Influence of Cell Dissociation Procedures on the
Tumorigenicity of Simian Virus 40 Transformed Fibroblasts¹

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¹ Research support received from Public Health Service Grant
CA15184-01 from the National Cancer Institute; National
Aeronautics and Space Administration Contract No. W12-792-6;
and U.S. Energy Research and Development Administration
Contract W-7405-eng-48 with the Lawrence Berkeley Laboratory.

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SUMMARY

Mouse fibroblasts transformed by Simian Virus 40 (SV40) were examined for tumor forming ability in syngeneic BALB/c mice following dissociation from tissue culture dishes by two procedures: (1) "Trypsin-EDTA": a 5 min exposure at 27°C to a combination of trypsin (2.5 mg/ml) and the divalent ion chelating agent EDTA (3 mM); (2) "EDTA": a 10 min exposure at 27°C to 3 mM EDTA alone. A significantly greater in vivo proliferative capacity was observed for cells dissociated by the trypsin-EDTA procedure, with the injected cell dose for tumor production in 50% of recipient mice (the TPD_{50}) being 16-fold lower than the TPD_{50} for cells dissociated by the EDTA procedure. Host immunosuppression with 300 rad whole-body irradiation led to a significant 7-fold decrease in the TPD_{50} for cells dissociated by the EDTA procedure, while no significant decrease in TPD_{50} was observed for cells dissociated by the trypsin-EDTA procedure.

INTRODUCTION

Tumor production in vivo has been reported for cells transformed in vitro by DNA viruses such as SV40, adenovirus, and polyoma (1-14). The extent of tumorigenicity observed in such tests is influenced by several biological and technical factors: (1) the extent of tumor-host interaction (syngeneic or allogeneic host); (2) host immune status (immunologically competent or immunosuppressed); (3) the number of cells in the challenge inoculum; and (4) the dissociation technique used to remove cells from tissue culture dishes prior to injection. The experiments reported here were designed to assess quantitatively the effects

of factors (2), (3), and (4) on the tumorigenicity of a clonal line of SV40 transformed mouse fibroblasts.

MATERIALS AND METHODS

Tumorigenicity tests were carried out with the following clonal lines of mouse fibroblasts: (1) 3T3, an established cell line derived from a BALB/c embryo culture; (2) SV-T2, an SV40 transformed established cell line derived from the same embryo culture as 3T3; (3) D3, a "revertant" cell line derived from an SV-T2 culture. The untransformed 3T3 line exhibits contact inhibition of cell division in vitro. The SV-T2 transformed line exhibits multilayered growth in vitro, and contains intranuclear T antigen as detected by a direct immunofluorescent assay with hamster antibody. The D3 revertant cell line exhibits contact inhibition of cell division, but contains intranuclear T antigen.

Growth medium used for all cultured cell lines was Dulbecco's Modified Eagle Medium (DMEM) (Grand Island Biological Co., Grand Island, N.Y.) containing 5% calf serum (Pacific Biological Co., Berkeley, Calif.). Cells were cultured in 100 mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.), and were maintained at 37°C in a humidified 95% air/5% CO₂ atmosphere.

Tumorigenicity assays were carried out with cells obtained from cultures in exponential growth phase. Prior to removal from tissue culture dishes cells were rinsed twice with DMEM (without added serum). Dissociation of cells from tissue culture dishes was then carried out by the following two procedures: (1) "Trypsin-

EDTA" procedure: Tris-saline buffer solution (pH 7.4) containing 2.5 mg/ml trypsin 1:250 (Difco Laboratories, Detroit, Mich.) and 3 mM ethylenediamine tetraacetic acid (EDTA) was added to culture dishes for 5 min at 27°C; (2) "EDTA" procedure: Puck's saline GM (Ca^{++} - Mg^{++} free) containing 3 mM EDTA was added to culture dishes for 10 min at 27°C. In both methods, the incubation time was chosen to be the minimum required to dissociate cells from their substrate. At the end of the incubation period, the dissociation medium was gently pipetted against the surface of the culture dishes to suspend any loosely adherent cells, and then was delivered into a chilled centrifuge tube containing sufficient calf serum to bring the final serum concentration to 10%. The cells were pelleted by centrifugation at 150 x g for 2 min, resuspended in DMEM (without added serum), counted in a hemocytometer, and the cell concentration adjusted to the desired level for injection into recipient mice. Cell viability was tested by measuring the percentage of cells able to exclude 0.5% nigrosin dye (Matheson, Coleman, and Bell, Norwood, Ohio).

