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Inactivation of Tumor Cell-associated Feline Oncornavirus for Preparation of an Infectious Virus-free Tumor Cell Immunogen[1]

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SUMMARY

Ultraviolet (UV) and thermal methods of inactivating the oncogenic potential of C-type particle-producing feline oncornavirus-induced tumor cells were developed. The techniques were evaluated by several parameters for their use in preparation of cellular immunogens. The UV inactivation dose required to reduce the number of focus-forming units per ml by 1 $\log_{10}$ for FL-74 lymphoblastoid cell-associated feline leukemia virus was 44,000 ergs/sq mm, and the thermal inactivation dose required to reduce the number of focus-forming units per ml by 1 $\log_{10}$ at 45° was 16 min. Inactivation of greater than 6 $\log_{10}$ of virus per ml associated with 4×10^8 cells required a UV dose of 270,000 ergs/sq mm, 100 min at 45° or 3 min at 56°. All three treatments concomitantly destroyed the replicating potential of FL-74 cells as shown by their inability to propagate under normal growth conditions and to incorporate [^{3}H]thymidine into nuclear DNA. UV inactivation and thermal inactivation at 45° allowed the best retention of feline oncornavirus-associated cell membrane antigen. A 50% loss in antigenic activity was observed as a result of 56° treatment, but this method was the only one that did not destroy the surface structural integrity of FL-74 cells.

INTRODUCTION

The ability of a host to resist tumor growth often appears to be the result of the immunological response of the host to tumor-associated antigens (7, 9). The protective effect of antitumor immunity against feline sarcoma virus-induced tumorigenesis is indicated by the presence of humoral antibody specific for FOCMA[2] in sera from cats with regressed tumors (2). The observed correlation between tumor regression and antibody to FOCMA suggests the use of FOCMA for immunoprophylaxis or immunotherapy.

Inoculation of experimental animals with nonviable tumor cells has been an approach in other oncogenic DNA and RNA systems for testing the feasibility of utilizing tumor membrane antigens as immunogens. Inactivated tumor cells can invoke tumor-specific cellular or humoral antibody responses or provide resistance to tumor formation or growth (6, 10, 11, 17, 19).

Methods of preparing tumor cells for injection must be carefully considered for their ability to eliminate tumor-forming potential while allowing retention of antigenicity. This study evaluates various techniques for inactivation of the oncogenic potential of feline oncornavirus-induced tumor cells which are continual producers of oncogenic RNA virus. In this system, inactivation of cell-associated virus as well as inactivation of the tumor cell-replicating potential is essential. Replicating potential, physical integrity, and FOCMA antigenicity of tumor cells are determined following inactivation of cell-associated virus by UV irradiation or heat treatment.

MATERIALS AND METHODS

Cells

FL-74 Cells. The FL-74 cell line, derived from a lymphoid neoplasm of a KT-FeLV-infected cat (22) was kindly provided by Dr. Myron Essex, Harvard University, Cambridge, Mass. The cells were grown in stationary suspension cultures using techniques described by Olsen *et al.* (15). Growth medium consisted of McCoy's Medium 5A (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% FBS (Reheis Chemical Co., Phoenix, Ariz.), gentamicin sulfate, 50 μg/ml (Gentocin; Schering Corporation, Kenilworth, N. J.), and nystatin, 10 units/ml (Mycostatin; E. R. Squibb and Sons, Inc., New York, N. Y.).

Feline Embryo Cells. Feline embryo cells were derived from whole 30- to 35-day-old embryos. The cells were grown in L-15 (Leibowitz) medium (Flow Laboratories, Rockville, Md.) supplemented with 15% FBS, 2 mM L-glutamine (Grand Island Biological), and antibiotics as above and were used between the 2nd and 15th passage.

Sarcoma-positive and Leukemia-negative Cells. A line of sarcoma-positive and leukemia-negative feline cells, derived from a Moloney murine sarcoma virus-transformed cat cell line, was kindly provided by Dr. Peter Fischinger, NIH, Bethesda, Md. (4). The cells, designated 81C, were grown in Falcon plastic tissue culture flasks in McCoy's Medium 5A supplemented with 15% FBS and antibiotics as above.

