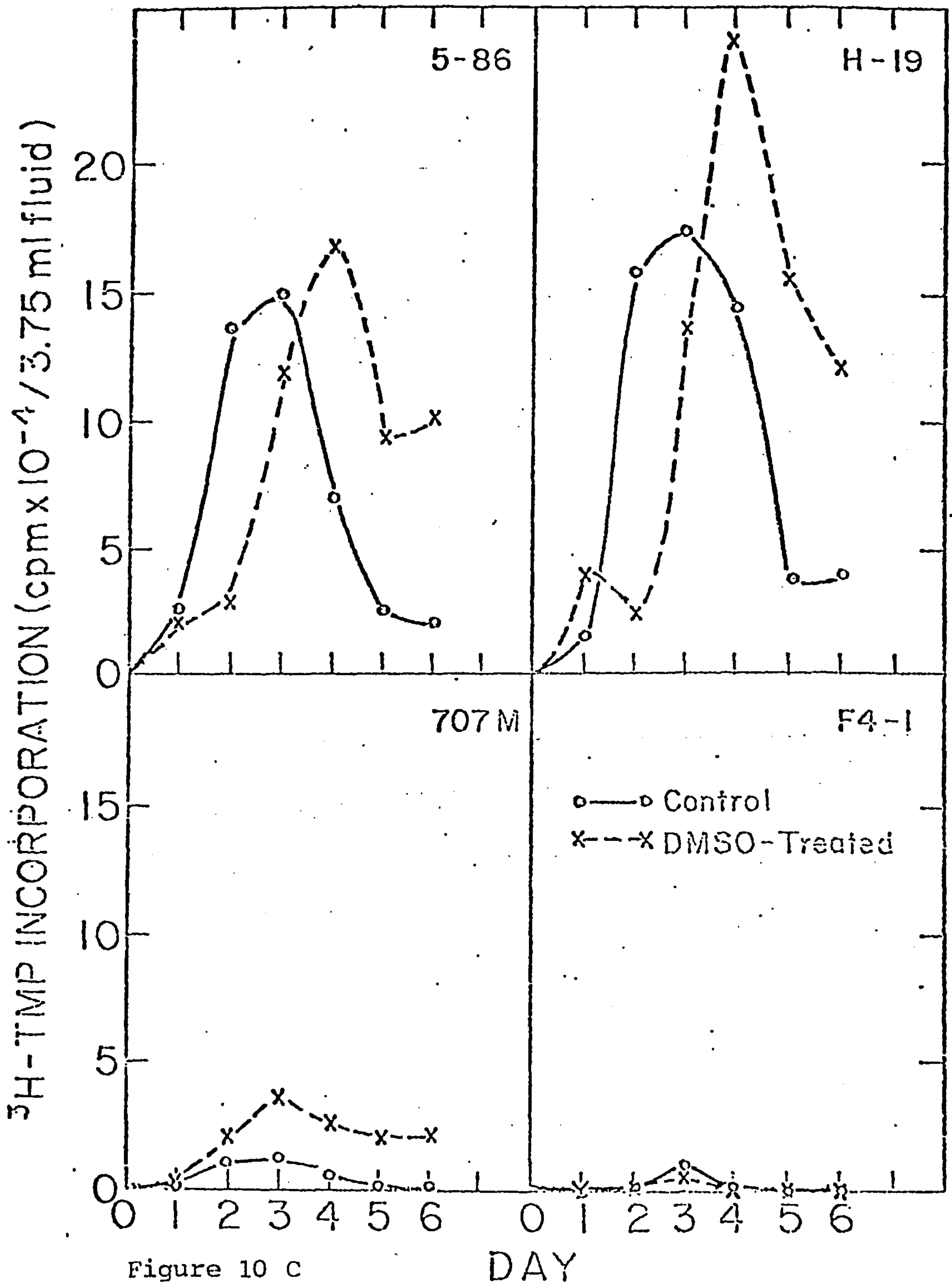
Figure 10E. Competitive radioimmunoassay of p 30 in conditioned medium of line 5-86, H 1.9, 707 R and F4-1. Samples of 0.2 ml. fluid were taken daily, clarified, and used to compete with the binding of goat antiserum. control DMSO



CELL GROWTH
CELLS/ml
1x10^5
2
4
6
8
1x10^6
2
5-86
H19
Control
DMSO-Treated
707 M
F4-1
0 1 2 3 4 5 6
DAY

Figure 10A


5-86
H-19
707M
F4-1
Control
DMSO-Treated
% of B+ CELLS
DAY
0
20
40
60
80
100
0 1 2 3 4 5 6
Figure 10 B



5-86
H-19
707 M
F4-I
Control
DMSO-Treated
³H-TMP INCORPORATION (cpm x 10⁻⁴ / 3.75 ml fluid)
DAY