Tumor forming ability of 3T3, SV-T2, and D3 cells was tested in syngeneic 6-week-old BALB/c male mice. The cell inoculum in a 0.1 ml volume of DMEM was injected subcutaneously into the right flank using a 25 gauge needle. Tumor producing ability of 3T3 and D3 cells was tested by injection of 10^7 cells into hosts that had been immunosuppressed with 300 rad of ^{60}Co whole-body irradiation. For SV-T2 cells, tumorigenicity in both normal and immunosuppressed hosts was quantitated by determining the TPD₅₀ level, i.e. the dose of cells producing progressively

growing tumors in 50% of recipient mice. The calculation of a TPD_{50} value was based on the percentage of tumor "takes" observed at two months post-injection in groups of mice receiving 10^3 , 2.5×10^3 , 5×10^3 , 10^4 , 5×10^4 , 10^5 and 10^6 cells per mouse. An average of 7 mice were used at each injected cell dose level. Tumor appearance was scored weekly for a period of 2 months following inoculation, and then at 4 and 6 months post-injection in order to detect late tumor growth. In two separate experiments, the percentage of mice developing tumors at each of the injected cell dose levels was found to be extremely reproducible. Calculation of TPD_{50} values and 95% confidence limits was carried out by the method of Litchfield and Wilcoxin (15).

BALB/c mice were immunosuppressed by administering 300 rad of ^{60}Co gamma radiation. The mice were irradiated with a 1400 Ci ^{60}Co source one day prior to inoculation with challenge cells. The exposure dose rate was 13 rad/min.

RESULTS

Figure 1 summarizes the results of experiments on tumor production in BALB/c mice following injection of SV-T2 single cell suspensions prepared by either the trypsin-EDTA or the EDTA dissociation procedure (see "Materials and Methods"). The injected dose of SV-T2 cells required for production of progressively growing tumors in 50% of recipient mice (the TPD_{50}) was found to be 16.2-fold lower for trypsin-EDTA dissociated cells than for EDTA dissociated cells (3180 vs 51,500 cells). Statistical analysis of the dose-response curves showed this difference

to be significant at the level $p < 0.01$ (15), thus indicating that the trypsin-EDTA dissociation procedure yields a higher percentage of cells capable of unlimited proliferation in vivo.

Based on ability to exclude the vital stain nigrosin, 99% of SV-T2 cells prepared by the trypsin-EDTA method were viable. In contrast, only 80% viability was observed for cells dissociated by the EDTA procedure. Suspensions of SV-T2 cells prepared for injection into mice were also cultured in vitro, and growth curves were measured over a 4 day period. From these growth curves, it was determined that the population of cells surviving either the trypsin-EDTA or the EDTA dissociation procedure have log phase growth characteristics in vitro with a 17.5 hr doubling time.

Immunosuppression of recipient mice with 300 rad ^{60}Co whole-body irradiation lowered the TPD_{50} 's for trypsin-EDTA and EDTA dissociated SV-T2 cells by 2.4-fold and 7.0-fold respectively (Figure 1). The reduction in TPD_{50} level for immunosuppressed relative to non-immunosuppressed mice was statistically significant ($p < 0.01$) only in the case of SV-T2 cells dissociated by the EDTA procedure.

In tumorigenicity tests with immunosuppressed hosts, the TPD_{50} for SV-T2 cells dissociated by the trypsin-EDTA procedure was 5.6-fold lower than the TPD_{50} for cells prepared by the EDTA method (Figure 1). This difference was statistically significant at the level $p < 0.01$. The lower TPD_{50} observed for SV-T2 cells dissociated by the trypsin-EDTA procedure is consistent with results described above for non-immunosuppressed mice.

Regardless of the dissociation procedure employed or the immune status of the host, the initial appearance of palpable tumor nodules (> 1 mm diameter) was rarely observed later than 4 weeks after inoculation of BALB/c mice with SV-T2 cells. The time of initial tumor appearance depended upon the number of SV-T2 cells injected, but following injection of an identical inoculum, tumors arose in immunosuppressed mice approximately one week earlier than in non-immunosuppressed mice. Once tumors reached a palpable size, they grew progressively and ultimately led to death of the host. No evidence was noted for tumor regression in any of the experiments reported here.

Tumor forming capability was also tested with normal 3T3 and revertant D3 cells. No tumor growth was noted during the 2 month assay period in either normal or immunosuppressed (300 rad) 6-week-old BALB/c mice following subcutaneous injection of 10^7 3T3 or 10^7 D3 cells. This result was obtained with 3T3 and D3 cells prepared by either the trypsin-EDTA or the EDTA dissociation procedure.