FeLV Focus Formation Assay

KT-FeLV was quantitated by a focus formation assay using 81C cells according to a modified procedure of Fischin-

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[2] The abbreviations used are: FOCMA, feline oncornavirus-associated cell membrane antigen; KT-FeLV, Kawakami-Theilen strain feline leukemia virus; FBS, fetal bovine serum; FeLV, feline leukemia virus; D_{10}, dose required to reduce the number of focus-forming units per ml by 1 $\log_{10}$.

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ger *et al.* (4). Briefly, cells were seeded in Falcon tissue culture cluster dishes at a concentration of 4×10^5 cells/well, incubated in a humidified 5% CO_2 atmosphere at 37° for 3 hr, and thereafter treated with DEAE-dextran for 30 min. FL-74 cell suspensions containing FeLV were diluted 10-fold and sonically dispersed for 1 min in a 10-kHz Raytheon Model DF101 sonic oscillator (Raytheon Co., Manchester, N. H.). Fivefold serial dilutions of each sonically dispersed sample were then added to the cluster dish wells. Following a 60-min virus adsorption at 37°, growth medium was added, and the dishes were incubated once again. The cell monolayers were maintained for 12 days after which they were fixed with 10% buffered formalin and stained with Giemsa.

Cytotoxic Antibody Adsorption

Cat sera were assayed by the microcytotoxicity assay as described by Mathes *et al.* (13). High-titer sera were used for FOCMA adsorption at 4-fold concentrations of their end-point titer dilutions. One tenth-ml volumes of diluted cat sera were added to 0.1-ml volumes of diluted FL-74 cell suspensions (undiluted concentration, 4×10^8 cells/ml) in 12- × 75-mm polypropylene capped tubes (Falcon). All cell samples were stored frozen at −20° and thawed immediately prior to testing. Adsorption was carried out at 4° for 16 hr with constant agitation, and the serum was clarified by centrifugation (800 × *g* for 10 min) for subsequent testing in the microcytotoxicity assay.

Autoradiography

FL-74 cells were suspended at a concentration of 1.0×10^6 cells/ml in growth medium and continuously labeled with [^{3}H]thymidine, 0.5 μCi/ml (specific activity, 6 Ci/mmole; Schwarz/Mann, Orangeburg, N. Y.), for 24 hr at 37°. Following the [^{3}H]thymidine pulse, the cells were washed 3 times in phosphate-buffered saline (0.01 M sodium phosphate:0.15 M NaCl), resuspended in 1% bovine serum albumin in phosphate-buffered saline at a concentration of 10^8 cells/ml, and spread on glass slides. Air-dried cells were fixed at room temperature for 10 min in a solution consisting of acetic acid:absolute ethanol (1:3), rinsed in 70% ethanol and 3 changes of 95% ethanol, and allowed to air dry once again.

Slides with fixed cells were dipped in prewarmed (45°) Eastman Kodak NTB 2-type nuclear track emulsion (Eastman Kodak Co., Rochester, N. Y.) (14) and placed in light-tight slide boxes containing Drierite (W. A. Hammond Drierite Co., Xenia, Ohio). Autoradiographs were exposed for 4 days at 4°, developed in D-19 developer at 16° (Eastman Kodak) for 3 min, washed in distilled water, and fixed in Kodak acid fixer.

Cells were then stained with Erlich's glycerin alum hematoxylin for 4.5 min, washed with 2 rinses of distilled water, and stained with 1% (w/v) alcoholic eosin for 3 min. Prior to mounting of the coverslips with Permount, the slides were rinsed in 95% ethanol, air dried overnight, and dipped in xylene.