Figure 10 C

COMPETITIVE RADIOIMMUNOASSAY FOR FLV-gp71

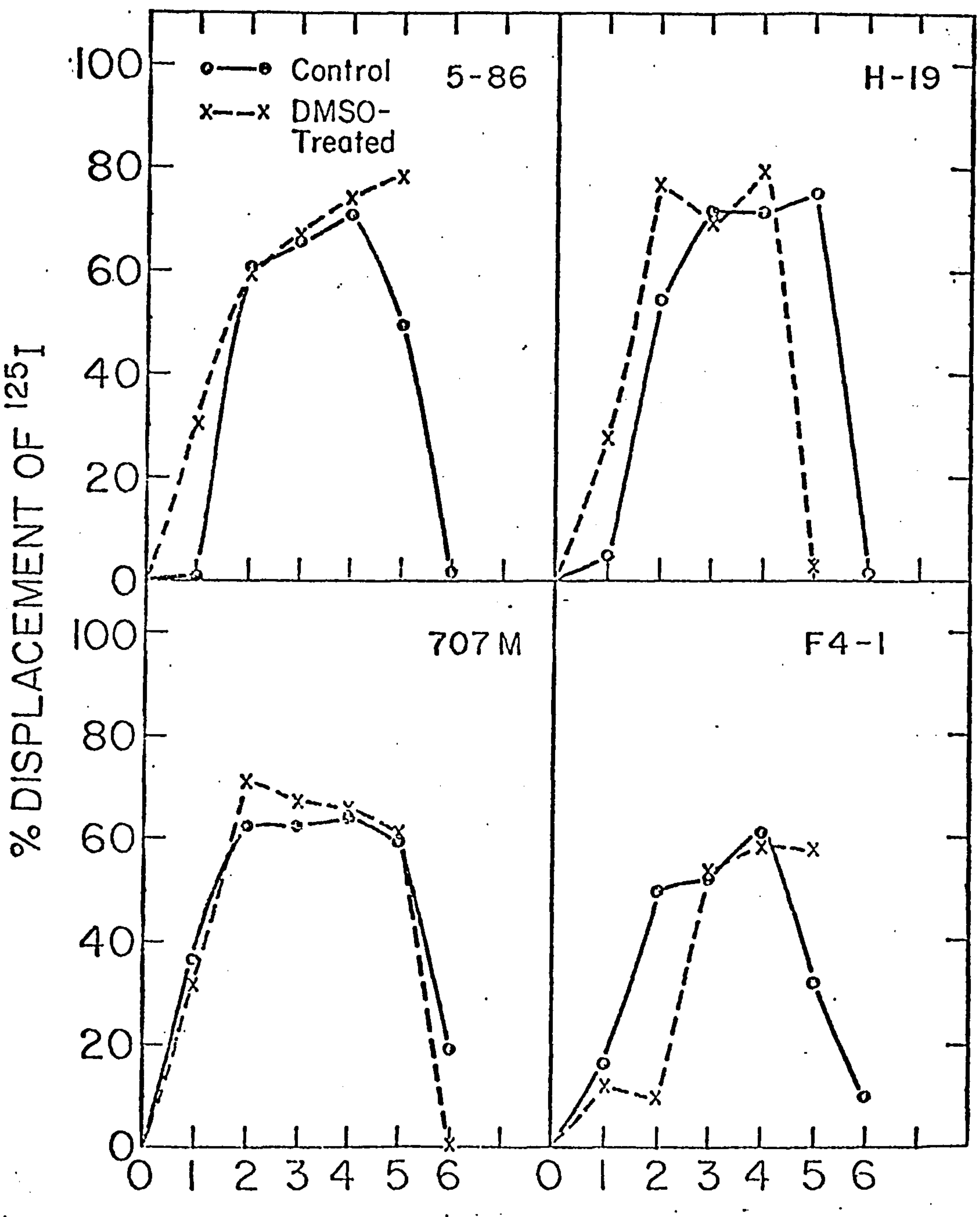


Figure 10 D

COMPETITIVE RADIOIMMUNOASSAY FOR FLV-P30

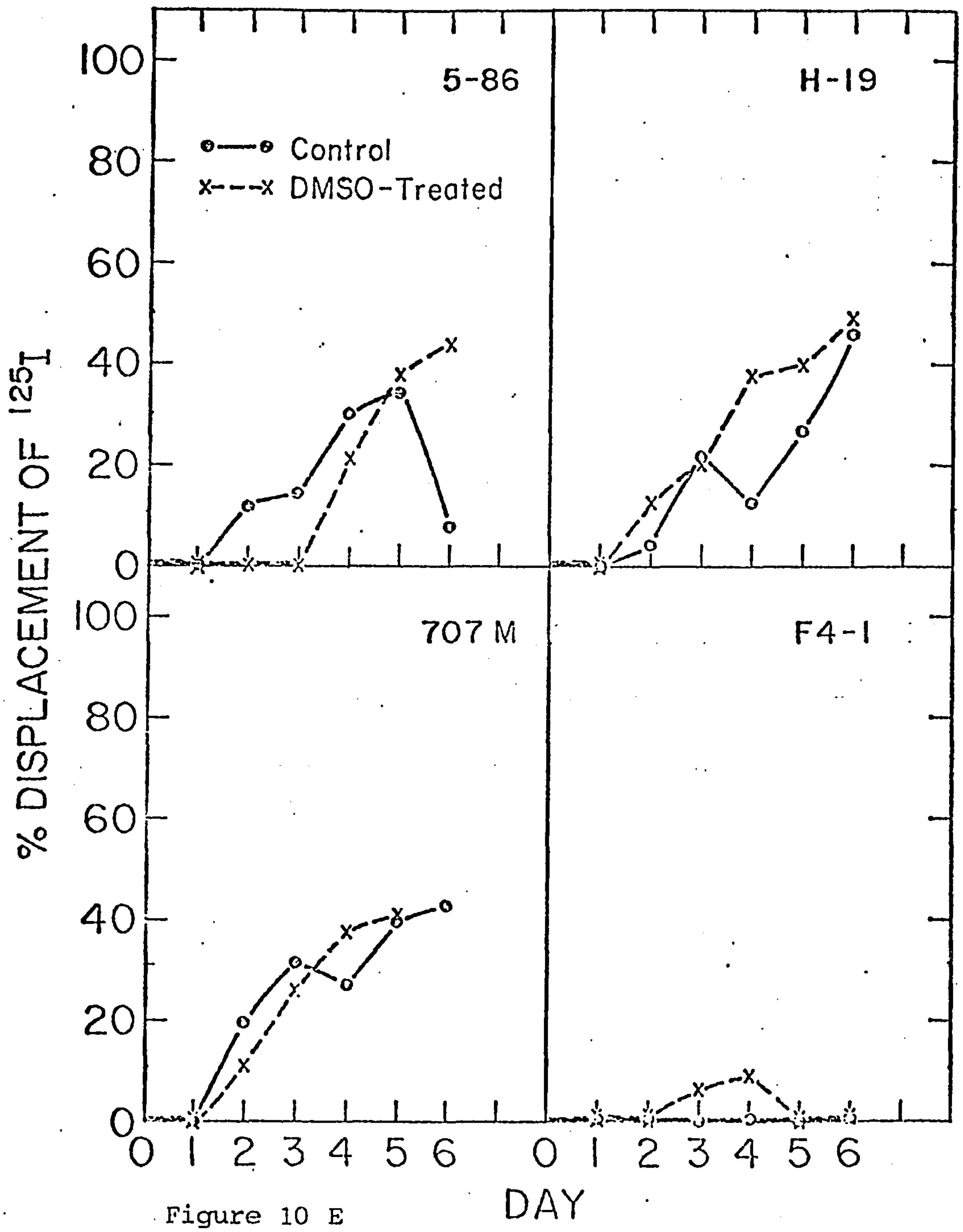


Figure 10 E

of all untreated cultures were comparable. For all four lines, there was log phase growth within the first 3-4 days after seeding 10^5 cells/ml. and an average doubling time of 12-15 hrs. When the cells were seeded with 1.9% DMSO, the DMSO-resistant variant H-1.9 showed the same early lag in growth as its DMSO-sensitive parent line, and reached stationary phase within 4-5 days. In the DMSO-resistant line 707M and inducible line F4-1, however, cell growth was not affected by the presence of DMSO in the medium.

Hemoglobin synthesis: (Fig. 10B)

In cultures without DMSO, all four lines had low levels of B+ cells, ranging from 0-3%. When grown in medium with DMSO, the inducible lines 5-86 and F4-1 showed an increase of B+ cells starting 2-3 days after treatment and reached a level of 85-90% B+ cells by the 6th day. In comparison, H-1.9 and 707R responded poorly to DMSO stimulation and less than 5% of the cells were B+ by the 6th day.

Virus release: (Fig. 10C)

Viral RT activity in the culture fluid was measured as an indication of virus release from FL cells. Poly rA.dT_{10} was used as the template-primer. The same pattern of virus release was found in line 5-86 and its DMSO-resistant derivative, H-1.9. The control cultures of these two lines showed an exponential increase in virus release during the early log phase of cell growth. Virus accumulation peaked on the 3rd day and decreased sharply after the cells reached stationary phase. When the cells were treated with DMSO, there was an initial lag in virus release, which paralleled cell growth. Virus release peaked on the 4th day at a level higher than the peak for cells grown in control medium, and decreased after the cultures reached the stationary phase. Both the non-inducible line 707R and the inducible line F4-1 produced very low amounts of virus.

Release of viral antigens:

The presence of viral antigens was assayed in the culture fluid of these four lines by competitive radioimmunoassay for FLV-gp71 and p30 with monospecific sera. Determinations are plotted as the percent displacement of ^{125}I-labeled viral antigen by 200 µl of culture fluid. Lines 707R and F4-1 produce little or no virus as measured by reverse transcriptase activity. However, the level of gp 71 detected in the culture fluid of these cells was comparable to that detected in the 5-86 and H 1.9 cultures. There was no change in the amount of gp 71 released as a result of treating the cells with DMSO (Figure 10D).

The two virus-producing lines, 5-86 and H 1.9 and the low virus-producer, 707 R, shed similar amounts of viral antigen p 30 into the medium and these levels did not change when the cells were treated with DMSO. Little or no p 30 was detected when F4-1 cells were grown in unsupplemented medium. When line F4-1 was treated with DMSO, a low level of p 30 antigen was detected (Figure 10E).

Comparison of the Tumorigenicity of 4 Cell Lines

The average survival of mice innoculated with line 5-86 cells grown in control medium or in 1.9% DMSO in the experiments where different cell lines were compared (Table 14) was similar to that in the third experiment where the different treatments were compared. Mice treated with cells grown in control medium survived 45.8 $\pm$ 4.6 and 48.0 $\pm$ 3.5 days while mice treated with cells which had been incubated with 1.9% DMSO survived 56.0 $\pm$ 3.4 and 56.8 $\pm$ 10.2 days.

Values for the L.D. 50 for mice innoculated with line 5-86 cells were 1.6 x 10^5 /mouse and 1.8 x 10^4 cells/mouse for cells grown in 1.9% DMSO or unsupplemented medium respectively (Table 15).

Table 14

Comparison of Tumor Incidence and Host Survival After Innoculation of Control and DMSO-treated Cells of Four Cell Lines in Syngeneic Mice

Experiment 1: DBA/2J Females, 2.5×10^5 cells/mouse

Cell Line	Medium Treatment	# Mice with Tumor/Total		Mean Days Survival
5-86	Control	5/5	9/10	45.8 ± 4.6
	1.9% DMSO	4/5		56.0 ± 3.4
H1.9	Control	2/5	2/10*	77.0 ± 14.4
	1.9% DMSO	0/5		
707	Control	1/5	3/10**	65
	1% DMSO	2/5		86 ± 7.07
F4-1	Control	4/5	7/10	67.8 ± 13.9
	1.5% DMSO	3/5		83.3 ± 10.5

Experiment 2: DBA/2J Males, 1×10^5 cells/mouse

Cell Line	Medium Treatment	# Mice with Tumor/Total		Mean Days Survival
5-86	Control	4/5	9/10	48 ± 3.5
	1.9% DMSO	5/5		56.8 ± 10.2
H1.9	Control	2/5	3/10*	84 ± 11.3
	1.9% DMSO	1/5		75
707R	Control	3/5	3/10*	77 ± 6.2**
	1% DMSO	0/5		
F4-1	Control	4/5	7/10	55.5 ± 11.8
	1.5% DMSO	3/5		57.0 ± 9.6

*χ^2 significantly different from 5-86 cells at $p \leq 0.5$ without Yates' correction.