DISCUSSION

In conducting tumorigenicity assays with transformed cell lines, plasma membrane alterations occur during the process of dissociating the cells from their tissue culture substrate prior to injection into animal hosts. Membrane damage of this nature could lead to a reduction in the percentage of viable cells in the transplant inocula. In addition, the degree of host-transplant interaction may be affected by virtue of alterations in

the expression of cellular transplantation antigens. The extent to which these effects occur might be expected to vary depending upon whether cells are dissociated by an enzymatic procedure such as trypsinization, or by exposure to a divalent ion chelating agent such as EDTA or EGTA (ethylenedis(oxyethylenenitrilo) tetraacetic acid). In a previous study, Inbar et al. demonstrated that polyoma and SV40 transformed hamster cells have a greater tumor forming ability when dissociated with trypsin than with EDTA (11). The tumorigenicity studies reported here for the SV-T2 line of SV40 transformed BALB/c fibroblasts confirm this observation, and provide the following additional information:

(1) The lower tumor forming capability of EDTA dissociated cells appears to be a consequence of the prolonged incubation with EDTA that is required in order to achieve a single cell suspension. This follows from a comparison of the results of experiments in which SV-T2 cells were exposed to the same 3 mM concentration of EDTA during a 5 min incubation with trypsin-EDTA and a 10 min incubation with EDTA alone. The latter dissociation procedure led to a 16-fold higher TPD₅₀ value, thus indicating that the lengthened time of exposure to EDTA resulted in a significantly lower proliferative capacity of SV-T2 cells in vivo. Evidence that EDTA produces extensive cellular damage was also provided by the observation that only 80% of SV-T2 cells dissociated by the EDTA procedure retained their viability as judged from exclusion of nigrosin dye. A further indication of the deleterious effect of EDTA on cellular structure is provided by the finding of Culp and Black that a loss of surface glycoprotein

and intracellular protein and RNA occurs when SV-T2 cells are exposed to 0.5 mM EDTA at 37°C for a period as short as 5 min (16).

Two alternative explanations can be given for the lower tumorigenic potential of SV-T2 cells dissociated by the EDTA procedure: (a) A mitogenic effect by trypsin could provide a growth advantage for cells dissociated by the trypsin-EDTA method in comparison with cells dissociated by EDTA alone (17,18). This possibility appears unlikely in view of the similar in vitro growth characteristics observed for cells dissociated by the trypsin-EDTA and the EDTA procedures. (b) The action of trypsin may lead to a rapid dissociation of cells from their substrate during the trypsin-EDTA procedure, with a resultant protection against more severe cellular damage resulting from detachment by EDTA. In order to test this possibility, additional tumorigenicity tests should be performed in which SV-T2 cells are dissociated by a 5 min exposure to trypsin alone, and by a 10 min exposure to the trypsin-EDTA mixture. A comparison of the TPD_{50} 's obtained for cells dissociated by these methods with the TPD_{50} values measured in the present series of experiments should demonstrate whether the action of trypsin serves to prevent colateral EDTA-induced cellular damage.

(2) The TPD_{50} for SV-T2 cells dissociated by the EDTA procedure was significantly (7-fold) lower in immunosuppressed relative to non-immunosuppressed mice. In contrast, host immunosuppression was not found to have a significant effect on the tumorigenicity of cells dissociated by the trypsin-EDTA procedure. These findings indicate that prolonging the period of

exposure to EDTA results in a greater immunogenicity of the transformed cells, and thereby leads to a heightened transplantation resistance in syngeneic hosts.

It should also be noted that tryptic digestion of cellular surface material could conceivably lead to a reduced antigen expression in SV-T2 cells dissociated by the trypsin-EDTA procedure, with a result that tumorigenic potential of these cells is independent of host immune status. This possibility appears unlikely, however, on the basis of the observation that the native cellular surface coat is regenerated within a few hours following trypsin treatment (19).

In summary, the preliminary observations reported here indicate that the tumorigenic potential of SV40 transformed cells is strongly dependent on the method chosen to detach cells from their tissue culture substrate. The present results indicate that a dissociation method involving brief exposure to a trypsin-EDTA mixture is superior for preservation of in vivo cellular proliferative capacity, as compared with a dissociation method requiring prolonged exposure to EDTA alone.

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CAPTION FOR FIGURE 1

TPD₅₀ values are presented graphically for SV-T2 cells prepared by the trypsin-EDTA and the EDTA dissociation procedures, and injected into normal or immunosuppressed (300 rad) syngeneic 6-week-old BALB/c male mice. Bars represent 95% confidence limits for the TPD₅₀ values.

FIGURE 1