UV Irradiation of FL-74 Cells

FL-74 cells were harvested, washed 3 times in Hanks' balanced saline solution supplemented with 5% calf serum, and resuspended at a concentration of 4×10^8 cells/ml in Hanks' balanced saline solution. Open 60-mm Falcon plastic tissue culture Petri dishes, each containing 1 ml of cell suspension, were exposed to a germicidal low-pressure mercury lamp with an emitting wavelength of 2537 Å. The dose rate, measured by a Blak-Ray UV intensity meter (Ultraviolet Products, Inc., San Gabriel, Calif.), was 152 ergs/sq mm/sec at the sample surface. Irradiation was performed over ice and the dishes were rotated continuously during exposure.

Thermal Inactivation of FL-74 Cells

FL-74 cells were prepared as above for the UV irradiation and dispensed in 3-ml quantities in 5-ml vials. The cells were incubated in a 45 or 56° water bath with constant agitation. To stop the reaction, the cells were transferred immediately to a 4° ice bath.

RESULTS

UV Inactivation of FL-74 Cell-associated KT-FeLV. The survival curve of FL-74 cell-associated KT-FeLV following UV irradiation is shown in Chart 1. The inactivation dose D_{10} was 44,000 ergs/sq mm, and the accumulated surface dose necessary for total inactivation of greater than 6 $\log_{10}$ of FeLV per ml was determined by extrapolation to be 270,000 ergs/sq mm. To establish the validity of this calculation,

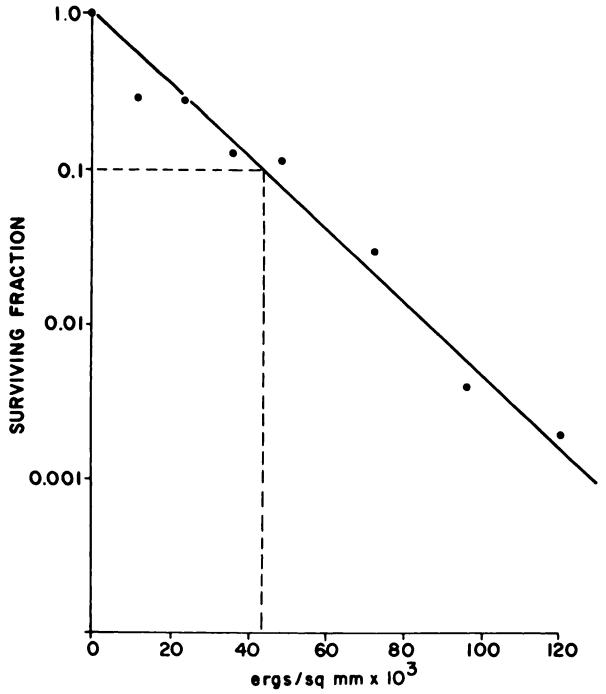

Chart 1. UV inactivation of FL-74 cell-associated KT-FeLV focus formation activity. The inactivation dose, D_{10}, is indicated by the *dashed line* drawn to the *abscissa*.

samples were exposed to a dose of 270,000 ergs and assayed for virus activity. No focus formation was observed on susceptible cells after exposure of the cell-bound virus to this irradiation dose.

Thermal Inactivation of FL-74 Cell-associated KT-FeLV. Complete thermal inactivation of 2.2×10^6 focus-forming units/ml of FL-74 cell-bound KT-FeLV was accomplished by a 3-min 56° incubation. No decrease in *in vitro* transformation activity was observed with samples incubated for up to 2 min, although the number of focus-forming units of FeLV decreased by nearly 3 $\log_{10}$ after a 2.5-min incubation.

Heat treatment (45°) was also effective in inactivating cell-associated virus. FL-74 cells were incubated for varying time intervals from 0 to 50 min, and the resulting virus thermal inactivation curve is illustrated in Chart 2. The thermal D_{10} was 16 min and the time required completely to eliminate virus focus formation activity in these samples was determined to be approximately 100 min.