**significantly different $p \leq .05$ from survival of mice injected with 5-86 control cells.

Table 15

Data Used to Calculate L.D. 50 of Line 5-86 Cells

Medium	Number of cells innoculated x 10^{-4} 25 dead/total	10 dead/total	2.5 dead/total
control	4/5	5/5	3/5
1.9% DMSO	4/5	1/5	1/5

Line F4-1, which also develops B+ cells after four days of treatment with DMSO, also tended to decrease in malignancy after treatment with DMSO. In the first experiment, mice innoculated with cells grown in control medium survived 67.8 ± 13.9 days and mice innoculated with cells grown in 1.5% DMSO (the optimal concentration for this line) survived 83.3 ± 10.5 days. In the second experiment (Table 14) survival times were 55.5 ± 11.8 and 57.0 ± 9.6 days respectively.

It should be noted that unlike the three other lines of cells used for these experiments, which were derived in Friend's laboratory and which synthesize virus of low leukemogenicity for adult mice,[29] Line F4-1 was derived from the Ostertag line which is leukemogenic.[90] Although the reverse transcriptase assay and the radioimmunoassay for p 30 antigen indicate that line F4-1 is defective in its ability to produce virus, tests of the cells and the supernatant medium in vivo indicate that some leukemogenic virus is produced. Seven mice out of 14 mice which developed tumors after innoculation with line F4-1 also had enlarged spleens, a hallmark of virus infection. Only two mice of 29 which developed tumors after treatment with the other cell lines developed enlarged spleens. One mouse of two innoculated i.p. with 0.2 ml. filtered control medium in which line F4-1 cells had grown for four days developed an enlarged spleen with histology indicative of typical Friend leukemia. The hematocrit of this mouse was 75 and it was killed 53 days after innoculation. A 20% spleen homogenate was innoculated into 7 SPF (specific pathogen free) DBA/2J mice, one of which died with an enlarged spleen 158 days after innoculation, and 5 Taconic mice none of which showed any sign of disease.

If the paired data (average survival time of mice innoculated with control vs. DMSO treated cells) for lines 5-86 and F4-1 is evaluated by Wilcoxon's non-parametric ranking test,[91] the difference between cells treated with DMSO and control cells is significant at $p \leq 0.05$.

Tumor incidence in mice innoculated with cells of the DMSO-resistant lines is significantly lower (5 mice or 6 mice out of 20 for line H 1.9 or 707 R respectively) than in mice innoculated with cells of line 5-86 (18 mice out of 20) when the data is pooled by cell line and evaluated by the χ^2 test. This reflects lower tumor incidence after DMSO treated cells of the DMSO-resistant lines were innoculated, since the χ^2 value for control cells is not significant ($\chi^2 = 3.13$, $0.1 > p > .05$). Those mice which developed tumors after innoculation of line H 1.9 or 707R cells tended to survive longer than mice innoculated with line 5-86 cells.

Immunogenicity of Cells of Line H 1.9 and 707R

The DBA/2J mice, innoculated with H 1.9 or 707 cells, which did not develop tumors, or mice innoculated with tissue culture medium were divided into two groups. One group was innoculated with FLV-P (diluted 10^{-2}) 108 days after the original innoculation with tumor cells (Table 16). Only one mouse out of eight innoculated developed an enlarged spleen and it died 71 days after virus innoculation. This mouse had been innoculated with line 707 cells grown in DMSO.

In contrast to the DBA/2J mice challenged after innoculation with syngenic cells, DBA/2J mice were not protected by previous treatment with tissue culture fluid (Table 17). Six mice of seven innoculated with the same pool of virus died at an average of 80 days after virus innoculation.

Table 16

Response of DBA/25 Female Mice Innoculated Subcutaneously with Isogenic Cells to Challenge with FLV-P (10^{-2}) 108 Days Later

Cell line	Treatment of cell line	Cell injection # dead/ # innoc.	Day of death post innoc.	Virus challenge # dead/ # inj.	Day of death post challenge	Challenge control # dead/ # not inj.
H 1.9	Control	2/5	67,87	0/1		0/3
	DMSO	0/5		0/3		0/3
707R	Control	1/5	65	0/2		0/2
	DMSO	2/5	82,91	1/2	71	0/1
Pooled	Control	3/5		0/3		
	DMSO	2/5		1/5		0/3
Total		5/20		1/8*		0/8

*Corrected χ^2 comparing these mice and mice injected with tissue culture supernatants (Table 17) = 5.34 $p \leq .02$

Table 17

Response of DBA/2J Female Mice Treated with 0.2 ml. Tissue Culture Supernatants to Challenge with FLV-P 108 Days Later

Cell line	Treatment of cell line	Super-natant # dead/ # innoc.	Day of death post innoc.	Virus challenge # dead/ # inj.	Day of death post challenge	Challenge control # dead/ # not inj.
5-86	C	0/2		1/1	29	0/1
	D	0/2		1/1	144	0/1
H 1.9	C	0/2		0/1		0/1
	D	0/2		1/1	62	0/1
707	C	0/2		1/1	123	0/1
	D	0/2		1/1	64	0/1
F4-1	C	1/2	53*	N.D.		0/1
	D	0/2		1/1	56	0/1
Pooled	C	1/8		2/3		
Pooled	D	0/8		4/4		0/8
Total		1/16		6/7		

*This mouse had an enlarged spleen and a hematocrit of 75. It was killed and pathology (2F4066) showed strong FL in the spleen and liver and moderate FL in the sternum. Immediately after the mouse was killed a 20% spleen homogenate was made and injected into 7 SPF DBA/2J female mice and 5 Swiss females. One DBA mouse died of an enlarged spleen 158 days after injection.