Retention of FOCMA on FL-74 Cells after UV or Thermal Inactivation. Antibody adsorption studies were used to determine whether FOCMA was retained by FL-74 cells following exposure to UV light or heat. Both treated and untreated cells were tested for their ability to adsorb specific antibody from 2 reference feline antisera previously shown to be cytotoxic to FL-74 cells when incubated with complement. Table 1 gives the number of adsorption units present in cell suspensions that were UV irradiated or heat treated with the minimum doses necessary for virus inactivation. Cell samples irradiated with a dose of 270,000 ergs/sq mm or exposed to a temperature of 45° for 100 min retained a number of adsorption units equivalent to that of untreated cell samples at the same cell concentration. The sample exposed to 56° for 3 min demonstrated a loss of 1 adsorption unit.

The specificity of the adsorption reaction was examined. When nontransformed feline embryo cells, handled similarly to the FL-74 cells, were incubated with reference serum they did not remove cytotoxic activity. For investigation of the possibility that the decrease in cytotoxic activity of FL-74 cell-adsorbed reference sera was due to anticomplementary activity, FL-74 cell lysates derived from freezing and thawing were incubated with complement which was subsequently clarified and used to lyse sensitized sheep RBC. Cell lysates diluted 2-fold were found to be slightly anticomplementary, whereas more dilute lysates were not anticomplementary. This test showed that complement-inhibitory activity could not account for the loss of cytotoxic activity demonstrated by sera adsorbed with FL-74 cell suspensions diluted 4- to 8-fold (Table 1).

Replicating Potential of FL-74 Cells after UV and Thermal Inactivation. Two criteria were established for determining FL-74 cell replicating potential following inactivation of cell-associated KT-FeLV: *(a)* the ability of treated cells to propagate under normal growth conditions; and *(b)* their ability to incorporate [^{3}H]thymidine into nuclear DNA as shown by autoradiography.

Immediately following exposure to UV irradiation or high temperatures, FL-74 cells were seeded in Falcon tissue culture flasks in growth medium at a concentration of 1.0×10^6 cells/ml, incubated at 37° for 3 days, and examined by a trypan blue dye exclusion test. According to this criterion, the cells lost their replicating potential after exposure to the minimum doses required for virus inactivation (Table 1). After 3 days in culture, all cells were nonviable. Lesser doses than those presented in Table 1 were also capable of destroying the ability of the cells to maintain normal functions and to propagate. For example, such a loss of function was observed as a result of a UV dose of 12,000 ergs/sq mm or 10 min at 45° (this was the shortest incubation time tested at 45°). Although cells exposed to 56° for a period ≤1.5 min were able to propagate, they failed to grow if incubated at the same temperature for ≥2.0 min.

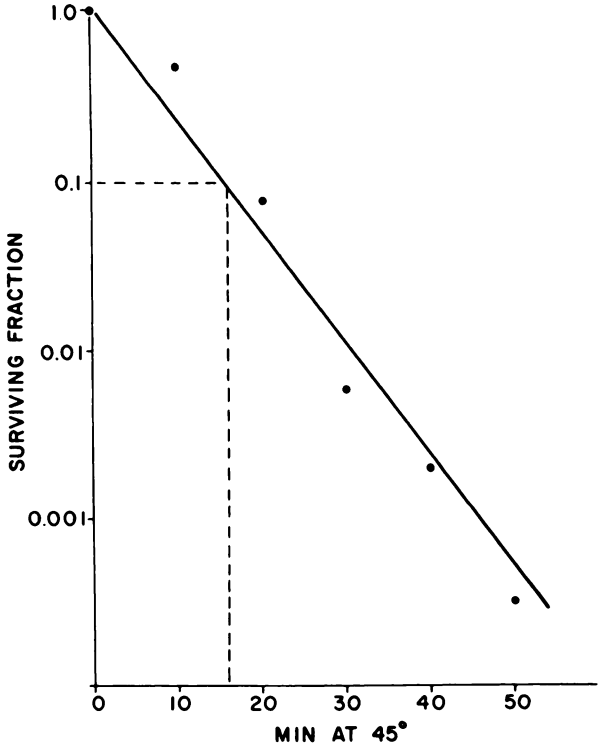

Chart 2. Thermal inactivation of FL-74 cell-associated KT-FeLV focus formation activity. The approximate time required to inactivate 1 $\log_{10}$ of focus formation units is indicated by the *dashed line* to the *abscissa*.