The difference between the group innoculated with cells before virus injection and the group innoculated with supernatant medium before virus injection is significant (adjusted $\chi^2 = 5.34$ $p \leq .02$.)

Swiss mice which had been innoculated with cells from each of the lines which had been grown in control or DMSO supplemented medium and tested with the same pool of virus, were also susceptible to the virus infection (Table 19). Six of 19 mice injected with virus died and four of them survived less than 30 days. None of the Swiss mice developed a tumor or leukemia after innoculation of the cells alone.

Twenty-seven Swiss mice (Table 19) were challenged with the same pool of FLV-P after innoculation with tissue culture supernatants. Ten of these mice died at an average of 63 days after virus challenge.

The pool of virus used for these challenges was also titered in Swiss mice (Table 20). Four of the five mice innoculated with the dilution used for the experiments (10^{-2}) developed leukemia. Three of them survived an average of 23 days after injection. The fourth mouse developed an enlarged spleen approximately 70 days after innoculation and died 184 days after innoculation. Including this mouse increases the average survival of this group to 63.5 days. All the mice innoculated with virus diluted 10^{-3} developed leukemia and died at an average of 40 days after innoculation. Two mice out of five innoculated with virus diluted 10^{-4} developed leukemia and they died at an average of 85 days after innoculation.

Table 18

Response of Swiss Mice Innoculated Subcutaneously

with 10^6 Allogenic Cells to Challenge

with FLV-P (10^{-2}) 66 Days Later

Cell line	Treatment of cell line	Cell injection # dead/ # innoc.	Virus challenge # dead/ # inj.	Day of death post challenge	Challenge control # dead/ # not inj.
5-86	Control	0/5	0/2		0/3
	DMSO	0/5	0/3		0/2
H1.9	Control	0/5	0/2		0/3
	DMSO	0/5	1/3	17	0/2
707R	Control	0/5	0/2		0/3
	DMSO	0/5	2/3	70,98	0/2
F4-1	Control	0/5	1/2	20	0/3
	DMSO	0/3	2.2	21,29	0/1
Pooled	Control	0/20	1/8*		0/12
	DMSO	0/18	5/11		0/7
Total		0/38	6/19		0/19

Table 19

Response of Swiss Female Mice Treated with Tissue Culture Supernatants to Challenge with FLV-P 108 Days Later

Cell line	Treatment of cell line, amt. used (ml.)		Super-natant innoc. # dead/ # innoc.	Day of death post innoc.	Virus challenge # dead/ # inj.	Day of death post challenge	Challenge control # dead/ # not inj.
5-86	C	0.2	0/5		1/3	27	0/2
		0.5	0/3		1/2	63	1/1
	D	0.2	0/5		0/2		0/3
		0.5	0/3		1/1	105	1/2
H 1.9	C	0.2	0/5		0/3		0/2
		0.5	0/3		0/2		0/1
	D	0.2	0/5		2/2	38,73	0/3
		0.5	0/3		0/1		0/2
707	C	0.2	0/5		0/3		0/2
		0.5	0/3		2/2	57,77	0/1
	D	0.2	0/5		1/2	56	1/3
		0.5	0/3		0/1		0/2
F 4-1	C	0.2	0/5		0/3		0/2
		0.5	0/3		0/2		0/1
	D	0.2	0/5		1/2	38	0/3
		0.5	0/3		1/1	99	0/2
Pooled	Control		0/32		4/15	56	1/2
	DMSO		0/32		6/12	68.2	2/20
Total			0/64		10/27**	av. 63.3	3/32*

**Significantly different from mice not injected with virus

$\chi^2 = 6.6$ $p \leq .02$

Not significantly different from titration (Table 20) $\chi^2 = .91$ $p \geq .05$

Not significantly different from mice innoculated with cells (Table 18) $\chi^2 = 0.15$ $p \geq .5$

* Note: microscopic examination of tissues from mice which died in challenge control group indicated these mice did not die of leukemia

Table 20

Titration of FLV-P in Swiss Female Mice

Dose	#Dead/#Innoc.	Day of death post innoc.	
10^{-2}	3/5*	20, 24, 26	$\bar{x}$ = 23.3
	(4/5	20, 24, 26, 184	$\bar{x}$ = 63.5)
10^{-3}	5/5	24, 20, 46, 48, 54	$\bar{x}$ = 40.4
10^{-4}	2/5	60, 110	$\bar{x}$ = 85
10^{-5}	1/5	146	

*One mouse developed an enlarged spleen approximately 72 days p.i. and died 184 days p.i. Average survival time of mice which developed leukemia is calculated with this mouse in parenthesis.

Discussion

These studies suggest that growth may play two apparently contradictory roles in the differentiation of Friend cells. An increasing percentage of the cells differentiates with continued growth in the presence of DMSO, yet glycerol, which reduces the growth rate, has a synergistic effect on differentiation when added with a sub-optimal concentration of DMSO.