Eighty-seven % of untreated FL-74 cells incorporated [^{3}H]thymidine into nuclear DNA, whereas none of the UV- or heat-inactivated cells examined incorporated the labeled nucleotide (Table 1). The differential ability for incorporating [^{3}H]thymidine is demonstrated in Figs. 1 to 4. Numerous silver grains can be seen over the nuclei of control cells (Fig. 1), but not above UV- or heat-inactivated cells (Figs. 2 to 4).

Physical Integrity of FL-74 Cells after UV and Thermal Inactivation. Although the procedures for inactivation of FL-74 cell-bound virus invariably destroyed the replicating potential of cells, they did not always incur damage to cellular morphological integrity. For determination of the portion of intact cells remaining in treated samples, a trypan blue dye exclusion test was performed immediately subsequent to UV irradiation or incubation at elevated temperatures. Table 1 gives the percentage of intact cells (those excluding trypan blue) following the minimum exposures required for virus inactivation in FL-74 cells. Cell suspensions exposed to 56° for 3 min maintained an equal percentage of cells excluding trypan blue as did untreated cell suspensions. Exposure to 45° for 100 min resulted, how-

Table 1
Antigenicity, morphological integrity, and replicating potential of FL-74 cells following inactivation of cell-associated virus

All cell samples were exposed to UV light or heat at a concentration of 4×10^8 cells/ml.

Inactivation treatment	FOCMA (adsorption units[a])	% cells excluding trypan blue[b]	% cells incorporating [^{3}H]thymidine[c]	Ability to propagate[d]
None	3	>85	87	+
UV irradiation (270,000 ergs/sq mm)	3	27	0	−
56° (3 min)	2	>85	0	−
45° (100 min)	3	<5	0	−

[a] One unit corresponds to a 2-fold dilution of FL-74 cell suspension capable of adsorbing cytotoxic antibody from Sera 30B and Q150.
[b] Determined by a trypan blue dye exclusion test performed immediately subsequent to inactivation.
[c] Determined by autoradiography.
[d] Cells were seeded at a concentration of 1.0×10^6 cells/ml in growth medium and examined 3 days later by a trypan blue dye exclusion test. +, trypan blue exclusion in greater than 90% of the cells; −, uptake of trypan blue in 100% of the cells.

ever, in a complete or nearly complete loss of cellular integrity in the sample population. Additional experimentation showed that the percentage of intact cells decreased gradually from greater than 85 to less than 5% as a consequence of 45° heating for time periods ranging from 0 to 60 min. Although a high percentage of UV-irradiated cells remained intact after exposure to at least 120,000 ergs/sq mm, only 27% of the cells excluded trypan blue after 270,000 ergs/sq mm, the minimum dose for virus inactivation.

DISCUSSION

The feasibility of inducing protective tumor-specific immunity can be tested *in vivo* by injection of whole tumor cells into animals either prophylactically or during the oncogenic latency period. Cells that are highly oncogenic, such as feline oncornavirus-induced tumor cells, and are to be used in immunization experiments must be inactivated prior to inoculation. The purpose of this study was to develop practical methods of inactivating feline tumor cells and their associated oncornaviruses while allowing optimum retention of viral or tumor-specific surface antigenicity.

The FL-74 cell line was used in the tumor cell inactivation study because of its potential as a source of immunogen. FL-74 cells are continual producers of KT-FeLV containing A, B, and C serotypes (18) and demonstrate FOCMA (2). The *in vitro* propagation of the FL-74 cell line has replaced the production of sarcomas in cats and has become the method of choice for obtaining large numbers of highly antigenic cells (15, 24).

The results have shown that inactivation of cell-bound KT-FeLV can be accomplished expeditiously by UV irradiation or exposure of the cells to elevated temperatures. Furthermore, cells subjected to these treatments are unable to synthesize DNA or to replicate.