In these experiments, the effects of growth are illustrated by the fact that line H 1.9, which is not inducible in stationary cultures, produces B+ cells when the cells are cloned in DMSO or allowed to grow by passage in fresh, treated medium. Furthermore, when cells of line 5-86 are treated with half the optimal concentration of DMSO, the percentage of differentiated cells increases if the cells are seeded at low density and can grow for a longer time, or if they are transferred to fresh, treated medium. The stimulatory effect of growth in the presence of an inducer have also been observed in experiments in which cells have been exposed to DMSO[26] or HMBA[92] for increasing lengths of time and then plated in semi-solid medium in the absence of inducer and in experiments in which cells were allowed to grow in sub-optimal concentrations of HMBA.[41]

On the other hand, the addition of 3.5% glycerol along with half the optimal concentration of DMSO clearly increased the average percentage of B+ cells above that observed with 0.95% DMSO alone from 2% to 37% in stationary cultures of line 5-86, and from 6% to 20% in transferred cultures of line H 1.9 and from 78% to 89% in transferred cultures of line 5-86.

These observations may be reconciled by the finding that during treatment with DMSO, the stability of globin message and the decrease of mRNA synthesis contribute to a relative increase in globin mRNA.[93,94] This effect, together with the increase in rate of production of globin mRNA[17,95] leads to the observed rate of hemoglobin accumulation. Thus, the fact that the optimal dose of DMSO is close to the toxic level[34] may be related to the decreases in transport[23,37] and RNA and protein synthesis[30] which are associated with treatment. Since glycerol and DMSO have similar effects on cell membranes[96,97] and microtubules[98,99] and compete for the same facilitated diffusion mechanism in the human red cell membrane,[100] they may have similar effects on the transport of glucose and amino acids, and consequently on cell metabolism.*

Treatment of line 5-86 with the combination of 3% glycerol and 2% DMSO restricted growth of the culture and induced differentiation of approximately 20% of the cells. Studying this treatment with the thymidine suicide technique[27] or autoradiography might indicate whether growth was limited to those cells which differentiated.

These experiments also bear on the relationship between DMSO-resistant variant lines and DMSO-sensitive lines. An apparently DMSO-resistant line, H 1.9, is capable of being induced to produce hemoglobin detected after seven days of treatment with 1.9% DMSO under conditions which allow growth. In contrast, hemoglobin can be detected in the parent line, 5-86, after three days of growth. However, the highest percentage of B+ cells observed after treatment of line H 1.9 was consistently less than a third of the percentage observed after identical treatment of line 5-86.

Unlike line 5-86, the efficiency of cloning of line H 1.9 is similar in 1.9% DMSO or unsupplemented medium. After 11 days of growth in DMSO, half the clones consisted of more than 20% B+ cells and less than half the clones survived for 20 days. Similar heterogeniety of response has been noted by Orkin.[14]

DMSO-resistant cell lines have been called variants rather than mutants. They arise more often than might be expected on the basis of mutation rates and are frequently unstable. It has been suggested that some of them are epigenetic. Results of the Delbruck fluctuation test indicated that treatment of cells with DMSO did not alter the number of variants observed. The mutagen ICR 191 may increase the number of variants although ethyl methane sulfonate was thought to be ineffective.[1]

Recent experiments have indicated the DNA which codes for the globin sequences is sensitive to DNA-ase I in both Friend cell lines with varied levels of response to DMSO and lymphoma line L 5178Y. This suggests that the sequences are available for transcription in contrast to cell lines in which these sequences are not degraded[101] by DNA-ase I. Variant lines FW[1] and F4N+2[24] and somatic cell hybrids between DMSO-responsive Friend cells and lymphoma line L 5178Y[1] are not inducible by DMSO alone but respond if exogenous heme is added. Since many of the steps of the heme pathway take place in the mitochondria, these lines may differ from responsive lines in the quantity or function of mitochondria.

Cybrids consisting of the nucleus of a responsive cell and the cytoplasm of a fibroblast apparently illustrate a different cytoplasmic effect. When the sensitivity of globin sequences to DNA-ase I of a cybrid culture was tested, the value was intermediate between the values for responsive and unresponsive cells. When the cybrids were cloned, some individual clones were responsive to DMSO stimulation and some were not. This heterogeniety was interpreted as the effect of a cytoplasmic factor which had changed the conformation of the DNA in the non-responsive cells.[101]

On the other hand, some variants respond to one inducer but not another,[1] suggesting a difference in receptor, metabolism or transport. Line H 1.9, which responds to butyric acid, would be part of this group.

The differences among variants, and the experiments with somatic cell hybrids and cybrids, suggest that DMSO-resistant variants may include several classes of cells: 1) mutants of nuclear genetic material, 2) mutants of cytoplasmic genetic material, 3) variants which differ in cytoplasmic elements as a result of unequal distribution and 4) variants which differ because of environmental factors. The clones of line H 1.9 which survived cloning and growth in DMSO and the cells which survived after continued passage in DMSO and DMSO plus glycerol could be used for future studies along these lines.

Spudich and Koshland have suggested that stochastic effects may be the result of an unequal distribution of enzymes within a population of cells.[102]

The different patterns of response of lines 5-86 and H 1.9 can be explained in terms of a stochastic model and may fit into class three. Differentiation may be dependent on a factor or factors which are randomly distributed in each line but which are at a higher level on the average in line 5-86. Such a factor or factors would have to be increased by treatment with DMSO under conditions where prolonged growth occurs and be stable enough to reach the effective level after several days of treatment. This model is consistent with either a nuclear or a membrane effect of inducing agents. The hypothesis could be tested using a cell line which responds faster than line 5-86 (i.e. line 745-PC-4)[68] and which would be expected to differentiate more readily. It is not known whether this line is responsive to lower concentrations of inducing agents or whether a relatively large percentage of the cells might respond to DMSO in the presence of an inhibitor of DNA synthesis. In a situation which may be analagous, the level of NAD has been implicated as the factor which determines the development of mesodermal cells into muscle or cartilage.[103]

The relationship between differentiation and growth arrest is not as clear in these experiments as it is in cloning experiments[25,26,79,80] or experiments using thymidine suicide.[27] Growth has been discussed in terms of population doublings since the changes observed might have been associated with decreases in the growth fraction (as has been observed in cloning experiments) or a continued slowing of growth. These factors could be identified by cloning after different intervals of exposure to the inducers. However, it is clear that cultures of line 5-86 treated with 1.9% DMSO or 0.95% DMSO plus 3.5% glycerol which are transferred to fresh treated medium are growth arrested after twelve days of treatment.