Since it is generally accepted that a lethal effect of UV light (2537 Å wavelength) upon viruses is due to adsorption of photons within nucleic acids or nucleoproteins (21), we propose that this is the primary mechanism of feline oncornavirus inactivation. Inactivation of RNA tumor viruses by non-ionizing irradiation may, in part, be a consequence of viral RNA-directed DNA polymerase damage as well (12). The UV inactivation dose, D_{10}, for virus in cells at a concentration of 4.0×10^8 cells/ml was approximately 10 to 20 times greater than the D_{10} for non-cell-associated murine leukemia and sarcoma viruses (25) and feline sarcoma virus (unpublished data). A higher dose of irradiation is required to inactivate a cell-bound RNA tumor virus because UV penetration through samples containing high concentrations of cellular material is greatly reduced.

In this study, FeLV was inactivated at 2 different temperatures. Thermal inactivation studies of Rous sarcoma virus (1) and a number of nononcogenic viruses (23) have shown that thermal inactivation rate constants are dependent on temperature, indicating that different temperatures inactivate different viral components. Protein and enzyme thermal inactivations are associated with a high heat of activation, and a high entropy of activation and macromolecular damage occurs through hydrogen bond breakage resulting in unfolding of native molecules (20, 23). Inactivation of RNA is not associated with high heat and entropy of activation. RNA inactivations probably occur through cyclic phosphotriester link formations which lead to hydrolysis of the RNA chain (5, 23). Since the energy of activation is high for enzyme and protein inactivation and low for RNA inactivation, it might be speculated that at 56° feline oncornavirus inactivation occurs through denaturation of the reverse transcriptase enzyme whereas, at 45°, inactivation is brought about by rupture of the RNA molecule.

Rapid inactivation of virus occurred at 56°. There appeared to be an exponential drop in viral activity with incubation from 2.0 to 3.0 min, and cell suspensions incubated for 3.0 min were free of *in vitro* transformation activity. A lag in virus inactivation up to 2.0 min indicated that, during the initial incubation, the virus did not fully absorb the maximum heat incurred at this temperature. This was probably due to the tempering effect of high concentrations of cellular organic material, particularly protein, in the sample.

In spite of the fact that 56° inactivation was somewhat damaging to the antigenic properties of FL-74 cell membranes, this was the only technique that allowed maintenance of cellular surface structure as shown by a resistance to the permeability of trypan blue. The importance of maintaining surface integrity in cells that are to be used for immunization is evident with a consideration that the host immune response to membrane antigens may depend upon the proper distribution of antigenic determinants on the cell surface. In addition, it has been shown that the reaction of thymus-derived cells to antigen-carrier molecules is often necessary for effective stimulation of the immune response (3). Loss of gross surface morphology may coincide with alterations in carrier molecular structure and such alterations cannot be detected by antibody adsorption studies.

We recommend the use of UV irradiation or heat treatment at 45° for inactivation of tumor cells and their associated feline oncornavirus since these methods allowed the best retention of FOCMA activity. Although 56° inactivation is probably not as effective in destroying viral infectious nucleic acid and causes some loss in antigenicity, it does have excellent potential for rapid processing of cells for immunization of experimental animals.

There is preliminary evidence that 56° heat-treated FL-74 cells are immunogenic in the cat and may provide protection against FeLV-related neoplasms (16). These *in vivo* investigations confirm that the thermal treatment of cells destroys their oncogenic potential; in this case the effectiveness of the procedure is demonstrated in the susceptible host. The efficacy of using FL-74 cells for specific immunostimulation has also been reported recently by Jarrett *et al.* (8) who immunized cats against development of FeLV viremia.