When line 5-86 and H 1.9 are compared, treatment with 1.9% DMSO or 3.5 glycerol plus 0.95% DMSO seems to have a greater effect on growth than it does on hemoglobin synthesis in line H 1.9. The clearest example of this is that in experiment 3, line H 1.9 stopped growing on day 11 although at that time less than 25% of the cells were benzidine positive. This was not due to increased toxicity of treatment in this line since appreciable numbers of dead cells were first observed on day 15. Furthermore, the average percentage of dead cells of line 5-86 in experiment 2 was 0.2%, 1.6%, 2.0%, 8.5% and 14.6% when the cells were grown in unsupplemented medium, 3.5% glycerol, 0.95% DMSO, 0.95% DMSO plus 3.5% glycerol and 1.9% DMSO respectively and the average percentages of dead cells for line H 1.9 listed in the same order were 0.5%, 1.3%, 4.3%, 5.1% and 11.4%. This suggests that the effect of treatment is similar in both lines. This was also reflected in the bioassay experiments. Only one mouse out of 10 injected with DMSO-treated line H 1.9 cells developed a tumor as compared to four mice out of ten injected with untreated cells.

In line 5-86, growing the cells in 1.9% DMSO was the only treatment which affected tumor incidence. This may be correlated with the higher percentage of differentiated cells associated with this treatment. However when mice are injected with different levels of cells and the L.D. 50 is calculated, cells treated with DMSO differ from untreated cells by almost one log. This corroborates the relationship found in line 707.[10] Additional data may substantiate the decrease in tumor incidence and increase in host survival time after treatment of other cell lines.

It should be noted that although line H 1.9 and line 5-86 both produce virus, they differ in ability to cause tumors. This suggests that virus production is not an important factor in the malignancy of these cells. Although tests of conditioned medium from line F 4-1 revealed gp 71 and little or no other virus components, some virus was seen in photographs taken with the electron microscope and the fact that mice treated with cells of this line developed enlarged spleens indicates that some leukemogenic virus was produced. Previous studies have shown that three innoculations of tissue culture cells protected Swiss mice against subsequent virus injection.[105] The finding in these studies that a single innoculation protected syngeneic mice but not Swiss mice from subsequent virus injection may stimulate further study of the relationship between the immune response to infected cells and virus.

The effect of treatment with DMSO and other inducers on clonigenicity[25,26,79,80,92] and tumorigenicity[10,82] has been noted by other investigators. Treatment of the cells was found to decrease cloning capacity and increase the survival time of an injected host. Furthermore, line 745D, a DMSO-resistant line, appeared to be less malignant than line 745 clone IV and its malignancy was further decreased after treatment with DMSO in one out of two experiments.[82] Although the results were given in terms of median survival, it may be concluded that, in general, malignancy decreased with treatment.

These studies have not resolved the relationship of growth and differentiation. They do, however, indicate the need for some macromolecular synthesis as in the case where line 5-86 cells are treated with 3% glycerol and 2% DMSO. They also indicate that globin mRNA may have accumulated under conditions where growth is slowed as in the presence of 0.95% DMSO and glycerol. This effect of glycerol supports Lowenhaupt and Lingrel's finding that the relative amount of globin message contributes to hemoglobin synthesis.[93,94] It has also been suggested that the IP25 protein which is found in the nucleus of treated cells may be associated with a decrease in growth which in turn promotes differentiation.[104] The accumulation of hemoglobin during a period of prolonged slow growth would also explain the development of B+ cells in line H 1.9 after cloning in DMSO or a series of transfers in fresh, treated medium.

* On the other hand, changes in the membrane might allow the entrance of components of the medium which are involved in the expression of differentiated function. Investigation of the relative proportions of beta major and beta minor chains produced after treating the cells with glycerol,DMSO, and combinations of DMSO and glycerol might resolve these questions.

Summary

I. Effects of treatment with both DMSO and glycerol

1. Treatment of line 5-86 with increasing concentrations of glycerol decreases the growth rate and slightly increases the percentage of cells which produce hemoglobin.

2. Addition of 3% glycerol together with the optimal concentration of DMSO (1.9%) decreases both cell growth and the percentage of differentiated cells.

    a) During treatment with 1.9% DMSO the cells double a little more than four times in four days.

    b) When the cells were treated with 3.0% glycerol and 1.9% DMSO together the growth rate was reduced to one doubling in four days.

    c) When the cells were treated with 1.9% DMSO, 50.5% became benzidine positive (B+).

    d) When the cells were treated with 3.0% glycerol and 1.9% DMSO together only 20% became B+.

    e) Differentiation of cells grown in medium containing 3.0% glycerol and 1.9% DMSO is not affected by seeding densities up to 8×10^5 cells.

3. Addition of 3.5% glycerol along with half the optimal concentration of DMSO (0.95%) increases the percentage of differentiating cells above the level observed after treatment with 0.95% DMSO alone. This indicates that these compounds have a synergistic effect.

II. Preliminary study of a DMSO-resistant variant cell line designated H 1.9.

A. Line H 1.9 differs from its parent line, 5-86 in several respects.

1. The percentage of B+ cells does not increase above background level when cells of line H 1.9 are grown in medium supplemented with concentrations of DMSO or HMBA which are optimal for induction of differentiation in line 5-86.