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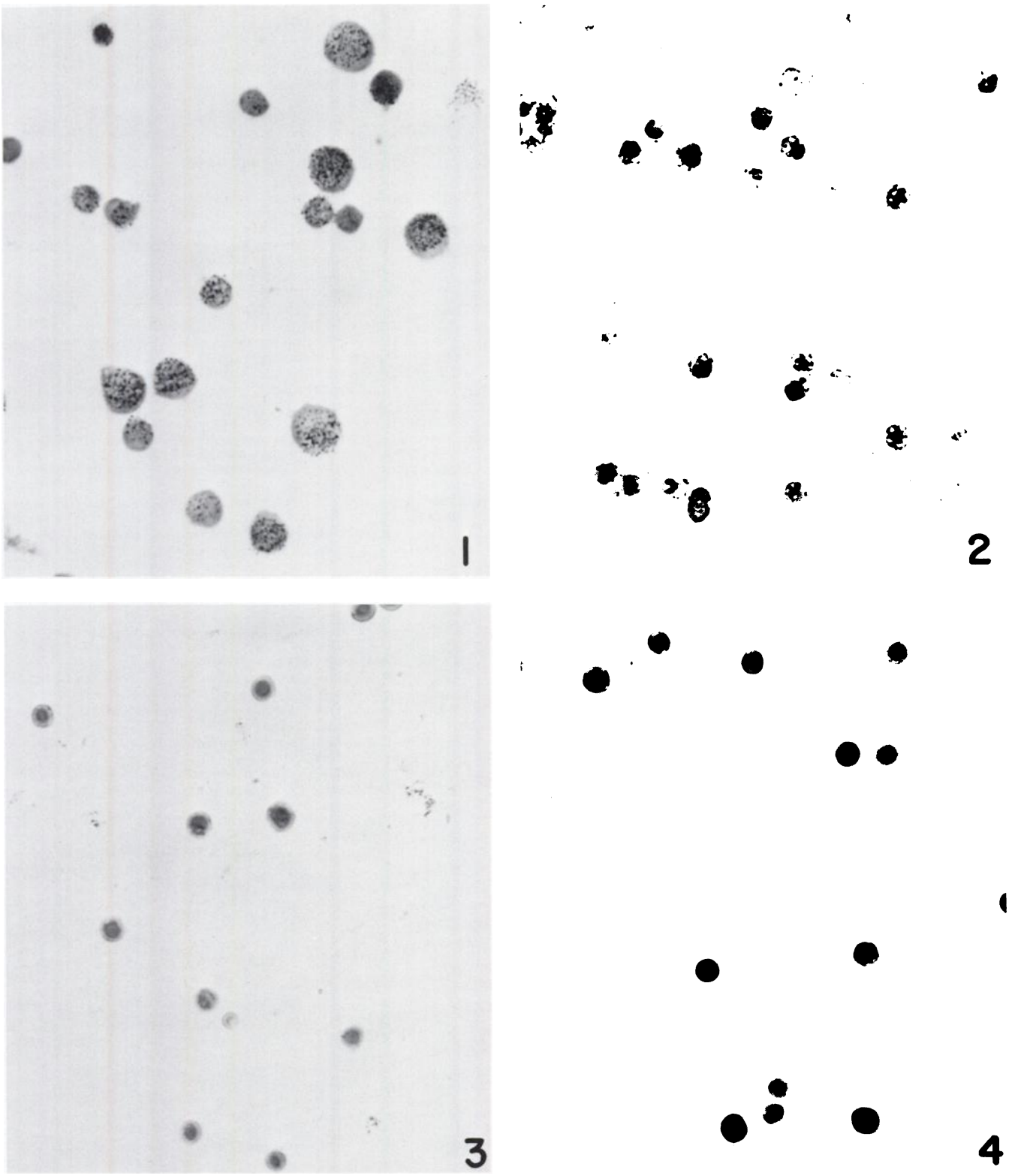

Fig. 1. Incorporation of [^{3}H]thymidine in untreated FL-74 cells. H & E, ×500.
Fig. 2. Incorporation of [^{3}H]thymidine in UV-irradiated (accumulated surface dose, 270,000 ergs/sq mm) FL-74 cells. H & E, ×500.
Fig. 3. Incorporation of [^{3}H]thymidine in FL-74 cells subsequent to incubation in a 45° water bath for 100 min. H & E, ×500.
Fig. 4. Incorporation of [^{3}H]thymidine in FL-74 cells subsequent to incubation in a 56° water bath for 3 min. H & E, ×500.