2. Line H 1.9 was less sensitive to the effect of butyric acid and the percentage of B+ cells was less than half that observed after treatment of the parent line.

B. Line H 1.9 is similar to line 5-86 as follows:

1. The growth rate in BME/E with 15% fetal bovine serum is the same for both lines.

2. The changes in growth pattern due to treatment with DMSO, HMBA, or butyric acid are similar.

III. In order to observe the effects of inducers without any possible effect of depletion of a factor in the medium, a set of experiments was done where the cell number was adjusted to 1×10^5 by transferring the cells to fresh, treated medium every other day. The following observations were made under these experimental conditions:

A. Effects of treatment on line 5-86.

1. Treatment with 1.9% DMSO, 0.95% DMSO plus 3.5% glycerol, or 0.95% DMSO alone led to increasing percentages of B+ cells which reached a maximum on days 6-8. At that point, the average percentages of B+ cells were 92, 89, and 78 respectively.

2. Further treatment led to a decrease in the percentage of B+ cells. This may be due to the selection of cells which are less likely to differentiate.

3. Prolonged treatment with 1.9% DMSO or the synergistic combination of glycerol and DMSO, (3.5% glycerol and 0.95% DMSO) led to growth arrest. The population failed to double within 48 hours after approximately 8 days of treatment. The remaining cells may be DMSO resistant.

4. Prolonged treatment with 0.95% DMSO led to a decrease in growth rate followed by recovery to close to control level. The minimum growth rate was reached between days 8-10.

5. Cells treated with 3.5% glycerol, which did not induce the production of B+ cells, showed changes in growth rate which were parallel to, but not as large as, those observed during treatment with 0.95% DMSO.

B. Effect of treatment on line H 1.9.

1. Line H 1.9 was not completely resistant to inducing compounds tested previously when the cells are treated using this procedure.

2. Treatment with 1.9% DMSO or the synergistic combination of glycerol and DMSO led to increases in B+ cells which reached a maximum around day 10. At that point, approximately 27% and 20% of the cells were B+ respectively.

3. Treatment with 0.95% DMSO led to a maximum of 6% of B+ cells. This level was reached twice, once after approximately 8 days and once at approximately 14 days.

4. The decrease in percentage of B+ cells after the maximum point was more gradual than that observed in line 5-86.
5. Growth arrest after treatment was not as clear in these cells. In one experiment, cells treated with 1.9% DMSO or the synergistic combination reached a minimum rate of growth after approximately 12 days of treatment. At that point, they doubled slightly more than once in two days. In the other experiment, the cells failed to double within 48 hours after treatment with 1.9% DMSO for approximately 12 days. Cells treated with the synergistic combination appeared to reach growth arrest after approximately 6 days.
6. Cells treated with 0.95% DMSO and 3.5% glycerol showed parallel fluctuations in growth.

IV. Studies of Tumorigenicity of FL cell lines. In these experiments cells were grown for four days in the medium noted and equal numbers of cells were injected into syngeneic (DBA/2J) mice within each experiment.

A. Growth of line 5-86 cells in 1.9% DMSO, 0.95% DMSO plus 3.5% glycerol, 0.95% glycerol alone, 3.5% glycerol alone, 3% glycerol plus 1.9% DMSO, or unsupplemented medium led to the following observations after the cells were injected.

1. Mice injected with cells treated with 1.9% DMSO were less linely to develop tumors than mice innoculated with cells which had grown in unsupplemented medium.
2. Mice survived longer after injection of cells which had grown under optimum conditions for differentiation (1.9%

DMSO or 0.95% DMSO plus 3.5% glycerol) than after injection of cells which had grown in unsupplemented medium.

3. Mice injected with cells grown under less than optimum conditions for differentiation tended to survive longer than mice injected with cells which had grown in unsupplemented medium.

B. Comparison of tumorigenicity of cells of lines differing in hemoglobin induction in response to treatment with DMSO and in virus production.

1. The four cell lines used in these experiments have been characterized as follows:

   a) Lines 5-86 produces hemoglobin and an increased level of virus after treatment with DMSO.

   b) Line H 1.9 does not produce hemoglobin after treatment with DMSO when the cells are not transferred to fresh treated medium. This line does produce an increased level of virus under the growth conditions used in this experiment.

   c) Line 707 R produces a low level of virus as measured by reverse transcriptase activity but produces the same level of virus antigens gp 71 and p 30 as lines 5-86 and H 1.9. When this line is treated with DMSO, no change in production of hemoglobin, virus or virus antigens is observed.

   d) Line F4-1 is defective in virus production as measured by reverse transcriptase or p 30 in the medium. Treatment with DMSO leads to hemoglobin production and production of a low level of p 30 but no increase in reverse transcriptase activity.

2. Mice injected with cells of line H 1.9 or 707R were significantly less likely to develop tumors than mice injected with cells of line 5-86. It is possible, though not readily explicable, that DMSO-resistant variant lines grow better in vitro than in vivo.
3. The mice injected with cells of line H 1.9 or 707R which did develop tumors survived longer than mice which developed tumors after injection of cells of line 5-86 or F4-1.
4. Production of high levels of virus by lines 5-86 and H 1.9 did not correlate with their tumorigenicity.
5. Mice injected with cells of line 5-86 or F4-1 which had been treated with DMSO tended to survive longer than mice injected with cells which had grown in unsupplemented medium.
6. Syngeneic mice which did not develop tumors after the injection of cells of lines H 1.9 or 707R tended not to develop erythroleukemia after subsequent injection of leukemogenic levels of polycythemic Friend virus.

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